Toxoplasma gondii: prevalence in species and genotypes of British bats (*Pipistrellus pipistrellus* and *P. pygmaeus*)

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ABSTRACT

Few studies have investigated *Toxoplasma gondii* infections in bat populations and none have reported its presence in protected British bat species. Using a collection of dead/euthanased bats collected from Lancashire, UK, two species of bats (*Pipistrellus pipistrellus* and *P. pygmaeus*) were tested using a highly sensitive SAG1-PCR method specific for detection of *T. gondii* DNA ($n = 77$; 71 *P. pipistrellus* and 6 *P. pygmaeus*). While some potential bias may exist in the sampling strategy, an overall prevalence of 10.39% (± 6.06%; 95%CI) was detected. All *P. pipistrellus*, were also genotyped using eleven polymorphic microsatellite loci to determine their local population structure. The programme STRUCTURE revealed that the majority of individuals (83%) were derived from one interbreeding population, and the remaining individuals (17%) had mixed genetic origins. There was no significant difference in the frequency of *T.gondii* infection or geographical distribution between subclusters. As all British bats are insectivorous, the routes of infection with *T. gondii* remain elusive. However, the locally large and panmictic gene pool suggests that intraspecies transmission could be applicable.

1. Introduction

Toxoplasma gondii is a globally distributed, obligate intracellular parasite with a complex lifecycle. It is of significant medical and economic importance as it causes abortion and congenital disease in humans and domestic animals. Three routes of transmission are thought to occur: ingestion of tissue cysts by intermediate hosts consuming raw infected meat, ingestion of oocysts shed by the feline definitive host into the environment and the transplacental transmission of tachyzoites from mother to foetus (Dubey, 2009). Due to these multiple potential transmission routes, and global distribution, it is highly prevalent and is thought to be able to infect almost any warm-blooded animals if they have the opportunity to encounter an infective stage (Dubey, 2009). This is largely due to the ability of the parasite to propagate both sexually, through the cat as the definitive host, and asexually (Dubey, 2009; Hide et al., 2009).

Bats are one of the most abundant, widely distributed and diverse vertebrate groups, and exhibit a variety of behaviours, including living in close proximity, which may make them highly suitable for parasite transmission and dispersion (Nicholls and Racey 2006). They carry a wide range of pathogens including viruses, fungi, bacteria, protozoa and helminths (Gardner and Molyneux, 1988; Mayen, 2003; Nathwani et al., 2003; Lord et al., 2012). Bats are recognised as important vectors of zoonoses (Nathwani et al., 2003) and as potentially rich sources of novel emerging viruses (Calisher et al., 2008). The recent emergence of the fungal pathogen, White Nose Syndrome, has also been of considerable concern for bat conservationists (Blehert, 2012).

Despite concerted effort in other areas of bat parasitology, driven by emerging infectious diseases in humans, livestock and bat populations themselves, very few studies on *T. gondii* infection in bats have been reported. In addition, despite the close contact between individual bats, there is no obvious mechanism of initial infection that easily complies with the accepted transmission routes (Hide et al., 2009). Using serological techniques, Zetun et al., (2009) failed to detect *T. gondii* in vampire bats (*Desmodus rotundus*) in Brazil, while Smith and Frenkel (1995) did not detect *T. gondii* in the big brown bat (*Eptesicus fuscus*) in Kansas, USA. *Toxoplasma* was, however,

isolated from the tissues of two bat species from Kazakhstan, (Galuzo et al., 1965). In two separate surveys of zoo animals, a serologically positive Indian flying fox was reported in Seoul, Korea, (Choi et al., 1987) and two clinical cases of toxoplasmosis were reported in black flying foxes in Australia (Sangster et al., 2012). Cabral et al., (2013) were the first to isolate and genotype *T. gondii* from bats using bioassays in mice. They found two infected individuals from a collection of 369 bats. Using a PCR detection system, a recent study of 550 insectivorous bats, collected in southern Myanmar, showed a prevalence of 29.3% infected with *T. gondii* (Sun et al., 2013). In another recent study from China (Yuan et al., 2013), *T. gondii* was detected by serology in 18.4% of a sample of 217 bats representing five species. To our knowledge no other studies, as yet, have reported *Toxoplasma* in bats, and no studies have so far been conducted in Europe (but see Dodd et al., 2010, based on preliminary results from a limited subset of animals used in the current study).

