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INFECTION PRESSURE IS NECESSARY, BUT NOT SUFFICIENT BY ITSELF, TO EXPLAIN *TOXOPLASMA GONDII* SEROPREVALENCE IN INTERMEDIATE HOST SPECIES

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ABSTRACT

Parasite infection pressure is suggested to be a strong driver of transmission within ecosystems. We tested if infection pressure drives seroprevalence in intermediate host species for *Toxoplasma gondii*. We defined *Toxoplasma* infection pressure to intermediate host species as the combined influence of cat abundance, environmental conditions, and its prevalence in the cat population. We sampled and tested 2 species of rodent and collated information on *Toxoplasma* seroprevalence in koalas, wallabies, kangaroos, and sheep. All species were sampled using equivalent methods, within a 2-yr period, and from adjacent regions of low and high *Toxoplasma* infection pressure. The seroprevalence of *Toxoplasma* in kangaroos scaled with infection pressure, but we observed no statistical difference in seroprevalence for any other species between these 2 regions. Within the region of low infection pressure, *Toxoplasma* seroprevalence did not differ between species. However, within the region of high *Toxoplasma* infection pressure, we observed large variation in seroprevalence between species. Our results demonstrate that infection pressure is not sufficient by itself, but merely necessary, to drive *Toxoplasma* seroprevalence in intermediate host species. Where *Toxoplasma* seroprevalence in an intermediate host species is already low, further reducing infection pressure will not necessarily further decrease seroprevalence in those species. This has important ramifications for the mitigation of parasite infections and suggests that reductions in *Toxoplasma* infection pressure, intended to reduce infections, may be most effective and applicable to species that are known to experience high rates of infection.

Toxoplasmosis is a disease of significant health concern for humans, livestock, and wildlife (Canfield et al., 1990; Epiphanio et al., 2001; Dubey, 2009a, 2016). The disease is caused by the cat-borne protozoal parasite *Toxoplasma gondii*. Felids are the definitive host of the parasite and shed the oocyst stage in their feces, predominately following their first infection (Zulpo et al., 2018). Oocysts are particularly resistant to environmental conditions and can remain infective for at least 18 mo under favorable conditions, but they can be killed rapidly via desiccation (Yilmaz and Hopkins, 1972; Frenkel et al., 1975; Dubey, 1998). Intermediate hosts of *T. gondii* can be any endotherm, which become infected via two major routes of transmission, oocyst or bradyzoite consumption (Aramini et al., 1999; Hill and Dubey, 2002; Dubey, 2016; Zulpo et al., 2018). Accordingly, based on the major routes of *T. gondii* transmission to intermediate hosts, we would expect the parasite's infection pressure to be largely dependent on oocyst contamination in the environment. Similarly, oocyst contamination in the environment should be dependent on cat density, environmental conditions, and the prevalence of *T. gondii* infection in the cat population (Frenkel and Dubey, 1973; Dubey, 1998, 2009b; Zulpo et al., 2018).

Many studies have shown that ecological or physiological characteristics of an intermediate host species, such as age or body mass, can influence *T. gondii* seroprevalence (Afonso et al., 2007; Tagel et al., 2019). However, the relative contribution of *T. gondii* infection pressure to explaining seroprevalence in intermediate host species is not well known. This has important ramifications for the mitigation of *T. gondii* infection via reductions in infection pressure.

We investigated if *T. gondii* infection pressure is sufficient, by itself, to explain the observed seroprevalence in intermediate host species. We defined *T. gondii* infection pressure to intermediate hosts as the combined influence of cat density, environmental conditions, and the seroprevalence of the parasite in the cat population. If *T. gondii* infection pressure is sufficient to drive seroprevalence in intermediate host species, then we would expect

that seroprevalence will scale with infection pressure for all species tested. If the relationship between *T. gondii* seroprevalence and infection pressure is linear, whereby an increase in infection pressure results in a proportional increase in seroprevalence, then the proportional differences in seroprevalence among species will remain constant across regions of differing infection pressure.

