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Review

Is *Toxoplasma gondii* a threat to the conservation of free-ranging Australian marsupial populations?

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A B S T R A C T

It has often been asserted that Australian marsupial species are particularly susceptible to *Toxoplasma gondii* infection and to clinical toxoplasmosis following infection. This implicates *T. gondii* as a potential threat to marsupial population viability, and contrasts to what is known of *T. gondii* in populations of several other host species. We reviewed the literature, and found a lack of scientifically robust evidence addressing the occurrence of *T. gondii* infection in free-ranging populations of Australian marsupial species, and the impacts of the infection on population health. Key limitations included a lack of studies in free-ranging marsupial populations, study findings susceptible to substantial chance influences, and selection, misclassification and confounding biases. The lack of scientifically robust data available on this topic indicates that assertions that free-ranging populations of Australian marsupials are particularly susceptible to *T. gondii* infection and to toxoplasmosis are premature. The threat of *T. gondii* to the viability of free-ranging marsupial populations should therefore be regarded, at this stage, as a hypothesis.
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1. Introduction

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

There has also been speculation as to the role of introduced infectious disease as a contributing factor to population declines of Australian marsupials (e.g., Freeland, 1994; Abbott, 2006; Thompson et al., 2010).

Toxoplasma gondii is arguably the most broadly implicated infection, prompted by reports of cases and outbreaks of severe clinical toxoplasmosis in captive populations of Australian marsupial species. From the published literature, there appears to be a perception that marsupials are particularly susceptible to infection with *T. gondii* and to toxoplasmosis. For example: "Toxoplasmosis is a significant disease of Australian marsupials commonly causing mortality in captive and free-ranging populations…" (Obendorf et al., 1996); "Marsupials and new world monkeys are among the most susceptible animals for developing the clinical disease toxoplasmosis…" (Skerratt et al., 1997); "Australasian marsupials, especially wallabies, are highly susceptible to acute toxoplasmosis" (Dubey and Crutchley, 2008); "Australian marsupials are among the most susceptible hosts for *T. gondii*…" (Parameswaran et al., 2009a); "*T. gondii* is of concern for Australian native marsupials, which appear to be particularly susceptible to acute infection…" (Hollings et al., 2013).

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

1.1. *T. gondii* infection and toxoplasmosis

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

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

2. The frequency of *T. gondii* infection in free-ranging populations of Australian marsupial species

No published studies have investigated the incidence of *T. gondii* infection in free-ranging populations of Australian marsupials. Surveys have provided estimates infection prevalence and seroprevalence; these are summarised in Tables 1 and 2.

2.1. *T. gondii* infection surveys undertaken in free-ranging populations of Australian marsupials

Evidence of *T. gondii* infection has been found in free-ranging populations of red kangaroos (*Macropus rufus*), western grey kangaroos (*Macropus fuliginosus*), common wallaroos (*Marcopus robustus*) and woylies (*Bettongia penicillata*) (Table 1). Findings suggestive of *T. gondii* infection (histopathological evidence without confirmatory testing) have also been obtained from long nosed bandicoots (*Perameles nasuta*), eastern barred bandicoots (*Perameles gunnii*), southern brown bandicoots/quenda (*Isoodon obesulus*), quokka (*Setonix brachyurus*), brushtail possums (*Trichosurus vulpecula*), brush-tailed phascogales (*Phascogale tapoatafa*) and kowari (*Dasyuroides byrnie*) (Table 1).

Prevalence estimates are all limited by uncertain external validity, due to the use of non-proportionate sampling methods: reliance on culled animals, road kill or trapping for study subjects may entail selection bias. Surveys involving small sample sizes have low power to detect the presence of infection, and marked imprecision in prevalence estimates (Table 1). Commonly, the use of diagnostic methodology that is known to be of poor sensitivity and/or specificity in other species leaves apparent prevalence estimates subject to misclassification bias.

In marsupial surveys, the most commonly used diagnostic test has been the mouse bioassay, which lacks sensitivity (Piergili Fioretti, 2004). None of the surveys of Australian marsupials using this technique also used immunohistochemistry or PCR to confirm identification of *T. gondii* bradyzoites. Thus, the specificity of the mouse bioassay may be compromised, as *T. gondii* bradyzoites can appear very similar to those of *Neospora caninum* under light microscopy (Dubey et al., 2009). As with the mouse bioassay, sample inoculation into cell culture (in this case 13-day old chick embryos (Table 1)) lacks sensitivity, often because of laboratory error (Piergili Fioretti, 2004).

Histopathological examination of host tissues, without confirmatory immunohistochemistry or PCR, was used in a number of marsupial surveys (Table 1). However, this is also an insensitive screening tool, particularly in identifying low burden *T. gondii* infections (Piergili Fioretti, 2004). Specificity of these results might also be compromised by misidentification of other protozoan parasites as *T. gondii* (Dubey et al., 2009).

