Prevalence of *Toxoplasma gondii* in localised populations of *Apodemus sylvaticus* is linked to population genotype not to population location.

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SUMMARY

*Toxoplasma gondii* is a globally distributed parasite infecting humans and warm-blooded animals. Although many surveys have been conducted for *T. gondii* infection in mammals, little is known about the detailed distribution in localised natural populations. In this study, host genotype and spatial location were investigated in relation to *T. gondii* infection.Wood mice (*Apodemus sylvaticus*) were collected from 4 sampling sites within a localised peri-aquatic woodland ecosystem. Mice were genotyped using standard *A. sylvaticus* microsatellite markers and *T. gondii* was detected using 4 specific PCR based markers: SAG1, SAG2, SAG3 and GRA6 directly from infected tissue. Of 126 wood mice collected, 44 samples were positive giving an infection rate of 34.92% (95% CI: 27.14%-43.59%). Juvenile, young adults and adults were infected at a similar prevalence, respectively, 7/17 (41.18%), 27/65 (41.54%) and 10/44 (22.72%) with no significant age-prevalence effect (*P* = 0.23). Results of genetic analysis of the mice showed that the collection consists of four genetically distinct populations. There was a significant difference in *T. gondii* prevalence in the different genotypically derived mouse populations (*P*=0.035) but not between geographically defined populations (*P*=0.29).These data point to either a host genetic/family influence on parasite infection or to parasite vertical transmission.

Key words: *Toxoplasma gondii*; *Apodemus sylvaticus*; transmission; vertical transmission; transmission cycles; populations; genotypes; wild mammals; rodents; PCR

KEY FINDINGS

*Toxoplasma gondii* was detected at a prevalence of 34.92% in a population of *Apodemus sylvaticus*

Microsatellite genotyping of the host, *A. sylvaticus,* identified 4 genetically distinct populations

*T. gondii* infection was significantly associated with host genotype but not location of host capture

This suggests *T. gondii* infection is influenced by vertical transmission or by host genetic differences

INTRODUCTION

*Toxoplasma gondii* is an apicomplexan parasite with a global distribution which can cause significant disease in many species including humans (Dubey, 2010; Dubey and Jones, 2008). Members of the family Felidae are the only known definitive hosts (Frenkel *et al.*, 1970; Hutchison, 1965) but it also infects a whole range of warm-blooded vertebrates, including domestic, wild- and marine mammals, birds and humans (Dubey, 2010). After ingesting the sporulated oocysts or tissue cysts they serve as intermediate hosts, in which asexual reproduction occurs. There are three main routes of transmission: via oocysts shed in faeces of the definitive hosts, ingestion of tissue cysts and congenital transmission. The relative importance of each of these transmission routes is not fully understood (Hide *et al.* 2009) but infection by oocysts derived from felids is generally considered the most important (Tenter *et al.* 2000). In humans, ingestion of tissue cysts, from raw meat may be the main route of transmission in developed countries whereas ingestion of oocysts may be more significant in developing countries (Dubey and Jones, 2008). As prey or carrion, rodents may be a significant intermediate host in the transmission to other animals and high frequencies of infection have been observed (Marshall *et al.* 2004, Meerburg *et al.* 2012) including in areas that appear to be relatively free of cats (Thomasson *et al*. 2011). The importance of congenital transmission is low in humans ranging between 0.01 and 1% of live births (Tenter *et al.* 2000) but the importance is more controversial in other mammalian species such as sheep (Hide *et al.* 2009; Innes *et al*. 2009). However, in rodents this route of transmission may be more important. Beverley (1959) first suggested that vertical transmission can play an important role in the transmission of the parasite using mice as a model system. Vertical transmission has been experimentally confirmed in laboratory conditions in murids (*Apodemus sylvaticus, Mus domesticus*) (Dubey *et al*. 1995; Elsaid *et al*. 2001; Owen and Trees, 1998: Stahl *et al*. 2002), rats (*Rattus norvegicus*) (Dubey *et al*. 1997) and shown to be significant in a natural population of domestic mice (*M. domesticus*) (Marshall *et al.* 2004). Furthermore, a high prevalence of infection was found in *A. sylvaticus* sampled in an area relatively free of cats (Thomasson *et al*. 2011) suggesting that *Toxoplasma* can also be maintained within rodent populations in the putative absence of cats.