To address this question, we sampled and tested house mice (*Mus musculus*) and bush rats (*Rattus fuscipes*), and we collated recently published information on *T. gondii* seroprevalence in koalas (*Phascolarctos cinereus*), tammar wallabies (*Macropus eugenii*), western grey kangaroos (*Macropus fuliginosus*), and sheep (*Ovis aries*) from a region of low infection pressure and an adjacent region of high infection pressure (Taggart et al., 2019b, 2019c, 2020b). We obtained *T. gondii* seroprevalence estimates for all species and tested for effects of infection pressure, species, and their interaction on *T. gondii* seroprevalence. Here, we discuss the role of *T. gondii* infection pressure in driving seroprevalence in intermediate host species and the implication of this result for mitigating *T. gondii* infection via reductions in infection pressure, and we hypothesize other factors that may contribute to the observed results.

MATERIALS AND METHODS

Study sites

Study regions included the Fleurieu Peninsula (35°35′18.96″S, 138°11′54.96″E) on mainland South Australia and the adjacent Dudley Peninsula on Kangaroo Island (35°48′5.4″S, 137°58′30.72″E). Both regions experience similar Mediterranean climates (Jenkins, 1985; Schwerdtfeger, 2002) and comprise similar land uses and vegetation types (cropping and pasture land interspersed with patches of native vegetation, largely low *Eucalyptus* spp. woodlands). These 2 regions are located only 13.5 km apart. This assisted in controlling for any potentially confounding climatic conditions that may contribute to the differential survival of *T. gondii* oocysts in the environment, and consequently differences in *T. gondii* seroprevalence in our study species. Cat (*Felis catus*) abundance on the Fleurieu Peninsula, South Australia, is reported to be approximately 10 times lower than that on the adjacent Kangaroo Island (Bengsen et al., 2011; Taggart et al., 2019a; Hohnen et al., 2020). Similarly, the seroprevalence of *T. gondii* in cats on the Fleurieu Peninsula would be expected to be lower than that in insular cats, based on its lower seroprevalence in cats from the wider Australian mainland and the known high seroprevalence in insular cats (89.4% seropositive) (O'Callaghan et al., 2005; Fancourt and Jackson, 2014). We, therefore, assumed the Fleurieu Peninsula to be a region of low *T. gondii* infection pressure and Kangaroo Island to be a region of high *T. gondii* infection pressure. This assumption is supported by previous work, which has shown a lower seroprevalence of *T. gondii* in sheep (10%) on the South Australian mainland relative to that on Kangaroo Island (sheep 23%) and a low seroprevalence of *T. gondii* in rabbits from this region as well (O'Donoghue et al., 1987; McKenny et al., 2020; Taggart et al., 2020b). Hereafter, we refer to the Fleurieu Peninsula on the South Australian mainland as a region of low *T. gondii* infection pressure (low cat abundance, expected low seroprevalence in cats) and Kangaroo Island as a region of high *T. gondii* infection pressure (high cat abundance, high seroprevalence in cats).

We sampled house mice and bush rats at 4 sites in the region of low *T. gondii* infection pressure and 4 sites in the region of high *T. gondii* infection pressure. Study sites within each region were a subset of the same study sites used to assess cat abundance in each region in Taggart et al. (2019a). These sites were chosen to be representative of the remnant vegetation types in these regions and were all surrounded by agricultural grazing land.

Animal capture

House mice and bush rats were trapped in cages or Elliot traps baited with peanut butter and rolled oats, or in unbaited pitfall traps. Traps were deployed along linear transects within native vegetation patches at intervals of 20 m. Trapping occurred between January 2017 and April 2018.

Blood sampling and processing

We collected blood from conscious house mice via venipuncture of the facial vein (Francisco et al., 2015) and from anesthetized bush rats via jugular venipuncture (Parasuraman et al., 2010). All captured rodents were marked to facilitate the identification of recaptures and to avoid individuals being sampled multiple times.