The discovery of high prevalences of *T.gondii* in insectivorous bat species (Dodd et al., 2010; Sun et al., 2013; Yuan et al., 2013) raises interesting questions about parasite acquisition. Transmission could occur during contact within roosts, by incidental infection with oocysts from the environment or perhaps by vertical transmission from mother to offspring. These questions could be addressed by investigating the distribution and genetic relationships of *T. gondii* infected bats in a sympatric population. Some recent studies have investigated bat population structure in relation to zoonotic disease infection (Turmelle et al., 2009; Atterby et al., 2010; Smith et al., 2011). None, however, have investigated the population structure of bats in relation to *T. gondii* infection

The objectives of our study were to investigate the prevalence of *T. gondii* in a population of pipistrelle bats in the UK, to investigate host population structure and to investigate the prevalence of infection in relation to any substructuring observed within the bat population.

2. Methods

2.1 Bat sample collection

A collection of two species of British bats *Pipistrellus pipistrellus* (*n* = 71) and *P. pygmaeus* (*n* = 6) was provided by The South Lancashire Bat Group (Registered Charity No. 1109519). These animals were collected over a two year period across the sampling area (South Lancashire, UK). The *P. pipistrellus* (n=71) were collected throughout the Lancashire area with the majority of individuals found between Bolton and Burnley and a few within Manchester city centre. *Pipistrellus pygmaeus* (*n* = 6) were all collected at the same location (Walkden). In UK law, bats are protected species making it extremely difficult to catch and kill bats without very good reason. For this reason, all bats were found dead, later died during rehabilitation or were euthanased by a veterinary surgeon. (We recognise that this approach may have an impact on potential collection bias and the sample size). All procedures were conducted in accordance with UK law and had no impact on conservation (Lord et al., 2012). The heads from the bats were tested by the Veterinary Laboratories Agency (VLA, Weybridge) to screen for the presence of European bat lyssavirus as required by law in the UK. No bats were found to be positive. UK Health and Safety law, required us to keep bat tissues frozen, in a secure location until absence of lyssavirus infection was confirmed, thus preventing from the isolation of parasites for genotyping. The location where the bats were collected was georeferenced to allow distribution mapping. Within the *P. pipistrellus* collection, a total of 28 (39.4%) females and 43 (60.6%) males were represented revealing a non-significant male bias (χ^2 = 2.024, df = 1, *P* = 0.1548). The study received ethical approval from the University of Salford Research Governance and Ethics Committee (RGEC Reference: REP09/095).

2.2 DNA Extraction, PCR detection of T. gondii and DNA sequencing

Due to the large proportion of the brain removed for lyssavirus testing, this study used sections of CNS tissue that were approximately 2 mm^3 in size. For DNA extraction, samples were placed in 400 µl lysis buffer, incubated overnight in Proteinase K and extracted with Tris buffered phenol:chloroform:isoamyl alcohol 25:24:1 pH 8.0 (VWR

