Investigating the role of wild carnivores in the epidemiology of bovine neosporosis

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(*Received 3 June 2012; revised 17 August 2012; accepted 29 August 2012; first published online 15 October 2012*)

SUMMARY

Neospora caninum is a protozoan parasite, primarily associated with bovine abortion. The only definitive hosts discovered to date are carnivores. This study aimed to identify the role of mammalian carnivores in the epidemiology of bovine neosporosis. A sample bank of serum, fecal and brain samples was established: American mink (*Mustela vison*), red foxes (*Vulpes vulpes*), pine martens (*Martes martes*), badgers (*Meles meles*), stoats (*Mustela erminea*), otters (*Lutra lutra*) and feral ferrets (*Mustela putorius*). Approximately 1% of mink and 1% of fox samples were positive by IFAT. According to PCR analysis of DNA extracted from brain tissue, 3% of the mink, 4% of the otters and 6% of the foxes examined were infected with *N. caninum*. All fecal samples tested negative for *N. caninum* DNA ($n = 311$), suggesting that the species that tested positive were intermediate not definitive hosts. This is the first time that tissues from mustelids have tested positive for *N. caninum*. The need to test 2 relatively large (~ 200 mg) targeted parts of the brain to avoid false negatives was also identified. The relatively low prevalence of *N. caninum* in Irish carnivores suggests that the local ecology of a species has an important influence on its epidemiological role.

Key words: *Neospora caninum*, wildlife, carnivore, mustelid.

INTRODUCTION

Neospora caninum is an intracellular protozoan parasite which causes the disease neosporosis. *Neospora* infection is primarily associated with abortions in cattle (Thilsted and Dubey, 1989) and hind limb paralysis in dogs (Dubey *et al.* 1988; Barber and Trees, 1996). It is estimated that world-wide economic losses attributed to *N. caninum*-induced abortions run into hundreds of millions of dollars per year (Dubey *et al.* 2007). An efficacious vaccine is currently unavailable (Reichel and Ellis, 2009). Domestic dogs (Dubey *et al.* 1988), coyotes (*Canis lupus dingo*) (Gondim *et al.* 2004*a*), wolves (*Canis lupus*) (Dubey *et al.* 2011) and dingoes (*Canis latrans*) (King *et al.* 2010) have been identified as definitive hosts to date, while many domestic and wild mammal species have been identified as intermediate hosts (Gondim, 2006). Intermediate hosts become infected either by ingesting oocysts shed in the feces of final hosts (De Marez *et al.* 1999; Gondim *et al.* 2002) or by hunting and scavenging tissues from infected hosts (Gondim *et al.* 2002, 2004*a*). *Neospora* infection can also be transmitted vertically, a common transmission route in cattle and dogs.

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As carnivores are at the top of the food chain, measuring their prevalence of *N. caninum* can give an indication of the presence of *N. caninum* infections lower down the food chain (Jakubek *et al.* 2001; Lindsay *et al.* 2001). However, there is evidence that the level of *N. caninum* infection acquired by a carnivore varies depending on the intermediate host consumed (Gondim *et al.* 2002, 2004*b*; Dubey *et al.* 2007). Levels of exposure to the disease in carnivores vary in different habitats also. For instance, dogs have been found to have higher seroprevalence in rural areas where they have more contact with infected bovine tissue (Basso *et al.* 2001; Antony and Williamson 2003; Lasri *et al.* 2004). By investigating which aspects of a carnivore's ecology are associated with the variation of prevalence between and within different species, we can further improve our understanding of carnivores' role in the epidemiology of bovine *Neospora* infection.

The wild mammal carnivores found in Ireland are red foxes (*Vulpes vulpes*), American mink (*Mustela vison*), European badger (*Meles meles*), Irish stoat (*Mustela erminea hibernica*), European otter (*Lutra lutra*), pine marten (*Martes martes*), feral ferret (*Mustela putorius furo*) and feral cats (*Felis catus*). The aim of this study was to investigate how common *Neospora* infection is in wild carnivore hosts in Ireland and to use this information in conjunction with the knowledge about the ecology of these

Parasitology (2013), **140**, 296–302. © Cambridge University Press 2012
doi:10.1017/S0031182012001588

https://doi.org/10.1017/S0031182012001588 Published online by Cambridge University Press

Table 1. Number of samples collected for each species of animal

Species	Full cadaver	Serum	Brain	Feces	No. of individuals
Fox	109	101	151	91	156
Mink	0	114	197	82	221
Badger	0	51	50	50	51
Stoats	41	34	33	15	41
Otters	36	34	24	25	36
Pine martens	12	9	8	48	60
Feral ferrets	0	4	0	0	4

animals to identify their role in the epidemiology of bovine neosporosis.