Blood samples were centrifuged, and sera samples were collected and stored at –20 C as described by Taggart et al. (2019c). Sera samples experienced 2 freeze/thaw cycles before testing. Long-term sera storage does not significantly modify the interpretation of *T. gondii* serology (Dard et al., 2017).

Toxoplasma gondii antibody test

Sera samples were tested for *T. gondii* immunoglobulin G (IgG) antibodies using equivalent methods for all individuals and species (Taggart et al., 2019b, 2019c, 2020b). We used a commercially available modified agglutination test (MAT) (Toxo-Screen DA, bioMérieux, Marcy-l'Étoile, France) as described in detail by Taggart et al. (2019c). The MAT is based on the direct agglutination of fixed *T. gondii* tachyzoites of the RH strain, with sera pre-treated with 2-mercaptoethanol to neutralize IgM antibodies (Desmonts and Remington, 1980; Dubey and Desmonts, 1987). IgG antibodies typically take greater than 1 wk to develop, but once developed, they persist for the remainder of the animal's life. Consequently, this test may give false-negative results within the first week of infection (Dubey and Crutchley, 2008). Sera were screened at 1:40 and 1:4,000 dilutions and classified as positive if agglutination occurred at either dilution. This minimized possible type 2 error due to the prozone effect. With each assay, we included positive and negative control sera from known infected (intra-peritoneal inoculation with *T. gondii* tachyzoites) and uninfected mice and a negative phosphate-buffered saline (PBS, pH 7.2) control. A serum sample was classified as positive when agglutination of *T. gondii* formed a mat covering about half or more of the well base.

The MAT has not been evaluated in the study species, but we assumed its diagnostic sensitivity and specificity to be satisfactory based on appropriate quality controls and the high sensitivity and specificity of the assay reported in other species (Mainar-Jaime

Table I. *Toxoplasma gondii* seroprevalence (with 95% confidence intervals [CIs]) by species from a region of low infection pressure and a region of high infection pressure.

	Low infection pressure		High infection pressure		
Species	n	Seroprevalence % (95% CI)	n	Seroprevalence % (95% CI)	Source
House mouse	19	0.0 (0.0, 16.8)	36	0.0 (0.0, 9.6)	This study
Bush rat	169	0.0 (0.0, 2.2)	46	2.2 (0.1, 11.3)	This study
Koala*	63	0.0 (0.0, 5.7)	94	0.0 (0.0, 3.9)	Taggart et al. (2019b)
Tammar wallaby	—	—	76	14.5 (8.3, 24.1)	Taggart et al. (2019c)
Western grey kangaroo	61	0.0 (0.0, 5.9)	156	20.5 (14.9, 27.5)	Taggart et al. (2019c)
Sheep	—	—	560	56.8 (52.7, 60.8)	Taggart et al. (2020b)

* Koalas were sampled from the Mt. Lofty Rangers on mainland South Australia, just northeast of the Fleurieu Peninsula.

and Barberan, 2007). To our knowledge cross-reactivity with the MAT is low (Dubey, 2016; Gondim et al., 2017).

Data analysis

Collated information: We combined our data on *T. gondii* seroprevalence in house mice and bush rats with previously published data on *T. gondii* seroprevalence in koalas, tammar wallabies, western grey kangaroos, and sheep (Taggart et al., 2019b, 2019c, 2020b). All of these species were sampled within a 2-yr period and tested using equivalent methods from the described regions of low and high infection pressure (Taggart et al., 2019b, 2019c, 2020b). House mice, bush rats, koalas, and western grey kangaroos were sampled in the same regions of low and high *T. gondii* infection pressure. Tammar wallabies and sheep were only sampled from the region of high *T. gondii* infection pressure.

Statistics: We used generalized linear models (GLMs) to investigate the association between *T. gondii* seroprevalence and the predictor variables infection pressure (low vs. high) and species, and their interaction. In each GLM, *T. gondii* seroprevalence in an animal was treated as a binary response, and the default logit link was used. Statistical significance was determined using a score test (Lagrange multiplier) and model parsimony assessed using the Akaike information criterion (AICc). We considered a model to have substantial support if P values for predictor variables were <0.05 and ΔAICc was <2 (Burnham and Anderson, 2002). Due to zero infections within the region of low infection pressure, the GLM exhibited quasi-complete separation, and so the conventional Wald's test was not useful for posthoc comparisons between species within locations (Mansournia et al., 2018).