Mice probably play an important role in *T. gondii* transmission, often prey to cats, yet little is known of *T. gondii* prevalence and genotypes in wild mouse populations. The majority of epidemiological studies on mice have been based on serological diagnostic methods which detect current and historical infection (Dubey *et al*. 1995; Franti *et al*. 1976; Hejlicek and Literak, 1998; Hejlicek *et al*. 1997; Jackson *et al*. 1986; Jeon and Yong, 2000; Smith and Frenkel, 1995; Yin *et al*. 2010). Studies conducted using PCR–based methods as a diagnostic tool have generally shown higher prevalence in some wild mice populations with prevalences ranging from 10.4% (Vujanic *et al.* 2011), 13.6% (Kijlstra *et al*. 2008), 29% (Zhang *et al*. 2004) up to 40.78% (Thomasson *et al*. 2011) and 59% (Marshall *et al.* 2004). With the exception of a study of urban mice (*M. domesticus*) showing different levels of infection within different mouse populations (Marshall *et al.* 2004; Murphy *et al.* 2008), information on the distribution of infected mice within natural populations of wild mice are generally lacking. The aims of this study were to investigate the detailed distribution of *T. gondii* infection in a series of localised populations of *A. sylvaticus* collected in a systematic manner (Boyce *et al.* 2012; 2013). These populations reside in an area relatively free of cats (< 2.5 cats per km2)(Hughes *et al*. 2008) but where previous studies have demonstrated a high prevalence (Thomasson *et al*. 2011). The objectives were to investigate host genotype and spatial location in relation to *T. gondii* infection. We show that parasite infection is linked to host population genotype not population location.

MATERIALS AND METHODS

A total of 126 wood mice (*Apodemus sylvaticus*) were collected and euthanased from four sites located within the boundaries of the Malham Tarn Nature Reserve, North Yorkshire, UK (Figure 1) as described previously (Boyce *et al.* 2012; 2013; Morger *et al*. 2014). Collection points at these sites, labelled Tarn Woods, Tarn Fen, Ha Mire and Spiggot Hill, were recorded using GPS position fixing (WGS84). All appropriate permissions were obtained (Boyce *et al.* 2012; 2013) and ethical approval was granted by the University of Salford Research Ethics and Governance Committee (CST 12/36). Mice were examined for a range of parameters including sex, weight and length. Mice weighing less than 14 g were considered juveniles (Higgs and Nowell, 2000). The brains were dissected out, using sterile technique, and transferred into sterile tubes containing 400 μl of lysis buffer (0.1 M Tris pH 8.0, 0.2 M NaCl, 5 mM EDTA, 0.4% SDS) and stored at *−*20 °C until DNA extraction. Due to the freezing in lysis buffer, it was not possible to conduct serological tests for *Toxoplasma gondii* infection nor was it possible to isolate viable parasites.

