High prevalence of the digenean *Plagiorchis* sp. in the wood mouse *Apodemus sylvaticus* in a periaquatic ecosystem.

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SUMMARY

The prevalence of the digenean *Plagiorchis* sp. was investigated in a natural wood mouse population (*Apodemus sylvaticus*) in a periaquatic environment. Classical identification was complemented with the use of molecular differentiation to determine prevalence and verify species identity. Use of the complete ITS1-5.8S rDNA-ITS2 and partial 28S rDNA gene sequences have confirmed that the species reported at this location was *Plagiorchis elegans* and not *Plagiorchis muris* as reported previously, illustrating the difficulties in identification of these morphologically similar parasites. *P. elegans* is typically a gastrointestinal parasite of avian species but has also been reported from small mammal populations. The occurrence of this digenean in *A. sylvaticus* in the UK is rare however in the area immediately surrounding Malham Tarn, Yorkshire, it had a high prevalence of 23% and a mean worm burden of 26.6±61.5. The distribution of *P. elegans* followed a typically overdispersed pattern and both mouse age and sex were determined to be two main factors to be associated with prevalence. Male mice harboured the majority of worms carrying 688 of 717 recovered during the study and had a higher prevalence of 32.4% in comparison to only 8.7% in the small intestine of female mice. A higher prevalence of 43% was also observed in adult mice compared to 14% for young adults. No infection was observed in juvenile mice. These significant differences are likely to be due to differences in the foraging behaviour between the sexes and age cohorts of wood mice.

Key words: Plagiorchiidae; *Plagiorchis muris*; *Plagiorchis elegans*; wood mouse; *Apodemus sylvaticus*; DNA; aquatic environment.

INTRODUCTION

Wild rodent populations are commonly examined for their helminth assemblages and studies conducted worldwide have revealed a plethora of nematode, cestode and trematode species being harboured by various rodent fauna. In the UK, several commonly identified helminth species have been documented in rodents from different sites. The most frequently reported digeneans of the wood mouse *Apodemus sylvaticus* tending to be *Corrigia vitta* and *Brachylaemus recurvum* typically found infecting the pancreatic ducts and the small intestine of their host respectively (Elton *et al*., 1931; Lewis, 1968; Lewis and Twigg, 1972; Behnke *et al.,* 1999; Abu-Madi 2000). The parasites of *A. sylvaticus* and other small mammals have been extensively studied in populations located in a periaquatic environment at the Malham Tarn Nature Reserve, North Yorkshire, UK, including the discovery of a new parasite species (Allan *et al* 1999; Hughes *et al* 2006, 2008; Rogan *et al* 2007; Hide *et al* 2009; Thomasson *et al* 2011; Boyce *et al* 2012). Specifically, Rogan *et al*., (2007) reported the occurrence of the intestinal digenean *Plagiorchis muris* in the wood mouse *A. sylvaticus* over a 13-year period with an overall prevalence of 16.9%.

The occurrence of *P. muris* within the UK is rare. As far as can be determined, the first report of *P. muris* in the UK occurred in Oxford during a study on the health of a wild mouse population which took place from September 1925 to January 1928 (Elton *et al.,* 1931). During this study the digenean *Lepoderma muris* (syn. *P. muris*) was described from the small intestine of the wood mouse *A. sylvaticus* at a very low prevalence of 0.1%. *L. muris* was furthermore reported in the brown rat *Rattus norvegicus* in Cambridgeshire, UK (Baylis, 1939) and later an occurrence of *P. muris* was recorded in Scotland in 1963 when the digenean was unexpectedly recovered from the intestine of a Scottish Hill sheep during a parasitological necropsy (Fahmy and Rayski, 1963). These studies, including

those of Rogan *et al.,* (2007) were all based on classical parasite identification using morphology and have not benefited from the greater precision available for DNA sequencing analysis.

Members of the genus *Plagiorchis* are cosmopolitan and tend to demonstrate low definitive host specificity. Species of this genus have been previously described from the intestines of reptiles and birds in addition to mammals (Janssen and Bock, 1990; Biserkov and Kostadinova, 1998; Ito and Itagaki, 2003). *P. muris* is no exception. This species was originally described from the small intestine of the black rat, *Rattus rattus* and the brown rat, *Rattus norvegicus* by Tanabe in Kyoto, Japan (Tanabe, 1922) and has since been considered to be predominantly a digenean of wild rodents (Elton *et al.,* 1931; Seo *et al*., 1964; Ito and Itagaki, 2003; Chai *et al*., 2007; Rogan *et al*., 2007). Definitive host variability for *P. muris* has, however been, frequently recorded including that of the domestic dog, *Canis familiaris* in Japan (Saito *et al*., 1995), the feral Japanese raccoon, *Procyon lotor* (Yamada, 2000; Sato and Suzuki, 2006) and the Mexican Greater funnel-eared bat; *Natalus mexicanus* (Perez-Ponce de Leon *et al*., 1996) in addition to several cases of natural avian infection in the USA (McMullen, 1937; Cort and Ameel, 1944; Secord and Canaris, 1993). Typically, *P. elegans* has been considered foremost in the genus *Plagiorchis* for infecting birds (Shimalov, 2002) however this species has also been reported to parasitize several species of rodent, including *A. sylvaticus* (Montgomery and Montgomery, 1990a), the yellow necked mouse, *Apodemus flavicollis* (Hildebrand and Zaleśny, 2009), the striped field mouse, *Apodemus agrarius* (Shimalov, 2002; Hildebrand and Zaleśny, 2009), and the bank vole, *Myodes glareolus* (Hildebrand and Zaleśny, 2009).