International Ltd, UK) as previously described (Thomasson et al., 2011). Extracted DNA was visualised using agarose gel electrophoresis to confirm presence, quality and amount. Quality of extracted DNA samples, for PCR analysis, was ensured by amplification of the mammalian alpha-tubulin gene (F: 5'CGTGAGTGCATCTCCATCCA- 3' and R: 5'GCCCTCACCCACATACCAGTG-3') as previously described (Morley et al., 2005, 2008). The detection of *T. gondii* was based on a nested PCR method targeting the surface antigen 1 gene, (Thomasson et al., 2011). Extensive precautions were taken to avoid PCR contamination as described previously (Williams et al., 2005; Hughes et al., 2008). Bands obtained by nested PCR were sequenced to ensure that they were the correct 519bp product. PCR amplification products were purified prior to being sequenced (Source Bioscience (UK)) using a Geneflow (UK) Q-Spin gel extraction purification kit. The data was compared to published *T. gondii* sequences by multiple sequence alignment using the CLUSTAL W software alongside a reference SAG1 sequence taken from the Genbank database (Accession number: GQ253086.1). Also the BLAST algorithm search (http://www.ncbi.nlm.nih.gov/BLAST/) was used to confirm that sequences derived from PCR amplified bands were SAG1 products.

2.3 Genotyping of Pipistrellus sp.

The population genetic structure of the bat samples was determined using 11 microsatellite loci identified from the literature (Burland et al., 1998; Castella and Ruedi, 2000; Mayer, et al., 2000; Racey et al., 2007; Vonhof et al., 2002). Primer sets and details are shown in Table 1. PCR conditions (Vonhof et al., 2002) consisted of an initial denaturation step (5 min at 94°C) followed by 35 cycles (denaturing at 94°C, 30s; annealing at 52°C, 30s; extending at 72°C, 30s) and a final extension step (72°C, 30 min). The final PCR reaction volume was 25µl and contained 1µl of DNA sample, 2.5µl of 10x reaction buffer (Bioline Ltd, UK, 160 mM $(NH_4)2SO_4$, 670mM Tris-HCl (pH 8.8 at 25° C), 0.1 % stabilizer), 0.25µl of 25mM of each dNTP (except microsatellite *P*219, for which we used 15µl), 2.5µl of 10pmol/µl of each primer (except microsatellites *P*219 and Paur05, for which we used 1.25µl) and 0.25µl of Taq (5 units/µl). We used 2µl of 50mM $MgCl_2$ for microsatellite primers *EF*1 and *P*13, and 1µl for all others. All reaction

mixtures were made up to 25µl with sterile molecular grade water. The samples were genotyped using an ABI3130 genetic analyser (Applied Biosystems) and procedures were carried out according to the manufacturer's instructions. Serial dilutions of test PCR products were conducted and run on the genotyper to ascertain optimal concentrations for the best peak discrimination. Using these test PCR products as indicators of optimal concentration, gel electrophoresis was used to demonstrate successful amplification and to judge the dilution factor needed for sample PCR products for loading on the ABI3130. Once individual sample PCR products were diluted with an appropriate amount of molecular grade water, as judged by gel electrophoresis, they were combined with up to 4 other PCR products for genotyping. Discrimination of specific microsatellites was achieved by using combinations with different expected allele sizes and different fluorescent dyes attached to primers. In addition to 1µl of combined PCR products, 0.1µl of GeneScan™ 500 LIZ™ size standard and 5µl of formamide was made up to 10µl of solution with molecular grade water and denatured at 95ºC for 5 minutes (as described in the manufacturer's instructions). The size of each peak was determined via Peak Scanner software version 1.0 (Applied Biosystems). Alleles were then scored initially by eye (true allele size calling) and converted into workable integers (allele binning) using the program Tandem v1.08. Integers were further analysed using the program MicroChecker to check for the presence of scoring errors and null alleles.