MATERIALS AND METHODS

Collection of samples

Cadavers were collected as road kill, donations by anonymous hunters or donations by other research and wildlife organizations. All samples collected except for red foxes were mustelids. Feral cats were not included in this study due to difficulty in distinguishing feral cats from domestic cats. In total, 109 foxes, 197 American mink, 41 stoats, 36 otters, 12 pine martens and 4 feral ferrets were collected throughout Ireland. Road kill was only collected if it occurred within the last 24 h. Animals from hunters were normally shot or trapped during the night and collected the following day. Wherever possible post-mortem and sampling were carried out immediately, otherwise the animal was stored at −20 °C until the post-mortem and sampling could take place. Blood, brain and fecal samples were made available from 52 badgers, sampled throughout Ireland as part of the tuberculosis testing programme. Fifty pine marten scats collected during a national pine marten census (O' Mahony, 2012) and 50 fox heads from throughout Northern Ireland, sampled for a study on rodenticide in non-target animals (Tosh *et al*. 2011) were also made available.

Due to the varying states of condition of the animals collected it was not always possible to retrieve all 3 types of samples required (Table 1). Blood was collected from the body cavities of animals and serum was separated by centrifugation. Fecal samples were extrudated from the rectum. Where it was not possible to obtain enough fecal matter from the rectum, material was extruded from higher up the digestive tract. Whole brain and serum samples were stored at −20 °C, fecal samples were stored at 4 °C until examination.

IFAT of serum samples

Serum samples were tested using a commercial indirect fluorescent antibody test (IFAT) according to the manufacturer's instructions (*Neospora caninum* FA Substrate Slide VMRD Inc, Washington, USA). *Neospora caninum* positive control – fox origin (VMRD Inc, Washington, USA) was used as a positive control and buffer contained within the kit was used as a negative control. FITC-labelled rabbit anti-fox IgG was used as secondary antibody in the fox assays and FITC-labelled goat anti-ferret IgG for all mustelid samples. Antibodies were used at a dilution of 1:80. All serum samples were first screened at a concentration of 1:20 and if found to be positive were repeated at 1:20 and 1:40. The status of all samples that showed fluorescence was confirmed by a second reader. Samples were only considered positive if fluorescence was observed at 1:40. Any positive serum samples were also analysed for the presence of *Toxoplasma gondii* antibodies using a latex agglutination test (LAT) (Toxoreagent, Mast Diagnostics, Bootle, UK) according to the manufacturer's recommendations.

PCR analysis of brain samples

Brains were allowed to defrost at room temperature and weighed. Where possible a 200 mg sample was taken from the mid-cerebrum and another 200 mg sample was taken from the corpora quadrigemina. If the brain was too damaged to confidently identify the mid-cerebrum or corpora quadrigemina 2 × 200 mg samples were taken from any tissue that could confidently be identified as brain tissue. This was the case in 30 out of the 151 fox brains, 91 out of 197 American mink brains, 32 of the 50 badger brains, 8 out of the 33 stoat brains, 13 out of the 24 otter brains and 5 out of the 8 pine marten brains.

Neospora caninum DNA was extracted from the tissue samples according to the methods described by Boom *et al*. (1990) and McLauchlin *et al*. (1999). To avoid any cross-contamination, the utensils used were either disposable or sterilized by flame alcohol between samples. DNA extractions took place in a separate laboratory to the dissections, in a laminar airflow hood that was sterilized between extractions. Although, McLauchlin *et al*. (1999) used zirconia beads for oocyst disruption in feces, it was found that the beads also effectively homogenized the brain tissue and liberated the *Neospora* DNA. Detection of *N. caninum* DNA was carried out by a nested PCR following the method of Buxton *et al*. (1998). The

https://doi.org/10.1017/S0031182012001588 Published online by Cambridge University Press

Table 2. Prevalence of antibodies to *Neospora caninum*, brain tissue with detectable *N. caninum* DNA and oocysts in feces of Irish carnivores ± 95% CI

