Transmission Ecology of Gastrointestinal Trematodes of Small Mammals, Malham Tarn, North Yorkshire, UK

Kellyanne Boyce

Centre for Parasitology and Disease Research,
School of Environment and Life Sciences,
University of Salford, Salford, UK.

Submitted in Partial Fulfilment of the Requirements of the Degree of Doctor of Philosophy, April 2013.

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Acknowledgments

First and foremost this study would not have been possible without the funding and support provided from The University of Salford graduate teaching assistantship program for which I am truly indebted. I would like to give special thanks to my supervisors Professor Philip Craig and Professor Mike Rogan for whom I have much admiration. Your support, advice and expertise throughout the project have given me a positive and inspired experience and I thoroughly appreciate your continued encouragement. I would furthermore like to thank Professor Geoff Hide, who has given me an endless amount of his time and invaluable guidance. I hold you in high esteem.

I would also like to give special thanks to The National Trust for granting the licence which enabled this work to be carried out and to all of the staff at the Malham Tarn FSC Field Centre, and in particular the former warden Mr Adrian Pickles, the current Warden Mr Mike Cawthorn and Miss Kate Martin who has provided me with a great deal of support and assistance during the sampling period. I furthermore have a great appreciation for all the technical support that I have received at The University of Salford, in particular I would like to thank Mrs Helen Bradshaw for always providing a shoulder to cry on during times of stress, and Dr Tony Bodell and Dr Belgees Boufana for not only their technical advice but also for their continued words of encouragement and for just being good friends.

I am also appreciative to Professor Richard Birtles and Professor Jerzy Behnke for advice with species identification and to David Hodgson for providing very important information from the sampling location. I am also very appreciative to fellow PhD students Jaroslav Bajnok for providing data used within this thesis and Michelle Hussein for laboratory and sampling assistance during the summer of 2011, and additionally to the many undergraduate and postgraduate students who have contributed to the autumn fieldwork.

Finally, I have uttermost thanks to my family, especially my sister Cheryl for all her help and assistance during sampling and without whom it would have been very cold and lonely in the Yorkshire dales, My mum and Ever for their patience and my beautiful daughter Svava Ósk for her extreme amount of patience at such a young age for a mum who has been very busy over the past few years, I love you dearly!

Abstract

In 2007, Rogan *et al*. reported the rare occurrence of the digenean *Plagiorchis muris* from the wood mouse *Apodemus sylvaticus* at Malham Tarn Nature Reserve in North Yorkshire, UK. The life cycle of this trematode is not well understood. Therefore, in order to investigate the occurrence of this digenean and attempt to understand its transmission ecology at Malham Tarn, 180 rodents (117 *A. sylvaticus*, 54 *Myodes glareolus* and 9 *Microtus agrestis*) trapped between January 2010 and October 2011 were examined for helminths by dissection. The following helminth species were identified, including the following nematodes (overall prevalence rates and mean intensity in parentheses): *Heligmosomoides polygyrus* (67.5%, 9.2±12.7), *Heligmosomoides glareoli* (3.17%, 44±55.2), *Syphacia stroma* (29.1%, 32.5±37.2), *Syphacia* sp. (11.1%, 15.2±13.2) and *Aoncotheca murissylvatici* (7.6%, 3.0±2.7), the following cestode: *Hymenolepis diminuta* (7.9%, 1.0±0) and the following digeneans: *Brachylaemus recurvum* (1.7%, 2.0±1.4), *Plagiorchis elegans* (23%, 26.6±61.5) and *Notocotylus malhamensis* (14.3%, 65.6±104.6). The use of DNA sequencing of the partial 28S rRNA gene and the complete Internal Transcribed Spacer (ITS1-5.8SrDNA-ITS2) region confirmed the presence of *Plagiorchis elegans* at this location and not *P. muris* as originally reported. *P. elegans* was recorded in the wood mouse population but was not identified from any of the vole species despite careful observations. *P. elegans* was dispersed across the study site and demonstrated seasonal variation that is thought to be linked to the development of intermediate host species involved in transmission. Both mouse age and sex were determined to be associated with prevalence, with adult male mice carrying a statistically higher worm burden and prevalence. These significant differences are likely to be due to differences in the foraging behaviour between the sexes and age cohorts of the wood mouse. *My. glareolus* and *Mi. agrestis* were infected with *N. malhamensis;* this digenean was identified as a new species based upon morphological inspection and DNA analyses of the 28S rRNA gene and the complete ITS region. *N. malhamensis* was not identified from the wood mouse despite thorough examination. The *N. malhamensis* ITS sequence (GenBank: JQ766940) is the first to be published for a *Notocotylus* species and infection of *My. glareolus* furthermore represents a new UK host record. *N. malhamensis* appeared to be site-specific, being identified only from a wetland location with a prevalence of 66.7% and mean intensity of 94.6±119.4 in the bank vole and 50% prevalence and mean intensity of 4.3±2.9 in the field vole. This digenean did not demonstrate seasonality, rather infection is considered to be related to the ecology of this site and its associated flooding. Finally, PCR-based methodologies for detecting larval digeneans within their intermediate host were developed in an attempt to identify the invertebrate species involved in the transmission of both *P. elegans* and *N. malhamensis*. The use of morphological analysis, coupled with DNA sequencing, identified the larval stages of *Haplometra cylindracea* and *Notocotylus* sp. from Lymnaeid snails. However, the larval stages of *P. elegans* and *N. malhamensis* were not identified despite careful observations and the limitations encountered during this process are discussed.

CHAPTER 1:

General introduction

1.1 General overview

Parasitic organisms are ubiquitous in nature and form an integral component of all ecosystems. Parasites typically generate feelings of aversion stemming from the fact that many species cause significant human disease and are furthermore responsible for great economic losses and as such are typically seen to reduce host fitness and represent a poor ecosystem (Marcogliese, 2005). As pointed out by Marcogliese (2005) however, this feeling of abhor is in part, due to the lack of understanding and appreciation for the complex biology involved in parasite transmission and their important role within ecosystems. The concept of ecosystem health is highly complex and in a societal view has been typically regarded as one that is free of disease (Costanza and Mageau, 1999). In reality however, the criteria used for determining the state of an ecosystem may vary according to a particular interest and as such the definition of 'ecosystem health' may be considered ambiguous (Marcogliese, 2005). According to Hudson *et al*. (2006) this definition focuses on the functioning of a whole community, and should be used to describe an ecosystem that is sustainable, in that it can maintain structure and function over time and is resilient to adverse affects (Costanza and Mageau, 1999).

Historically, the first parasitic tapeworms and roundworms were recognised well before the dawn of the Christian era (Dickson, 2000; Cox, 2002). The first recorded description of a trematode species, which is now known to be *Fasciola,* was made by Jehan de Brie, a French shepherd in 1379 (Dawes, 1968). Although parasitic systems have endured and undergone host co-evolution (Koella *et al*., 1988), these systems have in the past been largely overlooked in terms of community ecology. In particular, parasites have been highly neglected in the study of food web dynamics (Hudson *et al*., 2006). As stated by Freeland and Boulton (1990), "Of the 113 food webs summarised, none includes a single parasitic species. This has occurred in spite of all organisms being subject to some form of parasitism, and in spite of there being more species of parasite than any other trophic class." Estimates made by Dobson *et al*. (2008) suggest that there are at least 50% more parasitic helminth species than there are vertebrate hosts worldwide and that as many as 75% of the links in food webs actually involve parasites. They emphasised that the

actual number of parasites could be much higher with many host groups such as fish being hugely under sampled, in addition to the rate of discovery of new species appearing to have grown linearly for some well studied helminth groups (Dobson *et al*., 2008). The reason the role of parasite fauna in ecosystem functioning has been largely neglected is because their relative biomass has been considered to be low in comparison to other trophic groups (Hudson *et al*., 2006). Studies conducted by Kuris *et al*. (2008) in three estuaries on the Pacific coast of California and Baja California however, have indicated parasites to have a substantial biomass in these ecosystems being comparable to that of top predators and interestingly they calculated the annual production of trematode free living larval stages to be greater than the combined biomass for all other parasite species and the biomass for birds.

Nonetheless, in many cases, parasites do not cause significant disease but understanding the transmission dynamics that underpin the parasitic system means that they can be highly informative indicators of host ecology and the interactions that take place within an ecosystem. According to Hudson *et al*. (2006) there is a growing body of evidence that parasite-mediated effects can be significant in shaping community structure by affecting interspecific competition, energy flow and driving biodiversity. In particular, helminth parasites often possess complex life cycles that are dependent upon both environmental stability for the survival of free-living larval stages and trophic interaction for successful transmission (Marcogliese and Cone, 1997). As such, parasite abundance and composition can be reflective of biodiversity, host trophic position and the food web dynamics within any ecosystem (Marcogliese, 2005). Taking these factors into consideration, Hudson *et al*. (2006) regarded a healthy ecosystem to be, "One that is rich in parasite species".

1.2 Parasitic systems

In recent years parasites have become widely investigated in terms of population systems and community links (Granovitch, 1999). For the successful maintenance of a basic parasitic system, a minimum of two populations are necessary, that of the host and that of the parasite (Figure 1.1A). This type of system is typical of species with monoxenous life cycles and strict host specificity. In reality however, more than one host species may be utilised thus several different host communities may be involved in the

life cycle of a single parasite species (Granovitch, 1999). On the contrary, the use of two, three or four hosts may be necessary for the successful development of heteroxenous parasites to their adult form (Figure 1.1B). In such instances, the complexity involved in maintaining 'community links' is greatly increased as successful development becomes dependent upon the interaction between several host communities within a single ecosystem. To a greater extent several spatially separated host populations may furthermore be interlinked by the same parasitic system in which a host species that is capable of a wide spatial distribution is used (Granovitch, 1999). Due to their nature in transmission, monoxenous parasites are far more likely to endure and thrive within ecosystems that are subject to high perturbation than parasites that necessitate a heteroxenous existence (Dzikowski *et al*., 2003).

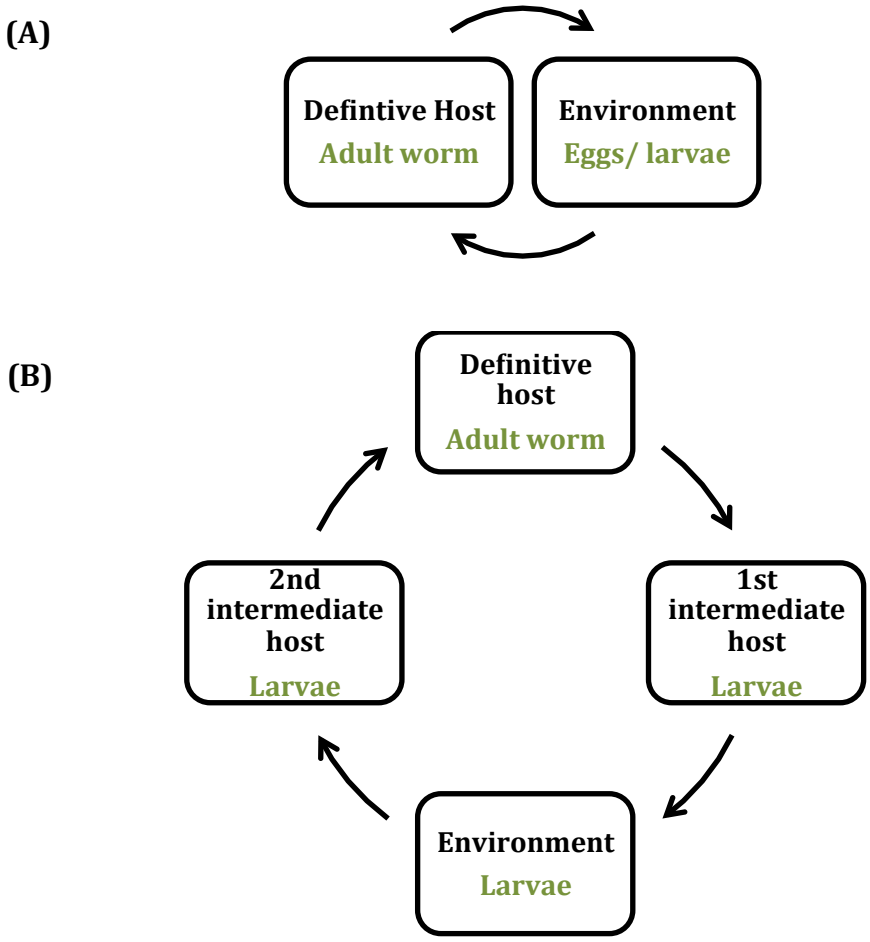


Figure 1.1 Examples of parasite systems. **(A)** In the monoxenous life cycle the parasite infects a single host species. The adult parasite inhabits the definitive host and eggs/larval stages develop within the environment. **(B)** In the heteroxenous life cycle more than one host species is utilised. The adult parasite inhabits the definitive host but larval stages undergo obligatory development within the environment and within up to three intermediate host species.

1.3 Parasites as environmental indicators

Due to the complexity of the heteroxenous life cycle, a vast amount of ecological information regarding the state of an ecosystem can be acquired. Digenean trematodes in particular are a very informative group. These helminths possess a life cycle that involves a highly complex series of transmission events that involves up to three intermediate hosts, is dependent on environmental stability for the survival of free-living larval stages and is reliant upon trophic interactions for successful transmission (Pearson, 1959; Möhl *et al*., 2009). The occurrence of digenetic species within an ecosystem is therefore reflective of the presence of both suitable intermediate host species and the ability of free-living larval stages that are typically considered to be sensitive and fragile (Pietrock and Marcogliese, 2003), to survive and be capable of infecting these hosts.

Marcogleise (2005) emphasised the importance of parasitic systems as key components of ecosystems and concluded that the use of parasitology for environmental assessment and inclusion into biotic keys should be encouraged. Several studies have employed the use of digenetic parasites as key indicators to evaluate ecosystems. Huspeni and Lafferty (2004) used larval trematodes to evaluate a restoration project of a salt marsh. They used a before and after control strategy over a six year period to monitor changes to the larval trematodes infecting the California horn snail *Cerithidea californica.* Following restoration, an increase in trematode prevalence from 12 to 43% and species richness from 4.5 to 9 was observed. It was concluded that this increase was a direct consequence of a larger number of birds and bird species using the salt marsh following the project.

On the contrary, parasitic systems can be used as a means to monitor anthropogenic induced modifications. Aquatic parasitic systems are commonly employed to monitor water pollution and eutrophication. A study by Dzikowski *et al*. (2003) compared a highly polluted marine ecosystem against that of a relatively unspoiled site using differences in fish parasite assemblages. They identified a significantly lower number of trematode metacercariae, an absence of gastrointestinal helminths possessing a heteroxenous life cycle and a lower species richness in fish derived from the polluted site.

It is highly likely therefore that changes observed in parasite composition are reflective of changes within the environment. Not all parasite species will respond similarly however to environmental perturbations. As indicated by Dzikowski *et al*. (2003), the reduction in the species richness of heteroxenous parasites at the polluted site was counteracted by a higher species richness of monoxenous parasites. Therefore, it may become apparent that in some instances, parasite richness may remain consistent although one species that is better adapted to the environmental change may replace another. As such, although species richness is a good indicator for differences between locations it is recommended that changes to specific taxa within an ecosystem furthermore be monitored (Marcogliese, 2005).

Parasite distribution may also reflect changes in host exposure. Host species may undergo niche shifts, by which exposure rates to infective stage parasites can increase or decrease and alter patterns of infection within the population. This was demonstrated in a study by Marcogliese *et al*. (1986) who assessed the effects of eutrophication on the prevalence of *Crepidostomum cooperi* metacercariae, a fish trematode at a shoreline habitat. Eutrophication caused deeper waters to become anoxic forcing the migration of the second intermediate host, the burrowing mayfly *Hexagenia limbata* to shallower water. This niche-shift resulted in the overlap of the mayfly with the first intermediate host, the sphaeriid clam. This change in the spatial distribution of the two intermediate host species resulted in an increased exposure rate of *H. limbata* with *C. cooperi* following release from the molluscan host and ultimately resulted in a significant increase in metacercarial prevalence in the second intermediate host.

Changes in parasite abundance and composition may furthermore be an indication of modifications to host populations present within an ecosystem. The interaction between parasite and host can be highly specific. In such cases, transmission is dependent upon the stable existence of important population systems. Reduced survival of vital host communities could have profound effects upon the continuation of the parasitic system (Granovitch, 1999). For example, molluscs are typically regarded as 'keystone taxa' being inextricably linked to the life history of digenean trematodes (Poulin and Cribb, 2002). Omission, reduction or changes to species present may be reflected in the abundance and composition of digenetic species within the definitive host population.

Alternatively, changes in the environment or to important intermediate host populations may inadvertently increase definitive host exposure, should parasites that demonstrate reduced or a lack of host specificity undergo a host-shift, increasing their dispersion within an ecosystem by infecting animals with which contact may not normally be common (Granovitch, 1999).

1.4 The digenea

The digenea is the largest subclass of the class Trematoda (Bray, 2002) and encompasses the most successful group of internal metazoan parasites (Nolan and Cribb, 2005). The digenea includes 148 recognised families, almost 2800 nominal genera and an estimated 18,000 nominal species (Bray, 2008). As many as 55 described families are parasites of mammals of which only 25 are considered to exclusively infect this group (Bray, 2008). The digenea are primarily intestinal parasites although in tetrapods variants have furthermore been identified extra-intestinally, and may be found inhabiting the circulatory system, lungs, oesophagus, urinary bladder, liver, eye and ovary (Cribb *et al*., 2003).

1.4.1 Morphological classification of the digenea

Traditionally, the methods employed to classify helminth parasites have principally utilised the morphology of the adult structure. Information regarding larval structure, life histories, and physiology and host relationships has only typically been considered when available (Haley, 1962). However, as pointed out by Cribb *et al*. (2003) sexually mature adult specimens of phylogenetically distinct taxa may resemble one another although differentiation may be obvious in their larval stages of development and therefore collection of such data is similarly important for taxonomic purposes. The extent of intra-specific variation in helminth parasites has also been recognised. As such, it is highly recommended that morpho-anatomic measurements of the worm body or its internal organs are not used for species differentiation (Kinsella, 1971). This type of data should be used as a general guideline being considered subjective and only key taxonomic features that remain constant across a species and enable replicable identification should be utilised (Lal, 1935; Simon-Vicente *et al*., 1985a). With respect to the digenea, adult specimens are principally differentiated by the form and positioning of the suckers, the digestive tract and the reproductive system (Figure 1.2). The most

extensive works for digenean trematodes in the UK have been compiled by Dawes (1968), and more recently on a worldwide capacity the 'Keys to the Trematoda' (Gibson *et al*., 2002; 2005; 2008) which is a very useful key for the identification of members of this group, although these keys typically provide information for adult structure and to the genus level only and therefore requires further location of species descriptions made by original authenticators.

With respect to larval stages, it has been recommended that identification based on larval morphology alone should also be considered suggestive, as this approach has furthermore resulted in many identification errors within the literature (Cort, 1914). Classically, identity could only be certain following the experimental rearing of adults from larval stages or by the examination of larvae generated from eggs isolated from adults (Cort, 1914). The study of larval stages however is a more difficult task. Cercariae for example are small in size, highly mobile and have a considerable capacity to change shape (Cort, 1914). This idea of classification based purely on morphology has therefore been subject to much debate (Harper, 1929). In the early cercarial studies, Lühe (1909) used tail morphology to group species. Subsequent researchers however have questioned this approach, which takes the morphology of the larvae only into account and is independent of life history. They have thereafter attempted to group cercariae based upon whether they develop inside sporocysts or redia thus considering life history to be most important (Lebour, 1912). As is evident from the current literature nonetheless, there is still a paucity of information regarding the life history of many digenean species and therefore this approach alone may neither be suitable for the study of many identified trematode species.

In view of this information, many studies now use morphological structures that are modified the least during transition from larvae to adult; essentially the excretory system, as the internal structure of the cercariae is considered to resemble that of the adult into which it will develop (Harper, 1929). In addition to the excretory system, more recent studies have employed several variations in structure that are considered to be of taxonomic importance, and in many instances reflective of the adaptations to a specific lifecycle (SRG, 2010). These features include tail morphology, the number of suckers, the presence/absence and number and positioning of spines, the

presence/absence of a stylet and its structure and the number of penetration glands (Faltynkova *et al*., 2007) (Table 1.1 & Figure 1.3). Other larval forms such as the metacercarial stages can also be extremely difficult to identify and have very often been impossible to differentiate in the absence of experimental studies (Stunkard, 1966). Differentiation based upon the shape, size, wall thickness, and internal structures such as spines, floating stylet following release from the cercarial buccal cavity and the excretory vesicle are all considered important (Faltynkova *et al*., 2007). These details nonetheless can be indicative of genus only, and further speciation using these criteria may be considered an extremely difficult task which in many instances has led to the misclassification of species (Tkach *et al*., 2000a). The advent of molecular tools however has provided an additional method to the classical means of differentiation enabling many of the uncertainties previously experienced to be overcome. These new approaches have enhanced the robustness of species distinctions and furthermore highlighted the many helminth species that have been previously collated as cryptic species (Morgan and Blair, 1995; Vilas *et al*., 2005).

1.4.2 Molecular characterisation of the digenea

Morphological classification remains an important aspect of taxonomic resolution (Poulin and Leung, 2010), however when used in combination with molecular differentiation, a more reliable and robust method for species characterisation is created (Nolan and Cribb, 2005). The molecular analysis of specimens enables digenean species to be compared without the consideration of confounding factors that could typically hinder identification such as age, intraspecific variation, host species and geographical location (Nolan and Cribb, 2005). The use of the ribosomal genes (rDNA) for parasite identification is now well established and these are the most commonly employed targets for trematode studies (Ryu *et al*., 2000; Tkach *et al*., 2000a; Tkach *et al*., 2000b; Dzikowski and Levy, 2009). Ribosomal DNA comprises both coding and non-coding regions. The coding regions have relatively slow evolutionary rates and are therefore highly conserved, which is ideal for the identification of familial relationships. The non-coding regions on the other hand are more rapidly evolving and are therefore appropriate for species level distinction (Nolan and Cribb, 2005). It has been emphasised however that these gene targets should only be used for species discrimination when genetic differences are consistent and when analysed in

combination with both the morphology and the biology of the digenean under study. Several studies have demonstrated the successful combination of such data in the analysis of digenean trematodes (Morgan and Blair, 1995; Jousson *et al*., 1999; Tkach *et al.,* 2000a; Overstreet *et al*., 2002).

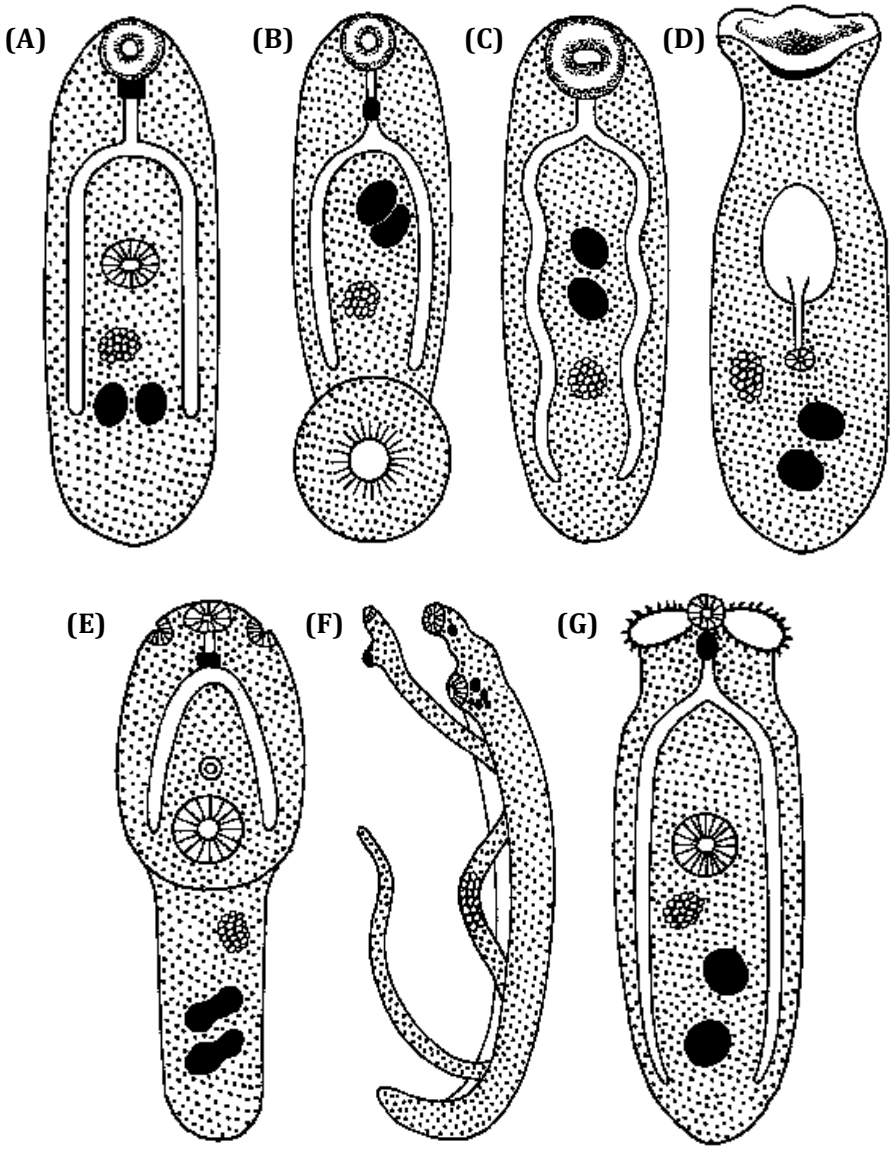


Figure 1.2 Common digenean body plans showing the form and positioning of the suckers, the digestive tract and the reproductive systems. Images adapted from Mehlhorn (2001). **(A) Distome**: this is the most common body plan possessing both an oral and a ventral sucker. The ventral sucker is positioned anywhere on the ventral surface but not at the extreme posterior. **(B) Amphistome**: these have large fleshy bodies that possess a prominent sucker at the posterior end of the body. **(C) Monostome**: the ventral sucker is absent. **(D) Gasterostome**: the gut is very simple and represents a sac like structure attached to a mouth located near to the centre of the body. **(E) Holostome**: the body is obviously divided into two separate regions. The anterior section may hold an additional adhesive organ. **(F) Schistosome**: the only group in which separate sexes are present. The female helminth is enveloped within the gynaecophoric canal of the male. **(G) Echinostome**: these are similar in form to distomes but possess a prominent collar of spines surrounding the oral sucker. This information has been adapted from SRG (2010).

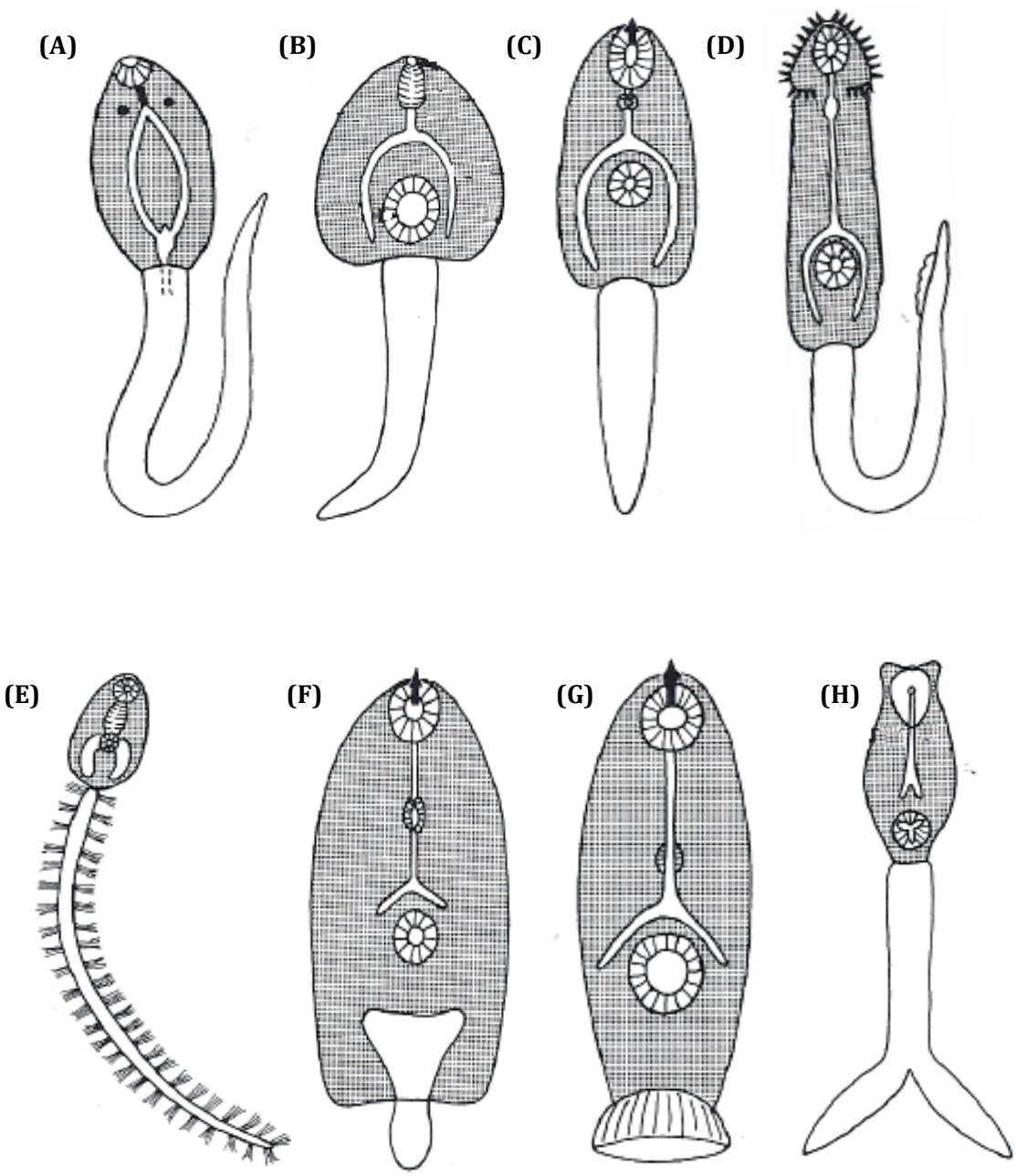


Figure 1.3 The three major groups of cercariae: **(A)** **Monostome cercariae**: The specimens lack a ventral sucker, possess a simple tail and are found to develop within redia. **(B)** **Amphistome cercariae**: The large ventral sucker of these specimens is situated at the base of a slender simple tail. This type of cercariae also develops within redia. **(C - H)** **Distome cercariae**: This is the most commonly identified group and also the most diverse: **(C)** Xiphidiocercariae; **(D)** Echinostome cercariae; **(E)** Trichocercous cercariae; **(F)** Microcercous cercariae; **(G)** Cotylocercous cercariae; **(H)** Furcocercariae. Image adapted from Mehlhorn (2001).

Table 1.1 Cercarial classification of commonly observed groups based on gross morphology. Information compiled from Olsen (1986) and SRG (2010).

Major group	Subdivision 1	Subdivision 2
Monostome cercariae: The specimens lack a ventral sucker and possess a simple tail and are found to develop within redia.		
Amphistome cercariae: The larger ventral sucker of these specimens is situated at the base of a slender simple tail. This type of cercariae also develops within redia.		
Distome cercariae: This is the most commonly identified group and also the most diverse and can be subdivided into seven groups.	**Leptocercous cercariae**: possess straight tails that are narrower in diameter than the body and are further subdivided into three groups:	**Gymnocephalous cercariae**: Possess suckers of equal size and is the most common form being found to develop within redia. **Xiphidiocercariae**: The oral sucker is equipped with a stylet used for penetration of the second intermediate host. These cercariae develop within sporocysts. **Echinostome cercariae**: Possess a ring of spines at the anterior end. These develop within redia.
	Trichocercous cercariae: Possess long tails equipped with rings and bristles. Marine species and develop within redia.	
	Cystocercous cercariae: Possess a highly enlarged tail into which the cercarial body may be retracted. These develop within sporocysts.	
	Microcercous cercariae: Possess vestigial tail and may develop within either redia or sporocyst.	
	Cotylocercous cercariae: Possess short cup-like tails that function as an adhesive organ. Marine species.	
	Cercariaea cercariae: Do not possess a tail, is not free swimming. May develop within either redia or sporocyst.	
	Furcocercariae: Possess forked tail at the end. These develop within sporocyst.	

1.4.3 The complex digenean life cycle

The life cycle and geographical distribution of digenean trematodes is diverse and is considered to be characteristic for each species (Chai, 2007). Despite this diversity, the typical digenean life cycle includes free living and parasitic stages, has sexual and asexual multiplication and furthermore alternates between mollusc and vertebrate hosts (Cribb *et al*., 2003). The complexity of the digenean life cycle is characteristic to this group, typically entailing a series of improbable events. Typically, the life cycle involves three main transmission challenges. Firstly, the miracidium released from the egg must locate and infect a suitable molluscan intermediate host. Secondly, the cercariae released from the mollusc must find a suitable second intermediate host to encyst as metacercariae and lastly the metacercariae must be ingested by an appropriate definitive host (Poulin and Cribb, 2002).

In reality however, there are many deviants from the typical life cycle (Figure 1.4). Poulin and Cribb (2002) in particular have discussed the effects of truncation on these complex pathways emphasising the benefits of a simpler life cycle that is easier to complete. The first possibility of truncation increases the efficiency of transmission by omission of the miracidial stage. In this situation the egg is ingested by the first intermediate host, which is thereafter penetrated internally. This mode of transmission has been well established for the Chinese liver fluke *Clonorchis sinensis* (Komiya, 1992) and evidence suggests that it may be similarly important in the transmission of *Plagiorchis* spp. (Gorman, 1980; Bock, 1984; Zakikhani and Rau, 1992) and *Notocotylus* spp. (Murrills *et al*., 1985).

Murrills *et al*. (1985) examined the internal structure of the eggs of *Notocotylus attenuatus* in some detail determining the absence of the miracidium and the presence of a sporocyst and an opercular cord instead. They suggested that this was indicative of a truncated life cycle and established that just one-day post ingestion, the sporocyst can be found lying freely within the haemocoel of the snail within the vicinity of the intestine. They proposed that this occurred via mechanical injection and that the opercular cord functions to inject the sporocyst through the gut wall of the snail.

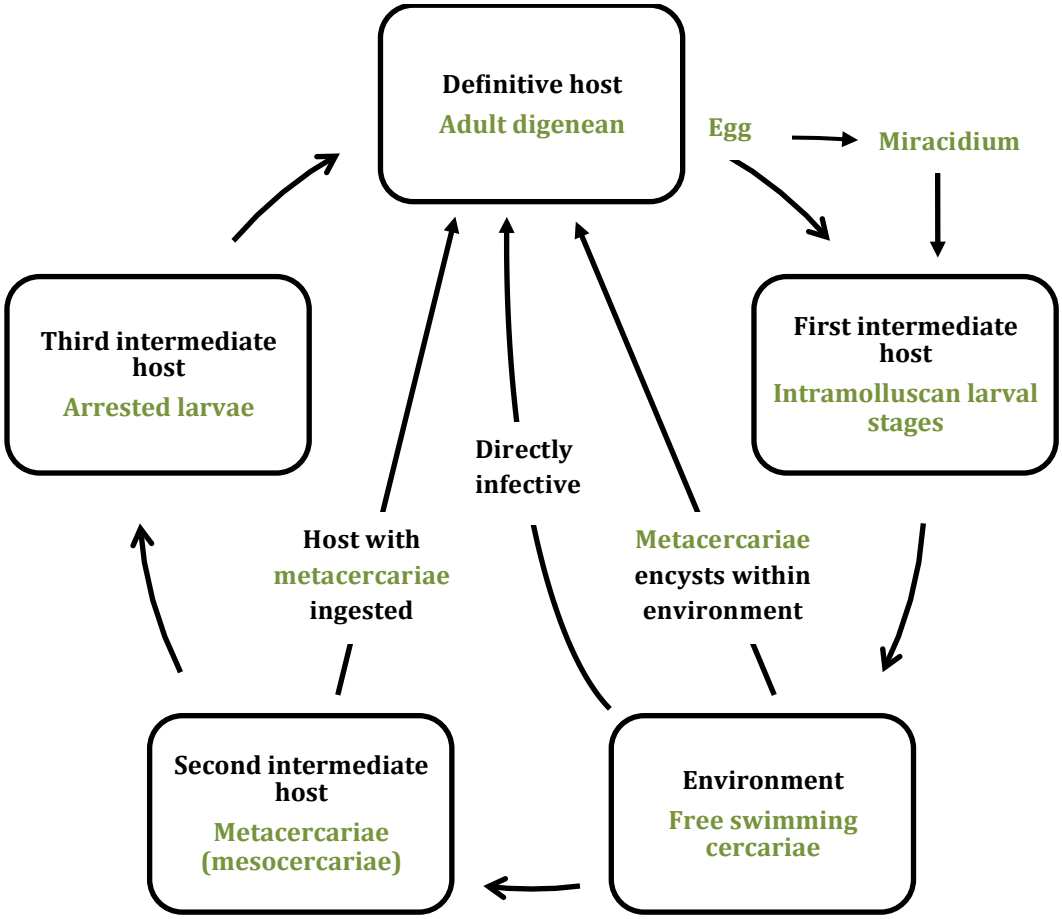


Figure 1.4 The complex developmental pathways that can be followed by digenean trematodes. Image adapted from SRG (2010).

The most common forms of variation however, occur inside of the first intermediate host. The development of intramolluscan generations is highly complex and several common pathways have been recognised (Figure 1.5). Cribb *et al*. (2003) however have indicated the development and the distinction between daughter sporocysts and redia to not be as clear cut as the literature once suggested. This statement has stemmed from the fact that many variations to these common forms have been demonstrated. For example, in the two families Heronimidae and Bucephalidae, it is thought that only the mother sporocyst stage occurs (Cribb *et al*., 2003). Dönges (1971) furthermore demonstrated how the redia of echinostomatids that were producing cercariae, were capable of reverting back to producing more redia when transplanted into a non-infected molluscan host. A further example contributing to this confusion was described by Barker and Cribb (1993), who indicated life cycle truncation in the sporocysts of

some members of the cyathocotylids, digenea of waterfowl, to release miracidia that are capable of emerging from the snail and infecting other molluscan hosts.

Poulin (2003) furthermore suggested that the transmission strategies of digenean trematodes is more plastic than originally thought enabling the omission of one host from the life cycle when limited opportunities for transmission are perceived. According to Poulin and Cribb (2002) there are a further two types of life cycle truncation that can occur in the first intermediate host (Figure 1.6). Precocious development occurs when the first intermediate host is also used as the second intermediate host. This occurs because the cercariae fail to emerge. Instead they lose their tail and differentiate into a metacercariae within the mollusc, which is thereafter similarly infective to the definitive host. This shortened cycle therefore evades host finding by the free swimming cercarial stage and has been recognised in several digenean families including the Echinostomatidae, Gymnophallidae and the Microphallidae (Poulin and Cribb, 2002).

Experimental studies by Poulin (2003) indicate precocious development to occur in the absence of chemical cues from the definitive host and furthermore when mortality of suitable intermediate hosts species is increased. Lastly, the entire digenean life cycle has been shown to occur within the first intermediate host. In these circumstances the sexually mature adult develops within the sporocyst or the redia and produces eggs that are capable of hatching into miracidia. This mode of transmission appears to be quite common, with members of eight of the 32 families listed by Poulin and Cribb (2002) demonstrating this behaviour, including members of the Plagiorchiidae.

Further modes of life cycle truncation have been found to occur in the second intermediate host (Poulin and Cribb, 2002). Progenesis is in fact the most common method for shortening the life cycle of digenean trematodes with as many as 79 digenean species belonging to 50 genera and 24 families being known to utilise this method (Lefebvre and Poulin, 2004). In progenesis, the need for the vertebrate definitive host is eliminated and the metacercariae develop precociously into an adult stage producing eggs within the metacercarial cyst (Poulin and Cribb, 2002). This type of development is considered beneficial to the trematode in that all metacercariae can potentially breed, whereas typically a large number of metacercariae either die within

their intermediate host or fail to establish in their definitive host (Lefebvre and Poulin, 2004). In the typical digenean life cycle, the adult trematode is most often located in the intestinal tract of the definitive host and eggs are dispersed into the environment within host faeces. During progenesis however, eggs are produced in an unusual site and the question of egg release into the environment has been a topic of question. Lefebvre and Poulin (2004) have identified five methods employed by trematodes for the release of progenetic eggs. These include host death, along with host eggs and excretory products, by host predation and by host skin rupture.

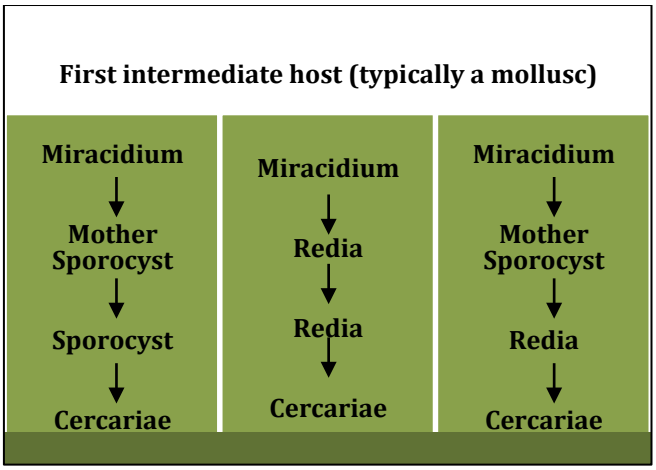


Figure 1.5 Common developmental pathways followed by digenean intramolluscan stages.

The final truncation strategy employed by digeneans is the sequential use of the second intermediate host as the definitive host (Poulin and Cribb, 2002). This strategy is commonly employed by digeneans of amphibians where the metacercarial cyst is retained by the same host. For example, in the life cycle of the Plagiorchiid *Haplometra cylindracea*, the cercariae penetrate the skin at the antero-ventral region of the tadpole, migrate to the throat region and encyst in the buccal mucosa. Following growth after a few days, the cyst bursts and the worms migrate to the lungs following metamorphosis of the host where they mature into sexual adults thereby utilising the same host species sequentially (Grabda-kazubska, 1970).

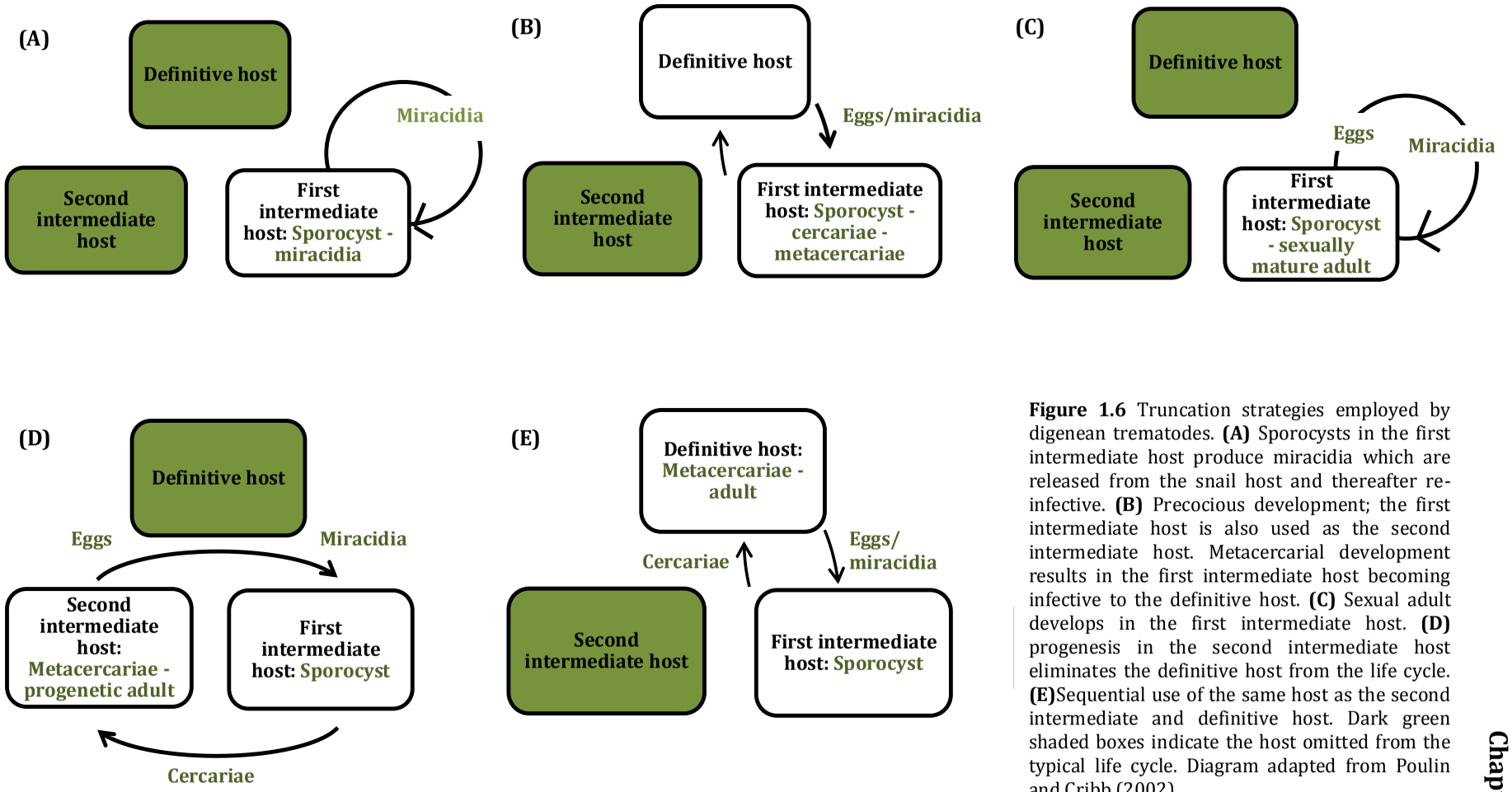


Figure 1.6 Truncation strategies employed by digenean trematodes. **(A)** Sporocysts in the first intermediate host produce miracidia which are released from the snail host and thereafter re-infective. **(B)** Precocious development; the first intermediate host is also used as the second intermediate host. Metacercarial development results in the first intermediate host becoming infective to the definitive host. **(C)** Sexual adult develops in the first intermediate host. **(D)** progenesis in the second intermediate host eliminates the definitive host from the life cycle. **(E)**Sequential use of the same host as the second intermediate and definitive host. Dark green shaded boxes indicate the host omitted from the typical life cycle. Diagram adapted from Poulin and Cribb (2002).

1.4.4 Trematode distributions

As part of their complex developmental strategies, digenean trematodes infect a diverse range of hosts and as such are found to be cosmopolitan in their distribution. Being the most abundant group of parasitic helminths, hosts belong to all vertebrate groups, and represent most terrestrial, freshwater and marine invertebrates (Niewiadomska and Pojmanska, 2011). Their distribution however is highly variable and typically aggregated within these host populations with the majority of hosts harbouring just a few helminths and a small number harbouring the majority of organisms (Wilson *et al*., 2002). There are several factors thought to be important in determining the spatial and temporal heterogeneity seen in wild animal populations including differences in host exposure, susceptibility, competition and predation and the abundance and behaviour of host species (Skirnisson *et al*., 2004; Fredensborg *et al*., 2005). Factors that predict focal distributions are generally considered to be reflective of the movement of host species that may be integral components of life cycles and necessary for propagation. Haukisalmi *et al*. (1988) suggested that seasonal changes were a result of a temporary restriction in the transmission of digeneans by the presence or absence of 'keystone' intermediate hosts; however this may similarly be reflective of definitive host species.

Due to the great mobility and wide spatial distribution of birds in comparison with other host species, birds are considered to be the main dispersal agents of trematodes and are assumed to be responsible for the large scale diversity observed in the spatial infection of larval trematodes in their first intermediate hosts (Smith, 2001; Skirnisson *et al*., 2004; Fredensborg *et al*., 2005). Birds however are highly variable in time and space and therefore so can be the transmission of trematode eggs from infected birds to snail hosts (Fredensborg *et al*., 2005). Whilst avian definitive hosts may be considered to emanate spatial patterns in the snail host, these patterns may also be altered by snail migration. This can have two effects upon the dispersal of associated helminth populations, either homogenisation or increased aggregation (Smith, 2001). The study of helminths in wild birds however is subject to numerous constraints with the main difficulty being the collection of specimens for examination (Wobeser, 2008). Avian digeneans can furthermore be difficult to assess. Due to the migratory behaviour of their hosts, determining digenean origin can be difficult with wild birds foraging from a variety of

locations and habitats, and as such much of the information available for bird digenea focuses upon descriptive accounts (Wobeser, 2008).

On a more local scale, wild rodent populations are commonly examined for their parasite assemblages and studies conducted worldwide have revealed a plethora of nematode, cestode and trematode species being harboured by various rodent fauna (Elton *et al.,* 1931; Lewis, 1968a; 1968b; Tenora *et al.,* 1983; Abu-Madi *et al*., 2000; Shimalov, 2002; Hildebrand, 2008; Mazeika *et al*., 2009; Chechulin, 2010). Rodents tend to be ideal for the demonstration of parasite flow within an ecosystem and due to their non-migratory behaviour as seen in avian communities, enable temporal and spatial heterogeneity in infection to be demonstrated at a specific locality (Behnke *et al*., 1999; Abu-Madi *et al.*, 2000).

1.4.5 UK reports of digeneans

In the UK, digenean trematodes have been reported from a variety of intermediate and definitive host species. Overall, waterfowl are considered to harbour species rich helminth communities and in particular a wide range of trematode species (Beverly-Burton, 1961; Beverley-Burton, 1972). Contrastingly, however, the communities of wild rodents are considered to be depauperate in comparison (Behnke *et al*., 2009). Examples of rodent digenea and digeneans recorded from UK snail populations have been listed (Table 1.2 & 1.3). Although these lists may not be exhaustive, this data provides an overview of the digenean species previously identified within UK wildlife. What is evident from the compiled lists nonetheless is that *Brachylaemus* spp. and *Corrigia vitta* appear to be the most commonly reported digeneans from rodents (Table 1.2) and *Lymnaea peregra* the most frequently utilised first intermediate host (Table 1.3) in the British Isles. In 2007 however, Rogan *et al*. reported the rare occurrence of the intestinal trematode *Plagiorchis muris* in the wood mouse *Apodemus sylvaticus* at Malham Tarn in North Yorkshire, UK. At this location *P. muris* had been previously recorded each September for a 13-year period, with a mean prevalence of 16.9%. As far as can be determined, this was only the fourth report of *P. muris* in the UK (Elton *et al.,* 1931; Baylis, 1939; Fahmy and Rayaki, 1963), which also generated the highest prevalence rate from these previous records.

Table 1.2 A compilation of digenean species recorded within wild UK rodents. The location of the report and rodent species infected has been indicated. This data has been compiled by the use of the Natural History Museum host parasite database (Gibson *et al*., 2005) in addition to a thorough literature search (see individual references below).

Digenean species	Founders	Host species		Location	Reference
		Scientific name	Common name		
Brachylaemus recurvum	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	South England	Behnke *et al*., 1999
Brachylaemus recurvum	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Wales	Lewis, 1968a
Brachylaemus recurvum	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	North Yorkshire	Behnke *et al*., 2009
Brachylaemus recurvum	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Oxford	Elton *et al*., 1931
Brachylaemus recurvum	Dujardin, 1845	*Apodemus flavicollis*	Yellow necked mouse	UK	Lewis, 1987
Brachylaemus recurvum	Dujardin, 1845	*Rattus norvegicus*	Brown rat	UK	Lewis, 1987
Brachylaemus migrans	Dujardin, 1845	*Sorex araneus*	Common shrew	Oxford	Baylis, 1928b
Brachylaemus oesophagei	Shaldybin, 1953	*Sorex araneus*	Common shrew	UK	Lewis, 1987
Brachylaemus oesophagei	Shaldybin, 1953	*Sorex minutus*	Eurasian pygmy shrew	UK	Lewis, 1987
Corrigia vitta	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Surrey, England	Behnke *et al*., 1999
Corrigia vitta	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Isle of Wight	Abu-Madi *et al*., 2000
Corrigia vitta	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Wales	Lewis, 1968a
Corrigia vitta	Dujardin, 1845	*Apodemus sylvaticus*	Wood mouse	Oxford	Elton *et al*., 1931
Corrigia vitta	Dujardin, 1845	*Apodemus flavicollis*	Yellow necked mouse	UK	Lewis, 1987
Corrigia vitta	Dujardin, 1845	*Microtus agrestis*	Field vole	Surrey, England	Lewis and Twigg, 1972
Corrigia vitta	Dujardin, 1845	*Myodes glareolus*	Bank vole	Surrey, England	Lewis and Twigg, 1972
Corrigia vitta	Dujardin, 1845	*Arvicola terrestris*	European water vole	England	Gibson *et al*., 2005
Dicrocoelium soricus	Diesing, 1858	*Sorex araneus*	Common shrew	UK	Lewis, 1987
Dicrocoelium soricus	Diesing, 1858	*Sorex minutus*	Eurasian pygmy shrew	UK	Lewis, 1987
Fasciola hepatica	Linnaeus, 1758	*Ondatra zibethica*	Muskrat	England	Gibson *et al*., 2005
Maritrema apodemicum	Lewis, 1966	*Apodemus sylvaticus*	Wood mouse	Wales	Lewis, 1968a
Notocotylus noyeri	Joyeux, 1922	*Microtus agrestis*	Short tailed vole	Cambridgeshire	Baylis, 1939
Notocotylus noyeri	Joyeux, 1922	*Arvicola amphibious*	Water vole	Cambridgeshire	Baylis, 1928b
Plagiorchis elegans	Rudolphi, 1802	*Apodemus sylvaticus*	Wood mouse	Northern Ireland	Montgomery *et al*., 1990a
Plagiorchis muris	**Tanabe, 1922**	***Apodemus sylvaticus***	**Wood mouse**	**North Yorkshire**	**Rogan *et al*., 2007**
Plagiorchis muris	Tanabe, 1922	*Apodemus sylvaticus*	Wood mouse	Oxford	Elton *et al*., 1931
Plagiorchis muris	Tanabe, 1922	*Rattus norvegicus*	Brown rat	Cambridgeshire	Baylis, 1939
Quinqueserialis quinqueserialis	Barker & Laughlin, 1911	*Ondatra zibethica*	Muskrat	River Severn	Baylis, 1928b
Rubenstrema exasperatum	Rudolphi, 1819	*Sorex araneus*	Common shrew	Oxford	Baylis, 1928b

Table 1.3 An overview of digenean species recorded within wild UK Freshwater snail populations. The location of the report and snail species infected has been indicated. This data has been compiled by the use of the Natural History Museum host parasite database (Gibson *et al*., 2005) in addition to a thorough literature search (see individual references below).

Digenean species	Founders	Host species		Location	Reference
		Scientific name	Common name		
Apatemon gracilis	Rudolphi, 1819	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Apatemon minor	Yamaguti, 1933	*Lymnaea peregra*	Wandering pond snail	Scotland	Gibson *et al*., 2005
Cotylurus cornutus	Rudolphi, 1808	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Diplostomum spathaceum	Rudolphi, 1819	*Lymnaea stagnalis*	Great pond snail	Lower Thames Valley	Morley *et al.,* 2003
Diplostomum spathaceum	Rudolphi, 1819	*Lymnaea peregra*	Wandering pond snail	Lower Thames Valley	Morley *et al.,* 2003
Diplostomum spathaceum	Rudolphi, 1819	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Diplostomum gasterostei	Williams, 1966	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Diplostomum phoxini	Faust, 1918	*Lymnaea peregra*	Wandering pond snail	Scotland	Gibson *et al*., 2005
Echinoparyphium recurvatum	Listow, 1873	*Valvata piscinalis*	European stream valvata	Scotland	Harper, 1929
Echinoparyphium recurvatum	Listow, 1873	*Lymnaea peregra*	Wandering pond snail	West Sussex, England	McCarthy, 1999
Echinoparyphium recurvatum	Listow, 1873	*Lymnaea peregra*	Wandering pond snail	Lower Thames Valley	Morley *et al.,* 2003
Echinoparyphium recurvatum	Listow, 1873	*Physa fontinalis*	Common bladder snail	West Sussex, England	McCarthy, 1999
Echinoparyphium recurvatum	Listow, 1873	*Planorbis planorbis*	Ramshorn snail	West Sussex, England	McCarthy, 1999
Fasciola hepatica	Linnaeus, 1758	*Lymnaea truncatula*	Dwarf pond snail	England	Gibson *et al*., 2005
Haplometra cylindracea	Zeder, 1800	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Hypoderaeum conoideum	Bloch, 1782	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Notocotylus attenuatus	Rudolphi, 1809	*Lymnaea peregra*	Wandering pond snail	Scotland	Williams, 1966
Notocotylus attenuatus	Rudolphi, 1809	*Lymnaea peregra*	Wandering pond snail	Lower Thames Valley	Morley *et al.,* 2003
Notocotylus imbricatus	Looss, 1839	*Bithynia tentaculata*	Mud bithynia	Cardiff, South Wales	Pike, 1969
Notocotylus triserialis	Diesing, 1839	*Lymnaea peregra*	Wandering pond snail	Cardiff, South Wales	Pike, 1969
Notocotylus seineti	Fuhrmann, 1919	*Lymnaea peregra*	Wandering pond snail	Scotland	Harper, 1929
Plagiorchis sp.	-	*Lymnaea peregra*	Wandering pond snail	West Sussex, England	McCarthy, 1999
Sanguinicola inermis	Plehn, 1905	*Lymnaea peregra*	Wandering pond snail	Lower Thames Valley	Morley *et al.,* 2003
Tylodelphis clavata	Nordmann, 1832	*Lymnaea peregra*	Wandering pond snail	Rutland, England	Moody and Gaten, 1982
Xiphidiocercariae	Luhe, 1909	*Lymnaea peregra*	Wandering pond snail	Scotland	Harper, 1929

1.5 *Plagiorchis muris*

1.5.1 The definitive host

P. muris was first described by Tanabe in 1922 in Kyoto, Japan. This digenean was originally recovered from the black rat, *Rattus rattus* and the brown rat, *Rattus norvegicus* (Seo *et al*., 1981) and has since been found in wild rodents from several locations (Elton *et al.,* 1931; Seo *et al*., 1964; Ito and Itagaki, 2003; Chai *et al*., 2007; Rogan *et al*., 2007). There has however been much debate as to whether *P. muris* is synonymous with *P. elegans* (Gorman, 1980; Langley and Fairley, 1982) which is foremost parasitic in birds but has furthermore been reported from rodents (Montgomery and Montgomery, 1990a; Shimalov, 2002; Hildebrand and Zaleśny, 2009; Mazeika *et al.,* 2009). This may not however be surprising since members of the genus *Plagiorchis* Lühe, 1899, typically demonstrate low definitive specificity (Janssen and Bock, 1990; Biserkov and Kostadinova, 1998; Ito and Itagaki, 2003). *P. muris*, has furthermore been recorded from a variety of non-rodent definitive host species including humans (Asada *et al*., 1962; Hong *et al*., 1996), the domestic dog *Canis familiaris* (Saito *et al*., 1995), feral raccoons *Procyon lotor* (Yamada, 2000; Sato and Suzuki, 2006), bats (Perez-Ponce de Leon *et al*., 1996) and several avian hosts (McMullen, 1937b; Cort and Ameel, 1944; Secord and Canaris, 1993).

1.5.2 Intermediate hosts

The life cycle of *P. muris* is a triheteroxenous cycle that utilises a molluscan first intermediate host and an arthropod second intermediate host. The molluscan host involved in transmission appears to vary geographically although Lymnaeid species appear to be most commonly reported. There does not however appear to be any clear indication of a particular species involved in transmission. Paucity in the literature indicates several possibilities with *Lymnaea pervia* (Hong *et al*., 1996) *Lymnaea japonica* (Gibson *et al*., 2005) *Lymnaea peregra* (Gibson *et al*., 2005) and the common beach snail *Stagnicola emarginata angulata* which belongs to the Lymnaeidae family all functioning as the first intermediate host for this species. Natural infections of *P. elegans* however have also been reported from *Lymnaea stagnalis* (Bock, 1984; Väyrynen *et al*., 2000), *L. peregra* (Väyrynen *et al*., 2000; Gibson *et al*., 2005), *Lymnaea elodes* (Gibson *et al*., 2005), *Lymnaea zazurensis* (Gibson *et al*., 2005) and *Lymnaea truncatula* (Manga-Gonzalez *et al*., 1994).

The second intermediate host for *P. muris* is a range of aquatic insect larvae (Hong *et al*., 1998; Hong *et al.,* 1999) although freshwater fish including *Liobagrus andersoni*, *Odontobutis platycephala* and *Puntungia herzi* have all been furthermore implicated as hosts (Hong *et al*., 1996). The most commonly reported insect hosts found to harbour *P. muris* are members of the order Odonata (Hong *et al*., 1998; Hong *et al*., 1999; Gibson *et al*., 2005; Hong, 2009). Other than for this group, there is limited information available with the majority of reports stemming from experimental infection studies. The experimental infection of mosquito larvae, caddisfly larvae, dragonfly nymphs, long-horned fly larvae, beetle larvae, chironomids and the water hog louse *Asellus aquaticus* have all been demonstrated (McMullen, 1937b; Macy, 1960; Bock, 1984; Webber 1987; Zakikhani and Rau, 1991).

1.5.3 Distribution

Plagiorchis species are considered to be cosmopolitan in their distribution. Differences in domain however can be recognised upon examination of the literature (Table 1.4). Although this list is far from comprehensive, it provides an overview to the geographical distributions of both *P. muris* and *P. elegans*. Except for the reports from Nigeria (Udonsi, 1989), Vietnam (Gibson *et al*., 2005) and India (Gibson *et al*., 2005), all other reports for both species seem to originate from within the Palaearctic and Nearctic regions. Only 10 records for *P. elegans* infection and 11 for *P. muris* infection were recovered from the Nearctic zone (Gibson *et al*., 2005).