DNA was isolated, from *A. sylvaticus* brain tissue, using proteinase K lysis followed by phenol/chloroform extraction as previously described (Duncanson *et al*. 2001). Extracted DNA was tested for mammalian tubulin to ensure the viability for PCR (Terry *et al*. 2001) and appropriate protocols to prevent cross contamination were followed (Williams *et al*. 2005; Hughes *et al.* 2006; Morley *et al*. 2008). Detection of *T. gondii* was carried out using nested PCR amplification of the surface antigen genes 1 (SAG1) (Savva *et al*. 1990) as modified by Morley *et al.* (2005). Positive amplification was confirmed by nested PCR amplification with three other sets of *T. gondii* specific primers (SAG2, SAG3 and GRA6) as described by Su and colleagues (Su *et al*. 2006; Shwab *et al*. 2013). All samples were tested a minimum of three times with the SAG1-PCR and occasional samples which showed a sporadic positive amplification were further tested until they either attained the criteria of three positive SAG1-PCR amplifications or they were then considered negative. Samples passing the SAG1 PCR criteria were then also confirmed with a minimum of three positive amplifications using each of the four other markers before the mouse brain was considered positive for *T. gondii* infection. In addition to being used for parasite detection, these three genes (SAG2, SAG3 and GRA6) were used as RFLP markers for direct genotyping of PCR positive brain tissues as described (Su *et al*. 2006; Shwab *et al*. 2013). Typically, *T. gondii* genotyping is carried out using DNA taken from isolated viable parasite strain cultures using a total of 10 genetic markers. In this study, viable parasite isolation was not possible and consequently genotyping was conducted on DNA extracted directly from tissue. This is known to be difficult due to low infection levels and as a consequence, only three markers could be reliably amplified for genotyping (SAG2, SAG3 and GRA6). Amplification and RFLP analysis, directly from tissues has been reported to be very difficult to achieve and, the same was true in this study. Multiple PCR reactions were necessary to build up the RFLP results. We recognise the limitations of this approach over parasite isolation, particularly for the detection of genotypes due to partial digestion with restriction enzymes, and have taken precautions to ensure maximum reliability. All PCR reactions were performed using published primer sequences (see below for specifics). Sheep DNA was used as a positive control for tubulin PCR, *T. gondii* DNA strains RH (Type I), SR (Type II – isolated from a goat, Slovakia (Spisak *et al.* 2010) and checked as Type II for all 10 markers, this paper – data not shown) and C56 (Type III) were used as positive controls for diagnostic PCRs and genotyping. Sterile water was used as a negative control and interspersed throughout experiments to ensure that contamination would be detectable. For the SAG1 PCR, each sample was tested at 2 concentrations of DNA (1/5 and 1/10 dilution as the ratio of parasite to host DNA was unknown). All PCR reactions were performed using a Stratagene ROBOCYCLERTM (La Jolla, California, USA). PCR products were run on 1.5% agarose TBE gel containing GELRED and visualized on a Syngene G-BOX Gel Documentation and Analysis System (Cambridge, UK). For the majority of genotyping reactions, the Type II strain was used as the positive control (as this is the most predominant type in Europe) so that the occurrence of unusual Type I and Type III strains being derived from contamination by the control could be ruled out. To avoid confusion by partial digestion, careful analysis of band sizes, from all markers, was carried out in relation to published marker DNA sequences and other studies. The SAG2 locus has two polymorphic sites at 3´ and 5´ ends for type II and type III (Howe *et al*. 1997) and amplification of the ends of this locus were performed separately. The two PCR reactions for the SAG 2 gene were optimized as described by Fuentes *et al.* (2001). Amplification was carried out in a final volume of 20 μl containing 2.7 µl of KCL buffer (containing 15 mM MgCl2 manufactured by Bioline), 0·32 μl of dNTP mix (100 mM), 1 μl of (10 pM/μl) forward primer and reverse primer and 0.4 µl of 5 units Biotaq polymerase (Bioline). Two microliters of DNA were used as a template. The thermal cycling conditions consisted of an initial denaturation step of 4 minutes at 95°C. This was followed by 20 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes, and a final elongation step of 72°C for 10 minutes. The resulting amplification products were diluted 1/10 in water and a second amplification of 35 cycles was performed using 1 µl of the diluted product as template. The annealing temperature for the second round primers was 60˚C but all other conditions remained the same as the first round (Fuentes *et al.* 2001).

 For the PCR reaction targeting the 3’end of SAG2 gene, correct predicted product sizes of 300 bp for the first round and 222 bp for the second round of amplification are expected. In control *Toxoplasma* DNA, both products could be seen but in positive mouse brain DNA samples, products could only be seen in the second round. For the PCR reaction targeting the 5’ end of SAG2 gene, first and second round products of, respectively, 340 bp and 241 bp were the correct predicted band sizes. Again, both could be seen in control DNA samples but only the second round product was observed in positive mouse brain DNA. Positive PCR reactions were further analysed by restriction enzyme digestion with each of the restriction enzymes *Sau3Al* (5’-end products) and *HhaI* (3’-end products) using 8·5 μl of PCR product, 1 μl of the manufacturers recommended buffer and 0·5 μl of enzyme. These were incubated at 37 °C for a minimum of 2 hours. Products were visualized by gel electrophoresis on a 2·5% agarose gel. Typing was achieved by combining 5’ and 3’ RFLP patterns (Howe *et al*. 1997).