PCR amplification of *T. gondii* DNA in tissue samples is generally considered a sensitive indicator of infection (Burg et al., 1989; Su et al., 2010). PCR has only been used in two surveys of Australian marsupials, which collectively sampled four species (Parameswaran et al., 2010; Pan et al., 2012). A high proportion these animals tested positive for *T. gondii* by this methodology. Though these findings are limited by small sample sizes, they sharply contrast to collective findings of similar species surveyed histopathologically, where infection was rarely identified (Table 1). As the latter studies were based on different species in different

Table 1
Toxoplasma gondii infection surveys undertaken in free-ranging populations of Australian marsupial species. Except where noted, no prior clinical suspicion of toxoplasmosis existed in sampled animals.

Species	Study location (study)	Sampling timeframe	Diagnostic test[a]	No. infected/ no. tested (%)	95% CI[b]
Eastern grey kangaroo (*Macropus giganteus*)	Roma, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/4 (0%)	0 −52.2%
Eastern grey kangaroo	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/3 (0%)	0 −60.2%
Western grey kangaroo (*M. fuliginosus*)	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	5/5 (100%)	54.1 −99.6%
Western grey kangaroo-adults	Perth, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain and tongue)	9/12 (75%)	46.2 −90.9%
Western grey kangaroo-pouch young	Perth, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain, heart, skeletal muscle, liver, lung, small intestine, kidney and spleen)	2/17 (11.8%)	3.6 −34.7%
Common wallaroo (*M. robustus*)	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/5 (0%)	0 −45.9%
Common wallaroo	Kynuna, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/3 (0%)	0 −60.2%
Common wallaroo	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	5/5 (100%)	54.1 −99.6%
Red kangaroo (*M. rufus*)	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/5 (0%)	0 −45.9%
Red kangaroo	Longreach, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/6 (0%)	0 −41.0%
Red kangaroo	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	6/6 (100%)	59.0 −99.6%
Woylie[c] (*Bettongia penicillata*)	Manjimup, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain and heart)	2/4 (50%)	14.7 −85.3%
Long-nosed potoroo (*Potorous tridactylus*)	Winkleigh, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/1 (0%)	0 −84.2%
Quokka (*Setonix brachyurus*)	Rottnest Island, Western Australia (Gibb et al., 1966)	Nov 1961	Histopathology (left lateral femoral muscle biopsy)	4/92 (4.3%)	1.8 −10.6%
		Nov 1963		14/20 (70%)	47.8 −85.4%
		Jan 1964		1/20 (5%)	1.2 −23.8%
		Feb 1964		6/18 (33.3%)	16.3 −56.6%
Quokka[d]	Rottnest Island, Western Australia (Gibb et al., 1966)	Nov 1963 −Feb 1964	Mouse bioassay (suspension of host skeletal muscle, +/− liver, brain and heart, injected IP and combined IP & IC; impression smears ± histopathological examination of mice) 13-day old chick embryo inoculation (suspension of host skeletal muscle, cardiac muscle and brain)	2/28 (7.1%)	2.2 −22.8%
Brushtail possum (*Trichosurus vulpecula*)	Brisbane, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	2/7 (28.6%)	8.5 −65.1%
Brushtail possum	Dalby, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain or pooled organs, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/3 (0%)	0 −60.2%
Brushtail possum	Cressy, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain or pooled organs, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/9 (0%)	0 −30.8%
Western Ringtail possum (*Pseudocheirus occidentalis*)[e]	Western Australia (Parameswaran, 2008)	Not specified	PCR (heart of one possum, skeletal muscle of another)	0/2 (0%)	
Brush-tailed phascogale (*Phascogale tapoatafa*)	Burleigh, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1/1 (100%)	15.8 −98.7%
Eastern pygmy possum (*Cercartetus nanus*)	Wilmot, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/1 (0%)	0 −84.2%
Southern brown bandicoot (*Isoodon obesulus*)	Brisbane & Innisfail, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	5/10 (50%)	23.4 −76.6%
Southern brown bandicoot	Brisbane, Queensland (Pope et al., 1957a)	August 1955 −February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	16/38 (42.1%)	27.8 −57.9%
Southern brown bandicoot	Mt Glorius, Queensland (Pope et al., 1957a)	August 1955 −February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	0/2 (0%)	0 −70.8%
Southern brown bandicoot	North Queensland (Pope et al., 1957a)	August 1955 −February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1/3 (33.3%)	6.8 −80.6%

(*continued on next page*)

Table 1 (*continued*)