In comparison, the vast majority of reports are derived from the Palaearctic. Within this region however differences can also be observed. For *P. elegans* as many as 184 records originating from Europe and European Russia were identified in comparison to only 15 reports from Asian Russia, a single occurrence listed from India and no records from any Far Eastern country or from the oriental region (Gibson *et al*., 2005). For *P. muris* on the other hand, European reports appear to be far more limited with only 10 records being identified (Elton *et al*., 1931; Fahmy and Rayaki, 1963; Gibson *et al*., 2005; Rogan *et al.,* 2007) from this region combined with European Russia. In fact, overall dispersion of this species appears to be far less than that observed for *P. elegans*, with almost all cases being reported from the Far East with as many as 24 records from various mammal and invertebrate fauna being evident.

Table 1.4 An overview of the distribution of *P. muris* and *P. elegans*. The data has been compiled by the use of the Natural History Museum host parasite database (Gibson *et al*., 2005) in addition to a thorough literature search (see below).

Digenean Species	Location	Reference
P. muris	**India**	Gibson *et al*., 2005.
	Iraq	Al-Zihiry and Awad, 2008
	Japan	Tanabe, 1922/ Ito and Itagaki, 2003/ Saito *et al*., 1995
	Kazakstan	Gibson *et al.,* 2005
	Korea	Hong *et al*., 1996; 1999/ Seo *et al*., 1999/ Chai *et al.,* 2007
	Mexico	Perez-Ponce de Leon *et al*., 1996
	Nigeria	Udonsi, 1989
	USA	Cort and Olivier, 1943/ Secord and Canaris, 1993
	United Kingdom	Elton *et al*., 1931; Rogan *et al.,* 2007/ Fahmy and Rayaki, 1963.
	Vietnam	Gibson *et al*., 2005.
P. elegans	**Alaska**	Kinsella *et al*., 2007
	Austria	Faltynkova *et al*., 2007
	Belarus	Shimalov, 2002
	Canada	Hoberg and McGee, 1982/ Kinsella *et al*., 2007
	Czech Republic	Faltynkova *et al*., 2007
	Finland	Tenora *et al*., 1983/ Väryryen *et al*., 2000
	Germany	Faltynkova *et al*., 2007
	Greenland	Kapel and Nansen, 1996
	Iceland	Skirnisson *et al*., 1993
	India	Gibson *et al*., 2005
	Iraq	Gibson *et al*., 2005
	Ireland	Langley and Fairley, 1982 ; Montgomery and Montgomery, 1990a
	Russia	Kobishev, 1970
	Slovak Republic	Faltynkova *et al*., 2007
	Spain	Manga-Gonzalez *et al*., 1994
	Poland	Faltynkova *et al*., 2007/ Hildebrand and Zaleśny, 2009
	Lithuania	Mazeika *et al*., 2009

1.5.4 Taxonomy

A high degree of morphological diversity displayed between members of the family Plagiorchiidae has previously created a great complexity in obtaining a naturally defined classification system within the family (McMullen, 1937c). A continued disagreement over reliable diagnostic criteria has exacerbated the problem and it is not surprising therefore that the systematics of the family has been continuously reviewed (Tkach, 2008). Further controversies surround the type-genus *Plagiorchis* Lühe, 1899. This genus has been synonymised with *Lepoderma* Looss, 1899 which was published in a different journal on the very same day. This resulted in decades of arguments over taxonomic priority with one or the other being considered a senior synonym by various

researchers (Tkach, 2008). There has been further confusion surrounding the status and morphology of the type species *P. vespertilionis* Müller, 1784, which is primarily parasitic in bats (Tkach *et al*., 2000a). Currently however the taxonomic status of *Plagiorchis muris* according to The Bay Science Foundation Inc. (2013) is generally accepted as follows:

Kingdom: Animalia	Linnaeus, 1758
Phylum: Platyhelminthes	Gegenbaur, 1859
Class: Trematoda	Rudolphi, 1808
Subclass: Digenea	Carus, 1863
Order: Plagiorchiida	
Family: Plagiorchiidae	Lühe, 1901
Genus: *Plagiorchis*	Lühe, 1899
Species: *muris*	Tanabe, 1922

1.5.5 Life cycle and morphological development

There appears to be a paucity of information on the life history of many *Plagiorchis* species with the life cycle of some species remaining completely unknown (Radomyos *et al*., 1989; Chai, 2007). What is known regarding the development of these digeneans however indicates a complex transition through five morphologically distinct stages, including the adult digenean, the miracidium, the sporocyst, the cercariae and the metacercariae (Bock, 1984). The following account discusses what is currently known regarding morphology and development.

1.5.5.1 The adult digenean

The outer tegument of *P. muris* functions to maintain homeostasis during the host-parasite interphase. The tegument is covered with cytoplasmic processes that are minutely spined and possess sensory papillae (Hong, 2009). This tegumental spination occurs over the entire body but decreases in size towards the posterior end of the body (Hong, 2009). The adult form is elongated attenuating towards both the posterior and anterior extremities (Hong *et al*., 1998) and typically ranges in size from a minimum recorded length of 0.80mm to 3.00mm by 0.24mm to 0.95mm in width (Tanabe, 1922;

Seo *et al*., 1964; Hong *et al*., 1996; Rogan *et al*., 2007; Chae *et al*., 2010). *P. muris* is distomatic possessing both an oral and ventral sucker both of which are spherical in shape and have a typical ratio of 1:1 (Seo *et al*., 1964) to 1:48 (McMullen, 1937b). The oral sucker connects to the pharynx via a short pre-pharynx following which the oesophagus bifurcates approximately midway between the oral and ventral suckers and two blindly ended caeca extend towards the posterior extremity of the body (Chae *et al*., 2010).

The genital aperture is situated posterior to the ventral sucker although the single ovary is located just mid level and almost contiguous with the ventral sucker (Hong, 1998). The cirrus sac is dextral and extends posteriorly beyond the ventral sucker (Chae *et al*., 2010). There are two testes, which are circular to oval in shape and almost contiguous to one another. The uterus forms a characteristic S-shape, which extends from the ovary and intertesticularly towards the posterior end of the body (Seo *et al*., 1964) also extending forwards to the genital pore that is located between the pharynx and the ventral sucker. As many as 700 eggs have been observed to fill the entire length of the uterus in gravid specimens (McMullen, 1937b). Vitelline glands that secrete yolk for egg development have been reported to extend from the level of the oral sucker to the posterior end of the body as well as laterally thereby filling the entire width of the body (Tanabe, 1922; Seo *et al*., 1964). The adult fluke, occupying the small intestine of the definitive host, releases eggs into the host lumen, which are subsequently passed into the environment via the host faeces. The unembryonated eggs develop within water, a process that is temperature dependent, becoming infective upon development of the contained miracidium (Bock, 1984).

1.5.5.2 The miracidium

The egg of *P. muris* is golden-brown and elliptical in shape, measuring 32-38μm by 20-24μm, is conspicuously operculated at one end and possesses a small knob at the opposite end (Hong *et al*., 1996). In fresh faeces, the egg of *Plagiorchis* is unembryonated containing a clear germ cell and four vitelline gland cells. Following maturation within water after approximately 30 to 50 days (temperature dependent) the mature miracidium, which is the infective stage to the first intermediate molluscan host can be observed to move convulsively within the egg (Bock, 1984). The lifecycle of *Plagiorchis*

species is not very well understood and experimental infection using *Lymnaea* snails has indicated that infection may occur via ingestion of the infectious egg containing the miracidial stage (Gorman, 1980; Bock, 1984; Zakikhani and Rau, 1992).

1.5.5.3 The sporocyst

Within the molluscan intermediate host, the miracidium undergoes differentiation into the mother sporocyst stage. Mother sporocysts are round to oval in shape and are discrete structures that adhere firmly to the intestinal wall (Cort and Olivier, 1943). The mother sporocyst is surrounded by an outer wall composed of irregular cells, several layers of which form to create a base for attachment to the intestinal wall (Cort and Ameel, 1944). Each mother sporocyst can be divided into several lobes. Each lobe contains germ cells that are able to develop into any number of daughter sporocysts and as many as 500 daughter sporocyst embryos can be present at any one time (Cort and Olivier, 1943). Daughter sporocysts contained within the mass are more elongated in shape and possess an outer coat comprised of material derived from the mother sporocyst wall and as such each of the daughter sporocysts tend to be adjoined by the cells of their outer coat (Cort and Ameel, 1944).

Following full development, individual daughter sporocysts separate and migrate towards the digestive gland of the snail host where they become firmly attached to snail tissues (Cort and Ameel, 1944). Within the daughter sporocyst, germ balls develop into cercariae that eventually escape via the terminal birth pore (Cort and Ameel, 1944). Despite such, the daughter sporocyst remains active throughout the life of the snail and germ balls at varying stages of development can be continuously observed, thus once a snail is infected, it appears to be infected for the remainder of its life (Cort and Olivier, 1943).

1.5.5.4 The cercariae

The cercariae of *P. muris* are xiphidiocercariae. On average the body of the xiphidiocercariae measures 240µm by 92µm and the tail 190µm and 27µm and possesses both an oral and ventral sucker. The oral sucker is armed with a stylet in the anterior rim that is used during active penetration of a second intermediate host (McMullen, 1937b). There are penetration glands located on each lateral side of the

cercariae that possess ducts which extend anteriorly to open adjacent to the tip of the stylet (Gorman, 1980). Once the xiphidiocercariae of *P. muris* are shed from the molluscan intermediate host, they move actively through the water column to locate a second intermediate host (McMullen, 1938). Experimental data has shown that the cercariae of *Plagiorchis* remain active for approximately 12 hours at room temperature with the majority dying after 24 hours (Bock, 1984). Cercariae therefore have a limited time period in which to locate a suitable second intermediate host, penetrate and encyst within the tissues to differentiate into the metacercarial stage (McMullen, 1938).

1.5.5.5 The metacercariae

Metacercarial development typically occurs within aquatic insect larvae (Hong *et al*., 1998; Hong *et al.,* 1999) and freshwater fish (Hong *et al*., 1996) and the lifecycle of *Plagiorchis* is complete when the second intermediate host containing the metacercariae is ingested by a definitive host. Precocious development has also been observed by McMullen (1938) who observed metacercarial development inside sporocysts within the molluscan intermediate host. This suggests that snails may also be a source of infection if consumed by the definitive host.

The metacercariae is elliptical to spherical in shape and has been observed to have an average size of 165µm by 185µm (Hong *et al*., 1998). The stylet armed on the rim of the oral sucker of the cercariae is typically shed during this stage and can often be seen floating within the cyst cavity which can be observed sub-terminally. The tail is furthermore lost at this stage (McMullen, 1938). Additionally, the ventral sucker, which is slightly smaller than that of the oral sucker, can be seen centrally adjacent to a black Y-shaped excretory bladder. No primordial genital organs have been observed in the metacercarial stage. Primordial genitalia only appear following excystation and can be visualised as thick masses surrounding the ventral sucker in just one-day-old flukes (Hong *et al*., 1998). Once inside the definitive host, the metacercariae which is surrounded by a hyaline thin wall (Hong *et al*., 1999) excysts within the duodenum of the small intestine and the flukes become ovigerous five days post infection and at this point are regarded as adult flukes that can release eggs into the intestinal lumen and continue the lifecycle of the parasite (Hong, 2009).

1.6 The study site

Malham Tarn is located in North Yorkshire, Northwest England at an altitude of 375m above sea level (Grid reference SD892866). Malham Tarn and its associated wetlands and woodlands were declared a 'National Nature Reserve' by Natural England in 1992 and is a 'Site of Special Scientific Interest' (SSSI). The tarn itself is the only upland marl lake within Britain and furthermore one of only eight in Europe (Rogan *et al*., 2007).

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. The lake is located predominantly upon carboniferous limestone thus all groundwater entering the tarn is received from limestone or limestone rich drift giving the tarn its alkaline nature (Woof and Jackson, 1988). The lake is a running water system with the largest inflow of water entering at the northwest corner and a smaller inflow from the northeast. There is one major outflow of water that moves in a southerly direction as a surface stream for approximately 500m before sinking into the Great Scar Limestone at several locations (Woof and Jackson, 1988).

The boundaries of Malham Tarn Nature Reserve were originally delimited to encompass as many representative examples of different habitat types as possible (Sinker, 1960). The tarn itself occupies the vast majority of the reserve and is surrounded by several main habitats including limestone pavements, cliff and scree, plantations, improved, limestone and drift grasslands, calcareous marsh, fen and carr, raised bog and ponds and streams (Sinker, 1960). This complex range of habitats has resulted in a wide plant and invertebrate diversity and Malham Tarn is now considered to host more than 1000 dipteran species (Rogan *et al*., 2007).

The exact reason for the occurrence of *P. muris* at Malham Tarn is unclear, however the animal fauna at this location have been previously studied for a range of host-parasite systems (Kennedy & Burrough, 1978; Allan *et al*., 1999; Hughes *et al*., 2006; Rogan *et al*., 2007; Hughes *et al*., 2008; Behnke *et al*., 2009; Thomasson *et al*., 2011) and it is speculated that the high diversity of animal life present at Malham Tarn may be important in the complex life cycle and transmission ecology of helminths, in particular *P. muris* at this location.

Figure 1.7 Aerial view of Malham Tarn Nature Reserve, © Janet Wright Photography UK.

1.7 Aims and objectives

Although the digenea encompasses the most successful group of internal metazoan parasites, there remains a paucity of information regarding the life history strategy of many species. In order to integrate the effects of parasites into current community structures, understanding the mechanisms that underlie their recruitment into host populations is highly important. Little is known about the exact life cycle of *Plagiorchis muris* infecting the rodents at Malham Tarn and, in particular, the identity and role of intermediate host species. The high prevalence reported by Rogan *et al*. (2007) suggests that the presence of water, waterfowl and aquatic organisms might be key factors. In order to investigate the rare occurrence of *P. muris* at this location, prevalence and intensity of adult stages collected from rodents trapped at defined woodland sites around this upland lake will be examined. This study will be carried out quarterly over a two year sampling period and encompass several objectives:

i. The original report by Rogan *et al*. (2007) discussed data generated from the same time each year. This investigation will include quarterly sampling over a

two year period in order to examine seasonal changes in the prevalence and intensity of adult flukes.

ii. The original report furthermore described the prevalence and intensity of *P. muris* from a single locality therefore this research intends to conduct a more extensive survey of the Malham Tarn area.

iii. The presence of all other helminth parasites will be recorded and factors that may contribute in defining the helminth communities of rodents at Malham Tarn, including host sex and age and both seasonal and site-specific variation will be investigated. Component communities will also be compared.

iv. The identity of *P. muris* at this location has been based on classical parasite identification using morphology and has not yet benefited from the greater precision of DNA sequencing analysis. DNA sequencing of the ribosomal genes will therefore be performed in order to confirm the identity of *P. muris*.

v. Attempts will be made to identify the intermediate host species involved in the transmission of *P. muris* at this location. Aquatic snails and insect larvae will be collected on a seasonal basis from water bodies within the area and will be examined for trematode larval stages. Species identification will be confirmed by linking the specific identity between larval and adult stages using DNA sequence data.

vi. Attempts will be made to design and optimise a robust molecular detection assay (PCR) based on the DNA sequences generated during the study, for the detection of *P. muris* larval stages within its intermediate host species.

CHAPTER 2:

Materials and Methods

2.1 Field methods

2.1.1 Study site

The primary aim of this study is to investigate the digenean species of rodents trapped from woodland sites that are located nearby to freshwater habitats. A permit was granted by the National Trust to allow sampling of areas located within the boundaries of The Malham Tarn reserve (Figure 2.1). Plantations occupy only a small fraction of the land and are represented by Tarn Woods, Ha Mire Plantation and Spiggot Hill. Ownership boundaries form part of the reserve perimeter causing sampling to be restricted in part. All three plantations were selected for rodent trapping in addition to a fourth site, Tarn Fen, an area of raised bog covered by deciduous woodland. Because water is generally considered to be an important aspect in the life cycle of digenean species, suitable rodent trapping sites were carefully selected that were located in close proximity to each border of the tarn (Figure 2.1).

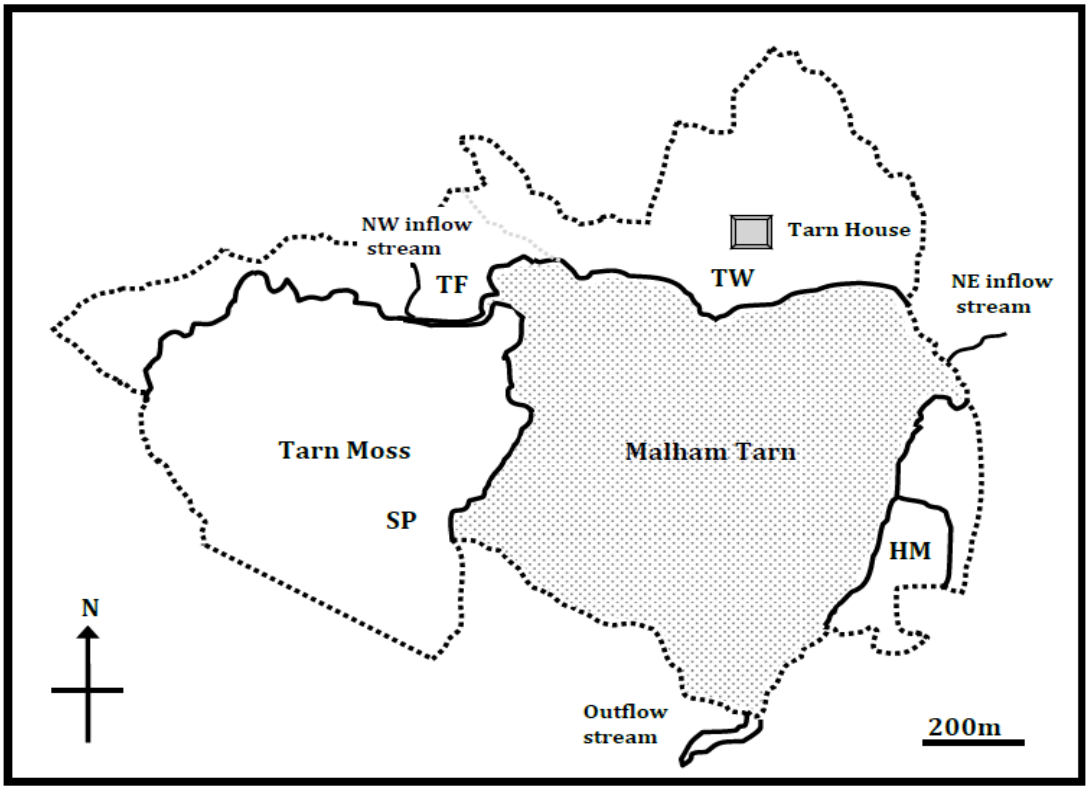


Figure 2.1 Map of the Malham Tarn Nature Reserve and adjacent woodlands. Redrawn from Shorrock and Sutton (2010). **Key = TW:** Tarn Woods; **TF:** Tarn Fen; **SP**: Spiggot Hill; **HM**: Ha Mire Plantation. The black dotted line represents the reserve boundary. The grey dotted line indicates the boundary between Tarn Fen and Tarn Woods.

2.1.1.1 Tarn Woods

Tarn Woods is located on the north shore of the tarn (Grid reference SD893267) and is the largest plantation covering approximately 40 acres of mixed deciduous and coniferous woodland (Williamson, 1968), including larch (*Larix* spp.), spruce (*Picea* spp.), and beech (*Fagus* spp.). Ground cover appears diminished in parts covered by a thick leaf litter and fallen logs but is dominated by dog's mercury (*Mercurialis perennis*) elsewhere (Sinker, 1960). The sampling site is located approximately 120m north of the tarn shore, beyond Tarn Close which is located on a moderate slope adjacent to the tarn margin and is separated by a narrow shingle beach.

2.1.1.2 Ha Mire Plantation

Ha Mire plantation is located on the southeast shore of the tarn (Grid reference SD897266). The plantation was established during the 19th century (Seaward and Pentecost, 2001) and is mixed deciduous and coniferous woodland, predominantly composed of mature sycamore (*Acer pseudoplatanus*), larch (*Larix* spp.), alder (*Alnus* spp.) and birch (*Betula* spp.). Ground flora is mostly grasses (*Festuca* spp. and *Agrostis* spp.) with dog's mercury (*Mercurialis perennis*), bracken (*Pteridium* spp.), bramble (*Rubus* spp.) and fallen logs interspersed throughout (Sinker, 1960). The site covers an area of approximately 10 acres (Williamson, 1968) and is walled off to the north, east and south with only the west border being openly exposed to the tarn margin which is separated only by a very narrow shingle beach.

2.1.1.3 Spiggot Hill

Spiggot Hill is located at the southwest junction of the tarn's south and west shores (Grid reference SD886966). Spiggot Hill is an exposed prominent drift hillock that is composed of mixed deciduous and coniferous woodland with larch (*Larix* spp.), beech (*Betula* spp.) and sycamore (*Acer pseudoplatanus*). Ground flora is sparse during the winter months consisting mainly of leaf litter and fallen logs but dense during the spring and the summer with grasses, dog's mercury (*Mercurialis perennis*) and meadowsweet (*Filipendula ulmaria*) (Sinker, 1960). The Hillock is surrounded by the ombrotrophic peat surfaces of Tarn Moss. Several small peat pools are located to the northwest of Spiggot Hill within these surfaces (Sinker, 1960). The water level of these pools tends to fluctuate with changes in rainfall.

2.1.1.4 Tarn Fen

Tarn Fen is located at the northwest junction of the tarn's north and west shores (Grid reference SD887067). Tarn Fen is one of a few sites of its kind remaining within the UK and is composed of several microhabitats including sedge swamp, rich fen, poor fen, carr and marsh meadow (Sinker, 1960). The entire area includes West Fen, Middle Fen and East Fen and covers an area of approximately 26 acres (Williamson, 1968). The sampling site lies approximately 170m west of the tarn margin where Middle Fen and East Fen meet (Cooper and Proctor, 1998). Middle Fen and East Fen are both relatively sheltered areas comprising deciduous woodland dominated by birch (*Betula* spp.), willow (*Salix* spp.) and alder (*Alnus* spp.) (Sinker, 1960). The sampling site comprises an open and relatively level area located at the east end of Middle Fen and the west border of East Fen where ground flora typically consists of grasses, (*Deschampsia flexuosa, Molinia* spp.), hair moss (*Polytrichum* spp.) and rotting tree stumps (Sinker, 1960). The broad inflow stream passes directly through the border of Middle and East Fen and carries an abundance of aquatic flora, predominantly the common water-starwort (*Callitriche* spp.) and characean algae (*Nitella* spp.). The stream is furthermore fringed in parts with the common water-crowfoot (*Rananculus aquatilis*) (Sinker, 1960). The main inflow stream is highly prone to flooding during periods of heavy rainfall, which often results in the adjacent woodlands and grasslands becoming submerged. Flooding of the Tarn Fen area was frequently observed during the current study. Tarn Fen furthermore adjoins the west border of Tarn Woods and is an area highly exposed to considerable amounts of water (Sinker, 1960).

2.1.1.5 Aquatic sampling sites

Freshwater snails and aquatic insect larvae were collected from several sites surrounding the tarn. Water sampling areas were selected on the basis of accessibility and their proximity to rodent sampling sites. Aquatic invertebrates were collected from the two inflow streams located at the northwest and northeast corners of the tarn, Spiggot Hill peat pools when levels permitted, and at various accessible points of the tarn margin that were considered to be in close proximity to rodent trapping points. The main outflow stream located southeast of the tarn runs through private grounds and for such reason could not be sampled.

2.1.2 Sampling periods

Sampling was conducted quarterly from each of the sampling locations between January 2010 and October 2011 (8 sampling sessions in total). The exact date of each season was determined by weather conditions and accessibility to the tarn area, although every effort was made to conduct sampling within time periods of close proximity.

2.1.3 Rodent trapping

Rodents were trapped using Longworth small mammal traps. Trapping was carried out over a period of four nights each season using a method adapted from Giraudoux *et al*. (2008). Four trap lines consisting of 15 traps were set up with a 3m distance between two traps. Traps were set in straight lines when feasible and when possible were placed strategically alongside and resting on top of natural objects such as fallen tree trunks and branches and within shrubs and grass tussocks. Traps were also set up adjacent to identified burrow entrances and were covered with leaf litter and grass. Traps were placed at an angle to prevent rain entering the nest box. Each set of traps was identifiable by a marker cane (taped red at the top) placed within a 1m distance of the trapping point. Hay was placed inside the nest box of each trap and dried food (grains, seeds and fruits) was used as bait according to Behnke *et al*. (1999). Bait was placed inside the nest box and a small amount of pre-bait was also placed at the tunnel entrance.

Traps were inspected early each morning during the sampling period. Closed traps were broken open within a clear polythene bag containing cotton wool impregnated with an anaesthetic according to the 'Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals (Scientific Procedures) Act 1986, according to Rogan *et al*. (2007). The position of the catch was recorded using a Garmin GPS 60 set to the co-ordinate framework WGS84. Each specimen was labelled and placed within a small plastic bag for transportation to the Centre for Parasitology and Disease Research at The University of Salford. Rodents were refrigerated at 4°C for examination within one day of their capture, otherwise were frozen at -20°C for later analysis.

2.1.4 Invertebrate collection

Two methods were employed for the collection of freshwater invertebrates depending on the habitat type. A qualitative and quantitative assessment was carried out at each of the sampled water bodies. Both snails and insects were easily collected from the two inflow streams located northeast and northwest of the tarn using a D-frame aquatic dip net and kick sample technique. Ten sample dips in total from each of these locations were taken when feasible (depending on water levels). The net was placed on the waterbed and the substrate immediately upstream was disturbed allowing snails and aquatic insect larvae to be carried downstream and into the net by the water current. The net was also swept back and forth through vegetation to disturb any invertebrates that could be attached. The contents of the net were emptied into a light coloured tray where invertebrates could be picked on-site using tweezers.

Invertebrates were furthermore handpicked from the stems of vegetation and the underlying surfaces of rocks, particularly at specific points along the tarn margin that consisted primarily of a rocky substrate. All aspects of water habitat within the area were sampled including regions of flowing water, standing water and weeded and rocky areas to ensure that all possible habitats within the site were equally sampled. Attempts were also made to identify temporary water bodies during each sampling session. Care was taken as to not remove any rare or endangered species of invertebrate (Joint committee for the conservation of British Invertebrates). All organisms were placed into plastic screw top pots for transportation. For snails, the pot was lined with damp vegetation and water derived from the sampling site. Insect larvae were placed directly into 70% ethanol. Invertebrates were transported back to the Centre for Parasitology and Disease Research at The University of Salford. Invertebrate lists were compiled to identify the type and number of invertebrate fauna collected from each location.

2.2 Laboratory methods

2.2.1 Rodent examination

Rodents were morphologically identified to species level using the criteria of Sargent and Morris (2003). The morphology of the two vole species trapped from Malham Tarn was verified using molecular identification (see 2.2.8.4) according to Pfunder *et al.*

(2004). Rodent weight in grams and length in centimetres from nose to anus was recorded prior to dissection/freezing. Rodents were aged according to their weight/length (Watts, 1968; Behnke *et al*., 1999). Rodents were sexed during dissection following the removal of the abdominal contents.

The fur on the ventral surface of the animal was dampened with distilled water. The abdominal cavity was opened down the mid-ventral line using scissors, and a scalpel was used to pry away the fur and skin from the underlying membrane. The membrane was opened using scissors to uncover the viscera. The rib cage was opened and the heart was removed and placed into a 1.5ml eppendorf tube containing 400µl lysis buffer (100mM NaCl, 25mM ethylene diamine tetraacetic acid, 0.5% (w/v) sodium dodecyl sulphate, 20mM Tris pH 8.0) (Terry *et al*., 2001). The stomach and the intestinal tract were removed from the abdominal cavity and examined at ×16 under a dissecting microscope (Wild M3B, Heerbrugg, Switzerland).

The stomach was opened in order to examine the dietary fragments. Items within the stomach contents were sorted and morphologically examined. The intestinal tract was sectioned into the small intestine, the large intestine and the caecum and was submerged into phosphate buffered saline (PBS) along with the mesenteries and pancreatic duct. The intestinal tract including the caecum was opened longitudinally and was examined along with the mesenteries and pancreatic duct for the presence of helminth parasites. Each rodent was numbered accordingly and listed into an excel database along with the trapping point, date, species, weight (g), length (cm), sex and the number and identification of parasite fauna recorded.

Additionally, the entire brain tissue was aseptically removed and placed into an eppendorf tube containing 400µl lysis buffer (Terry *et al*., 2001), and frozen at -20°C. Both the heart and the brain tissue were supplied to Jaroslav Bajnok of the Molecular Epidemiology Research Group at the University of Salford for the detection of *Toxoplasma gondii* and for the genotyping of Malham Tarn's *Apodemus sylvaticus* population (currently unpublished data).

2.2.2 Snail examination

Snails were speciated according to Macan and Cooper (1960). They were examined upon return to the laboratory. However, when longer maintenance of snail collections was required, snails were housed in 4 litre glass covered tanks containing a prepared 'instant pond water salt' mixture (Sciento, Manchester, UK), maintained at a temperature of 4°C and fed washed lettuce *ad libitum* according to Voutilainen *et al*. (2009). Snails were stored in separate tanks on the basis of their collection point. For helminth examination, a snail crushing method was employed according to Caron *et al*. (2008). The crushing method is preferred over an induced shedding technique for the detection of recent trematode infections that may carry few fully developed cercariae (Voutilainen *et al*., 2009). For examination, snails were individually placed into a watch glass, submerged in 1×PBS and the shell examined at ×40 under a dissecting microscope (Wild M3B, Heerbrugg, Switzerland) to check for the presence of metacercarial cysts. The entire snail was subsequently held between a pair of tweezers, crushed using a spatula, and seekers were used to tease the internal structures of the snail apart. Digenean larval stages were primarily identified microscopically.

Each snail was given a number and was listed into an excel database along with its collection point, date, species and the type and intensity of larval stages present. For specimens in which the infection was too great to record an actual number of parasites, "+100" was indicated in the database.

2.2.3 Arthropod examination

Insect larval stages were identified according to Kimmins (1962), Hickin (1967), Macan (1970), Hynes (1977), Croft (1986) and Wallace (2006). One insect from each species was stored in a 1.5ml eppendorf tube containing 70% ethanol to be maintained as type specimen and are currently held at the School of Environment and Life Sciences, The University of Salford, Salford Crescent, Manchester, UK. Insects collected from each location were counted, dated and stored separately according to genus (or species when feasible) in 30ml screw cap sample pots containing 70% ethanol. Each insect was individually placed into a petri dish and submerged in 1×PBS. The surface of the insect larvae was initially examined for digenean metacercariae. For the internal examination, the insect was held with a pair of tweezers and opened down the mid-ventral surface

using a scalpel. Seekers were used to separate the internal contents of the insect and the body cavity was examined under a dissecting microscope at x 16 (Wild M3B, Heerbrugg).

2.2.4 Helminth examination

A selection of each adult helminth species recovered from rodent hosts, were photographed using a Nikon Eclipse TS100 and attached Nikon Digital Sight (DS-L2, Nikon, Japan). Morphology was examined using a Leica DM500 microscope and measurements were recorded using an eyepiece graticule (Leica) that had been previously calibrated using a 1mm stage micrometer (Graticules Ltd, Kent, UK). Helminths were identified to genus level and species level when feasible and their morphology was cross-referenced against available literature to verify species identification (Hussey, 1957; Avery 1974; Lewis, 1987; Justine and De Roguin, 1990). Adult helminths were stored in vials containing 70% ethanol and were labelled according to the number of their host animal for future reference.

Larval stage parasites collected from invertebrates were isolated from host tissues using a 20µl pipette and their morphology and measurements recorded as conducted for adult parasites. Methylcellulose was used to minimise movement of cercarial stages during examination. A sample of each identified larval stage was placed into a 1.5ml eppendorf tube containing 200µl lysis buffer in preparation for DNA extraction (see 2.2.6).

2.2.5 Staining

Adult specimens were relaxed in distilled water overnight, fixed in 5% formalin and flattened under light cover slip pressure. The specimens were stained with borax carmine and mounted in Canada balsam adapted from Gurr (1963). Formalin fixed samples were transferred into a 30ml screw cap sample pot containing 50% ethanol for 2 hours. The ethanol was removed and the samples were submerged in borax carmine stain (boil together 2g carmine, 8g borax, and 200ml distilled water. Cool and make up to 200ml and add 200ml 70% ethanol) for a period of 3 hours.

After 3 hours the stain was removed and each specimen was washed in 3% acid alcohol (3ml Hydrochloric acid (HCl 37% (w/w) and 97ml 70% (v/v) ethanol) repeatedly to

extract colour. Samples were transferred into 70% ethanol for 5 minutes to wash away the acid solution. The samples were dehydrated by submersion into 80% ethanol for 5 minutes, 90% ethanol for 5 minutes and 100% ethanol for 5 minutes. The samples were mounted in Canada balsam. Neither histoclear nor clove oil was used as both caused tissue degradation.

2.2.6 DNA extraction from parasite tissue

DNA was extracted from parasite tissue using a standard phenol: chloroform method adapted from Terry *et al*. (2001). For adult helminths, DNA was extracted from individual ethanol fixed specimens. Parasites were placed into a 1.5ml eppendorf tube containing 200µl lysis buffer (as 2.2.1) and 5µl proteinase K (20mg/ml) and incubated overnight at 56°C.

200µl phenol: chloroform: isoamyl (25: 24: 1) was added to each sample and each tube was inverted regularly for 10 minutes prior to centrifugation for 10 minutes at 13,000rpm using an AccuSpin Micro 17 centrifuge (Thermo Fisher Scientific, Germany). The supernatant was subsequently transferred to a fresh 1.5ml eppendorf tube and the phenol: chloroform stage was repeated a further two times. DNA was precipitated by the addition of 36µl sodium acetate (3M, pH5.2) and 500µl 100% ice-cold ethanol and stored overnight at -20°C.

Each sample was subsequently centrifuged for 20 minutes at 13,000rpm. The supernatant was removed and 200µl 70% ethanol was placed onto the DNA pellet and the sample centrifuged at 13,000rpm for a further 10 minutes. The supernatant was discarded and the eppendorf tube was inverted to remove excess ethanol and air-dry the DNA pellet. DNA was re-suspended in 50µl 1×Tris-EDTA (TE) buffer and stored at 4°C prior to PCR analysis.

2.2.7 DNA extraction from rodent tissue

DNA was extracted from voles to verify morphological identification. A $1cm^2$ section of thigh muscle was aseptically removed using a scalpel and scissors from each of the vole species that had been phenotypically identified. The muscle tissue was placed into an

eppendorf tube containing 400µl lysis buffer. DNA extraction was carried out as in 2.2.6 except that reagents were doubled at each stage of the protocol.

2.2.8 DNA quantification and PCR amplification

The concentration of each DNA sample prepared for PCR amplification was determined by spectrophotometry (Jenway Genova, UK). Where required, samples were diluted using TE buffer to provide a DNA template of 50µg/µl. All PCR reactions were carried out using a Robocycler 96 PCR machine (Stratagene, La Jolla, California, USA) and visualised on a 1% (w/v) Tris-borate-EDTA (TBE) agarose gel stained with gel red using a G:Box gel imaging system (Syngene, Cambridge, UK). If DNA sequencing was to be performed PCR products were visualised on a 1% (w/v) Tris-acetate-EDTA (TAE) agarose gel.

2.2.8.1 28S rRNA gene /ITS region from parasite DNA

The 28S rRNA gene was amplified by PCR using the forward digenean specific primer dig12 (5'AAGCATATCACTAAGCGG3') and the reverse universal primer Lo (5'GCTATCCTGAGRGAAACTTCG3') according to Tkach *et al.* (2000b).

The ITS1, 5.8S, ITS2, and flanking regions of the 18S rRNA and 28S rRNA gene were amplified by PCR using the forward universal primer BR (5'GTAGGTGAACCTGCGGA3') and reverse digenean specific primer dig11 (5'GTGATATGCTTAAGTTCAGC3') according to Tkach *et al.* (2000a).

Each 50µl PCR reaction comprised 5µl 10X DreamTaq buffer (including 2mM $MgCl_2$), 0.5µl deoxynucleotide triphosphate (dNTPs; 100mM, Bioline, London, UK), 2.5µM forward primer, 2.5µM reverse primer, 36.5µl molecular grade PCR water, 1µl DreamTaq DNA polymerase (5u/µl) and 2µl DNA template (50µg/µl).

The cycling profile consisted of an initial denaturation step of 1 cycle at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C, annealing at 54°C and elongation at 72°C for 1 minute each, and one final cycle at 72°C for 10 minutes.

2.2.8.2 Plagiorchiid PCR

Larval stage DNA was amplified by PCR using primers designed in the current study (see chapter 6): PlagF ($_{5'}$CTACGTACAGTCATATATCGGCA$_{3'}$) and PlagR ($_{5'}$ TGTCGAGGGCAATGATCCA$_{3'}$).

Each 25µl reaction contained 2.5µl 10X DreamTaq buffer (including 2mM $MgCl_2$), 0.25µl dNTPs (100mM, Bioline, London, UK), 1.25µM forward primer, 1.25µM reverse primer, 18.25µl molecular grade PCR water, 0.5µl DreamTaq DNA polymerase (5u/µl) and 1µl DNA template (50µg/µl).

The cycling profile consisted of an initial denaturation of 1 cycle at 95°C for 5 minutes, 30 cycles of denaturation at 95°C, annealing at 60°C and elongation at 72°C for 30 seconds each, and one final cycle at 72°C for 10 minutes.

2.2.8.3 Notocotylid PCR

Larval stage DNA was amplified by PCR using primers designed in the current study (see chapter 6): NotoF ($_{5'}$GCGGTATTCGTTACAACTGTG$_{3'}$) and NotoR ($_{5'}$AGGCGAACCCATCCATC$_{3'}$).

Each 25µl reaction contained 2.5µl 10X DreamTaq buffer (including 2mM $MgCl_2$), 0.25µl dNTPs (100mM, Bioline, London, UK), 1µM forward primer, 1µM reverse primer, 18.75µl molecular grade PCR water, 0.5µl DreamTaq DNA polymerase (5u/µl) and 1µl DNA template (50µg/µl).

The cycling profile followed the same pattern as the Plagiorchiid protocol except for an annealing temperature of 66°C.

2.2.8.4 Cytochrome oxidase I from vole DNA

The cytochrome oxidase I gene was amplified from vole DNA by PCR using the forward primer RonM ($_{5'}$GGMGCMCCMGATATRGCATTCCC$_{3'}$) and the reverse primer NancyM ($_{5'}$CCTGGGAGRATAAGAATATAWACTTC$_{3'}$) according to Pfunder *et al.* (2004).

Each 25µl reaction contained 2.5µl 10X DreamTaq buffer (including 2mM $MgCl_2$), 0.25µl dNTPs (100mM, Bioline, London, UK), 1µM forward primer, 1µM reverse primer,

18.75µl molecular grade PCR water, 0.5µl DreamTaq DNA polymerase (5u/µl) and 1µl DNA template (50µg/µl).

The cycling profile consisted of an initial denaturation of 1 cycle at 95°C for 15 minutes, 45 cycles of denaturation at 95°C for 40 seconds, annealing at 50°C for 40 seconds and elongation at 72°C for 1 minute, and one final cycle at 72°C for 7 minutes.

2.2.8.5 Snail specific PCR

The large subunit (16S) mitochondrial rRNA gene was amplified from *Lymnaea peregra* and *Lymnaea stagnalis* to test the amplifiability of the extracted DNA samples using the forward primer $_{5'}$CGCCTGTTTATCAAAAACAT$_{3'}$ and the reverse primer $_{5'}$CCGGTCTGAACTCAGATCACGT$_{3'}$ according to Remigio and Blair (1997).

Each 25µl reaction contained 2.5µl 10X DreamTaq buffer (including 2mM $MgCl_2$), 0.25µl dNTPs (100mM, Bioline, London, UK), 1µM forward primer, 1µM reverse primer, 18.75µl molecular grade PCR water, 0.5µl DreamTaq DNA polymerase (5u/µl) and 1µl DNA template (50µg/µl).

The cycling conditions consisted of an initial denaturation of 1 cycle at 94°C for 4 minutes. This was followed by a single cycle of 95°C for 3 minutes, 50°C for 2 minutes, 72°C for 1 minute 30 seconds, four cycles of 93°C for 15 seconds, 50°C for 15 seconds, 72°C for 1 minute 30 seconds and 25 cycles of 93°C for 5 seconds, 50°C for 8 seconds and 72°C for 1 minute. A final extension period of 10 minutes at 72°C was conducted.

2.2.9 PCR purification/DNA sequencing

The target DNA was excised from the agarose gel using a UVP transilluminator (Chromato-vue, CA) and purified using a PCR purification kit (Geneflow, Staffordshire, UK) according to the manufacturer's instructions. 10µl of the purified product was re-run on a 1% (w/v) TAE gel to ensure the persistence of the target amplicon prior to packaging. Samples were commercially sequenced in both directions (Source Bioscience, Nottingham, UK). Forward and reverse primers were included with the samples (10µl/reaction) at a concentration of 3.1pmol/µl for both forward and reverse sequencing to be completed.

Sequence data was analysed and regions of ambiguity were verified using Finch TV trace viewer (Geospiza, Seattle, WA). DNA sequence data was compared against the NCBI (National Center for Biotechnology Information) database using the BLAST program (www.ncbi.nlm.nih.gov/BLAST/).

2.3 Data analysis

2.3.1 Host analysis

The overall trapping success for each season was calculated by the number of successful trappings divided by the number of traps laid out during each sampling session. For each rodent species, the percentage of males and females caught according to location and season was determined. Rodent weight in grams, length in centimetres and age structure at each location and during each season was furthermore compared. For determination of rodent age, three age cohorts were established according to Behnke *et al*. (1999). Rodents weighing 1 to 12g were considered juvenile, 13 to 19g young adults and more than 20g adults. Where weight information was lacking (n = 5), rodents were aged using the 'length criteria' established by Watts (1968).

The frequency distribution of *Apodemus sylvaticus* and *Myodes glareolus* was determined. This was not possible for *Microtus agrestis* due to a limited number of samples (n = 9). Length was used as a determinant because weight data was missing for five samples. The mean, standard deviation, median, mode and skew values were calculated using Microsoft Excel (2007) and measures of skewness were interpreted according to the values described by Brown (2011).

2.3.2 Adult helminths

Comparison of helminth prevalence and mean intensity was examined by sampling location, trapping season, host species, host sex and host age. Prevalence was used to indicate the percentage of infected individuals within the sample and was calculated by dividing the number of hosts infected by the number of hosts examined. Species with a prevalence less than 10% were considered rare and ≥10% common, according to Bush *et al.* (1990). 95% confidence limits were applied to all prevalence data using Graphpad Software (http://graphpad.com/quickcalcs/ConfInterval1.cfm). Prevalence data

between two sample sets was analysed by 2 × 2 contingency tables using Fisher's exact test available from Graphpad Software (http://www.graphpad.com/quickcalcs/contingency1.cfm). More than two data sets were analysed using chi-squared test for heterogeneity according to Holmes *et al*. (2006). Intensity was used to indicate the number of helminths recovered from an infected host and mean intensity was calculated by dividing the total number recovered of a particular helminth species by the number of hosts infected with that species. Intensity data was compared using Mood's median test (Rózsa *et al*., 2000).

The association between *Plagiorchis* prevalence and rodent length (cm), weight (g) and rainfall data (mm) were analysed using Spearman's rank of correlation (Fowler *et al*., 1998, p138). Monthly rainfall data were provided by Malham Tarn Field Centre. Prevalence determined each season over a two-year period was analysed in relation to the previous three months rainfall (mm) adapted by Rogan *et al*. (2007).

Helminth dispersion was investigated according to Fowler *et al*. (1998, p65). A dispersion index was calculated using the variance to mean ratio (σ^2/μ). For *Plagiorchis,* expected frequencies were calculated and superimposed onto observed frequencies according to a negative binomial model (Fowler *et al*., 1998, p70). A goodness of fit test was conducted to test the agreement of the selected model (Fowler *et al*., 1998, p116).

2.3.3 Larval digeneans

Comparison of larval digenean prevalence was examined by snail species, location and season. For prevalence, 95% confidence limits were calculated using Graphpad Software (http://graphpad.com/quickcalcs/ConfInterval1.cfm). To test for an association between larval digeneans and snails, 2 x 2 Fisher's exact test was used for the analysis of two sample sets and chi-squared test for heterogeneity when more than two sets were examined (Holmes *et al*., 2006). In order to determine the significance of zero prevalence, for snails that were examined for infection with *Plagiorchis elegans*, binomial confidence intervals were calculated (p = <0.05, two-tailed test) based on standard methods (http://statpages.org/confint.html).

2.3.4 Phylogenetic analysis

Phylogenetic analysis of members of the Plagiorchiidae and the Notocotylidae accessible from NCBI, in addition to those specimens identified during the current study was undertaken using partial sequences of the 28S rRNA gene and the Internal Transcribed Spacer (ITS) regions. Sequences with corresponding GenBank accession numbers have been indicated (Table 2.1 & 2.2). All sequences were trimmed to provide block sequences of the same size allowing for sequence gaps. During analysis, gaps were treated as missing bases. The UPGMA rooted distance method (Phylip 3.69) was employed and nodal support was determined using 1000 bootstrap replicates. Results were displayed by a rooted rectangular cladogram using TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Table 2.1 Internal Transcribed Spacer DNA sequences used for phylogenetic analysis.

Digenean species	NCBI Accession number	Size of DNA fragment (bp)
Plagiorchis maculosus	AF316152	901
Plagiorchis koreanus	AF151944	908
Plagiorchis vespertilionis	AF151949	937
Plagiorchis muelleri	AF151947	953
Brachylaemus recurvum	Chapter 6	1020
Diplostomum spathaceum	Chapter 6	927
Notocotylus malhamensis	Chapter 6	956
Tylodelphys scheuringi	FJ469596	925
Diplostomum indistinctum	GQ292508	924
Diplostomum huronense	GQ292507	922
Aptorchis sp.	AM932523	936

Table 2.2 Partial 28S ribosomal DNA sequences used for phylogenetic analysis.

Digenean species	NCBI Accession number	Size of DNA fragment (bp)
Haplometra cylindracea	Chapter 6	1071
Plagiorchis koreanus	AF151944	1071
Plagiorchis vespertilionis	AF151949	1071
Plagiorchis muelleri	AF151947	1071
Paramonostomum anatis	AF184258	1073
Notocotylus attenuates	AF184259	1072
Notocotylus sp. UK	AY222219	1071
Notocotylus sp. BH	EU712725	1072
Catatropis indicus	AY222220	1072
Notocotylus malhamensis	Chapter 6	1072
Notocotylus cercariae	Chapter 6	1075

CHAPTER 3:

Helminth communities of rodents from Malham Tarn, North Yorkshire, UK.

3.1 Introduction

The early study of parasitology in the UK gave little attention to the ecological aspect of helminth infection in wild animals (Lewis, 1968a). Although comprehensive reference lists documenting the helminth findings of earlier workers were well established (Nicoll, 1923; Baylis, 1928b; Baylis 1939), the extent of information provided in these lists tends to be deficient and in most instances, simply provides a host name, location and helminth name and tends to lack further reference. Many reports also focused solely on species descriptions (Harper, 1929; Beverley-Burton, 1961) with little or no indication of prevalence or intensity. In 1931, Elton *et al*. conducted an extensive study into the helminth parasites infecting the wood mouse *Apodemus sylvaticus* and the bank vole *Myodes glareolus* in Oxfordshire, UK. As far as can be determined, this was the first UK report to examine seasonal variation in helminth communities and indicate a positive correlation between an increase in host age and the level of parasitism.

The helminth fauna of British rodents has since been well documented (Lewis 1968a; Lewis and Twigg, 1972; Lewis, 1987; Behnke *et al.,* 1999; Abu-Madi *et al*., 2000) with the most comprehensive of these studies tending to not only analyse prevalence and intensity data but also focus upon the factors that may potentially affect the distribution and composition of the helminth community (Behnke *et al*., 1999; Abu-Madi *et al*., 2000; Behnke *et al*., 2009). Helminth communities are typically studied at three hierarchal levels. The infracommunity refers to all of the parasite species harboured by a single host specimen, the component community is all the parasite species within a host population and the compound community examines all of the parasite species within an ecosystem (Holmes and Price, 1986).

The most recent advances in the study of parasite community ecology have been made at the infracommunity level whereby the interactions between infrapopulations are analysed (Poulin, 2001). As reviewed by Poulin (2001), the most common type of interaction to occur is the competition for food and space. The different responses of the helminth infrapopulations during concomitant infections have been identified to be numerical or functional, whereby the size of one or both infrapopulations reduce in

response to the presence of the second population or where the realised niche is adjusted to prevent overlap between the two respectively. According to Poulin (1999), what determines which species is negatively affected during a concomitant infection may simply be a result of which species establishes first within the host or an intrinsic biological property such as body size. Despite the procurement of a large body of knowledge regarding the patterns and processes that function in determining the structure of these communities, there is still no general consensus regarding the importance of interspecific interaction (Poulin, 2001).

Generally, the ecological aspect of studying helminth infection in wild mammal populations, tends to focus on the importance of intrinsic (host sex and host age) and extrinsic (season and location) factors in shaping the helminth community (Abu-Madi *et al*., 2000). According to Lewis (1968a), the variation in parasite abundance and composition frequently observed is due to several contributing factors that in addition to host sex and age, is highly influenced by the correlation between the life history of the respective parasite and changes in the density, feeding habit and behaviour of the host population.

3.1.1 Host sex

The effect of host sex on helminth infestation rates has been frequently addressed. Lewis and Twigg (1972) reported adult male wood mice and adult male bank voles to harbour larger worm burdens of a wider range of helminth species than their female counterparts. They suggested that the immune response to helminths was weaker in males than females resulting in male mice having a greater susceptibility to infection. In his earlier work, Lewis (1968a) had previously reported female wood mice to be more resistant to infection with the nematodes *Heligmosomoides polygyrus* and *Syphacia stroma* than males. He suggested that these differences were related to the age and the sex of the host which depicted their differences in movement and foraging habits, in addition to differences in hormones between the two sexes. As discussed by Poulin (1996) there are intrinsic biological differences between males and females and high testosterone levels in males can ultimately cause immunosuppression, which results in males being more prone to infection. Additionally, the female hormone, oestrogen has

been documented to increase resistance against helminth infection (Haukisalmi *et al*., 1988).

Poulin *et al*. (2011) coupled parasite dispersion with the spatial ecology of the host. It is evident that the home range of male rodents is much greater than for females (Langley and Fairley, 1982; Wolton, 1985; Attuquayefio *et al*., 1986), in particular during the breeding season (Corp *et al*., 1997). Males have furthermore demonstrated much more foraging activity than females (Buesching *et al*., 2007) where females have a greater dependency on resources and tend to establish mutually exclusive breeding territories (Flowerdew, 1993). According to Lewis (1968a), male rodents are more likely to become infected with helminths such as digenean species that utilise intermediate host species whilst more actively foraging in the field. In a study including digenean trematodes in the west of Ireland however, Langley and Fairley (1982) found that female mice were more heavily infected than their male counterparts, which they suggested was due to females having a greater food requirement and demonstrating a greater foraging behaviour of invertebrates during the breeding season.

In other studies conducted by Abu-Madi *et al*. (1998; 2000) and Hildebrand (2008) however, host sex was not considered to be a significant determinant in the variation observed in abundance and distribution of any of the examined helminth species. According to Poulin (1996), statistically significant inequalities between the sexes and helminth infestation are not very commonly reported and the observation of one sex bias over another is generally considered to be caused by the particulars of the host-parasite system under study. He did however suggest that the many non-significant sex biased reports found in the literature may in fact provide a significant result if pooled, as sex bias may not always be evident from a single host sample.

3.1.2 Host age

The relationship between rodent age and the level of parasitism has also been well documented with several studies indicating an increase in worm burden with an increase in host age (Elton *et al*., 1931; Lewis, 1968a; Lewis and Twigg, 1972; Langley and Fairley, 1982; Behnke *et al*., 1999). Langley and Fairley (1982) commented on the lack of knowledge regarding the rate at which helminths are expelled by the host's

immune response and suggested that the assessment of findings from each age cohort should be speculative. Studies by Hong *et al.* (1998) have since identified the efficiency of the immune system in expelling the digenean trematode *Plagiorchis muris* from the small intestine of albino laboratory rats. They determined that up to 96% of *P. muris* worms were expelled from the small intestine within 28 days post infection. This data suggests that infections identified from the field are highly likely to have occurred within the preceding month.

Langley and Fairley (1982) stated that older mice are likely to be more parasitized than their younger cohorts simply because they have had a longer time in which to acquire infection. Lewis (1968a) suggested that this is because adult rodents demonstrate a greater foraging activity than juveniles. As pointed out by Lewis (1968a) however, infection may be related to the life history of the particular parasite species under examination. For helminths with direct life cycles such as *Syphacia* spp., transmission may be reliant upon host contact and therefore infection rates may be density dependent and as such rodents of all ages may have an equal chance of acquiring infection (Lewis, 1968a). For helminth species with indirect life cycles, a greater difference in prevalence and intensity between the different age cohorts is likely. For example, transmission of digenean trematodes and some cestodes is dependent upon the development and the density of important intermediate host species and their location. For rodent infection to occur movement of the host species will be required and therefore older rodents that demonstrate a greater foraging activity (Bueshing *et al*., 2007) are more likely to harbour infection.

3.1.3 Seasonal variation

Since the work of Elton *et al*. (1931), seasonal fluctuation in helminth abundance and composition has been well established (Lewis, 1968a; Langley and Fairley, 1982; Chai *et al.,* 2007). According to Lewis (1968a), yearly variation in parasite abundance and composition is influenced by a combination of factors including the association between the parasite's developmental cycle and population changes of the host. For parasites with indirect life cycles such as digenean trematodes, Haukisalmi *et al*. (1988) noted that seasonal variation may result from a temporary restriction in transmission by the occurrence or absence of 'keystone' intermediate host species. Langley and Fairley

(1982), found digeneans to be most common during the summer and decline in the autumn, a pattern that they speculated was following a period of invertebrate feeding by wood mice during the early summer. Chai *et al*. (2007) also reported the highest mean worm burden of the digenean *P. muris* to occur during the summer although the peak prevalence occurred in the autumn. It was postulated that these peaks coincide with the development of insect intermediate hosts to their adult forms that can be consumed by rodents during these periods, and that the lack of infection observed during the spring was due to few insects surviving the cold winter months in northern Gyeonggi-do Province, Korea.

In the case of helminths with direct life cycles, the infective stages may vary in response to seasonal conditions of temperature and humidity and as such it may be expected that the number of infective eggs or larvae will decrease in the winter months and increase during the summer (Lewis, 1968a). In a study conducted by Hildebrand (2008), helminths were observed more frequently during the summer and autumn which was considered to be influenced by the seasonal activity of small rodents. In the studies of Abu-Madi *et al*. (1998; 2000) seasonal fluctuation and site-specific variation were deemed the most important determinants for the differences observed in worm burden and prevalence of the intestinal helminths *H. polygyrus*, *S. stroma* and *Catenotaenia pusilla* in an *A. sylvaticus* population in the south-east of England. During this study, worm burden for these helminths was contrastingly highest during the spring and the winter with reduced burdens occurring during the summer and the autumn. Slight differences in peak periods were however observed between sampling locations suggesting that more than a single independent effect may be involved in influencing the helminth community at any one time, and that rather there may be a combination of interactive effects involved in influencing transmission (Behnke *et al*., 1999).

3.1.4 Site-specific variation

The helminths of small mammals have been studied worldwide from a large variety of habitat types including rough grasslands, woodland (Lewis, 1968a), irrigation fields and water distribution areas (Hildebrand, 2008; Hildebrand and Zaleśny, 2009), pastures and agricultural areas (Shimalov, 2002) and even from domestic environments (Seo *et al.,* 1964). It has been well established that helminth community structure can vary in

response to site-specific conditions (Abu-Madi *et al*., 1998; Behnke *et al*., 2001; Brouat *et al*., 2007; Hildebrand, 2008). Abu-Madi *et al*. (1998) compared three contrasting habitat types. Although similar patterns in the helminth fauna were observed from two sites, differences were observed in the third. At the third location the soil was more acidified, and it was suggested that this resulted in a lower survival rate of free living larval stages that require optimal conditions of soil structure, moisture, temperature, and pH for development.

It may be hypothesised that different locations of comparable habitat quality may support similar helminth communities. In a study by Behnke *et al*. (2001) the influence of intrinsic factors were found to be less important in shaping the component community and in fact it was determined that different sites were responsible for the variation observed in community structure even when the different sampling locations were in close proximity and of similar habitat type. Hildebrand (2008) furthermore found that the differences observed in helminth composition and abundance at both the component and compound community levels were attributed by site-specific conditions.

Studies that examine the helminth communities of different rodent species that live in sympatry have also revealed differences in helminth composition and abundance. Brouat *et al*. (2007) examined two species of closely related *Mastomys* rodents that although are sympatric, differ in their habitat choice. Many helminth species were recorded from both hosts; however the variation in prevalence and intensity of some helminths was correlated with habitat factors such as the presence of a 'water body', indicating habitat characteristics to have a profound impact on the overall helminth community structure. Another study conducted by Simoes *et al.* (2011) examined three closely related rodents that also live sympatrically in a coastal forest in Brazil. Although differences were observed between infracommunities, similarities were observed at the component community level. The data suggested that helminth specificity in addition to host habitat were significant factors in structuring the helminth community.

3.1.5 Host behaviour

In habitats where different rodent species occur sympatrically, the differences exhibited in aspects of their biology including habitat choice and feeding habits may ultimately

affect the abundance and composition of their helminth community (Žąsityté, 2001). Lewis (1987) furthermore suggested that the composition of helminth fauna in any small mammal population is dependent upon the food intake and feeding habits of the host. Hildebrand and Zaleśny (2009) demonstrated both qualitative and quantitative differences in the digenetic helminth fauna of small rodents in Lower Silesia, Poland which they also speculated was due to differences in the feeding habits of the host species involved.

The most extensively studied UK rodents tend to be the wood mouse *A. sylvaticus*, the bank vole *My. glareolus* and the field vole *Microtus agrestis* (Elton *et al*., 1931; Lewis 1968a; Lewis 1968b; Lewis and Twigg, 1972; Abu-Madi *et al*., 1988; Behnke *et al*., 1999; Abu-Madi *et al*., 2000). *A. sylvaticus* is typically granivorous which through a complex system of tunnels both above and below ground, has a wide foraging range. During periods when seeds are scare, the diet of *A. sylvaticus* will change from granivorous to insectivorous (Lewis and Twigg, 1972; Montgomery and Montgomery, 1990b).

My. glareolus on the other hand has a more limited home range and tends to restrict its foraging to hedgerows and areas where cover is thick (Lewis, 1987). Although this species will consume small amounts of animal food, its diet is predominantly folivorous. *Mi. agrestis* however occurs mainly in rough grasslands and has a very small home range in comparison. This species is strictly herbivorous and predominantly consumes grass (Lewis, 1987). It has been suggested that the difference in diet between host species will in turn be reflected in the abundance and composition of the parasite fauna harboured (Lewis, 1987). For instance, it may be expected that a higher rate of cestodes and digeneans that encompass invertebrate intermediate hosts in their life cycle will be observed in the wood mouse rather than either of the vole species.

This is typical of insectivorous mammals, in which the helminth fauna tends to consist predominantly of a larger number of trematode and cestode species (Lewis, 1968b). Lord *et al*., (2012) conducted a study into the helminth communities of bats collected from the Greater Manchester and Lancashire regions in England. Digenean trematodes were the only helminth group identified and this was considered to be due to the foraging and dietary habits of the species examined. Other studies, have found the

helminth fauna of shrews to be dominated by cestodes and particularly those of the Hymenolepidae that utilise insect intermediate hosts for transmission (Haukisalmi, 1989). The helminth communities of shrews are typically found to be species-rich. In another study of common shrews conducted by Shimalov (2001), 95.3% of hosts were reported to be infected. In this study 32 helminth species were identified which included 11 trematodes, 11 cestodes and 10 nematodes. The majority of helminths identified in this study were considered to be specific parasites to insectivorous mammals.

Examination of this information suggests that differences in the helminth communities of various rodent fauna at any location would be apparent. It may be speculated that the helminth fauna of voles that are predominantly herbivorous will consist mainly of monoxenous nematodes whilst wood mice are far more likely to harbour heteroxenous parasites such as digenean and cestode species that are transmitted via insect intermediate hosts.

3.1.6 Study site

Malham Tarn Nature Reserve is a site of Special Scientific Interest (SSSI) located in North Yorkshire at an altitude of 375m above sea level, and has been the location of an annual parasitology field course conducted by The University of Salford since 1993. The parasites of the wood mouse *A. sylvaticus* and other animals at this location have been extensively studied for a range of host parasite systems (Kennedy & Burrough, 1978; Allan *et al*., 1999; Hughes *et al*., 2006; Rogan *et al*., 2007; Hughes *et al*., 2008;; Behnke *et al*., 2009; Thomasson *et al*., 2011). Specifically, Rogan *et al.* (2007) reported the rare occurrence of the intestinal digenean *P. muris* in *A. sylvaticus* over a 13-year period with an overall prevalence of 16.9%. Typically, *Corrigia vitta* and *Brachylaemus recurvum* (syn. *Brachylaima recurva*) that infect the pancreatic ducts and the small intestine of their host respectively are the most commonly identified digeneans of UK rodents (Elton *et al*., 1931; Lewis, 1968a; Lewis and Twigg, 1972; Benhke *et al.,* 1999; Abu-Madi *et al*., 2000). To date, *C. vitta* has not been recorded from Malham Tarn, however Lewis (1968a) indicated this species to demonstrate seasonal variation and it may be suggested that this digenean has been overlooked at this location predominantly upon this basis.

3.1.7 Aims

So far, the investigations conducted into the parasites of small mammals by Salford University have only been carried out during September and typically from one site (Tarn Woods). In a comparative study conducted by Behnke *et al.* (2009), the helminth fauna of Malham Tarn appeared slightly depauperate in both richness and abundance in comparison to other sites. It has been recognised that ecological studies can be confounded by sampling effort. As total species richness is considered to strongly correlate with sampling effort (Walther *et al*., 1995), this chapter aims to contribute a continuation of the work made by Salford University, by extending the scope of analysis to other sites within the nature reserve. It is furthermore the intention to examine factors that may contribute in defining the helminth communities of rodents at Malham Tarn, including host sex and age and both seasonal and site-specific variation. The component communities of Malham Tarn will furthermore be compared, given that the differences in feeding habits and behaviour may influence the helminth fauna harboured by each host population.