Little is known about the exact life cycle of *Plagiorchis spp.* that infect these rodents and, in particular, the identity and role of intermediate hosts. The high prevalence reported in this periaquatic site (Rogan *et al.,* 2007) suggests that the presence of water and aquatic organisms might be key factors. The unique nature of this site and the

development of molecular tools for detection and identification of these parasites presents an opportunity to dissect the ecology of this parasite and its hosts.

In the current study we investigate the rare occurrence of *Plagiorchis muris* at Malham Tarn, Yorkshire, UK (Rogan *et al*., 2007) by examining prevalence, intensity and seasonality of adult stages collected from rodents trapped at defined woodland sites around this upland lake. We furthermore highlight the difficulty in distinguishing *P. muris* and *P. elegans* in the absence of molecular tools and investigate life cycle indicators within this periaquatic environment.

MATERIALS AND METHODS

The study was carried out at Malham Tarn Nature Reserve located in North Yorkshire, Northwest England at an altitude of 375m above sea level and is an area that has previously been investigated for a range of host parasite systems (Kennedy and Burrough, 1978; Allan *et al*., 1999; Hughes *et al*., 2006, 2008; Rogan *et al*., 2007; Behnke *et al*., 2009; Hide *et al*., 2009; Thomasson *et al*., 2011; Boyce *et al*., 2012).

Rodent trapping and examination was conducted according to the methods described by Boyce *et al*., (2012). In total 117 wood mice (*Apodemus sylvaticus*) and 63 voles (54 bank voles *Myodes glareolus* and 9 field voles *Microtus agrestis*) were trapped from four sampling sites around Malham Tarn: Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation between January 2010 and October 2011 (**Figure 1**). A permit was granted by the National Trust to allow sampling within the boundaries of the reserve. Plantations within these boundaries are represented by Tarn Woods, Ha Mire Plantation and Spiggot Hill. Ownership boundaries form part of the reserve perimeter restricting sampling in part. Tarn Fen is an area of raised bog covered by deciduous woodland located within the reserve boundaries. As water is considered to be an important aspect in

the life cycle of *Plagiorchis* these four trapping sites were selected in order to cover a vast area of woodland throughout the reserve, which is located in close proximity to each of the tarn's borders.

Morphological examination of the digenean specimens recovered from the small intestine of *A. sylvaticus* was carried out according to the methods described by Boyce *et al*., (2012). The majority of the specimens were fixed in 70% ethanol suitable for molecular analysis. DNA was extracted from 12 individual worms collected from different host specimens of *A. sylvaticus* when feasible using a phenol: chloroform method modified from Thomasson *et al*., (2011) which encompassed halving the amount of reagent at each stage of the protocol. DNA was extracted from three individual worms isolated from three different wood mice trapped from each of the four sampling sites. Only one wood mouse however was found to be infected from Spiggot Hill therefore three individual worms from the same host specimen needed to be used in this instance.

The Internal transcribed spacer (ITS), including the ITS1, 5.8S, ITS2 and flanking regions of the 3' end of the 18S and 5' end of the 28S were amplified using the forward universal primer BR ($^{5'}$GTAGGTGAACCTGCGGA$^{3'}$) and reverse digenean specific primer dig11 ($^{5'}$GTGATATGCTTAAGTTCAGC$^{3'}$) according to Tkach *et al.,* (2000a). The partial 28S rDNA gene region was amplified using the forward digenean specific primer dig12 ($^{5'}$AAGCATATCACTAAGCGG$^{3'}$) and the reverse universal primer Lo ($^{5'}$GCTATCCTGAGRGAAACTTCG$^{3'}$) according to Tkach *et al.,* (2000b).

Each 50µl PCR reaction contained 5µl 10X DreamTaq buffer including 2mM $MgCl_2$ (Fermentas, Life Sciences), 0.05µmol dNTPS (100mM, Bioline), 2.5µM forward primer, 2.5µM reverse primer, 5U DreamTaq DNA polymerase and 2µl DNA template. All PCR reactions were performed using a Robocycler 96 PCR machine (Stratagene, CA) and visualised on a 1% (w/v) Tris-acetate-EDTA (TAE) agarose gel stained with gel red using a G: Box gel imaging system (Syngene, UK). The amplification profile consisted of 1 cycle at

