

Toxoplasma gondii coinfection with diseases and parasites in wild rabbits in Scotland

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SUMMARY

In wild rabbits (*Oryctolagus cuniculus*) on an estate in Perthshire, central Scotland, the seroprevalence of *Toxoplasma gondii* was 18/548 (3.3%). The wild rabbit could be a *T. gondii* reservoir and it has potential value as a sentinel of *T. gondii* in environmental substrates. *Toxoplasma gondii* was associated with female sex ($P < 0.001$) and with relatively heavy infections by *Eimeria stiedae* ($P = 0.036$). It was not associated with the intensity of coccidial oocysts, the severity of myxomatosis caused by the virus *Myxomatosis cuniculi*, the intensity of roundworm eggs, the year or season, rabbit age or distance from farm buildings. Coinfections could have been affected by gestational down regulation of type 1 T helper cells. A sudden influx or release of *T. gondii* oocysts might have occurred. This is the first report of *T. gondii* in any wild herbivore in Scotland and also the first report of lapine *T. gondii* as a coinfection with *E. stiedae*, *M. cuniculi* and helminths.

Key words: *Toxoplasma gondii*, *Eimeria stiedae*, rabbit, coinfection.

INTRODUCTION

The apicomplexan parasite *Toxoplasma gondii* is widespread and zoonotic. It has been found in every warm-blooded vertebrate so far studied (Dubey, 2008). Its definitive host is any felid, where it reproduces sexually, leading to the excretion of oocysts in cat feces. Oocysts environmentally resistant and can be transported in substrates such as soil, water and vegetation. Any non-felid warm-blooded vertebrate can be an intermediate host, where *T. gondii* tachyzoites can cause acute toxoplasmosis. Thereafter *T. gondii* persists as bradyzoites in tissue cysts. Hosts can be infected prenatally by tachyzoite transfer, or postnatally by bradyzoite or oocyst ingestion.

Asymptomatic *T. gondii* infections have been reported both in wild and captive rabbits, and worldwide reports were summarized previously (Dubey and Beattie, 1988; Dubey, 2010). Rabbits are considered an important intermediate hosts because cats that prey on them can excrete *T. gondii* oocysts. The wild rabbit is also a source of *T. gondii* for wild carnivores worldwide, including in Scotland (Burrells *et al.* 2013), carnivorous and omnivorous wild birds in Spain (Darwich *et al.* 2012), game trappers in Wales (Beverley *et al.* 1954) and liver patients in Mexico (Alvarado-Esquivel *et al.* 2011). The grazing and burrowing

wild rabbit could be exploited as a sentinel of *T. gondii*. Associations have been reported between *T. gondii* seroprevalence in wild rabbits and environmental variables likely to affect oocyst exposure in Spain (Almería *et al.* 2004) and Australia (Cox *et al.* 1981).

Here we examined *T. gondii* seroprevalence in wild rabbits on the Pitroddie Estate in Perthshire, central Scotland, where hill and lowland fields are used for livestock and arable crops, and feral cats are occasionally seen. The wild rabbit population has been sampled by shooting nearly every week since 1977 (Boag *et al.* 2013). These rabbits carry other apicomplexan parasites, including *Eimeria stiedae*, the cause of lapine hepatic coccidiosis. They are infected also by *Myxomatosis cuniculi* and by helminths, including the roundworms *Trichostrongylus retortaeformis* and *Graphidium strigosum*.

MATERIALS AND METHODS

Samples

Sera and data were collected from 548 rabbits shot between January 2007 and December 2008 at a study site in central Scotland. The sampling area is 12–286 m above sea level. It contains Pitroddie Farm buildings (PF) at ordnance grid reference NO 2148 2520 and Nether Durdie cottages at ordnance grid reference NO 2140 2450. Within the sampling area, Pitroddie Crags (northwest of PF) and PF land (north of PF) are mostly marginal farmland, while Nether Durdie (southwest of PF) is mostly

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arable farmland. A disused quarry separates Pitroddie Crags from PF. A burn (stream) flows west to east from the quarry and passes slightly south of PF. An access road runs mostly alongside the burn but passes slightly north of PF.