3.2 Materials and Methods

3.2.1 Rodent sampling

Rodent trapping was conducted quarterly between January 2010 and October 2011 from four woodland sites surrounding the tarn (Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation). Rodents were trapped using Longworth small mammal traps according to the methods described in 2.1.3. Rodents were initially identified by morphology (see 3.2.2). Molecular differentiation was furthermore employed to verify the identity of the morphologically similar bank vole *Myodes glareolus* and field vole *Microtus agrestis* (see 3.2.3). Rodents were processed and dissected as describe in section 2.2.1.

3.2.2 Morphological identification

Rodents were identified on the basis of fur colour and markings, ear size and tail length (Table 3.1). *Apodemus sylvaticus* was distinguished from the morphologically similar yellow necked mouse *Apodemus flavicollis* by its chest markings. For *A. sylvaticus* the underside is white in comparison to *A. flavicollis* which possesses an unbroken yellow band across the chest and forelegs (Sargent and Morris, 2003). Separation of the two vole species at this location were primarily differentiated by tail length with the tail of *My. glareolus* measuring 50% of the head and body length, and the tail of *Mi. agrestis* measuring only 30% in comparison (Sargent and Morris, 2003).

Table 3.1 Morphological features used to identify rodents trapped from Malham Tarn. Information retrieved from Sargent and Morris (2003).

Rodent species	Markings	Tail length	Ears
A. sylvaticus	Reddish-brown with white chest	Long with black fur on top	Large and prominent
My. glareolus	Reddish-brown	50% the length of the head and body	Prominent from body fur
Mi. agrestis	Greyish-brown	30% the length of the head and body	Difficult to visualise from body fur

3.2.3 Molecular identification

The morphological identification of *My. glareolus* and *Mi. agrestis* was verified by molecular analysis. DNA was extracted from a piece of rodent thigh muscle according to

methods described in section 2.2.7. DNA barcoding of the cytochrome oxidase I gene was conducted using the methods explained in 2.2.8 and 2.2.8.4. The target DNA was purified and commercially sequenced in both directions (2.2.9) and results were compared with those already present within the NCBI database.

3.2.4 Host population genetics

Analysis of the population structure of *A. sylvaticus* at Malham Tarn between January 2010 and October 2011 was conducted by Jaroslav Bajnok of the Molecular Epidemiology Group at The University of Salford, using samples which I provided to him during this time period. These methods were not employed for either vole population due to both financial constraints, in generating genotyping primers, and small sample sizes. Genotyping using fluorescent microsatellite markers of ten loci (1A, 2A, 3A, 4A, 7S, 8S, 9A, 12A, TNF, MS19) was conducted using a Hitachi 3130 genetic analyser (Applied Biosystems, UK).

3.2.5 Data analysis

Helminths were morphologically examined and identified according to the methods presented in section 2.2.4. Comparisons made for prevalence and intensity according to host sex, host age, host population, seasonal variation and site-specific differences were analysed using the methods described in 2.3.2.

3.3 Results

3.3.1 Host trapping success

A total of 180 rodents including *A. sylvaticus* (n = 117), *My. glareolus* (n = 54) and *Mi. agrestis* (n = 9) were successfully trapped and examined (Table 3.2).

Table 3.2 The percentage of each rodent species trapped in the present study. The percentage of males and females trapped by species has been indicated.

Rodent species	% caught	95% Confidence Limits		Sex	Number	% caught	95% Confidence Limits	
		Lower	Upper				Lower	Upper
A. sylvaticus (117/180)	65	57.78	71.60	**M**	71	61	51.62	69.07
				F	46	39	30.93	48.38
M. glareolus (54/180)	30	23.77	37.07	**M**	34	63	49.60	74.60
				F	20	37	25.40	50.40
M. agrestis (9/180)	5	02.52	09.36	**M**	6	67	35.09	88.27
				F	3	33	11.73	64.91

Both *A. sylvaticus* and *My. glareolus* were trapped from all four sampling locations whereas *Mi. agrestis* was trapped from Tarn Woods and Tarn Fen only (Figure 3.1). For *A. sylvaticus* more males were examined from each site except for Tarn Fen but for *My. glareolus* this was the reverse with more females being trapped from each site except for Tarn Fen where the yield of male rodents was slightly greater. For *Mi. agrestis* a greater number of females were caught from Tarn Fen but more males were trapped from Tarn Woods.

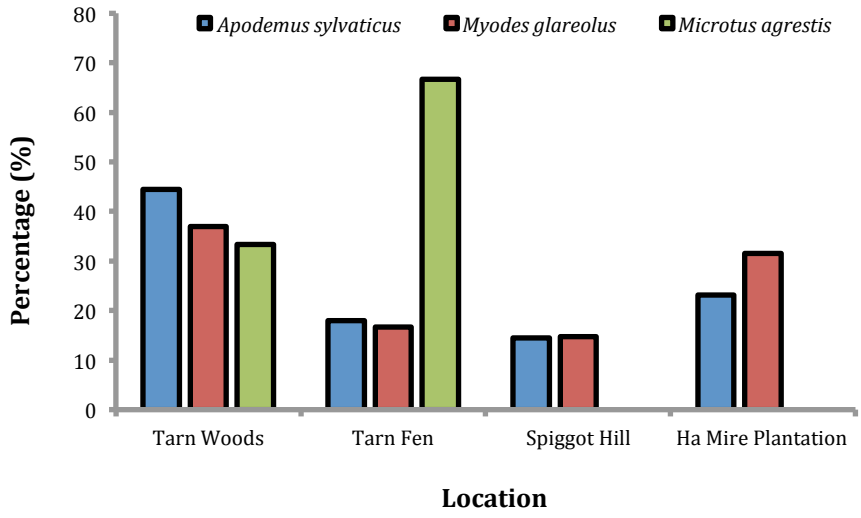


Figure 3.1 The percentage of each rodent species trapped by sampling site.

Both *A. sylvaticus* and *My. glareolus* were trapped every season whereas *Mi. agrestis* was trapped during the summer and the autumn periods of 2011 only (Figure 3.2). For *A. sylvaticus,* winter and spring yielded the lowest number of samples with the highest catch rate occurring during the autumn. More males were caught during the winter, summer and autumn and an equal number of male and females were trapped during the spring.

Similarly, the trapping rate for *My. glareolus* was lowest during the winter and the spring and peaked during the autumn. Contrastingly however, more female than male bank voles were caught each season except for spring when male trappings dominated. For *Mi. agrestis*, an equal number of male and female field voles were trapped during the summer but far more females were caught during the autumn.

A breakdown of the percentage of male and female rodents trapped from each location and during each season has been presented in appendix A.

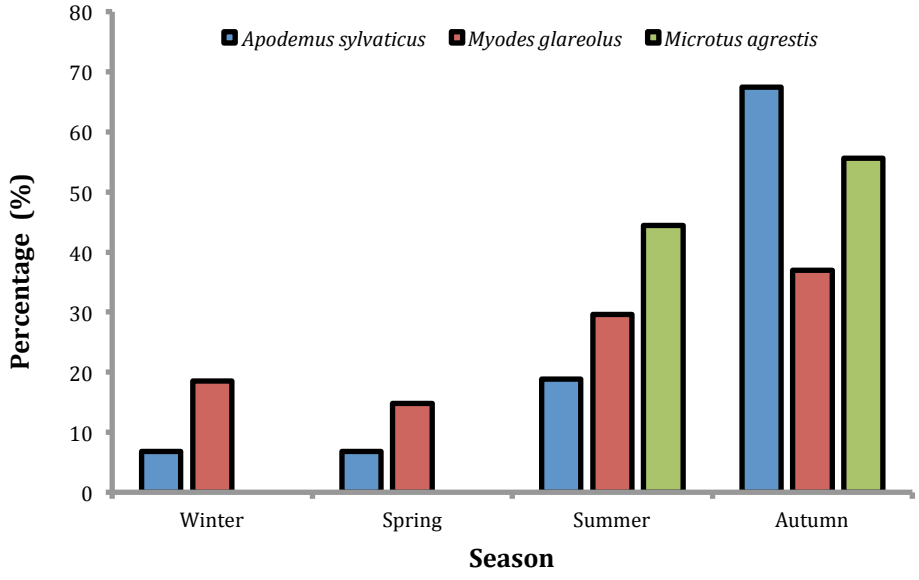


Figure 3.2 The percentage of rodents trapped each season.

3.3.1.1 Host identification

Rodents were successfully identified morphologically using the criteria of Sargent and Morris (2003) (Figure 3.3). Identification of the two vole species was furthermore confirmed by molecular barcoding of a 439bp sequence of the Cytochrome Oxidase 1 gene (Figure 3.4). In both cases, animals identified by morphology as *My. glareolus* and *Mi. agrestis* were confirmed by 99% sequence homology with NCBI reference sequences (*My. glareolus* AY332679 and *Mi. agrestis* AY332684). Sequences from Malham voles generated during the present study have been deposited into GenBank under accession numbers (*My. glareolus* JQ794805 and *Mi. agrestis* JQ794806).

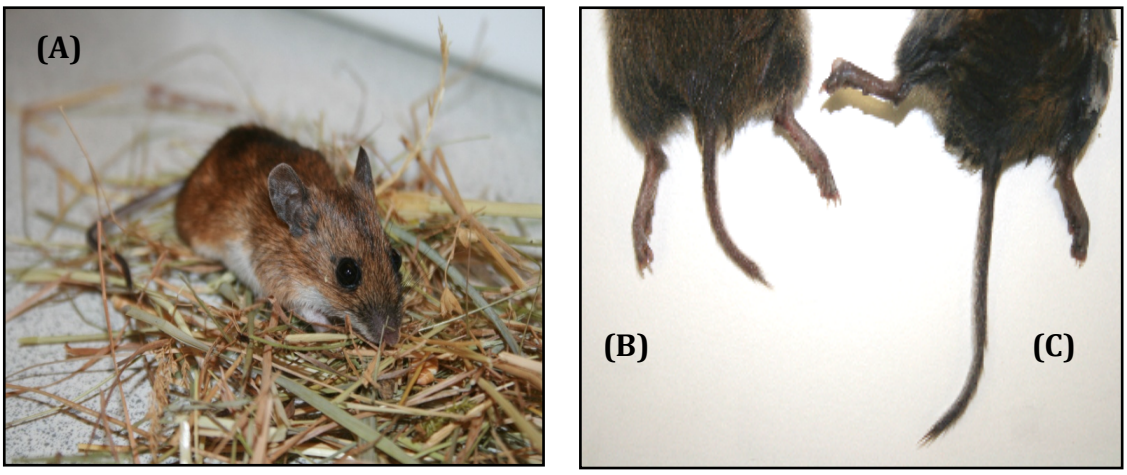
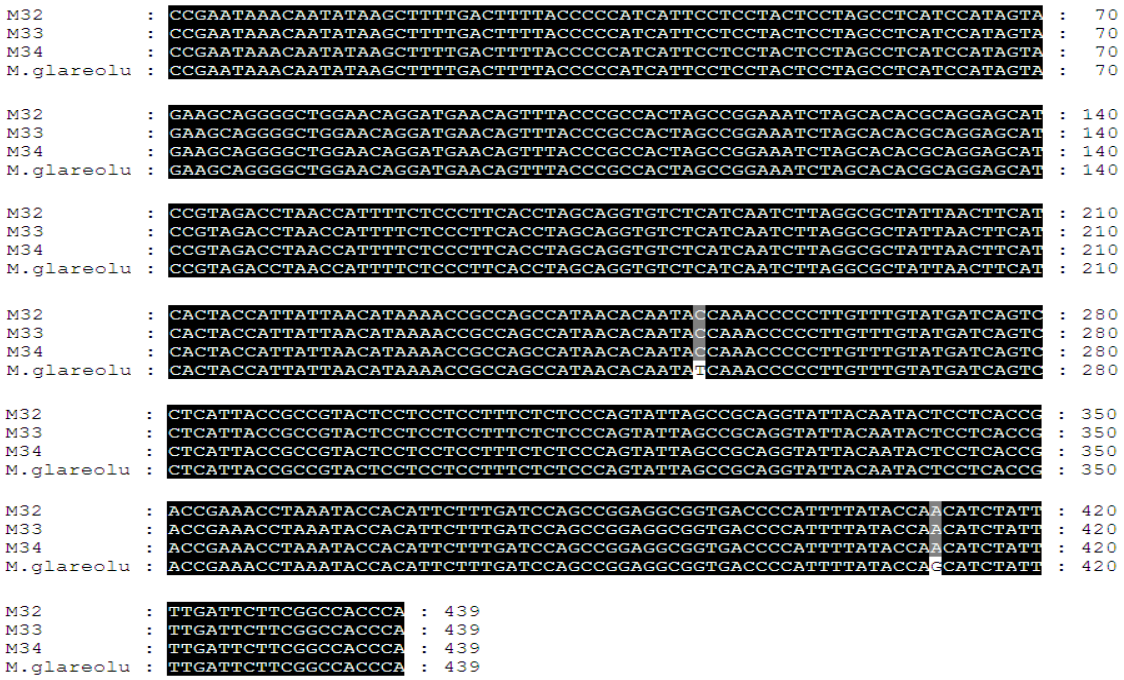


Figure 3.3 Morphological identification of rodents trapped from Malham Tarn during the present study. **(A)** The wood mouse *Apodemus sylvaticus*. **(B)** The short-tailed field vole *Microtus agrestis* and **(C)** the bank vole *Myodes glareolus*.

(A)



(B)

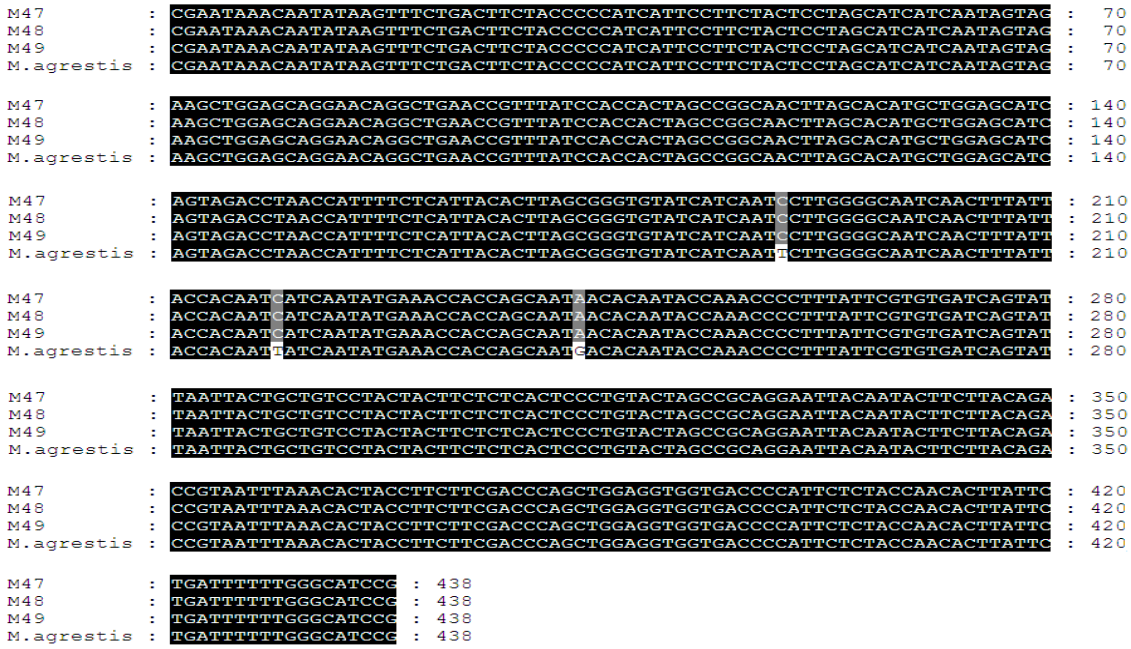


Figure 3.4 A partial comparison of the mitochondrial cytochrome oxidase I gene of the species identified as **(A)** *My. glareolus* and **(B)** *Mi. agrestis,* against sequences obtained from NCBI (Accession: *Mi. agrestis* AY332684.1; *My. glareolus* AY332679.1). Data has been presented using GeneDoc alignment tool. Black shading indicates regions of conserved homology. Sample numbers M32 to M34 were morphologically identified as *My. glareolus* and sample numbers M47 to M49 were morphologically identified as *Mi. agrestis*.

3.3.1.2 Host population genetics

In total, 10 microsatellite loci were examined. Using the programme STRUCTURE, Jaroslav Bajnok (Ph.D student, University of Salford) used the microsatellite profile of 108 wood mice and identified three (K = 3) main phylogenetically different population clusters at Malham Tarn (Figure 3.5). Gene flow between these three populations can be observed and dispersion of the wood mouse samples throughout the area infers migration and interbreeding of mice between the various sampling sites. Each population cluster does however appear to reflect the geographical origins of the examined samples. The largest cluster (population 3 - blue) was identified from all four sites but predominantly from the southern area of the tarn (Figure 3.6). This microsatellite profile was also the only type to be found at Spiggot Hill suggesting the origin of this genotype to be from this location. The second main population (population 1 - red) appears to be the dominant profile originating from Tarn Woods with 31 rodents at this location carrying this genotype. Only five other mice carrying this profile were identified from other locations, four from Tarn Fen and one from Ha Mire Plantation. The smallest cluster (population 2 - green) was found to be equally distributed between Tarn Woods and Ha Mire Plantation and contained only a single wood mouse from Tarn Fen.

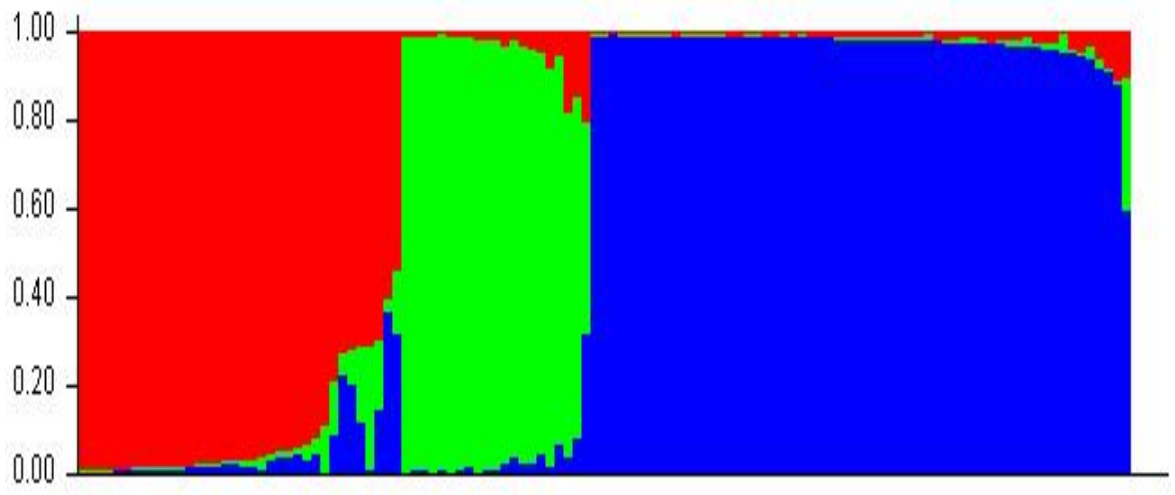


Figure 3.5 Estimated population structure of the Malham Tarn wood mice using STRUCTURE software (Pritchard *et al.*, 2000) to interpret microsatellite genotyping data (K=3). Each K population is indicated by a colour segment. **Key**: **population 1** = red; **population 2** = green; **population 3** = blue**.** Data analysis and image courtesy of Jaroslav Bajnok (Ph.D student, University of Salford).

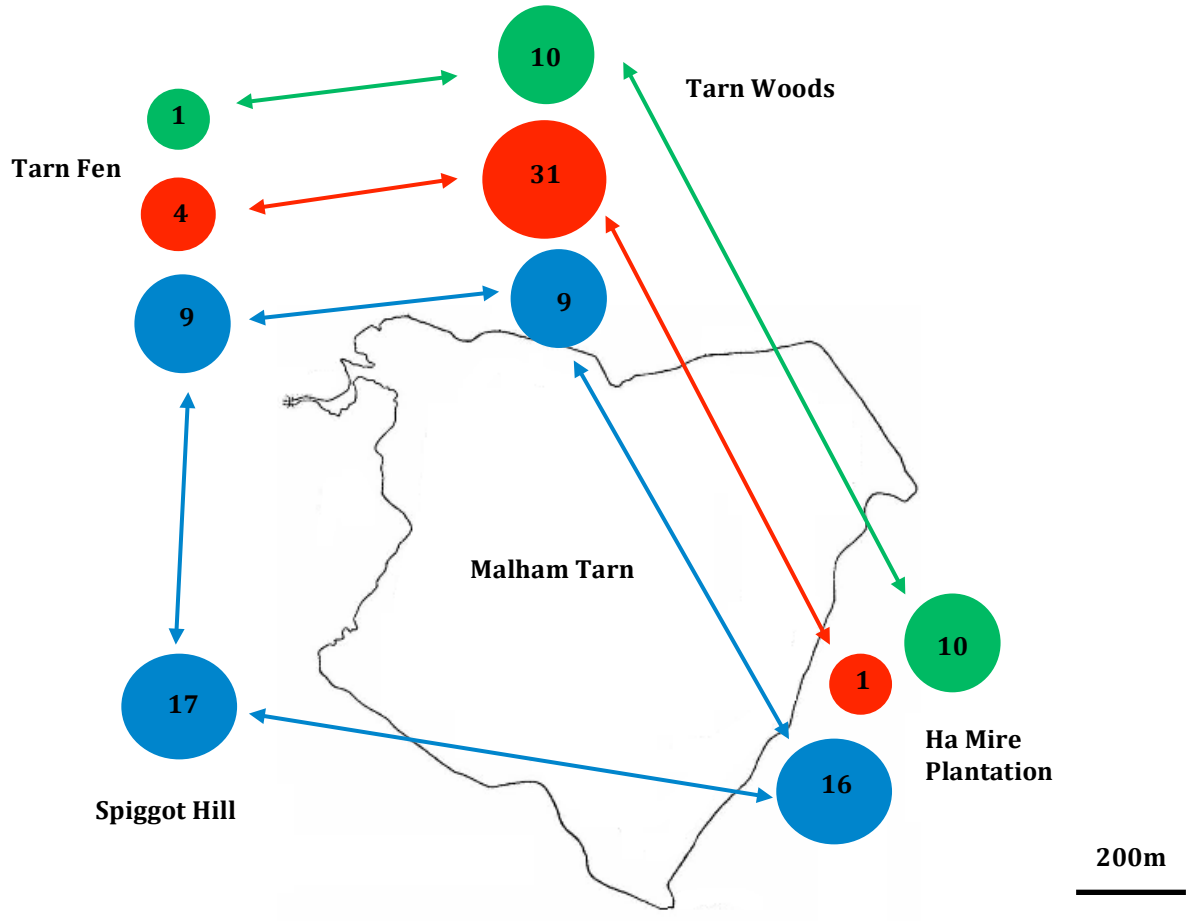


Figure 3.6 Dispersion of K populations throughout the Malham Tarn sampling sites. K=3. Each K population is indicated by a colour segment. **Key**: **population 1** = red; **population 2** = green; **population 3** = blue. Numbers represent the number of rodents carrying the main microsatellite profile at each location. Data is missing for two rodents trapped from Tarn Woods and seven from Tarn Fen.

3.3.1.3 Host frequency distribution

The frequency distributions of *A. sylvaticus* and *My. glareolus* were established based on rodent length (cm). Frequency distribution analysis could not be performed for *Mi. agrestis* due to the limitation in sample size (n = 9).

The body length of *A. sylvaticus* ranged from 4.5 to 9.8cm for females and 5.8 to 9.5cm for males. The distribution of wood mice appeared to fit the criteria of a normal distribution with the mean length (7.9cm) almost equalling the median (8cm) and mode (8cm) and a total of 78% (91/117 × 100) of the observations falling within the range of x ± 1s (7.9 ± 0.92). Calculation of skewness demonstrates a moderate negative skew (γ^3 = -0.75) indicating the sample set to contain a greater number of mice with longer length.

This was reinforced with 56% of wood mice measuring above the mean value (Figure 3.7A).

The body length of *My. glareolus* ranged from 6.3 to 9.5cm for females and 6.5 to 9.4cm for males. Bank voles had a mean length of 7.9cm, a median value of 8.05cm and a mode of 8.5cm. Only 65% (35/54 × 100) of observations were seen to fall within $x \pm 1s$ (7.9 ± 0.93). Calculation of skew (γ^3 = -0.21) indicated the sample set to demonstrate slightly more approximate symmetry than wood mice although 54% of voles still measured above the mean value (Figure 3.7B).

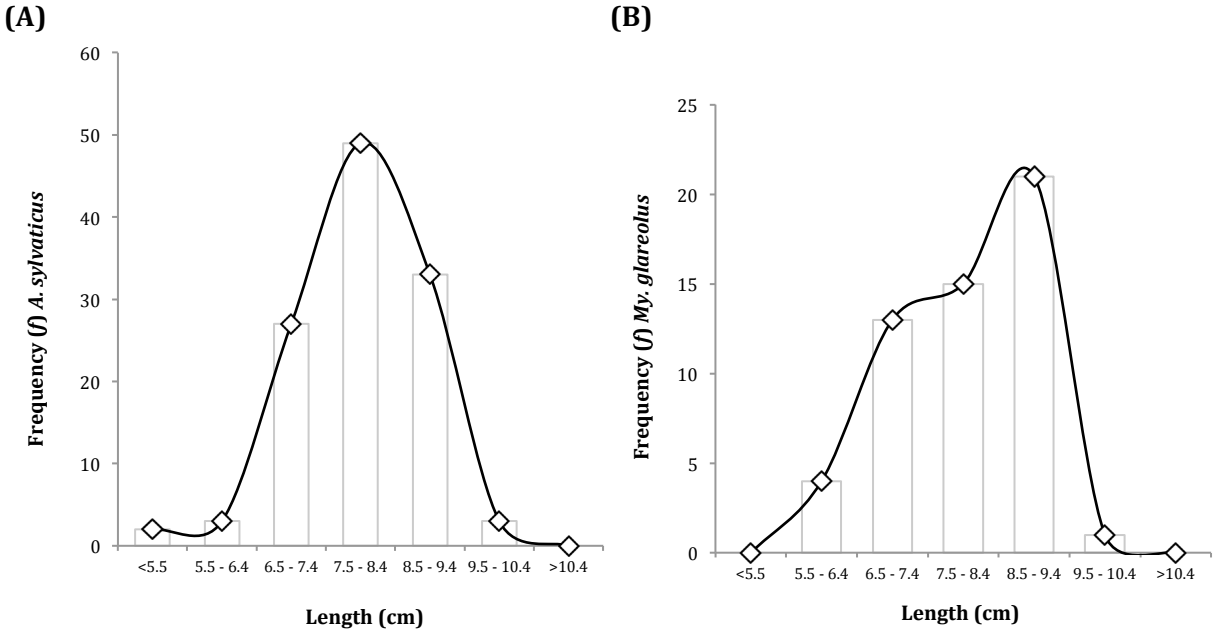


Figure 3.7 Frequency distribution of **(A)** *A. sylvaticus* (n = 117); **(B)** *My. glareolus* (n = 54).

3.3.1.4 Host age structure

Using weight and length data accumulated during the study, the age structure of rodents was established using the criteria of Watts (1968) and Behnke *et al*. (1999) (Table 3.3). A breakdown of mean values for rodent weight and length trapped at each location and during each season has been indicated in appendix B.

The age structure of rodents trapped each season and from each location has furthermore been presented in appendix C.

Table 3.3 The overall percentage of each rodent species examined within the three established age groups based on the criteria of Watts (1968) and Behnke *et al*. (1999).

Rodent species	Age group	Percentage caught (%)	95% Confidence Limits	
			Lower	Upper
A. sylvaticus	**Juvenile**	14.5	09.18	22.14
(n = 117)	**Young adult**	47.9	39.02	56.84
	Adult	37.6	29.35	46.66
My. glareolus	**Juvenile**	20.8	11.84	33.63
(n = 54)	**Young adult**	54.7	41.45	67.35
	Adult	24.7	14.81	37.69
Mi. agrestis	**Juvenile**	11.1	<00.01	45.67
(n = 9)	**Young adult**	33.3	11.73	64.91
	Adult	55.6	26.63	81.16

3.3.2 Helminth findings

3.3.2.1 Species richness

In total, nine intestinal helminth species were identified which included three digenean species, five nematode species and one cestode (Table 3.4 and Figure 3.8). Species richness differed by host species, sampling site, age group and season (Figure 3.9). *Brachylaemus recurvum* was recovered from Tarn Fen only which added to the greater richness observed at this location, however it should be emphasised that *B. recurvum* was extremely rare (<10%) being recovered from only two rodents throughout the duration of the study and has been previously recorded from *A. sylvaticus* trapped from Tarn Woods (Salford University unpublished data). Furthermore, although male rodents harboured nine helminth species compared to seven for females, this was due to both *H. glareoli* and *B. recurvum* being found in male rodents only. Both sample sizes however were extremely small and *B. recurvum* has previously been reported parasitizing female wood mice at Tarn Woods (Salford University unpublished data).

Table 3.4 Helminth richness at Malham Tarn between January 2010 and October 2011. **Key = T:** Trematoda**; N:** Nematoda**; C:** Cestoda**.**

	Rodent species		
	A. sylvaticus	***My. glareolus***	***Mi. agrestis***
T	*Plagiorchis elegans* *	-	-
	-	*Notocotylus malhamensis* *	*Notocotylus malhamensis* *
	Brachylaemus recurvum	-	-
N	*Heligmosomoides polygyrus*	*Heligmosomoides glareoli*	*Heligmosomoides glareoli*
	Syphacia stroma	*Syphacia* sp.	-
	Aoncotheca murissylvatici	*Aoncotheca murissylvatici*	-
C	-	*Hymenolepis diminuta*	*Hymenolepis diminuta*

* *Plagiorchis elegans* and *Notocotylus malhamenesis* have been identified on the basis of other work conducted within this thesis (see chapters 4 and 5).

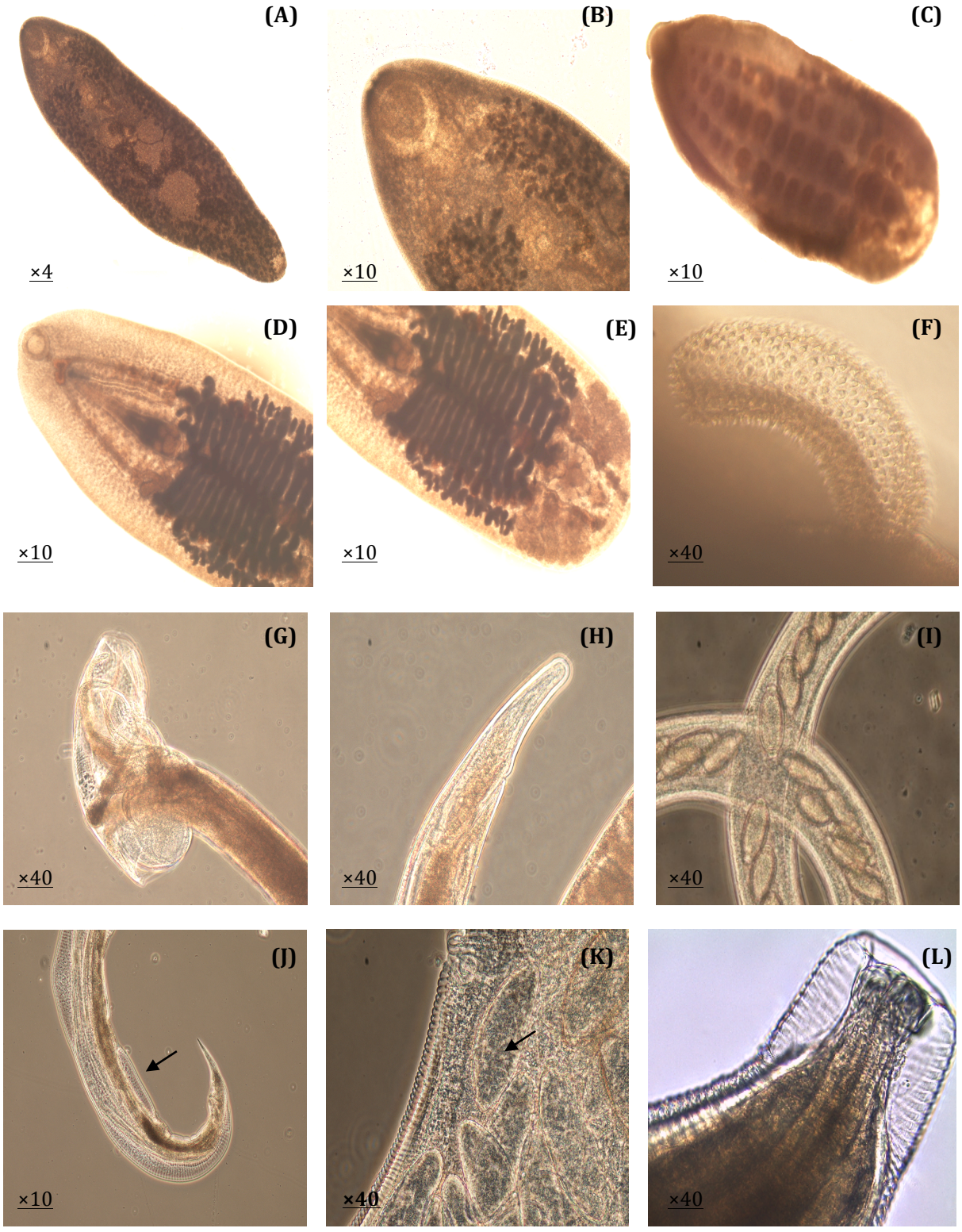


Figure 3.8 Photographed Species identified during the current study. **(A)** *Plagiorchis elegans*, entire specimen; **(B)** anterior region indicating oral and ventral suckers and the extent of the vitelline glands; **(C)** *Notocotylus malhamensis* ventral surface; **(D)** anterior view; **(E)** posterior view; **(F)** heavily spinulated cirrus; **(G)** *Heligmosomoides polygyrus,* copulatory bursa of a male nematode; **(H)** posterior end of a female; **(I)** *Aoncotheca murissylvatici*. eggs inside female nematode; **(J)** *Syphacia stroma,* cuticular mamelon of a male specimen; **(K)** asymmetrical eggs from a gravid female; **(L)** *Syphacia* sp.

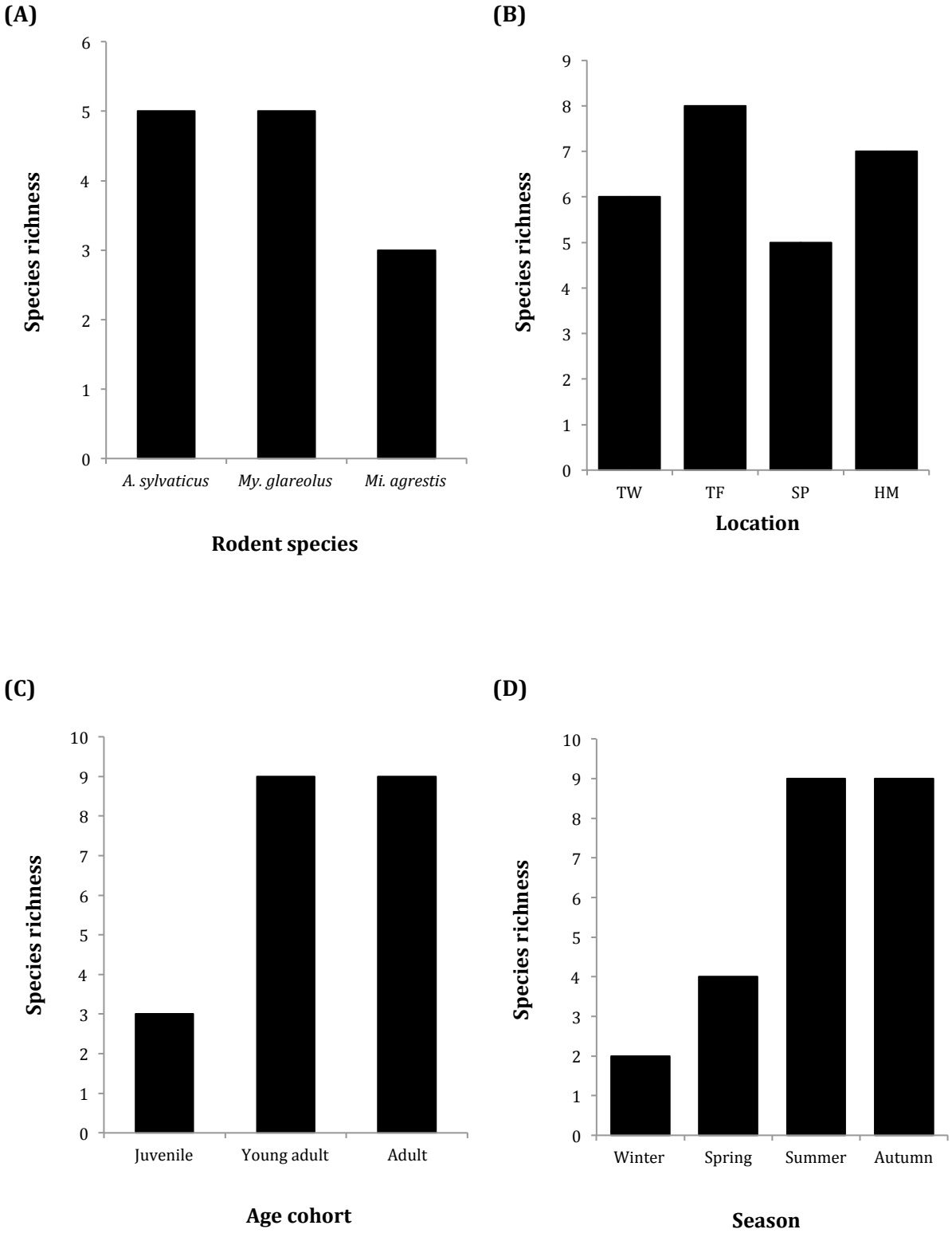


Figure 3.9 Variation in the total species richness observed (including all three host species) during the study period. **(A)** Total species richness observed for each of the three rodent hosts. **(B)** Total species richness by site. **Key = TW**: Tarn Woods; **TF**: Tarn Fen; **SP**: Spiggot Hill; **HM**: Ha Mire. **(C)** Total species richness observed in each age cohort. **Key = Juvenile:** 1-12g; **Young adult:** 13-19g; **Adult:** 20g+ according to Behnke *et al*. (1999). **(D)** Total species richness observed each season.

3.3.2.2 Dispersion

From 180 rodents sampled across the two year study, 115 (63.8%) were infected with at least one of the recorded helminths. *A. sylvaticus* was the most commonly infected rodent species with as many as 78.6% (92/117) harbouring at least one species in comparison to 35.2% (19/54) for *My. glareolus* and 44.4% (4/9) for *Mi. agrestis.*

For *A. sylvaticus* the total worm burden ranged from 0 to 275 with a mean intensity of 28.01±41.9 (2661/95). Parasite distribution appeared to be overdispersed within the population (variance to mean ratio: $\sigma^2/\mu = 67.8$) with 18.8% (95% Cl: 12.69% – 26.90%) of wood mice being uninfected in comparison to just 5.9% (95% Cl: 2.72% - 12.04%) harbouring more than 100 worms and conforming to a negative binomial distribution (Figure 3.10).

For both *My. glareolus* and *Mi. agrestis* the total worm burden ranged from 0 to 294 with a mean intensity of 36.9±64.3 (998/27). Parasite distribution also appeared to be overdispersed ($\sigma^2/\mu = 130.9$) with 57.1% (95% Cl: 44.85% – 68.61%) of voles being uninfected in comparison to just 3.17% (95% Cl: 0.23% - 11.50%) harbouring more than 100 worms and also conforming to a negative binomial distribution (Figure 3.11).

Individually, all helminth species were found to be overdispersed (>1) at Malham Tarn. The *dispersion indexes* of individual species have been presented (Table 3.5).

Table 3.5 The *dispersion indexes* of individual helminth populations at Malham Tarn.

Helminth species	*Dispersion Index* (σ^2/μ)
Plagiorchis elegans	158.9
Brachylaemus recurvum	2.5
Notocotylus malhamensis	209.4
Heligmosomoides polygyrus	20.5
Heligmosomoides glareoli	78.8
Syphacia stroma	64.9
Syphacia sp.	23.8
Aoncotheca murissylvatici	12.6
Hymenolepis diminuta	10.1

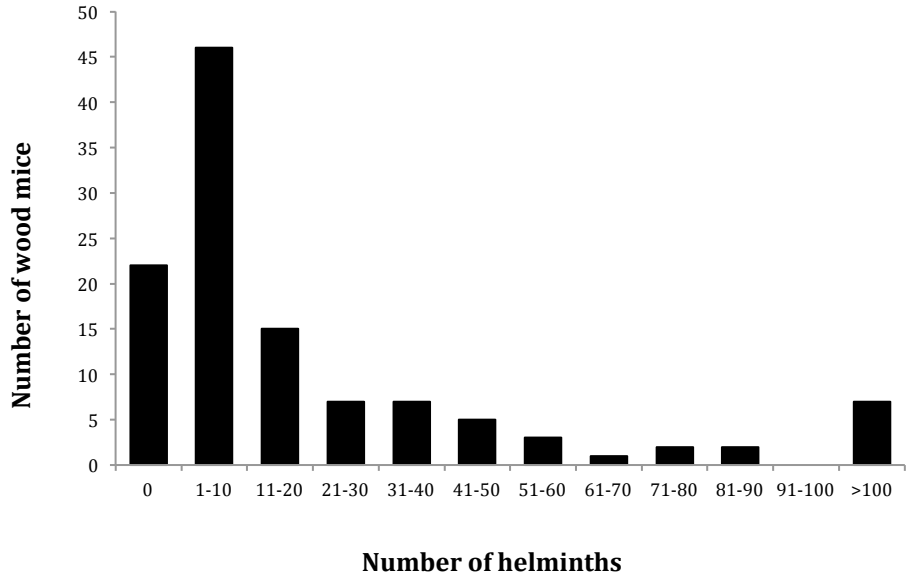


Figure 3.10 Frequency histogram indicating the total number of helminths per wood mouse.

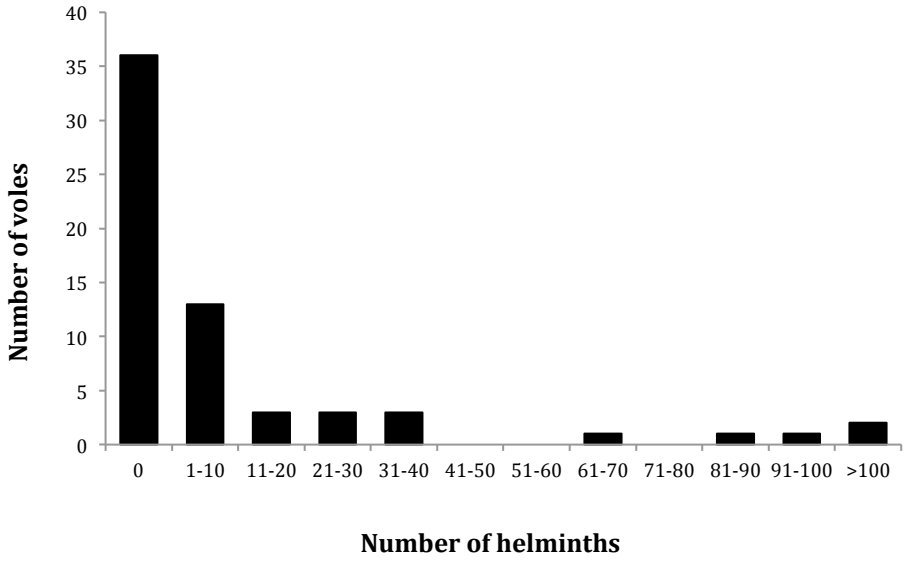


Figure 3.11 Frequency histogram indicating the total number of helminths per vole.

Multiple infections were observed in all three host species. This was most commonly observed in *A. sylvaticus* with 33.3% (39/117) of mice carrying two helminth species, 5.1% (6/117) carrying three species and a single rodent (0.8%) being simultaneously infected with five helminth species (Figure 3.12). The greatest number of helminths simultaneously infecting both vole species was two, with 7.4% (4/54) of *My. glareolus* and 11.1% (1/9) of *M. agrestis* carrying two helminths (Figure 3.13).

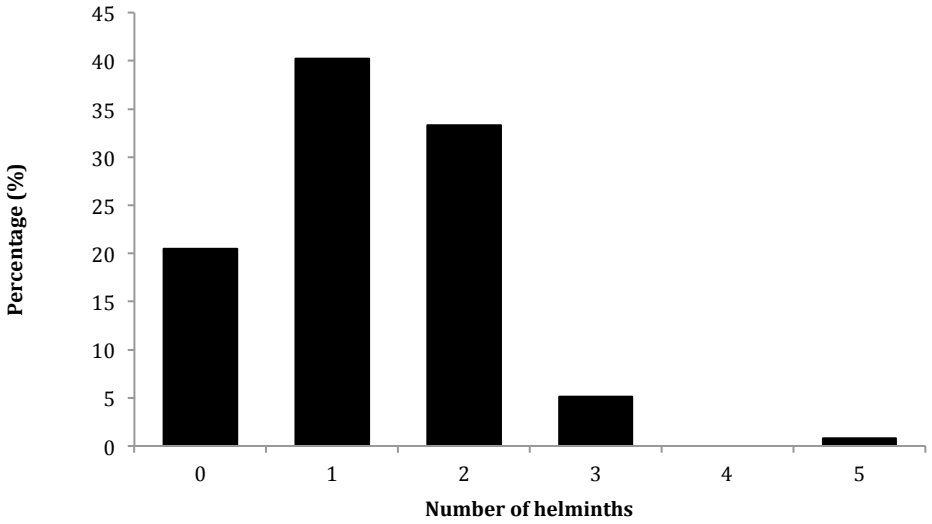


Figure 3.12 The number of helminth species per wood mouse.

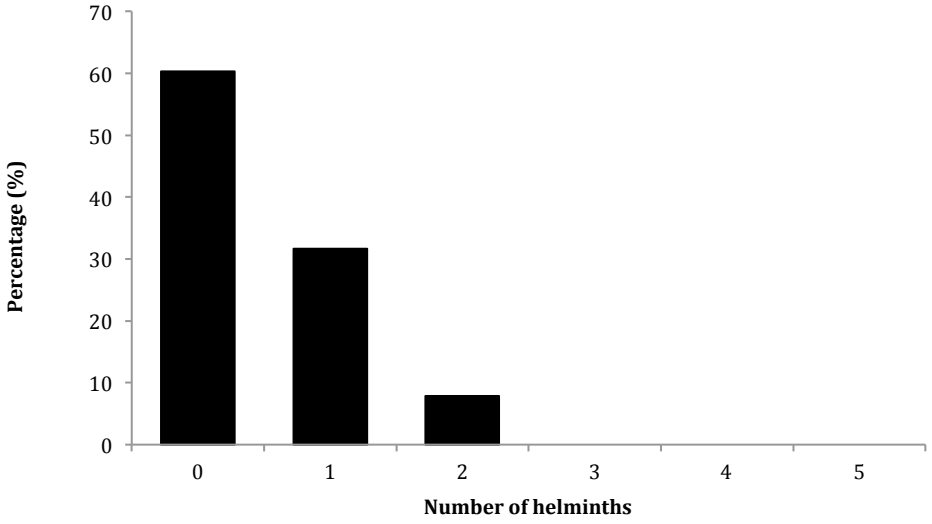


Figure 3.13 The number of helminth species per vole.

3.3.2.3 Overall prevalence and mean intensity

In total, two trematodes: *P. elegans* and *N. malhamensis* and three nematodes: *H. polygyrus, S. stroma* and *Syphacia* sp. were common (≥10%) within the rodent populations. All other helminths, including the trematode *B. recurvum,* the nematodes *H. glareoli* and *A. murissylvatici* and the cestode *H. diminuta* were deemed to be rare (<10%) (Table 3.6).

Table 3.6 The overall prevalence and mean intensity of helminth species identified at Malham Tarn. **Key** = (**A)**: *Apodemus sylvaticus*; **(M)**: *Myodes glareolus*.

Helminth species	Overall prevalence (%)	95% Confidence limits		Mean intensity ± SD
		Lower	Upper	
P. elegans	23.00	16.33	31.54	26.6±61.5
B. recurvum	01.70	00.09	06.40	2.0±1.41
N. malhamensis	14.30	07.48	25.20	94.7±119.4
H. polygyrus	67.50	58.58	75.35	9.2±12.7
H. glareoli	03.17	00.23	11.50	46.5±58.70
S. stroma	29.10	21.58	37.87	32.5±37.2
Syphacia sp.	11.10	04.83	22.55	15.2±13.2
A. murissylvatici (A)	06.80	03.32	13.10	3.0±2.7
A. murissylvatici (M)	09.30	03.60	20.33	9.4±9.4
H. diminuta	07.90	0.305	17.66	3.4±5.4

For *B. recurvum*, *H. glareoli*, *A. murissylvatici* and *H. diminuta*, no further analysis has been conducted. The following sections include the individual analysis of the helminth species categorised as 'common' only. Tabulated prevalence rates by host sex, age and by site and season have been presented in appendix D.

3.3.2.4 Association with host sex

Overall, more male than female wood mice were infected with helminths, however this difference in prevalence did not prove to be statistically significant using 2 × 2 Fisher's exact test (exact p value = 0.2490) (Table 3.7). In total, male mice harboured 2043 worms compared to only 534 worms for female mice. The total mean intensity was furthermore greater in males and this was considered to be significantly different using Mood's median test ($X^2 = 8.971$, $p = 0.05$, $v = 1$).

Interestingly, for both vole species, females had a higher prevalence than males. Due to the small sample size of *Mi. agrestis,* all voles were treated as a single group for statistical analysis (n = 63). Overall, prevalence in male and female voles was 11.1% (7/63) and 25.4% (16/63) respectively however this difference was not statistically significant using 2 × 2 Fisher's exact test (exact p value = 0.0635). Only 366 helminths were recovered from male voles compared with 458 from females. The mean intensity in male voles however was higher than for females, although this result was not found to be significantly different using Mood's median test ($X^2 = 0.002$, $p = 0.05$, $v = 1$).

Table 3.7 Total helminth infection of male (**M**) and female (**F**) rodents of each species.

Rodent species	Host sex	n	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
				Lower	Upper	Lower	Upper	
A. sylvaticus	M	71	83.1	72.58	90.22	0	275	34.7±48.5
	F	46	73.9	59.61	84.53	0	108	17.1±24.8
My. glareolus	M	20	30.0	14.32	52.13	0	175	45.0±63.9
	F	34	38.0	23.86	55.00	0	294	35.6±75.3
Mi. agrestis	M	3	33.3	05.63	79.76	0	83	44.0±55.2
	F	6	50.0	18.76	81.24	0	32	17.3±13.3

Individually, *P. elegans*, *H. polygyrus* and *S. stroma* all had a higher prevalence in male rodents. The prevalence of *N. malhamensis* however was higher in females and *Syphacia* sp. was only recorded in female voles (Figure 3.14). Statistical significance was only observed between males and females infected with *P. elegans* (Table 3.8).

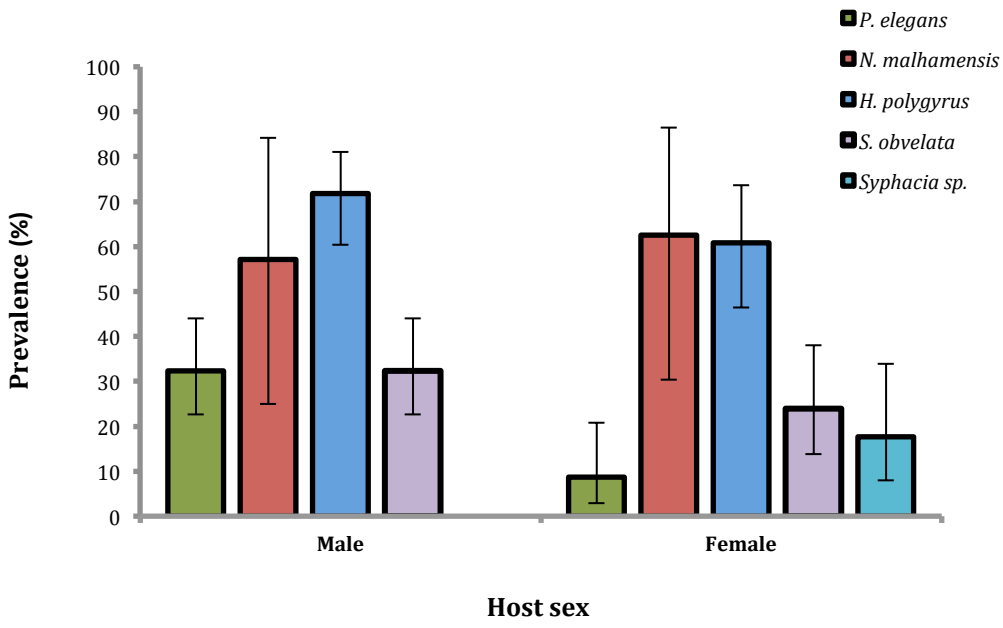


Figure 3.14 Prevalence of individual helminth species in male and female rodents. 95% confidence limits have been indicated by error bars.

Male rodents had a higher mean intensity of *P. elegans*, *N. malhamensis* and *H. polygyrus* but *S. stroma* was higher in females (Figure 3.15). None of these differences were statistically significant (Table 3.8). For *Syphacia* sp., no comparison could be made as this species was only recorded from female voles.

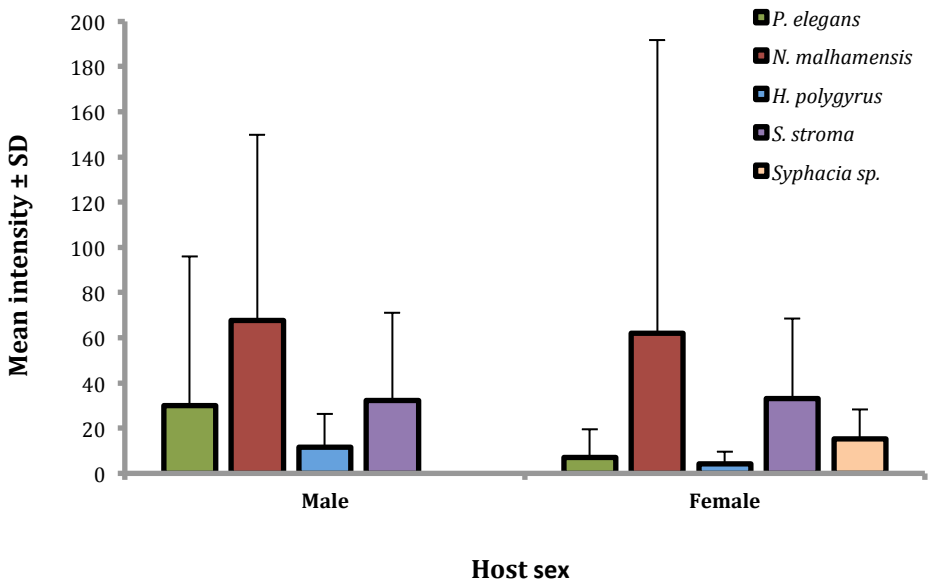


Figure 3.15 Mean intensity of individual helminth species in male and female rodents. Standard deviation has been indicated by error bars.

Table 3.8 Results of statistical analysis of prevalence and mean intensity between male and female rodents infected with each helminth species. Statistical significance indicated by p = <0.05.

Helminth species	Fisher's exact p value	Mood's median test (X^2), V = 1
P. elegans	0.003	0.213
N. malhamensis	0.713	0.056
H. polygyrus	0.232	1.953
S. stroma	0.406	0.134
Syphacia sp.	**NA**	**NA**

3.3.2.5 Association with host age

For *A. sylvaticus* and *My. glareolus* adult rodents carried both the higher prevalence and a higher worm burden (Table 3.9). Using chi squared test for heterogeneity, the difference in prevalence between the age groups for *A. sylvaticus* was proved to be significant (X^2 = 27.13, p = 0.05, v = 2), however for voles no significance was determined (X^2 = 0.11, p = 0.05, v = 2).

In total, 1472 helminths were recovered from adult wood mice compared with 956 from young adults and just 149 from juveniles. The highest mean intensity occurred in adult mice. Juveniles however harboured a greater mean intensity than young adults which is likely to be due to a single juvenile specimen harbouring 100 *S. stroma* worms. These differences in mean intensity were proved not to be significant using Mood's median test (X^2 = 5.59, p = 0.05, v = 2).

Adult voles also harboured the majority of helminths with 573 worms being collected from this cohort compared with 185 from young adults and just 66 from juveniles. Adult voles also had the highest mean intensity and juveniles again had a greater mean intensity than the young adult group. The differences observed in mean worm burden between the three groups was not proved to be statistically significant using Mood's median test ($X^2 = 2.16$, $p = 0.05$, $v = 2$).

Table 3.9 Total helminth infection of adult, young adult and juvenile rodents of each species. **Key** = **A**: adult; **Y**: young adult; **J**: juvenile.

Rodent species	Age	n	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
				Lower	Upper	Lower	Upper	
A. sylvaticus	A	44	95.5	84.03	99.58	0	275	35.06±53.06
	Y	56	80.4	67.98	88.83	0	121	21.24±28.85
	J	17	35.3	17.17	58.84	0	101	24.83±39.49
My. glareolus	A	13	46.2	23.19	70.87	0	294	81.67±124.2
	Y	31	29.0	15.94	46.75	0	89	17.67±28.17
	J	10	40.0	16.71	68.84	0	37	16.05±15.37
Mi. agrestis	A	5	20.0	02.03	64.04	0	83	-
	Y	3	100.0	38.28	100.0	0	14	08.67±04.62
	J	1	00.0	00.00	89.66	0	-	-

Individually, *P. elegans*, *H. polygyrus* and *N. malhamensis* had a higher prevalence in adult rodents than those of younger age cohorts. Both *P. elegans* and *N. malhamensis* were the only two species not recovered from juvenile rodents. The only helminth to have a greater prevalence in juvenile rodents was *Syphacia* sp. (Figure 3.16). Statistical significance however was only observed between the age cohorts for *P. elegans, N. malhamensis* and *H. polygyrus* (Table 3.10).

P. elegans, *H. polygyrus* and *N. malhamensis* all had higher mean intensities in adult rodents than in those of younger age groups however *S. stroma* had a greater mean intensity in juvenile rodents. Only the difference in intensity between the age groups for *H. polygyrus* and *S. stroma* were statistically significant (Table 3.10). For *Syphacia* sp. mean intensities could not be compared as there was only one adult rodent infected with 4 worms and one young adult with 21 worms. Juvenile rodents appeared to harbour the majority of helminths for this species with four rodents carrying 66 worms and having a mean intensity of 16.5±15.37 (Figure 3.17).

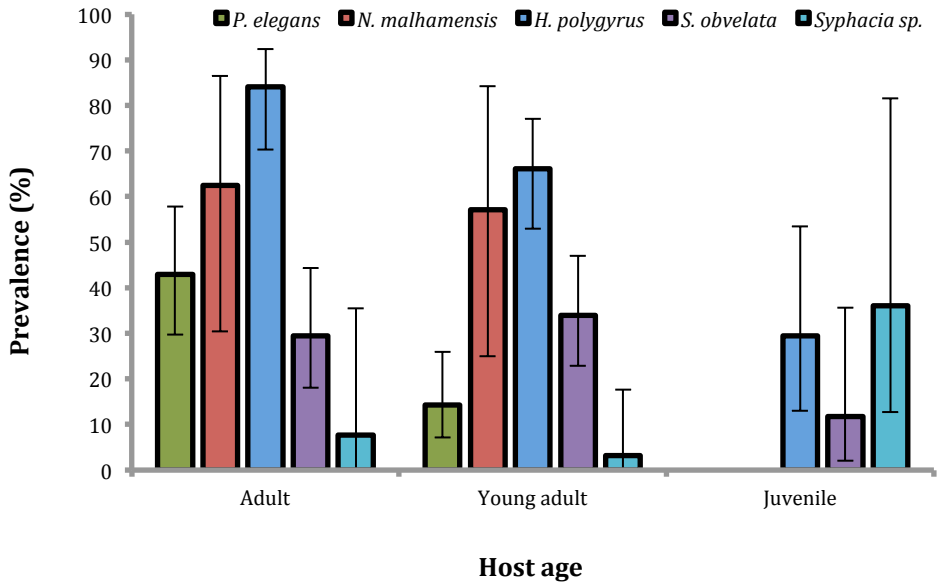


Figure 3.16 Prevalence of helminths in adult, young adult and juvenile rodents. 95% confidence limits have been indicated by error bars.

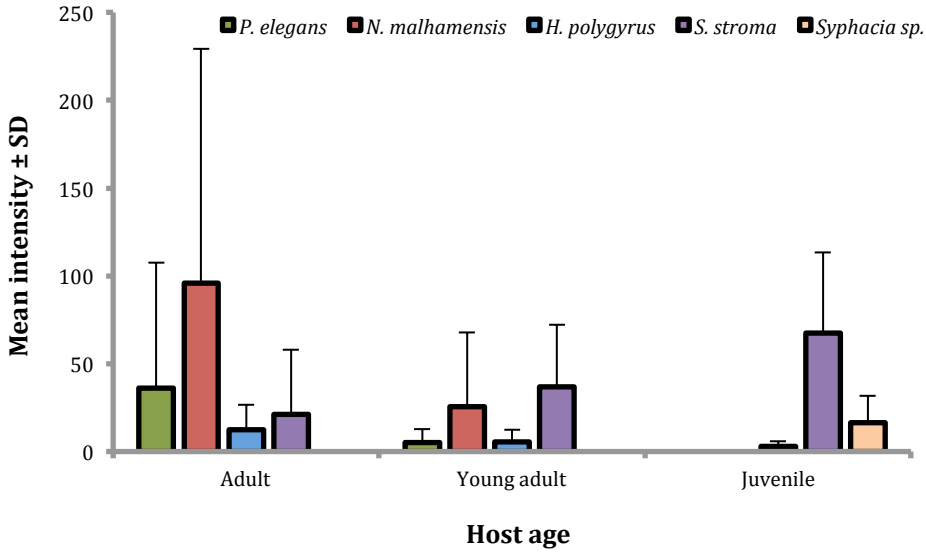


Figure 3.17 Mean intensity of helminths in adult, young adult and juvenile rodents. Standard deviation has been indicated by error bars.