Species	Study location (study)	Sampling timeframe	Diagnostic test[a]	No. infected/ no. tested (%)	95% CI[b]
Southern brown bandicoot	Brisbane, Queensland (Pope et al., 1957b)	1951	Histopathology (brain, lungs, liver, spleen, kidney, retroperitoneal lymph node); Mouse and guinea pig bioassays (suspension of host liver, spleen and brain injected IP and IC; histopathological examination of mouse brain, heart, lungs, spleen, skeletal muscle, kidneys, pancreas, blood)	1/1 (100%)	15.8 −98.7%
Long-nosed bandicoot (*Perameles nasuta*)	Mt Glorius, Queensland (Pope et al., 1957a)	August 1955 −February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1/2 (50%)	9.4 −90.6%
Long-nosed bandicoot	Northern Queensland (Pope et al., 1957a)	August 1955 −February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	2/4 (50%)	14.7 −85.3%
Eastern barred bandicoot (*P. gunnii*)	Longford, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/1 (0%)	0 −84.2%
Eastern barred bandicoot[f]	Tasmania (Obendorf et al., 1996)	July 1992 −March 1995	Histopathology (brain, heart, lung and skeletal muscle) with serology	7/8 (87.5%)	51.8 −97.2%
Greater bilby (*Macrotis lagotis*)	Birdsville, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	0/1 (0%)	0 −84.2%
Tasmanian devil (*Sarcophilus harrisii*)	Gladstone, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/3 (0%)	0 −60.2%
Eastern quoll (*Dasyurus viverrinus*)	Gladstone, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/7 (0%)	0 −36.9%
Spotted-tailed quoll (*D. maculatus*)	Wilmot, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/1 (0%)	0 −84.2%
Northern quoll[g] (*D. hallucatus*)	Kakadu National Park, Northern Territory (Oakwood and Pritchard, 1999)	1993–1995	Histopathology (brain, heart, lungs, diaphragm, “gut”, liver, tongue, hindleg muscle)	0/28 (0%)	0 −11.9%
Kowari (*Dasyuroides byrnie*)	Queensland (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, “gut”, spleen, pancreas, parapancreatic node)	4/17 (23.5%)	9.7 −47.6%
Dusky Antechinus (*Antechinus swainsonii*)	Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/− liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0/1 (0%)	0 −84.2%
Brown Antechinus (*A. stuartii*)	Not specified (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, “gut”, spleen, pancreas, parapancreatic node)	0/3 (0%)	0 −60.2%
White-footed dunnart (*Sminthopsis leucopus*)	Not specified (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, “gut”, spleen, pancreas, parapancreatic node)	0/7 (0%)	0 −36.9%

[a] Regarding mouse bioassays-SC = subcutaneous inoculation, IP = intraperitoneal inoculation, IC = intracerebral inoculation.
[b] Calculated by review author using Jeffrey's 95% confidence interval.
[c] One (infected) woylie exhibited neurological symptoms prior to death.
[d] Survey undertaken in wild quokka with serological or histological evidence of *T. gondii* infection.
[e] Wild possums found with neurological symptoms.
[f] One bandicoot obtained via trap death-previously identified as seropositive for *T. gondii*; origin of the other bandicoots unclear.
[g] Study subjects obtained via roadkill.

locations, the results may simply reflect heterogeneity in the distribution of infection. However, such discordant results do lend support to the hypothesis that histopathological surveys are insensitive in estimating prevalence of infection in marsupial populations showing no clinical signs of toxoplasmosis. PCR techniques have been demonstrated to have high specificity (Burg et al., 1989): given the use of appropriate negative controls and sequencing in both studies, it seems unlikely that false positives would have substantially influenced the findings of the PCR surveys.

2.2. *T. gondii* exposure in free-ranging populations of Australian marsupials

T. gondii serosurveys have found *T. gondii* antibodies in populations of western grey kangaroos, eastern grey kangaroos (*Macropus giganteus*), Bennett's wallabies (*Macropus rufogriseus*), bridled nailtail wallabies (*Onychogalea fraenata*), Tasmanian pademelons (*Thylogale billardierii*), brush tailed rock wallabies (*Petrogale penicillata*), quokkas, woylies, Tasmanian devils (*Sarcophilus harisii*), common wombats (*Vombatus ursinus*), eastern quolls (*Dasyurus viverrinus*), spotted-tail quolls (*Dasyurus maculatus*), chuditch (or western quolls, *Dasyurus geoffroii*), brushtail possums, western ringtail possums (*Pseudocheirus occidentalis*), southern brown bandicoots, eastern barred bandicoots, long-nosed bandicoots and bilbies (*Macrotis lagotis*) (Table 2).