Species	Seroprevalence	Infected brain tissue	Oocysts in feces
Fox	1% ± 1·9 (n = 101)	6% ± 3·8 (n = 151)	0% (n = 91)[b]
Mink	0·9% ± 1·7 (n = 114)	3·1% ± 3·4 (n = 197)	0% (n = 82)[a]
Badger	0% (n = 51)	0% (n = 50)	0% (n = 50)[c]
Stoat	0% (n = 34)	0% (n = 33)	0% (n = 15)[a]
Otter	0% (n = 30)	4·2% ± 7·93 (n = 24)	0% (n = 25)[a]
Pine marten	0% (n = 9)	0% (n = 8)	0% (n = 48)[d]
Feral ferret	0% (n = 4)	–	–

[a] DNA extracted directly from 200 mg of feces.
[b] All samples examined by microscopy, DNA extracted from concentrated oocysts from 13 samples.
[c] All samples examined by microscopy, DNA extracted from concentrated oocysts from 7 samples.
[d] All samples examined by microscopy, DNA extracted from concentrated oocysts from 2 samples and directly from 200 mg of feces in 6 samples.

targeted region for DNA amplification was the internal transcribed spacer (ITS1) gene region (which can be used to differentiate between *N. caninum, Hammondia hammondi, Hammondia heydorni* and *Toxoplasma gondii,* Dubey and Schares, 2006) using outer primers NN1 (5′-tca acc ttt gaa tcc caa-3′) and NN2 (5′-cga gcc aag aca tcc att-3′) to amplify an external 425 base pair region and inner primers NP1 (5′-tac tac tcc ctg tga gtt g-3′) and NP2 (5′-tct ctt ccc tca aac gct-3′) to amplify the internal 249 base pair region. DNA extracted from placental tissues from aborted calves (kindly provided by Dr Luis Miguel Ortega Mora) was used as the positive control; negative controls were performed in the absence of template DNA. Extractions and PCR on the positive control tissue yielded *N. caninum* DNA each time they were carried out.

The amplification was carried out in a thermal cycler with 50 μl of a reaction mixture consisting of 0·15 μM of each primer, 0·2 mM of dNTP mixture, 1·5 mM $MgCl_2$, 400 ng/μl BSA, 1X Buffer (GoTaq Flexi buffer, Promega), 2 units/50μl GoTaq DNA polymerase, Promega and 4 μl of DNA template for the external amplification. The reaction mixture was prepared in a cabinet that was sterilized between preparations with UV light to prevent contamination. For the internal amplification the concentration of the primers was increased to 0·2 μM and the DNA template concentration reduced to 2 μl. Cycling conditions for the first amplification step consisted of an initial denaturation at 95 °C for 5 min followed by 45 cycles of denaturing at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. The second amplification step was identical except that both annealing and extension times were shortened by half. For both PCR assays the final extension was carried out at 72 °C for 5 min.

The presence of the *N. caninum* product was visualised by a 2% agarose electrophoresis gel using SYBR Safe DNA gel stain fluorescence (Invitrogen). PCR was repeated on all positive samples to ensure they were not a result of contamination. All positive samples were purified using a High Pure PCR product purification kit (Roche) and sent to be sequenced (GATC, Germany) for confirmation that they were *N. caninum* and not another cross-reactive species.

PCR analysis of fecal samples

The methods employed to test the fecal samples for *N. caninum* depended on the amount of fecal matter available. If less than 1·7 g of faecal material were present in the sample, DNA was extracted directly from 0·2 g of the feces. If over 1.7 g of faecal material were available, samples were first screened microscopically for the presence of *N. caninum* oocysts following concentration by flotation in Sheather's sugar solution (sp. gr. 1·18 at 4 °C). If oocyst-like structures were detected, DNA was extracted and screened using PCR. In the case of otter fecal samples, DNA was extracted directly from 0·2 g as its jelly-like consistency prevented reliable flotation of oocysts. The DNA extraction technique and PCR assay used were the same as that used to test the brain samples.

The association between the relative frequency of animals of different species being infected was examined using a Chi squared test.