Table 3.10 Results of statistical analysis of prevalence and mean intensity between adult, young adult and juvenile rodents infected with each helminth species. Statistical significance indicated by p = <0.05.

Helminth species	Chi squared test for heterogeneity (X^2), V = 2	Mood's median test (X^2), V = 2
P. elegans	18.1	2.3
N. malhamensis	9.9*	1.2
H. polygyrus	16.9	10.9
S. stroma	3.0	7.1
Syphacia sp.	5.5	**NA**

3.3.2.6 Seasonal variation

The overall helminth prevalence across the four sampling seasons was compared. In *A. sylvaticus,* a peak prevalence of 100% was observed during the winter. This value lowered and remained almost consistent throughout the spring and summer reducing slightly during the autumn (Table 3.11). The high prevalence rates observed throughout the year were undoubtedly contributed to by infection with the nematode *H. polygyrus*. These differences were not statistically significant using chi squared test for heterogeneity (X^2 = 4.04, p = 0.05, v = 3). Although the lowest prevalence in *A. sylvaticus* was observed during the autumn, the highest numbers of helminths (1789) were retrieved during this period compared to only 90 from the winter, 372 from the spring and 326 from the summer. Despite observable differences in mean intensity across the seasons, no significance was proved using Mood's median test (X^2 = 0.12, p = 0.05, v = 3) (Table 3.11). Contrastingly, the lowest prevalence in voles occurred during the winter. Prevalence increased during the spring and remained relatively consistent throughout the summer, peaking by the autumn (Table 3.11). These differences were however not proved to be statistically significant using chi squared test for heterogeneity (X^2 = 4.04, p = 0.05, v = 3). Mean intensity was at its lowest during the winter with only 3 specimens being recovered compared with 476 during the spring, 125 during the summer and 220 during the autumn. From the spring collection however, 470 of the specimens were *N. malhamensis* accumulated from just three bank voles which contributed to the highest mean intensity of the study. Despite these noticeable differences in mean intensity, none were not proved to be statistically significant using Mood's median test (X^2 = 1.38, p = 0.05, v = 3).

Table 3.11 Total helminth infections during each season in rodents of each species. **Key** = **Wi**: winter; **Sp**: spring; **Su**: summer; **Au**: autumn.

Rodent species	Season	n	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
				Lower	Upper	Lower	Upper	
A. sylvaticus	Wi	8	100	62.78	100.0	1	26	11.25±09.97
	Sp	8	87.5	50.78	99.89	0	185	53.14±71.38
	Su	22	86.4	65.82	96.10	0	76	17.16±17.70
	Au	79	74.7	64.04	83.04	0	275	30.32±45.10
M. glareolus	Wi	10	10.0	<00.01	42.60	0	3	-
	Sp	8	37.5	13.49	69.62	0	294	158.7±144.2
	Su	16	37.5	18.37	61.47	0	19	07.00±06.40
	Au	20	45.0	25.81	65.81	0	89	21.60±28.15
M. agrestis	Su	4	25.0	03.41	71.09	0	83	-
	Au	5	60.0	22.91	88.40	0	14	08.70±04.62

Individually, *H. polygyrus* and *S. stroma* were detected throughout the year and were the only two species recovered during the winter (Figure 3.18). Both *P. elegans* and *N. malhamensis* were first observed during the spring. *P. elegans* appeared to demonstrate a pattern of seasonality, being absent during the winter, at its lowest prevalence during the spring and peaking during the summer and autumn for the first and second sampling years respectively. *N. malhamensis* however did not appear to demonstrate seasonality, with relatively high prevalence rates occurring during the spring and autumn and a reduction being observed during in summer. All five helminths were collectively present during the summer and the autumn only. None of these differences in prevalence were statistically significant (Table 3.12). For *Syphacia* sp. prevalence could not be compared as this helminth was only collected during the summer and autumn only.

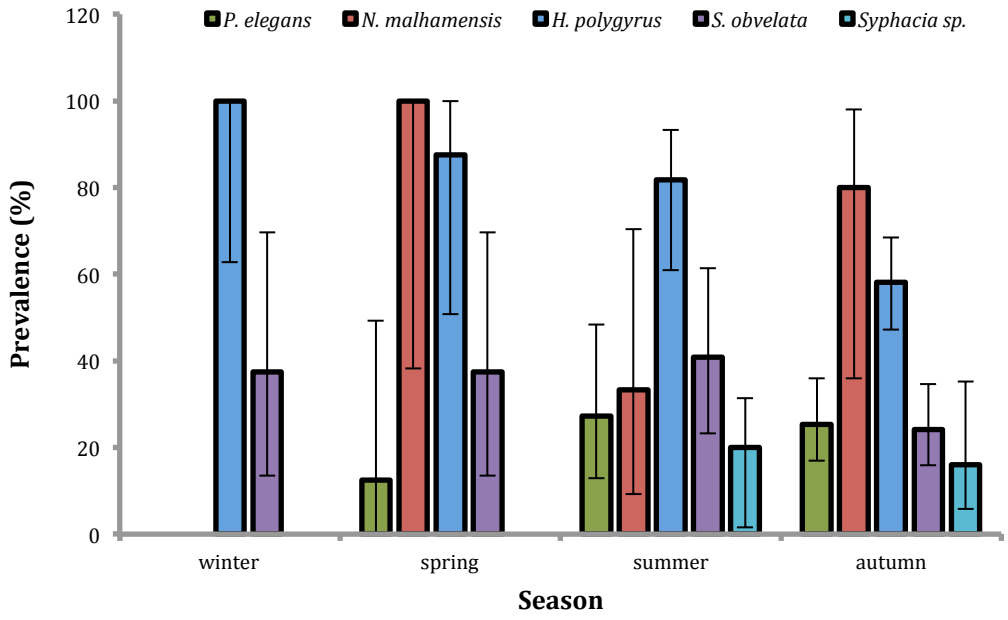


Figure 3.18 Prevalence of individual helminth species during each season. 95% confidence limits have been indicated by error bars.

Both *H. polygyrus* and *N. malhamensis* had higher mean intensities during the spring whereas the greatest mean intensity for *S. stroma* and *P. elegans* occurred during the autumn (Figure 3.19). As mentioned, the mean intensity for *Syphacia* sp. between seasons cannot be compared. None of the differences observed in mean intensity were statistically significant (Table 3.12).

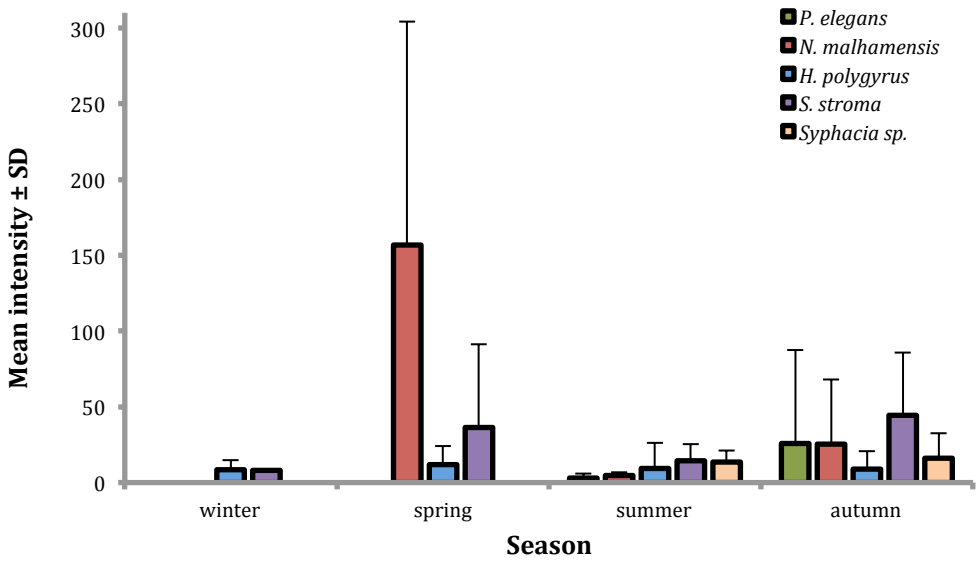


Figure 3.19 Mean intensity of individual helminth species during each season. Standard deviation has been indicated by error bars.

Table 3.12 Results of statistical analysis of prevalence and mean intensity during each season. Statistical significance indicated by p = <0.05.

Helminth species	Chi squared test for heterogeneity (X^2), V = 3	Mood's median test (X^2), V = 3
P. elegans	3.38	1.64
N. malhamensis	6.14	0.75
H. polygyrus	5.19	0.18
S. stroma	3.09	0.31
Syphacia sp.	**NA**	**NA**

3.3.2.7 Site- specific variation

The overall helminth prevalence across the four sampling locations was compared (Table 3.13). In *A. sylvaticus,* prevalence was relatively high at all four sites and as such there was no significant difference determined using chi squared test for heterogeneity (X^2 = 2.49, p = 0.05, v = 3). In total, 1255 helminths were recovered from wood mice trapped at Tarn Woods compared with 320 from Tarn Fen, 671 from Spiggot Hill and 331 from Ha Mire Plantation. The greater number of helminths collected from Tarn Woods was undoubtedly due to the larger host sample number acquired from this location as the highest mean intensity was observed from wood mice trapped at Spiggot Hill. The apparent difference in mean intensity observed between the four locations was not proved to be statistically significant using Mood's median test (X^2 = 0.83, p = 0.05, v = 3) (Table 3.13).

In voles, the overall helminth prevalence was extremely similar between Tarn Woods, Spiggot Hill and Ha Mire Plantation. Although the prevalence at Tarn Fen was noticeably much higher, this difference was only found to be borderline significant using chi squared test for heterogeneity (X^2 = 7.69, p = 0.05, v = 3). The greater prevalence observed at this location was unequivocally due to infection with *Notocotylus malhamensis* where as many as 60% of voles were seen to harbour this parasite. The greatest number of helminths from voles was also recovered from Tarn Fen with 683 worms being collected in comparison to just 64 from Tarn Woods, 22 from Spiggot Hill and 55 from Ha Mire Plantation. From the 683 specimens collected from Tarn Fen, as many as 581 were *N. malhamensis*. Despite an observable difference in mean intensity between sites, this was not deemed to be statistically significant using Mood's median test (X^2 = 0.15, p = 0.05, v = 3).

Table 3.13 Total helminth infection in rodents from each sampling location. **Key** = **TW**: Tarn Woods; **TF**: Tarn Fen; **SP**: Spiggot Hill; **HM**: Ha Mire Plantation.

Rodent species	Site	n	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
				Lower	Upper	Lower	Upper	
A. sylvaticus	TW	52	84.6	72.21	92.26	0	185	28.52±39.13
	TF	21	80.9	59.41	92.92	0	121	18.82±30.00
	SP	17	76.5	52.23	90.95	0	275	51.62±73.74
	HM	27	70.4	51.35	84.32	0	68	17.42±20.74
M. glareolus	TW	20	30.0	14.32	52.13	0	26	10.67±09.77
	TF	9	66.7	35.09	88.27	0	294	95.67±118.5
	SP	8	25.0	06.31	59.91	0	21	11.00±14.14
	HM	17	29.4	12.99	53.43	0	37	11.00±14.61
M. agrestis	TW	3	00.0	00.00	61.75	-	-	-
	TF	6	66.7	29.57	90.75	0	83	27.25±37.36

There was no significant difference in the prevalence of *P. elegans*, *H. polygyrus* or *Syphacia* sp. between sampling locations. For *S. stroma* however, a significant difference in the prevalence found at Ha Mire Plantation and Tarn Woods was determined (Table 3.14). *Notocotylus malhamensis* was recovered from Tarn Fen only. This helminth was not recorded from any of the three plantations despite the careful examination of a further 48 voles (45 *My. glareolus* & 3 *Mi. agrestis*). The detection of *N. malhamensis* at Tarn Fen only was determined to be highly significant (Table 3.14). Despite observable differences in mean intensity between locations, none of the recorded differences were deemed to be statistically significant (Table 3.14).

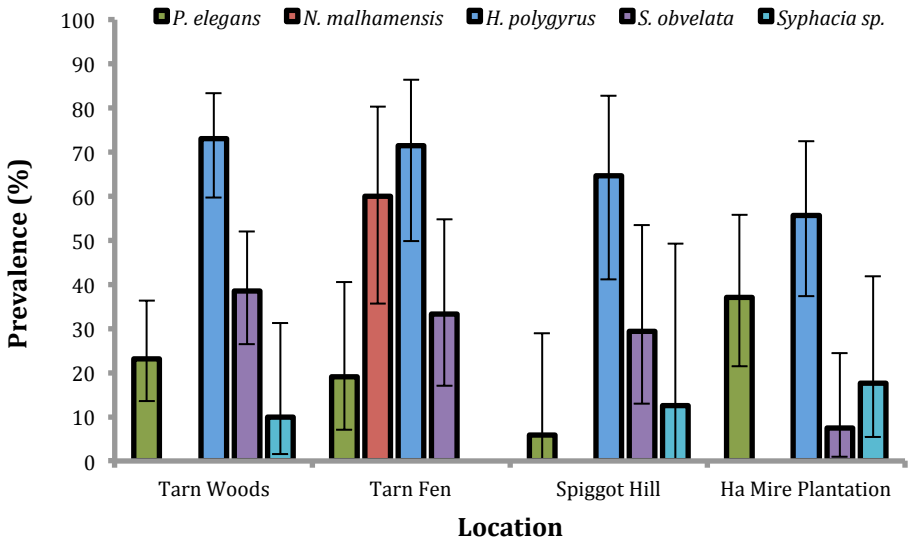


Figure 3.20 Prevalence of individual helminth at each location. 95% confidence limits have been indicated by error bars.

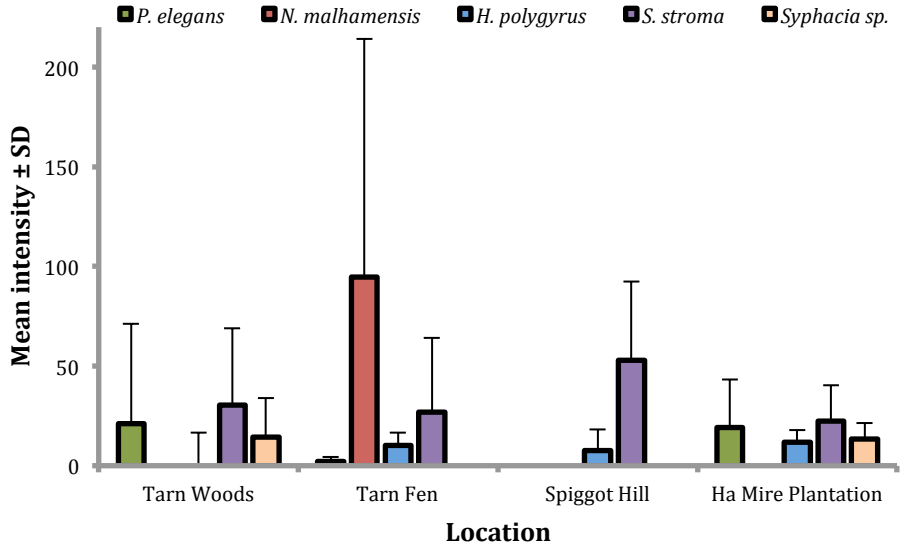


Figure 3.21 Mean intensity of individual helminth species at each location. Standard deviation has been indicated by error bars.

Table 3.14 Results of statistical analysis of prevalence and mean intensity at each location. Statistical significance indicated by p = <0.05.

Helminth species	Chi squared test for heterogeneity (X^2), $V = 3$	Mood's median test (X^2), $V = 3$
P. elegans	3.79	1.93
N. malhamensis	31.47	**NA**
H. polygyrus	2.66	0.48
S. stroma	8.40	1.04
Syphacia sp.	0.84	1.42

3.3.2.8 Association with K Populations

The prevalence and mean intensity of helminths in relation to each K population can only be analysed for those collected from *A. sylvaticus*. There was no significant difference in the overall prevalence of helminths between the three population clusters determined using chi squared test for heterogeneity ($X^2 = 4.44$, $p = 0.05$, $v = 2$), nor was there any difference in overall intensities using Mood's median test ($X^2 = 0.92$, $p = 0.05$, $v = 2$). The only significant difference identified between the populations was for *H. polygyrus* prevalence between population number 1 and number 2 (Table 3.16). *B. recurvum* was included in the initial analysis in this instance to include all identified helminths of *A. sylvaticus* however this helminth was only recorded from a single adult male during the summer and a young adult male during the autumn, both of which belonged to the same population cluster. For such reason, this species could not be statistically compared.

Table 3.15 Total helminth infection in rodents from each wood mouse population.

Population	n	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
			Lower	Upper	Lower	Upper	
1	36	83.3	67.73	92.51	0	76	10.8±15.4
2	21	71.4	49.79	86.44	0	117	26.2±38.6
3	51	82.4	69.52	90.66	0	275	20.3±42.7

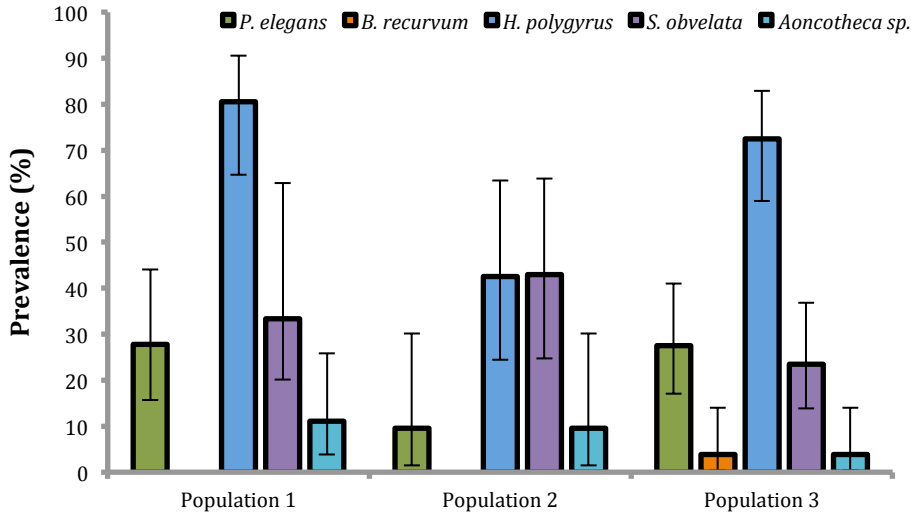


Figure 3.22 Prevalence of individual helminth in each K population. 95% confidence limits have been indicated by error bars.

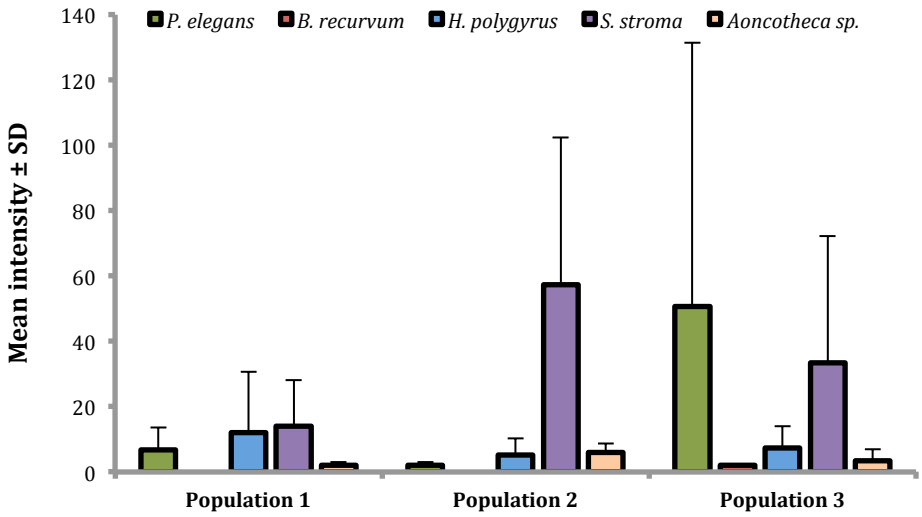


Figure 3.23 Mean intensity of individual helminth in each K population. Standard deviation has been indicated by error bars.

Table 3.16 Results of statistical analysis of prevalence and mean intensity in each K population. Statistical significance indicated by p = <0.05.

Helminth species	Chi squared test for heterogeneity (X^2), V = 2	Mood's median test (X^2), V = 2
P. elegans	3.03	2.61
B. recurvum	**NA**	**NA**
H. polygyrus	9.37	0.11
S. stroma	2.82	0.95
Aoncotheca murissylvatici	1.75	0.53

3.4 Discussion

The current study tested the hypothesis that an increase in sampling effort would inevitably increase species richness (Walther *et al*., 1995). The findings of the current study however are concurrent with that reported by Behnke *et al*. (2009) who described the helminth fauna at Malham Tarn to be depauperate in comparison to other UK sites. In his report, Behnke (2009) listed eight helminth species from *Apodemus sylvaticus* at Malham Tarn; however this study revealed only five from this species of rodent. In the present study, the nematode *Trichuris muris* or the larval cestodes *Cladotaenia globifera* or *Taenia taeniaformis* were not identified. The data presented in the report by Behnke (2009) however stemmed from a larger sample size of 296 mice examined over a 16 year period and these three helminths were recorded respectively at low prevalence rates of 0.3%, 1% and 0.7% only. Expansion of the original study site to include other woodland areas within the nature reserve did nonetheless lead to the discovery of a new digenean species; *Notocotylus malhamensis* (see Chapter 5). The helminth fauna of

rodents at Malham Tarn typically appears to be dominated by monoxenous nematodes with, prior to this study, only two digeneans, *Plagiorchis elegans* and *Brachylaemus recurvum* being recorded. Both of these digenean species possess triheteroxenous life cycles which involve two intermediate hosts. *P. elegans* utilises a freshwater snail as the first intermediate host (Manga-Gonzalez *et al*., 1994; Väyrynen *et al*., 2000; Ghobadi and Farahnak, 2004; Faltýnková *et al*., 2007) and an arthropod as the second intermediate host (Hong *et al*., 1999) whereas *B. recurvum* uses two terrestrial molluscs (Gracenea and González-Moreno, 2002). Both species were recorded from *A. sylvaticus* as would be expected for digenean species that utilise invertebrate intermediate hosts that are predisposed to foraging by wood mice.

Only two specimens of *B. recurvum* were however recovered during the present study which is undoubtedly due to the terrestrial transmission cycle of this digenean. Other studies have also found this helminth to have a low prevalence in the wood mouse from other UK locations (Lewis, 1968a; Behnke *et al*., 2009). Typically in the UK, *Corrigia vitta* is found at a relatively high prevalence in the wood mouse (Abu-Madi *et al*., 2000; Behnke *et al*., 2009). The life cycle of *C. vitta* is not well understood. According to the Natural History Museum host-parasite database, only two species of terrestrial mollusc, the two toothed door snail *Clausilia bidentata* and the plaited door snail *Cochlodina laminata* have been listed in the transmission of this digenean (Gibson *et al*., 2005). Although both of these invertebrate species have been previously recorded in the Malham area (Cameron, 1978), and despite increasing the sampling effort in addition to adding seasonal examination of the helminth fauna, this species of digenean was still not identified at Malham Tarn. Both invertebrate species have however been recorded in woodland sites located northwest of the current study location (Cameron, 1978) and it is therefore possible that wood mice at these locations harbour *C. vitta*.

There were three phylogenetically different population clusters of wood mice identified at Malham Tarn. Population genetics however did not appear to be important in terms of shaping the helminth community with a significant difference in the prevalence of *H. polygyrus* between population one and population two only being determined. There were no other differences in the helminth fauna between these two populations and it may therefore be suggested that genetic susceptibility is not an important factor for

determining helminth distributions in this instance. Similarly to the studies of Abu-Madi *et al*. (1998; 2000) and Hildebrand (2008), host sex was not identified as an important determinant of prevalence or mean intensity for most identified helminths. Statistical significance was determined for wood mice carrying *P. elegans* only where male mice were found to harbour a significantly higher prevalence than females. As discussed in chapter 4, this difference is likely to be due to the transmission ecology of the parasite which can be coupled with the spatial ecology of male wood mice.

Overall, juvenile rodents had the lowest helminth richness. Helminth prevalence in juvenile wood mice was also deemed to be statistically lower than older age cohorts although no significance between any age group was observed for voles. Individual analysis revealed a significant difference in prevalence between adult and all younger mice infected with *P. elegans*, adults and young adults against juveniles infected with *H. polygyrus* and adults against all younger voles infected with *N. malhamensis*. It should be mentioned however that *N. malhamensis* was only trapped from Tarn Fen and no juvenile voles were trapped from this location. Although a highly statistical difference was determined between adults and younger cohorts harbouring this digenean, this statistical difference was lost when site was taken into account and the difference in prevalence between adult and young adult voles at this location only was analysed. Generally however, these results are as expected with older mice being more likely to be infected because they have had a longer time in which to acquire infection (Langley and Fairley, 1982) and because they demonstrate a greater foraging behaviour. For differences in intensity, significance was only proved for *H. polygyrus* in which adult wood mice carried a significantly greater number of worms compared to younger groups and for *S. stroma* in which juvenile mice had the greatest mean intensity. Although not significant, *Syphacia* sp. also had a greater mean intensity as well as prevalence in juvenile voles than any other age group.

As discussed by Lewis (1968a) however, infection of the host can be related to the life history of the particular parasite under study. Transmission of *Syphacia* spp. is not reliant upon dispersion within the environment; rather, transmission of this species is more likely to occur through host contact (Lewis, 1968a). The infective eggs of *Syphacia* spp. are typically deposited on the fur and the peri-anal region of the host and infection

can be passed on directly from contaminated individuals. Although infection can also be acquired indirectly from contaminated materials within the environment (Taffs, 1976), rodents of all ages are considered to have an equal chance of acquiring infection. The greater infestation rate of juveniles in the present study is therefore not surprising. It is likely that these infection levels are due to high contact levels in the nest during breeding season (Lewis, 1968a) and contributed to by retro-infection, which is furthermore an important aspect in the transmission biology for this group of helminths (Taffs, 1976).

Seasonality was not considered to be an important determinant in the present study, with no significant result being indicated for any of the identified helminths, despite only the two nematodes *S. stroma* and *H. polygyrus* being recorded during the winter. This is in disagreement with Abu-Madi *et al*. (2000) who found significant seasonal variation in prevalence and intensity of *H. polygyrus* and *S. stroma* in the south-east of England. It should be emphasised however that the sample sizes analysed in the present study are relatively small (n = 117 compared with n = 399) in comparison and as discussed by Poulin (1996), statistically significant inequalities may not always be evident from a single host population.

Both *S. stroma* and *H. polygyrus* nonetheless demonstrate transmission strategies appropriate for a continual transmission to occur throughout the year. As previously discussed, the transmission of *S. stroma* is host density dependent and wood mice have been shown to demonstrate high overwintering populations (Flowerdew, 1972). The prevalence and mean intensity of *H. polygyrus* was relatively similar throughout the year indicating a continual mode of transmission at this location. This species possesses a direct life cycle and is therefore, similarly to *S. stroma,* not reliant upon the presence of intermediate hosts for transmission. Wood mice become infected with this nematode by ingesting the L3 larval stage from the environment during foraging. The development of *H. polygyrus* occurs within the environment after the eggs are passed with host faeces and as such is generally dispersed in accordance with the foraging range of the infected animal (Lewis, 1968a). The infection foci can therefore be vast or limited and can be scattered randomly or aggregated within the foraging area which can be indicated by the type of dispersion within the wood mouse population.

All helminths recorded within this study were found to be overdispersed and represent a negative binomial distribution. As suggested by Lewis (1968a) this type of dispersion does indicate the infective eggs, larvae or intermediate hosts to be aggregated within the study area. Site-specific variation did appear to be an important determinant for shaping the digenean communities at Malham Tarn. Although not found to be significant *P. elegans* had the highest prevalence at Ha Mire Plantation. Ha Mire Plantation is a relatively small sampling site in comparison to the other locations and is closed off by dry stone walling to the north, east and south. The west border is openly exposed to the tarn margin but separated by a narrow shingle beach. As such it is highly likely that the boundaries of this site contribute to the higher prevalence rates observed here with both hosts and infective stages being confined to a more limited space. A similar situation was reported by Abu-Madi *et al*. (2000), who found that wood mice confined to hedgerows on the Isle of Wight were generally more heavily infected with helminths than other sites where they were not confined. They considered this effect to be due to the confinement of both host and helminth transmission stages which are therefore more likely to encounter one another.

The greatest impact of site on component community structure was observed at Tarn Fen where the highest helminth richness was identified with eight of the nine identified species being recorded here. Tarn Fen was furthermore the only location from where the new digenean species *N. malhamensis* was discovered and this result was highly significant. Species belonging to the genus *Notocotylus* utilise only a single freshwater snail for transmission (Williams, 1966; Manga-Gonzalez *et al*., 1994; Väyrynen *et al*., 2000; Morley *et al*., 2003). The ecology associated with Tarn Fen seems to be ideal for propagating this digenean which encompasses only one intermediate host with restricted motility within its life cycle. The detailed life cycles of most *Notocotylus* species are currently unknown. Typically, a brackish or freshwater lymnaeid or hydrobiid becomes infected by eating eggs previously released into water with faeces of the definitive host (Murrils *et al*., 1988). Following development in the snail, actively swimming eyed cercariae are released which quickly encyst onto nearby solid objects (Simon-Vicente *et al*., 1985b). This is typically a rapid process and usually occurs on the shell of the host snail from which the cercariae has emerged or the shell of another snail

within close proximity (Stunkard, 1966). The usual definitive hosts for *Notocotylus* species are waterfowl that are capable of plucking snails infected with metacercariae on their shell from aquatic plants in open water bodies (Beverley-Burton, 1961; 1972). On the contrary, small semi-aquatic mammals such as voles may have a limited access to these infected stages, typically being restricted to feeding at the water's edge (Webber *et al.,* 1987). Metacercariae however, furthermore develop on the leaves of aquatic plants and as shown by Simon-Vicente *et al*. (1985b) the metacercariae of *Notocotylus neyrai* Gonzalez-Castro, 1945, a parasite of rodents have been found encysting on the water-starwort *Callitriche stagnalis* in Spain. The broad inflow stream that passes directly through Tarn Fen carries an abundance of *Callitriche* spp. and although the life cycle of *N. malhamensis* is currently unknown, it may be postulated that this is also the scenario and route of infection at Tarn Fen and that small mammals typically become infected when feeding on this aquatic vegetation. The transmission ecology of this helminth therefore appears to be ideal for voles that are highly herbivorous in nature.

It was speculated at the beginning of this study that due to the insectivorous nature of *A. sylvaticus,* this rodent was most likely to harbour helminths that utilise arthropod intermediate hosts for transmission. This hypothesis was proved in the respect that *P. elegans* and *B. recurvum* were recovered from wood mice only. The cestode *Hymenolepis diminuta* was not recorded from the wood mouse but only from both vole species. This is surprising since this helminth utilises the beetle *Tenebrio molitor* as an intermediate host (Hurd *et al*., 2001), yet *Mi. agrestis* is considered to be strictly herbivorous. The only field vole infected however was from Tarn Fen which carried 13 worms. According to the Natural History Museum host-parasite database, *Mi. agrestis* has furthermore been implemented as a host for *H. diminuta* in the Czech Rebublic and in Russia (Gibson *et al*., 2005). The new digenean species *N. malhamensis* also appeared to demonstrate a marked specificity for voles at Tarn Fen and to date, no infection has been observed in the wood mouse despite careful examination. Simon-Vicente *et al*. (1985b) have however demonstrated the susceptibility of *A. sylvaticus* to infection by *N. neyrai*, although at a lower rate than that of the southern water vole *Arvicola sapidus* in Spain.

Both the result of Simon-Vicente *et al*. (1985b) and the result acquired from Tarn Fen in the current study are unequivocally due to differences in the ethology between wood

mice and voles. A study by Buesching *et al*. (2007) noted that *A. sylvaticus* was significantly more arboreal than *My. glareolus.* The wood mouse is a considerably agile animal in comparison with well developed senses and as such is most capable of manoeuvring from predators. *A. sylvaticus* has an omnivorous diet and is particularly insectivorous (Lewis and Twigg, 1972; Montgomery and Montgomery, 1990b). Buesching *et al*. (2007) suggested that arboreality in the wood mouse is most likely due to foraging for seeds and invertebrate animals that are prosperous within the tree canopy. With such agility, *A. sylvaticus* can afford to spend much more time within the tree canopy than can *My. glareolus*. The diet of *My. glareolus* is eminently folivorous although this species will consume small amounts of animal matter whereas that of *Mi. agrestis* is strictly herbivorous (Lewis, 1987). Although arboreality has also been observed by the bank vole, this tends to be at a much lower rate and is most typical in woodlands with a dense understory and under certain selection pressures (Buesching *et al*., 2007). Studies by Kikkawa (1964), demonstrated the movement and activity of both *A. sylvaticus* and *My. glareolus* in Wytham Woods, Berkshire, UK. He found that the distribution of the bank vole was closely associated with ground cover. During winter months as the amount of cover reduced the distribution of the vole became far more limited.

N. malhamensis was recorded from *My. glareolus* and *Mi. agrestis* trapped between spring and late autumn. No infection was identified during the winter sampling period. The winter sampling period however is often shortcoming due to adverse weather conditions at the Tarn. Heavy snowfall and reduced temperatures have been known to reduce the capture rate of the Tarn's small mammal populations (Shorrock and Sutton, 2009). During the present study, only a single bank vole was captured and examined during the winter complying with that discussed by Kikkawa (1964). The trapping pattern from Tarn Fen does appear to be consistent with the literature. Only *My. glareolus* and *Mi. agrestis* have been trapped from the dense shrubbery and grassland adjacent to the inflow stream. All specimens of *A. sylvaticus* were captured from the woodland areas that are set back from the water's edge. The stream at Tarn Fen is highly prone to flooding during periods of heavy rainfall in which ground flora is exposed to considerable amounts of water and the adjacent woodlands and grasslands become submerged. During episodes of flooding it is most likely that all rodent species take to

the trees to avoid drowning. It may be postulated that infection of *Mi. agrestis* with *H. diminuta* occurred during such periods either via accidental ingestion or due to a reduced access to vegetative matter. *A. sylvaticus* however has been recorded at greater heights than the bank vole, climbing as high as 220cm (Buesching *et al*., 2007) in comparison to a maximum of 150cm. Buesching *et al*. (2007) suggested that vertical separation of the two species reduces interspecific competition. Additionally, as the diet of *A. sylvaticus* constitutes mainly seeds and insects typically found at such heights it is likely that the wood mouse spends longer durations away from the flood zone. With the vole species being preferably herbivorous, it is likely that they will return to ground level almost imminently following the receding of the water to its normal limits. The main inflow stream encompasses several snail species that are potentially involved in the life cycle of *N. malhamensis* (see chapter 6). During periods of flooding, invertebrate species inhabiting the water column are likely to be conveyed into the typical foraging range of the vole communities along with floating aquatic vegetation. As the water recedes, both snails and plants infected with metacercariae are likely to be left on the ground. Aquatic plants have been observed littering this area on several occasions, a factor that will contribute in increasing the exposure of the voles to infective metacercariae during subsequent bouts of foraging.

The metacercariae of *N. attenuatus* Rudolphi, 1809 possesses two cyst walls, a tough inner wall and an outer mucoid envelope. Graczyk and Shiff (1994) observed that during desiccation, the mucoid envelope progressively shrank towards that of the inner wall playing an important role in survival. Studies by Southgate (1971) indicated that the outer mucoid envelope contains water-soluble glycoproteins that remain on the cyst when it is exposed to air. These glycoproteins are thought to have a protective effect by providing a humid atmosphere when the cyst is emerged from water. Graczyk and Shiff, (1994) concluded that the metacercariae of *N. attenuatus* are very well adapted to overwintering and cysts that are located close to the water surface or stored just under cool and moist conditions could remain viable for up to six months post encystment. Simon-Vicente *et al*. (1985b) however noticed that when removed from water the cyst wall of *N. neyrai* quickly loses its transparency and buckles by drying. The capabilities in survival of the cysts of *N. malhamensis* have yet to be determined. Despite such, frequent bouts of flooding in the area may be important in providing several windows of

opportunity whereby following the receding of the stream water, the environment above water level remains sufficiently damp and humid to facilitate infection. Dave Hodgson, an independent researcher to the area in the study of the water chemistry of Malham Tarn, has provided information, that has recorded flooding at Tarn Fen in September 2010, February 2011 and August 2011 (Personal communication), corresponding to a pattern of 5 to 6 months between each flood. For species such as *N. attenuatus* in which the cyst is capable of surviving for such prolonged periods, this is ideal for maintaining a continuous cycle of infection (Graczyk and Shiff, 1994). The environmental conditions at Tarn Fen therefore appear to be highly conducive for the transmission of *N. malhamensis* and furthermore demonstrate the significant effect of site-specific variation on component helminth communities. This aspect of the study is in agreement of Abu-Madi *et al*. (2000) who emphasised how site-specific differences in the prevalence and intensity of helminths can be pronounced even between locations of close proximity and in the same climatic zone.

3.5 Summary

In conclusion, nine species of helminth were identified during the present study. The helminth fauna of Malham Tarn is predominantly composed of monoxenous nematodes, with only two digeneans previously being recorded. In addition to furthering the knowledge regarding prevalence and intensity of helminths at this location, increasing the sampling effort additionally led to the discovery of the new digenean species *Notocotylus malhamensis*. Host sex was considered to be an important determinant for the prevalence of *P. elegans* only. An increase in host age was only influential upon the prevalence of *P. elegans* and *H. polygyrus* which is likely due to differences in foraging activity between the different age groups of wood mice. No significant seasonal effects were determined, although the absence of digeneans during the winter months was evident, probably resulting from the lack of keystone intermediate hosts at this time of year. Only *N. malhamensis* appeared to be site-specific, with transmission appearing to be related to the ecology of the Tarn Fen site and it's associated flooding. The extent of flooding observed at Tarn Fen was not seen at any of the three plantations, supporting the hypothesis that it is the ecological aspects associated with this area that are conducive to the transmission of *N. malhamensis*.

CHAPTER 4:

A molecular and ecological analysis of the trematode *Plagiorchis elegans* in the wood mouse *Apodemus sylvaticus* from a periaquatic ecosystem in the UK.

4.1 Introduction

Wild rodent populations are commonly examined for their parasite assemblages and studies conducted worldwide have revealed a plethora of nematode, cestode and trematode species being harboured by various rodent fauna (Elton *et al.,* 1931; Lewis, 1968a; Lewis, 1968b; Tenora *et al.,* 1983; Abu-Madi *et al*., 2000; Shimalov, 2002; Hildebrand, 2008; Mazeika *et al*., 2009; Chechulin *et al*., 2010). 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* tend to be *Corrigia vitta* and *Brachylaemus recurvum* (syn. *Brachylaima recurva*) typically found infecting the pancreatic ducts and the small intestine of their host respectively (Elton *et al*., 1931; Lewis, 1968a; Lewis and Twigg, 1972; Behnke *et al.,* 1999; Abu-Madi *et al*., 2000). In 2007, Rogan *et al*. (2007) reported the occurrence of the intestinal digenean *Plagiorchis muris* in the wood mouse *A. sylvaticus* at Malham Tarn Nature Reserve, North Yorkshire, UK. This species had been recorded each September 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 intestine of the wood mouse *A. sylvaticus*. Due to a very low prevalence of 0.1% (n = 692) however, it was decided not to proceed with further description of the species, which was beyond the scope of the investigation (Elton *et al*., 1931). *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 Rayaki, 1963). To date, *P. muris* at Malham Tarn has been recorded from one location (Tarn Woods) and during September only (the consequence of an annual University of Salford undergraduate parasitology field course

in September) (Rogan *et al.,* 2007). This chapter therefore aims to investigate the occurrence of this digenean at Malham Tarn by examining the prevalence and intensity of *P. muris* seasonally and also from other woodland areas at this location.

4.1.1 *Plagiorchis* Lühe, 1899

Plagiorchis Lühe, 1899, is the type genus of the family Plagiorchiidae Lühe 1901. This family has been said to have the most complex taxonomic history of all digeneans and has at different times included as many as 150 genera (Tkach, 2008) and hundreds of described species (McMullen, 1937c), the majority of which have over time been synonymised or transferred to other families. Members of the genus *Plagiorchis* are cosmopolitan and tend to demonstrate low definitive host specificity, being deemed capable of infecting a wide range of host species. *Plagiorchis* species 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).

The genus *Plagiorchis* itself has undergone taxonomic controversy stemming as far back as the late nineteenth century when two synonymous genera; *Lepoderma* Looss, 1899 and *Plagiorchis* Lühe, 1899 were described almost simultaneously and as such were published in journals that were printed on the very same day (Talbot, 1934). The genus *Plagiorchis* was printed in *Zoologischer Anzeiger* on the 28th December 1899 and sent to the subscriber on the 29th December 1899. *Lepoderma* was published in *Zoologischer Jahrbücher* also on the 28th December 1899 but was not sent to the subscriber until 30th December 1899 (Kharoo, 2011).

This situation led to much dispute in terms of taxonomic priority, and ultimately divided the scientific community into two (Byrd, 1935). Many subsequent researchers however have prioritised the genus *Plagiorchis* based on the fact that the name *Lepoderma* did not reach the subscriber until one day later (Tkach, 2008). As such, early publications may refer to either genera but ultimately regard the same species of digenean.

There has been further confusion regarding the status and morphology of the type species *Plagiorchis vespertilionis* Müller, 1784, a parasite of chiropterans (Tkach *et al*., 2000a). This digenean was the first of the genus to be described by Müller in 1784.

Unfortunately, the type material of *P. verspertilionis* examined by Müller was lost and the original description furthermore deemed inadequate for species verification by subsequent researchers (Tkach *et al*., 2000a). As such, forms differing from the originally described morphotype were frequently reported, resulting in the misidentification of the vast majority of *Plagiorchis* species reported from European and Holarctic bats during the 20th century (Tkach *et al*., 2000a).

In 2000 however, Tkach *et al*., (2000a) re-described the type species *P. vespertilionis*. In doing so, they established a neotype in order to create nomenclatural stability for the species and both morphologically and molecularly defined it from other *Plagiorchis* species known to infect bats. The study entailed the creation of a dichotomous key primarily to define *P. vespertilionis* from both *Plagiorchis koreanus* Ogata, 1938, and *Plagiorchis muelleri* Tkach and Sharpilo, 1990, and additionally utilised *Plagiorchis elegans* Rudolphi, 1802, a species typical of infecting birds as an out-group.

4.1.2 *Plagiorchis muris* Tanabe, 1922

Plagiorchis muris, the species recorded by Rogan *et al.* at Malham Tarn in 2007, was originally described by Tanabe in 1922, who recovered the digenean from the small intestine of the black rat, *Rattus rattus* and the brown rat, *Rattus norvegicus* in Kyoto, Japan. *P. muris* 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), however, there has been considerable variability in the recorded definitive hosts including that of the domestic dog, *Canis familiaris* in Japan (Saito *et al*., 1995) and feral Japanese raccoons, *Procyon lotor* which have been reported on more than one occasion (Yamada, 2000; Sato and Suzuki, 2006). Further afield, *Plagiorchis* infection of the Mexican Greater funnel-eared bat; *Natalus mexicanus* has been recorded in the Neotropical region of Mexico (Perez-Ponce de Leon *et al*., 1996) in addition to several cases of natural avian infection in the United States of America. Infection of the robin, *Turdus migratorius*, the nighthawk, *Chordeiles minor*, the herring gull *Larus smithsonianus*, and the spotted sandpiper *Actitis macularius,* have been observed at Douglas Lake in Michigan State (Cort and Ameel, 1944; McMullen, 1937b), in addition to

the Greater yellowleg, *Tringa melanoleuca* in Texas and New Mexico (Secord and Canaris, 1993).

Typically, *P. elegans* has been considered foremost in the genus *Plagiorchis* for infecting birds (Shimalov, 2002). There have however been numerous reports of *P. elegans* also parasitizing several rodent hosts, 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). Infection of larger mammals with *P. elegans* has furthermore been identified and hosts as diverse and remote as the arctic fox, *Alopex lagopus* in Iceland (Skirnisson *et al*., 1993) and Greenland (Kapel and Nansen, 1996) to the wild muskrat, *Ondatra zibethicus* in Lithuania (Mazeika *et al.,* 2009) have been reported.

4.1.3 Human infection

P. muris has furthermore been recognised as a known zoonotic pathogen but its public health importance has been largely ignored as human infection is very rare (Ito and Itagaki, 2003). As far as can be determined, in total as little as 12 cases of natural human plagiorchiasis have been described (Ghobadi and Farahnak, 2004; Guk *et al*., 2007). Human infection is acquired similarly to that of the definitive host; by ingestion of freshwater fish or aquatic insect larvae infected with the metacercarial stage of the parasite. To date, natural human infection has been reported in Japan (Asada *et al*., 1962), The Republic of Korea (Hong *et al*., 1996; Guk *et al.*, 2007), The Philippines, Indonesia (Waikagul, 1991), and Thailand (Radomyos *et al*., 1989), all of which are countries where food-borne trematodiasis remains a significant public health problem due to a continued growth in aquaculture (Keiser and Utzinger, 2005).

Only two of the 12 reported cases of human plagiorchiasis have been due to infection by *P. muris* (Table 4.1). Asada *et al.* (1962) reported the first known incident of a natural human plagiorchiasis case by *P. muris* in Japan in 1962 and assumed chironomid larvae to be the source of infection. Three specimens of *P. muris* have since been reported from the diluted faecal sediment of a Korean man in the Republic of Korea, who was

additionally infected with one specimen of the intestinal digenean *Echinostoma hortense* and 862 specimens of *Metagonimus takahashii* and had a history of eating various kinds of freshwater fish from a local stream (Hong *et al*., 1996). Other *Plagiorchis* species that have been recognised to cause human plagiorchiasis are *P. philippinensis* Sandground, 1940*, P. javensis* Sandground, 1940, *P. harinasutai* Radomyos, 1989, and *P. vespertilionis* (Table 4.1).

P. philippinensis was identified in the province of Llocos Sur in the Philippines, when five specimens were recovered from the human intestine of a native. For *P. javensis,* only a single specimen was recovered from the contents of the small intestine of a Javanese native during a pathological examination at the Institute of Batavia in Indonesia (Sandground, 1940). *P. harinasutai*, was designated as a new species and was fully described by Radomyos *et al*. (1989) following the recovery of six worms from four opisthorchiasis patients in Thailand (Radomyos, 1989). The most recent report of human plagiorchiasis however occurred in the Republic of Korea when a single adult specimen of *P. vespertilionis* was identified from the intestine of a 34 year old man who had regularly eaten the raw flesh of snakehead mullet and gobies (Guk *et al*., 2007).

Table 4.1 Five *Plagiorchis* species currently known to cause human plagiorchiasis.

***Plagiorchis* species**	**Founder**	**Location**	**Reference**
P. muris	Tanabe, 1922	Japan/ Republic of Korea/ USA (experimental)	Asada *et al*. 1962/ Hong *et al*. 1996/ McMullen 1937a
P. philippinensis	Sandground, 1940	Philippines	Sandground, 1940
P. javensis	Sandground, 1940	Indonesia	Sandground, 1940
P. harinasutai	Radomyos, Bunnag & Harinasuta, 1989	Thailand	Radomyos *et al*. 1989
P. vespertilionis	Muller, 1784	Republic of Korea	Guk *et al*., 2007

4.1.4 Morphological differentiation of adult *Plagiorchis*

Unfortunately, species within the genus *Plagiorchis* display a high morphological similarity in the adult form and consequently can often be difficult to distinguish (Bock, 1984) (Figure 4.1). Problems often encountered in species identification are exacerbated by poor original descriptions and illustrations by authenticators in addition to a paucity of type material and a lack of knowledge on the life history of many species (McMullen, 1937c).

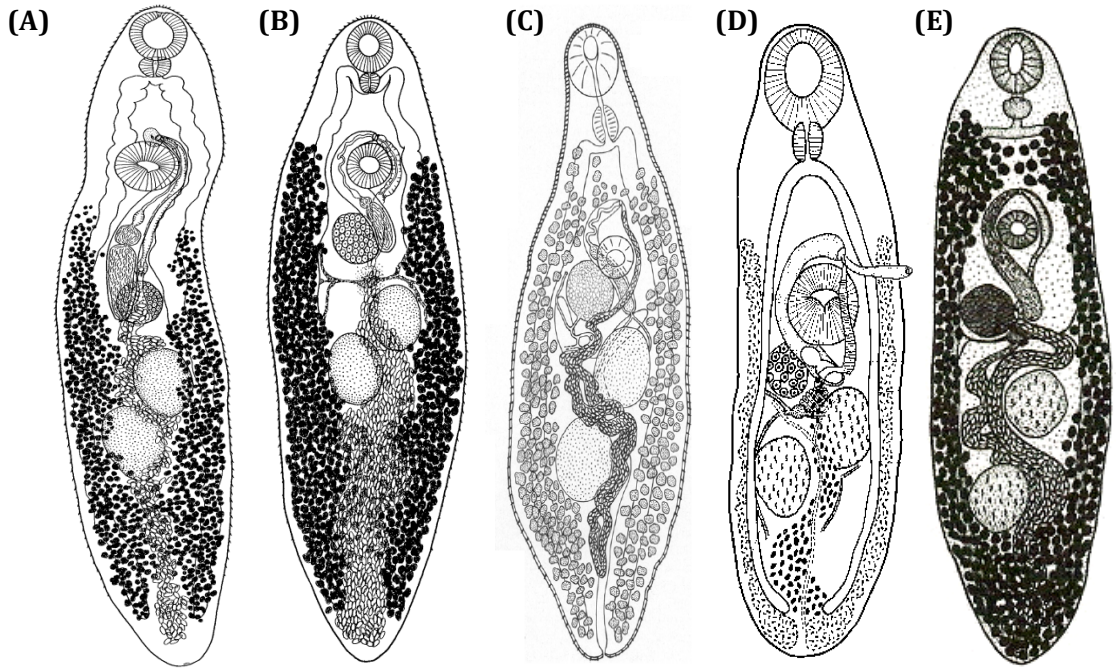


Figure 4.1 Examples of morphologically similar *Plagiorchis* species**. (A)** *Plagiorchis vespertilionis* (Tkach *et al*., 2000a); **(B)** *Plagiorchis muelleri* (Tkach *et al.,* 2000a); **(C)** *Plagiorchis elegans* (Bock, 1984); **(D)** *Plagiorchis maculosus* (Angel, 1958); **(E)** *Plagiorchis muris* (McMullen, 1937a). Body sizes have not been presented to scale.

Classical diagnosis of *Plagiorchis* species usually encompasses morphological characteristics, including the location and morphology of the cirrus and cirrus sac, the testes, ovary, and mehlis gland, and sucker ratio (Guk *et al*., 2007). Tkach *et al*. (2000a) used the relative size of the oral and ventral suckers, extent and confluency of the vitelline glands, the distance between the ovary and the centre of the ventral sucker and the body length to width ratio to differentiate the bat-associated species of *Plagiorchis* (including *P. elegans* considered a facultative parasite of bats) from one another.

The use of morphological criteria to differentiate species is not however without fault. Gorman (1980) described *P. muris* as a synonym *of P. elegans*, stating that due to the amount of information available for *P. muris,* she was unable to distinguish these two species amongst others based on available morphological information alone. Even Tkach *et al*. (2000a) placed emphasis upon the fact that his criteria may not be applied to all studied specimens due to the high variability seen in *Plagiorchis* species, in particular for younger specimens and those that may become distorted during the fixation process

(Tkach *et al*., 2000a). The use of morphological diagnosis should therefore be treated cautiously as delineation of *Plagiorchis* species by taxonomists has often been complex and many species within the genus have been ultimately synonymised (Tkach, 2008).

In some instances, founders have based their description of new digenean species on a single specimen (Sandground, 1940; Nath and Pande, 1963; Gupta & Jehan 1977; El-Naffar and Khalifa 1983). This situation vitally lacks a type specimen and essentially fails to consider the extent of intraspecific variation that may be observed. Other authors have designated new species based upon host specificity (Blankespoor, 1974) however there are several *Plagiorchis* species that have been recognised to be polyxenous (McMullen, 1937b; Kinsella, 1971; Blankespoor, 1974; Gorman, 1980). As pointed out by Blankespoor (1974), these kind of situations raise doubt to the validity of many species within the genus. Furthermore, as the effects of different definitive host species upon parasite development and final overall morphology has been established by several sets of experimental data (Kinsella, 1971; Blankespoor, 1974; Gorman, 1980) it may be suggested that there is in fact no difference between certain Plagiorchiids known to occur in both avian and mammalian hosts (Blankespoor, 1974). It may be postulated that experimental data generated by Blankespoor (1974) and Gorman (1980) can invalidate much of the morphological criteria set out by Tkach *et al*. (2000a) and Guk *et al*. (2007) by the demonstration of intraspecific variability in most morphological aspects other than the ratio of the oral and ventral sucker. It could be suggested therefore that differentiation of *Plagiorchis* species based purely upon morphological criteria alone should therefore be treated with caution.

Table 4.2 Anatomical measurements typically used to describe *Plagiorchis muris* in the literature.

Body length (mm)	Body width (mm)	OS to VS ratio	Location	Reference
0.80 – 2.20	0.24 – 0.80	1.10:1 – 1.11:1	Japan	Tanabe, 1922
1.85 – 2.05	0.59 – 0.63	1.05:1 – 1.09:1	Korea	Chae *et al*. 2010
1.46 – 1.77	0.36 – 0.45	1:1	Korea	Seo *et al*. 1964
2.85 – 2.99	0.82 – 0.95	Not stated	Korea	Hong *et al.* 1996
~2.67	~0.52	~1.48:1	USA	McMullen, 1937b
2.00 – 3.00	Not stated	~1.25:1	UK	Rogan *et al*. 2007

4.1.5 Molecular differentiation of the genus *Plagiorchis*

As discussed, the classical approach to species identification may not always be feasible. It has during recent years become apparent that morphology alone may not always be a sufficient tool for the determination of many species (Morgan and Blair, 1995), and more recently the use of molecular differentiation has become foremost for clearing up such situations. Tkach *et al*. (2000a) analysed the sequence divergence between four members of the genus *Plagiorchis: P. vespertilionis, P. koreanus*, *P. muelleri* and *P. elegans* and found that each DNA sequence analysed, clearly defined the presence of a distinct species despite morphological similarities. There is however, a current paucity of DNA data for *P. muris* within the DNA databases, with only two partial sequences currently available; one generated from the mitochondrial cytochrome oxidase subunit 1 (CO1) gene and a second sequence from the ribosomal 28S gene. These sequences for *P. muris* had been generated from metacercariae isolated from dragonflies and adult specimens collected from rodent intestines (Lee *et al*., 2004).

4.1.6 Aims

The aim of this chapter is to investigate the occurrence of *P. muris* at Malham Tarn by morphological and molecular analysis of specimens collected from rodents trapped within the Malham Tarn area. Further investigation will aim to examine any differences in seasonal prevalence and intensity rates and utilise four separate sampling sites located on each border of the tarn to identify whether this digenean species occurs in sites other than Tarn Woods. Investigation into factors that could influence infection rates including intrinsic (sex, age, and host species) and extrinsic (location and season) factors will furthermore be examined.

4.2 Materials and Methods

4.2.1 Rodent trapping

Seasonal trapping of rodents was conducted quarterly between January 2010 and October 2011 from Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation according to the methods described in 2.1.3 and rodents were processed and dissected according to section 2.2.1

4.2.2 Examination of adult specimens

Ten *Plagiorchis* specimens recovered from the small intestine of *Apodemus sylvaticus* were relaxed in distilled water and fixed in 5% formal saline under light cover slip pressure. The morphology of the specimens was examined, photographed and measured in micrometers using the methods outlined in section 2.2.4. The drawing of *Plagiorchis* was manually created by tracing the photographs.

4.2.3 Molecular analysis

DNA was extracted from 12 *Plagiorchis* specimens using the phenol: chloroform method described in 2.2.6. Three specimens from each sampling location were used for molecular analysis and when feasible, these were recovered from different hosts. Only one wood mouse from Spiggot Hill however was infected, therefore three individual worms from the same host specimen needed to be used in this instance. The complete ITS and a partial 28S rDNA gene region were amplified and commercially sequenced (see 2.2.8.1 and 2.2.9). The 12 DNA sequences were primarily aligned to check for sequence homology from each of the four sampling sites and FinchTV trace viewer was utilised in order to verify any regions of ambiguity. Both the resolved ITS and the 28S rDNA sequences were thereafter inputted into the NCBI BLAST program as in section 2.2.9 for molecular identification.

4.2.4 Phylogenetic analysis

Both the amplified ITS and 28S rDNA sequences generated from Malham Tarn were aligned using clustalW (http://www.genome.jp/tools/clustalw/) against similar sequences that were obtained from the NCBI database in addition to sequences of other

digenean species found at Malham Tarn during this study (Tables 2.1 & 2.2). Phylogenetic analysis was performed using methods described in section 2.3.4.

4.2.5 Ecological analysis

Prevalence data between two sample sets was analysed by 2 × 2 contingency tables using Fisher's exact test (two-tailed) available from Graphpad Software (http://www.graphpad.com/quickcalcs/contingency1.cfm). More than two data sets were analysed using chi-squared test for heterogeneity according to Holmes *et al*. (2006). Intensity data was compared using Mood's median test (Rózsa *et al*., 2000). Associations between *Plagiorchis* and other helminth species and between the presence of adult insect remains in the stomach contents of wood mice and *Plagiorchis* prevalence, rodent sex and rodent age were also investigated using pairwise analysis by 2 × 2 Fisher's exact test (two-tailed).

4.3 Results

Plagiorchis muris was successfully recovered from the small intestine of *Apodemus sylvaticus* trapped from all four locations: Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation indicating this digenean species to be dispersed throughout the Malham Tarn area. *P. muris* was not detected from any of the vole species examined despite careful observation (n = 63). In total 717 specimens of *P. muris* were recovered from all four sampling sites between January 2010 and October 2011 with an overall prevalence rate of 23% (95% Cl: 16.33% - 31.54%; n = 117) and mean intensity of 26.6±61.5 (717/27) and indicating *P. muris* to be the dominant intestinal digenean within this host species. This rate is higher than that previously reported by Rogan *et al*. (2007) who determined an overall prevalence of 16.9% and mean intensity of 2.03 although it should be emphasised that this figure was derived from wood mice trapped from the Tarn Woods area and in September only. Individual prevalence rates from each of the sampling sites have been discussed in section 4.3.3.2.

4.3.1 Morphology of adult *Plagiorchis* from Malham Tarn

The morphological description of the *Plagiorchis* specimens recovered from Malham Tarn is based upon the analysis of 10 flattened specimens (Figure 4.2A & 4.2B). The

morphology of the *Plagiorchis* specimens collected from all four sampling sites has been crossed referenced for consistency to create the following description.

4.3.1.1 External features

The body is distomatic in form and dorso-ventrally flattened. The anterior region of the body is bluntly pointed and the posterior end tapers to create a somewhat oval outline that extends with movement in live specimens to adopt a more elongated appearance. The dimensions of the body range from 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 that can be seen covering the entire surface. The dorsal surface appears to be obscured by a vast array of vitelline glands that fail to maintain confluency within the anterior region.

4.3.1.2 Alimentary canal

The oral sucker is roughly spherical in shape with a dimension of 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 appearing to be more spherical in form that that of the oral sucker. These measurements indicate an oral to ventral sucker ratio of 1.52: 1 (width ratio) – 1.57: 1 (length ratio).

4.3.1.3 Reproductive system

Two circular shaped testes are situated posterior to a single ovary. The testes are obliquely positioned with the anterior teste situated slightly right of the median line and the most posterior teste 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 the 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 the posterior vitellarian commissure. On the ventral surface, 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.

Table 4.3 The range and mean value of morpho-anatomic features of *Plagiorchis* collected from the small intestine of *Apodemus sylvaticus* at Malham Tarn.

***Plagiorchis* measurement (n = 10)**	**Range in measurements (µm) (n = 10)**	**Mean value (µm)**
Body Length	1896 - 3120	2642
Maximum body width	500 - 768	577
Oral sucker width	215 - 300	246
Oral sucker length	210 - 300	256
Ventral sucker height	144 - 200	163
Ventral sucker width	144 - 200	163

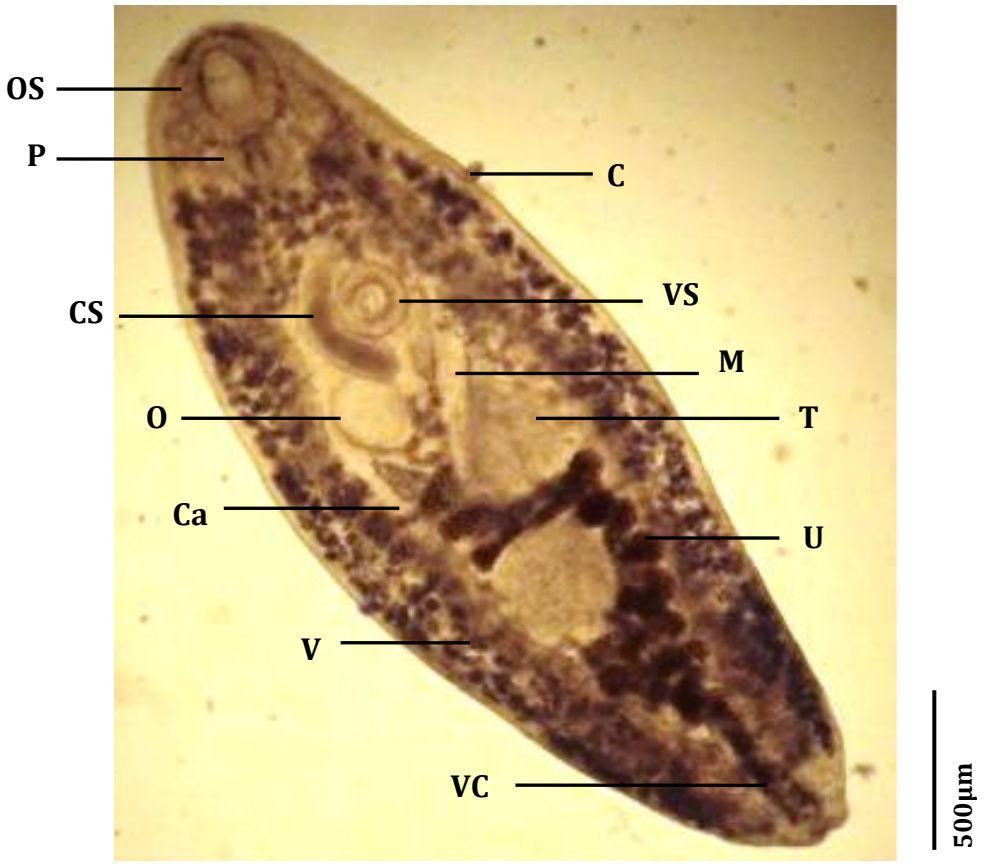


Figure 4.2A Photograph of *Plagiorchis* specimen recovered from the small intestine of *Apodemus sylvaticus* at Malham Tarn. Abbreviations: **OS**, oral sucker; **P**, pharynx; **C**, cirrus; **CS**, cirrus sac; **VS,** ventral sucker; **M**, metraterm; **O**, ovary; **T**, testi; **Ca,** caecum; **U**, uterus; **V**, vitellaria; **Vc,** vitellarian commissure.