Global Positioning System data were collected for each shot rabbit and used by triangulation to calculate distance from PF. Those calculated distances were between five and 240 m. Vegetation surrounding each rabbit was classed as woody (coniferous trees, deciduous trees, gorse and hawthorn) or non-woody (bracken, garden, grass and nettles). The season of shooting was classed as spring (March–May), summer (June–August) or autumn/winter (September–February) because rabbits rarely breed in winter. Rabbits were sexed, weighed entire and classed as neonate (200–749 g), juvenile (750–1249 g) or adult (≥ 1250 g) as described (Boag *et al.* 2001).

Sample size did not differ between 2007 and 2008, but it was relatively small in autumn/winter (detail not shown). Sampled rabbits included 100 kittens, 134 juveniles and 314 adults. There was no sex bias in any age class, vegetation type or distance from PF (detail not shown).

Infections

Freshly shot rabbits were examined in the field for overt myxomatosis. In a laboratory, the liver was examined for gross lesions consistent with coccidiosis. As required for different analyses, myxomatosis and coccidiosis were considered as binary variables or classed as absent, light, moderate, heavy or very heavy as described (Boag *et al.* 2013). A 2 g sample of each rabbit's feces (rectal contents) was examined for helminth eggs and coccidial oocysts. Due to laboratory limitations, egg counts [eggs per gram (epg)] were pooled for the roundworms *T. retortaeformis* and *G. strigosum*. Oocyst counts [oocysts per gram (opg)] were pooled without species identification. As required for different analyses, egg and oocyst counts were considered as binary variables, or natural log-transformed, or classed as zero, low (1–99), medium (100–999) or high (≥ 1000).

Sera were tested for specific anti-*T. gondii* IgG using the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987), end-titrating at twofold dilutions starting at 1:20. Transformations of the inverse MAT titre did not normalize variance, so the MAT result was considered as a binary variable. An inverse titre of 1:20 was considered sufficient to indicate infection. Rabbits were classed as low seropositive (inverse titre 20 or 40) or high seropositive (inverse titre ≥ 80). A titre of 1:25 has been considered sufficient to indicate infection for wild rabbits in Spain (Almería *et al.* 2004; García-Bocanegra *et al.* 2010a, b) and for domestic rabbits in Brazil (Dubey *et al.* 2011) and Mexico (Alvarado-Esquivel *et al.* 2013). No formal validation of MAT for rabbits has been

reported but the test has been extensively for the detection of *T. gondii* antibodies in many species of animals, including rabbits (Dubey, 2010).

Statistical analysis

Seropositive rabbits were examined as case studies. Statistical analyses were performed in R v2.12.2 (Foundation for Statistical Computing, 2011). Counts of rabbits were compared using the χ^2 test or Fisher's Exact Test, and factors associated with seropositivity were sought using additive logistic regression with binomial errors. That model was fitted using the likelihood ratio test (LRT) by χ^2 . For the whole dataset ($n = 548$) candidate explanatory factors were the rabbit's sex and age class, the year and season of shooting and the distance from PF, the intensity class of myxomatosis, and log-transformed counts of coccidial lesions and roundworm epg. The same model, with log-transformed coccidial opg as a further candidate explanatory factor, was fitted for the 499 rabbits whose feces had been examined.

RESULTS

Apicomplexa

Antibodies to *T. gondii* were found in 18/548 (3.3%) rabbits with titres of 1:20 in nine, 1:40 in three, 1:80 in two, 1:160, 1:10 240, 1:20 480 and 1:81 920 each rabbit. Figure 1 shows the distribution of shot rabbits and their *T. gondii* infection status. The six high seropositives were shot within 103 m of PF, between January 2007 and May 2008, five of them before the end of June 2007. Visual inspection of the map did not suggest that seropositive rabbits were associated with the burn but there might be a north–south linear pattern in those rabbits' locations.