Seroprevalence surveys are prone to limitations regarding inferring infection prevalence, similar to those affecting surveys of *T. gondii*. In particular, the potential influence of misclassification is an important consideration. While the use of serological surveys avoids invasive tissue sampling (which usually necessitates euthanasia or opportunistic sampling of otherwise dead animals in wildlife populations), it is important that the serological test(s) have been validated at the cut off titre used to differentiate infected and non-infected animals, in the species being studied. Identifying whether or not the serological test accurately reflects the infection status of the tested host is essential in estimating the true prevalence of infection from apparent seroprevalence data. Very few serological tests have been adequately validated for use in Australian marsupial species (Supplementary Tables 1–3).

Table 2
Toxoplasma gondii seroprevalence surveys undertaken in free-ranging populations of Australian marsupial species. Except where noted, animals were sampled or taken from the wild via trapping or culls, and no prior clinical suspicion of toxoplasmosis existed in sampled animals.

Species	Study location (study)	Sampling timeframe	Serological test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive/ no. tested (%)	95% CI
Western grey kangaroo (*Macropus fuliginosus*)	Perth, Western Australia (Parameswaran et al., 2009a; Parameswaran, 2008)	May 2005–May 2007	ELISA (IgG only) *OD ≥0.636*[a]	34/219 (15.5%)	10.7–20.3%
Eastern grey kangaroo (*M. giganteus*)	Roma, Queensland (Parameswaran, 2008)	2004–2005	ELISA (IgG only) *OD ≥0.636*[a]	0/112 (0%)	0–3.2%[b]
Eastern grey kangaroo	Sydney, New South Wales (Parameswaran, 2008)	May 2006	ELISA (IgG only) *OD ≥0.636*[a]	2/65 (3.1%)	0–7.3%
Macropus spp[c]	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥1:8*[a]	0/31 (36.7%)[d]	0–10.9%[b]
Bennett's wallaby (*M. rufogriseus rufogriseus*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥1:16*[a]	0/1 (0%)	0–84.2%[b]
Bennett's wallaby	Tasmania[f] (Johnson et al., 1988)	Not specified	ELISA (IgG only) *OD ≥0.25*[a]	5/151 (3.3%)	1.5%–7.5%[b]
Bennett's wallaby[g]	Tasmania[c] (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	2/25 (8.0%)	2.4–25.1%[b]
Tasmania pademelon (*Thylogale billardierii*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥1:16*[a]	3/7 (42.9%)	15.7–75.5%[b]
Tasmanian pademelon	Tasmania[f] (Johnson et al., 1988)	Not specified	ELISA (IgG only) *OD ≥0.25*[a]	15/85 (17.7%)	11.0–27.1%[b]
Tasmanian pademelon[g]	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	28/228 (12.3%)	8.6–17.2%[b]
Bridled nailtail wallaby (*Onychogalea fraenata*)	Taunton National Park, Queensland (Turni and Smales, 2001)	1996	Latex agglutination test	6/39 (15.4%)	7.3–29.8%[b]
Black footed rock wallaby (*Petrogale lateralis*)	South Western Australia (Jakob-Hoff and Dunsmore, 1983)	1979	Indirect haemagglutination inhibition test	0/26 (0%)	0–12.8%[b]
Brush tailed rock wallaby (*P. penicillata*)	South east Queensland (Barnes et al., 2010)	July 2004–August 2005	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	3/64 (4.7%)	1.7–12.9%[b]
Banded hare wallabies (*Lagostrophus fasciatus*)	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/5 (0%)	0–45.9%[b]
Spectacled hare wallabies (*L. conspicillatus*)	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/3 (0%)	0–60.2%[b]
Quokka (*Setonix brachyurus*)	Rottnest, Western Australia (Gibb et al., 1966)	1964	Sabin-Feldman dye test *Titre >1:8*[e]	13/37 (35.1%)	21.8–51.4%[b]
Burrowing bettong/boodie (*Bettongia lesueur*)	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/28 (0%)	0–11.9%[b]
Burrowing bettong/boodie	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/14 (0%)	0–21.8%[b]
Brush-tailed bettong/woylie (*B. penicillata*)	Upper Warren region, Western Australia (Parameswaran, 2008)	March 2006	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	9/153 (5.8%)	3.2–10.8%[b]
Brush-tailed bettong/woylie	Dryandra Nature Reserve, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/12 (0%)	0–24.7%[b]
Brush-tailed bettong/woylie	Batalling Forest, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/17 (0%)	0–18.5%[b]
Brush-tailed bettong/woylie	Tutanning Nature Reserve, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/8 (0%)	0–33.6%[b]
Brush-tailed bettong/woylie	Venus Bay Island, South Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/14 (0%)	0–21.8%[b]
Brush-tailed bettong/woylie	St Peters Island, South Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	1/73 (1.4%)	0.3–7.3%[b]
Brushtail possum (*Trichosurus vulpecula*)	Sydney (Eymann et al., 2006)	Nov 2002–April 2005	Modified agglutination test (not IgM) *Titre ≥1:25*[a]	9/135 (6.3%)	3.6–12.2%[b]
Brushtail possum	Myall Lake National Park, New South Wales (Eymann et al., 2006)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:25*[a]	0/7 (0%)	0–36.9%[b]
Brushtail possum	Taronga Zoo grounds (non captive)-Sydney (Hill et al., 2008)	Feb 2005–May 2006	Modified agglutination test (not IgM) *Titre ≥1:25*[a]	6/126 (4.8%)	2.2–10.0%[b]
Brushtail possum	Blue Mountains, New South Wales (Hill et al., 2008)	Oct 2005 & May 2006	Modified agglutination test (not IgM) *Titre ≥1:25*[a]	0/17 (0%)	0–18.5%[b]
Brushtail possum	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥1:8*[e]	1/7 (14.3%)[d]	3.2–52.7%[b]
Brushtail possum	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	0/14 (0%)	0–21.8%[b]
Brushtail possum[g]	Kangaroo Island (O'Callaghan and Moore, 1986)	March–April 1985	Indirect haemagglutination test	0/30 (0%)	0–11.2%[b]
Brushtail possum[h]	Western Australia (Clarke, 2011)	2006–2008	Direct agglutination test Modified agglutination test (not IgM)	0/95 (0%) 0/95 (0%)	0–3.8%[b] 0–3.8%[b]
Brushtail possum	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/6 (0%)	0–41.0%[b]
Ringtail possum (*Pseudocheirus convolutor*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥1:16*[a]	0/3 (0%)	0–60.2%[b]
Western ringtail possum (*P. occidentalis*)[h]	Western Australia (Clarke, 2011)	2006–2008	Direct agglutination test *Titre ≥1:64*[e]	2/99 (2.0%)	0.6–7.0%[b]