94°C for 10 minutes, followed by 35 cycles of 1 minute at 94°C, 1 minute at 54°C and 1 minute at 72°C and one final cycle at 72°C for 10 minutes. The target bands were excised from the gel using a UV transilluminator and purified using a PCR purification kit (Geneflow) according to the manufacturer's instructions. Samples were commercially sequenced in both directions (Source Bioscience, Nottingham, UK). The 12 DNA sequences for the ITS gene were primarily aligned using the multiple sequence alignment program ClustalW (www.genome.jp/tools/clustalw/) to check for sequence homology between specimens from each of the four sampling sites. This procedure was repeated with the 28S rDNA data. In both instances FinchTV trace viewer (Geospiza, Seattle, WA) was utilised in order to verify any regions of ambiguity. The two DNA sequences generated for the ITS and 28S rDNA from Malham Tarn were compared with those held in the National Center for Biotechnology Information (NCBI) database using the BLAST program (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Differences in prevalence observed between sampling sites and seasons were investigated using Chi-squared test for heterogeneity. Host sex and host age were statistically analysed using 2 x 2 contingency tables using Fisher's exact test (http://www.graphpad.com/quickcalcs/ConfInterval2.cfm). For determination of age, rodents were split into three age cohorts according to Behnke *et al*., (1999). Associations between prevalence and host length (cm), weight (g) and rainfall data (mm) were analysed using Spearman's rank of correlation. Monthly rainfall data was provided by Malham Tarn Field Centre. Prevalence calculated during each season over a two-year period was analysed in relation to the previous three months rainfall (mm) adapted from Rogan *et al*., (2007).

In an attempt to identify life cycle indicators of *Plagiorchis* sp. at Malham Tarn, molluscan species were also collected quarterly between January 2010 and October 2011 in order to examine for intramolluscan stages. Several water bodies located within close

proximity to the rodent trapping sites including the tarn margin were selected for analysis. Snails were collected using a D-frame aquatic dip net and kick sample technique and were also hand-picked from the stems of vegetation and underlying surfaces of rocks. Snails were speciated according to Macan and Cooper (1960) and housed in the laboratory in 4 litre glass covered tanks containing pond water. Snails were maintained at 4°C and fed washed lettuce *ad libitum* according to Voutilainen *et al*., (2009). For the examination of *Plagiorchis* sp. intramolluscan larval stages, a snail crushing method was employed according to Caron *et al*., (2008). Binomial confidence intervals on parasite prevalences were calculated (*P* = 0.05, two-tailed test), based on standard methods (http://statpages.org/confint.html).

RESULTS

Five helminth species were recorded from the sampled wood mouse population; *Heligmosomoides polygyrus, Syphacea oblevata, Capillaria murissylvatici, Brachylaemus recurvum* and *Plagiorchis* sp. with species richness varying from 0 to 5 in individual animals. *Plagiorchis* sp. was not detected from any of the vole species despite careful observations.

In total, 717 *Plagiorchis* worms were successfully recovered from the small intestine of 27 of 117 examined wood mice between January 2010 and October 2011. An overall prevalence rate of 23% was determined from all four sampling locations: Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation indicating this digenean species to be dispersed throughout the Malham Tarn area. Worm burden ranged from 0 to 275 with a mean of 26.6±61.5 (717/27) appearing to be typically overdispersed in distribution (Variance to mean ratio (VMR): $\sigma^2/\mu = 158.89$) with 84.6% of wood mice being infected with zero or just a single worm in comparison to just 1.7% harbouring the majority of parasites (>100 worms). A goodness of fit indicated a good agreement with the negative

binomial distribution ($G_{calculated}$ = 21.17, *df* = a – 3 (12 – 3) is 21.67, *p* = 0.01). *Plagiorchis* sp. was identified following the microscopical analysis of 10 fixed and unstained specimens.

The adult worm (**Figure 2**) measured 1.66 to 2.93mm (mean 2.64mm) in length by 0.46 to 0.76mm (mean 0.58mm) in width at the widest part (across the region of the most anterior testis). The tegument possesses minute spines covering the entire surface. The ventral surface appears to be obscured by a vast array of vitelline glands that fail to maintain confluency within the anterior region. The oral sucker is roughly spherical in shape and measures 200 to 300µm in length (mean 256µm) by 200 to 300µm (mean 247µm) in width. The oral sucker lies anterior to the pharynx from which the intestinal bifurcation occurs. The intestine appears short and indistinct and thereafter extends into two very long blindly ending caeca that are often difficult to observe, commonly being masked by a copious mass of vitelline glands, but reaching the near posterior extremity of the body. The ventral gland is smaller in size than that of the oral sucker measuring 130 to 200µm (mean 163µm) in length by 130 to 200µm (mean 163µm) in width. These measurements indicate an oral to ventral sucker ratio of 1.52: 1 (width ratio) – 1.57: 1 (length ratio). Two oval shaped testes are situated posterior to a single ovary. The testes are obliquely positioned with the anterior testis situated slightly right of the median line and the most posterior testis to the left. The ovary lies just posterior to the ventral sucker, separated by the cirrus sac, which curves in a posterior direction along the left hand side of ventral sucker. The metraterm can be visualised to curve posteriorly along the right hand side of the ventral gland adjoining the anterior region of the uterus when not obscured by the vitelline glands. The uterus extends to the posterior extremity of the body presenting a characteristic s-shape that reaches from the region of the ovary and continues intertesticularly towards a posterior vitellarian commissure. The vitelline glands continue along both lateral sides, from the far extremity of the hind body and into the

forebody surpassing the ventral sucker and creating confluency often up to the posterior border of the pharynx. The morphological features described in this section hold a sufficient similarity for both *P. muris* and *P. elegans* therefore the use of morphology alone could be considered ambiguous and the use of molecular differentiation be regarded an important means to complement species classification in this case.