A nested PCR was used to detect the SAG3 gene (Grigg *et al.* 2001; Su *et al*. 2006). Amplification was carried out in a final volume of 50 μl containing 5 μl of 10 × HT PCR buffer (HT Biotechnologies ) (100 mM Tris HCl (pH 9·0), 15 mM MgCl2, 500 mM KCl, 1% TritonX-100, 0·1% (w/v) stabilizer), 0·5 μl of dNTP mix (100 mM), forward primer Fext (5′ CAACTCTCACCATTCCACCC 3′) and 2·5 μl of (10 pM/μl) reverse primer Rext (5′ GCGCGTTGTTAGACAAGACA 3′) and 2·5 units Biotaq polymerase (Bioline). DNAse-free water made the final volume to 50 μl. All samples were tested 3 times at 1 μl, 2 μl and 1 μl 1:5 dilution of sample DNA. Amplification was carried out using a Stratagene Robocycler as follows: an initial denaturation step of 5min at 94 °C was followed by 35 cycles of PCR performed for 40 sec at 94 °C, 40 sec at 60 °C and 60 sec at 72 °C, with a final extension step of 10 min at 72 °C. Second-round PCR was carried out using the same reaction and cycling conditions as the first round with the exception of the primers which were Fint (5′ TCTTGTCGGGTGTTCACTCA 3′) and Rint (5′ CACAAGGAGACCGAGAAGGA 3′). A volume of 2 μl of first-round product was added to act as a template. Amplification products (10 μl) were visualized by agarose gel electrophoresis on a 2% agarose gel containing GelRed. Positive PCR reactions (226 bp) were further analysed by restriction enzyme digestion with each of the enzymes *NciI* and *AlwNI*, 13 μl of PCR product, 1.5 μl of buffer 4 (NEB) and 0·5 μl of enzyme. These were incubated at 37 °C for a minimum of 2 hours.

GRA6 nested PCR was performed using published sequences: F1: 5’ ATTTGTGTTTCCGAGCAGG 3’,R1:5’ GCACCTTCGCTTGTGGT 3’ and F2: 5’ TTCCGAGCAGGTGACC 3’, R2: 5’ GCCGAAGAGTTGACATAG 3’ (Su *et al.* 2006). A 344 bp product at the end of the second round was considered to be positive. For RFLP typing, 5 µl of nested PCR products were treated with *MseI* in total volume of 20 µl at 37˚C for 1 h and then digested samples were resolved on a 2.5% agarose gel to reveal banding patterns (Khan *et al*. 2005).

A total of 126 DNA samples from *A. sylvaticus* were prepared for microsatellite genotyping. Nine *A. sylvaticus* loci were amplified and genotyped using primers and conditions previously described (Makova *et al*. 1998). One *M. domesticus* microsatellite, which amplifies from *A. sylvaticus* was also used (MS19) (Primers - Forward: 5’ TGCTCACTGATTTGAGCCTGTGCA 3’, Reverse: 5’ ATAAATACAGAGCAAAGC 3’). The PCR reaction mixture (20 µl) consisted of 2 µl Bioline buffer (excluding MgCl2), 0.6 µl Bioline MgCl2 (50 mM), 2 µl of each primer (10 pM/µl), 0.2 µl dNTP mix (25 mM each), 0.2 µl *DNA* Taq Polymerase(5U) and 12 µl PCR water.