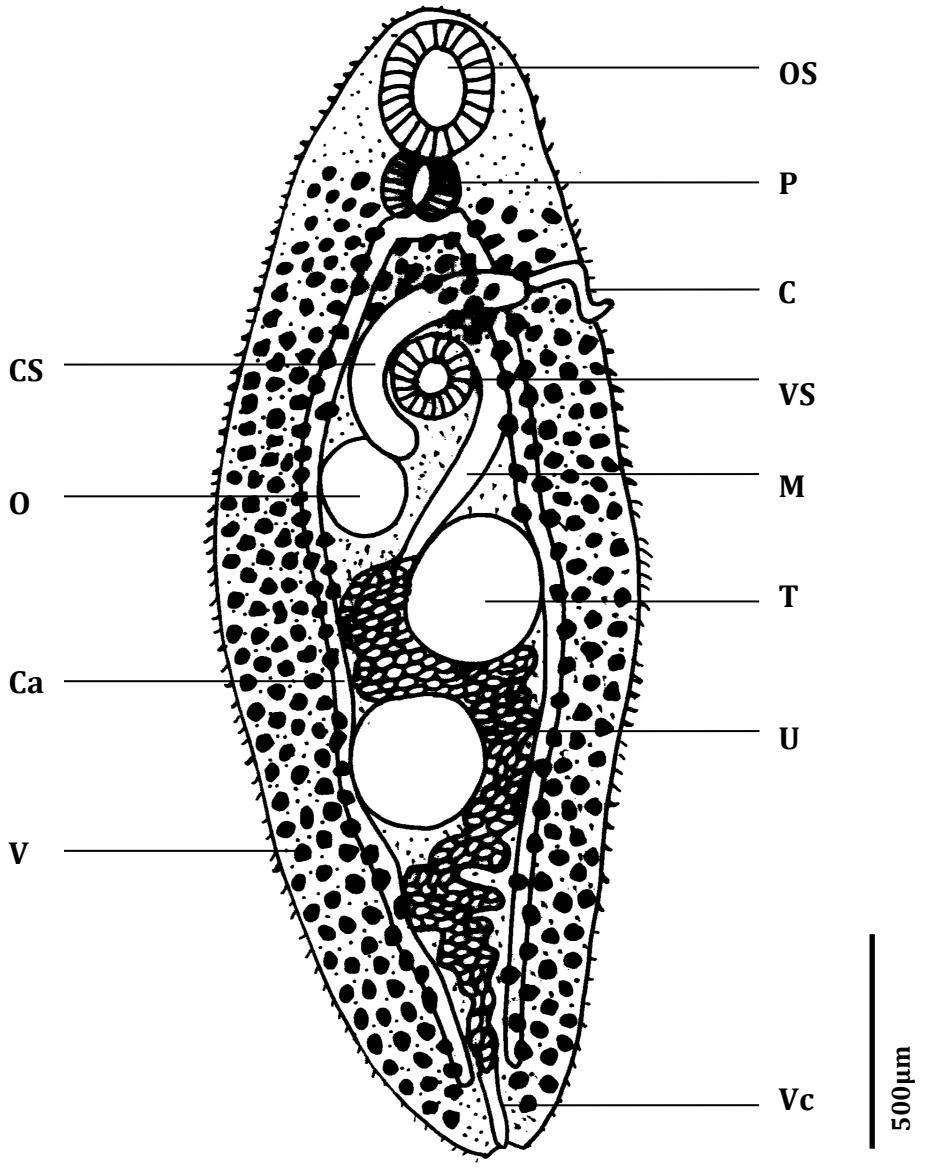


Figure 4.2B Diagrammatic representation of *Plagiorchis* specimen recovered from the small intestine of *Apodemus sylvaticus* at Malham Tarn. Abbreviations: **OS**, oral sucker; **P**, pharynx; **C**, cirrus; **CS**, cirrus sac; **VS,** ventral sucker; **M**, metraterm; **O**, ovary; **T**, testi; **Ca,** caecum; **U**, uterus; **V**, vitellaria; **Vc,** vitellarian commissure.

4.3.2 Molecular analysis

DNA was successfully extracted and amplified from 12 individual *Plagiorchis* specimens and both the complete ITS and partial 28S rDNA gene sequences were successfully generated (Figures 4.3 & 4.4). The DNA sequence data available in the NCBI sequence database for the genus *Plagiorchis* is currently limited, with as little as 19 nucleotide sequences for various genes available for comparison. From these 19 sequences, 11

sequences are for the Internal Transcribed spacer regions (including 5 repeated isolates), 6 partial sequences for the 28S rDNA and 2 partial sequences for the cytochrome c oxidase subunit 1 gene. Furthermore, 2 sequences have been matched with *Plagiorchis* at the genus level only, as they were derived from incompletely identified material (Marin Perez, 2006 unpublished; Moszczynska *et al*., 2009).

4.3.2.1 Ribosomal ITS region

The 1213bp sequence for the ITS region of *P. muris* recovered from Malham Tarn was inputted into the NCBI BLAST program for comparison against five available *Plagiorchis* DNA sequences: *P. maculosus* adult (AF316152) collected from the Chaffinch, *Fringilla coelebs* (Snyder and Tkach, 2001), *P. elegans* adult (AF151952) collected from a Red-Backed Shrike, *Lanius collurio, P. koreanus* adult (AF151944) from Kuhl's pipistrelle, *Pipistrellus kuhli*, the common noctule, *Nyctalus noctula* and Daubenton's bat *Myotis daubentoni*, *P. vespertilionis* adult (AF151949) from *M. daubentoni* and *P. muelleri* adult (AF151947) from the serotine bat *Eptesicus serotinus* (Tkach *et al*., 2000a) all obtained within the Ukraine. The ITS sequence for *P. muris* collected from Malham Tarn shared a 100% sequence homology with that of *P. elegans,* with only one omission of adenosine at site 571 (Figure 4.3). This omission was observed in all 12 generated DNA sequences. The *P. muris* sequence generated from Malham Tarn shared only 94% sequence homology with *P. maculosus,* 89% with *P. koreanus*, 91% with *P. vespertilionis*, and 90% with *P. muelleri*. The ITS sequence generated during the current study has been deposited into GenBank under accession number JX522536.

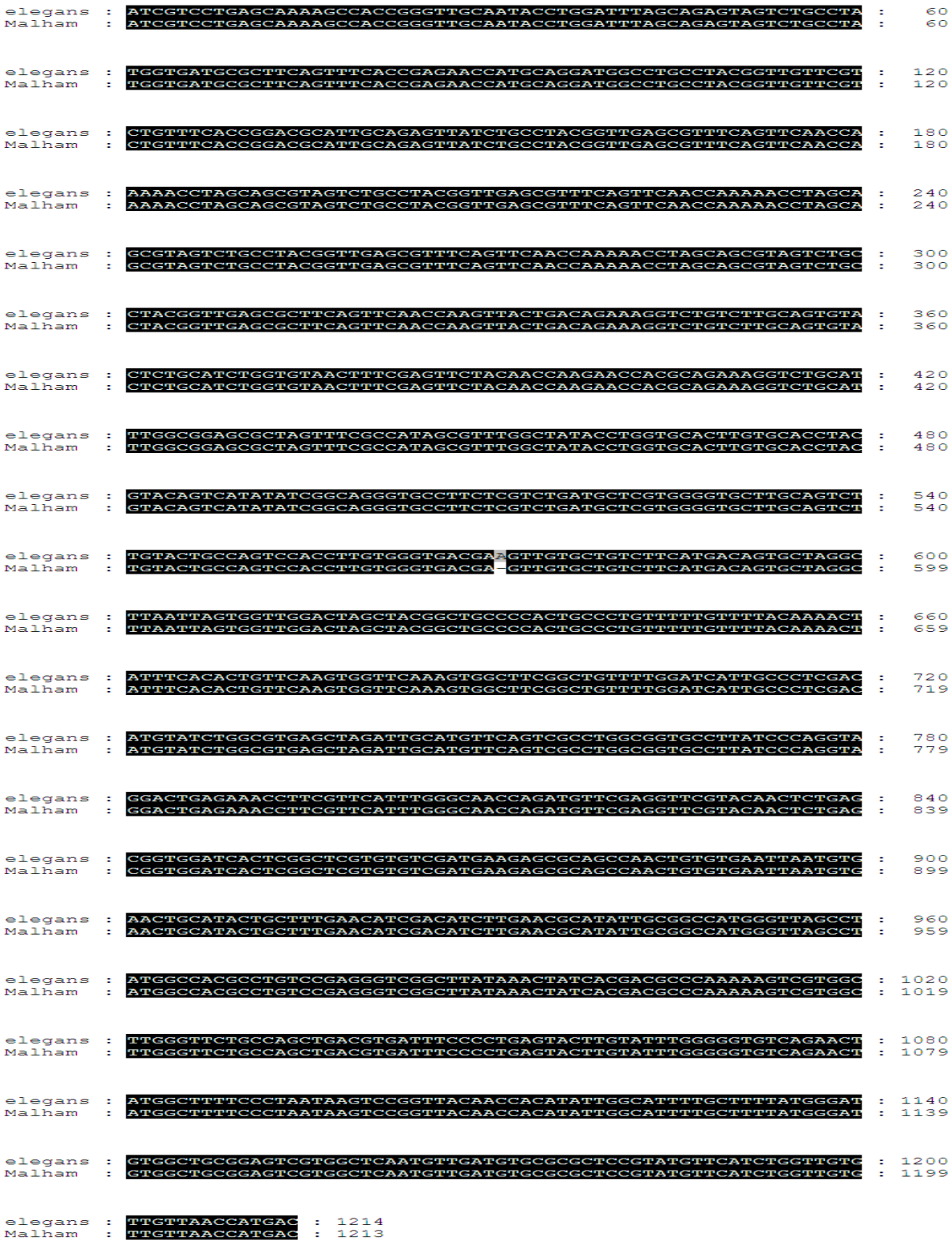


Figure 4.3 Comparison of the complete ITS DNA sequence of *Plagiorchis* collected from Malham Tarn against the ITS DNA sequence for *P. elegans* (AF151952) retrieved from NCBI, using GeneDoc alignment tool. Black shading indicates regions of conservation between the two digeneans. An omission of adenosine at a single site can be observed at position 571 of the Malham Tarn sequence.

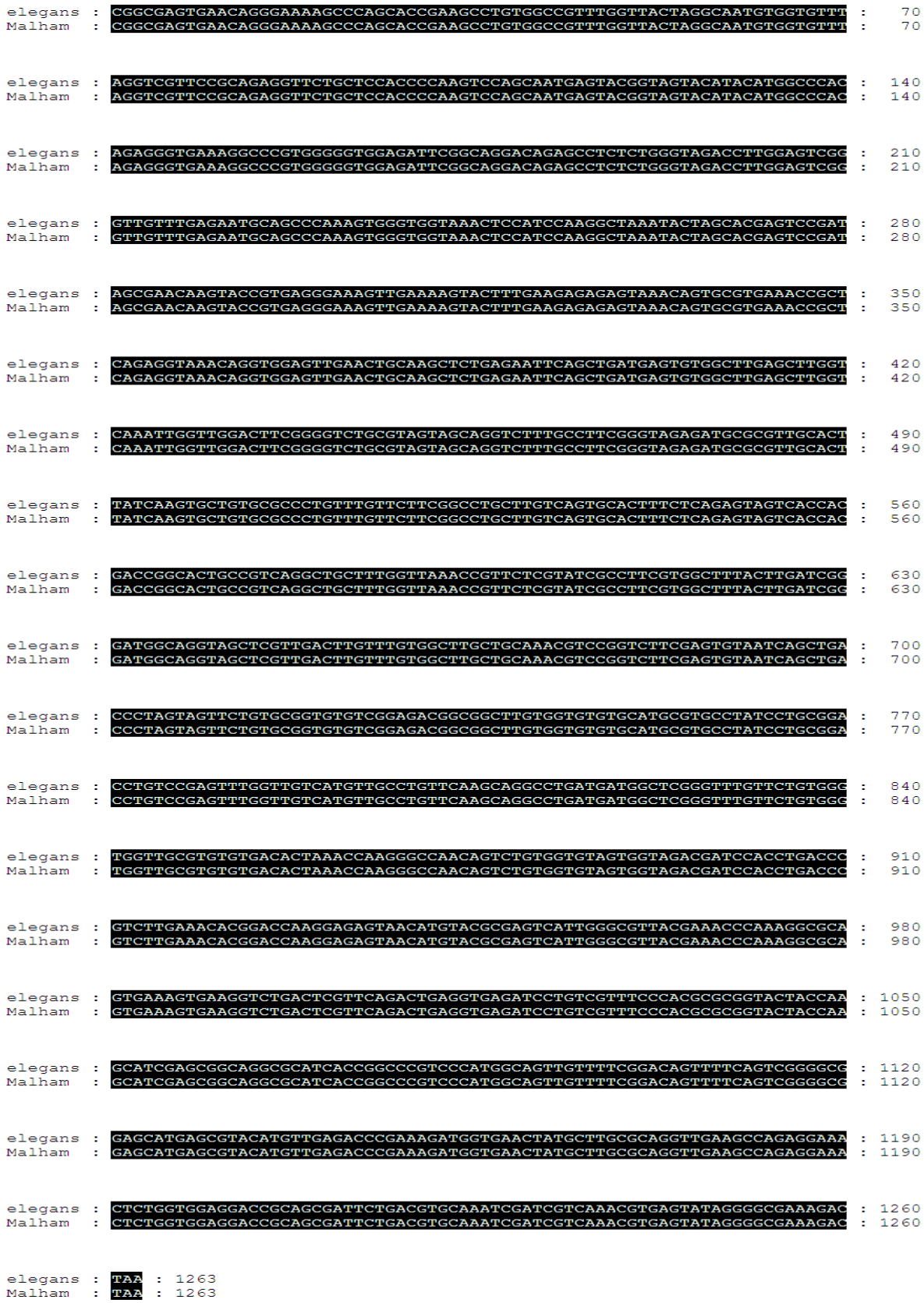


Figure 4.4 Comparison of the partial 28S rRNA sequence of *Plagiorchis* collected from Malham Tarn against the 28S rRNA sequence for *P. elegans* (AF151911) retrieved from NCBI, using GeneDoc alignment tool. Black shading indicates regions of conserved homology between the two digeneans.

4.3.2.2 28S rRNA gene

The 1263bp partial DNA sequence generated for the 28S rRNA gene of *P. muris* recovered from Malham Tarn was also inputted into the NCBI BLAST program for comparison against five available *Plagiorchis* 28S rRNA sequences: *P. elegans* (AF151911) (Tkach *et al*., 1999), *P. muris* adult (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* adults collected from *N. noctula* in the Sumy region of the Ukraine and *M. daubentoni* in the vicinity of Kiev, Ukraine (AF151931) (Tkach *et al*., 2000b).

A 100% sequence homology between the 12 specimens examined from Malham Tarn and the DNA sequence for *P. elegans* (AF151911) was observed using the full1263bp 28S rRNA sequence (Figure 4.4). Only a 304bp partial sequence for *P. muris* was available in the NCBI database for comparison (Lee *et al*., 2004). All available 28S rRNA sequences available for *Plagiorchis* spp. including the sequence generated from Malham Tarn were therefore trimmed and aligned with the sequence for *P. muris* (AF096222). The 28S rRNA sequence for *P. muris* collected from Malham Tarn shared a 100% sequence homology with *P. elegans*, 98% with *P. muelleri*, *P. koreanus* and *P. vespertilionis* and only a 95% match with *P. muris* (Figure 4.5a). The same 28S rRNA gene sequence was used to compare the 12 specimens collected from the four sampling sites at Malham Tarn (Figure 4.5b). The sequences from all four locations were 100% identical to *P. elegans* and shared only 95% homology with *P. muris*. This data in combination with the 100% sequence homology match demonstrated by the ribosomal ITS gene region (see 4.3.2.1) questions the identity of *P. muris* at Malham Tarn suggesting that this species may in fact be *P. elegans*. The specimens recovered from Malham Tarn therefore will be referred to as *Plagiorchis elegans* herein. The 28S rRNA sequence generated during the current study has been deposited into GenBank under accession number JX522535.

4.3.2.3 Phylogenetic analysis

The same phylogenetic relationship between *P. elegans* and the other available *Plagiorchis* species was inferred from both the ITS region and 28S rRNA genes with the majority of branches showing significant bootstrap support (>90%) (Figure 4.6 & Figure

5.7). In both cases, *P. elegans* clusters into a well-supported clade with other members of the Plagiorchiidae included in the analysis inferred by 100% bootstrap support. Both genes furthermore divide the genus *Plagiorchis* into two well-divided clades also indicated by 100% bootstrap support with the bat-associated species: *P. vespertilionis*, *P. koreanus* and *P. muelleri* appearing to be most closely related. The ITS gene infers *P. elegans* from Malham Tarn to be most closely related to *P. maculosus* (95% bootstrap support), a species that similarly tends to predominantly infect birds and small mammal species.

(A)

(B)

Figure 4.5 (A) Comparison of the partial 28S rRNA sequence of *Plagiorchis* collected from Malham Tarn against other *Plagiorchis* species retrieved from the NCBI database: *P. elegans* (AF151911); *P. koreanus* (AF151930); *P. muelleri* (AF184250); *P. vespertilionis* (AF151931)*; P. muris*. (AF096222). **(B)** Comparison of the partial 28S rRNA sequence of *Plagiorchis* collected from four different sites at Malham Tarn (Tarn Woods, Spiggot Hill, Tarn Fen, and Ha Mire Plantation) against *P. elegans* and *P. muris.* Comparison has been made using GeneDoc alignment tool. 100% sequence homology between the Malham Tarn specimens and *P. elegans* can be observed and 18 nucleotide differences can be observed between the Malham Tarn specimens and *P. muris*.

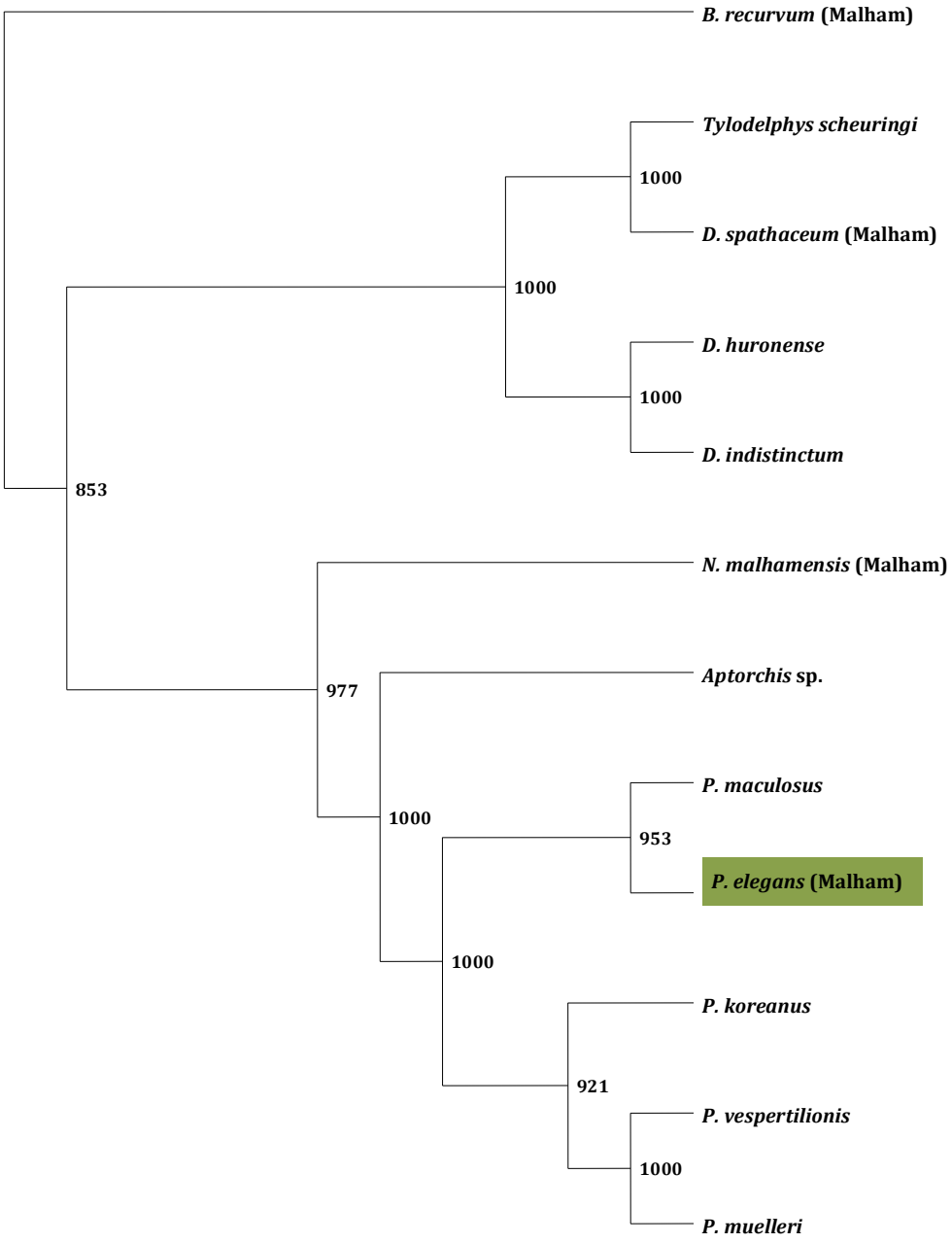


Figure 4.6 UPGMA rooted rectangular cladogram based on the complete ITS region of *P. elegans* collected from Malham Tarn, depicting the relationship between *P. elegans* and available ITS sequences of other members of the genus *Plagiorchis*: *P. maculosus* (AF316152), *P. koreanus* (AF151944), *P. vespertilionis* (AF151949) and *P. muelleri* (AF151947). The DNA sequence for *P. elegans* from the NCBI database has not been included as it 100% identical to that generated from the Malham Tarn samples. Figure to the right of each node represent percentage boostrap support based on 1000 replicates (Phylip 3.69). Other digenean species found at Malham Tarn have also been included in the analysis: *Brachylaemus recurvum*, *Diplostomum spathaceum* and *Notocotylus malhamensis*. All three sequences were generated during this study. Other digenean species found to be closely related to digenean species collected from Malham Tarn following an NCBI blast search have also been included: *Tylodelphys scheuringi* (FJ469596), *Diplostomum indistinctum* (GQ292508), *Diplostomum huronense* (GQ292507), and *Aptorchis* sp. (AM932523).

4.3.3 Ecological analysis

4.3.3.1 Dispersion

Dispersion of *P. elegans* within the wood mouse population (n = 117) was determined. Worm burden ranged from 0 to 275 with parasite distribution appearing over dispersed within the population and conforming to that of a negative binominal distribution (variance to mean ratio (VMR): $\sigma^2/\mu = 158.89$) with 76.9% (95% Cl: 68.46% - 83.67%; n = 117) of wood mice being uninfected in comparison with 1.7% (95% Cl: 0.11% - 7.44%) harbouring the majority of parasites (>100 worms). This data was reinforced by superimposition of the observed and expected frequencies (Figure 4.7). 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).

Table 4.4 The observed **(O)** and expected **(E)** frequencies of *P. elegans* in *A. sylvaticus* at Malham Tarn based on a negative binomial distribution according to Fowler *et al*. (1998).

	Number of *P. elegans*											
	0	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	>100
O	90	19	1	2	1	1	0	1	0	0	0	2
E	86.64	12.78	1.95	1.31	0.98	0.79	0.6	0.56	0.49	0.43	0.38	0.35

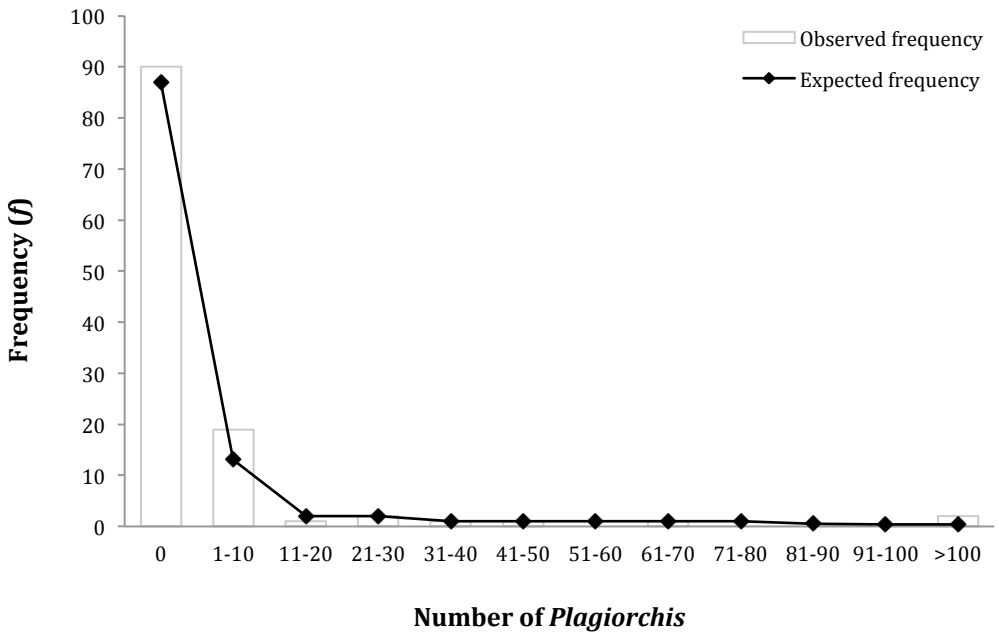


Figure 4.7 Frequency distribution of *P. elegans* within the *A. sylvaticus* population at Malham Tarn (n=117) indicating the observed and expected frequencies calculated according to a negative binomial distribution (Fowler *et al.,* 1998). Histogram represents observed frequencies and line graph expected frequencies.

4.3.3.2 Prevalence and mean intensity by sampling site

The prevalence and mean intensity of *P. elegans* between the four selected sampling sites: Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation were compared (Table 4.6). The greatest prevalence was observed at Ha Mire Plantation in which 37% (10/27) of sampled wood mice carried a mean intensity of 19±24.2 worms (187/10). The prevalence of *P. elegans* at Tarn Woods was less with 23.1% (12/52) of wood mice infected despite a slightly higher mean intensity of 21±50.33 (246/12). A prevalence of 19.1% (4/21) was observed at Tarn Fen and the lowest mean intensity of the study of just 2±2.5 (9/4). The figure derived from Spiggot Hill should be treated cautiously as this is due to a single rodent infection carrying 275 worms despite this site having the lowest prevalence rate of the study at just 5.9% (1/17). There was no significant difference in prevalence using a chi-squared test for heterogeneity ($X^2 = 3.79$, $p = 0.05$, $v = 3$), or mean intensity ($X^2 = 1.93$, $p = 0.05$, $v = 3$) using Mood's median test between the four locations.

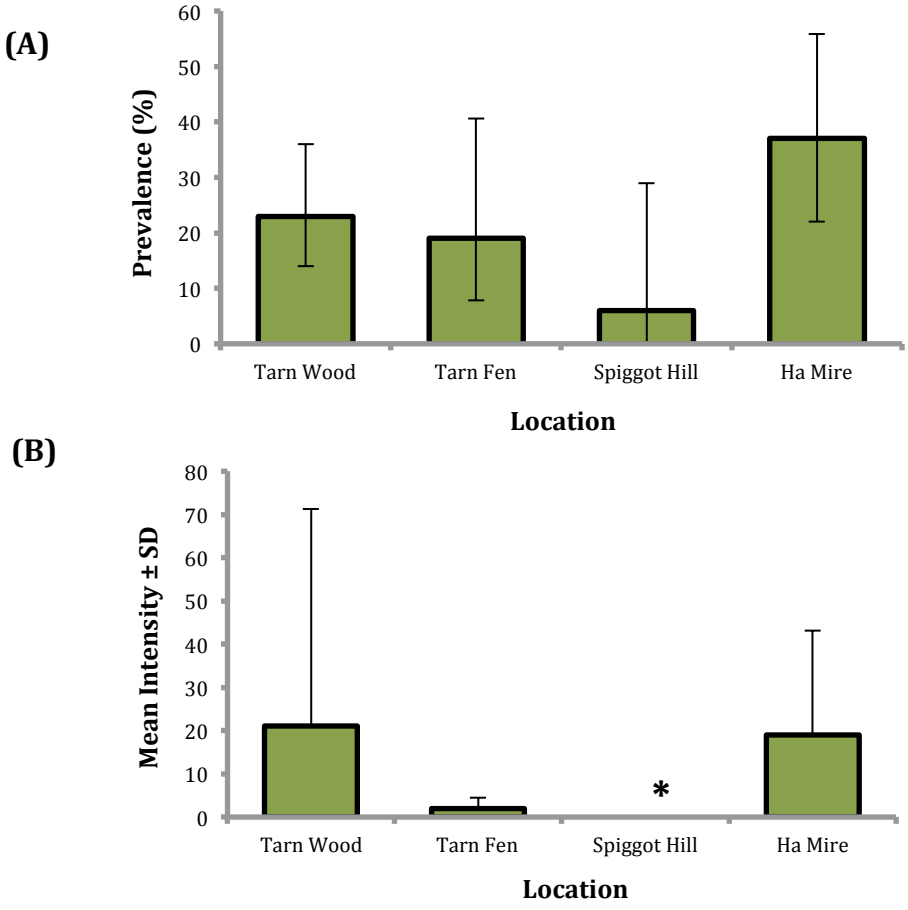


Figure 4.8 (A) Prevalence indicating 95% confidence limits and **(B)** mean intensity indicating standard deviation for *P. elegans* in the *A. sylvaticus* (n = 117) population collected from the four sampling sites between January 2010 and October 2011. The mean intensity of *P. elegans* from Spiggot Hill has not been included due to a single infection of 275 worms*.

4.3.3.3 Seasonal variation in prevalence and mean intensity

The prevalence and mean intensity of *P. elegans* each season was compared (Table 4.6). Both mean prevalence and mean intensity of *P. elegans* were zero during the winter (January) sampling periods (0/8), but increased to 12.5% (1/8) with a mean intensity of 179 during the spring (April). This intensity rate however was generated from a single wood mouse harbouring that number of worms and is therefore not an indication of a mean value. The highest mean prevalence rate was observed during the summer sampling period (July). A prevalence of 27.3% (6/22) was recorded with a mean intensity of 3±2.94 worms (20/6). Despite a slight decrease in prevalence in the autumn (September/October) to 25.3% (20/79), the mean intensity increased considerably to 26±61.46 worms (518/20) for this period. There was no significant difference in prevalence using a chi-squared test for heterogeneity ($X^2 = 3.38$, $p = 0.05$, $v = 3$), or mean intensity ($X^2 = 1.64$, $p = 0.05$, $v = 3$) using Mood's median test between the seasons.

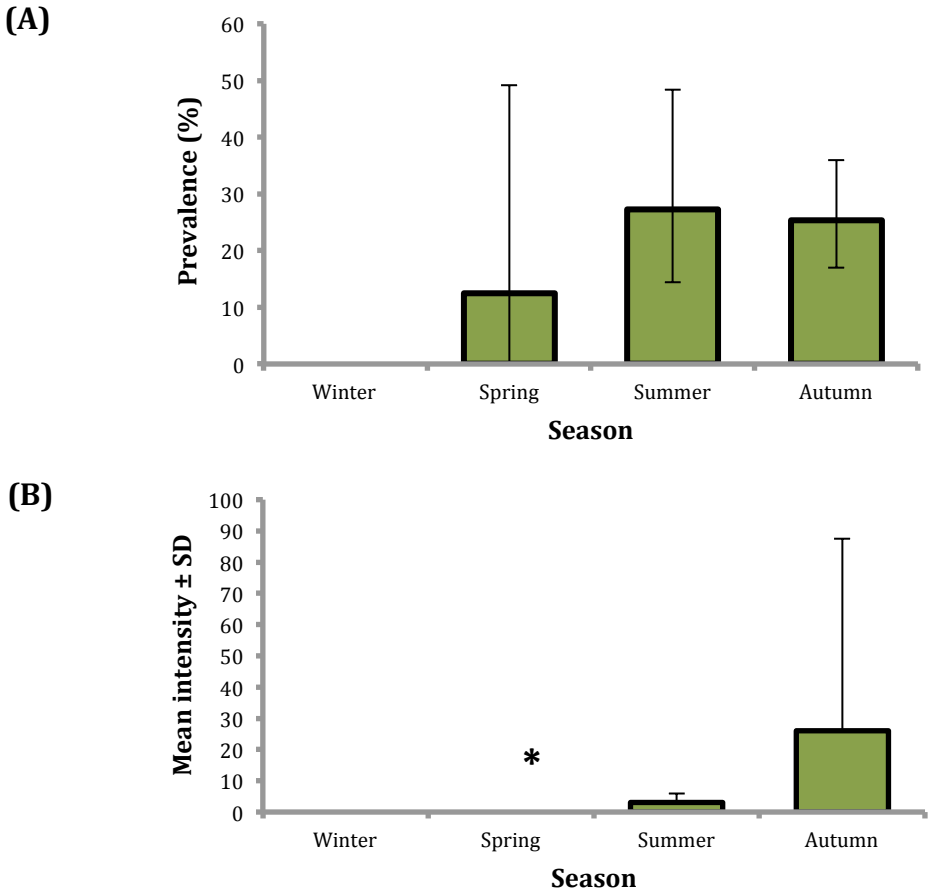


Figure 4.9 (A) Mean prevalence indicating 95% confidence limits and **(B)** mean intensity indicating standard deviation for *P. elegans* in *A. sylvaticus* collected each season at Malham Tarn between January 2010 and October 2011 (n = 117). The mean intensity of *P. elegans* in spring has not been included due to a single mouse infection carrying 179 worms*.

4.3.3.4 Association with rainfall

Rogan *et al*. (2007) suggested that rainfall could play an important factor in *Plagiorchis* prevalence by providing temporary water bodies around the tarn area in which insect larvae may develop. *Plagiorchis* prevalence was therefore examined in relation to total monthly rainfall data (Figure 4.10A). The prevalence of *P. elegans* was statistically analysed in relation to the previous three months rainfall (mm) using Spearman's rank of correlation (Table 4.5). A very weak but significant correlation (r_s = 0.095, p = <0.05, n = 27) between the two factors was determined (Figure 4.10B).

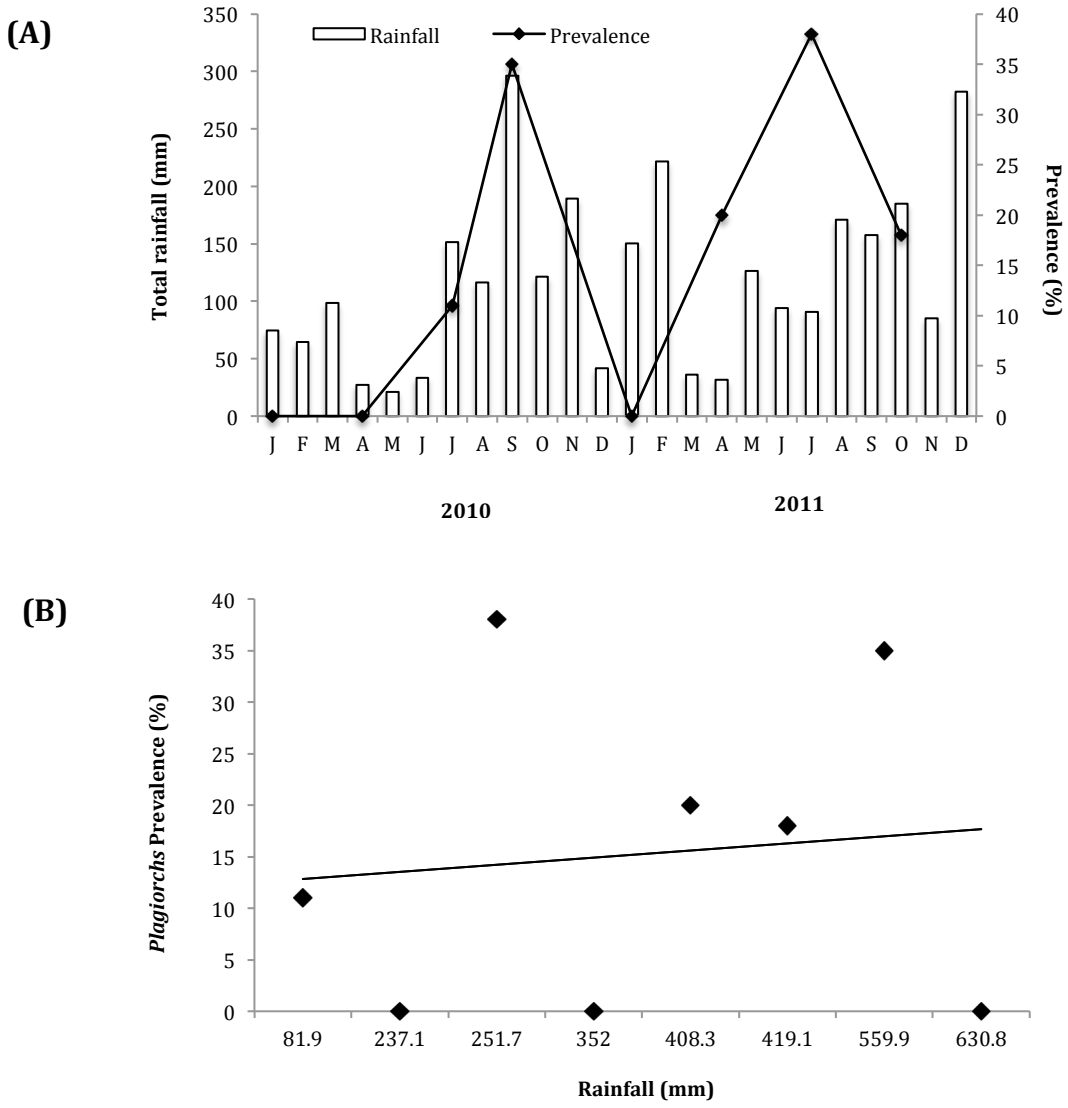


Figure 4.10 *Plagiorchis* prevalence in relation to total monthly rainfall (mm) over the two-year sampling period. The histogram represents the amount of total monthly rainfall (mm) and the line graph *P. elegans* prevalence. Eight observations of *P. elegans* prevalence in total were recorded. (**B**) A very weak correlation (r_s = 0.095) observed between the prevalence of *Plagiorchis* and the total amount of rainfall (mm) recorded during each three month period prior to sampling. Black marker points represent *P. elegans* prevalence and the black line is a representative trendline of correlation.

Table 4.5 Rainfall data used to analyse the correlation between the total amount of three months rainfall (mm) and *P. elegans* prevalence (%) by Spearman's rank of correlation. Rainfall data was supplied by Malham Tarn Field centre. Emboldened numbers are the figures that have been used within the statistical analysis.

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			

4.3.3.5 Prevalence and mean intensity by host sex

In total, 71 male mice and 46 female mice were examined (Table 4.6). The prevalence in male mice was 32.4% (23/71) in comparison to only 8.7% (4/46) for female mice (Figure 4.11A). Male mice harboured the majority of worms carrying 688 of 717 *P. elegans* recovered and having a mean intensity of 30±66.09 (688/23) against 7±12.5 (29/4) for female mice (Figure 4.11B). Although this difference in prevalence was found to be highly statistically significant using 2 x 2 Fisher's exact test (exact p value = 0.003), no statistical difference in mean intensity between the two groups was identified using Mood's median test ($X^2 = 0.213$, $p = 0.05$, $v = 1$).

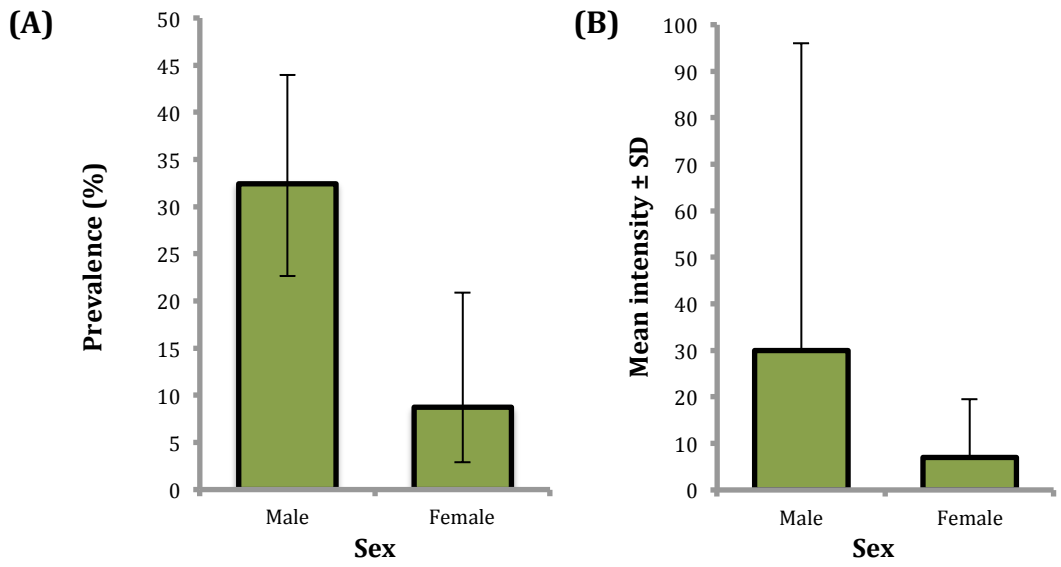


Figure 4.11 (A) The total prevalence indicating 95% confidence limits and **(B)** mean intensity indicating standard deviation for *P. elegans* collected from each sex of wood mice (n = 117) at Malham Tarn. Sex categories include mice of all age cohorts.

4.3.3.6 Prevalence and mean intensity by age group

P. elegans was recorded in a total of 19 out of 44 adult wood mice examined giving a prevalence of 43% (Table 4.6). This figure was less in young adults (14%, 8/56) and no infection was detected in juvenile mice (0/17) (Figure 4.12a). A similar pattern was observed for mean intensity with adult wood mice harbouring the vast majority of infection (range 0 to 275 worms) carrying a mean worm burden of 36±71.72 (677/19) in comparison with 5±7.86 (40/8) for young adults (range 0 to 24) and zero for juveniles (Figure 4.12b). Using chi squared test for heterogeneity this prevalence between the age cohorts was found to be highly significant (X^2 = 18.1, p = 0.05, v = 2). Pairwise analysis using 2 x 2 Fisher's exact test was therefore conducted determine where they significance lay. No significant difference was observed between the juvenile and young adult age categories (exact p value = 0.185) however the difference between adult wood mice and young adults (exact p value = 0.002) and adult and juvenile (exact p value = 0.001) age cohorts was found to be highly statistically significant. No statistical difference in mean intensity was identified between any of the groups using Mood's median test (X^2 = 2.33, p = 0.05, v = 2).

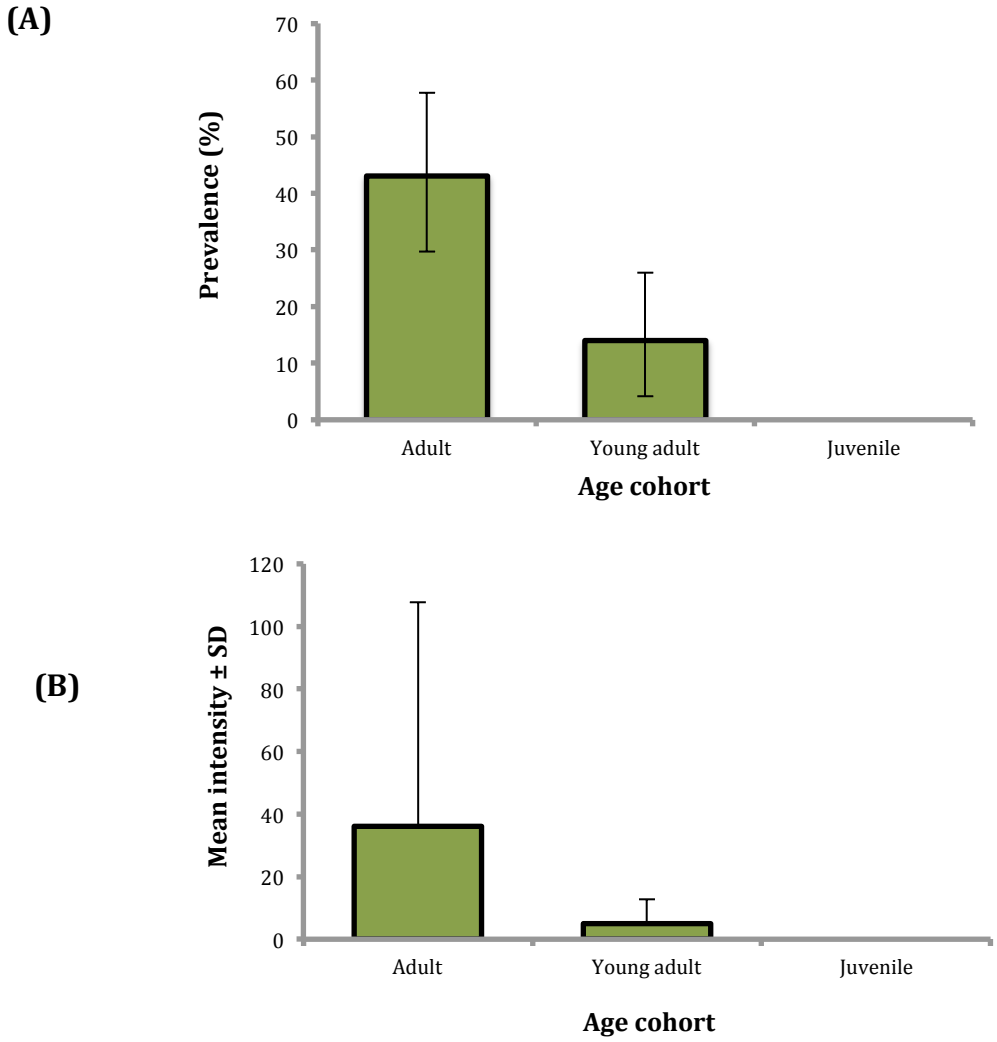


Figure 4.12 (A) The prevalence indicating 95% confidence limits and **(B)** mean intensity indicating standard deviation for *P. elegans* collected from each age cohort of wood mice (n = 117) at Malham Tarn, using the age criteria of Behnke *et al*. (1999); **Juvenile**: 1 to 12g, **Young adults**: 13 to 19g, **Adults**: >20g. No infection was observed in juvenile wood mice.

Spearman's rank of correlation was furthermore used to determine if there was any association with *P. elegans* prevalence and mouse weight (g) and mouse length (cm). Mouse weights established during the study ranged from 4.0 to 29g. Mice were categorised into seven weight classes in increments of 4.0g ranging from 4.0g or less to mice weighing more than 25g. A very strong positive correlation was determined between an increase in *P. elegans* prevalence and an increase in mouse weight (r_s = 0.929, p = <0.05, n = 113) (Figure 4.13).

Mouse lengths established during the study ranged from 4.5cm to 9.8cm. Mice were furthermore categorised into seven length classes in increments of 0.8cm ranging from

less than 5cm to mice measuring more than 9.4cm. A very strong positive correlation between *P. elegans* prevalence and an increase in mouse length was also determined using this criterion (r_s = 0.955, *P* = <0.05, n = 117) (Figure 4.14).

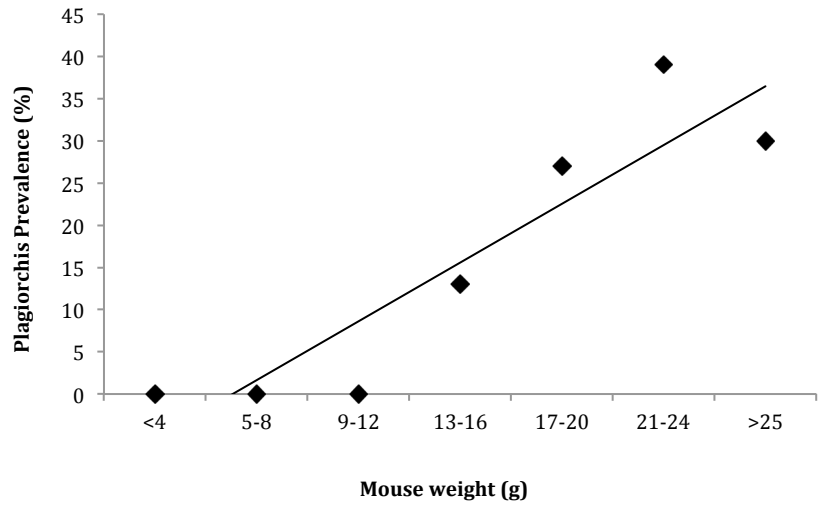


Figure 4.13 A very strong positive correlation (r_s = 0.929) observed between the prevalence of *Plagiorchis* and mouse weight (n = 113). Black marker points represent *Plagiorchis* prevalence rates calculated for each weight class and the black line is a representative trendline of correlation.

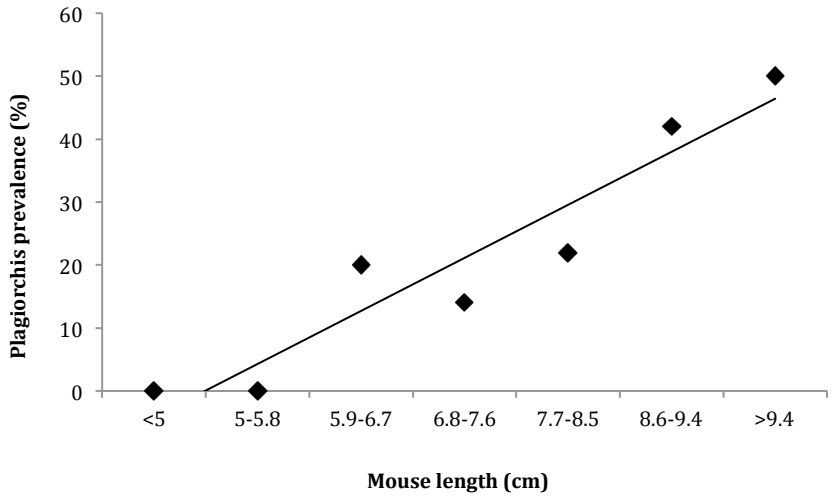


Figure 4.14 A very strong positive correlation (r_s = 0.955) observed between the prevalence of *Plagiorchis* and mouse length (n = 117). Black marker points represent *Plagiorchis* prevalence rates calculated for each length class and the black line is a representative trendline of correlation.

Table 4.6 Summarised prevalence (with 95% confidence limits) and mean intensity data for *P. elegans* in *A. sylvaticus* from Malham Tarn. * Figure derived from a single host infection.

	Prevalence (%)	95% Confidence limits		Mean intensity ± S.E.M
		Lower	Upper	
Overall	23.00	16.33	31.54	27 ± 61.5
Location				
Tarn Woods	23.1	13.58	36.28	21± 50.33
Tarn Fen	19.1	07.80	40.59	2 ± 2.5
Spiggot Hill	5.9	00.01	28.92	275*
Ha Mire	37.0	21.47	55.84	19 ± 24.2
Season				
Winter	0	0	37.22	0
Spring	12.5	00.11	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	02.90	20.86	7 ± 12.5
Age cohort				
Adult	43.2	29.67	57.79	36 ± 71.72
Young adult	14.3	07.16	26.00	5 ± 7.86
Juvenile	0	0	21.63	0

The difference in prevalence and mean intensity was calculated to identify the number of male and female mice infected in each age cohort (Figure 4.15). Prevalence in adult males was 50% (95% Cl: 34.07% - 65.93%; n = 34) in comparison to only 20% (95% Cl: 4.59% - 52.06%; n = 10) in adult females. This difference was not statistically significant using 2 x 2 Fisher's exact test (exact p value = 0.1448). Male mice in the adult age cohort also carried the vast majority of worms harbouring 650 with a mean intensity of 38±75.49 (650/17) against female mice that carried 27 worms with a mean intensity of 13.5±17.68 (27/2). The difference in mean intensity between the sexes in the adult age group was not found to be statistically different using Mood's median test ($X^2 = 0.006$, $p = 0.05$, $v = 1$).

Young adult males also carried a higher prevalence of 20% (95% Cl: 9.14% - 37.67%; n = 30) compared with 7.7% (95% Cl: 1.02% - 25.26%; n = 26) for females in the same age cohort. This difference was not found to be statistically significant using 2 × 2 Fisher's exact test (exact p value = 0.2529). The number of worms recovered from this age

cohort was much lower than that of the adult group with male mice harbouring just 38 worms (mean intensity 6.3±8.82) in comparison to female mice with a mere 2 worms and mean intensity of 1±0 (2/2). This difference was furthermore not found to be statistically different using Mood's median test ($X^2 = 0.178$, $p = 0.05$, $v = 1$).

Using 2 x 2 Fisher's exact test, statistical significance was identified in prevalence between adult males and young adult males (exact p value = 0.0177) and adult males and young adult females (exact p value = 0.0005) only. No significance in worm burden was determined between any of the groups using Mood's median test (adult males and young adult males $X^2 = 0.015$, $p = 0.05$, $v = 1$ and adult males and young adult females $X^2 = 0.268$, $p = 0.05$, $v = 1$).

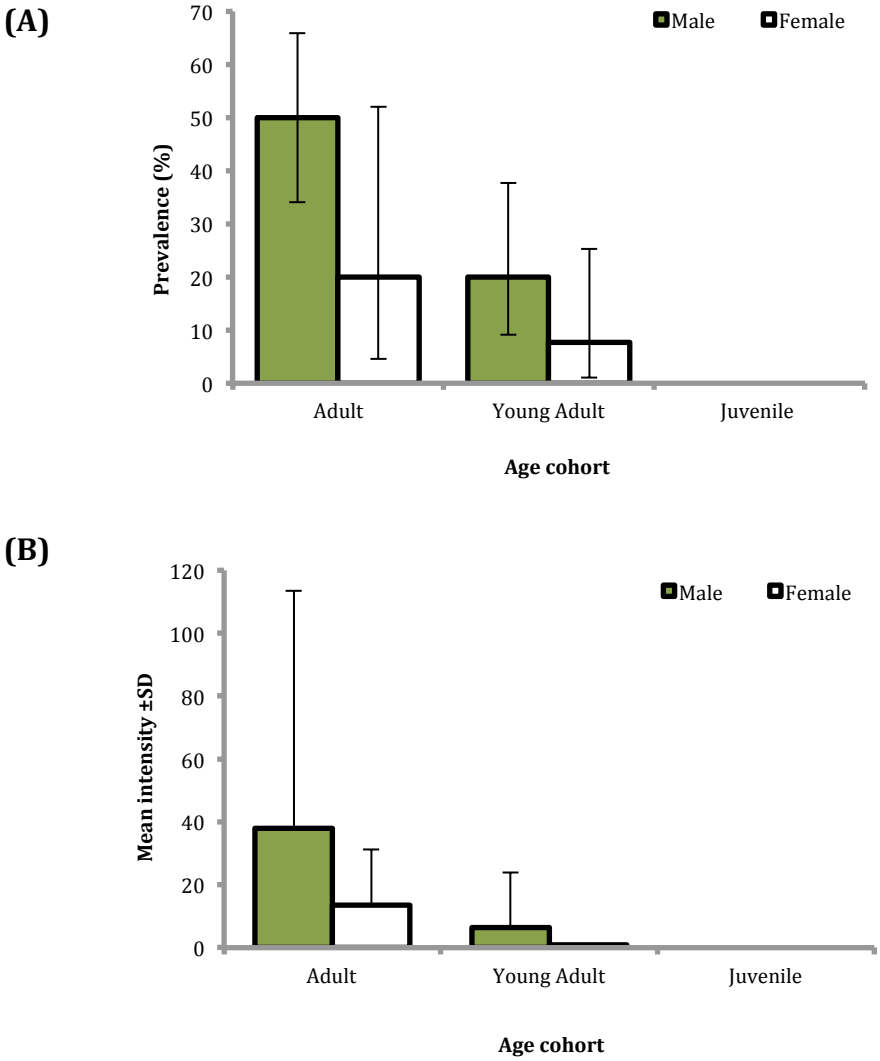


Figure 4.15 (A) The prevalence indicating 95% confidence limits and **(B)** mean intensity indicating standard deviation for male and female wood mice (n = 117) infected with *P. elegans* in each age cohort according to Behnke *et al*. (1999); **Juvenile**: 1 to 12g, **Young adults**: 13 to 19g, **Adults**: >20g.

4.3.3.7 Co-infection

Apodemus sylvaticus was infected with a further four species of helminth. From the 27 wood mice infected with *P. elegans*, 21 were furthermore infected with *Heligmosomoides polygyrus,* seven with *Syphacia stroma*, one with *Aoncotheca murissylvatici.* and one with *Brachylaemus recurvum.*

B. recurvum was detected in only two of the 117 wood mice examined and was therefore eliminated from analysis. For the wood mice not infected with *P. elegans* (n = 90), *H. polygyrus* was recovered from 58, *S. stroma* from 27 and *Aoncotheca murissylvatici* from just seven. None of these associations were proved to be statistically significant using 2 x 2 Fisher's exact test with exact p values of 0.6136, 0.8204 and 0.6824 respectively.

4.3.3.8 Stomach contents

From the 117 examined samples, 93 appeared to be composed mostly of plant material. The remains of adult insects (parts of exoskeleton, antennae and entire legs) were observed in 24 wood mice. From these 24 samples, 15 were infected with *Plagiorchis*. An extremely significant association between the presence of adult insect remains within stomach contents and *Plagiorchis* prevalence was determined using 2 x 2 Fisher's exact test (exact p value = 0.0005). Unfortunately, molecular identification of insect fragments could not be performed due to time constraints. This may however be suggested for future work.

From the 24 samples containing adult insect remains, 75% were from male mice (18/24) in comparison to just 25% for female mice (6/24), although this difference was not statistically significant using 2 x 2 Fisher's exact test (exact p value = 0.1589). From the total number of wood mice examined, 25% (18/71) of males had recently eaten adult insect remains in comparison to just 13% (6/46) of female mice. This difference was also not found to be statistically significant using 2 x 2 Fisher's exact test (exact p value = 0.2470). From the wood mice containing adult insect remains in their stomach contents, 19 were categorised as adult, four as young adults and only one as a juvenile. The difference between adults and young adults (exact p value = 0.0001) and adults and juveniles (exact p value = 0.0057) was statistically significant. There was no difference however between the young adult and juvenile age categories (exact p value = 1.0000).

4.4 Discussion

In the present study the use of molecular differentiation was employed to investigate further the occurrence of *Plagiorchis muris* at Malham Tarn. The results indicate that the currently identified specimens of *P. muris* at this location are *Plagiorchis elegans*. As far as can be determined 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). Elton *et al.* (1931) reported a prevalence of *P. muris* in the wood mouse *A. sylvaticus* in Oxford, UK and Baylis (1939) simply listed an occurrence of the digenean in the brown rat *Rattus norvegicus* in Cambridgeshire, UK. Neither author described the morphology of the parasite involved nor was DNA sequencing an aspect of biological surveys at that time. The third report made by Fahmy and Rayski in 1963, described *P. muris* from the intestine of a Scottish Hill sheep recovered during a parasitological necropsy. This was a short report which provided no written account of the digenean, 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*. Although very difficult to assess, retrospectively, it is possible that these earlier authors misclassified the species and were also looking at *P. elegans*.

Previously, Fahmy (1954) described a new species of *Plagiorchis* from the otter *Lutra lutra* killed off the coast of Edinburgh, Scotland which he coined *Plagiorchis lutrae*. In his report, Fahmy stated that this new species closely resembled the description of *P. muris,* but was differentiated on the basis of size with *P. lutrae* appearing to be much smaller. The measurements provided for *P. lutrae* however indicated a range in length of 0.9-1.28mm, which overlaps with those from the original description of *P. muris*, described by Tanabe in 1922 (0.80-2.20mm). Fahmy however differentiated *P. lutrae* on the basis that this specimen was much smaller than the *P. muris* described by McMullen (1937b) from Douglas Lake in Michigan State, USA rather than using the original description for the species by Tanabe (1922). Fahmy (1954) furthermore used the vitelline glands as a means to differentiate the two species. The vitellaria of *P. lutrae* extend from the middle of the oral sucker to the posterior extremity of the body whereas in the description of Tanabe (1922) the vitellaria of *P. muris* reach only as far as the pharynx.

Interestingly, in the report of *P. muris* from the Scottish hill sheep made by Fahmy and Rayski in 1963, the diagram of *P. muris* furthermore indicates the vitelline glands to extend from the middle section of the oral sucker to the posterior extremity of the body, similarly to the diagram presented in the paper of Fahmy in 1954. The identity of *P. muris* described by Fahmy and Rayski (1963) may therefore also be considered doubtful due to both the discrepancy in the diagrammatic representation of the species involved and additionally because the specimen was inadequately described to allow a comparison to be made for confirmation.