DNA was successfully extracted and amplified from 12 individual *Plagiorchis* worms recovered from the small intestine of 10 *A. sylvaticus* mice trapped from the four different sites. Amplification of the internal transcribed spacers (ITS) including the ITS1, 5.8S and ITS2 generated a sequence of 1213bp (GenBank accession: JX522536). The 12 ITS sequences generated from all four sampling sites were 100% identical. The ITS sequence generated from Malham Tarn was compared against five DNA sequences generated from *Plagiorchis* adults that were held in the NCBI database: *P. maculosus* (AF316152) collected from the Chaffinch, *Fringilla coelebs* (Snyder and Tkach, 2001), *P. elegans* (AF151952) collected from The Red-Backed Shrike, *Lanius collurio, P. koreanus* (AF151944) collected from Kuhl's pipistrelle, *Pipistrellus kuhli*, the common noctule, *Nyctalus noctula* and Daubenton's bat *Myotis daubentoni*, *P. vespertilionis* (AF151949) from *M. daubentoni* and *P. muelleri* (AF151947) from the serotine bat *Eptesicus serotinus* all obtained within the Ukraine (Tkach *et al*., 2000a). The ITS sequence from Malham Tarn shared a 100% sequence homology with that of *P. elegans* with only one omission of adenosine at site 571. This omission was observed in all 12 generated DNA sequences. The Malham Tarn sequence shared only 94% sequence homology with *P. maculosus,* 89% with *P. koreanus*, 91% with *P. vespertilionis*, and 90% with *P. muelleri*.

Amplification of the 28S rDNA gene generated a partial sequence of 1263bp (GenBank accession: JX522535). The 12 28S rDNA sequences generated from all four sampling sites were also 100% identical. This sequence was also compared against five available DNA sequences from the NCBI database: *P. elegans* (AF151911) (Tkach *et al*.,

1999), *P. muris* (AF096222) obtained from the intestine of a rat in the Republic of Korea (Lee *et al*., 2004), *P. muelleri* (AF184250) (Tkach *et al*., 2001), *P. koreanus* (AF151930) and *P. vespertilionis* (AF151931) collected from *N. noctula* in the Sumy region of the Ukraine and *M. daubentoni* in the vicinity of Kiev, Ukraine (Tkach *et al*., 2000b). The partial 28S rDNA sequence available for *P. muris* (AF096222) was only 304bp in length (Lee *et al*., 2004). All other available 28S sequences for *Plagiorchis* species including the sequence generated from Malham Tarn were therefore trimmed and aligned with this sequence for *P. muris*. The 28S sequence generated for specimens collected from Malham Tarn again shared a 100% sequence homology with that of *P. elegans*, 98% with *P. muelleri*, *P. koreanus* and *P. vespertilionis* and only a 95% match with *P. muris* (**Figure 3**). This data in combination with the 100% sequence homology match demonstrated by the ITS region, questions the identity of *P. muris* at Malham Tarn and infers that the species present at this location is in fact that of *P. elegans*. For the remainder of this paper, references to *Plagiorchis* sp. in this study should be read as *P. elegans*. The prevalence of *P. elegans* at this location was examined.

Prevalence was analysed in relation to both extrinsic and intrinsic factors. During the study a comprehensive data set was established which recorded trapping location, date, host sex, host weight and host length. All prevalence data, 95% confidence limits and mean intensities have been summarised in **Table 1**.

To determine whether there was an association with prevalence and a particular trapping site, the rate of prevalence was examined between the four sampling sites using chi squared test for heterogeneity. The greatest prevalence was observed at Ha Mire Plantation in which 37.03% (n = 27) of sampled wood mice carried a mean intensity of 19±24.2 worms (187/10). The prevalence at Tarn Woods which is the original sampling site reported by Rogan *et al*., (2007) was less with only 23.07% (n = 52) of wood mice being infected despite a slightly higher mean intensity of 21±50.33 (246/12). At Tarn Fen, only

four of the 21 examined wood mice were infected with *P. elegans* giving a prevalence of 19.05%. A very low worm burden of just 2±2.5 (9/4) was also observed at this site. Only a single wood mouse was infected with *P. elegans* at Spiggot Hill providing the lowest prevalence of the study at 5.88% (n = 17). This mouse however harboured 275 worms, the highest number recorded during the study. No significant heterogeneity was found between the prevalence of *P. elegans* and any of the four sites ($X^2 = 3.79$, $p = 0.05$, $v = 3$).