(continued on next page)

Table 2 (*continued*)

Species	Study location (study)	Sampling timeframe	Serological test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive/ no. tested (%)	95% CI
			Modified agglutination test (not IgM)	0/99 (0%)	0–3.6%[b]
Common planigale (*Planigale maculata*)	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/5 (0%)	0–45.9%[b]
Common wombat (*Vombatus ursinus*)	Southern Tablelands, New South Wales (Hartley and English, 2005)	3/8/2001–25/2/2002	Latex agglutination test *Titre ≥1:32*[a]	1/23 (4.35%)	1.0–21.1%[b]
			Direct agglutination test *Titre >1:64*[a]	6/23 (26.1%)	12.6–46.7%[b]
			Modified agglutination test (not IgM) *Titre >1:64*[a]	6/23 (26.1%)	12.6–46.7%[b]
Southern brown bandicoot (*Isoodon obesulus*)	southern Western Australia (Jakob-Hoff and Dunsmore, 1983)	1979	Indirect haemagglutination inhibition test	0/3 (0%)	0.0–60.2%[b]
Southern brown bandicoot	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥1:8*[a]	35/89 (39.3%)[d]	29.8–49.7%[b]
Southern brown bandicoot	Brisbane (Pope et al., 1957a)	Feb 1951	Complement fixation test *Titre ≥1:8*[e]	1/1 (100%)	15.8–98.7%[b]
Southern brown bandicoot	Brisbane, North Qld, Mt Glorius-Queensland (Pope et al., 1957a)	August 1955–February 1956	Complement fixation test *Titre ≥1:8*[e]	15/35 (42.9%)[d]	27.9–59.2%[b]
Golden bandicoots (*I. auratus*)	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/11 (0%)	0–26.5%[b]
Eastern barred bandicoot (*Perameles gunnii*)	Huon Valley, Tasmania (Obendorf et al., 1996)	July 1992–March 1995	Direct agglutination test and modified agglutination test[i] *Both titres ≥1:64*[a]	10/150 (6.7%)[j,k]	3.7–11.8%[b]
Western barred bandicoot (*P. bougainville*)	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥1:40*[a]	0/2 (0%)	0–70.8%[b]
Long-nosed bandicoot (*P. nasuta*)	Queensland (Pope et al., 1957a)	August 1955–February 1956	Complement fixation test *Titre ≥1:8*[e]	1/1 (100%)	15.8–98.7%[b]
Long-nosed bandicoot	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥1:8*[a]	2/7 (28.6%)	8.5–65.1%[b]
Greater bilby (*Macrotis lagotis*)	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥1:8*[a]	0/1 (0%)	0–84.2%[b]
Northern quoll[g] (*Dasyurus hallucatus*)	Kakadu National Park, Northern Territory (Oakwood and Pritchard, 1999)	Feb 1993–May 1995	Latex agglutination test	0/22 (0%)[l]	0–14.8%[b]
Eastern quoll (*D. viverrinus*)	Cradoc & Judbury, Tasmania (Fancourt et al., 2014)	May 2011–July 2013	Modified Agglutination Test (not IgM) *Titre ≥1:64*[a]	Ranged from 77.3% to 100%[m]	
Eastern quoll	Bruny Island, Tasmania (Fancourt et al., 2014)	May 2011–September 2013	Modified Agglutination Test (not IgM) *Titre ≥1:64*[a]	Ranged from 9.4 to 29.4%[m]	
Eastern quoll	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	13[n]/24 (54.2%)	34.9–72.2%[b]
Spotted-tailed quolls (*D. maculatus*)	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	5[n]/7 (71.4%)	34.9–91.5%[b]
Western quoll/chuditch (*D. geoffroii*)	Batalling Forest, Western Australia (Haigh et al., 1994)	Not specified	Latex agglutination test	2/17 (11.8%)	3.6–34.7%[b]
Western quoll/chuditch	Julimar state forest, Western Australia (Parameswaran, 2008)	June 2007	Modified Agglutination test (not IgM) *Titre ≥1:40*[a]	3/23 (13.0%)	0–26.8%
Tasmanian devil (*Sarcophilus harisii*)	Tasmania[c] (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥1:64*[a]	6[n]/18 (33.3%)	16.3–56.6%[b]