Given this separation issue and the finding that there were highly significant species effects in our GLM (see Results), we compared *T. gondii* seroprevalence between species within the region of high infection pressure only. For each 2×2 cross-tabulation of counts of 2 species by *T. gondii* seropositivity, we used either a Fisher's exact test or chi-squared test with the $(n-1)$ adjustment (Campbell, 2007), depending on the count of positive animals in the groups being tested. To correct for multiple comparisons, we determined statistical significance according to a Bonferroni adjusted alpha value of 0.003. However, we acknowledge that this correction will be conservative due to known dependence between tests (Abdi, 2007). We present *T. gondii* seroprevalence and binomial exact 95% confidence intervals. All statistical analyses were performed in base R version R 3.5.1 (R Core Team, 2020).

RESULTS

We sampled 19 mice and 169 rats in the region of low infection pressure and 36 mice and 46 rats in the region of high infection pressure, totaling 270 individuals (Table I). We additionally compiled *T. gondii* seroprevalence information on another 124 animals across another 2 species from the region of low infection pressure, and another 886 animals across another 4 species from the region of high infection pressure (Table I). The apparent seroprevalence of *T. gondii* for both mice and rats from the region of low infection pressure, and for mice from the region of high infection pressure was 0% (Fig. 1; Table I). From the region of high *T. gondii* infection pressure, a single rat was positive, giving an apparent seroprevalence of 2.2% (Fig. 1; Table I).

Our most parsimonious GLM supported the inclusion of infection pressure and species as fixed effects (infection pressure: Rao score $= 16.84$, df $= 1$, $P \leq 0.001$; species: Rao score $= 228.01$, df $= 5$, $P \leq 0.001$; Suppl. Data, Table S1). The seroprevalence of *T. gondii* was substantially higher within the region of high infection pressure relative to that within the region of low infection pressure, and large variation in seroprevalence existed between species generally. The interaction infection pressure*species was not supported in GLMs due to the absence of *T. gondii* infection in all species within the region of low infection pressure. However, we found large variation in *T. gondii* seroprevalence among species within the region of high infection pressure using chi-squared and Fisher's exact tests (Table II). Within the region of high infection pressure, the seroprevalence of *T. gondii* in sheep was greater than the seroprevalence of *T. gondii* in all other species; similarly, the seroprevalence in kangaroos was greater than that in mice, rats, and koalas, and the seroprevalence in wallabies was greater than that in koalas. For all other pair-wise comparisons we found no difference in *T. gondii* seroprevalence according to our Bonferroni adjusted alpha value of 0.003. We did not test for differences in *T. gondii* seroprevalence between species within the region of low infection pressure because we observed no infection in any species and hence no variation in seroprevalence between species.

DISCUSSION

If *T. gondii* infection pressure is sufficient, by itself, to drive seroprevalence in intermediate host species, then seroprevalence