Logistic regression (Table 1) showed that *T. gondii* seropositivity was associated with female sex and relatively heavy coccidiosis. Univariate comparisons confirmed that *T. gondii* seroprevalence was relatively high in females ($P = 0.001$ by Fisher's Exact Test; Fig. 2) that it varied with the severity of coccidiosis ($P = 0.002$ by Fisher's Exact Test; Fig. 2) and showed that it did not depend on age, year, season or vegetation type (detail not shown).

The prevalence of hepatic coccidiosis was 179/548 (32.7%). Coccidiosis was light in 148 rabbits (11 seropositive for *T. gondii* at titres between 1:20 and 1:20 480), moderate in ten (all seronegative for *T. gondii*), heavy in 17 (one seropositive for *T. gondii* at titre 1:81 920) and very heavy in four (one seropositive for *T. gondii* at titre 1:80). The prevalence of coccidiosis did not differ between sexes but it was relatively high in kittens and juveniles (detail not shown). It was relatively high in

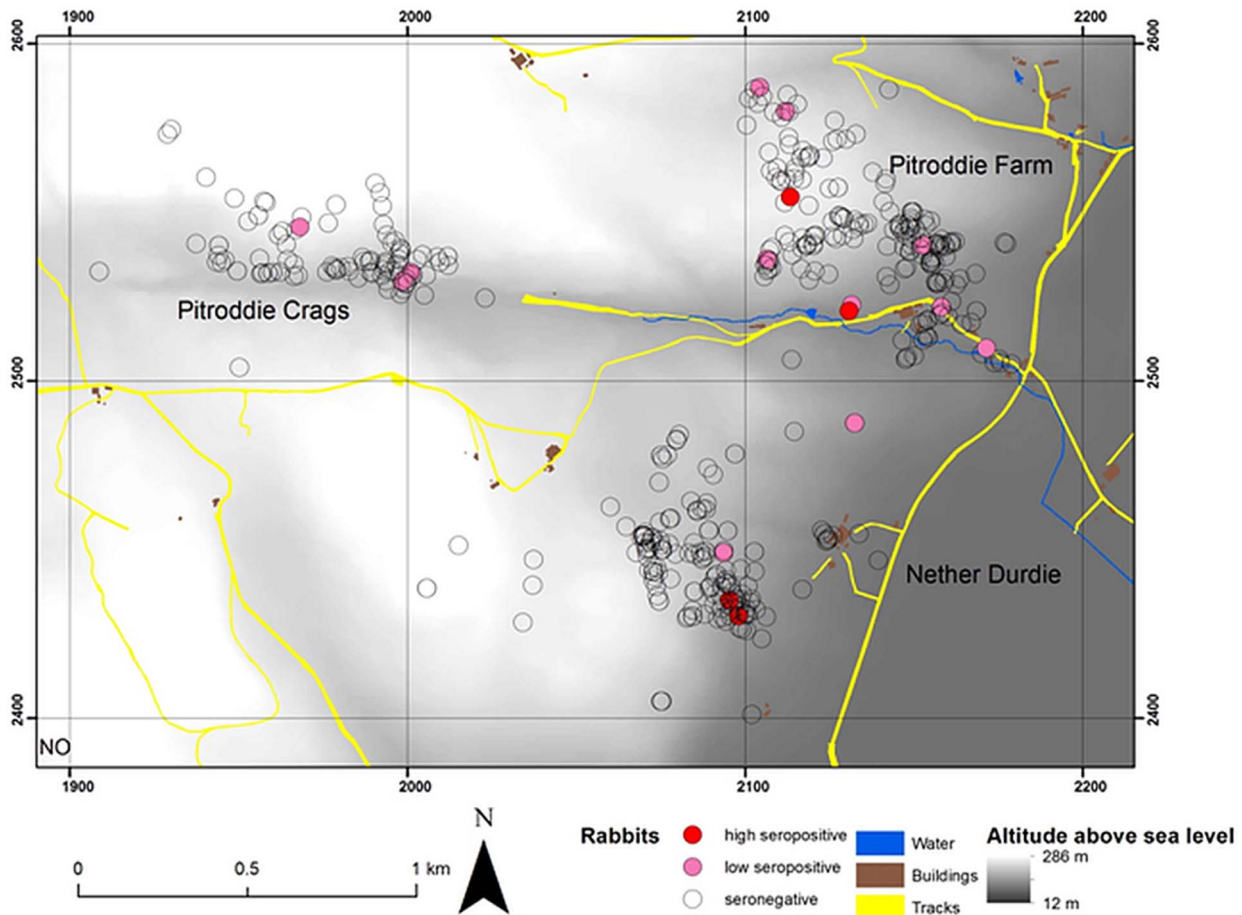