Thermal cycling conditions used were an initial 5 min denaturation at 95 °C followed by 35 cycles at 95 °C for 40 sec (denaturation), 30–45 s at the annealing temperature, 72 °C for 30–60 s (extension), concluding with a final 30 min extension at 72 °C (Makova *et al*. 1998). Amplification products (10 μl) were visualized by agarose gel electrophoresis on a 1.5% agarose gel containing GelRed. PCR products were then mixed with formamide and LIZTM standard marker and genotyped on the GENOTYPER (Applied Biosystems 3130 Genetic Analyser) according to the manufacturer to gain allele sizes relative to an internal size standard. Each sample for each locus was scored with Peak Scanner TM v1.0 to identify peaks and fragment sizes for application-specific capillary electrophoresis assays (Dodd *et al.* 2014). The program *Structure 2.3.3* (Pritchard *et al*. 2000; Evanno *et al*. 2005)was used to analyse multi-locus microsatellite genotype data to investigate population structure. In some cases, the program STRUCTURE cannot detect subgroups with weaker probabilities when all data is included. It is common practice (e.g. Gelanew *et al*. 2010) to rerun each initial group separately through STRUCTURE to investigate substructuring. This was carried out on the R, G and B groups and indeed did reveal further structuring in the R Group (see results). Program COLONY v2.0.5.0 (Wang, 2004) was used to estimate the full- and half-sib relationships of mice. The program MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004) was used to check for the absence of microsatellite null alleles and scoring errors. None were found.

RESULTS

The study site (Malham Tarn, Yorkshire Dales, UK) yielded 126 *A. sylvaticus* from 4 spatially separate sampling sites (Figure 1). To examine the prevalence of *T. gondii* in this population of *A. sylvaticus*, a series of PCR reactions were conducted using *T. gondii* specific PCR primers. DNA was successfully isolated from 126 mice brains and tested for the absence of PCR inhibition using PCR amplification of the mammalian α-tubulin gene. Forty four samples from 126 gave positive reactions with four *T. gondii* specific markers SAG1, SAG2, SAG3 and GRA6 (three replicates each) (Table 1). Thus an infection rate of 34.92% (95% CI: 27.14%-43.59%) was found. A total of 24/76 (31.58%, 95% CI: 22.19%-42.74%) of male and 20/50 (40%, 95% CI: 27.59%-53.84%) of female mice were found to be positive for *T. gondii.* No significant difference was found in prevalence in males and females (*χ2* =0.863, D.F. = 1, *P* = 0.353). A total of 17 juveniles, 65 young adults and 44 adults were present in this cohort of 126 of which 7 (41.18%), 27 (41.54%) and 10 (22.72%), respectively, were PCR positive for *T. gondii.* Therewas no significant age prevalence effect (*P* = 0.23).

To investigate parasite diversity present in infected animals, it is usual to determine the genotypes of isolated viable *T. gondii* strains by restriction fragment length polymorphism (RFLP) mapping using a standard set of 10 markers (e.g. Su *et al*. 2006; Shwab *et al*. 2013). As the possibility did not exist to isolate viable parasites from this set of samples, direct genotyping from brain tissue DNA was carried out. Due to presumed low parasite intensity levels, we were only able to directly genotype all of the positive mice using 3 genetic markers. Other genetic markers could not be consistently amplified to levels sufficient for RFLP mapping. Table 2 presents the RFLP results for all 44 positive mice using genetic markers GRA6, SAG2, and SAG3 as described elsewhere (Su *et al*. 2006; Shwab *et al*. 2013). Using the SAG2 (both ends) and the GRA6 genes, both Type II and Type III banding patterns were detected. The digestion of the SAG3 PCR products with *AlwNI* and *NciI* revealed Type II and III patterns, and in a number of mice, a combination of all three types (I, II, and III). An example gel of a mouse exhibiting Type I, II and III SAG3 RFLP patterns is presented in Figure 2. Detailed analysis of the RFLP patterns, in combination with published DNA sequences for the three loci showed, that a mixture of strains was a possible interpretation for some of these combinations. Mixtures of parasite strains have not been widely reported for *T. gondii* infections and, although requiring genotyping of isolated viable parasites for complete confirmation, suggests that this in an interesting phenomenon that should be explored further.