2.4 Population genetic analysis of Pipistrellus sp.

Allele ranges and allele frequencies were analysed using Genepop v4.0. Deviations from Hardy-Weinberg equilibrium were quantified using the probability test implemented in MicroChecker. Genepop v4.0 was also used to check for linkage disequilibrium across the loci. Two pairs of loci (ppip04/ppip06 and p217/p219) showed significant linkage disequilibrium suggesting they are, for example, inherited within the same haplotype block. The lack of linkage disequilibrium in all other loci, taken together with successful previous use of ppip04/ppip06 and p217/p219 (Mayer et al., 2000; Racey et al., 2007), suggested that the obtained data were suitable for analyses. The individual samples were assigned to clusters using the algorithm implemented in STRUCTURE

2.3 (Pritchard et al., 2000). The lengths of the burnin period and the Markov Chain Monte Carlo (MCMC) repetitions were both set to 100,000.The ancestry model was set to the 'admixture model' as the samples were not expected to be a fully discrete population. The allele frequency model was set to 'independent model' as recommended (Pritchard et al., 2000). Separate simulations were run for models with different numbers of assumed genetic clusters (K = 1 - 7) averaged over 3 runs. The most likely K was inferred from graphical representations of K values against the estimated log posterior probability following recommendations of Evanno et al., (2005). Individuals were assigned to a cluster based on proportion (>50%) of membership (q) to the assigned cluster.

2.5 Statistical analyses

Comparisons of observed and expected data were conducted using a Chi- squared test with P<0.05 indicating significance. Tests of association were conducted using 2 x 2 contingency tables and Fishers Exact Test using P <0.05 to indicate significance. Analysis of distribution of bat collection locations were estimated using a Dispersion Index based on the variance to mean ratio (VMR) method using 16 non-overlapping replicates. Indices of dispersion were interpreted as follows: clumped >1; random = 1 and uniform = <1.

3. Results and Discussion

T. gondii was detected in eight (10.39% ± 6.06%; 95%CI) of 77 bats. Seven of the 71 *P. pipistrellus* individuals (prevalence 9.9% $\pm$7.0%; 95%CI) and one of the six *P. pygmaeus* were positive (prevalence 16.67% $\pm$29.8; 95%CI). Five infected individuals were female (four *P. pipistrellus* and one *P. pygmaeus*) and three were males (all *P. pipistrellus*).

To confirm that the PCR amplicons of the SAG1 gene amplification were indeed the correct products, they were sequenced. With only a single base pair difference from a Genbank database entry it was confirmed that the amplified products were the correct products and of *T. gondii* origin. The sequence of one of these, amplified from bat SA/06/05 (a female bat collected in Rochdale, UK), was deposited as accession number JX013908 in GenBank.

There was no significant association between *Toxoplasma* infection and sex (Fisher's exact test $P = 0.2654$) although the sample sizes were probably too small to be meaningful. For each bat, the body condition index (BCI) was calculated as the weight/forearm length ratio (Speakman and Racey, 1986). Four pregnant females were omitted. There was no significant difference between male and female BCIs (P = 0.1086). The mean value of the BCI for *T. gondii* uninfected bats was 0.125 and that for the *T. gondii* infected bats was slightly higher at 0.132 but there was no significant difference between the two populations (P = 0.55). No significant association between *T. gondii* infection and heterozygosity was found (Fisher Exact Test, P= 0.74),

No geographical clustering or significant patterns were observed in the distribution of the collection locations of *Toxoplasma* infected (Dispersion Index (DI), 0.9; random distribution) or uninfected (DI, 3.2; uniform distribution) *P. pipistrellus.*

All 11 microsatellite loci were successfully amplified from the *P. pipistrellus* samples. No evidence of errors caused by erroneously scoring stutter bands or other artefacts was detected. Loci EF4 and P13 showed a significant homozygote excess, which was possibly caused by null alleles. All loci were polymorphic and had between 9 (*Paur*05) and 26 alleles (*Ppip*06). Based on the majority of 9 loci (Table 1) the population was in Hardy-Weinberg equilibrium. The Bayesian assignment test using all 11 loci suggested

K = 4 (ln (PD) mean = -3350.3) as the most likely partition, however consisting of a single dominant cluster, and a small number of individuals with mixed genetic origins. This suggests that the majority of bats (n=59; 83.1%) represent a single interbreeding population. No geographic clustering was observed in the major cluster group (DI, 2.0; uniform distribution) or the mixed genotype group (DI, 1.4; uniform distribution). There was also no significant difference in the *T. gondii* infection prevalence when comparing the major cluster and the mixed cluster (P= 0.33).