[a] Cut off for seropositivity nominated by study authors.
[b] Calculated by review author using Jeffrey's 95% confidence interval.
[c] Seven red kangaroos (*Macropus rufus*) and 42 grey kangaroos (*M. giganteus*) sampled. Data as to which species the excluded anticomplementary sera were from was not reported.
[d] Anti-complementary sera excluded from results-18 macropod sera (Cook and Pope, 1959); one common brushtail possum serum (Cook and Pope, 1959); 35 southern brown bandicoot sera (Cook and Pope, 1959); two southern brown bandicoot sera (Pope et al., 1957a,b).
[e] Cut off applied to presented data *post hoc* by review authors.
[f] Pooled data from various locations in Tasmania.
[g] Some or all samples obtained via roadkill.
[h] Many possums were sampled on multiple occasions-if any individual tested positive at any time, it was included as 'positive'; all possums that tested negative on all occasions were included as 'negative'.
[i] DAT titre <64 and no reaction on MAT classed as negative serological result; DAT ≥64 and no reaction on MAT classed as suspicious serological result; DAT and MAT titres both ≥64 classed as positive serological result.
[j] A further 7/150 (4.7%) had 'suspicious' reactions.
[k] One seropositive bandicoot appeared to have neurological symptoms when subsequently retrapped (unclear whether this was a blinded observation); one seropositive bandicoot found dead in trap on retrapping, and had histopathological evidence of *T. gondii* infection.
[l] Two quolls had very low serological reactions (1:2 and 1:3) that the study authors thought were probably insignificant. Neither of these quolls had histological evidence of *Toxoplasma gondii* infection.
[m] Cohort study-seroprevalence measured approximately every second month.
[n] Estimated from graph in reference.

Factors that may impact the validity of using serological survey data to estimate infection prevalence include: 1) an antibody response may reflect exposure to an infection, but not necessarily establishment of infection in the host; 2) if the sampled animal is acutely infected, it may not yet have a detectable antibody titre; 3) if an infected animal has developed an IgM titre, but not yet IgG, serological tests that do not detect *T. gondii* IgM antibodies (eg the modified agglutination test, or some ELISAs) will not classify these

animals as infected; 4) in animals with long term chronic *T. gondii* infections, serological antibody titres may drop to levels that are below the cut off titre for differentiating infected vs uninfected animals, compromising sensitivity – waning serological titres have been demonstrated in chuditch (Haigh et al., 1994), brushtail possums (Eymann et al., 2006), woylies (A. Worth, Murdoch University-unpublished results) and in eastern grey kangaroos clinically suspected of *T. gondii* infection (Miller et al., 2003); 5) non-specific agglutination, which is known to occur with the direct agglutination test in other species (Dubey, 2010), may compromise test specificity; 6) anti-complementary sera require exclusion from serosurveys that use the complement fixation test, which may bias findings (e.g., Pope et al., 1957a; Cook and Pope, 1959); and 7) the complement fixation test, the Sabin Feldman dye test and ELISAs require species-specific reagents. ELISAs used in marsupial surveys were developed for use in the species involved. However, in studies where the Sabin-Feldman dye test or complement fixation test were used, there was no description of the test being adapted for the marsupial species surveyed (Pope et al., 1957a; Cook and Pope, 1959; Gibb et al., 1966; Munday, 1972).