As studies of *Plagiorchis* infection in natural rodent populations have demonstrated seasonal variation in prevalence and intensity, we examined this hypothesis at Malham Tarn. Mean prevalence and mean intensity was calculated each season over a two year period. Both mean prevalence and mean intensity were zero during the winter (January) sampling periods (n = 8). Only one mouse was infected during the spring (April) giving a prevalence of 12.5% (n = 8). This mouse however harboured 179 worms. Peak prevalence occurred during the summer (July) when 27.3% (n = 22) of mice carried a mean intensity of 3±2.94 worms (20/6). Prevalence thereafter decreased slightly to 25.3% (n = 79) during the autumn (September/October) however mean intensity increased considerably to 26±61.46 worms (518/20) for this period. Despite observable differences in prevalence no significant heterogeneity was found between the prevalence of *P. elegans* and season ($X^2 = 3.38$, $p = 0.05$, $v = 3$). A Spearman's rank of correlation also indicated a very weak but significant correlation ($r = 0.095$, $P = <0.05$) between the prevalence of *P. elegans* and rainfall for the three months preceding each sampling session (**Table 2**).

In total, 71 male mice and 46 female mice were examined. Prevalence in male mice was greater at 32.4% in comparison to only 8.7% for female mice. Male mice furthermore harboured the majority of worms carrying 688 of 717 recovered with a mean intensity of 30±66.09 (688/23) against only 7±12.5 (29/4) for female mice. A highly significant difference between *P. elegans* infection and *A. sylvaticus* sex was identified ($p = 0.003$).

The data was furthermore analysed to determine whether there was an age-dependent prevalence occurring at this location. *P. elegans* was recorded in a total of 19 out of 44 adult wood mice (43.2%) which was greater than that observed for young adult mice (14.3%, n = 56). No juvenile mice were infected during the present study (n = 17). A similar pattern was observed for mean intensity with adult wood mice harbouring the vast majority of infection carrying 677 of 717 worms recovered. Adult males (n = 34) however carried 650 of these worms in comparison to just 27 worms carried by adult female mice (n = 10). Mean worm burden in the adult group was 36±71.72 (677/19) in comparison to only 5±7.86 (40/8) for young adults and zero for juvenile mice. No significant difference was observed between the juvenile and young adult age categories (p = 0.185) however the difference between adult wood mice and young adults (p = 0.002) and adult and juvenile (p = 0.001) age cohorts was found to be highly statistically significant. Wood mice ranged in weight from 4.0 to 29g and length from 4.5 to 9.8cm. A Spearman's rank of correlation using these data against prevalence gave a very strong correlation in both cases (r = 0.929 P = <0.05 and r = 0.955, P = <0.05 respectively) indicating an age-dependent prevalence to be evident at this location.

In an attempt to identify life cycle indicators of *P. elegans* at Malham Tarn, a total of 2021 snails consisting of 11 species were examined for intramolluscan stages by crushing (**Table 3**). No larval stages of *P.* elegans were found in any of the snails despite careful observation and the frequent detection of other trematode larvae such as *Notocotylus*. Binomial confidence intervals were calculated (P = 0.05, two-tailed test) to establish whether zero prevalence was a significant result using these samples. The results however indicate that none of the examined snail species can be currently ruled out as a potential intermediate host for *P. elegans* with zero prevalence not being significant given the small sample sizes. Despite this for snail species where sample sizes were high, very low prevalences (of less than 1%) are likely. Further investigation is required.

DISCUSSION

In the present study we employed the use of molecular differentiation to investigate further the occurrence of *Plagiorchis muris* at Malham Tarn. Results indicate that the currently identified specimens of *P. muris* at this location are *Plagiorchis elegans*. As far as we can determine, our previous report (Rogan *et al*., 2007) was the fourth known report of *P. muris* in British wildlife (Elton *et al.,* 1931; Baylis 1939; Fahmy and Rayski, 1963) although the conclusion from this study indicates that this trematode was, in fact, *P. elegans*, and also raises the question as to whether other previous studies have correctly identified the species. Elton *et al.,* (1931) reported only a prevalence and Baylis, (1939) simply listed an occurrence of the digenean. Neither author described the morphology of the parasite involved nor was DNA sequencing an aspect of biological surveys at that time. The third report by Fahmy and Rayski (1963) was a short report which provided no written account, but rather incorporated a diagram as a means of description. Unfortunately, the diagram contained insufficient detail to clarify its status as *P. muris* from that of *P. elegans*.

Previously, Fahmy (1954) described a new species of *Plagiorchis, P. lutrae* from the otter (*Lutra lutra)* in Scotland. This new species closely resembled the description of *P. muris* but was differentiated on the basis of size. The measurements of this new species did however coincide with those provided in the original description of *P. muris* described by Tanabe in Kyoto, Japan in 1922; despite such *P. lutrae* was compared with *P. muris* described by McMullen (1937) from Douglas Lake in Michigan State, USA which was much larger in size.