RESULTS

IFAT of serum samples

One (0·99%) of 101 red foxes and 1 (0·88%) of 114 American mink tested positive for antibodies against *N. caninum* using IFAT (Table 2). None of the other samples were positive. One badger serum sample that gave a positive signal at 1:20 but was negative at a 1:40 dilution was considered negative. The fox sample was *T. gondii* positive with a titre of 1:128, the mink sample tested negative for *T. gondii* antibody.

https://doi.org/10.1017/S0031182012001588 Published online by Cambridge University Press

Negative and positive controls never resulted in false positives or false negatives.

PCR analysis of brain samples

Of the 151 fox brains tested *N. caninum* was detected in 9 (5·96%, Table 2). The majority of positive foxes came from Co. Galway where most of the fox samples (n=70) originated. Of the 197 mink brains tested *N. caninum* was detected in 6 (3·05%, Table 2). Only 1 (4·16%) otter out of 24 was found to be positive (Table 2). This animal had been found as road kill on a coast road outside Dungarvan, Co. Waterford. *Neospora caninum* DNA was not detected in any of the other species sampled (Table 2). *Neospora caninum* DNA was not detected in the brains of the fox or mink from which antibodies against *N. caninum* were detected by IFAT. No significant association was detected between the species that tested positive and prevalence (χ^2=1·7651, 2 D.F; P>0·05).

Out of all the 16 infected brains identified, 2 were positive at both the mid-cerebrum and the corpora quadrigemina (12·5%). In 6 (37·5%) only the corpora quadrigemina, but not the mid-cerebrum sample, were positive. While in 5 (31·25%) the mid-cerebrum, but not the corpora quadrigemina sample, was positive. The remaining 3 (18·75%) positive samples were too damaged to confidently identify the different parts. In 2 of these only 1 sample was positive. Negative and positive controls never resulted in false positives or false negatives.

PCR analysis of fecal samples

Neospora caninum DNA was not detected in any of the fecal samples tested (Table 2). Although oocysts, resembling *N. caninum,* were detected in 28 of the fecal samples this could not be confirmed by PCR. Negative and positive controls never resulted in false positives or false negatives.

DISCUSSION

Brain tissue was used in the present study because Ho *et al*. (1997) and Dubey and Schares (2006) identified it as the most successful tissue to identify the presence of *N. caninum* in infected animals. In only 3 (18·75%) of the 16 positive brains could *N. caninum* DNA be detected in both regions, the corpora quadrigemina and the mid-cerebrum. Therefore, studies testing only 1 area of the brain could result in an under-estimation of the prevalence of *N. caninum*. Hughes *et al*. (2008) found these 2 parts of the brain to be most frequently positive in rabbits. The only other molecular study of *N. caninum* in the brains of Irish foxes, besides the present study, did not detect the presence of the parasite in 148 brains examined (Murphy *et al*. 2007). The targeting of 2 specific regions of the brain in the present study may account for the identification of positive animals. De Marez *et al*. (1999) found that *N. caninum* did not have a uniform distribution in the tissue of its host. Ho *et al*. (1997) recommended that at least 3 samples of brain tissue from the 1 cow should undergo PCR amplification.

This is the first time that *N. caninum* has been identified in the tissue of a European otter. Five European otters previously tested for *N. caninum* antibodies (Sobrino *et al*. 2008) and 1 tested by molecular methods (Hůrková and Modry, 2006) were all found to be negative. The positive European otter in this study was found on a coastal road in Dungarvan, County Waterford. Attempts to ascertain the relative susceptibility of otters living on the coast to infection of *N. caninum* are difficult, as this was the only otter of the 33 tested that came from a coastal area. As the European otter is threatened internationally (Marnell *et al*. 2009) a more thorough investigation of the possible exposure of the European otter to disease as a consequence of food and habitat choice is warranted.

That red foxes were found to be hosts of *N. caninum* in Ireland is not surprising as their most common prey items in Ireland, rabbits and other small mammals, have also been shown to be a host by molecular methods (Hughes *et al*. 2006, 2008; Ferroglio *et al*. 2007; Jenkins *et al*. 2007; Thomasson *et al*. 2011). Carrion, which was also identified in the stomachs of foxes used in this study (Whelan, 2008), is a potential source of infection. Dogs have been observed to become infected after the ingestion of naturally infected bovine placenta (Dijkstra *et al*. 2001). As foxes are commonly observed to take afterbirths during calving (Sleeman *et al*. 2008) and exploit seasonally available food resources (Hewson, 1984), the placenta could be a very important source of infection for foxes.