Fig. 1. Map showing rabbit locations and serological results. Seronegative, inverse titre <20. Low seropositive, inverse titre 20 or 40. High seropositive, inverse titre ≥80 (© Crown Copyright/database right 2015). An Ordnance Survey/EDINA supplied service.

Table 1. Factors associated with *Toxoplasma gondii* infection in wild rabbits (*n* = 548 unless otherwise stated)

Explanatory factor	Upward trend	Logistic coefficient	S.E. of coefficient	LRT by χ^2	df	<i>P</i>
Sex	Female	1.82	0.64	10.92	1	<0.001
<i>Eimeria stiedae</i> (hepatic coccidiosis)	Relatively heavy coccidiosis	0.26	0.11	4.88	1	0.027
Coccidial oocysts (<i>n</i> = 499)	None	-0.01	0.06	0.01	1	0.906
<i>Myxomatosis cuniculi</i> (myxomatosis)	None	Light: 0.33	Light: 1.10	0.51	3	0.917
		Moderate: -0.03	Moderate: 1.09			
		Heavy: -13.38	Heavy: 1050.78			
Roundworms (<i>Trichostrongylus retortaeformis</i> and <i>Graphidium strigosum</i> eggs)	None	-0.07	0.11	0.37	1	0.543
Year	None	2008: -0.86	2008: 0.52	2.98	1	0.094
Season	None	Spring: -0.20	Spring: 0.66	0.36	2	0.837
		Summer: 0.16	Summer: 0.63			
Age	None	Kitten: -0.16	Kitten: 0.75	2.54	2	0.281
		Juvenile: 0.76	Juvenile: 0.56			
Distance from Pitroddie Farm buildings	None	-5.78	4.92	1.46	1	0.227