There appears to be much ambiguity in the literature that describes *P. muris* from various locations. The majority of reports describing the occurrence of *P. muris* from outside of Southeast Asia seem to indicate both a greater range in length and oral to ventral sucker ratio. The description provided by McMullen (1937b) in the USA was based upon adult digeneans recovered from a range of experimental hosts including man, rat, mouse and pigeon in addition to a range of naturally infected avian fauna. The average length and oral to ventral sucker ratio reported from this study, was beyond the maximum dimensions reported for the Southeast Asian *P. muris* (Tanabe, 1922; Seo *et al*., 1964; Chae *et al*., 2010). Similarly, the length and oral to ventral sucker ratio of *P. muris* described by Rogan *et al*. (2007) in addition to those recorded during this study exceed those dimensions reported in Asia. Intriguingly, the measurements described for *P. muris* by McMullen (1937b), Rogan *et al*. (2007) and during this study appear to overlap with the description of *P. elegans* generated by Gorman in 1980 (Gorman, 1980), who indicated a range in body length of 1.04-3.89mm and a sucker ratio of 1.16:1 – 1.63:1, much greater than that expected for *P. muris* (Tanabe, 1922).

As discussed nonetheless, the use of morphoanatomic measurements to delineate digenean species has often been considered a questionable approach (Lal, 1935a; Simon-Vicente *et al*., 1985a). Experimental evidence from a PhD study conducted by A. M. Gorman in 1980 identified the extent of intraspecific variation within a pure strain of *P. elegans* used to experimentally infect a range of animals including gerbils, hamsters, rats, pigeons, ducklings and LACA mice. The study identified various manifestations in several anatomical structures not only between the different definitive host species but furthermore within the same definitive host species. Manifestations included differences

in the extent of the vitellaria, aperture shape of both the oral and ventral suckers, the posterior extent of the caeca and uterus, outline of the ovary and testes and finally variation in size, shape and position of the cirrus sac (Gorman, 1980). The key to *Plagiorchis* species found in bats created by Tkach *et al*. (2000a), distinguishes *P. elegans* from other bat infecting species by the oral sucker being distinctly larger than that of the ventral sucker in combination with the vitelline glands being confluent or almost confluent in the forebody of the worm. As pointed out by Blankespoor (1974) and Gorman (1980) however the use of the vitelline glands for diagnosis may be deemed redundant due to the intraspecific variation of this feature observed in *Plagiorchis* species. 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; Chae *et al*., 2010) or in other the reports, the level of the oral sucker (Fahmy and Rayski, 1963; Seo *et al*., 1964). In one study alone, the vitelline glands were observed to reach as far forward as the level of the oral sucker in a seven day old *P. muris*, but did not reach this position in some younger and older specimens (Hong *et al*., 1998).

Hong *et al*. (1996) described *P. muris* from a human case of plagiorchiasis. In this report, the vitelline glands reached the level of the pharynx and were used as a means to morphologically differentiate the three recovered specimens 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 infecting rodents.

Fortunately, in order to overcome the difficulty highlighted in the morphological identification of *P. muris*, the use of DNA sequencing could be employed during this study to aid in confirming the identity of the Malham Tarn specimens. The use of the internal transcribed spacer region and the 28S rDNA gene indicate that in fact the specimens at Malham Tarn may be *P. elegans* and not *P. muris* as originally specified (Rogan *et al*., 2007). The 28S rDNA sequence of *P. muris* used for comparison in this study was generated in Korea using Asian material (Lee *et al*., 2004). Only a 95% match between the Korean DNA sequence and the Malham Tarn specimens was achieved

however 100% match was observed between the Malham Tarn *Plagiorchis* and the DNA sequence generated for *P. elegan*s recovered from within the Ukraine (Tkach *et al*., 1999).

Based on these results and taking into consideration the unreliability of the morphological characteristics typically used to differentiate the two species in question (Blankespoor, 1974; Gorman, 1980; Hong *et al*., 1998; Tkach *et al*., 2000a), it could be speculated that similarly to the Malham Tarn specimens, other reports describing *P. muris* from outside of Asia are in fact different to that reported from Asian wildlife (Lee *et al*., 2004) and identifications based purely on morphology have led to the common misidentification of *P. elegans*. In particular, for McMullen (1937b) who recovered the adult specimens of *Plagiorchis* from a range of naturally infected avian fauna that are typical hosts for *P. elegans* and commented that the cercariae used for experimental infection possessed seven or eight pairs of penetration glands typical of *P. elegans* as opposed to the four pairs originally described for *P. muris* by Tanabe (1922).

Despite the questionable identity of *Plagiorchis* at Malham Tarn, the occurrence of this digenean on an annual basis at this location since 1993 is considered rare for the UK (Rogan *et al*., 2007). The overall prevalence rate of 23% recorded during this study appears to be much greater than what has been observed in the literature. Other UK reports involving *P. muris* and *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* recovered from *A. sylvaticus* in Ireland have furthermore indicated very low prevalence rates of 1% or less (Langley and Fairley, 1982; O'Sullivan *et al*., 1984). Further afield, the prevalence of *P. muris* appears to remain lower than that determined at Malham Tarn. 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 however, does appear to be the species reported most often from small mammals within Europe. These reports are nonetheless lower than the prevalence found at Malham Tarn. Hildebrand and Zaleśny (2009) reported a prevalence of just

1.3% in the bank vole *Myodes glareolus* trapped from Lower Silesia in Poland and a single specimen of *P. elegans* recovered from *M. glareolus* in Pallasjärvi, Finland gave a prevalence of just 0.5% (Tenora *et al*., 1983). A slightly higher prevalence of 3.1% was reported by Shimalov (2002) from *A. agrarius* trapped from the banks of drainage channels in Belorussian Polesie, located in the southern part of Belarus.

The reasons for the occurrence of *Plagiorchis* at such a high prevalence of 23% 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; Chae *et al*., 2010). Being the only upland marl lake of its kind in Britain (Rogan *et al*., 2007), it could be postulated that Malham Tarn itself may play an important role by providing important breeding sites for intermediate host species involved in the transmission of *Plagiorchis* at this location. Information regarding the second intermediate host for *Plagiorchis* appears to be limited with little being known regarding the life cycle of many species within the genus. The most commonly reported insects found to be naturally infected by *P. muris* in the Republic of Korea are dragonflies typically of the genera *Pantala*, *Calopteryx*, and *Sympetrum* (Hong *et al.,* 1999; Chae *et al.,* 2010). Malham Tarn is home to several species of dragonfly including species of the genera *Calopteryx*, and *Sympetrum* (Shorrock and Sutton, 2010). Attempts to identify the first and second intermediate host species involved in the transmission of *Plagiorchis* at Malham Tarn have been addressed in chapter 6.

In the present study, *Plagiorchis* was recorded from all four sampling sites, located on each shore of the tarn. Despite differences observed in prevalence, no significant difference between any of the sites was identified. Rogan *et al.* (2007) identified a cyclic alternation of high and low prevalence values of *Plagiorchis* every two years over a 13-year period. They determined a positive correlation between a high spring/summer rainfall and a higher *Plagiorchis* infection during the autumn. A very weak association between the prevalence of *Plagiorchis* and the amount of rainfall however was identified

when examining these factors quarterly during the present study, suggesting that temporary water bodies may not actually be an important factor involved in transmission. It could be speculated therefore that infection rates are related to the presence of intermediate host species that breed within the tarn body itself and are accessible to wood mouse populations inhabiting all woodland sites.

Each trapping site is separated from the tarn by a narrow shingle beach and earth ridge, and although this is terrain that wood mice may be capable of crossing, a crude morphological examination of the stomach contents of *A. sylvaticus* at Malham Tarn demonstrated the presence of adult insect debris. A statistically significant association was determined between the presence of this debris and *Plagiorchis* infection. This data could suggest that the main source of infection for *A. sylvaticus* stems from adult insects infected with *Plagiorchis* metacercariae migrating into the home range of the wood mouse following emergence from the water, as opposed to wood mice attempting to drink tarn water containing aquatic insect larvae. 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 *Plagiorchis* at Malham Tarn, demonstrated a typical pattern of over-dispersion with few hosts harbouring the majority of parasites. Over-dispersion in parasite assemblages are typical of wild animal populations and are often associated with intrinsic and extrinsic factors (Behnke *et al*., 1999). In the present study, rodent age group and sex were the two main intrinsic factors found to be associated with *Plagiorchis* prevalence.

Khammes and Aulagnier (2007) used three age categories to examine the differential diet of *A. sylvaticus*. The results indicated arthropod remains to be more abundant in the stomach contents of adult mice than that of younger animals. Similar findings were observed in the current study in which adult insect debris was observed to be most abundant in adult mice than younger adults and juveniles, a difference that was found to be statistically significant. In the present study, the prevalence of *Plagiorchis* was also 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 with adult

mice being more likely to consume insect material than younger cohorts (Khammes and Aulagnier, 2007).

As pointed out by Lewis (1987) however, the diet of rodents varies throughout the year in response to variation within the habitat, population density and season. 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; typically during the spring and early summer months (Lewis, 1987; Montgomery and Montgomery, 1990b). Insects are the second intermediate host for *Plagiorchis* (Hong *et al*., 1996; Hong *et al.,* 1999; Chae *et al.,* 2010) and it is likely that the insects eaten by *A. sylvaticus* at this time of year may contain the infective metacercarial stages of *Plagiorchis*. A study conducted by Montgomery and Montgomery (1990b) at Clandeboye Estate and Tollymore Forest Park in County Down, Northern Ireland, compared the stomach contents of *A. sylvaticus* at the two locations. Clandeboye Estate is mixed deciduous woodland and Tollymore Forest Park is predominantly coniferous with remnants of secondary deciduous woodland. Despite differences observed in diet during the study period, animal material was seen to rapidly decline in both sampling sites after September. The consumption of insect material by *A. sylvaticus* between spring and autumn coincides with the peak prevalence and mean intensity period for *Plagiorchis* at Malham Tarn.

Chai *et al*. (2007) furthermore found seasonal differences in a study conducted to examine the infection rate of *P. muris* in a natural population of *A. agrarius* in northern Gyeonggi-do Province, Korea. They demonstrated peak prevalence during the autumn and zero prevalence during March and concluded that the peak prevalence at this time was due to many species of insect larvae developing into adults giving wild animals much more opportunity to consume adult insects that may be infected with *P. muris*. They furthermore explained the lack of infection during the spring as a result of few insects surviving the cold winter months in the sampling area. The literature appears to suggest that the peak prevalence of *Plagiorchis* within any sampling site is dependent upon the developmental cycle of the intermediate hosts involved in transmission. This concept can be reinforced by a laboratory based study conducted by Hong *et al*. (1998) which suggested that the likelihood of finding adult digeneans within the intestine of the

definitive host from the previous season to be low, with up to 96% of *Plagiorchis* worms being expelled from the intestine of albino laboratory rats within 28 days post infection.

As previously mentioned host sex was furthermore found to be associated with the prevalence of *Plagiorchis* at Malham Tarn. Male mice carried both a higher prevalence and higher worm burden than their female counterparts. This difference in prevalence was found to be statistically significant. Previous studies however have not identified any significant differences in the diet between male and female *A. sylvaticus* (Watts, 1968; Montgomery and Montgomery, 1990b; Khammes and Aulagnier, 2007). Although in the present study, adult insect remains were detected in 25% of male mice in comparison to only 13% of female mice this difference was not found to be statistically different. It should be noted then again, that the examination of stomach contents can only provide an indication of the recent dietary habits of the animal and enable speculation as to the source of infection. It is not possible to identify at what point the animal became infected with *Plagiorchis* and as such differences in the stomach content at the time of capture may not necessarily be related to the source of infection.

It is likely however that the differences observed in prevalence between males and females and adult and younger mice may be due to variation in behaviour between the various groups. As stated by Poulin *et al*. (2011), the dispersion of the parasite is coupled with the spatial ecology of the host. 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). In particular, during the breeding season which is typically from April until October (Bueshing *et al*., 2007), male wood mice have been observed to increase their home range by as much as five times greater than their normal range during the non-breeding season (Corp *et al*., 1997). Male wood mice are furthermore much more arboreal than female wood mice. Bueshing *et al*. (2007) found that a significant 70% of male mice were captured from within trees as opposed to only 30% of females in a study carried out in Wytham Woods in Oxfordshire, UK. From the 70% of male mice captured, as many as 88% were classed as adults. Bueshing *et al*. (2007) considered the insectivorous nature of wood mice to be one reason for arboreality in particular, as insects and other small invertebrates often inhabit the tree canopy. Mice were furthermore caught in trees each season, including the breeding

period, corresponding with the times when animal matter appears to make up much more of the mouse diet than usual (Lewis, 1987; Montgomery and Montgomery, 1990b). This aspect of wood mouse ecology could contribute to an increased exposure to parasitic organisms such as *Plagiorchis* that utilise insect intermediate hosts as part of their life cycle. As pointed out by Bueshing *et al*. (2007) climbing as a means to acquire food that may be plentiful on the ground appears to be energetically expensive. Female wood mice have a greater dependency on resources than males and have been observed to establish mutually exclusive breeding territories (Flowerdew, 1993). For such reason, female mice may be less likely to become infected with *Plagiorchis* due to a reduced exposure to metacercarial infected insects than their male counterparts that appear more prone to wanderlust.

4.5 Summary

In conclusion, the identity of *Plagiorchis* at Malham Tarn may now be deemed uncertain. Molecular sequencing suggests that the species involved at this location may in fact be that of *P. elegans*, a typical avian species that has also been commonly reported in rodents within Europe. It could furthermore be speculated that due to the ambiguity observed in the morphological criteria used to distinguish *P. muris* and *P. elegans*, that misidentification of either species has commonly occurred. The present study indicates *Plagiorchis* to be the dominant intestinal digenean of wood mice at Malham Tarn and suggests that both host sex and age are the two most important factors for acquiring infection with male adult mice being the most commonly parasitized specimens. Furthermore, the significant water body of Malham Tarn may be one of the most important factors involved in maintaining the continual host-parasite system at this location, by providing important breeding sites for intermediate hosts involved in transmission. Attempts to identify the intermediate hosts involved in transmission at Malham Tarn have been addressed in chapter six. Only by pin-pointing the intermediate host species involved may we fully understand the ecology of this digenean and appreciate its high success rate for parasitizing the wood mice of Malham Tarn, the occurrence of which does not appear to be apparent in other wood mice populations studied within the UK (Lewis, 1968a; Lewis and Twigg, 1972; Behnke *et al.,* 1999; Abu-Madi, 2000).

CHAPTER 5:

Identification of a new species of *Notocotylus* recovered from the bank Vole (*Myodes glareolus*) and the field Vole (*Microtus agrestis*) at Malham Tarn, UK.

5.1 Introduction

Wild murid populations are commonly examined for their helminth fauna and comprehensive reference lists documenting the helminth parasites of the UK have been established by earlier workers (Nicoll, 1923; Baylis, 1928b; Baylis 1939). Studies involving UK rodents tend to indicate a commonality in the occurrence of several helminth species from spatially separated populations, with the most commonly reported digeneans from the wood mouse *Apodemus sylvaticus* tending to be *Corrigia vitta* and *Brachylaemus recurvum* (Elton *et al*., 1931; Behnke *et al.,* 1999; Abu-Madi *et al.,* 2000). In 2007 however, an occurrence of the intestinal trematode *Plagiorchis muris* was reported from *A. sylvaticus* at Malham Tarn Nature Reserve located in North Yorkshire UK, which had been previously recorded over a 13-year period (Rogan *et al*., 2007).

The occurrence of *P. muris* at Malham Tarn has led to further investigation into the prevalence and life cycle of this species. During the current study a different species of intestinal adult digenean was recognised as *Notocotylus*. Reports of digeneans from UK vole populations appear to be more limited however these *Notocotylus* specimens were found infecting the caecum of both the bank vole *Myodes glareolus* and the field vole *Microtus agrestis*. In the comprehensive parasite lists devised by Baylis in 1928b and 1939, *Notocotylus noyeri* was recorded from the water-vole *Arvicola amphibius* (syn. *A terrestris*) and the short tailed field vole *Microtus hirtus* (syn. *Mi. agrestis*) in Cambridgeshire, UK. All other reports of species from the genus *Notocotylus* in the UK have involved life cycles incorporating waterfowl as definitive hosts. This study is the first to report a *Notocotylus* species from *My. glareolus* in the UK, as far as can be established and this chapter aims to identify these adult digeneans to species level.

5.1.1 *Notocotylus* Diesing, 1839

The genus *Notocotylus* Diesing, 1839 is cosmopolitan, parasitizing waterfowl, and mammals with an affinity for water. The genus has a very complicated taxonomic history. It was originally created to accommodate *Festucaria pedata* Schrank, 1786,

Fasciola verrucosa Frolich, 1789, *Fasciola anseris* Gmelin, 1790, and *Monostomum verucosum* Levinsen, 1881 (Bhutta and Khan, 1975), all of which are now regarded to be synonyms of *Catatropis verrucosa* (Kanev *et al*., 1994). Diesing later made slight modifications to his diagnosis emphasising the presence of three longitudinal rows of ventral glands and attempted to rename the genus *Notocotyle* (Harrah, 1922). Renaming of the genus by Diesing however appears to have been rejected by most subsequent workers who have chosen to retain the name *Notocotylus* in their studies.

Upon erecting the genus, Diesing placed *Notocotylus triserialis* Diesing, 1839, a parasite of wild and domestic ducks as type species. In 1846 however, Creplin considered *N. triserialis* to be synonymous with *Monostomum attenuatum* Rudolphi, 1809 (syn. *Notocotylus attenuatus*), which was accepted by subsequent authors and was transferred into the genus *Notocotylus* and designated as type species of the notocotylids of ducks by Kossack in 1911 (Dubois, 1951). The original material of *M. attenuatum* that was examined by Kossack however was poorly preserved and as a result he did not re-describe its morphology in his justification. The type material of this species was later destroyed in world war, leaving only the original diagnosis by Rudolphi as a means to descriptive information (Dubois, 1951). Rudolphi however, was very brief in his description and did not describe the full figuration of the worm (Rudolphi, 1809; 1819). Dubois (1951) suggested that *M. attenuatum* should be regarded as a 'species inquirenda' due the fact that the identity of the species cannot be undoubtedly verified and in his contention proposed the return of the type species to *N. triserialis* Diesing, 1839.

In 1935, Lal (1935a) reviewed the criteria used for the specific diagnosis of members of the genus *Notocotylus.* He emphasised the importance of identifying the composition of the ventral surface of the digenean as a means for differentiation. This use of the ventral surface as a mean of diagnosing genera has been previously subject to much debate, with many previous authors questioning the validity of the approach. Members of the genus *Catatropis* also possess three rows of glands, two lateral rows and a median glandular ridge (Martin, 1956). The ridge can often be difficult to visualise which has led to mistakes in identification, resulting in species being placed into the wrong genera. In 1915, Barker identified a new notocotylid species, *C. filamentis* from the American

muskrat *Fiber zibethicus*, (syn. *Ondatra zibethicus*). Because the ventral surface comprised three rows of flattened non-protrusible glands, Barker placed the species into the genus *Catatropis*. As later pointed out by Herber (1955), there were errors in the work of Barker. This new species was referred to as *Catatropis fimbriata* within the text but the figure was labelled as *C. filamentis*. According to Herber (1955) these specimens were described from very poorly preserved specimens of *N. urbanensis*. *C. filamentis* has since been relocated into the genus *Notocotylus* and is currently known as *N. filamentis* (Gibson *et al*., 2005).

Additionally, Noble (1933) identified a new trematode species from the American coot *Fulica americana* for which he coined the name *Catatropis pacifera.* In his original description, he stated that the ventral glands were present in three longitudinal rows with the median glands appearing larger in size than those of the lateral rows. He emphasised that the presence of glands was difficult to demonstrate, the full complement only being visible in fully mature specimens. The glands were however described as non-evaginable, discrete in form and the median glands did not fuse into a continuous line of cells. He stated that there was no evidence of a ventral ridge, yet he placed the species into the genus *Catatropis* rather than *Notocotylus* without explanation. This species has since been relocated (Harwood, 1939) and is now known as *Notocotylus pacifera*.

More recently, Cribb (1991) suggested the relocation of *Catatropis gallinulae* Johnston, 1928 into the genus *Notocotylus*. The original description made by Johnston in 1928 described two lateral rows of glands but noted that the median ridge was not visual (Martin, 1956). With the use of a differential interference microscope however, Cribb (1991) was able to determine the presence of three rows of ventral papillae including a median row of discrete glands, suggesting in fact that this species belongs to the *Notocotylus* Diesing, 1839 group and for which he has proposed the name *Notocotylus elnaffari.* This may not be surprising since Noble (1933) stated that, "*C. pacifera* appears to be more closely related to *C. gallinulae* than to other described members of the genus".

To complicate matters further, some species of *Catatropis* are devoid of ventral glands, only possessing the mid-ventral ridge (Cribb, 1991). Harwood (1939) suggested the possibility that some species of *Catatropis* may have been mistakenly described as *Paramonostomum* since members of this genus lack both ventral glands and ridges. It is such scenarios created by the slight overlap in the criteria used for differentiation, why Baylis (1928a), Duthoit (1931) and Noble (1933) regarded the composition of the ventral surface to not be a thoroughly dependable basis for generic differentiation.

Lal (1935a) furthermore regarded the positioning of the genital pore to be an important characteristic for differentiation by which he proposed that the complex genus of *Notocotylus* be sub-divided into three distinct genera: *Naviforma* Lal, 1935; *Hindia* Lal, 1935 and *Notocotylus* Diesing, 1839. Species with a genital pore anterior to the intestinal bifurcation would be placed into the genus *Naviformia* Lal, 1935 with the type species *N. naviformes*. Species with a genital pore located at the intestinal bifurcation would belong to the genus *Hindia* Lal, 1935, with type species *N. gibbus* and finally species that would remain in the genus *Notocotylus* Diesing, 1839, with type species *N. attenuatus* would have a genital pore located post-bifurcal (Lal, 1935a).

Subsequently, in 1936, Szidat proposed that a new genus *Kossackia* should be used for *Notocotylus* species possessing three rows of non-protrusible and weakly developed glands (Nath and Pande, 1963). Harwood (1939) rejected all genera proposed by Lal and Szidat regarding all to be synonyms of *Notocotylus*, despite suggesting that a natural group was formed by *N. gibbus* Mehlis, 1846, *N. pacifera* Noble, 1933, and two of his newly described North American species *N. porzanae* Harwood, 1939 and *N. regis* Harwood, 1939. All have been recorded from the caeca of members of the family Rallidae and possess relatively short, oval bodies and ventral glands that are flat and non-protrusible (Beverley-Burton, 1961).

Dubois referred to the group suggested by Harwood (1939) as the ‘gibbus group’ and recommended that they be placed into the sub-genus *Hindia* Lal, 1935. According to the key of Dubois (1951) however, the sub-genus *Hindia* Lal, 1935 comprises species possessing only four to six ventral glands in the median row. *N. regis* Harwood, 1939 possesses 10 ventral glands in the median row and as such would not be eligible for the

group despite fitting all other criteria. On the contrary, *N. skrjabini* Ablasov, 1953 possesses a short, oval body and three rows of five ventral glands (Bisset, 1977). The ventral glands however are protrusible and as such this species is not eligible for inclusion into the sub-genus proposed by Dubois either.

The discussed examples demonstrate the complexity involved in attempting to divide the genus *Notocotylus* Diesing, 1839 into smaller sub-divisions. Nonetheless, Dubois (1951) attempted to promote the sub-genus *Hindia* Lal in his key to the notocotylids, which appears to have been disregarded by subsequent authors, such as Yamaguti (1958) who considered all previously suggested names as synonyms and retained the genus *Notocotylus* in his work.

In 1958, Beverley-Burton erected a new genus, *Uniserialis* to accommodate a new species *Uniserialis gippyensis* that she described from the intestinal caeca and bursa fabricii of three mallard ducks *Anas platyrhynchus* in Suffolk, England. The 49 specimens examined, possessed a single median row of five large ventral papillae. According to Bisset (1977), Baer and Joyeux (1961) synonymised *Uniserialis* with *Notocotylus*. This was done without provision of a valid argument for which Barton and Blair (2005) presumed was constituted by the arguments of earlier workers who did not regard the ventral surface to uphold a valid basis for differentiation.

Stunkard (1967) however reinstated the genus *Uniserialis* to include a newly described species *U. breviserialis* that was retrieved following the experimental infection of birds with cercariae isolated from *Hydrobia salsa* snails in Massachusetts, USA. *U. breviserialis* however possesses three rows of ventral glands, five in the median row and four in the two lateral rows. He pointed out that there were differences in the ventral surface composition but doubted the work of Beverley-Burton (1958), stating that the glands are rarely visible in fixed, stained specimens. Stunkard (1967) believed *U. breviserialis* to be congeneric with *U. gippyensis.* Rather than placing *breviserialis* into the genus *Notocotylus*, Stunkard (1967) attempted to differentiate the genera *Notocotylus* and *Uniserialis* by the site of host infection (bursa fabricii for both *U. gippyensis* and *U. breviserialis*) and by differences in the excretory system of the cercariae (Bisset, 1977).

Bisset (1977) described a new species *tadornae* from the New Zealand paradise shelduck *Tadorna variegata*. This species also possesses a single row of seven ventral glands however does not possess the pyriform body shape as does both *U. gippyensis* and *U. breviserialis* but rather has an elongated and parallel-sided body as occurs in other members of the genus *Notocotylus* (Bisset, 1977). Bisset did not place this species into the genus *Uniserialis* but instead named the species, *Notocotylus tadornae* despite the composition of the ventral surface. Bisset furthermore, recovered *U. gippyensis* Beverley-Burton, 1958 from the bursa fabricii and cloaca of three waterfowl species. He suggested that the intestinal caeca was not the usual site for infection as stated by Beverley-Burton (1958) and reported differences in the site of infection between this species and the newly described *N. tadornae*, which was almost exclusively a caecal-inhabitant. He emphasised the presence of large gaps in the knowledge of the notocotylids and as such claimed the basis for generic differentiation based purely on these criteria to be precarious agreeing with the opinion of Baer and Joyeux and placing both *N. tadornae* and *U. gippyensis* into the genus *Notocotylus*.

More recently, in agreement with the original work of Lühe (1909) who upon erection of the family Notocotylidae used the ventral surface composition as means of generic differentiation, the key of Barton and Blair (2005) has demonstrated the use of the ventral surface to be an appropriate means of dividing the family accordingly. In doing so *Uniserialis* is regarded to be a separate genus with *U. gippyensis* as type species as originally devised by Beverley-Burton in 1958 and containing *U. tadornae*. *Uniserialis breviserialis* has therefore been relocated into the genus *Notocotylus* and is now known as *N. breviserialis*. As such this concept has been adopted as a means of differentiation in this work and species of *Notocotylus* possessing three rows of discrete ventral glands only, both flattened and protrusible have been acknowledged as members of the *Notocotylus* Diesing, 1839 group (Table 5.1).

5.1.2 *Notocotylus* species

Misidentification of species within the genus *Notocotylus* has furthermore been commonplace and has often led to synonymisation of species and much confusion. These occurrences are primarily due to only small interspecific differences in morphology in

combination with the continued dispute over reliable diagnostic criteria (Nath and Pande, 1963). As a result many newly described species have been suppressed as synonyms and the systematics of the genus continuously reviewed (Lal, 1935a; Harwood, 1939; Dubois, 1951 and Stunkard, 1966; Simon-Vicente *et al*., 1985a). It is not surprising therefore that determining the number of valid species within the genus *Notocotylus* can be problematic, a fact that has been reflected in the variable number of species included in differential keys. Dubois (1951) recognised only 21 species in his key despite the fact that 32 species names had been listed prior to this date. Skrjabin (1953) recognised 24 species, Yamaguti (1958) 30 species from birds and Odening (1964, cited by Stunkard, 1966) included 26 species although as many as 42 species names had been proposed by this time.

More recent literature suggests that as many as 48 species parasitizing both birds and mammals may now belong to the genus (Kinsella and Tkach, 2005), however according to a recent literature search (valid until 11/08/11) as many as 68 different species-names coined by various authors between the years 1809 and 2011 have been identified, of which 12 species have been described from naturally infected rodents (Barker 1916; Lea *et al*., 1956; I-ping; 1965; Tenora *et al*., 1983; Simon-Vicente *et al*., 1985a; Cribb, 1991; Kinsella and Tkach, 2005; Chaisiri *et al*., 2011) (Table 5.1).

5.1.3 Synonymisation and Species inquirendae

There are several species of *Notocotylus* within the genus that are not considered to be valid, due to a lack of undisputable information and have therefore been regarded as 'species inquirendae'. For example, Rudolphi (1819) reported the digenean *Monostomum vespertilionis* (syn. *Notocotylus vespertilionis*) from the intestines of the bat *Vespertilionis lasiopteri*, now synonymised with the common noctule *Nyctalus noctula*. In his original work, Rudolphi (1819) did not provide a description of the species, a figure nor the exact information from where this species was discovered. *N. vespertilionis* is currently listed as a 'species inquirenda' by the Natural History Museum Host-Parasite database (NHM) and does not feature in any literature search other than for the retrieval of this amount of information.

Another example can be seen in the work of Szidat and Szidat who in 1933 isolated *Cercaria ephemera* Nitzch 1807, from the snail *Planorbis corneus* and reported its development into adult stage parasites. Breaching the rules of nomenclature they proposed a new species for the adult specimens; *Notocotylus thienemanni* (Stunkard, 1966), yet the NHM database maintains no records of this species and a Literature search yields very little information; Lal (1935a) indicates that the metraterm measures only 50% of the length of the cirrus sac, Ku (1937) describes a length up to 2.5mm and an ovary that is deeply lobed and Bhutta and Khan (1975) mentioned the presence of 12 glands in each of the three ventral rows. Today, *N. thienemanni* appears to be invalid and *N. ephemera* Nitzch, 1817 appears to be the name maintained by the NHM to describe species that have been isolated from *P. corneus* snails amongst others.

In 1963, Nath and Pande (1963) described a single specimen of *Notocotylus* that they isolated from the caecum of domestic fowl in India. They stated that the specimen appeared to be related to *N. attenuatus* but differed by several morphological characteristics. Firstly, the size of this specimen is much greater measuring up to 4.86mm in length by 1.57mm at the widest part in comparison to a maximum size of 2.96mm by 0.83mm (Beverley-Burton, 1961). Furthermore, the genital pore is positioned at the intestinal bifurcation as opposed to being post-bifurcal, there are 18 lateral glands in the ventral rows and additionally the range for the size of the eggs is slightly lower. It might be argued that the number of lateral glands or the size of the worm or its eggs may be variable traits. The positioning of the genital pore however, as shown by Lal (1935a) does stand as an important diagnostic criterion. Nath and Pande did emphasise that there was a need to review the type material of *N. attenuatus* but because the description was based on a single specimen they did not warrant its separation from this species.

Cribb, (1991) compared a newly described species *N. johnstoni* against the morphology of *N. nathipandei* Odening, 1964. The name *N. nathipandei* however does not return any literature and no records appear to be maintained in any database. It has been pointed out that *N. nathipandei,* coined by Odening in 1964 is a synonym of *N. attenuatus,* Nath and Pande (1963) (Anon, 2011).

Notocotylus duboisi was the name proposed by Stunkard (1966) to describe cercariae *imbricata* isolated from *Bithynia tentaculata*. This proposal came about because the same name was coined to describe two separately isolated cercariae: *C. imbricata* Looss 1893 from *B. tentaculata* in Germany and *C. imbricata* Looss 1896 from *Melania tuberculata* in Egypt. Understandably, two individual species within a genus cannot possess the same name. Dubois (1951) accepted the allocation of *C. imbricata* Looss 1893 as a validation of the 'species name' into the genus, as proposed by Szidat (1935) due to the fact that he used the combination *N. imbricatus* Looss, 1893.

According to Stunkard (1966) however the German version isolated from *B. tentaculata* was named only in a footnote and without figure or description and was therefore considered to be invalid. Stunkard only considered this name to be valid to describe the Egyptian specimens. Dubois (1951) however had already considered that *C. imbricata* Looss, 1896 was in fact the larval stage of *Notocotylus aegyptiacus*, a name already proposed by Odhner in 1905. *N. duboisi* therefore appears to be an invalid name returning no literature. According to the NHM database, references are maintained for *N. imbricatus* Looss, 1893 to describe the specimens isolated from *B. tentaculata* and *N. aegyptiacus* Looss, 1896 remains valid for species isolated from African waterfowl.

Finally, *N. ponticus* Tschiaberaschwili, 1964 was first reported following experimental studies by Tschiaberaschwili in Georgia. This new species was presented at the 16th scientific conference in Tbilisi, Georgia in 1964 (Tschiaberaschwili, 1964). There is very limited information available for this species and there appears to be much contradiction in the literature regarding its authenticity. *N. ponticus* does not appear on any zoological database including NHM. The only information available appears to be from Cribb (1991) who listed *N. ponticus* Tschiaberaschwili, 1964 and *N. zduni* Tschiaberaschwili & Dzhaveridze, 1968 as comparative species against *N. johnstoni*. In 1994, however Kanev *et al*. (1994) referred to *N. ponticus* Tschiaberaschwili, 1966 in their abstract whilst referencing the conference paper from 1964 (Tschiaberaschwili, 1964). This is confusing since it was Vassilev and Kanev (1984) who described the morphology and biology of *N. zduni* Tschiaberaschwili & Dzhaveridze, 1966 from Bulgaria which is now referred to as *N. zduni* Tschiaberaschwili and Dzhaveridze, 1968 by the NHM. In their 1994 paper, Kanev *et al*., describe the ventral surface of *N. ponticus*

as possessing 14-16 glands in the median row (most commonly 15) and 15-18 glands in the two lateral rows (most commonly 17). This information overlaps with their description of *N. zdun*i, 1966 in which the diagram indicates 15 glands in the median row and 17 glands in each of the lateral rows (Vassilev and Kanev, 1984). Although, it is possible the two species may possess the same number of ventral glands, contradiction of the dates by these authors may question the validity of two separate species. For such reasons *N. ponticus* Tschiaberaschwili, 1964 has been considered to be invalid as part of this study.

Table 5.1. A list of coined species names belonging to *Notocotylus* Diesing, 1839 in chronological order from 1809 until 2011 (n = 68). Key: (**ex**) = EXPERIMENTAL INFECTION.

Species	Founder	Year	Def. Host species	Location	Reference
N. attenuatus	Rudolphi	1809	Waterfowl	Cosmopolitan (**inc. UK**)	Baylis, 1928b; Eom and Rim, 1984; Beverley-Burton, 1961;
N. ephemera	Nitzsch	1817	Waterfowl/ unspecified mammal	Russia/ Bulgaria/ Lithuania/ Czechoslovakia	Gibson *et al*, 2005
N. linearis	Rudolphi	1819	Waterfowl	Poland/ Russia/USA/ Japan	Gibson *et al*, 2005
N. vespertilionis	Rudolphi	1819	Bat	Europe	Gibson *et al*, 2005
N. triserialis	Diesing	1839	Waterfowl	Cosmopolitan (**inc. UK**)	Pike, 1969
N. gibbus	Mehlis	1846	Waterfowl	**UK**	Baylis 1939; Beverley-Burton, 1961
N. imbricatus	Looss	1893	Waterfowl/ rodent	Belgium/**UK**/ Australia	Van Strydonck, 1965; Beverley-Burton, 1961; Cribb, 1991
N. aegyptiacus	Odhner	1905	Waterfowl	Egypt	Dubois, 1951
N. urbanesis	Cort	1914	Waterfowl	USA	Harrah, 1922
N. filamentis	Barker	1915	Rodent	USA	Barker, 1915
N. seineti	Fuhrmann	1919	Waterfowl/ Rodent	Ukraine/ China	Gibson *et al*., 2005; Fenglin 1993
N. noyeri	Joyeux	1922	Rodent	France /**UK**/Spain/Siberia	Joyeux, 1922; Baylis 1928b; Simon-Vicente *et al*., 1985a; Chechulin *et al*., 2010
N. chionis	Baylis	1928	Waterfowl	Patagonia	Baylis, 1928a; Dubois, 1951
N. tachyeretis	Duthoit	1931	Waterfowl	Patagonia	Duthoit, 1931
N. intestinalis	Tubangui	1932	Waterfowl	Philippines/ Russia	Tubangui, 1932; Besprozvannykh, 2010
N. naviformis	Tubangui	1932	Waterfowl	Philippines	Tubangui, 1932
N. pacifera	Noble	1933	Waterfowl	Cosmopolitan	Gibson *et al*., 2005
N. thienemanni	Szidat & Szidat	1933	Waterfowl	Europe	Stunkard, 1966
N. magniovatus	Yamaguti	1934	Waterfowl	Japan	Kamegai and Ichihara, 1973
N. parviovatus	Yamaguti	1934	Waterfowl	Japan	Kamegai and Ichihara, 1973
N. babai	Bhalerao	1935	Waterfowl	India/ Iran/ china	Bhalerao, 1935; Gibson *et al*., 2005; Fenglin, 1993
N. indicus	Lal (b)	1935	Waterfowl	India	Lal, 1935b
N. lucknowensis	Lal (a)	1935	Waterfowl	India	Lal, 1935a
N. ralli	Baylis	1936	Waterfowl	**UK**	Baylis 1936
N. anatis	Ku	1937	Waterfowl	China	Ku, 1937
N. orientalis	Ku	1937	Waterfowl	China	Ku, 1937
N. dafilae	Harwood	1939	Waterfowl	USA/ Russia	Harwood, 1939; Gibson *et al*., 2005
N. micropalmae	Harwood	1939	Waterfowl	USA	Harwood, 1939
N. regis	Harwood	1939	Waterfowl	USA	Harwood, 1939
N. porzanae	Harwood	1939	Waterfowl	USA	Harwood, 1939; Cooper, 1974
N. stagnicolae	Herber	1942	Waterfowl	USA	Herber, 1942
N. neyrai	Gonzalez-Castro	1945	Waterfowl	Spain	Simon-Vicente *et al*., 1985b
N. skrjabini	Ablasov	1953	Waterfowl	Russia/ Ukraine/ Armenia	Gibson *et al*., 2005
N. lopezneyrai	Dubois & Perez Vigueras	1953	Waterfowl	Cuba	Dubois & Vigueras, 1953
N. mamii	Hsu	1954	Rabbit (**ex**)	China	Hsu, 1954

N. solitaria	Singh	1954	Waterfowl	India	Singh, 1954
N. ratti	Lea *et al.*	1956	Rodent	China	Lea *et al.*, 1956
N. marinus	Ginetsinskaya & Naumov	1958	Waterfowl/ rodent	Russia	Ginetsinskaya & Naumov, 1958; Melnichenko, 1961
N. minutus	Stunkard	1960	Waterfowl	USA	Stunkard, 1960
N. duboisianus	Odening	1964	Waterfowl	India	Cribb, 1991
N. nathipandei	Odening	1964	Waterfowl	India	Nath & Pande 1963
N. ponticus	Tschiaberaschwili	1964	-	-	Cribb, 1991
N. wetlugensis	Schaldybin	1965	Rodent	Russia	Simon-Vicente *et al.*, 1985a
N. kiangsuensis	Shun I-Ping	1965	Rodent	China	I-Ping, 1965
N. atlanticus	Stunkard	1966	Waterfowl	USA	Stunkard, 1966
N. duboisi	Stunkard	1966	Waterfowl	Europe	Stunkard, 1966
N. breviserialis	Stunkard	1967	Waterfowl	Brazil	Stunkard, 1967; Claudio *et al.*, 1995
N. zduni	Tschiaberaschwili & Dzhaveridze	1968	Unspecified Mammal	Bulgaria	Mutafova & Dobriyanov, 1990
N. barmerensis	Gupta	1970	Waterfowl	India	Gupta, 1970
N. panjnadensis	Bhutta & Khan	1975	Waterfowl	Pakistan	Bhutta & Khan, 1975
N. anseri	Gupta & Gupta	1976	Waterfowl	India	Gupta & Gupta, 1977
N. casarcai	Gupta & Jehan	1977	Waterfowl	India	Gupta & Jehan, 1977
N. kanpurensis	Gupta & Gupta	1977	Waterfowl	India	Gupta & Gupta, 1977
N. poecilorhynchai	Gupta & Jehan	1977	Waterfowl	India	Gupta & Jehan, 1977
N. schmidti	Brooks and Heard	1977	Waterfowl	USA	Brooks & Heard, 1977
N. gallinulae	El-Naffer & Khalifa	1983	Waterfowl	Egypt	El-Naffer & Khalifa, 1983
N. vinodae	Gupta & Singh	1983	Waterfowl	India	Gupta & Singh, 1983
N. mcdonaldi	Gupta & Singh	1983	Waterfowl	India	Gupta & Singh, 1983
N. guptai	Gupta & Singh	1983	Waterfowl	India	Gupta & Singh, 1983
N. ajgaini	Gupta & Singh	1983	Waterfowl	India	Gupta & Singh, 1983
N. gonzalezi	Simon-Vicente *et al.*	1985	Rodent	Finland/ Spain	Tenora *et al.*, 1983; Simon-Vicente *et al.*, 1985a
N. paithanensis	Deshmukh	1985	Waterfowl	-	Deshmukh, 1985
N. lianhuaensis	Qiongzhang	1988	Waterfowl	China	Qiongzhang, 1988
N. johnstoni	Cribb	1991	Rodent	Australia	Cribb, 1991
N . polylecithus	Qiongzhang	1992	Waterfowl	China	Qiongzhang, 1992
N. biomphalariae	Flores & Brugni	2005	Waterfowl	Argentina	Flores & Brugni, 2005
N. fosteri	Kinsella & Tkach	2005	Rodent	USA	Kinsella and Tkach, 2005
N. loeiensis	Chaisiri *et al.*	2011	Rodent	Thailand	Chaisiri *et al.*, 2011

5.1.4 Rodent species of *Notocotylus*

Lucet recovered some trematodes from the caecum of the European water vole *Arvicola amphibius* in Courtenay, France in 1899 (Simon-Vicente *et al.,* 1985a). Joyeux later described these specimens in 1922 as a new species and named them *Notocotylus noyeri*. *N. noyeri* has since been described from several European countries including Great Britain (Baylis, 1928b; Baylis 1939), Hungary (Matskási *et al*., 1992), Lithuania (Mazeika *et al*., 2003) and the Czech Republic (Tenora, 2003) and has been regarded as the principal *Notocotylus* parasite of rodents in Europe.

Notocotylus neyrai Gonzalez-Castro, 1945 was first identified from the caecum of the southern water vole *Arvicola sapidus* in Granada, Spain (Simon-Vicente *et al*., 1985a). Upon examination Gonzalez-Castro realised that these specimens were morphologically distinct from those of *N. noyeri* that had been described by Joyeux (1922) and as such defined a new species. Dubois (1951) later reviewed the type material of *N. noyeri* and concluded that *N. neyrai* was a synonym of *N. noyeri* and that Joyeux had been incomplete in his original description; for example he fails to describe the surface composition of the cirrus.

In 1960, Melnichenko described trematodes from the caecum of *Arvicola terrestris* (syn. *A. sapidus*) in the Ukraine (USSR) that he identified as *Notocotylus marinus* Ginetsinkaya and Naumov, 1958. According to Simon-Vicente *et al*. (1985a), Sharpilo (1973) synonymised these specimens with *N. noyeri*. This is surprising since *N. marinus* only measures up to 1.8mm in length in comparison to *N. noyeri* which measures from 2.9 to 4.2mm and has a cirrus sac that measures twice as long as the uterine coils, a feature not observed in *N. noyeri* (Ginetsinkaya and Naumov, 1958).

Shaldybin (1976) furthermore described a rodent species of *Notocotylus* from the caecum of *Myodes glareolus* in the Gorki Region of the USSR. These specimens were similar in form to *N. noyeri* possessing a long and rectilineal metraterm that extends to more than 50% of the cirrus sac length but differed by the presence of caecal diverticles. The cirrus of this species was furthermore armed with spines, similarly to that of *N. neyrai*. He therefore coined a new species, *Notocotylus wetlugensis*. According to Simon-Vicente *et al*. (1985a), Yamaguti (1971) accepted *N. wetlugensis* as a valid species

however once again Sharpilo (1973) synonymised it with *N. noyeri*, which was also accepted by Ryzhikov *et al*. in 1978. Tenora *et al.* (1983) have since considered that *N. wetlugensis* may in fact be a valid species, since the co-existence of these three features has not been displayed by any other described species within the genus. Simon-Vicente *et al*. (1985a) referred to *N. wetlugensis* as a 'species inquirenda' stating that a review of type material was necessary.

Tenora *et al*. (1983), recovered trematodes from the caecum of three rodent species; the tundra vole *Microtus oeconomus*, the field vole *Microtus agrestis* and the bank vole *Myodes glareolus* in Finland. This trematode species was very similar morphologically to that of other species infecting European rodents but differed in possessing an unarmed cirrus in combination with a short metraterm that extended only as much as 25-33% the length of the cirrus sac. Simon-Vicente *et al*. (1985a) later had the opportunity of examining these specimens and confirmed that they were in fact the same as other specimens found infecting *A. sapidus* and Cabrera's vole *Microtus cabrerae* in Spain.

Following an extensive study into the morphology of *Notocotylus* species infecting rodents in Europe, Simon-Vicente *et al*. (1985a) concluded that three different forms of *Notocotylus* could be identified and in doing so re-validated *N. neyrai* to species level. They established that *N. neyrai* could be differentiated from *N. noyeri* by the morphology of the cirrus and the metraterm. *N. neyrai* possesses a heavily spinulated cirrus whereas *N. noyeri* has a smooth and unarmed cirrus. Additionally, the metraterm of *N. neyrai* is short forming only a final uterine funnel resulting in the presence of several convoluted uterine coils lying adjacent to the cirrus sac whereas that of *N. noyeri* is long and rectilineal extending to more than 50% the length of the cirrus sac. Additionally, they proposed the name *N. gonzalezi* to describe the specimens recovered from both Spain and Finland and which they designated as a third species, morphologically distinct from both *N. neyrai* and *N. noyeri* (Figure 5.1).

Due to the number of species previously synonymised with *N. noyeri*, it may now be questioned and as pointed out by Simon-Vicente *et al.* (1985a) as to what species of *Notocotylus* have been described by the many European authors during previous years. Only in the cases where a thorough species description is presented at the time of

publication will it be possible to decipher what species have actually been reported. Simon-Vicente *et al*. (1985a) analysed their morphological data against the information retrieved from a literature search. They found that only two reports of *N. noyeri* infecting rodents in Europe were deemed to be correct and in fact as many as nine reports had incorrectly diagnosed the species involved. In a personal letter from Odening to Mas Coma (1977), Odening confirmed that in two of his reports (Odening, 1964 and Odening and Bockhardt, 1965) he did not refer to the spinulation of the cirrus despite all of his specimens possessing a heavily spined cirrus (Simon-Vicente *et al*., 1985a). Simon-Vicente *et al*. (1985a) confirmed that in fact the species involved in all nine reports happened to be *N. neyrai* indicating *N. neyrai* to be the most frequently occurring *Notocotylus* species to infect European rodents. They concluded *N. gonzalezi* to be the second most occurring species although at a much lower rate and actually emphasised the rarity of *N. noyeri*.

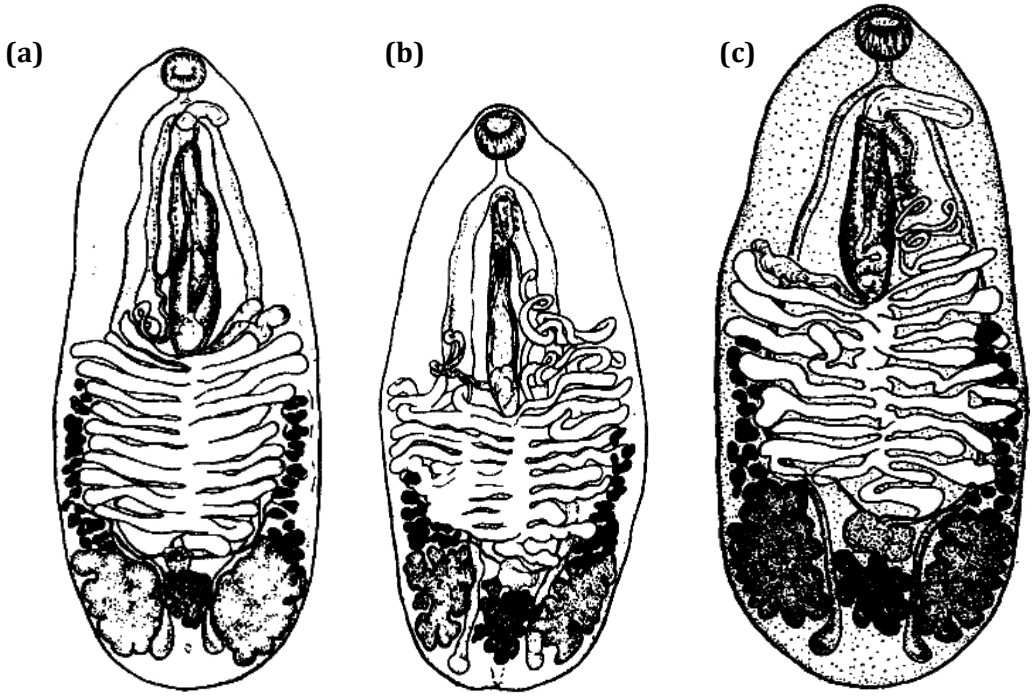


Figure 5.1 Morphological comparisons of the three valid *Notocotylus* species infecting rodents in Europe. Adapted from Simon-Vicente *et al*. (1985a). **(a)** *Notocotylus noyeri* Joyeux, 1922; **(b)** *Notocotylus neyrai* Gonzalez Castro, 1945; **(C)** *Notocotylus gonzalezi* Simon-Vicente *et al*. 1985. Differences in the size of the diagrams represent differences in mean length and width described by Simon-Vicente *et al.* (1985a), not to scale.

In 1919, Fuhrmann described a new species of *Notocotylus*, *N. seineti* from the caecum of the Garganey duck *Anas querquedula*. This species has been reported to infect the common eider *Somateria mollissima* as well as *Myodes glareolus* in the Ukraine (Gibson *et al*., 2005). According to the key of Dubois (1951), *N. seineti* differs from those species studied by Simon-Vicente *et al*. (1985a) by possessing a maximum of 12 ventral glands in the median row. Today, *N. seineti* is listed in the 'Fauna Europaea' database as a synonym of *N. imbricatus* (http://www.faunaeur.org/index.php). Furthermore, this species was not included in the review of, '*Notocotylus* species parasitizing rodents in Europe' written by Simon-Vicente *et al*. (1985a) and therefore currently appears to be invalid as a species and has been considered as a 'species inquirenda' in this study.

Synonymisation of *N. seineti* with *N. imbricatus* appears to be surprising since *N. imbricatus* possesses 16-17 lateral glands and 14-15 median glands on the ventral surface (Beverley-Burton, 1961). More recently, Cribb (1991) described *N. imbricatus* from the caecum and large intestine of the Australian water rat *Hydromys chrysogaster*. Despite morphological agreement however, Cribb concluded that the specimens described by him might be conspecific with *N. imbricatus* due to differences in both definitive and intermediate host specificity in the Tasmanian region, in addition to this being the first report of *N. imbricatus* infecting a mammal. Cribb furthermore described a new species of *Notocotylus* from this same rodent host, which he designated *Notocotylus johnstoni*. *N. johnstoni* is atypically found inhabiting the large intestine of its host. This species can be morphologically distinguished from European species by possessing a fewer number of ventral glands (10-13 lateral glands and 9-11 median glands) and by the pre-bifurcal positioning of the genital pore which is post-bifurcal in all European species (Simon-Vicente *et al*., 1985a).

The first report of a *Notocotylus* species infecting rodents outside of Europe was in 1915 when Barker described a new species, now known as *N. filamentis* from the duodenum of the American muskrat *Ondatra zibethicus*. *N. filamentis* differs from its European counterparts that infect rodents by having three rows of flat non-protrusible glands, a metraterm as long as the cirrus sac, cirrus covered with papillae and uterine coils that are confined to the intracaecal field. More recently another species, coined *Notocotylus fosteri* was described by Kinsella and Tkach (2005) from the rice rat *Oryzomys palustris*

in Florida, USA. This species also differs morphologically to the European species by having fewer number of ventral glands (10-13 in each row), and a genital pore that is located extremely pre-bifurcal so that it overlies the posterior margin of the ventral sucker.

Three species of *Notocotylus* infecting rats in the Far East have also been recognised; *N. ratti* from the black rat *Rattus alexandrines* in Shanghai, China (Lea *et al.,* 1956), *N. kiangsuensis* from the brown rat *Rattus norvegicus* in Kiangsu Province, China (I-ping, 1965) and *N. loeiensis* from the lesser rice field rat *Rattus losea* in Loei Province, Thailand (Chaisiri *et al*., 2011). All three species differ morphologically from the rodent infecting species of Europe, in particular by possessing fewer ventral glands. *N. ratti* possesses only 10-11 lateral glands and 5-6 median glands, *N. kiangsuensis* 10 lateral glands and 5 median glands and *N. loeiensis* has 9-11 glands in each of the three rows.

5.1.5 Morphological differentiation of *Notocotylus* species

Lal (1935a) considered the comparative size of the cirrus sac and metraterm, the arrangement of the uterine coils and the extent of vitellaria to remain constant across species. He emphasised that no reliance can be placed upon the size of the animal or its organs as fluctuations within narrow limits can occur due to fixation and pressure exerted during preparation of specimens. More recently, Simon-Vicente *et al*. (1985a) demonstrated the existence of four stable intraspecific characters across three *Notocotylus* species parasitizing rodents in Europe. These features included the cirrus and cirrus-sac, the metraterm, the uterine coils at the position of the cirrus sac level and the extent of the metratermic glands. In the same study, they considered morphoanatomic measurements as an important means of comparing species but did emphasise that differences in size may not be accepted as a valid argument due to variability and overlap in measurements between species.

Lal (1935a) also pointed out that variation in the actual number of ventral glands in each of the three rows could be regarded to be a constant trait of a species. Although the number of ventral papillae has been shown to differ according to the age of the animal (MacKinnon, 1982), the final number in each row does not appear to be affected by the size of the adult specimen. MacKinnon (1982) demonstrated *N. triserialis* Diesing, 1839

to possess a full complement of ventral glands at just five days post infection. Beverley-Burton (1961) provided an in-depth description of both *N. attenuatus* and *N. imbricatus*. She described fully mature specimens of *N. attenuatus* to measure up to 2.96mm in length and possess 14-15 median glands and 16-17 lateral glands. Despite *N. imbricatus* exceeding this length with measurements up to 4.73mm in the adult specimen, the number of ventral glands always remained the same in each row as for *N. attenuatus*.

Pike (1969) furthermore regarded the number of ventral glands to hold diagnostic value providing that numerous specimens are examined and both a range and an average number are verified. Pike (1969), reared *N. imbricatus* adults by experimentally infecting khaki campbell ducklings with cercariae collected from infected snails in South Wales. He acquired adult worms measuring up to 2.2mm. Even in specimens as small as 1.1mm he observed that the number of ventral glands within each row remained within the normal range expected for that particular species.

The use of several key taxonomic features when feasible for species identification is therefore advisable. Good observation of the ventral surface composition of mature animals in combination with other characteristics (Lal, 1935a; Simon-Vicente *et al*., 1985a) can provide credible data and be an important means of describing adult members of the Notocotylidae. This is the method that appears to be employed by current writers in the field who have recently described new species of *Notocotylus* (Cribb, 1991; Kinsella and Tkach, 2005; Chaisiri *et al*., 2011).

5.1.6 Aims

Despite an increase in the knowledge of the notocotylids over previous decades, difficulties in species identification is contributed to by a lack of understanding of the life cycles of many species, and furthermore to the fact that host specificity is not definite, as the same metacercariae has been shown to develop in various host species (Nath and Pande, 1963). The aim of this chapter is to identify to species-level, the *Notocotylus* specimens recovered from Malham Tarn using a classical morphological approach and to furthermore conduct DNA sequencing to provide a definitive tool for species identification in future studies of this kind. The specimens recovered from Tarn Fen will be referred to as *Notocotylus* malham herein.

5.2 Materials and methods

5.2.1 Rodent sampling

Rodents were trapped quarterly each season (January, April, July and end of September/October) from January 2010 to October 2011 from four sites surrounding Malham Tarn: Tarn Woods, Tarn Fen, Spiggot Hill and Ha Mire Plantation using methods described in section 2.1.3 and rodent were processed and dissected according to section 2.2.1

5.2.2 Faecal examination

Faecal pellets were isolated from the rectum of *N.* malham infected animals during dissection. Pellets were placed onto a microscope slide and homogenised in PBS solution. Eggs were observed using a Leica DM500 microscope and photographed using an attached Leica ICC50 digital camera. Egg measurements were recorded using an eyepiece graticule (Leica) that had been previously calibrated using a 1mm stage micrometer (Graticules Ltd, Kent, UK).

5.2.3 Examination, fixation and staining

Ten *N.* malham specimens recovered from the caecum of *My. glareolus* in September 2010 were relaxed in distilled water, fixed in 5% formalin and placed between two microscope slides to be flattened under light pressure. Specimens were examined, photographed and measured in micrometers as in 5.2.2 and drawings were made by tracing the photographs. These 10 specimens in addition to a further 10 specimens recovered from the caecum of *Microtus agrestis* were later stained with borax carmine and mounted in Canada balsam as in section 2.2.5. Morphology was cross-referenced with available published literature. Original species descriptions were used when available but this was not always feasible. In such cases second-hand information and differential keys were used for comparison. The morphology of the two different *N.* malham phenotypes collected from *My. glareolus* and *Mi. agrestis* were also cross-referenced with one another to ensure species identification.

5.2.4 Statistical analysis

The differences observed in morpho-anatomic measurements between the two phenotypes of *Notocotylus* malham recovered from *Myodes glareolus* and *Microtus*

agrestis were statistically analysed using a one-way parametric ANOVA with equal replicates (Holmes *et al*., 2006).

5.2.5 Molecular characterisation

DNA was extracted from individual *N.* malham animals using a standard phenol: chloroform method (2.2.6). DNA was extracted from three *N.* malham specimens recovered from the caecum of *My. glareolus* in September 2010. Both the ITS and the 28S rDNA gene were amplified and commercially sequenced according the methods in (2.2.8.1 and 2.2.9). Both DNA sequences were aligned against available data in NCBI**.** This procedure was conducted on three independent occasions (9 specimens in total). The ITS region of *N.* malham recovered from the caecum of *Mi. agrestis* was furthermore amplified and sequenced for comparison against the NCBI database in addition to those sequences generated from *My. glareolus*.

5.2.6 Phylogenetic analysis

Molecular phylogenetic analysis based on the partial 28S rDNA gene of *N.* malham was performed according to methods described in section 2.3.4. The 28S rDNA sequence of *N.* malham was analysed in relation to other closely related Notocotylids and *Plagiorchis* spp. whose sequences were available from the NCBI database in addition to the sequences of other digenean species found at Malham Tarn during this study (Table 2.2).

5.3 Results

Notocotylus malham was recovered from Tarn Fen only. In total 568 specimens of *Notocotylus* sp. were recovered from the caecum of *Myodes glareolus* and 13 from the caecum of *Microtus agrestis* trapped between July 2010 and October 2011, with prevalences of 66.7% (6/9) for *My. glareolus* and 50% (3/6) for *Mi. agrestis*. No infection was observed in either of these host species captured from Tarn Woods, Ha Mire Plantation or Spiggot Hill (0/48).

5.3.1 Morphology of *Myodes glareolus* specimens

The following description is based on 10 *Notocotylus* malham specimens collected from the caecum of *My. glareolus* at Tarn Fen in September 2010 (Figure 5.3).

5.3.1.1 External features

The body is monostomatous in form and dorso-ventrally flattened with lateral margins that fold dorsally to provide a curvature to the body and create a ventral concavity. The tegument is unspined. The anterior end of the body attenuates and is bluntly pointed in comparison to the posterior end, which is generally rotund. The body length ranges from 2.47 to 4.86mm (mean 4.04mm) and the maximum body width (midway across the uterus) from 1.17 to 1.53mm (mean 1.39mm). The ventral surface possesses three rows of protrusible glands, 42 - 47 in total, lateral rows consisting of 14 to 17 glands (mean 16) and a median row of 14 to 15 glands (mean 15). The first median gland is positioned half an interval behind the first lateral glands.

5.3.1.2 Alimentary canal

The dimension of the oral sucker ranges from 170 to 288μm in length (mean 234μm) by 180 to 300μm (mean 258μm) in width. The oral sucker opens into the oesophagus that measures between 150 to 170μm (mean 164μm) in length and bifurcates in front of the genital pore extending into very long blindly ending caeca. The caeca extend posteriorly underlying the uterine loops and curving in-between the laterally positioned testes and a single centrally located ovary. The caeca appear red in live specimens indicating possible blood feeding.

5.3.1.3 Reproductive system

The laterally positioned testes are large measuring between 650 and 800µm in length, lobed in form and extracaecal. The claviform cirrus sac extends from 840 to 1380µm in length with the proximal extremity positioned at 40 to 45% the length from the anterior. The length of the cirrus was difficult to measure appearing to be coiled in relaxed specimens when everted (Figure 5.2A). The width of the cirrus measured from 84 to 86µm in diameter at the distal end, and is densely spinulated over its entire surface.

The common genital pore lies behind the intestinal bifurcation. The uterus is transversely coiled between the base of the cirrus sac and the anterior border of the vitelline reservoir. There are 12 to 14 uterine loops that overflow the caecal field. Three to four uterine coils lie ahead of the vitelline glands. The anterior border of the vitelline glands is positioned at 48 to 50% from the anterior of the body length. Two lateral groups of 14 – 18 follicles extend to the posterior border of the uterus. All principal uterine loops are posterior to the base of the cirrus sac however secondary uterine loops can be observed on the lateral side of the cirrus sac, which adjoin the metraterm. The metraterm is long and rectilineal in form and measures from 67 to 82% the length of the cirrus sac. Metratermic glands can be observed along the full length of the metraterm. The egg measures 20µm × 10µm and bears two polar filaments, one on either side. The filaments range from 60µm to 180µm (n = 10), and were often seen to be unequal in length (Figure 5.2B).