Gondim *et al*. (2002) reported that dogs fed infected calf tissue produced significantly more oocysts that those fed infected mice, suggesting that calves are a more efficient intermediate host. Gondim *et al*. (2004*b*) and Sobrino *et al*. (2008) found higher seroprevalence rates in wolves than smaller canids and speculated that this was related to the greater proportion of ungulates in the diet of wolves. Although experimentally infected mice have been shown to infect dogs that ingest them (McAllister *et al*. 1998; Lindsay *et al*. 1999), it may be that parasite burdens in naturally infected small mammals are too low to make them an efficient source of infection. The low prevalence of *N. caninum* observed in foxes in Ireland may be a result of small mammals making up a greater proportion of their diet, in comparison to scavenging from ungulates carcasses or placenta (Fairley, 2001).

This is the first time that *N. caninum* has been identified in the tissue of an American mink. As this

https://doi.org/10.1017/S0031182012001588 Published online by Cambridge University Press

species' global range is still increasing (Ibarra *et al.* 2009), it could introduce *Neospora* infections into new areas and expose immunologically naive animals. The lack of any positive inland European otters in this or other studies (Hůrková and Modry, 2006; Sobrino *et al.* 2008) suggests that the mink may not be coming into contact with *N. caninum* when hunting in fresh water but, instead, they become infected when searching for food in terrestrial habitats.

Two of the 9 foxes and 1 of the 6 mink that were positive for the presence of *N. caninum* in their tissue were from the Boora Bog in County Offaly. In contrast, none of the 38 stoats sampled from that area were positive for *N. caninum*. However, this does not necessarily indicate that stoats are not susceptible to infection by *N. caninum*. Polecats and other mustelids have been found to be seropositive (Dubey *et al.* 2007) and both otters and American mink have been shown to be hosts for *N. caninum* in this study.

Foxes and mink both generally have greater territory sizes than the stoat. On the other hand, the pooled territory size of the high number of stoats sampled from the area would be expected to compensate against this. Irish stoats primarily prey on small mammals (Sleeman, 1992; Fairley, 2001). That a specialist predator on small mammals, from an area where *N. caninum* is known to occur, were not infected, further suggests that naturally-infected small mammals may be inefficient intermediate hosts, or not hosts at all.

Similarly, the lack of *N. caninum* infection observed in pine martens and badgers may be linked to their omnivorous diet in Ireland (Fairley, 1967; Boyle and Whelan, 1990; Fairley 2001; Cleary *et al.* 2009), thus limiting their exposure to *N. caninum* (Melo *et al.* 2002). Hůrkova and Modry (2006) also did not detect *N. caninum* DNA in badgers or pine martens. In contrast, Sobrino *et al.* (2008) found both badgers and pine martens to be seropositive for *N. caninum* in Spain. This may be due to habitat differences between Spain and Ireland, or the presence of wolves in this area of Spain. Wolves being a definitive host for *N. caninum* (Dubey *et al.* 2011) and a provider of potentially infected ungulate carrion, for animals that are ordinarily unable to prey on deer (Selva, 2004).

The seropositive mink identified is the first case with detectable antibodies against *N. caninum* discovered in an American mink. One seropositive fox was also detected. Both originated from regions where *N. caninum* DNA was also detected in other animals. The *N. caninum* seropositive mink sample was negative for *T. gondii*, while the seropositive fox sample was also positive for *T. gondii*. However, these two parasites are antigenically different (Dubey *et al.* 2007) and no significant cross-reactivity between *N. caninum* and *T. gondii* when using an IFAT has been observed in other studies (Dubey *et al.* 1996; Buxton *et al.* 1997). The seroprevalence for antibodies to *N. caninum* in 0·99% of foxes observed is similar to the low seroprevalence of 1·4% (Wolfe *et al.* 2001) and 3% (Murphy *et al.* 2007) previously recorded in red foxes in Ireland. These findings are similar to the low seroprevalence in red foxes found in the UK (6% Simpson *et al.* 1997; 2% Barber *et al.* 1997; 0·9% Hamilton *et al.* 2005). Interestingly a low prevalence of *T. gondii,* a biologically similar parasite was also found in red foxes in the UK (Smith *et al.* 2003). That none of the ferrets were found to be positive may be a result of the small sample size ($n=4$) as polecats (the wild type of ferrets) have been found to be seropositive in Spain (Sobrino *et al.* 2008).