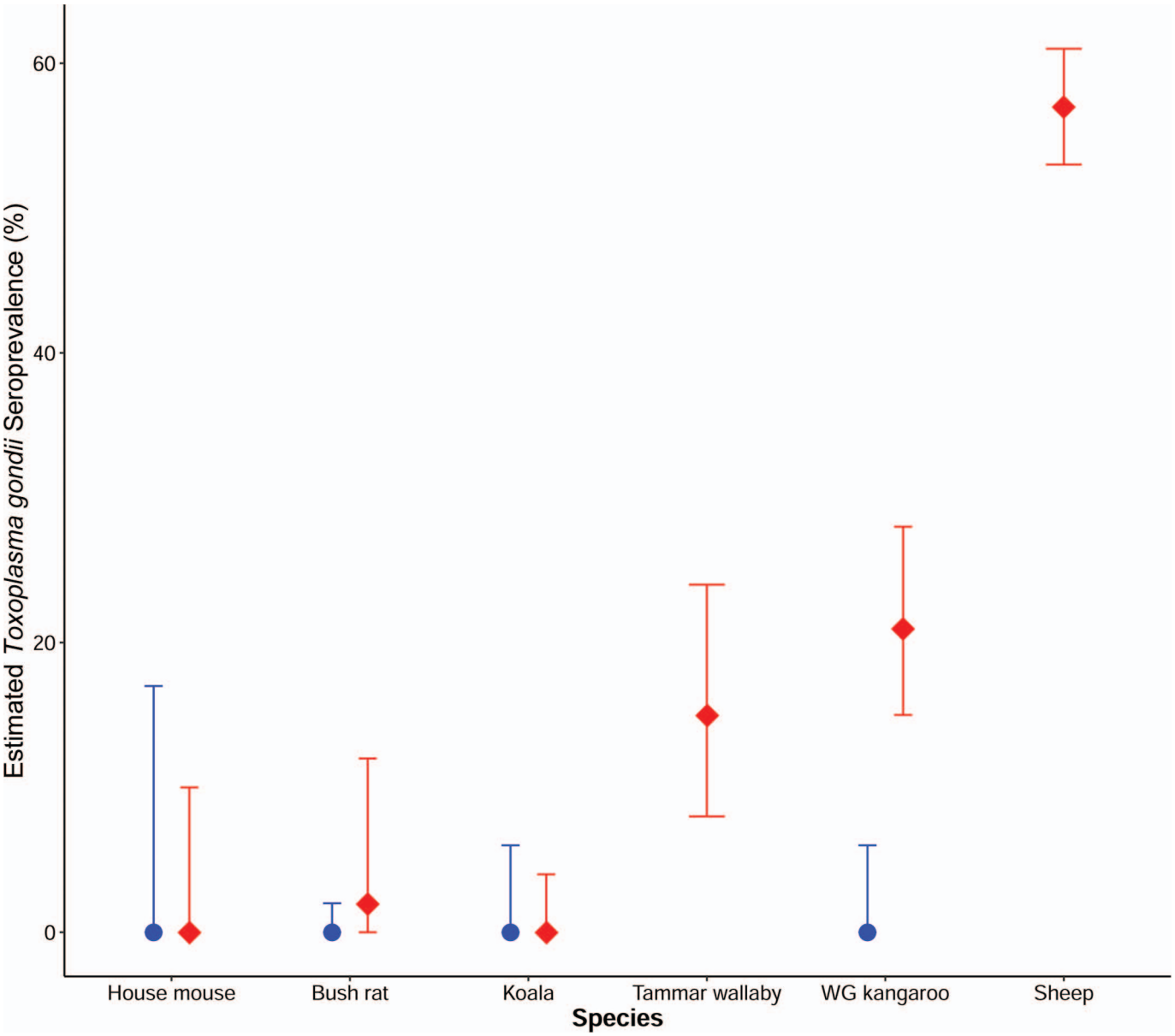

Figure 1. *Toxoplasma gondii* seroprevalence estimates (with binomial 95% confidence intervals) by species from a region of low infection pressure (blue circles) and a region of high infection pressure (red diamonds). Color version is available online.

Table II. *P* values for the comparison of *Toxoplasma gondii* seroprevalence across species within the region of high infection pressure. Statistically significant comparisons are listed in bold format and were determined based on a Bonferroni adjusted alpha level of 0.003. Numbers in parentheses indicate χ^2 values where a chi-squared test was used for comparison instead of a Fisher's exact test.