There are few reports on the presence of *T. gondii* in bats and our data show an overall high prevalence of infection (10.39% ± 6.06%) in these two major species of UK bats (*Pipistrellus pipistrellus*, 9.9% ±7.0%; *P. pygmaeus*, 16.67% ±29.8%). While previous studies only revealed sporadic detection of *T. gondii*, the present study is among the first to gain prevalence estimates, using PCR, for *T. gondii* infection in bats (see also Cabral et al., 2013; Sun et al., 2013) and the first to relate infection to host genotype or to gain prevalence data in British/European bat species. The prevalences of *T. gondii* infections in these three studies, and our study, are widely divergent, ranging from 29.3% and 18.4% (Myanmar and China) to 10.39% (UK) and 0.54% (Brazil). Large differences in *T. gondii* prevalence have previously been found both in different mammalian species and different geographical locations (e.g. Dubey, 2009). For example, in pregnant women, the approximate average prevalences in China, UK and Brazil are reported to be 10%, 10% and greater than 40% respectively (Gao et al., 2012; Pappas et al., 2009). Interestingly, the opposite is true for bats as the prevalence of infection in Brazil is much lower than China and the UK (Cabral et al., 2013). Thus bats are possibly not a major reservoir or influence on human health and infection. Differences in *T. gondii* prevalence in the four bat studies could be due to factors such as differences in study design, feeding locations, climatic conditions or background levels of *T. gondii* oocyst contamination. We acknowledge that the sample size of our bat collection is small and that, due to using dead or euthanased, injured, bats, has potential inherent bias. However, within the scope of our study, this does not detract from the demonstration of moderately high frequencies of infection in this collection and the presence of *T. gondii* in British bats. There are inherent difficulties with working with legally protected species, such as UK bats, which makes it difficult to gain large,

designed, sample collections from freshly captured and euthanased animals.

Little is known of the role of *T. gondii* in the health of bats or in the transmission to other hosts. In megabats, disease associated with *T. gondii* has been clearly shown (Sangster et al., 2012) however disease in microbats has not been reported. It is possible that by sampling dead or injured bats, we were inadvertently sampling from a population of diseased bats. We were unable to determine tissue cyst burden due to the paucity of remaining brain tissues after lyssavirus testing – which could have given an insight into pathology in the infected bats. We also found no evidence of ill health as measured by body condition index (BCI) comparing infected and uninfected bats. A large number of our bats had been injured by cat attacks there is the possibility – as has been shown in small rodents - that infected bats could be behaviorally attracted to cats.

The mechanism of transmission of *T. gondii* in the cohort of British bats used in this study is not known. The high prevalence found in this study is surprising, as the species screened are insectivorous. Of the three recognised routes of transmission of *T. gondii,* transmission of the bradyzoite stage by carnivory is unlikely due to the insectivorous dietary behaviours of the bats. This leaves oocyst ingestion deposited by cats in the environment (soil or water) and congenital transmission as other likely sources. In this study, many of the collected bats were casualties of incidents with cats. While this brief contact is unlikely to cause direct transmission of *T. gondii* to the bat, it is indicative that these bats are sampled from an area frequented by cats. Pipistrelle bats mainly employ an aerial hawking feeding strategy, form maternity colonies and mating territories in a wide variety of raised shelters and therefore are not frequently associated with the ground during their life cycle. However, during the summer months they do drink frequently from ponds and other water sources which could potentially be contaminated with *T. gondii* oocysts. They may feed on aquatic insects such as midges, caddis flies and mosquitoes by foraging over waterways and marshland, but in the UK tend to forage over hedgerows and open woodland (Swift et al. 1985). As these have aquatic larval stages, it is possible that the aquatic link is significant.