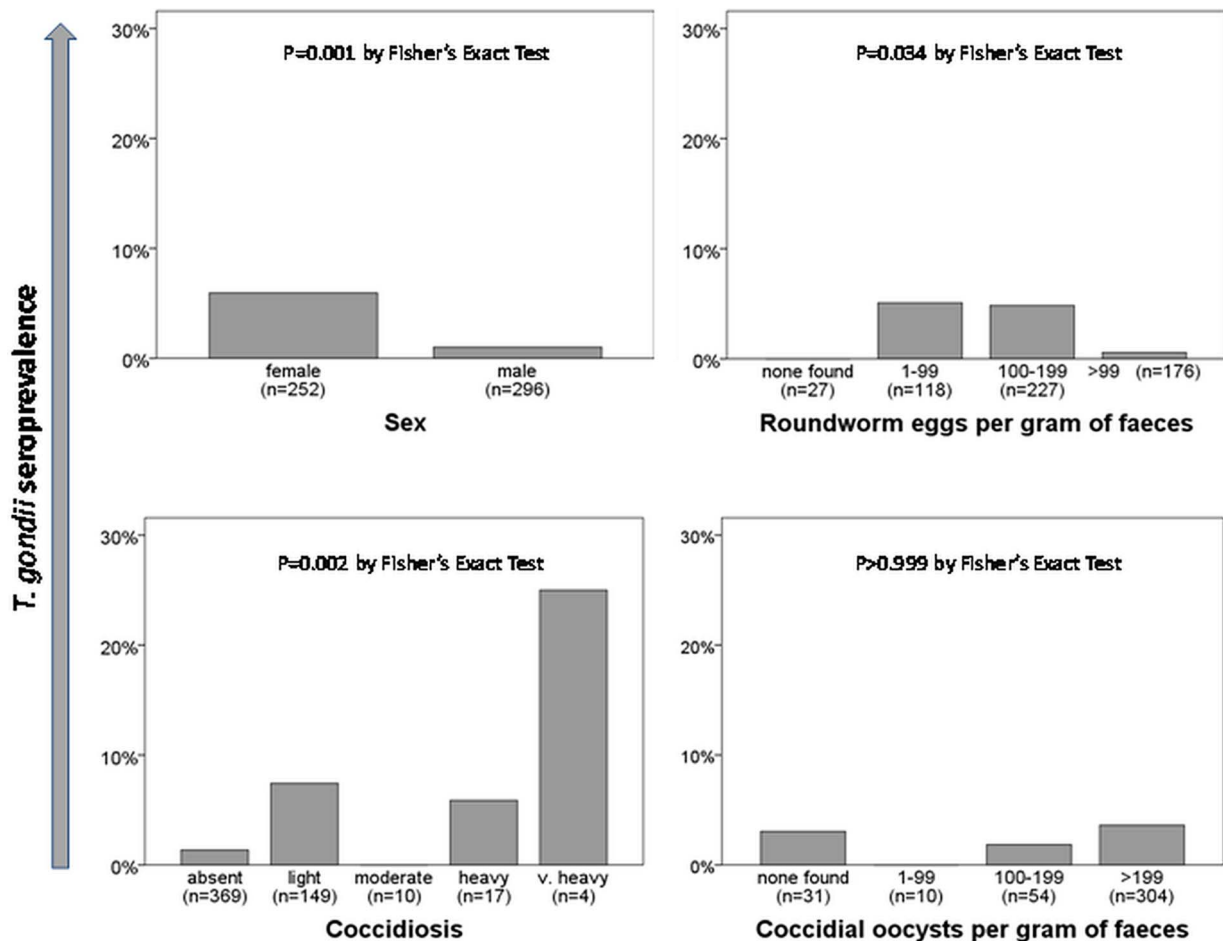


Fig. 2. *Toxoplasma gondii* seroprevalence in relation to sex, roundworms and *Eimeria steidae*. Univariate comparisons are shown.

2007 and in summer but it did not depend on vegetation type (detail not shown).

The prevalence of coccidial oocyst excretion was 368/499 (73.7%). Coccidial opg was low in ten rabbits (all seronegative for *T. gondii*), medium in 54 (one seropositive for *T. gondii* at titre 1:20) and high in 304 (11 seropositive for *T. gondii* at titres between 1:20 and 1:20 480). *Toxoplasma gondii* seropositivity was not associated with oocyst shedding ($P > 0.999$ by Fisher's Exact Test), a result also found for seroprevalence when multiple factors were considered (Table 1). The prevalence of coccidial oocysts did not differ between sexes but it was relatively high in kittens and juveniles (detail not shown). It was relatively high in spring and summer but it did not differ between years or between vegetation types (detail not shown). It was associated with coccidiosis ($\chi^2 = 17.39$, $df = 1$, $P < 0.001$): oocysts were shed by 143/167 (85.6%) rabbits with coccidiosis and 225/332 (67.8%) without coccidiosis.