	House mouse	Bush rat	Koala	Tammar wallaby	Western grey kangaroo
House mouse					
Bush rat	1.00				
Koala	1.00	0.33			
Tammar wallaby	0.02	0.03	**<0.001**		
Western grey kangaroo	**<0.001**	**0.002**	**<0.001**	0.27 (1.2)	
Sheep	**<0.001**	**<0.001**	**<0.001**	**<0.001 (47.9)**	**<0.001 (64.2)**

must scale with infection pressure for all species. If this relationship is also linear, then the proportional differences in seroprevalence among species will remain constant across regions of differing infection pressure. If intermediate host species seroprevalence scales with infection pressure, but proportional differences in seroprevalence among species are not constant across regions of differing infection pressure, then a sufficient, but non-linear, relationship between *T. gondii* seroprevalence and infection pressure will exist. However, if intermediate host species seroprevalence does not scale with infection pressure, then *T. gondii* infection pressure alone cannot be sufficient to drive seroprevalence, and seroprevalence must be strongly driven by other factors in addition to infection pressure. It is not possible for proportional differences in *T. gondii* seroprevalence among species to remain constant across regions of differing infection pressure if seroprevalence does not scale with infection pressure.

We found no evidence of *T. gondii* presence within our region of low infection pressure. Consequently, we cannot be certain that our specific study sites on the Fleurieu Peninsula represented a region of low infection pressure and did not represent a region of no/zero infection pressure. This has implications for the testing and interpretation of the conditions necessary to demonstrate a sufficient, or sufficient and linear, relationship between *T. gondii* infection pressure and intermediate host seroprevalence.

When testing if infection pressure scales with seroprevalence, it is irrelevant if *T. gondii* actively circulates in our region of low infection pressure or not. For mice, rats, and koalas, we found no evidence of a difference in *T. gondii* seroprevalence between our regions of low and high infection pressure. Thus, for these 3 species, our data provide evidence that *T. gondii* seroprevalence does not scale with infection pressure and is not sufficient, by itself, to drive seroprevalence.

When testing if proportional differences in seroprevalence among species remain constant across regions of differing infection pressure, it is important that *T. gondii* actively circulates to some extent within our region of low infection pressure. If it does not, then our test of the presence of a linear relationship between *T. gondii* infection pressure and intermediate host seroprevalence is invalid. Although we cannot conclusively demonstrate that *T. gondii* does circulate at our specific study sites within our region of low infection pressure, we suggest it is unlikely that *T. gondii* is completely absent from these study sites for the following reasons: (1) *T. gondii* antibodies have previously been detected in sheep, rabbits, and people on the broader Fleurieu Peninsula region, which encompasses our region of low infection pressure (Johnson et al., 1980; Berger and Gideon Informatics, 2010; Lanyon and O'Handley, 2020; McKenny et al., 2020); (2) our specific study sites within the region of low infection pressure are known to support a wild/feral cat population (Taggart et al., 2019a); (3) a recent systematic review and meta-analysis of *T. gondii* seroprevalence in felids worldwide and a regional review of *T. gondii* seroprevalence in cats in Australia both suggest that its seroprevalence in cats in Australia is among the highest worldwide (Fancourt and Jackson, 2014; Montazeri et al., 2020); (4) it is not uncommon for *T. gondii* seroprevalence values to be low, $<1\%$, but nonetheless *T. gondii* is still present and actively circulating (Dubey et al., 2006; Murata et al., 2018); (5) *T. gondii* is considered to be a ubiquitous parasite worldwide (Shwab et al., 2018); and (6) we are unaware of any studies that have demonstrated that *T. gondii* was absent from locations with a known cat population, climatic conditions conducive to the survival of oocysts in the environment, and known infections in multiple species within close proximity. If *T. gondii* does truly circulate at our specific study sites on the Fleurieu Peninsula, and this region can truly be considered to be a region of low infection pressure, then our data would also suggest that proportional differences in seroprevalence among species are not constant across regions of differing infection pressure, and infection pressure is not linearly related to seroprevalence in intermediate host species. This would be supported by the large difference in *T. gondii* seroprevalence between kangaroos and that in mice, rats, and koalas within the region of high infection pressure but no difference in the seroprevalence experienced by these species within the region of low infection pressure.