In order to investigate the host population genetics of this collection of *A. sylvaticus*, the mice were genotyped using 10 polymorphic microsatellite DNA markers. The level of genetic variation at the ten microsatellites loci was high, all ten microsatellites were polymorphic. The polymorphism varied between seven (locus 4A) and 15 (locus 12A) alleles per locus (Makova *et al*. 1998) with an average of 10.6 alleles per locus. Using the program MICRO-CHECKER 2.2.3 (Van Oosterhout *et al*. 2004) no evidence was found of null alleles and therefore all ten loci were included in the analysis. The results of the program STRUCTURE showed that the most probable number of clusters of individuals was three and that the whole wood mice population in the study site (Malham Tarn) consisted of three genetically distinct populations R, G and B (Table 3, Figure 3A). To evaluate the robustness of these three populations, STRUCTURE was rerun using K values of 4 and 5 (i.e. considering the probability of 4 and 5 groups) and the same three populations were identified showing that the groups are reliably assigned (Figure 3B). The sampling location was not used as prior information in the analysis so as not to bias any genetic interpretation. As the generation of groups by STRUCTURE is based on probability of an individual belonging to a group, sometimes groups with weaker probabilities can be masked during the first analysis. It is common practice (e.g. Gelanew *et al*. 2010) to re-examine genetically defined STRUCTURE groups by re-running each group (i.e. R, G and B) separately through STRUCTURE. Following this more detailed analysis using STRUCTURE, the R population could be divided into two genetically distinct populations R1 and R2 (Figure 4, Table 3). Again these groups were robust for higher values of K, data not shown). STRUCTURE was unable to divide the individuals from populations G and B into subpopulations so they were considered as individual populations. Based on these observations, we conclude that there are 4 genetically defined populations. These genetic populations, identified by STRUCTURE, were examined to see if there was any association between microsatellite derived populations and the trapping location of the mice. Testing the null hypothesis of no association, it was found that a significant association of mouse genetic group with trapping location was found (*χ2* = 46.6; D.F. = 9; *P* = 0.000). Patterns of distribution of the genetic groups show that this association is loose (e.g. the G group is predominantly found in the area designated Tarn Woods but that migration has occurred to neighbouring sites). This indicates that the habitat structure of the landscape allows a slow migration of mice from one population to another, perhaps with source and sink populations. The overall distribution of genetic groups in relation to location is presented in Figure 1.

To understand the distribution of *T. gondii* in mice from different collection sites, the prevalence was calculated for each site. While variation in prevalence was seen at each site, this was not significant (*P* = 0.29) (Table 4).

To understand the distribution of *T. gondii* in mice from different genetic populations, as inferred from STRUCTURE, the prevalence was calculated for each population. There was a significant difference between each genetic population (*χ2* =7.950, D.F. = 2, *P* = 0.018) (Table 5). This conclusion was also true when the three groups were also resolved at other values of K (i.e. 4 and 5, figure 3B).

Some previous studies have suggested that vertical transmission of *T. gondii* might be important in natural populations of mice (Owen and Trees, 1998; Hide *et al.* 2009; Thomasson *et al*. 2011) in which case a relationship should be detectable between infection and family line (Morley *et al.* 2005). Having established a relationship between infection and population structure, this raises the question as to whether these populations can be further analysed at the family level. To address this question, families were assigned to the mice using the program COLONY and the relationship with *T. gondii* infection examined. The COLONY program can be used to infer full- and half-sib relationships, assign parentage, infer mating system (polygamous/monogamous) and reproductive skew in both diploid and haplo-diploid species. Twenty two families of wood mice were identified in this collection in the study site. These comprised 4 families of full-sibs and 18 families of half-sibs based, as advised in the program literature, on mice being considered to be siblings if the likelihood probability was more than 0.85. Each identified family was quite small comprising only a few mice (n=2 to n=7). A range of prevalences were found in each of the families, for example, two families had 0% prevalence and one family had 85.7%. This suggests differences in prevalence of *T. gondii* between families and could support the notion of a mechanism that maintains transmission within families. However, due to the small numbers of mice this phenomenon could not be shown to be significant (*χ2* = 0.284, D.F. = 1, *P* = 0.5943).