None of the animals that were found to be *N. caninum* positive when testing DNA extracted from their brains, were seropositive. Many animals available to this study were already frozen and the thawing process may have resulted in the degradation of the serum (Anderson *et al.* 2007) resulting in false negatives. Consequently, these results should be viewed as lower estimates of seroprevalence. Alternatively, antibody levels in the animals that tested positive for *N. caninum* DNA may have been below the levels of detection by IFAT. *Neospora caninum* seroprevalence has been observed to increase at lower dilutions in wildlife samples examined by Wapenaar *et al.* (2007). There are no recommended cut-off values for *N. caninum* IFAT on wildlife, but a 1:40 dilution is the most commonly used (Dubey *et al.* 2007). Finally, it has long been established that *N. caninum* infection may not result in a detectable antibody response (De Marez *et al.* 1999). For instance, experimentally infected dogs shedding *N. caninum* oocysts and cattle with PCR detectable *N. caninum*-infected tissue have been found to be seronegative (McAllister *et al.* 1998; Lindsay *et al.* 1999 and Dijkstra *et al.* 2001; Ho *et al.* 1997; De Marez *et al.* 1999). The lack of seroconversion, even in experimentally infected animals, highlights how difficult it can be to detect the infection in naturally infected animals. Jenkins *et al.* (2007) when investigating natural infections also found a higher number of rats (*Rattus norvegicus*) and feral mice (*Mus musculus*) to be infected with *N. caninum* (40% and 10% respectively) when tested using molecular methods in comparison to results from the same animals when testing for antibodies by *Neospora* aggulation test (NAT) (4·6% and 5·1% respectively).

Based on the findings of this and other studies a nested PCR appears to be more sensitive at detecting *N. caninum* in wildlife than the IFAT test used, particularly when serum samples may have degraded during freezing and thawing, as is often the case when coordinating sample banks of many wildlife species. However, when DNA examinations are not possible serological surveys are a valuable tool in wildlife studies as long as the results are viewed as conservative.

https://doi.org/10.1017/S0031182012001588 Published online by Cambridge University Press

Despite *N. caninum* being detected in the tissue of 3 of the host species examined, none of the fecal samples tested positive for *N. caninum*. Similarly, Almeria *et al*. (2002) reported 10·7% ($n=122$) of red foxes to be positive by PCR for *N. caninum* but none of the fecal samples to be positive.

If Irish wild carnivores are only intermediate hosts, their predation on small, infected mammals could decrease the levels of *N. caninum*-infected animals available for consumption by definitive hosts. Hobson *et al*. (2005), in contrast to other studies, found the sighting of wild canids on the farm to be a factor in a lower risk of *N. caninum* abortion in dairy herds. They speculated that this was associated with wild canids avoiding a farm when farm dogs (a proven definitive host) were present. Moreover, the reduced abortion risk may also have been due to wild canids in the area suppressing the small mammal population. However, as discussed earlier small mammals may not be an efficient intermediate host.

In conclusion, differences in the ecology of animals may be a factor in their levels of exposure to *N. caninum*. The ability of naturally infected small mammals to infect definitive hosts is a key factor to understanding the sylvatic cycle of *N. caninum*. This study supports the theory that naturally infected small mammals may be inefficient intermediate hosts. Mustelids are confirmed hosts of *N. caninum* and therefore should be included in future wildlife studies. The higher sensitivity of molecular techniques means that they should be used instead of serological techniques where possible, particularly when samples have been frozen. Targeting specific areas of the brain when testing for *N. caninum* will further increase the sensitivity of tests.

ACKNOWLEDGEMENTS

The authors would like to thank all the people who made animals available, notably D. Buckley, L. O' Neill, W.I.T Mammal group, D. O'Mahony, U. Fogarty, N. McKenna, K. Buckley, R. Carden, P. Sleeman, D. Tosh and E. MacLoughlin and hunters throughout the country. Thanks to S. Hogan, D. Maguire and A. Lawless for advice on microscopy and photography. P. Stuart was partly funded under the Research Stimulus Fund Programme by the Irish Department of Agriculture and Food. Thanks to four anonymous reviewers for comments on the manuscript.

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