Myxomatosis

The prevalence of myxomatosis was 42/548 (7.7%). Myxomatosis was light in 18 rabbits (one seropositive for *T. gondii* at titre 1:81 920), moderate in 19

(one seropositive for *T. gondii* at titre 1:40) and heavy in five (all seronegative for *T. gondii*). No rabbit with very heavy myxomatosis was found. *Toxoplasma gondii* seropositivity was not associated with myxomatosis ($P = 0.451$ by Fisher's Exact Test), a result also found for seroprevalence when multiple factors were considered (Table 1). The prevalence of myxomatosis did not differ between sexes but it was relatively high in juveniles (detail not shown). It was relatively high in 2007 and in summer but it did not differ between vegetation types (detail not shown). Myxomatosis was associated with coccidiosis ($\chi^2 = 11.22$, $df = 1$, $P < 0.001$): coccidiosis was observed in 24/42 (57.1%) rabbits with myxomatosis and 155/506 (30.6%) without myxomatosis. Coccidial oocyst shedding was not associated with myxomatosis ($\chi^2 = 0.86$, $df = 1$, $P = 0.3545$): oocysts were shed by 34/42 (81.0%) rabbits with myxomatosis and 334/457 (73.1%) rabbits without myxomatosis.

Roundworms

The prevalence of roundworms was 521/548 (95.1%). Roundworm epg was 0 in 27 rabbits (all seronegative for *T. gondii*), low in 118 (six

seropositive for *T. gondii* at titres between 1:20 and 1:102 40), medium in 227 (11 seropositive for *T. gondii* at titres between 1:20 and 1:81 920) and high in 176 (one seropositive for *T. gondii* at titre 1:20). *Toxoplasma gondii* infection was associated with roundworm burden ($P=0.034$ by Fisher's Exact Test; Fig. 2) but that association was not found when multiple factors were considered (Table 1).

The prevalence of roundworms did not differ between sexes but it was relatively high in juveniles and adults (detail not shown). It was relatively high in summer and autumn/winter but it did not differ between years or vegetation types (detail not shown). Roundworms were not associated with coccidial oocyst shedding ($\chi^2=0.00$, $df=1$, $P=0.995$): 353/478 (73.8%) rabbits with roundworms shed oocysts while from 15/21 (81.0%) rabbits without a report of roundworms, oocysts were not observed.

DISCUSSION

The *T. gondii* seroprevalence in rabbits at Pitroddie is the lowest yet reported in wild rabbits. Previous authors found seroprevalences of 5% in the Czech Republic (Hejlíček *et al.* 1997), 11.9 and 14.2% in Spain (Almería *et al.* 2004; García-Bocanegra *et al.* 2010a, b), 17.4% in Australia (Cox *et al.* 1981), 21% in Norway and Sweden (Kapperud, 1978), 50% in Wales (Beverley *et al.* 1954), and a PCR prevalence of 68.4% in England (Hughes *et al.* 2008).

The absence of acute toxoplasmosis agreed with previous surveys of wild rabbits. That could imply that acutely ill wild rabbits remain underground or that they are vulnerable to predation. Very low seroprevalence at Pitroddie may be due to few feral cats being present. Previously, *T. gondii* oocysts or infections have been reported where cats were present (Sroka *et al.* 2006) and where traces of their feces could have been carried on straw (Faull *et al.* 1986) or in water (Cox *et al.* 1981; Aramini *et al.* 1999; Conrad *et al.* 2005), but rarely where cats were absent (Dubey *et al.* 1997). Straw is not routinely imported to Pitroddie and waterborne *T. gondii* has not been reported there.

Wild rabbits' frequent breeding could explain the associations between *T. gondii* and female sex, and heavy coccidiosis. In mammals with haemochorial placentation such as rabbits, mice and humans, gestational immunosuppression prevents rejection of the foetus (Moffett and Loke, 2006). The T helper cell type 1 (Th1) response to immune challenge is downregulated relative to the type 2 (Th2) response. This change impairs the pregnant female's defence against intracellular coccidia such as *T. gondii* and *E. stiedae*, and viruses such as *M. cuniculi*, while enhancing her defence against extracellular parasites such as helminths (Cox, 2001). Therefore pregnant rabbits could be relatively susceptible to infection by *T. gondii*, *E. stiedae* and *M. cuniculi*. In

managed wild rabbits in Spain, new coccidial infections were relatively frequent during the breeding season (Bertó-Moran *et al.* 2013).