Congenital transmission of *T. gondii* in mammals is generally considered to be rare, although recent studies on rodents have suggested that it can occur at higher frequencies than expected (Hide et al., 2009). This could be a possible mechanism for

propagating the parasite in bats following initial primary infection at low frequency from oocysts. To our knowledge this possibility has not been explored in bats but potentially could be explored by constructing detailed genealogies of bat families from microsatellite population genetic data.

In the population of bats used in this study, a total of 11 polymorphic alleles were identified from 71 individuals (*P. pipistrellus)* to determine their population structure. The program STRUCTURE assigned the bats into groups based on minimising deviations from Hardy-Weinberg Equilibrium. Two groups were identified with a majority group possessing most of the individuals (*n* = 59). The remainder (n=12) had mixed genetic origins. No genetic or geographic clustering of infected bats was observed with five of the seven *T. gondii* infected bats belonging to the major genetic cluster identified. The sampling regime, limited number of STRUCTURE-derived populations and low prevalence was not sufficient to allow exploration of congenital transmission of *Toxoplasma* in this population of bats.

The conclusion, from the genotyping studies, that the majority of these bats derive from the same interbreeding population is puzzling in the light of knowledge of pipistrelle behaviour. Unlike a number of other bat species, for example, *Myotis spp.*, which swarm (and mate) before hibernation, *P. pipistrellus* mating is reported to occur more randomly with males possessing individual courtship territories (Sachteleben and von Helversen, 2006). Our study also illustrates that pipistrelle bats can exhibit low genetic population structuring within the relatively large area occupied by South Lancashire (>300km^2), however, the generality of this observation in the UK is currently unknown. Whether the close genetic relationship is related to the spread of *T. gondii* amongst bats is not known.

The presence of *T.gondii* in insectivorous bats at a reasonably high prevalence is surprising and raises the question as to how they have become infected. The lack of any genetic or geographic substructuring in the population of infected bats points generally towards infection by environmental contamination but further studies are required to establish transmission routes. Apart from data provided by Sangster et al. (2012), there are no studies conducted to date that investigate whether there is a relationship between *T. gondii* infection and bat health. In most mammalian species, *T.*

gondii infection has little detrimental effect on the host (Dubey, 2009) and circulates at high prevalence in host populations. The same situation may also be the case for infection of *Pipistrellus spp.* in the UK.

Acknowledgments

References

Atterby, H., Aegerter, J., Smith, G., Conyers, C., Allnutt, T., Ruedi, M., MacNicoll, A. 2010. Population genetic structure of Daubenton's bat (*Myotis daubentonii*) in western Europe and the associated occurrence of rabies. European Journal of Wildlife Research 56, 67-81.

Blehert, D.S. 2012. Fungal disease and the developing story of bat white-nose syndrome. PLoS Pathogens 8, e1002779.

Burland, T.M., Barratt, E. M., Racey, P. A. 1998. Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross species amplification within the family Vespertilionidae. Molecular Ecology 7, 136-138.

Cabral, A.D., Gama, A.R., Sodré, M.M., Savani, E.S., Galvão-Dias, M.A., Jordão, L.R., Maeda, M.M., Yai, L.E., Gennari, S.M., Pena, H.F. 2013. First isolation and genotyping of *Toxoplasma gondii* from bats (Mammalia: Chiroptera). Veterinary Parasitology 193, 100-104.

Calisher, C.H., Childs, J.E., Field, H.E., Holmes, K.V., Schountz, T. 2006. Bats: important reservoir hosts of emerging viruses. Clinical Microbiology Reviews 19, 531-545.

Castella, V., Ruedi, M. 2000. Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). Molecular Ecology 9, 1000-1002.