The description of *P. muris* from various locations appears ambiguous. The majority of reports describing *P. muris* from outside of Southeast Asia indicate both a greater range in length and oral to ventral sucker ratio. The description of *P. muris* by McMullen (1937)

was based upon adult digeneans recovered from a range of experimental hosts in addition to a range of naturally infected avian fauna. The average length and oral to ventral sucker ratio of these specimens was however beyond the maximum dimensions reported for Southeast Asian *P. muris* (Tanabe, 1922; Seo *et al*., 1964).

Interestingly, the measurements provided for *P. muris* by McMullen (1937), Rogan *et al*., (2007) and during the current study overlap with the description generated for *P. elegans* from a PhD study conducted by Gorman at Leeds University in 1980. Experimental evidence provided by Gorman (1980) did however identify intraspecific variation within a pure strain of *P. elegans*. The study identified various manifestations in several anatomical structures, not only between different definitive host species but furthermore within the same definitive host including differences in the extent of the vitellaria and aperture shape of both the oral and ventral suckers (Gorman, 1980). Confluency of the vitelline glands have been used on several occasions as a means of differentiation for *Plagiorchis* species (Fahmy, 1954; Tkach *et al*., 2000a) however as pointed out by Blankespoor (1974) and Gorman (1980) use of the glands for diagnosis may not be appropriate due to the intraspecific variation observed in this feature. For instance the vitelline glands of *P. muris* have been reported to extend to either the posterior border of the pharynx (Tanabe, 1922; Hong *et al*., 1996) or the level of the oral sucker (Fahmy and Rayski, 1963; Seo *et al*., 1964; Hong *et al*., 1998). Hong *et al.,* (1996) nonetheless described *P. muris* from a human case of plagiorchiasis using the positioning of the vitelline glands to morphologically differentiate *P. muris* from both *P. vespertilionis* and *P. koreanus*. There was however no mention of *P. elegans* in this report despite this species appearing to display the most morphological similarity to *P. muris* in the distribution of the vitellaria. As far as can be determined there have currently been no reported cases of *P. elegans* infection in either Korea or Japan where *P. muris* appears to be considered the typical dominant *Plagiorchis* species found in rodents.

Currently, there appears to be ambiguity in the criteria used to morphologically differentiate *P. muris* and *P. elegans*. Fortunately, the use of DNA sequencing could be employed in the current study to confirm the identity of the Malham Tarn specimens. The use of the internal transcribed spacer regions and the 28S rDNA gene indicate the specimens recovered from Malham Tarn to be *P. elegans*. Based on these results and taking into consideration the unreliability of morphological differentiation for the two species in question (Blankespoor, 1974; Gorman, 1980; Hong *et al*., 1998), it could be speculated that other reports describing *P. muris* based purely on morphology have also misidentified the species involved. For example, in his report McMullen (1937) commented that the cercariae used for experimental infection possessed seven or eight pairs of penetration glands on either side of the stylet which is a combination typical of *P. elegans* (Faltýnková *et al*., 2007) as opposed to the four pairs originally described for *P. muris* by Tanabe (1922). This morphological description provided for *P. muris* by McMullen (1937) has since been a basis for morphological comparison made by some European authors (Fahmy, 1954; Rogan *et al*., 2007).

Despite the questionable identity over *Plagiorchis* at Malham Tarn, the occurrence of this digenean at this location within the UK is nonetheless considered rare, in particular with a consistent prevalence recorded since 1993 (Rogan *et al*., 2007). Furthermore, the overall prevalence rate of 23% recorded during this study appears to be much greater than that reported in the literature. Other UK reports involving either *P. muris* or *P. elegans* have encompassed very low prevalence rates of 0.1% and 0.05% respectively (Elton *et al*., 1931; Montgomery and Montgomery, 1990a). A further two reports of *P. muris* in the wood mouse *A. sylvaticus* in Ireland have also indicated very low prevalence rates of 1% or less (Langley and Fairley, 1982; O'Sullivan *et al*., 1984). Further afield, Ito and Itagaki, (2003) reported a prevalence of just 1.7% in the large Japanese field mouse *Apodemus speciosus* in Japan and Chai *et al*., (2007) recorded an overall prevalence of 5.3% in the

striped field mouse *Apodemus agrarius* in Korea. *P. elegans* does however appear to be the species reported most often from small mammals within Europe. Hildebrand and Zaleśny (2009) reported a prevalence of 1.3% in the bank vole *Myodes glareolus* trapped in Poland. A single specimen of *P. elegans* recovered from *M. glareolus* in Pallasjärvi, Finland, gave a prevalence of just 0.5% (Tenora *et al*., 1983) and a slightly higher prevalence rate of 3.1% was reported by Shimalov (2002) from *A. agrarius* in Belarus.