2.3. Prevalence data as a measure of infection frequency

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

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

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

3. Frequency of disease (toxoplasmosis) following infection with *T. gondii* in free-ranging populations of Australian marsupial species

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

It has also been suggested that various stressors may be relevant in the clinical manifestation of *T. gondii* infections-both acute and recrudescent-in marsupials (Obendorf and Munday, 1983; Johnson et al., 1988; Miller et al., 2000; Parameswaran et al., 2010; Fancourt et al., 2014), although there are no published explorations of this hypothesis. Some reports of toxoplasmosis amongst captive marsupial populations have noted exposure to potential stressors prior to the occurrence of disease. For example, immediately prior to an outbreak associated with high mortality, a population of captive sugar gliders (*Petaurus breviceps*) was exposed to repeated episodes of social disruption, suboptimal nutrition and suboptimal temperature (Barrows, 2006). Other cases or outbreaks of toxoplasmosis in captive wallabies have occurred following relocations (Wilhelmsen and Montali, 1980; Dubey and Crutchley, 2008; Bermundez et al., 2009) or social isolation (Adkesson et al., 2007). A study of *T. gondii* infection in dasyurids (65 wild-caught, 103 laboratory reared), using histopathology without confirmatory testing, found the widest dissemination of organisms consistent with *T. gondii* in two fat-tailed false antechinus (*Pseudantechinus macdonnellensis*) with leukaemia (Attwood and Woolley, 1973).

3.1. Longitudinal studies of the population effects of *T. gondii* in free-ranging populations of Australian marsupials

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

These challenges likely explain why only two such studies have been undertaken thus far. The first was on eastern barred bandicoots at two sites in southern Tasmania's Huon Valley. Sites were trapped every three months between July 1992 and March 1995. One hundred and fifty bandicoots were trapped over the period. Both the direct agglutination test (without 2-mercaptoethanol) (DAT) and modified agglutination test (that includes 2-mercaptoethanol) (MAT) were used. One hundred and thirty three bandicoots were negative on both serological tests, and 68% of these were recaptured at least once. Ten bandicoots were positive on both the MAT and DAT (both titres $\geq$64); five were not retrapped, while five had antibodies on two consecutive occasions (three months apart) and were not recaptured subsequently (Obendorf et al., 1996). These findings may reflect reduced survival times in eastern barred bandicoots infected with *T. gondii*, but there are substantial limitations in drawing such a conclusion. The generalities listed above apply, and lack of validation of the diagnostic test used is a potential influence on findings. Additionally, the small seropositive cohort size means that chance effects on the results cannot be confidently excluded, and confounding (by age, in

particular) cannot be ruled out, as the cumulative risk of *T. gondii* infection has been demonstrated to increase with age in other species (Dubey, 2010). A further seven bandicoots tested positive on the direct agglutination test (titre ≥64) in this study, but were negative on the modified agglutination test (titre <64); none of these seven bandicoots were recaptured (Obendorf et al., 1996). Again, while this may reflect a lower survival time in bandicoots acutely infected with *T. gondii*, this is not sufficient evidence to draw such a conclusion. The direct agglutination test has not been validated in eastern barred bandicoots, and it is known to have relatively poor specificity in other species (Dubey, 2010). Therefore, misclassification cannot be excluded, along with chance effects due to the small sample size.

The second study was undertaken in eastern quolls in Tasmania. Survival times of eastern quolls that were seropositive for *T. gondii* were compared to those that were seronegative, in a location where the quoll population was classified as 'stable' (Bruny Island) across a period of 2 years and 4 months. There was no evidence of a difference in survival time between seropositive and seronegative quolls (Fancourt et al., 2014). However, a lack of power cannot be excluded as an influence on study findings, due to the relatively small sample size involved. Regarding the use of these data to compare the survival time of *T. gondii* infected vs uninfected marsupials, the generalities described above apply, as does a lack of validation in the diagnostic methodology used to infer host *T. gondii* infection status from host serological data.

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

3.2. Using morbidity and mortality linked to toxoplasmosis in captive populations of Australian marsupials as an indicator of outcomes of T. gondii infection in free-ranging populations

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

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

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

Experimental studies under laboratory conditions are very useful for investigating the pathological processes of *T. gondii* infection and clinical toxoplasmosis in Australian marsupial species, and evaluating diagnostic methods. However, caution is required in extrapolating morbidity and mortality rates observed in such studies to free-ranging populations. For example, experimental infections undertaken in eastern barred bandicoots demonstrated the ability of *T. gondii* to cause fatal disease in this species (2/2 infected bandicoots died) (Bettiol et al., 2000). These findings may reflect eastern barred bandicoots being particularly susceptible to fatal toxoplasmosis following *T. gondii* infection. However, the widespread citation of this study as providing conclusive evidence of the high susceptibility of Australian marsupial species to toxoplasmosis is premature, for several reasons.