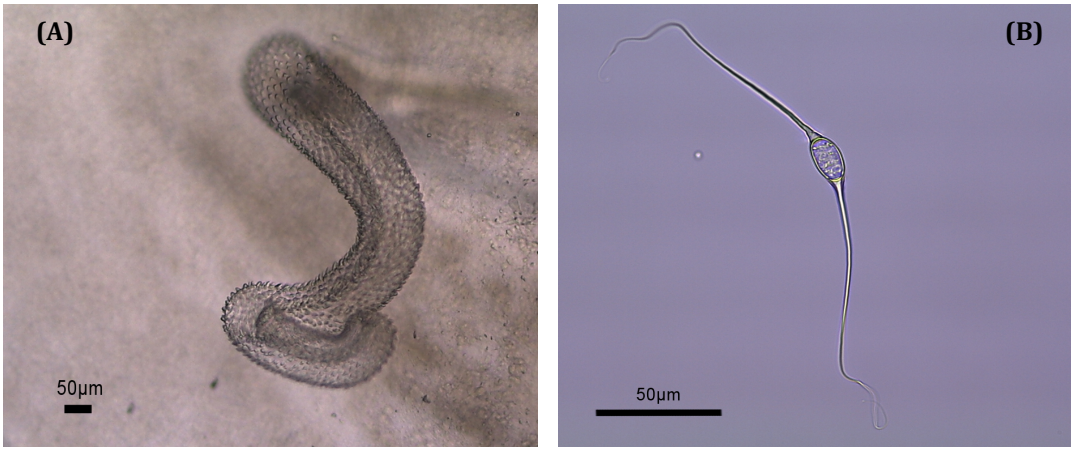


Figure 5.2 *Notocotylus* malham from Tarn Fen: **(A)** Spinulated cirrus × 10, **(B)** Eggs isolated from the faeces of *Myodes glareolus* ×40.

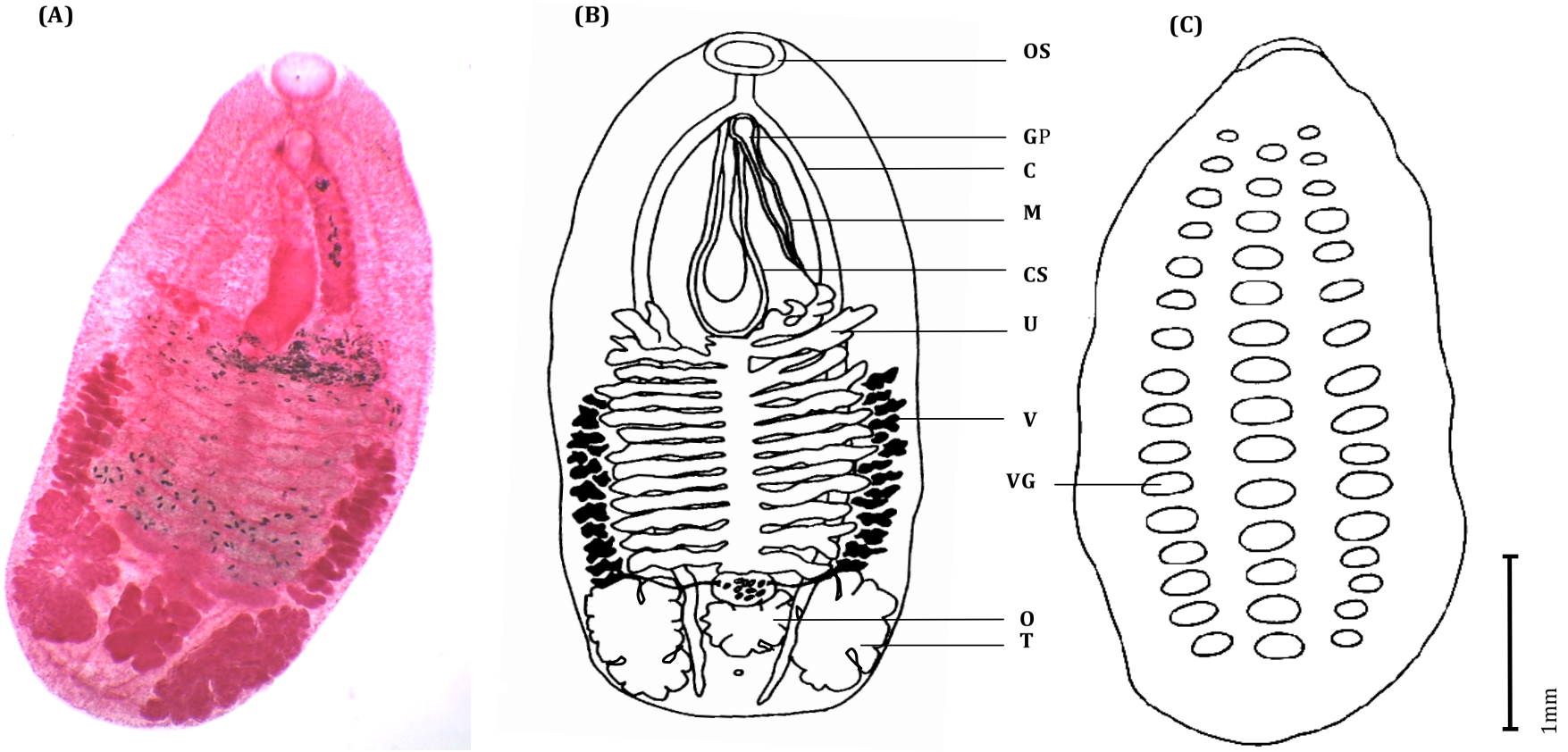


Figure 5.3 *Notocotylus* malham recovered from the caecum of *Myodes glareolus*. **(A)** Stained with Borax Carmine. **(B)** Dorsal view. **(C)** Ventral view. Abbreviations: **OS**, oral sucker; **GP**, genital pore; **C**, caecum; **M**, metraterm; **CS**, cirrus sac; **U**, uterus; **V**, vitelline glands; **VG**, ventral glands; **O**, ovary; **T**, testes.

5.3.2 Morphology of *Microtus agrestis* specimens

The following description is based on 9 *Notocotylus* specimens collected from the caecum of *Mi. agrestis* at Tarn Fen in October 2011 (Figure 5.4).

5.3.2.1 External features

The body of the *Microtus* specimens appears identical in form to those recovered from *My. glareolus* however these specimens appear to be more elongated with a body length ranging from 3.73 to 5.05mm (4.52mm) and a maximum body width (midway across the uterus) from 1.10 to 1.47mm (1.36mm). This elongation of the worms appears to create a more bluntly pointed posterior than seen in the *Myodes* specimens. The ventral surface composition was difficult to observe in several samples. Three protrusible rows of glands were however identified in three of the specimens which conformed to the pattern of glands observed in the *Myodes* worms, only differing by the last lateral glands extending slightly further towards the posterior and bypassing the last median gland. This arrangement appears to correspond with the elongation of the body.

5.3.2.2 Alimentary canal

The structure of the oral sucker is identical to that from the *Myodes* specimens. The oral sucker of the *Microtus* specimens however are slightly larger in width with a range of 210 to 300µm (286µm) in comparison to the length which is slightly shorter with a range of 170 to 230µm (213µm). The oesophagus is much more elongated in comparison ranging from 220 to 290µm (260µm) in length, bifurcating in front of the genital pore and extending into two blindly ending caeca which extend laterally curving in-between the laterally positioned testes and a single centrally located ovary. The caeca from this host furthermore appeared red in colour in live specimens.

5.3.2.3 Reproductive system

The lobed testes are extracaecal measuring between 700 and 900µm (800µm) in length. The claviform cirrus sac is much more elongated extending from 1050 to 1900µm (1610µm). The cirrus is densely spinulated as seen in the *Myodes* samples and appeared to coil when everted. The genital pore is located post bifurcal. The uterus is transversely coiled between the base of the cirrus sac and the anterior border of the vitelline reservoir. There are 12 to 14 primary uterine loops (most commonly 14) however the

majority of uterine loops are intracaecal with only a few loops slightly overlapping the caecal field. There is more space separating the uterine loops than seen in the *Myodes* specimens that appear more tightly packed. Three to four uterine coils lie ahead of the vitelline glands although in one specimen the vitelline glands extended as far as the anterior border of the uterine coils. Two lateral groups of 14 – 18 follicles lie outside of the caeca and extend to the posterior border of the uterus. The most forward uterine loop adjoins the metraterm on the lateral side of the cirrus sac. The metraterm is long and rectilineal. Despite being more elongated in the *Microtus* specimens with a range of 770 to 1460µm (1165µm), the metraterm to cirrus sac ratio appears to remain stable measuring 64 to 79% the length of the cirrus sac.

Table 5.2 Morpho-anatomic measurements of *Notocotylus* malham collected from the caecum of *Myodes glareolus* at Tarn Fen in comparison to the specimens collected from the caecum of *Microtus agrestis*. Data indicates the mean measurement with ranges in parentheses.

Measurements (µm)	***My. glareolus*** **n = 10**	***Mi. agrestis*** **n = 9**
Body Length	4039 (2472 – 4860)	4524 (3730 – 5050)
Maximum body width	1387 (1176 – 1536)	1356 (1100 – 1470)
Oral sucker width	234 (170 – 288)	286 (210 – 380)
Oral sucker length	258 (180 – 300)	213 (170 – 230)
Oesophagus length	164 (150 – 170)	260 (220 – 290)
Metraterm	909 (630 – 1100)	1165 (770 – 1460)
Cirrus sac length	1170 (840 – 1380)	1610 (1050 – 1900)
Metraterm/cirrus sac ratio (%)	77 (67 - 82)	72 (64 – 79)
Maximum width of uterine coils	1067 (640 – 1260)	781 (460 – 880)

5.3.3 Statistical analysis

The difference in morphoanatomic measurements between the two variant specimens recovered from *My. glareolus* and *Mi. agrestis* were analysed statistically using a one-way parametric ANOVA with equal replicates. Despite size variation seen between the two host species, these measurements were not found to be significantly different ($p = 0.5233$, $df = 1$, $F_{calculated} = 0.41$).

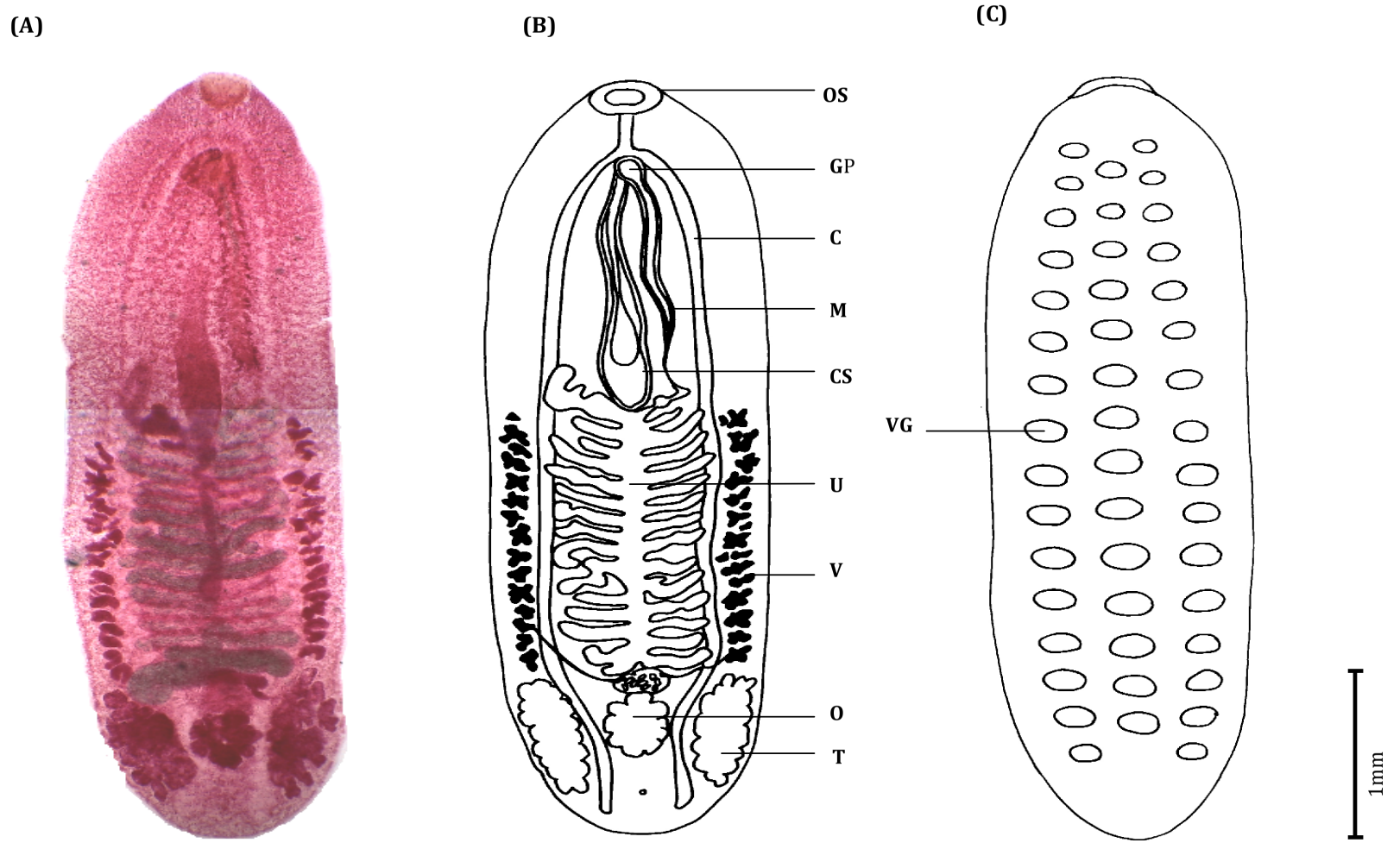


Figure 5.4 *Notocotylus* malham recovered from the caecum of *Microtus agrestis*. **(A)** Stained with borax carmine (merged photo) **(B)** Dorsal view. **(C)** Ventral view. Abbreviations: **OS**, oral sucker; **GP**, genital pore; **C**, caecum; **M**, metraterm; **CS**, cirrus sac; **U**, uterus; **V**, vitelline glands; **VG**, ventral glands; **O**, ovary; **T**, testes

5.3.4 Morphological comparison with literature

The morphology of *Notocotylus* malham was cross-referenced with morphological keys and original species descriptions from various literature sources. The criteria used for morphologically differentiating the Tarn Fen specimens from previously recognised species can be found in Appendix E.

5.3.5 Type specimens

45 specimens have been deposited at the Department of Zoology, Natural History Museum, Cromwell Road, London, UK (holotype NHMUK 2012.3.14.1, and paratypes NHMUK 2012.3.14.2-30 - 10 borax carmine stained specimens and 35 specimens stored in 70% molecular grade ethanol). A further 10 borax carmine stained specimens and 120 specimens stored in 70% molecular grade ethanol are held at The School of Environment and Life Science, The University of Salford, Salford Crescent, Manchester, UK.

5.3.6 Molecular Characterisation

The DNA of *Notocotylus* malham isolated from *My. glareolus* trapped at Tarn Fen was successfully amplified and sequenced. The following results were acquired following three independent PCR and sequencing reactions.

5.3.6.1 Ribosomal ITS region

A 1236bp fragment of the ribosomal ITS region was successfully amplified and sequenced (Figure 5.5). This appears to be the first ITS sequence generated from a *Notocotylus* species and cannot therefore be used for species comparison. This sequence however can be used as a definitive tool in future studies. The ITS region of a specimen recovered from *Mi. agrestis* was furthermore analysed and generated a 1231bp sequence for comparison. Sequences obtained from specimens recovered from the caecum of *My. glareolus* and *Mi. agrestis* were 100% identical (Figure 5.5). The ITS sequence of *Notocotylus* malham has been deposited into GenBank under accession number JQ766940.

5.3.6.2 28S rRNA gene

A 1268bp fragment of the 28S rRNA gene was successfully amplified and sequenced. The 28S rRNA sequence of *Notocotylus* malham was compared against *N. attenuatus* (AF184259) collected from an athyid duck in southern Ukraine (Tkach *et al*., 2001), *Notocotylus* sp. UK-PO-2003 (AY222219), based on sporocyst material from *Lymnaea* in the UK, and *Notocotylus* BH-2008 (EU712725) based on larval material from *Physa* from Nebraska, USA (Hanelt, 2009) (Figure 5.6). The sequence for *Notocotylus* malham appeared most closely related to *Notocotylus* BH-2008 sharing 99% sequence homology, followed by 98% with *Notocotylus* sp. UK-PO-2003 and only 96% with *N. attenuatus.* The Tarn Fen specimens cannot therefore be identified on the basis of DNA sequence data and must be identified using the classical method of morphological identification. The 28S rRNA sequence of *Notocotylus* malham has been deposited into GenBank under accession number JQ766939.

5.3.6.3 Phylogenetic analysis

Molecular phylogenetic analysis based on partial region of 28S rRNA was performed. Two main clades were determined using Phylip 3.69 (Figure 5.7). Members of the Plagiorchiidae were located into a separate clade from the Notocotylidae in which *Notocotylus* malham was clearly placed. The Notocotylidae was divided into two distinct clades in which *Notocotylus attenuatus* appears to be more closely related to *Catatropis indicus* than to other members of the genus *Notocotylus*. *Notocotylus* cercariae isolated from *Lymnaea peregra* snails appeared to be most closely related to the genus *Notocotylus* than to *Catatropis* or *Paramonostomum* confirming identification of the larval stages (see chapter 6). This information should however be treated precariously as analysis is based on a single gene region. No other DNA sequences for the ITS region of members of the Notocotylidae were available for comparison therefore only the 28S rRNA gene was examined.

Figure 5.5 Comparison of the ITS gene sequence of adult *Notocotylus* malham isolated from the caecum of *My. glareolus* and *Mi. agrestis* collected from Tarn Fen. Black shading indicates regions of conserved homology between the two specimens.

Figure 5.6 Alignment of the 28S rRNA gene sequence of adult *Notocotylus* malham from the caecum of *My. glareolus* at Tarn Fen against other *Notocotylus* sequences acquired from the NCBI database using a GeneDoc alignment tool: Accession: Sp.BH EU712725.1; sp.UK AY222219.1; *N. attenuatus* AF184259.1. Black shading indicates regions of conserved homology between the four species.

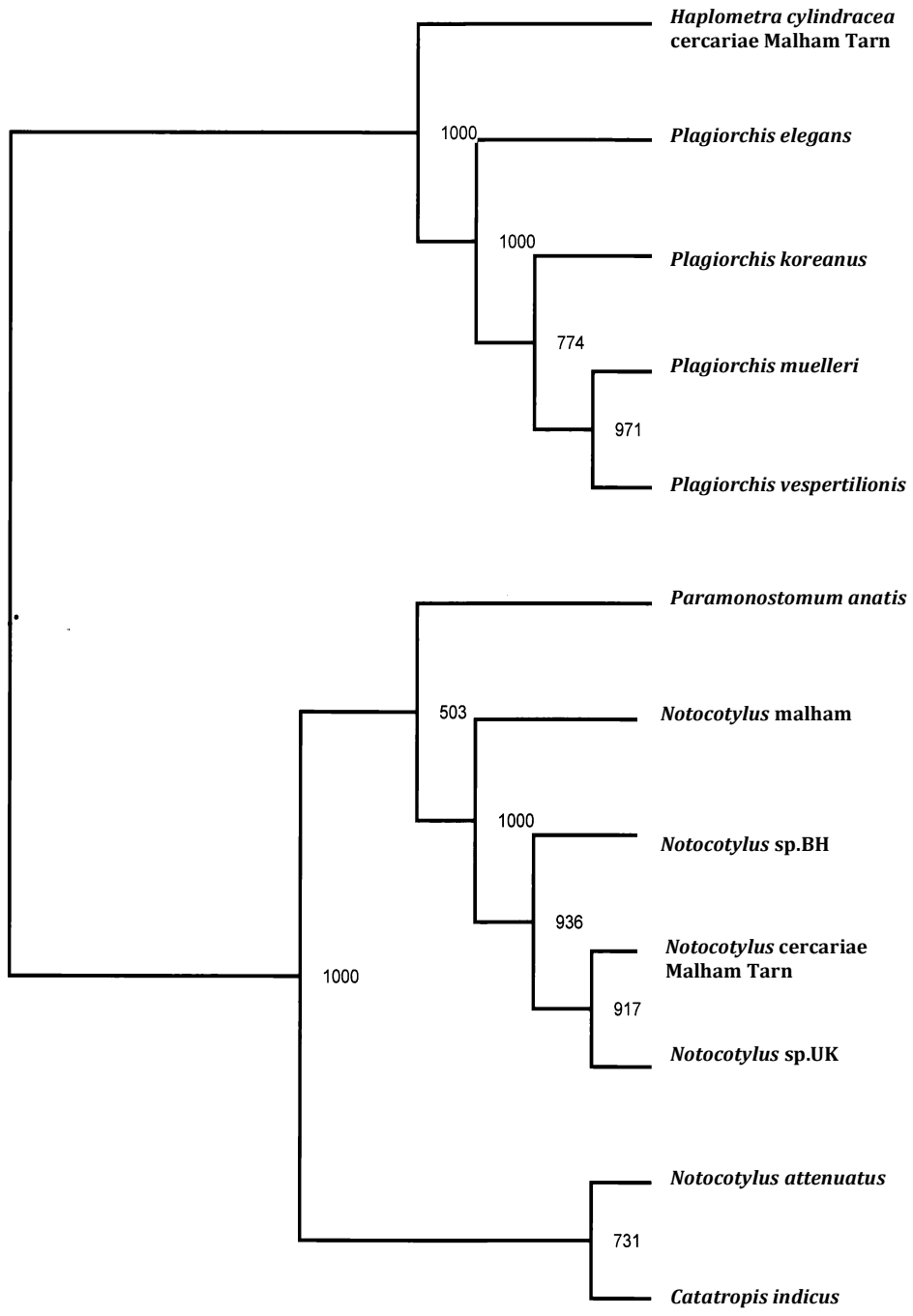


Figure 5.7 UPGMA rooted distance tree based on the 28S rRNA gene depicting the relationship between members of the Notocotylidae and the Plagiorchiidae. Figures to the right of each node represent percentage bootstrap support based on 1000 replicates (Phylip 3.69). NCBI accession numbers: *P. koreanus*: AF151944.1; *P. muelleri*: AF151947.1; *P. vespertilionis*: AF151949.1; *P. anatis*: AF184258; *N.* Sp.BH: EU712725.1: *N.* Sp.UK: AY222219.1; *N. attenuatus*: AF184259.1; *C. indicus*: AY222220.1.

5.4 Discussion

The specific identity of the adult *Notocotylus* specimens collected from Tarn Fen appears to be unknown. According to the literature only seven species of the genus *Notocotylus* Diesing, 1839 have been previously recorded in the UK. These include adult specimens of *N. attenuatus* Rudolphi, 1809 (Baylis, 1928b; Baylis 1939; Beverley-Burton, 1961), *N. noyeri* Joyeux, 1922 (Baylis, 1928b; Baylis 1939), *N. gibbus* Mehlis, 1846 (Baylis, 1939; Beverley-Burton, 1961), *N. ralli* Baylis, 1936 (Baylis, 1939) and *N. imbricatus* Looss, 1893 (Beverley-Burton, 1961). As pointed out by Simon-Vicente *et al*., (1985a) however the identity of *N. noyeri* may be doubtful and these reports from Baylis do not include an original morphological description for verification. Two species have furthermore been reported in their larval forms: *N. seineti* Fuhrmann, 1919 (Harper, 1929) and *N. triserialis* Diesing, 1839 (Pike, 1969) both of which were confirmed following an experimental infection of ducklings to retrieve adult specimens for examination.

The specimens were morphologically and molecularly confirmed as belonging to the genus *Notocotylus* and could be morphologically differentiated from all of the previously described UK descriptions using the criteria established by Lal (1935a), Dubois (1951) and Simon-Vicente *et al*. (1985a). *Notocotylus* malham appears most similar to *N. attenuatus* and *N. imbricatus* in the number of ventral glands with both species possessing 14 to 15 median glands and 16 to 17 lateral glands. The composition of the ventral glands of *N. imbricatus* is also similar with the first median gland being situated just half an interval behind the first lateral glands. This species can be differentiated however by possessing 17 to 20 uterine loops, all of which are confined to the intracaecal field and by also having a metraterm that is less than 50% the length of the cirrus sac (Beverley-Burton, 1961). For *N. attenuatus* the first median gland lies from one-half to one and a half intervals in front of the first lateral glands. This species overlaps in the number of uterine loops with the Tarn Fen specimens possessing 10 to 19 coils however these coils are also confined to the intracaecal space and the metraterm measures only a maximum of 70% of the length of the cirrus sac (Beverley-Burton, 1961).

The 28S rRNA gene sequence of *N. attenuatus* was furthermore available from NCBI for comparison for which only a 96% sequence homology with *N. malham* was achieved.

This result indicates the usefulness of DNA sequencing as an additional tool for use in conjunction with the classical approach to identification.

Notocotylus malham can furthermore be distinguished from *N. ralli*, which possesses 27 to 34 uterine loops on each side (Dubois, 1951) and *N. triserialis,* which although not discussed by Pike in the text, the figure presented indicates 19 uterine coils, in addition to a cirrus covered with large papillae and a metraterm that extends from 33 to 50% the length of the cirrus sac only (Pike, 1969). *N. gibbus* can be easily differentiated by the number and the composition of the ventral surface, possessing only 4 to 5 median glands and 11 lateral glands. *N. seineti* has been long considered a 'species inquirenda' however little data is still available for comparison. According to Lal (1935a) this species only measures up to 2mm in length. Furthermore, the key of Dubois (1951) indicates that this species only possesses a maximum of 12 ventral glands in the median row and 12 to 14 glands in the lateral rows and can therefore be ruled out on this basis.

The species for which *Notocotylus* malham appears to be most closely related is *N. noyeri,* which was one of the species reviewed by Simon-Vicente *et al.* (1985a). *Notocotylus* sp. bears resemblance to this species in the number and positioning of the uterine loops and the extent of the metraterm but differs not only in the number of ventral glands but also by the appearance of the cirrus. *N. noyeri* possesses 15 glands in each of the three ventral rows and also has a smooth, unarmed cirrus.

There appears to be 10 valid *Notocotylus* species infecting rodents worldwide including that of *N. noyeri*. *Notocotylus* malham however can be morphologically distinguished from all of them. *N. filamentis* Barker, 1915 has flattened and non-protrusible ventral glands, uterine coils confined intracaecal and a cirrus covered with papillae (Barker, 1915). *N. johnstoni* Cribb, 1991*, N. fosteri,* Tkach and Kinsella, 2005 and *N. loeiensis,* Chaisiri *et al.* 2011, all have a pre-bifurcal genital pore and both *N. ratti* Lea *et al*. 1956 and *N. kiangsuensis* I-Ping, 1965 can be distinguished on the basis of the ventral glands.

Notocotylus malham does appear to be most closely related to the other *Notocotylus* species known to infect rodents in Europe. All European species appear to bear much resemblance in the number and composition of ventral glands, the number and

positioning of the uterine coils and the positioning of the genital pore. *N. neyrai* possesses 12 to 15 median glands and 14 to 15 lateral glands. *N. gonzalezi* is identical to *N. noyeri*, possessing 15 glands in each of the three rows. All three species possess a post-bifurcal genital pore and an overlapping number of uterine coils that overflow the caecal field (Simon-Vicente *et al*., 1985a). *Notocotylus* malham can be distinguished from all three forms based on the number of ventral glands but bears great resemblance to all other features.

As shown by Simon-Vicente *et al*. (1985a) however the combination of the metraterm: cirrus sac ratio and cirrus composition can be used as a basis for differentiating the three species. These criteria can furthermore be used to distinguish *Notocotylus* malham from the three other European forms. *Notocotylus* malham is distinct from *N. gonzalezi* by both the metraterm: cirrus sac ratio and the composition of the cirrus surface. Although *Notocotylus* malham shares the structure and length of the metraterm with *N. noyeri*, it can be distinguished by the dense spinulation of the cirrus not present for *N. noyeri*. Finally, despite sharing the dense spinulation of the cirrus with *N. neyrai*, *Notocotylus* malham can be differentiated on the basis of the length and structure of the metraterm (Simon-Vicente *et al.*, 1985a).

It could be suggested that mixed infections by *N. noyeri* and *N. neyrai* occurs at Tarn Fen and that results represent an amalgamated description. Great care however has been taken in species identification and no mixed infection has been observed. All species examined from Tarn Fen exhibit the combined characteristics described in the results section.

Additionally, although *N. wetlugensis* Shaldybin, 1965 possesses the long and rectilineal metraterm and spinulation of the cirrus as seen in *Notocotylus* malham, this Russian species also possesses caecal diverticles which are absent in the Tarn Fen specimens.

Worldwide, there have been a further 46 reports of *Notocotylus* species infecting waterfowl and a further three species for which the definitive host remains obscure; *N. mamii* Hsu, 1954 was developed experimentally in a rabbit, *N. zduni* Tschiaberaschwili and Dzhaveridze, 1968 was developed in an unspecified mammal and according to

Kanev *et al*. (1994), *N. ponticus* Tschiaberaschwili, 1964 was described after experimental studies that had been conducted in Georgia. *Notocotylus* malham can be morphologically differentiated from all *Notocotylus* species for which a sufficient amount of descriptive information is available (Appendix E).

The 13 *Notocotylus* specimens collected at Malham Tarn in October 2011 were retrieved from the caecum of the field vole *Microtus agrestis*. All of these samples are morphologically comparable to the *Myodes* specimens when basing diagnosis on key taxonomic features. The *Microtus* specimens however are much more elongated in form with a greater range in length and smaller width. This elongation appears to have resulted in elongation of internal features as seen by the greater mean length of the oesophagus, cirrus sac and metraterm. Furthermore, when visible the last lateral glands appear to extend slightly further past the last median gland. This factor may also correspond with the elongation of the body. The greatest difference between the two specimen sets is the width of the uterine loops. In the *Myodes* specimens, the uterine loops consistently overflow the caecal field. In the *Microtus* specimens however the uterine loops are intracaecal only appearing to slightly overflow the caeca.

The extent of the uterine loops in the *Microtus* specimens may represent juvenile adults that are not yet fully developed. Kinsella (1971) demonstrated the growth and development of *Quinqueserialis quinqueserialis* a species closely related to *Notocotylus* in the Montane Vole *Microtus montanus*. At day 12 post infection the uterine coils of *Q. quinqueserialis* were confined within the caecal field. Only by day 15 post infection was overlapping of the caeca observed.

It is speculated that flooding of the Tarn Fen area is potentially important in terms of propagating the life cycle of *N. malham* and exposing the vole populations to the infective stage of the parasite such as metacercariae encysted on vegetation or other solid surfaces (see Chapter 3). According to Dave Hodgson, an independent researcher to the area, the last bout of flooding took place on the 13th October 2011 at which point the water level at Tarn Fen rose to 200mm above normal levels (personal communication). The *Microtus agrestis* samples were trapped on the 28th October 2011. If flooding is an important criterion for infection to occur then it might be speculated

that the estimated age of the *Notocotylus* samples recovered from *Microtus* would be less than 15 days old indicating that the uterine coils may not yet be fully developed.

The elongated appearance and increased range of internal measurements observed in the *Microtus* specimens may however represent their ability to grow to a much larger size in the *Microtus* host than in *Myodes*. In his study, Kinsella (1971) furthermore recorded the intraspecific variability of *Q. quinqueserialis* by experimentally comparing morphoanatomic measurements of the trematode from three rodent hosts: *M. montanus*, the meadow vole *Microtus pennsylvanicus* and the muskrat *Ondatra zibethicus*. He identified that adult specimens recovered from voles of the genus *Microtus* were consistently larger than those from *O. zibethicus* and concluded that morphoanatomic measurements were found to be host-dependent. This intraspecific variation observed in morphoanatomic measurements between the two host species at Malham Tarn reinforces the emphasis made by Lal (1935a), that no reliance can be placed upon the size of the worm or its internal organs for species differentiation and in fact key taxonomic features that remain constant across species should rather be used.

Several earlier researchers have questioned the causes of intraspecific variation in helminth parasites. In 1951, Read published an article discussing the 'crowding effect' in cestode parasites. It has since been recognised that the size of the worm decreases almost linearly with the number of worms present in a given infection. The effect of stunting on helminth parasites caused by crowding appears to be attributed by a combination of factors included intraspecific competition for resources and space (Bush and Lotz, 2000).

Only a maximum of six *Notocotylus* specimens were recovered from any given *Microtus* infection in October 2011, however as many as 294 worms were recovered from *Myodes* in a single host infection during the course of sampling. Studies by Lange and Staaland (1970) indicated the gross anatomy of the caecum of the two rodents to differ in size. The total length of the caecum of *Myodes glareolus* is only 13cm in comparison to 24cm for *Microtus agrestis*. This difference alone may contribute to 'crowding' during heavy infections. It is possible therefore that *Notocotylus* malham at Tarn Fen appear more stunted in the *Myodes* host due to adaptation in response to limitations in physical space.

5.5 Summary

In conclusion, infection of *Myodes glareolus* by *Notocotylus* malham at Tarn Fen appears to represent a new UK host record. Intraspecific variation observed in this digenean between the two rodent hosts *My. glareolus* and *Mi. agrestis* reinforces the unreliability of using only morpho-anatomic measurements as a means of species differentiation. Despite restrictions in the use of molecular differentiation during this study, the use of combining several key taxonomic features has proved useful for the identification of this digenean even from two different host species. *Notocotylus* malham should be identified on the basis of a long and rectilineal metraterm that extends from 64 to 82% the length of the cirrus sac or thereabouts in combination with a densely spinulated cirrus, a simple caeca and three rows of ventral glands possessing 14 to 17 lateral glands and 14 to 15 median glands. Based on the combination of these morphological characteristics it is considered that the *Notocotylus* specimens recovered from the two vole hosts at Tarn Fen represent a new species in the genus *Notocotylus* for which I propose the name *Notocotylus malhamensis.* Type specimens have been accepted by the Natural History Museum, London (holotype NHMUK 2012.3.14.1, and paratypes NHMUK 2012.3.14.2-30) and the description of the new species has been published in the refereed journal "Parasitology" (Boyce *et al*., 2012).

CHAPTER 6:

The use of molecular tools in the attempt to identify intermediate hosts involved in the transmission of digenean species at Malham Tarn, UK.

6.1 Introduction

The life cycle of digenean trematodes involves a highly complex series of transmission events that can involve up to three intermediate host species (Pearson, 1959; Möhl *et al*., 2009). The first intermediate host is usually a mollusc in which intramolluscan stage parasites must undergo obligatory development (Poulin and Cribb, 2002), however a limited number of exceptions have been identified in which the mollusc may be replaced by an annelid (Martin, 1952). In addition to the difficulties often encountered during the differentiation of adult digeneans, identification to species-level in the larval form can be extremely challenging for taxonomists in particular when differential characteristics are expressed by the adult morphology only (Kanev *et al*., 1994). It is very often the case therefore, that larval form parasites are identified to genus level only (Williams, 1966; Ghobadi and Farahnak, 2004). The study of larval digeneans typically encompasses the morphological examination of living parasites recovered either from naturally infected intermediate host species collected from the wild (Harper, 1929; Williams, 1966; Manga-Gonzalez, 1994; Hong *et al*., 1999; Ghobadi and Farahnak, 2004; Faltýnková *et al*., 2007) or from larval stages obtained following the experimental infection of laboratory reared intermediate hosts (McMullen, 1937b).

The use of DNA sequencing and molecular detection assays however are becoming more frequently employed as a means to determine the presence of and to identify digenean trematodes from within their intermediate hosts (Hamburger *et al*., 1998; Jousson *et al*., 1999; Nolan and Cribb, 2004). Although the use of morphological classification remains an important discipline for taxonomic resolution (Poulin and Leung, 2010), the use of molecular data provides the capacity to compare digenean species without having to consider confounding factors such as parasite age, intraspecific variability, host species and geographical location (Nolan and Cribb, 2005).

Three digenean species from small mammals have currently been recorded at Malham Tarn during the present study: *Brachylaemus recurvum*, *Plagiorchis elegans* and *Notocotylus malhamensis*. Typically species of the genus *Brachylaemus* demonstrate a triheteroxenous life cycle that typically encompasses two terrestrial molluscan hosts, the definitive host becoming infected by ingestion of an infected terrestrial snail (Gracenea and González-Moreno, 2002). The life cycle of both *Plagiorchis* and *Notocotylus* species however entails a freshwater system. *Plagiorchis* encompasses a triheteroxenous life cycle involving a freshwater snail as the first intermediate host (Manga-Gonzalez *et al*., 1994; Väyrynen *et al*., 2000; Ghobadi and Farahnak, 2004; Faltýnková *et al*., 2007) and an arthropod as the second intermediate host (Hong *et al*., 1999). Species of the genus *Notocotylus* however have a truncated life cycle (Poulin and Cribb, 2002) which involves only a single freshwater snail as the intermediate host (Williams, 1966; Manga-Gonzalez *et al*., 1994; Väyrynen *et al*., 2000; Morley *et al*., 2003). The life cycle of all three digenean species at Malham Tarn remain currently unknown. Only two specimens of *B. recurvum* were recovered during the present study. As this species possesses a terrestrial life cycle, no further investigation will be carried out. The aim of this chapter therefore is to attempt to identify the intermediate host species involved in the transmission of *P. elegans* and *N. malhamensis* at this location by the morphological and molecular examination of larval stage parasites recovered from freshwater snails and aquatic insect larvae collected from Malham Tarn.

6.1.1 Identification of larval digeneans

Digenean trematodes are small and soft-bodied and have been reported to possess few stable morphological characteristics, a problem that is exacerbated in larval stage parasites which posses even fewer morphological features than in their adult form (Moszczynska *et al*., 2009). The larval stages of parasites can furthermore be extremely challenging to work with. Cort (1914) stated that "Due to great mobility, small size and remarkable power of changing shape, some cercariae are very difficult objects to study". These difficulties can be exacerbated when more than one species within a genus utilises the same intermediate host and morphological similarity is vast. This situation was presented by Kanev *et al*. (2004) who identified that *Notocotylus imbricatus, N. parviovatus* and *N. ponticus* all use the same species of snail for their intermediate host

and in all cases the differential characteristics are expressed by the adult stage only (Kanev *et al*., 2004).

Further problems appear to stem from a lack of differential information in the current study of larval trematodes. For example, Faltýnková *et al.* (2007) created a dichotomous key for the identification of larval trematodes found parasitizing *L. stagnalis* across Europe. Within the key, monostomatous cercariae were listed as *Notocotylus attenuatus*. As seen in chapter 5 however there have been 63 taxa within this genus that appear to be valid, each of which is monostomatous in form and as such it cannot be specified that this particular cercarial stage is in fact *N. attenuatus*. Cort (1914) added that the identification of species based on larval morphology alone should in fact be considered suggestive and that many errors have occurred within the literature as a result of this method. Classical methods for identifying larval stages can only define a specific identity when either, adults are experimentally reared from the larval stages or the larvae are generated from eggs that have been recovered from an adult specimen (Cort, 1914). This statement has been reinforced by many subsequent researchers in the field such as Stunkard (1966) who verified that species identification of encysted metacercariae is extremely difficult if not impossible, and only by experimentally infecting a definitive host to generate adult parasites could identification be determined.

As a consequence of such difficulties encountered by traditional methods, the presence of cryptic species in recent years is becoming more apparent with the advent of molecular differentiation (Morgan and Blair, 1995). According to Nolan and Cribb, (2005) genetic variation provides the best insight into investigating inheritable traits and as such the application of DNA sequencing in co-operation with modern molecular techniques such as the polymerase chain reaction (PCR) (see 6.1.3), have now become commonplace in biological studies for the comparison of primary DNA sequences. In many instances the validity of the same or separate species is confirmed and furthermore the phylogenetic relationship between them is defined (Bartoli *et al*., 2000; Tkach *et al*., 2000a; Tkach *et al.,* 2000b; Olson *et al*., 2003; Lee *et al*., 2004; Moszczynska *et al*., 2009). Several gene families are commonly employed as a target for PCR amplification and DNA sequence comparison, for use in species differentiation and phylogenetic analysis of helminth parasites. The NADH dehydrogenase-1 and

cytochrome *c* oxidase I genes of mitochondrial DNA are commonly used (Ryu *et al*., 2000; Zhang *et al*., 2000; Lee *et al*., 2004; Vilas *et al*., 2005) however the most common gene target for trematode studies appears to be the ribosomal RNA gene (rDNA) (Ryu *et al*., 2000; Tkach *et al*., 2000a; Tkach *et al*., 2000b; Dzikowski and Levy, 2009).

6.1.2 Ribosomal DNA (rDNA)

The rRNA gene is a multigene family and as such there are hundreds of tandemly repeated copies located throughout the genome which creates a plenitude of template DNA for the detection and amplification of the gene by PCR (Nolan and Cribb, 2005). The rRNA gene has three coding regions and two non-coding regions. The coding regions transcribe the 18S, 5.8S and 28S subunits of ribonucleic acid (RNA) that are all essential structural components of the ribosomes. These regions of DNA have relatively slow evolutionary rates and as such are typically highly conserved (Nolan and Cribb, 2005). The two non-coding regions; the Internal Transcribed Spacer one (ITS1) and the Internal Transcribed Spacer two (ITS2) are flanked by the coding regions with ITS1 located between the 18S and 5.8S and the ITS2 between the 5.8S and 28S (Figure 6.1). The non-coding regions of DNA are more rapidly evolving (Nolan and Cribb, 2005). Over time these regions accumulate mutations that create interspecific variation, a factor that makes them ideal for the differentiation of congeneric species (Tkach *et al*., 2000a).

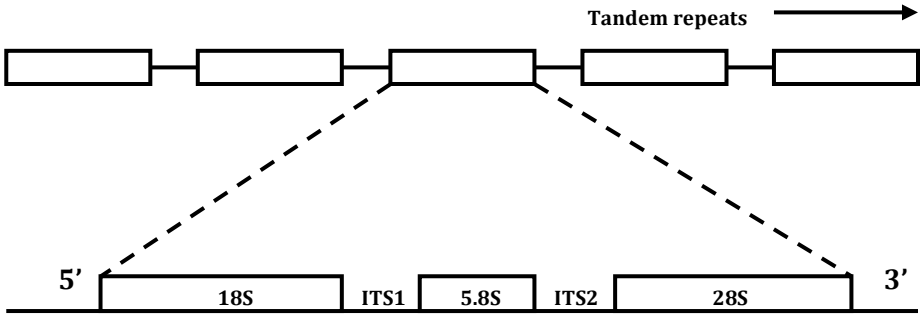


Figure 6.1. Structure of the ribosomal RNA gene (rRNA). The rRNA gene is tandemly repeated throughout the genome providing a vast amount of template DNA for detection by PCR. The non-coding regions; ITS1 and ITS2 used in primer design in the present study are flanked by the coding regions of the rRNA gene (Adapted from Nolan and Cribb, 2005).

The ITS region is often analysed as two individual components; ITS1 and ITS2 (Nolan and Cribb, 2005) although a limited number of studies have focused on the region as a whole (Morgan and Blair, 1995; Jousson *et al*., 1999; Tkach *et al.,* 2000a; Overstreet *et al*., 2002). Typically, the ITS1 region is considered to have a higher rate of interspecific variability than the ITS2 partly due to an array of tandem repeats located within the 5' end of the spacer (Nolan and Cribb, 2005). Nonetheless, the use of either the ITS1, ITS2 or the ITS region in its entirety, have all proven useful and been accepted as a means of analysis in systematic studies for indicating the presence or absence of interspecific and intraspecific variation by other researchers (Jousson *et al.,* 1999; Nolan and Cribb, 2004).

Species of the genus *Plagiorchis* can often be difficult to distinguish in their adult form (Bock, 1984) and furthermore demonstrate a lack of definitive host specificity contributing towards difficulties in species identification (Hong *et al*., 1996). Tkach *et al.,* (2000a), however analysed the sequence divergence between four members of the genus using the ITS regions (including the 5.8S ribosomal gene). Interspecific variation between the three bat species; *Plagiorchis vespertilionis, P. koreanus* and *P. muelleri* ranged from 2.9 to 12.8%. A greater divergence of 15.2% was observed between the three bat species and *P. elegans* typically found in birds. This study clearly defined the presence of distinct species despite morphological similarities.

Conversely, comparison of the ITS region has also highlighted identical sequences for putatively different species. In 1995, Morgan and Blair analysed the entire ITS region from three species of *Echinostoma*; *E. caproni, E. liei* and *E. sp*. II (African isolate) regarded by traditional methods of morphological identification to be distinct species. No interspecific variation was observed and as a consequence all three species were ultimately synonymised.

Intraspecific variability however is typically considered to be relatively stable within a species and studies comparing the ITS region of the same species obtained from different hosts and geographical locations have been successful in achieving 100% sequence homology (Cribb *et al*., 1998; Tkach *et al*., 2000a). Intraspecific variation has however been recognised in some digenean species although typically at low levels.

Sorensen *et al.* (1998) reported intraspecific variation according to geographical location. The rDNA sequence of three isolates of the North American *Echinostoma revolutum* were found to be identical, however this sequence differed from Eurasian isolates of the same species at nine bases of the 1006 bases aligned. Additionally, the rDNA sequences of two North American isolates of *Echinostoma trivolvis* were analysed in the same study. One of the isolates aligned identically with that of the already published sequence for the species however six nucleotide differences were recorded in the second isolate examined.

6.1.3 Polymerase Chain Reaction (PCR)

Since its development by Kary Mullis in 1985, PCR has revolutionised the world of molecular biology becoming central to several biological disciplines and as such has contributed towards a greater scientific understanding. In addition to its connection with DNA sequencing, PCR functions as a specific diagnostic application and has become common place in qualitative parasitological studies (Savva *et al*., 1990). The diagnostic function of PCR can be applied accordingly to the particular interest of the researcher and has frequently been used for generating prevalence data (Hughes *et al*., 2006), determining epidemiology (Marshall *et al.,* 2004; Williams *et al*., 2004) and furthermore in life cycle elucidation (Jousson *et al*., 1999; Bartoli *et al*., 2000; Nolan and Cribb, 2004).

For example, Nolan and Cribb (2004) utilised PCR and DNA sequencing to determine the intermediate host species involved in the transmission of *Paracardicoloides yamagutii* Martin, 1974, a digenean of the long-finned eel *Anguilla reinhardti* Steindachner, 1867, in Brisbane, Australia. They effectively sequenced and compared the ITS2 region of an adult *P. yamagutii* specimen and two putative sporocysts obtained from the hydrobiid gastropod, *Posticoba brazier* Smith. Alignment of the three sequences indicated one of the sporocysts to be identical to that of the adult trematode and as such the intermediate host used by this species was ultimately defined. This study was the first to determine the life history of this particular group using a molecular method.

Another study by Jousson *et al.* (1999) in Corsica, France incorporated similar methods to examine entire ITS regions to identify the developmental stages of digenean species belonging to the Opecoelidae family. The sequence of 16 adult trematode species was

compared with the sequence of 13 undescribed larval stages (9 cercariae and 4 metacercariae) isolated from various intermediate hosts. Nine of the 13 larval stages (6 cercariae and 3 metacercariae) were assigned to their corresponding adult forms identifying the relevant intermediate host species involved in transmission at that location.

Whatever the aim of the researcher, the basic methodology involved in the process of PCR remains consistent. PCR functions exponentially, to amplify a single copy of DNA by a factor of more than 10 million (Saiki *et al*., 1988). The process results in the acquisition of millions of copies of a fragment of an expected diagnostic size that can be visualised following agarose gel electrophoresis under a UV light source (Apte and Daniel, 2003).

Each cycle of PCR amplification involves three steps of DNA manipulation; denaturation of the double helix, hybridisation of primers and extension of the newly generated DNA strand. Initially, the DNA copy is heated to a temperature that induces denaturation of the double helix thus creating two single strands. During the subsequent step, the reaction temperature is lowered to enable two oligonucleotide primers; one forward and one reverse, to flank the region of target DNA that is to be amplified. An oligonucleotide primer is a single stranded sequence of DNA commercially synthesised to contain up to 30 nucleotide molecules and is designed to anneal specifically within the gene of interest. The forward primer, which is complementary to the sense strand (5'- 3') of the double helix, anneals to the 3' end of the antisense strand (3'- 5'). Conversely, the reverse primer is the reverse complement of the sense strand and as such anneals within the 3' end of this strand (Figure 6.2). The final step of PCR utilises a thermostable DNA polymerase (*Taq*) which is purified from the thermophilic bacterium *Thermus aquaticus. T. aquaticus* can withstand temperatures of up to 95°C and as such is ideal for use during PCR amplification in which prolonged temperatures as high as this are necessary for denaturation of the DNA helix (Saiki *et al.,* 1988). During this final step of the cycle, *Taq* polymerase extends the 3' end of the forward and reverse primers thereby synthesising a new complementary strand of DNA and thus creating two new double stranded copies of DNA.

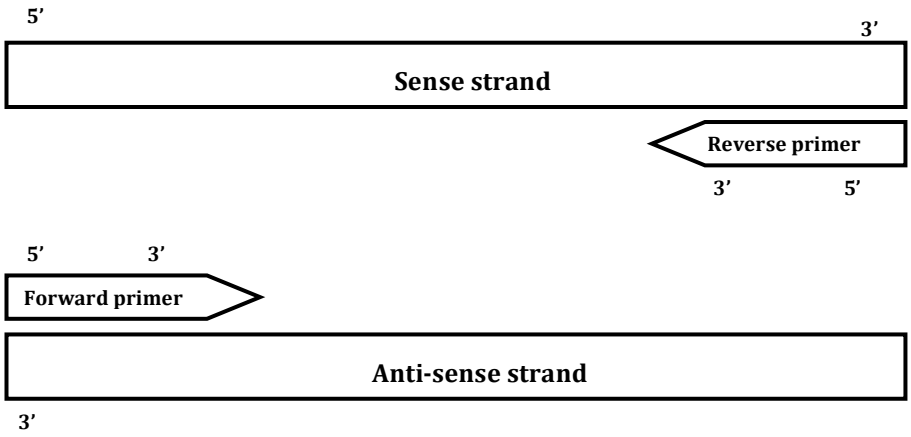


Figure 6.2 Primer annealing sites. The forward primer has a complementary sequence to the sense strand and as such anneals within the 3' end of the antisense strand (3' – 5'). The reverse primer which is the reverse complement of the sense strand anneals within the 3' end of this strand (5' – 3').

6.1.4 Aims

As far as can be determined, the use of molecular tools to aid in deciphering the life cycle of members of the genus *Plagiorchis* or *Notocotylus* have not yet been attempted. The aim of this chapter therefore is to attempt to use molecular identification methods to pin-point intermediate host species involved in transmission at Malham Tarn. Potential intermediate host species collected from accessible water bodies located throughout the tarn area will be examined for the larval stages of both *Plagiorchis* and *Notocotylus* and attempts will be made to link the specific identity between larval and adult stages of each digenean species using DNA sequence data. Attempts will also be made to design and optimise a robust molecular detection assay that can enable the presence or absence of larval staged parasites of each digenean within its host species to be determined.

6.2 Materials and Methods

6.2.1 Molecular design

Attempts were made to design primers that are capable of differentiating *P. elegans* and *N. malhamensis* from other digenean species including those that are known to be present at Malham Tarn: *Brachylaemus recurvum* and *Diplostomum spathaceum*.

6.2.1.1 Collection of information for the ITS gene region.

The National Center for Biotechnology Information (NCBI) database was screened for sequence data for the Internal Transcribed Spacer (ITS) gene for digenean species. According to NCBI, 2224 sequences based on the ITS regions of digenean trematodes have been deposited (search conducted 13.01.11) of which 52 different families were identified. From these 52 families, only 10 sequences for different *Plagiorchis* species had been deposited of which there was no information available for the ribosomal ITS region of *P. muris*. In order to design primers for diagnostic use, it was initially required to amplify and sequence this region (at the time of primer design the species considered to be at Malham Tarn was *P. muris*). This procedure was also carried out to generate the ITS sequences for *B. recurvum*, *D. spathaceum* and *N. malhamensis*. This was deemed necessary, as this information was also unavailable in the NCBI database and the DNA sequences for all four species are required for sequence differentiation during primer design.

6.2.1.2 DNA extraction and amplification

DNA was extracted from three individual specimens of each digenean species using a standard phenol: chloroform method as described in section 2.2.6 and the ITS gene region of each sample was amplified using primers described in 2.2.8.1. The ITS amplicon of each specimen was purified and commercially sequenced according to the methods described in section 2.2.9 and oligonucleotide primers specific to the ITS gene region of *Plagiorchis* and *Notocotylus* were designed according to the following methods.

6.2.1.3 Sequence analysis

The reverse complements of the generated reverse ITS sequences were obtained using a reverse complement program (www.bioinformatics/org/sms/rev_comp.html). The forward ITS sequences and the reverse complement ITS sequences were then aligned to

check for consensus using the multiple sequence alignment program ClustalW2 (http://www.genome.jp/tools/clustalw/). The sequences generated for each of the individual specimens were also aligned using ClustalW2 to compare results. Any inconsistencies were altered by examination of sequences displayed using Finch TV trace viewer (Geospiza, Seattle, WA). All DNA sequence data was presented using GeneDoc alignment tool (http://www.nrbsc.org/gfx/genedoc/)

The *Plagiorchis* sequence data was aligned with other available *Plagiorchis* species whose nucleotide sequences were obtained from the NCBI database (Accession: *P. maculosus* AF316152.1; *P. elegans* AF151952.1; *P. vespertilionis* AF151949.1; *P. muelleri* AF151947.1; *P. koreanus* AF151944.1) using Clustal W2 to identify regions of conserved homology. There were no ITS sequences available for other *Notocotylus* species to use in comparison. The generated ITS sequences were inputted into the Basic Local Alignment Search Tool (BLAST) for comparison against those sequences available in the database. All closely similar digenean sequences that were identified were aligned against *P. elegans, N. malhamensis*, *B. recurvem* and *D. spathaceum* using ClustalW2. From this information, sequence similarities were identified, and attempts to design two oligonucleotide primer sets for *Plagiorchis* (PlagF/PlagR) and *Notocotylus* (NotoF/NotoR) within regions of minimal sequence homology was carried out, according to the guidelines of Apte and Daniel (2003). Designated primer sequences were subjected to a primer blast search to compare against other available sequences within the database and to identify potential species that may incur a cross reaction during PCR.

6.2.1.4 Primer construction

The primer sequences were checked for hairpin formation using an oligo-calculator (www.basic.northwestern.edu/biotools/oligocalc.html). The melting temperature (T_m), Guanine/Cytosine (GC) content (%) and 3' stability (ΔG) were furthermore checked using the online Primer3Plus programme (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were commercially synthesised by Eurofins MWG Operon (Ebersberg, Germany). Primers were ordered in a lyophilised form and were re-suspended using molecular grade PCR water to a stock solution of 100pmol/µl.

Working aliquots of 10pmol/µl were generated using PCR water. Both stock primers and primer aliquots were stored at -20°C.

Primer sets were optimised to attain maximum specificity and sensitivity prior to diagnostic use. Primers were initially subjected to a temperature gradient reaction using a Robocycler Gradient 96 PCR machine (Stratagene, La Jolla, California, USA). Negative controls were included in all PCR reactions (PCR molecular grade water). Each primer set underwent a series of reactions set up in increments of two degrees Celsius across the thermoblock ranging from 52 to 72°C to identify the best annealing temperature for generating an optimal yield of PCR product. The optimum primer concentration per reaction mixture was furthermore selected prior to specificity testing. A primer panel set up in increments of 2.5pmol/µl ranging from 5pmol/µl to 12.5pmol/µl per reaction mixture was compared and the primer concentration that generated an optimum DNA yield was selected. It was not deemed necessary to optimise $MgCl_2$ concentration as the Dream *Taq* buffer used within the reaction mixture already contained a concentration of 2mM. This amount appeared adequate for generating an optimal amplification of product at the specified temperature and primer concentration in each instance.

6.2.1.5 Primer specificity

The specificity of the ITS primer sets were tested against a selected DNA panel. The DNA panel was generated using adult *P. elegans* and *B. recurvum* digeneans obtained from the small intestine of the wood mouse *Apodemus sylvaticus* and *N. malhamensis* taken from the caecum of the bank vole *Myodes glareolus* at Malham Tarn during the present study. DNA was also extracted from a *D. spathaceum* metacercariae (obtained from the eye lens of *Gasterosteus aculeatus* at Malham Tarn), and an adult *Dicrocoelium dendriticum* (provided by Professor Philip Craig). DNA extracted from the nematodes *Heligmosomoides polygyrus*, *Aoncotheca murissylvaticus* and *Syphacea stroma* (*A. sylvaticus*, Malham Tarn) was also included in the panel. In addition to parasite species, the DNA of *Lymnaea stagnalis* and *Lymnaea peregra* snails were included. Both snail species were obtained from the first generation of a laboratory bred colony (see 6.2.1.6) and are therefore regarded to be clean uninfected samples. DNA extracted from the larval stages of *Himasthla* sp. *Microphallus* sp. (both provided by an undergraduate final year project) and *Chaetognatha* sp. (obtained from wild *L. peregra* snails collected from

Malham Tarn) were also tested for cross-reactivity against each primer set. PCR amplification was tested using a Robocycler 96 (Stratagene, California, USA) according to the appropriate optimised protocol for each primer set. Specificity testing was repeated in triplicate.

6.2.1.6 Laboratory reared snails for DNA preparation

Two species of field collected snails: *Lymnaea stagnalis* and *Lymnaea peregra* were initially provided by Sciento (Manchester, UK). Snails were cleaned with a soft brush and laboratory prepared pond water prior to being housed in a 4 litre glass covered tank. Pond water was prepared from an 'instant pond water salt' mixture (Sciento, Manchester, UK). Snails were maintained at a temperature of 12°C and were fed washed lettuce *ad libitum* according to Voutilainen *et al*. (2009). Soil extract was prepared for addition to pond water to provide essential nutrients for optimum growth. Soil was placed into a one litre reagent bottle to approximately the 400ml mark to which 400ml of distilled water was added. The solution was autoclaved at 121°C for 30 minutes and left to settle for three days, following which 1ml of the solution was added to each tank for every 100ml of pond water based on guidelines provided by Sciento (Manchester, UK). Snail eggs were removed and placed into a separate tank and maintained under the same conditions as adult snails. Upon hatching, snails were fed an *Oscillatoria* culture according to Smith (1981) in addition to a mixed culture of diatoms and *Chlorella* to optimise growth. DNA was extracted from the first generation of laboratory-reared snails using a standard phenol: chloroform method (see 2.2.6) and was included in the DNA panel to test primer specificity (6.2.1.5) and sensitivity (6.2.1.7).

6.2.1.7 Primer sensitivity

The sensitivity of the two PCR assays was determined using a ten-fold serial dilution of parasite DNA to identify the minimum amplifiable concentration. The initial concentration of template DNA was determined by spectrophotometry (Jenway Genova, UK). Two DNA samples (*Plagiorchis* and *Notocotylus*) with equivalent DNA concentrations of 50ng/µl were selected. Parasite template DNA was diluted into pure snail DNA with a starting concentration of 250ng/µl obtained from *L. peregra* snails to simulate a typical testing scenario as would be expected to occur using field collected samples. A dilution series was produced with a concentration range of 50 to 5×10^{-8}ng/µl

and each dilution sample was subjected to PCR amplification using the appropriate optimised protocol for the primer set. Sensitivity determination was repeated in triplicate.

6.2.2 Invertebrate analysis

Invertebrate species were collected quarterly between January 2010 and October 2011 according to methods described in sections 2.1.1.5 and 2.1.4. The locations used for sampling have been indicated (Figure 6.3). Note that no invertebrate sampling was conducted on the southern margin of the tarn where the outflow stream is located as this area is private property and therefore inaccessible (reserve boundary has been indicated by white dashed line). Snails and insects were examined for parasite larval stages according to the methods in section 2.2.2 and 2.2.3. Primers designed in the present study were applied and tested on identified larval stages that were morphologically questioned to be *Plagiorchis* or *Notocotylus*. Amplified DNA fragments generated by use of either the Plag or Noto primer sets were excised, purified and sequenced according to section 2.2.9.

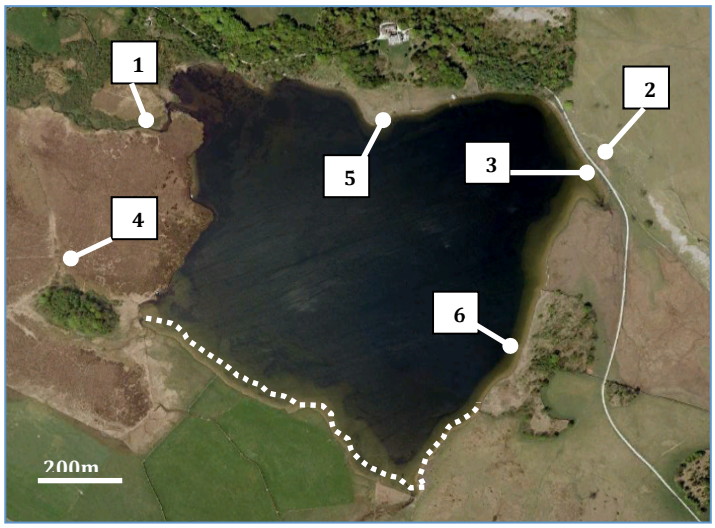


Figure 6.3 Invertebrate sampling sites used in the present study. **Key = 1**: The northwest inflow stream at Tarn Fen; **2:** Northeast inflow stream; **3:** Reed beds located on the northeast shore of the tarn; **4:** Spiggot Hill peat pools; **5:** Tarn Woods rocky shore; **6:** Ha Mire rocky shore. Image obtained from Google™ earth, 2012. The white dashed line indicates the reserve boundary.

6.2.2.1 Data analysis

Statistical analysis for prevalence rates of identified larval stages were analysed according to the methods described in section 2.3.3. The phylogenetic relationships of larval stage trematodes identified in the present study were examined against the 28S rDNA sequences of digenean species listed previously (Table 2.2) and using the methods described in section 2.3.4.