The reasons for the occurrence of *P. elegans* at such a high prevalence at Malham Tarn are unclear. Malham Tarn is a 'Site of Special Scientific Interest' (SSSI) boasting a vast array of plant and animal species. The surface area of the tarn is approximately 150 acres with an average depth of 2.4m and a maximum depth of 4.4m in various regions (Woof and Jackson, 1988). Similarly to this study, previous studies have observed the occurrence of *Plagiorchis* in regions of close proximity to significant water bodies (Cort and Olivier, 1943; Cort and Ameel, 1944; Bock, 1984; Hong *et al*., 1999; Hildebrand and Zaleśny, 2009). Being the only upland marl lake of its kind in Britain (Rogan *et al*., 2007), it could be speculated that Malham Tarn itself may play an important role by providing important breeding sites for intermediate host species. Molluscs of the genus *Lymnaea* are the dominant snails acting as the first intermediate host for *Plagiorchis* species worldwide (Tanabe, 1922; Velasquez, 1964; Bock, 1984; Manga-Gonzalez *et al*., 1994; Zakikhani and Rau, 1999; Väyrynen *et al*., 2000; Faltýnková *et al*., 2007). Four species of *Lymnaea* have been recorded at Malham Tarn including *L. stagnalis*, *L. peregra*, *L. palustris* and *L. truncatula* (Norris, 2003) although currently none of these hosts have been positively implicated in the life cycle of *P. elegans* at this location. Negative infections, in all of the 1603 *Lymnaea* specimens examined, suggest that these are unlikely to be intermediate hosts, but this is difficult to absolutely rule out. Insects act as the second intermediate host and dragonflies are the most commonly reported insects found to be naturally infected (Hong *et al.,* 1999). Malham Tarn is home to several species of dragonfly (Shorrock and

Sutton, 2010) however further study is required in order to identify first and second intermediate host species involved in transmission at this location.

In the present study, *P. elegans* was recorded from all four trapping sites and no association between prevalence and rainfall amount was identified suggesting that temporary water bodies may not be an important determinant for transmission and that rather infection is related to the presence of intermediate host species that breed within the tarn body itself. Each trapping site however is separated from the tarn by a narrow shingle beach and earth ridge, terrain that wood mice are unlikely to cross. A crude morphological examination of the stomach contents of *A. sylvaticus* (n = 117) at Malham Tarn demonstrated the presence of adult insect remains suggesting the main source of infection for *A. sylvaticus* to be adult insects infected with metacercariae that may migrate into the home range of the wood mouse following emergence from the tarn body. Other studies have also indicated the diet of *A. sylvaticus* to include various adult insects (Montgomery and Montgomery, 1990b; Khammes and Aulagnier, 2007).

The distribution of *P. elegans* at Malham Tarn demonstrated a typical pattern of over-dispersion with rodent age and sex being the two main factors associated with prevalence. Khammes and Aulagnier (2007) used three age categories to examine the differential diet of *A. sylvaticus*; juvenile, sub-adults and adults and indicated arthropod remains to be more abundant in the stomach contents of adult mice. In the present study, the prevalence of *P. elegans* was significantly greater in adult mice than younger age cohorts. This is likely due to differences in exposure to infective stage parasites through differences in diet as a result of adult mice demonstrating greater foraging behaviour (Lewis, 1968; Lewis and Twigg, 1972).

Several studies have indicated a change in the feeding habits of *A. sylvaticus* from a granivorous diet to one that consumes animal material during periods when seeds are scarce during the spring and early summer months (Lewis, 1987; Montgomery and

Montgomery, 1990b). A study by Montgomery and Montgomery (1990b) in County Down, Northern Ireland, compared the stomach contents of *A. sylvaticus* at two locations. In both sample sets animal material was seen to rapidly decline after September. The typical consumption of insect material by *A. sylvaticus* between spring and autumn coincides with the detectable prevalence period of *P. elegans* in the present study. Other studies have furthermore identified seasonal patterns of infection in natural rodent populations (Chai *et al*., 2007). Seasonality in prevalence is likely due to the developmental cycle of the intermediate host species involved in transmission. Hong *et al*., (1998) demonstrated that up to 96% of *Plagiorchis* worms were expelled from the intestine of albino laboratory rats within 28 days post infection indicating the likelihood of finding adult digeneans from the previous season to be low.

Male mice carried both a statistically higher prevalence and a higher worm burden than their female counterparts. It is likely that the differences observed between male and female and adult and younger adult mice are due to variation in behaviour between the various groups. It has been well documented that the home range of male rodents is much greater than that for female rodents (Langley and Fairley, 1982; Wolton, 1985; Attuquayefio *et al*., 1986), particularly during the breeding season between April and October (Bueshing *et al*., 2007) during which male wood mice have been observed to increase their home range by as much as five times (Corp *et al*., 1997). Male adult wood mice are also much more arboreal than their younger counterparts as well as female wood mice (Buesching, 2007). Arboreality in wood mice is considered to be due to their insectivorous nature, with insects and other small invertebrates often inhabiting the tree canopy. Climbing as a means to acquire food may however be energetically expensive (Bueshing *et al*., 2007) and female wood mice that have a greater dependency on resources have been observed establishing mutually exclusive breeding territories (Flowerdew, 1993) and as such are less likely to become infected due to a reduced

exposure to metacercarial infected insects than adult males that appear more prone to wanderlust.