Toxoplasma gondii infections usually progress to a chronic state (bradyzoite cysts) during which the host remains seropositive for life (Dubey, 2008). Therefore new *T. gondii* infections during pregnancy might have led to the non-seasonal but female-biased seroprevalence reported here. Sex-biased *T. gondii* seroprevalence was not observed in wild rabbits in Spain (Almería *et al.* 2004) or domestic rabbits in Mexico (Alvarado-Esquivel *et al.* 2013). Neither of those reports mentioned *E. stiedae*. Ours is the first report of *T. gondii* coinfecting with *E. stiedae* except in one captive rabbit (Dubey *et al.* 1992). Unlike *T. gondii*, *E. stiedae* is cleared within weeks (Barriga and Arnoni, 1981). It was detected in the present study by transient evidence: hepatic lesions and fecal oocysts. That transience may explain the lack of sex bias in those observations.

Once inside host cells, apicomplexan parasites subvert the immune response. They enhance their own survival and their chance of transmission by mechanisms which increase the Th1:Th2 ratio (Frölich *et al.* 2012; Graham *et al.* 2007). Subversion of the immune response by *E. stiedae* could limit any effect of pregnancy on rabbits' susceptibility to new *T. gondii* infections. That may have contributed to the observed lack of variation in *T. gondii* seroprevalence between age classes, which contrasted with a tendency for this parasite's prevalence to raise with age in many hosts (Dubey, 2008). On most farms oocyst exposure is inferred to be near-continuous for grazing animals, leading to an accumulation of chronic infections (Kijlstra and Jongert, 2009).

Association between oocyst excretion and hepatic coccidiosis indicated that many wild rabbits with overt coccidiosis at Pitroddie shed oocysts likely to represent the same parasite. Previously, experimental *E. stiedae* infection revealed its time course (Barriga and Arnoni, 1981). Those laboratory rabbits developed hepatic lesions in the third week post-infection, and they shed oocysts rapidly in the fourth and fifth weeks. But they shed some oocysts within the first 3 weeks, perhaps resembling wild rabbits at Pitroddie in which oocyst shedding and hepatic coccidiosis coincided. Those which shed oocysts without having overt coccidiosis might have cleared *E. stiedae* infection before being shot, or the oocysts in their feces might have been from other coccidia.

This is the first report of sex-independent coccidiosis in this population. The reduction in its prevalence with age and its seasonality were previously reported (Boag *et al.* 2013). For *E. stiedae* oocyst shedding, an Australian study of wild rabbits found sex-independence, falling prevalence with age and seasonality (Hobbs *et al.* 1999).

The MAT is a trusted method for many species and tissue types, measuring specific anti-*T. gondii* IgG (Dubey and Desmonts, 1987). However since Th2 cells are important in antibody responses, gestational reduction in the Th1:Th2 ratio might enhance females' production of specific IgG, leading to false positive MAT results. Future studies could use IgM serology and other methods, not dependent on antibodies, to detect new infections.

We speculate that the observation of six high-seropositive rabbits, close to one another in time and space, could have arisen from a sudden influx or release of oocysts near PF in late 2006. It is noteworthy that transformations of the inverse MAT titre did not normalize variance, reflecting an unusual distribution in that variable. The lack of statistical association between seropositivity for *T. gondii* and year, season, age or distance from PF did not disprove the hypothesis of oocyst influx or release. Oocysts could have been introduced by a cat or by passive transport, perhaps on a vehicle. Thereafter oocysts could have been transported throughout the site, and they could have been released from soil by changes in temperature or moisture. It is noteworthy also that relatively high prevalence in 2007 was observed for clinical signs of coccidiosis but not for coccidial oocyst shedding. These observations concerning *E. stiedae* arose within a few months of the putative influx or release of *T. gondii* oocysts, perhaps reflecting weather conditions. Future studies could seek *T. gondii* oocysts in wild rabbits' environment, and they could consider genetic relationships between rabbits.