As we did not observe a consistently lower seroprevalence of *T. gondii* in intermediate host species within the region of low infection pressure relative to the same species within the region of high infection pressure, our data suggest that *T. gondii* infection pressure, while necessary, is just one of many factors that influences seroprevalence in an intermediate host species. We cannot be 100% confident if this relationship between *T. gondii* infection pressure and intermediate host species seroprevalence is linear or not.

Our results have direct relevance to the mitigation of *T. gondii* infection via reductions in infection pressure, for example, via the removal of cats from an environment. Where *T. gondii* seroprevalence in an intermediate host species is already low, our results suggest that further reduction in the infection pressure will not necessarily decrease seroprevalence in those species. Instead, our results suggest that reductions in *T. gondii* infection pressure, intended to reduce infections, may be most effective and applicable to species that are known to experience high rates of infection.

Our data raise 2 additional questions. (1) In addition to *T. gondii* infection pressure, what drives seroprevalence in intermediate host species within our sampled regions? (2) Are the sampled species important to the epidemiology of *T. gondii* within the sampled regions? For kangaroos, wallabies, and koalas, *T. gondii* seroprevalence values and the importance of these species to the epidemiology of *T. gondii* within our sampled regions were discussed in detail by Taggart et al. (2019b) and Taggart et al. (2019c). However, rodent seroprevalence values are yet to be discussed.

Both mice and rats in our study experienced a low seroprevalence of *T. gondii*, even within our region of known high infection pressure. As seroprevalence, in general, is known to be driven by both the incidence of infection and duration of seroconversion in a species (Dohoo et al., 2009), it is likely that both ecological and physical traits of the mice and rats sampled contributed to the low *T. gondii* seroprevalence observed. Both of these species would be expected to spend a large proportion of their time foraging at or below ground level and could therefore ingest *T. gondii* oocysts in contaminated soil (Gleeson and Van Rensburg, 1982). However, both species are small, have small total energy requirements, and consume small total volumes of food. This would be expected to reduce the incidence of infection that these species would experience (Afonso et al., 2007). Both species are also short-lived ($\leq$1 yr), may suffer mortality or morbidity from toxoplasmosis, or may have an increased risk of

predation due to a reduction in their fear response from latent toxoplasmosis, all of which would be expected to reduce the duration of seroconversion that they experience (Matthewson et al., 1994; Dubey, 1996, 2006; White et al., 1996; Berdoy et al., 2000; Vyas et al., 2007; Boillat et al., 2020).

While there are several factors that make it logical for rodents to experience low *T. gondii* seroprevalence, large variability in seroprevalence in rodents is known to occur (Galeh et al., 2020). Our results are additionally consistent with others on islands where *T. gondii* infection pressure is known to be high but low seroprevalence values have been recorded in local rodent populations (Dubey et al., 2006; Murata et al., 2018). This may suggest that high infection pressure combined with short species generation times may facilitate rapid evolution and drive such species towards increased resistance to *T. gondii* infection and consequently a lower observed seroprevalence. While we are not aware of evidence to this effect for *T. gondii*, we suggest it would be worthy of further investigation in light of rapid evolutionary changes suggested in other species following intense selection pressures (Jolly et al., 2018; Jolly and Phillips, 2020).

Even in the instance that rodents experience a low *T. gondii* seroprevalence, recent simulations suggest that rodents are still highly important to the epidemiology of *T. gondii* due largely to the frequency with which they are consumed by felids (Taggart et al., 2020a). For example, the authors suggest that if felids consume an average of 1 rodent per day, then a *T. gondii* seroprevalence of only 0.2% (1/500 positive) in rodents will cause 51.9% of felids to be exposed to *T. gondii* annually.

Our study highlights the fact that *T. gondii* infection pressure is necessary, but not sufficient by itself, to explain seroprevalence in intermediate host species. This suggests that seroprevalence is strongly driven by factors other than infection pressure. When seroprevalence is already low, reductions in infection pressure may not achieve any further noticeable reduction in seroprevalence. Rather, reductions in *T. gondii* infection pressure intended to reduce infections may be most effective and applicable to species that are known to experience high rates of infection.

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