Choi, W. Yoo, J.E., Ho-Woo, N. 1987. *Toxoplasma* antibodies by indirect latex agglutination tests in zoo animals. The Korean Journal of Parasitology 25, 13-23.

Dodd, N., Lord, J.S., Brooks, D.R., Hide, G. 2010. An investigation into the prevalence of *Toxoplasma gondii* infection in British bats. XII International Congress of Parasitology, Proceedings 929, Melbourne, Australia.

Dubey, J.P. 2009. Toxoplasmosis of Animals and Man (2nd Edition). CRC Press, Boca Raton, Florida, pp1-305.

Evanno, G., Regnaut, S., Goudet, J. 2005. Detecting the number of clusters of individualsusing the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611-2620.

Galuzo, I.G., Golosov, V.I., Gorbunova, Z.I. 1965. Toxoplasmosis of cattle. *In*: Toxoplasmosis of animals. Alma-Ata 25-65.

Gao, X.J., Zhao, J.Z., He, Z.H., Wang, T., Yang, T.B., Chen X.G., Shen, J.L., Wang, Y., Lu, F.L., Hide, G., Lun, Z.R. 2012.. *Toxoplasma gondii* infection in pregnant women in China. Parasitology 139, 139-147.

Gardner , R.A., Molyneux, D.H. 1988. *Trypanosoma (Megatrypanum) incertum* from *Pipistrellus pipistrellus*: development and transmission by cimicid bugs. Parasitology

Hide, G., Morley, E.K., Hughes, J.M., Gerwash, O., Elmahaishi, M.S., Elmahaishi, K.H., Thomasson, D., Wright, E.A., Williams, R.H., Murphy, R.G., Smith, J.E. 2009. Evidence for high levels of vertical transmission in *Toxoplasma gondii*. Parasitology 136, 1877-85.

Hughes, J.M., Thomasson, D., Craig, P. S., Georgin, S., Pickles, A., Hide, G. 2008. *Neospora caninum*: detection in wild rabbits and investigation of co-infection with *Toxoplasma gondii* by PCR analysis. Experimental Parasitology 120, 255-260.

Lord, J.S., Parker, S., Parker, F., Brooks, D.R. 2012. Gastrointestinal helminths of pipistrelle bats (*Pipistrellus pipistrellus/Pipistrellus pygmaeus*) (Chiroptera: Vespertilionidae) of England. Parasitology 139, 366-374.

Mayen, F. 2003. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock industry and alternatives to an indiscriminate reduction of bat population. Journal of Veterinary Medicine Series B 50, 469-472.

Mayer, F., Schlotterer, C., Tautz, D. 2000. Polymorphic microsatellite loci in vespertilionid bats isolated from the noctule bat *Nyctalus noctula.* Molecular Ecology 9, 2208-2212.

Morley, E.K., Williams, R. H., Hughes, J. M., Terry, R. S., Duncanson, P., Smith, J. E., Hide, G. 2005. Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollias sheep. Parasitology 131, 181-185.

Morley, E.K., Williams, R.H., Hughes, J.M., Thomasson, D., Terry, R.S., Duncanson, P., Smith, J.E., Hide, G. 2008. Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. Parasitology 135, 169-73.

Nathwani, D., McIntyre, P.G., White, K. 2003. Fatal human rabies caused by European bat lyssavirus type 2a infection in Scotland. Clinical Infectious Diseases 37, 598–601.

Nicholls, B., Racey, P. 2006. Contrasting home-range size and spatial partitioning in cryptic and sympatric pipistrelle bats. Behavioral Ecology and Sociobiology 61, 131-142.

Pappas, G., Roussos, N., Falagas, M.E. 2009. Toxoplasmosis snapshots: Global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. International Journal of Parasitology 39, 1385-1394.