DISCUSSION

 In this study, we investigated the prevalence and genotypes of *T. gondii* in natural populations of wood mice (*Apodemus sylvaticus*) in an area relatively free of cats with less than 2.5 cats per km2 (Hughes *et al*. 2008). Despite the absence of cats, we observed a very high prevalence of the parasite, with 34.92% (95% CI: 27.14%-43.59%) of all mice being infected. This is similar to a previous study on a different set of *A. sylvaticus* caught in the same location which found that 40.78% of mice harboured the parasite (Thomasson *et al*. 2011). The results of both studies are, however, in contrast with a study on wild rodents, from Germany, where they found 0% prevalence in field mice, shrews and voles (Herrmann *et al*. 2012). The prevalences in small mammals in Europe vary, depending upon the rodent species, detection technique used and location where the study was conducted. In a similar, PCR based, recent study 0.7% of *Microtus* *arvalis* in Austria were positive (Fuehrer *et al.* 2010). Some serological studies show high prevalences in natural populations of small mammals, for example, in the Ardennes (France), the prevalence in shrews (*Sorex spp*.) was 60%, moles (*Talpa europaea*) 39% and voles (*Arvicola terrestris*) 39% (Afonso *et al*. 2007), with a later study by the same authors showing a lower prevalence in the aforementioned species and only 2.5% in *A. sylvaticus* (Gotteland *et al.* 2014). Higher seroprevalence was also observed in North America in *Peromyscus spp*., (between 23 – 31 %) while in another study the species *M. musculus* and *Microtus californicus* were negative (Dabritz  *et al*. 2008).

Little is known of genotypes of *T. gondii* circulating in wild populations of *Apodemus*. In our study, Type II and III banding patterns were the most common in the infected animals. However, interestingly, detailed analysis of banding patterns indicated that possible mixed strain infections might be occurring in this population of wood mice. It is commonplace to assign genotypes using a 10 RFLP system (Su *et al*. 2006; Shwab *et al*. 2013) however; it was not possible for us to assign such a system to these mice due to an inability to consistently amplify the other markers directly from these tissue samples. Furthermore, there was not the opportunity for us to isolate viable parasites to verify to presence of mixed strains. As mixed genotypes have rarely been reported before, this is potentially a very interesting observation but requires future investigation to explore in more detail.

The presence of putative mixed infections observed in these mice could possibly be due to recombinant genotypes produced if the definitive host is present within the transmission system. Interestingly, in the location studied here, this appears not to be the case as cats have been rarely reported (Hughes *et al*. 2008). The routes of transmission operating in this study location are unclear. The putative mixed genotypes are suggestive of infection of the *Apodemus* by ingestion of oocysts. However, if that were the case then we would expect an increase in prevalence with age due to older animals having a greater opportunity to contract the infection. But in our study no significant age prevalence effect was observed, although this could be due to the difficulty in establishing age in wild wood mice or to other confounding effects. Interestingly, also, our data suggest that the infection status of *A. sylvaticus* is more closely linked to genetic population structure rather than location. There are several possible interpretations for this. Firstly, different populations have some genetic differences in susceptibility of infection. As far as we can ascertain, there are no examples of this reported, in wild rodents, although laboratory studies have shown that inbred lines of rodents differ in their susceptibility to *T. gondii* (Li *et al*. 2012; Zhao *et al*. 2013; Lilue *et al*. 2013). Another interpretation is that vertical transmission is occurring and that *T. gondii* is being selectively transmitted through related lineages. In this study, a possibility of addressing this could have been available by tracking parasite genotypes through host genetic populations. However, the mixed genotypes we observed prevented us from testing any association of parasite genotype with host genotype. There is evidence that vertical transmission can occur in rodents (Marshall *et al.* 2004; Hide *et al.* 2009) including in *Apodemus* (Owen and Trees, 1998). Previous studies on sheep (Morley *et al*. 2005, 2008) have shown that when pedigree analysis is carried out on family lineages, there is evidence that the parasite is unevenly distributed amongst families even when they are exposed to the same environment (and therefore, presumably, at the same risk of infection from oocysts). In order to investigate this possibility further in this *Apodemus* population, population genetic data was analysed using a program called COLONY which can use microsatellite genotypes to assign sibling pairs to enable construction of family relationships. Unfortunately, many families were generated suggesting that it would not be possible to get large enough within family sample sizes to statistically address this question. Indeed, there was no significant difference in prevalence of *T. gondii* between families and the very large 95% confidence intervals suggested that this was almost certainly due to small sample sizes (n=2 to n=7) of mice in each family. To conduct future work on such a very detailed family based analysis like this, on natural populations of *Apodemus*, will involve sampling on a considerably greater scale.