This study sheds light on the detailed parasite-multihost interactions within this complex periaquatic ecosystem but demonstrates the difficulties associated with understanding these interactions. A key issue is clearly the need for accurate identification of all stages of the parasite, an issue that molecular tools can significantly enhance in particular for species such as *P. muris* and *P. elegans* that are difficult to distinguish by classical morphology alone. The greatest challenges lie in linking the life cycle stages together and to link transmission dynamics to the ecology of the parasites and hosts within the ecosystem. Further studies are required to fully understand these complex interactions.

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793 **Table 1**. Summarised prevalence and mean intensity data for *P. elegans* in *A. sylvaticus*

794 from Malham Tarn. ***** Figure derived from a single host infection.

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	Prevalence (%)	95% Confidence limits		Mean intensity ± S.E.M
		Lower	Upper	
Overall	23.0	16.33	31.54	27 ± 61.5
Location				
Tarn Woods	23.07	13.58	36.28	21± 50.33
Tarn Fen	19.05	7.80	40.59	2 ± 2.5
Spiggot Hill	5.88	0.01	28.92	275*****
Ha Mire	37.03	21.47	55.84	19 ± 24.2
Season				
Winter	0	0	37.22	0
Spring	12.5	0.11	49.22	179*****
Summer	27.3	12.88	48.43	3 ± 2.94
Autumn	25.3	16.96	35.96	26 ± 61.46
Sex				
Male	32.4	22.62	43.98	30 ± 66.09
Female	8.7	2.90	20.86	7 ± 12.5
Age cohort				
Adult	43.2	29.67	57.79	36 ± 71.72
Young adult	14.3	7.16	26.00	5 ± 7.86
Juvenile	0	0	21.63	0

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806 **Table 2.** Correlation of rainfall data and *P. elegans* prevalence (%). Total rainfall over a
807 three monthly period was analysed, using Spearman's rank of correlation, with parasite
808 prevalence Rainfall data was supplied by Malham Tarn Field centre. Numbers in bold are
809 the figures that have been used in the statistical analysis.
810

Month	**Total monthly rainfall (mm)**	**Total three months rainfall (mm)**	**Trapping month/ year**	***P. elegans* prevalence (%)**
2009				
October	128.3			
November	378.9			
December	123.6	**630.8**	January 2010	**0**
2010				
January	074.4			
February	064.4			
March	098.3	**237.1**	April 2010	**0**
April	027.2			
May	021.2			
June	033.5	**081.9**	July 2010	**11**
July	151.4			
August	116.4			
September	296.1	**559.9**	September 2010	**35**
October	121.4			
November	189.1			
December	041.5	**352.0**	January 2011	**0**

2011				
January	150.5			
February	221.4			
March	036.2	**408.3**	April 2011	**20**
April	031.4			
May	126.2			
June	094.1	**251.7**	July 2011	**38**
July	090.6			
August	170.8			
September	157.7	**419.1**	October 2011	**18**
October	NA			

824 **Table 3.** Prevalence of intramolluscan stages found in snail species. Confidence intervals
825 (95%, P = 0.05, two tailed test) were calculated using an online Binomial Confidence
826 Interval calculator (http://statpages.org/confint.html).
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Molluscan species	Number examined (n)	Prevalence (%)	95% Confidence interval	
			Lower	Upper
Lymnaea stagnalis	32	0	0.00	10.89
Lymnaea palustris	58	0	0.00	06.16
Lymnaea peregra	1270	0	0.00	00.29
Lymnaea truncatula	243	0	0.00	01.51
Anisus leucostoma	35	0	0.00	10.00
Bithynia tentaculata	10	0	0.00	30.85
Physa fontinalis	8	0	0.00	36.94
Planorbis sp.	7	0	0.00	40.96
Potamopyrgus antipodarum	188	0	0.00	01.94
Sphaerium corneum	33	0	0.00	10.58
Valvata cristata	137	0	0.00	02.66

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Figure 1. Map of Malham Tarn Nature Reserve and adjacent woodlands. Redrawn from Shorrock and Sutton (2010). **TW:** Tarn Woods; **TF** Tarn Fen; **SP** Spiggot Hill; **HM** Ha Mire Plantation. The dotted line represents the reserve boundary.

Figure 2. *Plagiorchis* specimen recovered from the small intestine of *Apodemus sylvaticus* at Malham Tarn. The drawing was made from a photograph taken with a Leica ICC50 digital camera attached to a Leica DM500 microscope. Abbreviations: **OS**, oral sucker; **P**, pharynx; **C**, cirrus; **CS**, cirrus sac; **VS,** ventral sucker; **M**, metraterm; **O**, ovary; **T**, testis; **Ca,** caecum; **U**, uterus; **V**, vitellaria; **Vc,** vitellarian commissure. **Scale bar = 500µm**.

Figure 3. Comparison of the partial 28S rDNA sequence of *P. muris* collected from Malham Tarn with other *Plagiorchis* species retrieved from the NCBI database: *P. elegans* (AF151911); *P. koreanus* (AF151930); *P. muelleri* (AF184250); *P. vespertilionis* (AF151931)*; P. muris* (AF096222). The comparison was made using the GeneDoc alignment tool. Black shading indicates regions of conserved homology. Grey indicates regions of conservation between four or more species.