Pritchard, J.K., Stephens, M., Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Racey, P.A., Barratt, E.M., Burland, T., Deaville, R., Gotelli, D., Jones, G., Piertney, S.B. 2007. Microsatellite polymorphism confirms reproductive isolation and reveals differences in population genetic structure of cryptic pipistrelle bat species. Biological Journal of the Linnean Society 90, 539-550.

Sachteleben, J., von Helversen, O. 2006.. Songflight behaviour and mating system of the pipistrelle bat (Pipistrellus pipistrellus) in an urban habitat. *Acta Chiropterologica* 8, 391-401.

Sangster, C.R., Gordon, A.N., Hayes, D. 2012. Systemic toxoplasmosis in captive flying-foxes. Australian Veterinary Journal 90, 140-142.

Smith, D., Frenkel, J. K. 1995. Prevalence of antibodies to *Toxoplasma gondii* in wild mammals of Missouri and east central Kansas: biologic and ecologic considerations of transmission. Journal of Wildlife Diseases 31, 15-21.

Smith, G.C., Aegerter, J.N., Allnutt, T.R., MacNicoll, A.D., Learmont, J., Hutson, A.M., Atterby, H. 2011. Bat population genetics and Lyssavirus presence in Great Britain. Epidemiology and Infection 139, 1463-1469.

Speakman, J.R., Racey, P.A. 1986. The influence of body condition on sexual development of male Brown long-eared bats (*Plecotus auritus*) in the wild. Journal of Zoology 210, 515-525.

Sun, H., Wang, Y., Zhang, Y., Ge, W., Zhang, F., He, B., Li, Z., Fan, Q., Wang, W., Tu, C., Li, J., Liu, Q. 2013. Prevalence and Genetic Characterization of *Toxoplasma gondii* in Bats in Myanmar. Applied Environmental Microbiology 79, 3526-3528.

Swift, S.M., Racey, P.A., Avery, M.I. 1985. Feeding ecology of *Pipistrellus pipistrellus* (chiroptera: vespertilionidae) during pregnancy and lactation. Journal of Animal Ecology 54, 217-255.

Thomasson, D., Wright, E.A., Hughes, J.M., Dodd, N.S., Cox , A.P., Boyce, K., Gerwash, O., Abushahma, M., Lun, Z-R., Murphy, R.G., Rogan, M.T., Hide, G. 2011. Prevalence and coinfection of *Toxoplasma gondii* and *Neospora caninum* in *Apodemus sylvaticus* in an area relatively free of cats. Parasitology 138, 1117-1123.

Turmelle, A.S., Olival, K.J. 2009. Correlates of viral richness in bats (order Chiroptera). EcoHealt. 6, 522-539.

Vonhof, M.J., Davis, C.S., fenton, M.B., Strobeck, C. 2002. Characterization of dinucleotide microsatellite loci in big brown bats (*Eptesicus fuscus*), and their use in other North American vespertilionid bats. Molecular Ecology Notes 2, 167-169.

Williams, R.H., Morley, E. K., Hughes, J. M., Duncanson, P., Terry, R. S., Smith, J. E. Hide, G. 2005. High levels of congenital transmission of *Toxoplasma gondii* in longitudinal and cross-sectional studies on sheep farms provides evidence of vertical transmission in ovine hosts. Parasitology 130, 301-307.

Yuan Z.G., Luo, S.J., Dubey, J.P., Zhou, D.H., Zhu, Y.P., He, Y., He, X.H., Zhang, X.X., Zhu, X.Q. 2013. Serological Evidence of *Toxoplasma gondii* Infection in Five Species of Bats in China. Vector Borne Zoonotic Diseases 13, 422-424.

Zetun, C.B., Hoffmann, J.L., Silva, R.C., Souza, L.C., Langoni, H. 2009. *Leptospira* spp. and *Toxoplasma gondii* antibodies in vampire bats (*Desmodus rotundus*) in Botucatu region, SP, Brazil. Journal of Venomous Animals and Toxins including Tropical Diseases. 15, 546-552.