Firstly, the study was restricted to eastern barred bandicoots, and thus assumptions that the results are applicable to all Australian marsupial species are not appropriate. Secondly, the small number of bandicoots infected is marked limitation in using the findings of this study to estimate the morbidity and mortality rates of toxoplasmosis post infection in this species. Thirdly, the inoculation dose and strain of *T. gondii* used in the experiment may not reflect the typical dose of infection, and virulence and pathogenicity, of the strains of *T. gondii* to which free-ranging populations of Australian marsupial species are commonly exposed. The bandicoots were orally infected with 100 cysts of the P89 strain of *T. gondii*, which is highly virulent in mice (Dubey et al., 1995). The authors noted that a previous attempt to induce infection in bandicoots with a lower dose of 10 oocysts of the same strain was unsuccessful (Bettiol et al., 2000). Finally, the morbidity and mortality rates observed in this study may have been biased by circumstances related to the experiment. In particular, the bandicoots studied may have been under stress. They were captured from the wild for the purposes of the study, then housed in captivity, fed a novel diet, and subjected to handling and procedures which included an oral inoculation procedure, repeated rectal

temperature monitoring and repeated blood tests. If stress-induced immunosuppression does indeed act as a causal complement in toxoplasmosis, stress may have facilitated the development of clinical disease in these bandicoots.

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

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

4. Other possible effects of *T. gondii* infection that may impact population viability of free-ranging Australian marsupials

4.1. Possible influences of T. gondii on behaviour

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

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

4.2. Possible effects of T. gondii infection on marsupial reproductive success

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

Available evidence strongly suggests that vertical transmission of *T. gondii* infection can occur in black-faced kangaroos (*Macropus fuliginosus melanops*), western grey kangaroos and woylies (Dubey et al., 1988; Parameswaran et al., 2009b). Vertical transmission of *T. gondii* from chronically infected western grey kangaroo dams to their young may occur commonly: two out of nine (22.2%; Jeffrey's 95% CI 6.7–55.6%) pouch young of chronically infected dams were positive for *T. gondii* via PCR (Parameswaran et al., 2009b). No study has investigated the impacts of infection on the survival of vertically infected young. If vertical transmission does occur relatively frequently, and commonly results in adverse impacts on the health of infected young, reproductive success in an infected population may be compromised.

Reproductive success of mature marsupials associated with *T. gondii* infection has been investigated in one study, of eastern quolls (Fancourt et al., 2014). The mean number of pouch young in July–September was higher in females that were seropositive for *T. gondii*, than those that were seronegative. Similarly, testicular volume during the mating season was higher in seropositive males, although the implications of this finding is unclear as a relationship between testicular volume and reproductive capacity in quolls has not been demonstrated. In addition, there were difficulties in accurately measuring and controlling for putative confounding variables, which complicates interpretation of the data (Fancourt et al., 2014). Anecdotally, high prevalence of *T. gondii* was observed in populations of western grey kangaroos, red kangaroos

and common wallaroos culled due to overpopulation (Parameswaran et al., 2010; Pan et al., 2012). While this suggests that *T. gondii* infection does not substantially impact reproductive capacity in these species, more specific investigations are required.

5. Conclusions

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

1) Validation of diagnostic test(s) used to detect the presence or absence of *T. gondii* infection in the species under study, to enable consideration of potential impacts of misclassification on analytical study findings (Boadella et al., 2011). Validation of tests in various marsupial species would also help to retrospectively clarify previous research findings.
2) The impact of sample sizes on results-ideally planning and undertaking studies where an adequate sample size for statistical extrapolations to a population level is realistically achievable, or where the novelty of descriptive findings would justify a study of only descriptive value. Measures of sampling error/precision should be included in results and considered in the interpretation of study findings if making analytical inferences (O'Brien et al., 2009; Boadella et al., 2011).
3) Sampling strategies, in an attempt to minimise the potential influence of selection bias (Nusser et al., 2008; Anderson, 2001; Boadella et al., 2011). Proportionate sampling is ideal, though often not realistically achievable in observational studies of wildlife.
4) Performing studies that involve temporal sequence on the same individuals (cohort studies), to compare morbidity and mortality rates of *T. gondii* infected vs non infected animals in free-ranging populations. Studies comparing findings in both stable populations and populations subject to stressors (such as habitat fragmentation) would be valuable in investigating the possibility of interaction between stressors and the impact of *T. gondii* infection on population health.
5) Controlling for confounding variables when making statistical extrapolations (for example, controlling for sex and age if using a Poisson regression to compare survivability of *T. gondii* infected and uninfected hosts). In most marsupial species, further research aimed at identifying and validating measures of potential confounders-particularly age-is required.
6) Potential influences of data clustering, which is potentially common in wildlife studies but rarely considered when making analytical inferences.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2015.12.002.

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