Despite the detailed analysis conducted here, it is clear that it will remain difficult to investigate the importance of the different transmission routes of *T. gondii* infection in natural populations of wild animals. The link, reported here, to host genotype is interesting. Whether this reflects some underlying vertical transmission of the parasite or differences in host genetic susceptibility will require further research. It also demonstrates the need for a deeper insight into the mechanics of *T. gondii* transmission, at a localised level, in natural populations of wild animals.

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FIGURE LEGENDS

Fig. 1. The population structure of *Apodemus sylvaticus* in four sampling areas (Spiggot Hill, Tarn Fen, Tarn Woods and Ha Mire) at the study site (Malham Tarn, Yorkshire, UK). The pie charts represent the percentage of each genotype at each location. The cross specifically localizes the sampling site and each colour represents individual populations (R1, R2, B and G). The overall distribution of genetic groups in relation to location is presented.

Fig. 2. Direct PCR amplification of a mixed genotype mouse (106) exhibiting Type I, II and III strain characteristics. Indicated band sizes are in base pairs. Lane 1. Hyperladder 50bp Marker (Bioline). Lane 2. Undigested SAG3 control (no enzyme added) – observed as a 226bp band. Lane 3. Mixture of Type I and III control DNA digested with *AlwNI*. This enzyme cuts only Type II sequences and not Types I or III. No digestion is observed in this mixture of Type I and III controls confirming that they do not contain Type II. Lane 4. Mixture of Type I and III control DNA digested with *NciI*. This enzyme cuts Type I strains twice generating three fragments of 99, 65 and 62bp. These bands are seen in this lane confirming the presence of Type I DNA. *NciI* cuts Type III DNA only once and generates fragments of 161 and 65bp. These bands are also seen in this lane confirming that it also contains Type III DNA and therefore confirms that this control sample is indeed a mixture of Type I and III. Lane 5. Mouse 106 DNA (recorded as a mixture of Type I, II and III) DNA digested with *NciI*. Bands observed at 99, 65 and 62bp confirm the presence of Type I DNA, bands at 161 and 65bp confirm the presence of Type III DNA while the presence of a further band at 226 indicates the presence of non-Type I or –Type III in this sample. Digestion with *AlwNI* confirms this is Type II DNA – see later. Lane 6. Mixture of Type I and III control DNA digested with *AlwNI*. This enzyme cuts only Type II sequences and not Types I or III. No digestion is observed in this mixture of Type I and III controls confirming that they do not contain Type II. Lane 7. Type II control DNA cut with *AlwNI*. This enzyme cuts Type II DNA once producing fragments of 128 and 98bp but does not cut either Type I or III. In this case the presence of bands at 128 and 98bp confirms the presence of the Type II control DNA. Lane 8. Mouse 106 DNA (recorded as a mixture of Type I, II and III) DNA digested with *AlwNI* shows bands of 128 and 98bp, confirming the presence of Type II DNA but also contains a band at 226bp confirming the presence of non-Type II DNA (shown earlier as Type I and Type III DNA).

Fig. 3

Panel A. Estimated population structure using microsatellite genotyping *(K*= 3). Analyses were performed using the admixture model provided by STRUCTURE software (Pritchard *et al.* 2000; Evanno *et al*. 2005). Each individual is represented by a vertical line broken into segments of different colours, with lengths proportional to its membership to each of the clusters (Red, Green and Blue).

Panel B. Evaluation of the robustness of the grouping by STRUCTURE. Summary structure diagrams are shown for K = 3 (see Panel A) and can be compared with diagrams generated when K =4 and K =5. The three main groups remain present despite “forcing” the program to generate more groups. Detailed analysis of the composition of mice confirms the identities of the groupings. (Note, STRUCTURE automatically assigns colours to groups and can result in changes in colour as values of K change).

Fig. 4.STRUCTURE plot showing that population R, when reanalysed by STRUCTURE, consists of two genetically distinct subpopulations, R1 (red) and R2 (green) (*K*=2). Analyses were carried out as described for Figure 3.

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