Another infectious agent subverting the lapine immune response is *M. cuniculi*. That virus is immunosuppressive, decreasing all T cells (García-Bocanegra *et al.* 2010a, b) especially Th1 cells (Jeklova *et al.* 2008). Therefore a rabbit with myxomatosis might resemble a pregnant rabbit in having a low Th1:Th2 cell ratio and hence being vulnerable to new *T. gondii* infections. If that is so, seropositivity for *T. gondii* could be associated with seropositivity for *M. cuniculi*. However, the present study did not test that hypothesis, examining *M. cuniculi* infection only as clinical myxomatosis which is transient.

Apicomplexan subversion of the immune response, increasing the Th1:Th2 cell ratio contrary to female rabbits' gestational Th1 downregulation, could affect rabbits' immune defences against nematodes (Boag *et al.* 2013). The present study did not distinguish biological effects of *T. gondii* and *E. stiedae*. It also did not distinguish between roundworm species, perhaps explaining the lack of sex bias in roundworm prevalence. *Trichostrongylus retortaeformis* epg is higher in breeding females than in non-breeding females and males in this population (Cattadori *et al.* 2005) and in a wild rabbit population in Spain (Molina *et al.* 1999), and it was higher in intact females than in sterilized females in a

managed wild rabbit population in Australia (Hobbs *et al.* 1999). In rabbits at Pitroddie, a biological interaction between *T. gondii* and roundworms remains plausible.

Future studies of rabbit populations with more abundant *T. gondii* could examine coinfections between it, *E. stiedae* and other parasites in this host. Molecular detection could be integrated with high-resolution genetic characterization (Su *et al.* 2010). Screening of wild rabbit populations could be complemented by experimental infections, including pregnant females. The use of body weight to estimate age could be reconsidered because coccidiosis and myxomatosis are known to slow growth in the rabbits at Pitroddie (Lello *et al.* 2005; Cattadori *et al.* 2008) and such an effect has been described also for coccidiosis in laboratory rabbits (Barriga and Arnoni, 1981).

People eating wild rabbit meat, or feeding it to their cats and other pets, are sometimes unaware of the need to cook or freeze meat thoroughly. Human *T. gondii* infections were reported in rabbit trappers in Wales (Beverley *et al.* 1954) and liver patients who had eaten rabbit meat in Mexico (Alvarado-Esquivel *et al.* 2011). A risk of infection was noted for workers handling farmed rabbits in Poland (Sroka *et al.* 2003) and for consumers of wild rabbit meat (Almería *et al.* 2004). Wild rabbit meat could be screened using ELISA as suggested for meat from farmed rabbits (Mecca *et al.* 2011) and consumers could be better informed through leaflets and advertisements.

The present study complements previous evidence that the wild rabbit can be used as an indicator species for *T. gondii* on land (Cox *et al.* 1981), as can wild carnivores (Burrells *et al.* 2013). It provides preliminary evidence that *T. gondii* in wild rabbits in Scotland behaves mostly as in other mammalian hosts. Gestational Th1 downregulation in the rabbit is probably involved. However in some rabbit populations, including the one studied here, outcomes are affected by another endemic apicomplexan parasite: *E. stiedae*. We suggest that *T. gondii* resembles *Eimeria* spp. in some of its effects on the immune system, including a rise in the Th1:Th2 ratio. Differences between the life cycles of *T. gondii* and *E. stiedae* may have led to different interactions with *M. cuniculi* and roundworms.

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