6.3 Results

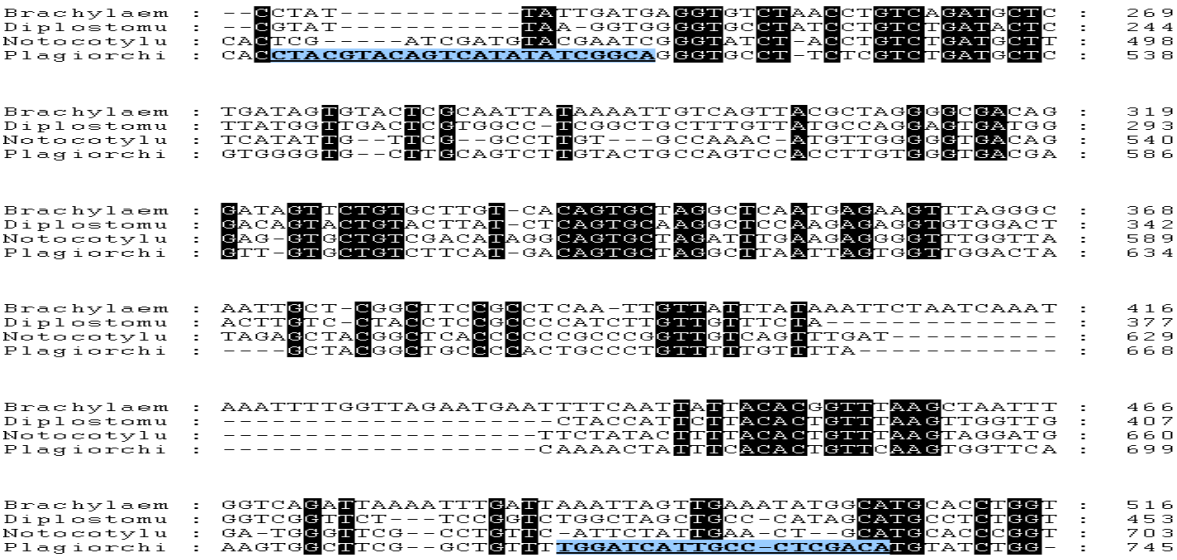
6.3.1 Primer design

The ITS gene region of all four digenean species recovered from Malham Tarn was successfully amplified and sequenced. The sequences were aligned and primers for *Plagiorchis elegans* (Figure 6.4A) and *Notocotylus malhamensis* (Figure 6.4B) were designed within regions of minimal sequence homology (Table 6.1). The ITS sequence for other *Notocotylus* species were unavailable from NCBI for comparison and as such this primer set (NotoF/NotoR) cannot be regarded to be species-specific at this time. Furthermore, the ITS sequence for *Plagiorchis* generated during this study shared more than 80% sequence homology with the other *Plagiorchis* species (100% with *P. elegans*, see chapter 4) located within the NCBI database. Consequently, difficulties were encountered in the design of species-specific primers and therefore currently this primer set (PlagF/PlagR) can also only be regarded to be genus specific at this time. No hairpin formation was detected for any of the primer sets using the online oligo-calculator and additionally primer-dimer formation was virtually non-existent during preliminary testing of all primers. Primer properties have been outlined in Table 6.1.

Table 6.1 *Plagiorchis* and *Notocotylus* primer sequences designed in the present study.

Primer name	Base pair (bp)	Primer sequence 5' – 3'	Primer properties		Product size (bp)
			Melting Temperature (°C)	GC content (%)	
PlagF	23	CTACGTACAGTCATATATCGGCA	53	43.5	**241**
PlagR	19	TGTCGAGGGCAATGATCCA	51	52.6	
NotoF	21	GCGGTATTCGTTACAACTGTG	52	47.6	**401**
NotoR	17	AGGCGAACCCATCCATC	49	58.8	

(A)



(B)

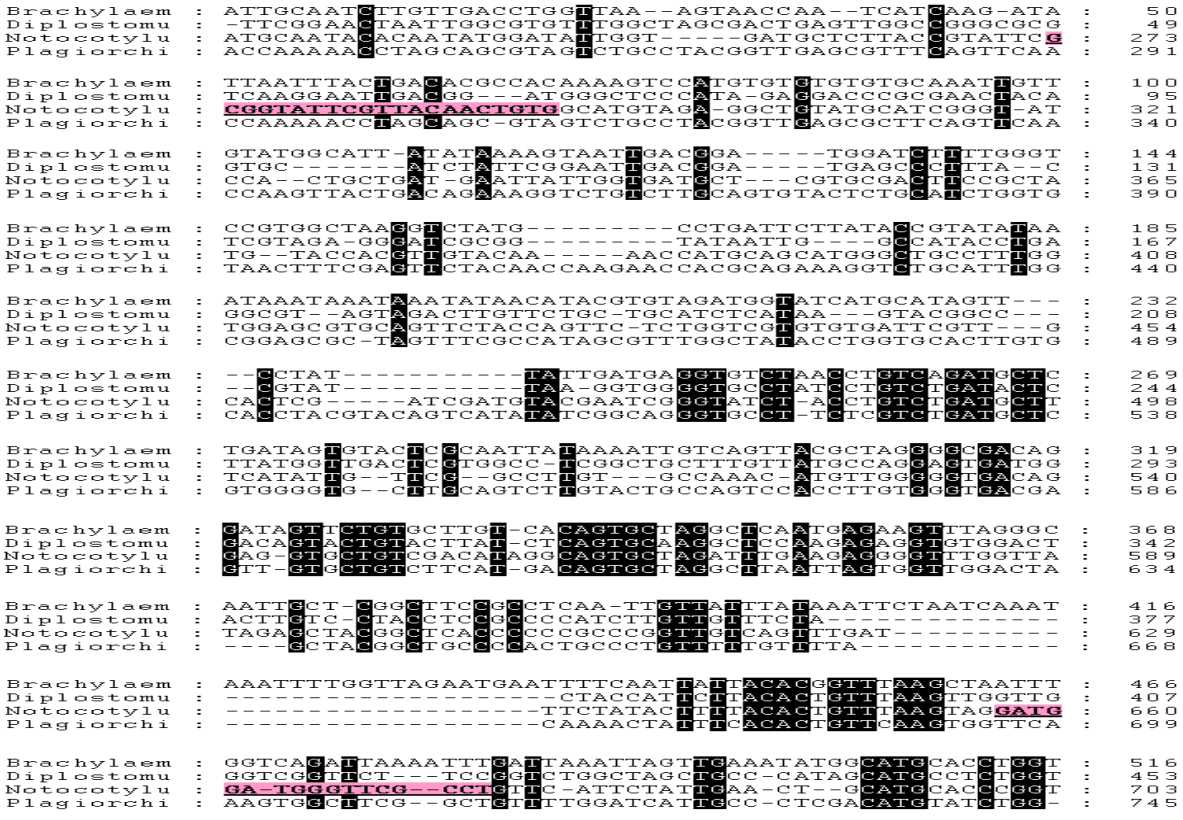


Figure 6.4 Sequence alignment of the ITS regions for all four digeneans collected from Malham wild life using GeneDoc Alignment tool. Black shading indicates regions of conservation between the four groups. **(A)** Annealing sites for the *Plagiorchis* primers highlighted in blue **(B)** *Notocotylus* primers highlighted in pink.

6.3.2 PCR optimisation

Preliminary testing of primers using standard concentrations and cycling conditions in accordance with Fermentas GmbH Life Sciences (Sankt Leon-Rot, Germany) general protocol (received with primers) produced a product of the expected size in all PCR reactions using DNA extracted from the appropriate parasite tissue. No PCR products were observed in the negative controls during any of the reactions. The temperature gradient PCR was successful in identifying the optimal annealing temperature for each primer set. The optimum annealing temperature was selected according to the highest temperature to consistently acquire the maximum yield of product. Additionally, a primer concentration gradient was performed and was successful in identifying the optimal primer concentration per reaction mixture. The finalised reaction conditions have been summarised (Table 6.2).

6.3.2.1 *Plagiorchis* primer optimisation

The optimum annealing temperature selected for the PlagF/PlagR primer set was 62°C. Above this temperature product yield substantially diminished and no yield was attained above 64°C (Figure 6.5a). The concentration of primer used in the reaction had a considerable effect on the yield of product. The maximum product yield was attained using 12.5pmol/µl per reaction mixture (Figure 6.5b).

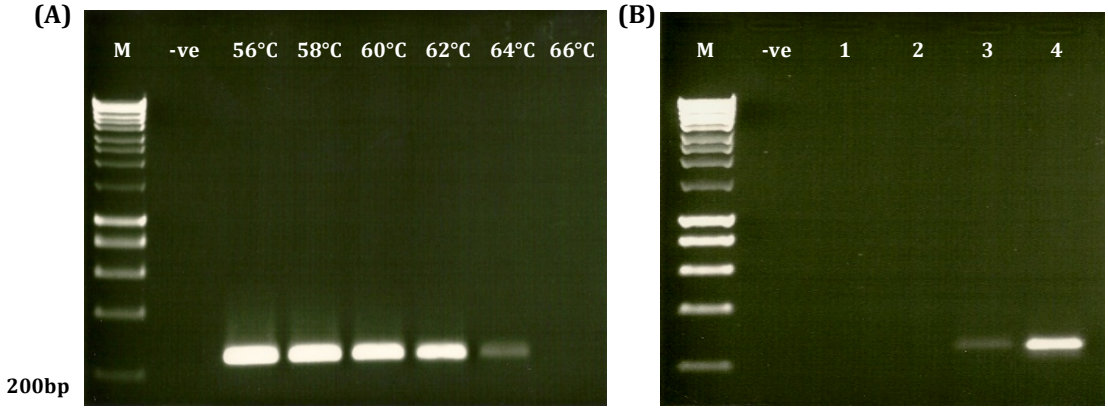


Figure 6.5 Amplification of a 241bp fragment from the ITS region of *Plagiorchis elegans* indicated on a 1% agarose (w/v) gel; **(A)** Temperature gradient from 56 to 66°C. **M**: Hyperladder 1; **-ve:** negative control (PCR grade water)**; (B)** Primer concentration gradient conducted at 62°C. **M**: Hyperladder 1; **-ve:** negative control (PCR grade water); **Lane 1**: 5pmol/µl; **Lane 2**: 7.5pmol/µl; **Lane 3**: 10pmol/µl; **Lane 4**: 12.5pmol/µl.

6.3.2.2 *Notocotylus* primer optimisation

The NotoF/NotoR primer set is extremely robust. Amplification was attained at temperatures ranging from 52 to 68°C above which there was zero yield (Figure 6.6A). The optimum annealing temperature selected for the NotoF/NotoR primer set therefore was 68°C. Additionally, amplification was evident at all primer concentrations although an optimal yield was attained at both 10pmol/µl and 12.5pmol/µl per reaction mixture (Figure 6.6B).

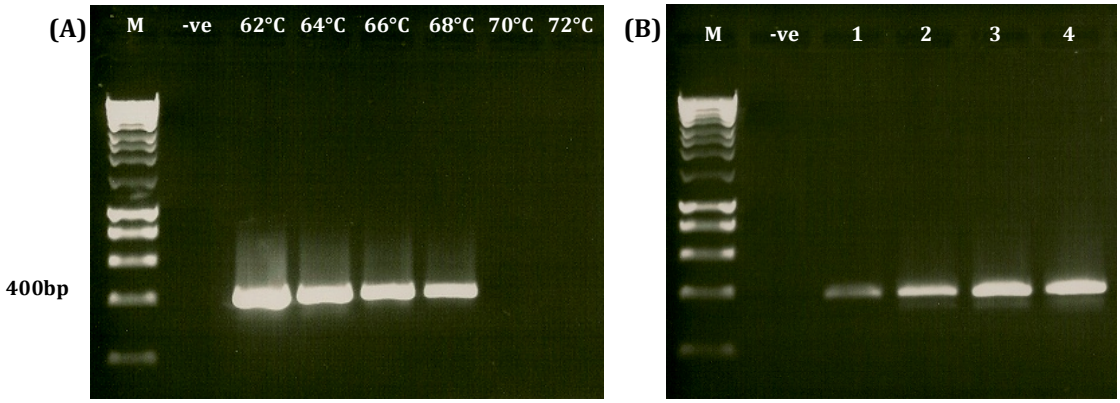


Figure 6.6 Amplification of a 401bp fragment from the ITS region of *Notocotylus malhamensis* indicated on a 1% agarose (w/v) gel; **(A)** Temperature gradient ranging from 62 to 72°C. **M**: Hyperladder 1; **-ve:** negative control (PCR grade water); **(B)** Primer concentration gradient conducted at 68°C. **M**: Hyperladder 1; **-ve:** negative control (PCR grade water); **Lane 1**: 5pmol/µl; **Lane 2**: 7.5pmol/µl; **Lane 3**: 10pmol/µl; **Lane 4**: 12.5pmol/µl.

6.3.2.3 Primer specificity

Each primer set was tested for specificity. No non-specific binding of primer to the DNA template was observed at the selected annealing temperatures. Furthermore, there was no cross reaction observed between the primers and any other parasite or intermediate host species included in the DNA panel (Figure 6.7A & 6.7B). Prior to testing with the Plag and Noto primers, the quality of intermediate host DNA was successfully tested for amplifiability according to Remigio and Blair (1997) (see 2.2.8.5). In each instance, the reaction yielded the expected size PCR product of 378 nucleotides. *Himasthla* sp. *Microphallus* sp. *Chaetognatha* sp. and *Syphacea oblevata* were tested at a later date when samples became available. No cross reactivity was observed between the primers and these four specimens. All reactions were conducted on three independent occasions.

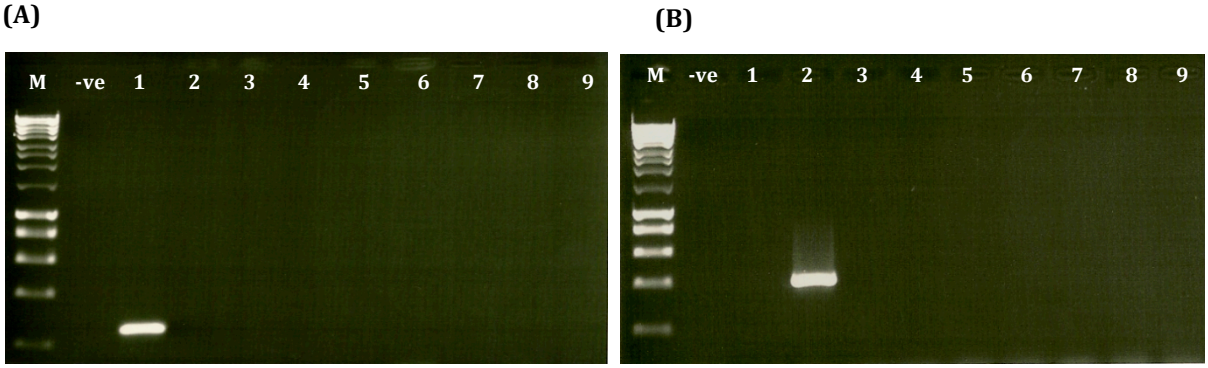


Figure 6.7 Primer specificity indicated on a 1% agarose (w/v) gel. **(A)** 241bp product of *Plagiorchis* at 62°C; **(B)** 401bp product of *Notocotylus* at 68°C. **M**: Hyperladder 1; **-ve:** negative control (PCR grade water); **Lane 1**: *Plagiorchis elegans*; **Lane 2**: *Notocotylus malhamensis*; **Lane 3**: *Brachylaemus recurvum*; **Lane 4**: *Diplostomum spathaceum*; **Lane 5**: *Dicrocoelium dendriticum*; **Lane 6**: *Aoncotheca* sp.; **Lane 7**: *Heligmosomoides polygyrus*; **Lane 8**: *Lymnaea stagnalis*; **Lane 9**: *Lymnaea peregra*.

6.3.2.4 Primer sensitivity

The most sensitive primer pair was NotoF/NotoR, which was able to detect *Notocotylus malhamensis* at a concentration of 5×10^{-3}ng/µl (Figure 6.8B). The PlagF/PlagR set were able to detect parasite DNA at a lowest concentration of 0.5ng/µl (Figure 6.8A). Cut off points have been included in the detection panel for comparison and all negative controls were clean following each reaction which was conducted on three independent occasions.

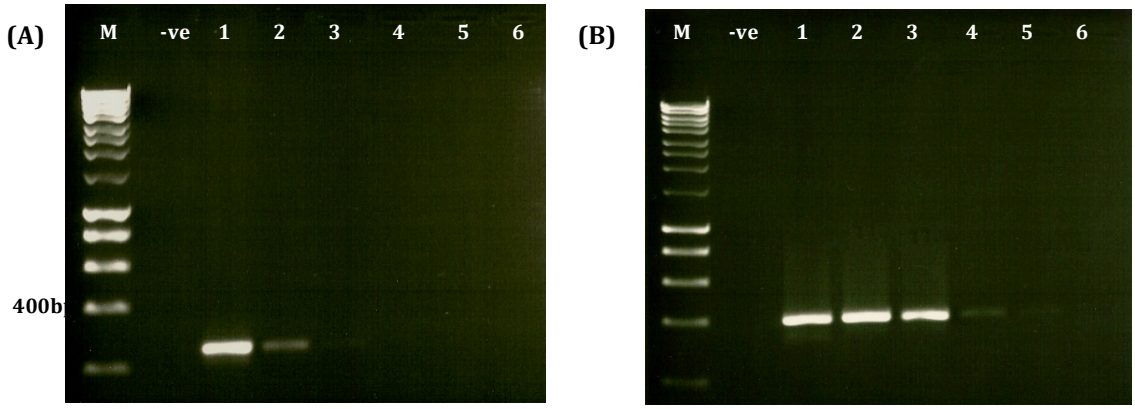


Figure 6.8 1% agarose (w/v) gel indicating primer sensitivity for *Lymnaea peregra* DNA at a concentration of 250ng/µl spiked with parasite DNA at a concentration of 50ng/µl. **(A)** *Plagiorchis elegans* at 62°C **(B)** *Notocotylus malhamensis* at 68°C. **M**: Hyperladder 1; **-ve**: negative control (PCR grade water); **Lane 1:** 50ng/µl; **Lane 2:** 5ng/µl; **Lane 3**: 0.5ng/µl; **Lane 4:** 0.05ng/µl; **Lane 5:** 5×10^{-3}ng/µl; **Lane 6**: 5×10^{-4}ng/µl.

Table 6.2 Finalised reaction conditions summarised after primer optimisation and testing.

Primer set	Selected annealing temperature (°C)	Optimal primer concentration (pmol/µl)	Sensitivity (ng/µl)
PlagF/PlagR	62	12.5	0.5
NotoF/NotoR	68	10.0	5×10^{-3}

6.3.3 Molluscan species

In the present study, a total of 2021 snails consisting of 11 species were collected from six different locations throughout the Malham Tarn area (Figure 6.9).

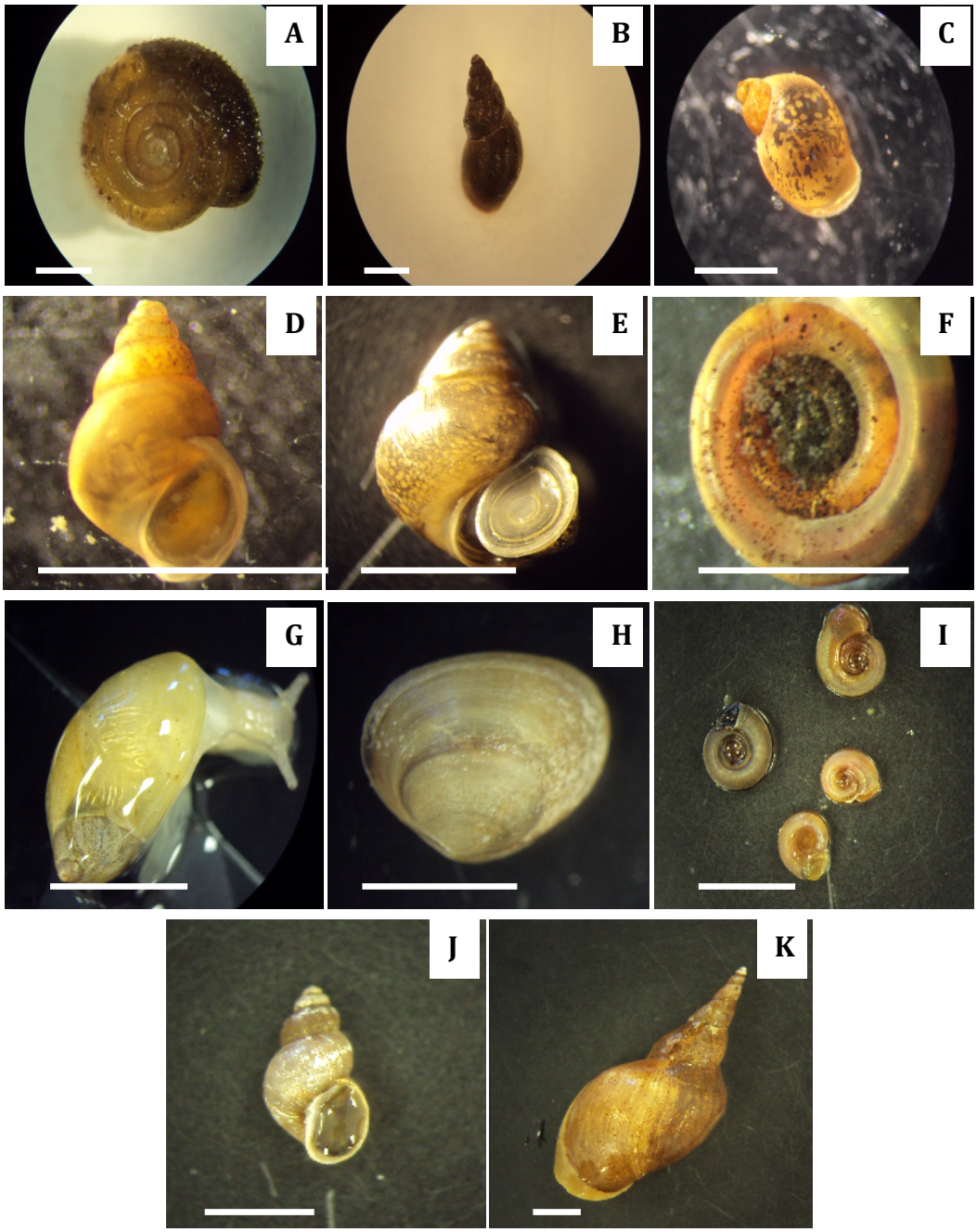


Figure 6.9 Snail species collected from the Malham Tarn area. **(A)** *Planorbis* sp.; (**B)** *Lymnaea palustris*; **(C)** *Lymnaea peregra*; **(D)** *Potamopyrgus antipodarum*; **(E)** *Bithnyia tentaculata*; **(F)** *Anisus leucostoma*;**(G)** *Physa fontinalis*; **(H)** *Sphaerium corneum*.; **(I)***Valvata cristata*. **(J)** *Lymnaea truncatula* **(K)** *Lymnaea stagnalis*. **White scale bars represent 5mm.**

Table 6.3 Differences observed in the seasonal collection of molluscs from six different sampling locations during the sampling period. **Key**: **W** = winter; **Sp** = spring; **Su** = summer; **A** = autumn; **TW** = Tarn Woods; **HM** = Ha Mire.

		Sampling site					
		NW inflow	NE inflow	Reed beds	Peat pools	Rocky shore TW	Rocky shore HM
species	**Season**						
Planorbis sp.	W	0	0	0	**7**	0	0
	Sp	0	0	0	0	0	0
	Su	0	0	0	0	0	0
	A	0	0	0	0	0	0
L. palustris	W	0	0	0	**3**	0	0
	Sp	0	0	**13**	0	0	0
	Su	0	0	**39**	0	0	0
	A	0	0	**2**	0	0	**1**
L. peregra	W	**108**	**288**	0	0	0	0
	Sp	**74**	**252**	0	0	0	0
	Su	**197**	**97**	**9**	0	**3**	**2**
	A	**116**	**117**	**3**	0	0	**4**
P. antipodarum	W	**20**	0	0	0	0	0
	Sp	**10**	0	0	0	0	0
	Su	**56**	0	0	0	0	0
	A	**102**	0	0	0	0	0
B. tentaculata	W	0	0	0	0	0	0
	Sp	0	0	0	0	0	0
	Su	**4**	0	0	0	0	0
	A	**6**	0	0	0	0	0
A. leucostoma	W	**2**	0	0	0	0	0
	Sp	**8**	0	0	0	0	0
	Su	**12**	0	0	0	0	0
	A	**13**	0	0	0	0	0
P. fontinalis	W	0	0	0	0	0	0
	Sp	0	0	0	0	0	0
	Su	0	0	**5**	0	0	0
	A	0	0	**3**	0	0	0
S. corneum	W	0	0	0	0	0	0
	Sp	0	0	0	0	0	0
	Su	0	0	**15**	0	**8**	**10**
	A	0	0	0	0	0	0
V. cristata	W	0	0	0	0	0	0
	Sp	0	0	0	0	0	0
	Su	0	0	**31**	0	**49**	**24**
	A	0	0	**9**	0	0	**24**
L. truncatula	W	**10**	**62**	**2**	0	0	0
	Sp	**1**	**60**	0	0	0	0
	Su	**4**	**35**	**4**	0	0	0
	A	0	**62**	**3**	0	0	0
L. stagnalis	W	0	0	0	0	0	0
	Sp	0	0	**2**	0	**9**	0
	Su	0	0	**6**	0	**11**	0
	A	0	0	**4**	0	0	0

6.3.4 Sampling difficulties

Although every effort was made to maintain consistency in sampling at all times (ten sample dips per site was conducted when feasible and each site was treated consistently), differences were observed in the seasonal collection of molluscs from different locations (Table 6.3). These differences do not necessarily depict changes in the developmental cycle and abundance of the examined species but may be reflective of the difficulties frequently encountered in their collection as a result of natural changes within the environment which included prolonged ice cover, flooding, high water and drought (Figure 6.10).

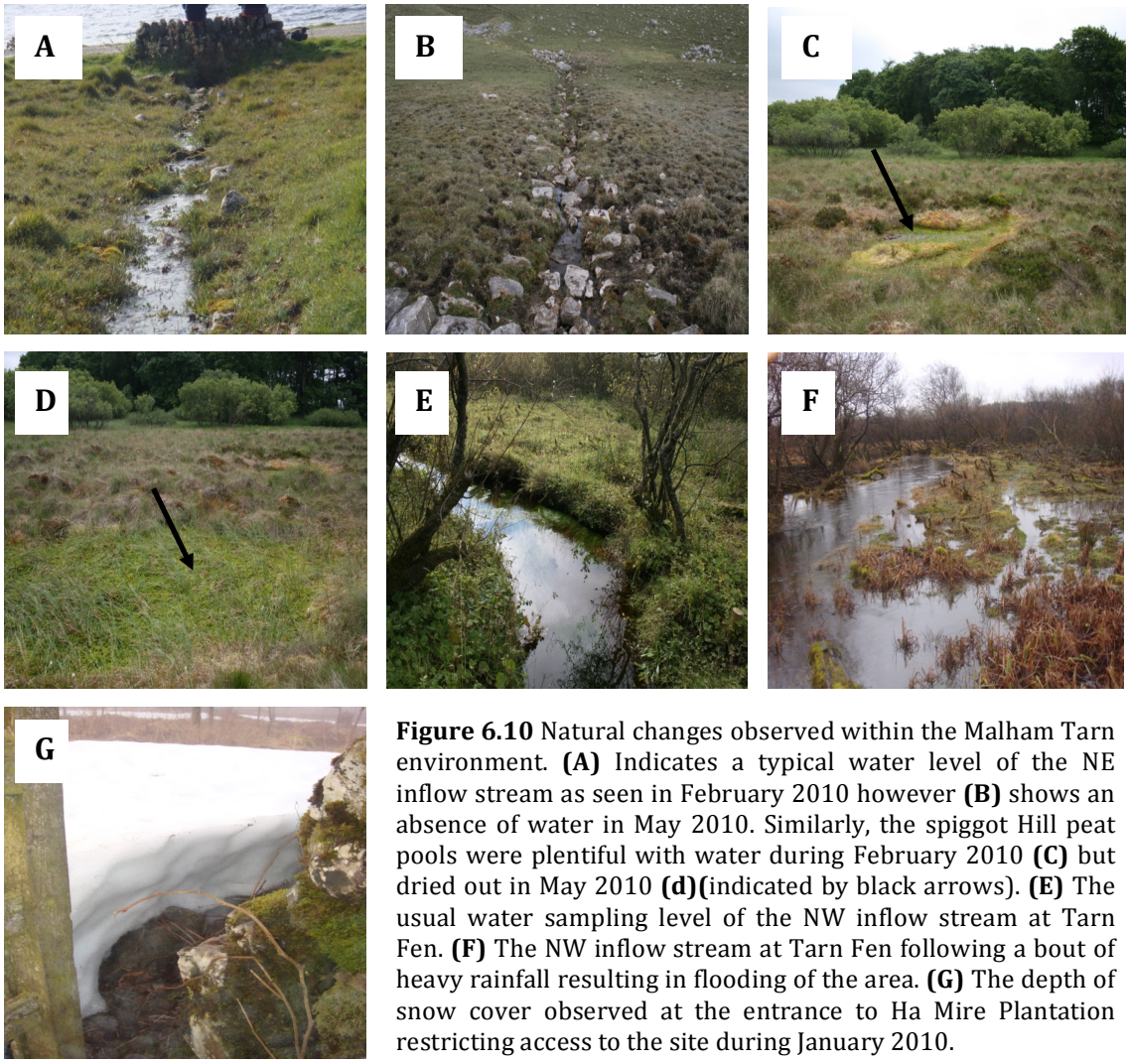


Figure 6.10 Natural changes observed within the Malham Tarn environment. **(A)** Indicates a typical water level of the NE inflow stream as seen in February 2010 however **(B)** shows an absence of water in May 2010. Similarly, the spiggot Hill peat pools were plentiful with water during February 2010 **(C)** but dried out in May 2010 **(d)(**indicated by black arrows). **(E)** The usual water sampling level of the NW inflow stream at Tarn Fen. **(F)** The NW inflow stream at Tarn Fen following a bout of heavy rainfall resulting in flooding of the area. **(G)** The depth of snow cover observed at the entrance to Ha Mire Plantation restricting access to the site during January 2010.

Prolonged ice cover of the Malham Tarn area during the winter sampling periods had detrimental effects on the collection of invertebrate species at this time of the year. Collections during this period were therefore limited to the inflow streams and a few standing water bodies where feasible. Furthermore, a lower than average amount of rainfall observed between January and late June 2010 resulted in the drought of several usual water sampling sites during the spring and summer sampling periods. Consequently, collection was restricted during these periods and in several instances only empty shell cases of molluscs were found. Accessibility was furthermore restricted at certain times throughout the study period. Periods of heavy rainfall during the autumn resulted in high water around the tarn margin and consequently made sampling of the tarns rocky shores inaccessible and on several occasions caused flooding of the Tarn Fen area making the collection of invertebrates much more difficult.

6.3.5 Examination of molluscs

In addition to parasitic helminths, the commensal *Chaetogaster* sp. belonging to the order Clitellata was frequently encountered. *Chaetogaster* sp. are small transparent freshwater annelids that live within the shell of molluscs and were recorded inhabiting the surface of seven snail species: *L. truncatula, L. peregra, L. palustris, L. stagnalis*, *A. leucostoma, P. antipodarum* and *V. cristata. Chaetogaster* sp. were included in the initial stages of primer design in order to eliminate the possibility of cross-reactivity in the testing primers on field collected samples.

Only two types of larval trematode were identified in the current study: xiphidiocercariae and monostome cercariae. Xiphidiocercariae possess a stylet positioned in the anterior rim of the oral sucker. Following morphological and molecular analysis, the xiphidiocercariae were identified as *Haplometra cylindracea*, a plagiorchiid of amphibians (Figure 6.11).

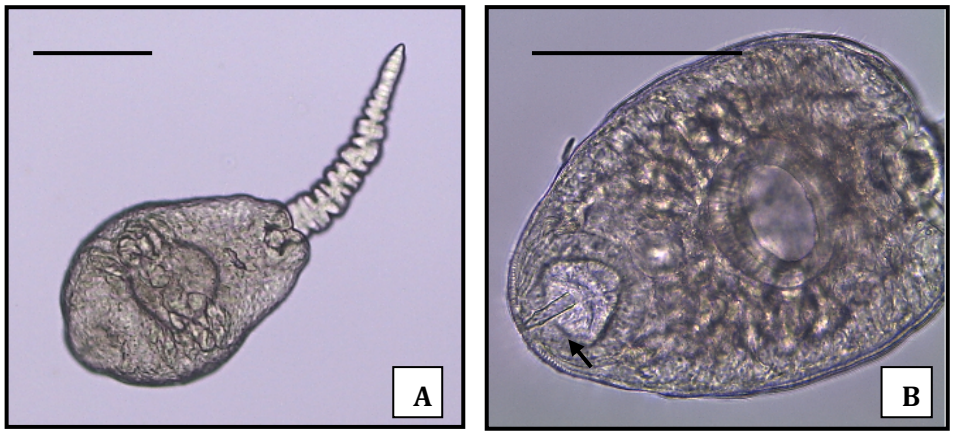


Figure 6.11 (A) *Haplometra cylindracea* isolated from the tissues of *Lymnaea truncatula* collected from the northeast inflow stream, × 10; **(B)** stylet present within the rim of the oral sucker (black arrow), × 40. **Scale bar represents 100µm.**

The Plag primer set was applied to verify the absence of *P. elegans* xiphidiocercariae. A 241bp product was successfully amplified and sequenced (Figure 6.12). Unfortunately, the amplicon shared only 86% sequence homology with *P. elegans* indicating the unspecific nature of the designed primers (Figure 6.13). A partial region of the 28S rRNA gene was subsequently amplified and sequenced in an attempt to molecularly identify the specimen which was later verified by 99% sequence homology (Accession: AF151933.1) to be *H. cylindracea* following an NCBI Blast search and sharing only a 94% sequence homology with that of *P. elegans* (Accession: AF151952.1). No ITS sequence data is currently available in NCBI for this digenetic species and therefore this information was not included during the initial stages of primer design.

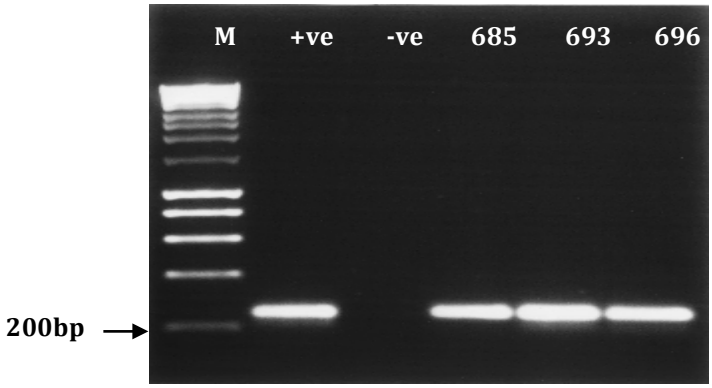


Figure 6.12 1% agarose (w/v) gel indicating amplification of a 241bp fragment from the ITS region of three samples of xiphidiocercariae (snails 685, 693 and 696) isolated the tissues of *Lymnaea truncatula* using the *Plag* primer set designed in the present study. **M**: Hyperladder 1; **+ve**: positive control (*Plagiorchis* adult, Malham Tarn); **-ve**: negative control (PCR grade water).

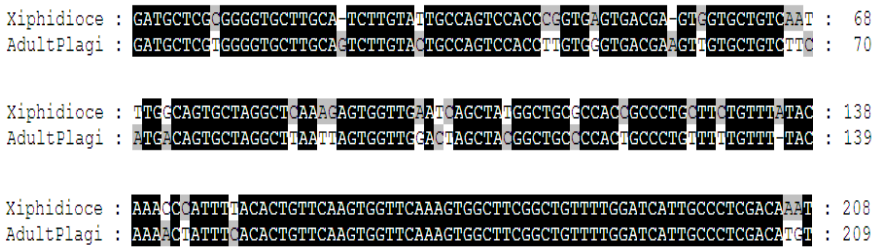


Figure 6.13 Comparison of the partial ITS sequence generated from the xiphidiocercariae isolated from *L. truncatula* using the Plag primer set designed in the present study against the same gene region generated for *Plagiorchis elegans* adults collected from Malham Tarn. Comparison made using GeneDoc Alignment tool. Black shading indicates regions of conservation between the two sequences. 86% sequence homology only is observed.

6.3.5.1 Monostome cercariae

The monostome cercariae were recovered predominantly from *L. peregra* collected from the northeast inflow and the northwest inflow streams. A single infection of *L. peregra* from the northeast reed beds and two infections of *Lymnaea truncatula* collected from the northeast inflow stream were furthermore detected. These monostome cercariae were identified following morphological (Figure 6.14) and molecular analysis (Figures 6.15 to 6.18).

6.3.5.2 Morphological analysis of monostome cercariae

The general body shape of the monostome cercariae changes with movement of the specimen, from round to more elongate in form. Measurements taken (n = 7) indicate the body to range in length from 200 to 650µm (mean 408µm) by 130 to 250µm in width (mean 191µm). The tail is typically as long as or slightly longer than the body measuring in range from 150 to 680µm (mean 431µm) in length by 40 to 90µm (mean 64µm) in width. These cercariae are trioculate possessing a pair of pigmented eyespots on the dorsal side of the body that lie peripheral to a third median spot which is positioned slightly anterior creating a triangular arrangement. The cercariae appear dark brown in colour and possess irregular patterns of pigmentation along the lateral margins that were observed to be darkest in the anterior region. The body possesses a single oral sucker which opens into a short oesophagus that bifurcates just posterior to the median eyespot. The oesophagus bifurcates into two very long caeca that extend to almost the posterior end of the body (seen in white). Two lateral excretory vessels (dark brown in colour) can also be observed to unite at the point of the excretory vesicle

located in the far posterior end of the body. The morphology of these cercariae represents members of the genus *Notocotylus*.

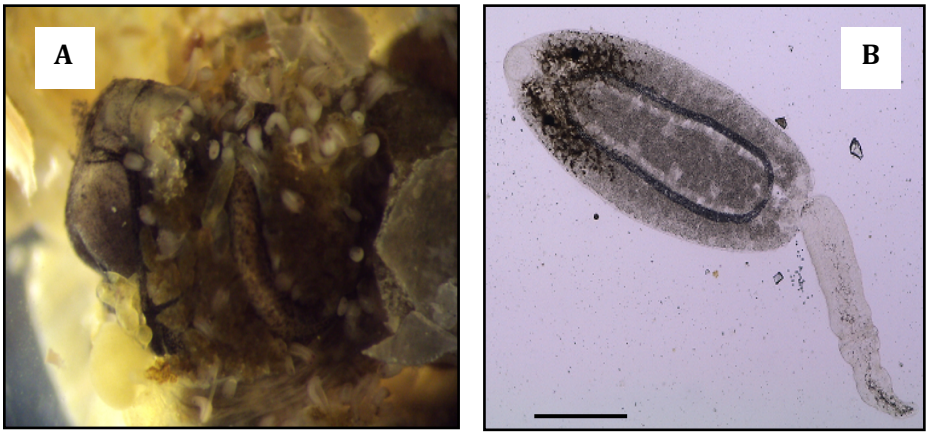


Figure 6.14 (A) *L. peregra* tissues infected with redia and cercariae following crushing. **(B)** Monostome cercariae released from snail tissue. **Scale bar represents 100µm**.

6.3.5.3 Molecular analysis of monostome cercariae

The Noto primer set was applied and successfully amplified a 401bp product as expected (Figure 6.15A). The amplicon was sequenced to verify identity (Figure 6.15B). Unfortunately, the amplicon shared only a 96% sequence homology with the *N. malhamensis* adult specimens indicating these primers to also have amplified from a non-specific source. During the initial stages of primer design no ITS sequence data was available in NCBI for any described *Notocotylus* species and therefore this information could not be included. Analysis of the complete ITS region of the monostome cercariae against *N. malhamensis* indicated a 98% sequence homology with a 27bp difference (Figure 6.18).

A partial region of the 28S rRNA gene was furthermore amplified, sequenced and subjected to a blast search to attempt identification (Figure 6.16). Results indicate the cercariae to belong to the Notocotylidae with the most closely related sequences (99% sequence homology) belonging to the genus *Notocotylus* (Table 6.4). All three cercarial sequences furthermore shared 100% sequence homology but only 99% homology with an 11bp difference with the *N. malhamensis* adults recovered from Tarn Fen (Figure 6.17).

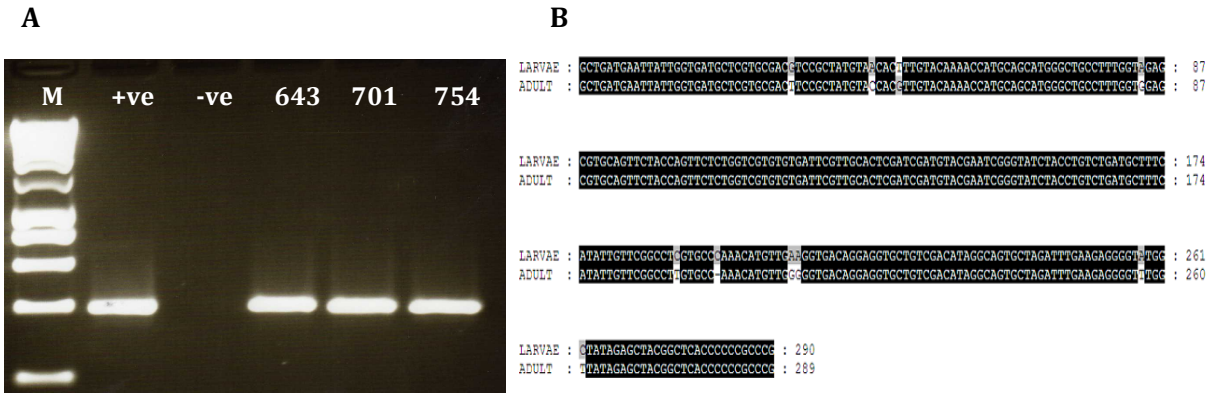


Figure 6.15 (A) 1% agarose (w/v) gel indicating amplification of a 401bp fragment from the ITS region of three samples of monostome cercariae (snails: 643 *L. peregra* from NE inflow; 701 *L. truncatula* from NE inflow; 754 *L. peregra* from NW inflow, Tarn Fen) using the *Noto* primer set. **M**: Hyperladder 1; **+ve**: positive control (*Notocotylus malhamensis* adult, Tarn Fen); **-ve**: negative control (PCR grade water). (**B**) Comparison of the partial ITS DNA sequence of cercarial isolates and *N. malhamensis* adults. Comparison made using GeneDoc Alignment tool. Black shading indicates regions of conservation between the two sequences. 96% sequence homology only is observed.

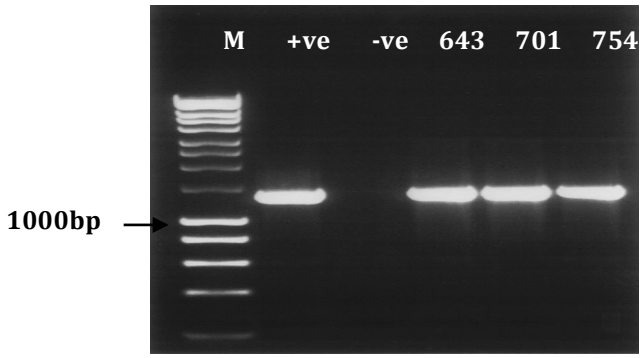


Figure 6.16 1% agarose (w/v) gel indicating amplification of a partial region of the 28S rRNA gene of the monostome cercariae isolated from snails **643** (*L. peregra* from NE inflow), **701** (*L. truncatula* from NE inflow) **754** (*L. peregra* from NW inflow, Tarn Fen) using primers according to Tkach *et al*. (2000b). **M**: Hyperladder 1; **+ve**: positive control (*Notocotylus malhamensis* adult, Tarn Fen); **-ve**: negative control (PCR grade water).

Table 6.4 Species of the Notocotylidae deemed to be most closely related to the monostome cercariae recovered from Malham Tarn using a partial gene region of the 28S rRNA gene, following an NCBI blast search. *Notocotylus malhamensis* recovered from Malham Tarn was also included in the alignment.

Digenean species	Accession number	Sequence homology (%)
Notocotylus malhamensis	Chapter 5	**99**
Notocotylus intestinalis	JQ890559.1	97
Notocotylus BH-2008	EU712725.1	**99**
Notocotylus UK-PO-2003	AY222219.1	**99**
Notocotylus attenuatus	AF184259.1	97
Quinqueserialis quinqueserialis	JQ670848.1	98
Paramonostomum anatis	AF184258.1	97
Catatropis indicus	AY222220.1	96

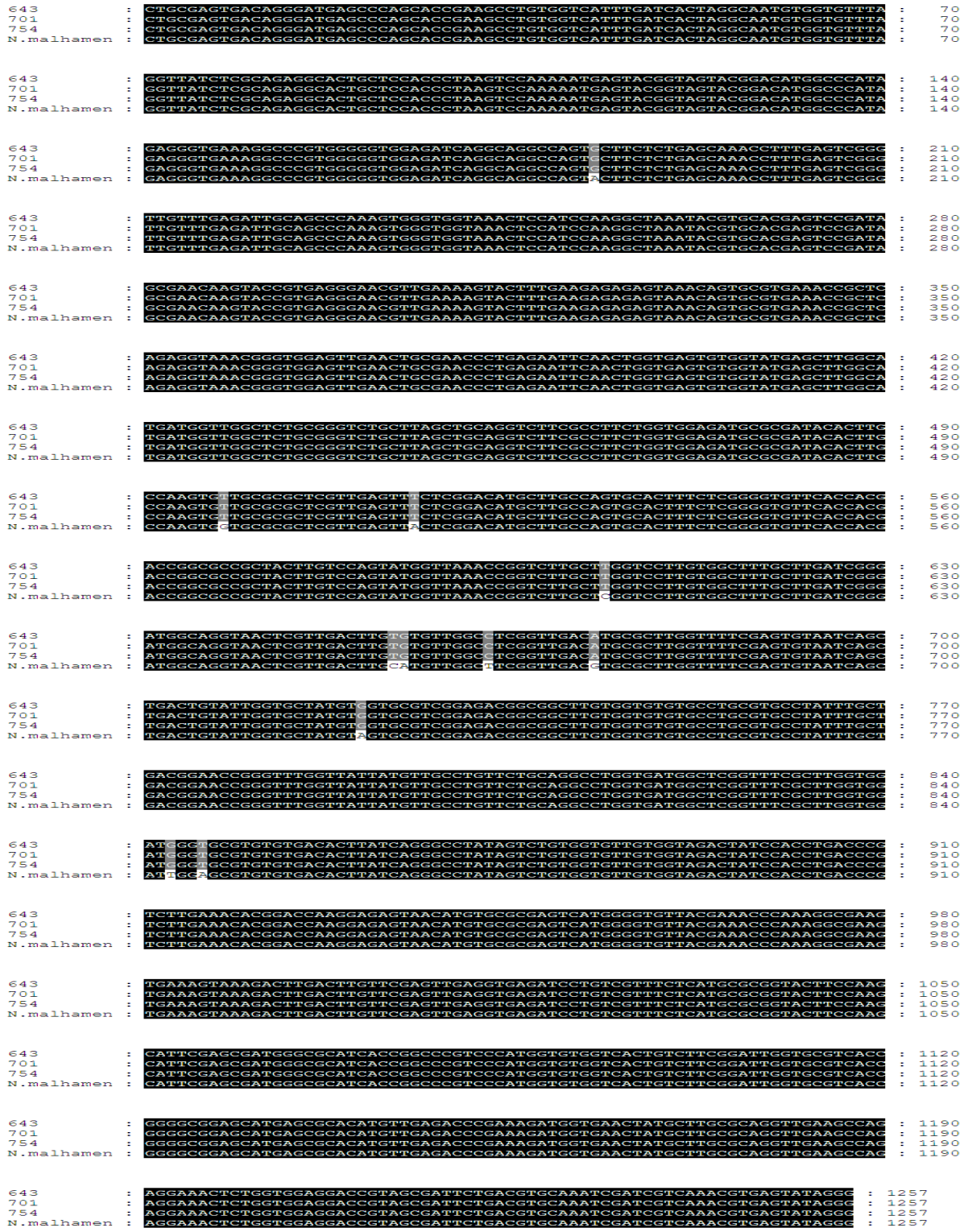


Figure 6.17 Comparison of the partial 28S rRNA gene sequence generated from the cercariae found in snails: 643 *L. peregra* from NE inflow; 701 *L. truncatula* from NE inflow; 754 *L. peregra* from NW inflow, Tarn Fen against *Notocotylus malhamensis* recovered from *Myodes glareolus* at Tarn Fen. 100% homology was observed between the cercarial stages but only 99% between the larval stages and the adult specimen. Comparison made using GeneDoc Alignment tool. Black shading indicates regions of conservation between sequences.

Figure 6.18 Comparison of the partial ITS gene sequence generated from the cercariae found in snails *L. peregra* from NW inflow, Tarn Fen against *Notocotylus malhamensis* recovered from *Myodes glareolus* at Tarn Fen. 98% homology was observed between the cercarial stage and the adult specimen (27bp difference). Comparison made using GeneDoc Alignment tool. Black shading indicates regions of conservation between sequences.

6.3.5.4 Morphological & molecular analysis of redia and metacercariae

The redia are dark brown and cylindrical in form (Figure 6.19C). The ends taper towards annular constrictions that divide the body into two or three regions. The redia contract and elongate with movement, ranging in length (n = 7) from 650 to 1240µm (mean 1015µm) and width from 180 to 280µm (mean 237). A pharynx is observed at the mouth end and a mass of germ cells that eventually develop into cercariae at the tail end. Internal structures are difficult to observe in mature specimens being masked by the developing cercariae.

The metacercariae were found attached to the surface of molluscan shells where they demonstrate a hemispherical form with a flattened base (Figure 6.19A). The cyst (n = 7) ranges in size from 220 to 250µm (mean 230µm) in width by 230 to 300µm (mean 246µm) in height (Figure 6.19B). The outer cyst wall typically measures from 10 to 20µm in thickness, most often being 20µm. The wall of the cyst is relatively transparent through which the coiled larvae and eyespots can be observed. The 28S rRNA gene of the redia and metacercariae was amplified and the results compared with the cercarial stage in order to demonstrate the specific identity between each of the larval stages. 100% sequence homology was observed between the three larval stages. Furthermore, 100% sequence homology was observed for the DNA sequence compared, from the metacercariae found on the surface of each of the different molluscan hosts (Table 6.7).

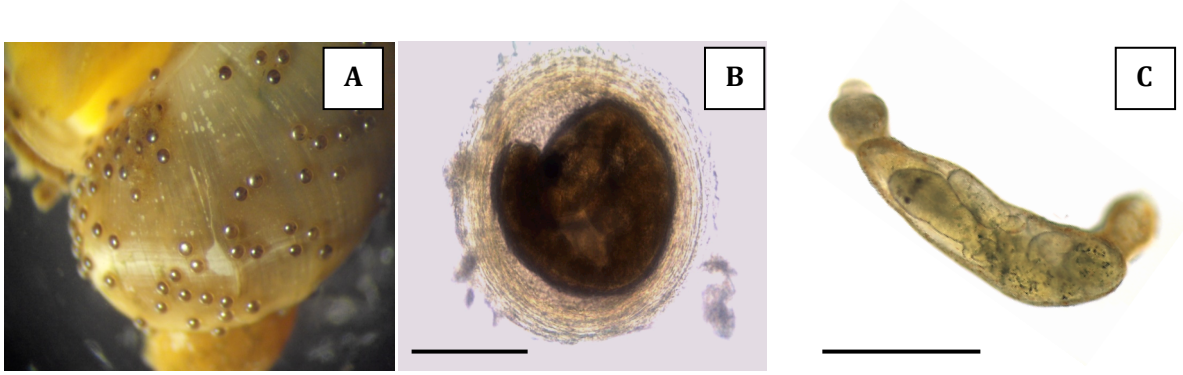


Figure 6.19 Notocotylid larval stages isolated from the tissues of *L. peregra* collected from the northeast inflow stream (sample 643). **(A)** Metacercarial stages encysted on the outer shell surface of *L. peregra*; **(B)** metacercarial stage. **Scale bar represents 100µm**. **(C)** Redial stage containing developing cercariae isolated from the tissues of *L. peregra* collected from the northeast inflow stream (sample 643). **Scale bar represents 500µm**.

6.3.5.5 Prevalence of *Notocotylus* sp.

The redia and cercariae of *Notocotylus* sp. was harboured by *L. peregra* and *L. truncatula* only. *L. peregra* located at the northwest inflow stream, northeast inflow stream and northeast reed beds was the most predominant species to harbour these larval stages with an overall prevalence of 4.7% (60/1270) compared with only two of the 243 (0.8%) examined *L. truncatula* collected from the northeast inflow. This difference was found to be very statistically significant using 2×2 Fisher's exact test (exact p value = 0.0036), indicating *L. peregra* to be the most important host for propagating this digenean at these locations.

The difference in prevalence of the redia and cercariae within *L. peregra* between the three infected locations was not considered significant using a chi-squared test for heterogeneity ($X^2 = 0.49$, $v = 2$, p = 0.7827) (Table 6.5). Seasonal differences observed in prevalence of the redia and cercariae within *L. peregra* from these three locations (Table 6.5) was however proved to be very statistically significant using chi-squared test for heterogeneity ($X^2 = 39.03$, $p = 0.05$, $v = 3$).

Table 6.5 Summarised prevalence (95% confidence limits) for the redia and cercariae of *Notocotylus* sp. isolated from *L. peregra* at Malham Tarn.

	Number	Prevalence (%)of redia & cercariae	95% Confidence limits	
			Lower	Upper
Overall				
L. peregra	1270	4.70	03.68	06.04
L. truncatula	243	0.80	00.03	03.15
Location				
Northwest inflow	495	4.40	02.92	06.67
Northeast inflow	754	4.90	03.56	06.71
Northeast reed beds	12	8.30	<0.01	37.53
Season				
Winter	396	0.50	00.15	02.31
Spring	326	9.20	06.49	12.87
Summer	303	7.60	05.07	11.18
Autumn	236	2.10	00.77	05.00

The metacercariae of *Notocotylus* sp. was the most abundant larval stage to be recovered. This stage of development appeared to be dispersed in a much greater area throughout the tarn. *Notocotylus* metacercariae were found encysted upon snails located at both inflow streams, the northeast reed beds, and the two rocky shores of Tarn Woods and Ha Mire Plantation. The overall metacercarial prevalence found on the

surface of molluscan shells throughout the duration of the study was 17.6% (355/2021), however differences in prevalence were observed according to both location and snail species. These differences in prevalence between the five different sites were highly significant using chi-squared test for heterogeneity (X^2 = 82.53, p = 0.05, v = 4). Pairwise analysis of prevalence between the various locations using 2 x 2 Fisher's exact test indicated there to be no significant difference between the northeast inflow stream and the northeast reed beds (exact p value = 0.2208) or the two rocky shores of Tarn Woods and Ha Mire plantation (exact p value = 0.7007). All other differences were however statistically different (Table 6.6).

Table 6.6 Pairwise analysis comparing the prevalence of the metacercariae of *Notocotylus* sp. on molluscan shell surfaces at each of the recorded locations using 2 Fisher's Exact Test.

Comparison		Fisher's exact p value
Location 1	**Location 2**	
Northwest inflow	Northeast inflow	0.0001
Northwest inflow	Rocky shore TW	0.0001
Northwest inflow	Rocky shore HM	0.0001
Northwest inflow	Northeast reed beds	0.0405
Northeast inflow	Rocky shore TW	0.0001
Northeast inflow	Rocky shore HM	0.0004
Reed beds	Rocky shore TW	0.0001
Reed beds	Rocky shore HM	0.0001

Despite *L. peregra* being the most predominant snail species to harbour the redial and cercarial stages of *Notocotylus* sp. at Malham Tarn, *L. truncatula* and *L. palustris* were the most commonly observed snails to carry the encysted stage on their shell surface. Metacercariae was collected from the shell surface of *L. peregra*, *P. antipodarum*, *A. leucostoma*, *L. truncatula*, *L. palustris* and *V. crista* (Table 6.7). The difference in prevalence between the snail species was significant using chi-squared test for heterogeneity (X^2 = 23.99, p = 0.05, v = 5). Pairwise analysis of prevalence between the various snail species using 2 x 2 Fisher's exact test indicated a significant difference between *L. truncatula* and *L. peregra* (exact p value = 0.0005), *L. truncatula* and *P. antipodarum* (exact p value = 0.0001) and *L. truncatula* and *V. cristata* (exact p value = 0.0005) only. The seasonal differences in prevalence were also found to be highly significant using chi-squared test for heterogeneity (X^2 = 284.03, p = 0.05, v = 3) (Table 6.7).

Table 6.7 Prevalence (95% confidence limits) of *Notocotylus* sp. metacercariae from Malham Tarn.

	Number	Prevalence (%) of metacercariae	95% Confidence limits	
			Lower	Upper
Location				
Northwest inflow	743	37.50	34.14	41.09
Northeast inflow	973	23.80	21.27	26.62
Northeast reed beds	150	28.70	22.01	36.38
Rocky shore TW	80	3.75	00.84	10.90
Rocky shore HM	65	6.20	01.98	15.22
Snail species				
L. peregra	1270	17.9	15.86	20.08
P. antipodarum	188	12.8	08.67	18.35
A. leucostoma	35	14.3	05.78	29.85
L. truncatula	243	28.0	22.71	33.95
V. cristata	137	12.4	07.80	19.07
L. palustris	58	24.1	14.85	36.64
Season				
Winter	502	0.6	00.12	01.83
Spring	429	40.8	36.24	45.51
Summer	621	20.0	17.01	23.30
Autumn	469	10.2	07.79	13.33

6.3.5.6 Phylogenetic analysis

The phylogenetic relationship between the *Notocotylus* larvae collected from *L. peregra* at Malham Tarn was analysed in relation to members of the genus Plagiorchiidae and other members of the Notocotylidae whose sequence data was available from NCBI (Table 2.2). A rooted dendrogram based on the partial 28S rRNA gene was established which placed *Notocotylus* sp. into a very well defined clade with other members of the Notocotylidae inferred by 100% bootstrap support (Figure 5.7). *Notocotylus* sp. appears to be most closely related to *Notocotylus* sp. UK (>90% bootstrap support), with 99% sequence homology observed between the two data sets with only a 4 nucleotide difference. The DNA sequence for *Notocotylus* sp. UK (AY222219) however was generated from incompletely identified sporocyst material isolated from *Lymnaea palustris* in the UK (Olson *et al*., 2003) and as such, species identification cannot be defined based upon these results.

6.3.6 Application of primers to wild snail samples

The two primer sets designed in the present study have been proven insufficient for species-specific use. Both primers amplified from an unspecific source. Limitations in the design have stemmed from a paucity of available DNA sequence data in the NCBI database for members of these genera. Ideally these primers would have been

developed further to enable their deployment directly onto field collected invertebrate samples, however the decision was made to not proceed with further development due to time constraints and the inability to collect the necessary information required to be able to ensure species-specific use.

6.3.7 *Plagiorchis* larval stages

Unfortunately, following the examination of 2021 snails (including 1603 Lymnaeids) there were no intramolluscan stages of *P. elegans* detected in the present study despite careful observation and the frequent detection of other trematode larval stages. Binomial confidence intervals were calculated (p = 0.05, two tailed test) to establish whether zero prevalence was a significant result using these representative samples (Table 6.8). 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, a very low prevalence (less than 1%) may be expected. Further investigation using a larger cohort of snails is required although time constraints limit this during the current study.

Table 6.8 Prevalence of intramolluscan stages of *Plagiorchis* found in snail species. 95% confidence intervals (p = 0.05, two-tailed test) were calculated using an online Binomial Confidence Interval calculator (http://statpages.org/confint.html).

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

6.3.8 Examination of insect larval stages

Twelve types of aquatic insect larvae dominated the collection during each sampling session (Table 6.9). Other insects such as *Bezzia* sp., *Tipula* sp., and members of the family Stratiomyidae were collected very sporadically during the study. In total, 2110 of the dominant insect larval and nymph stages were quantitatively analysed in an attempt to identify any trends in population changes throughout the year and identify any patterns of adult emergence that appear to correspond with mean peak prevalence and peak mean intensity of *P. elegans* at this location. Only four insect groups have emergence patterns that appear to overlap with both the mean prevalence and mean intensity of *Plagiorchis*: *Chironomid* spp., the uncased caddis *Plectrocnemia conspersa*, cased caddis flies and the mayfly *Baetis rhodani* (Table 6.9).

Table 6.9 Insects collected from Malham Tarn and their typical emergence pattern.

Insect	n	Emergence	Reference
Sialis lutaria	93	April to June	Elliott (1996)
Baetis rhodani	87	**April to July & September**	Humpesch (1979); Brittain (1990)
Nemoura sp.	266	March to May	Brittain (1990); Brittain (1993)
Simulium sp.	510	April to July	Davies (1966)
Plectrocnemia conspersa	14	**May to October**	Petersen *et al*. (1999)
Chironomid sp.	808	**April to October**	Learner and Potter (1794); Bay (2003)
Cased caddis sp.	332	**May to October**	Holmes (no date)

As explained in section 6.3.6 molecular analysis is not feasible for the detection of digenean larval stages in these circumstances. Insect larvae were therefore dissected and attempts to morphologically identify metacercarial cysts within the body cavity was performed. In total, 1472 insect larval and nymph stages were dissected and systematically examined. These included 30 specimens of *S. lutaria*, 29 *B. rhodani*, 117 *Nemoura* sp., 158 *Simulium* sp., and all sampled *Chironomids* and cased caddis fly larvae. Unfortunately, no metacercariae were found inside the body cavities despite careful observations. The metacercariae of *Notocotylus* sp. were however identified on the exterior regions of the caddis fly larvae *Potamophylax latipennis* (Figure 6.20). In total, 27% (4/15) of the *Potamophylax latipennis* larvae collected from the rocky shore of Ha Mire Plantation in June 2011 were infected with this digenean. Identification of *Notocotylus* sp. was confirmed by DNA sequencing of the 28S rRNA gene.

Figure 6.20 A metacercarial cyst of *Notocotylus* sp. (**black arrow**) attached to the forleg of the cased caddis fly larvae *Potamophylax latipennis* collected from the rocky shore of Ha Mire Plantation.

6.4 Discussion

The use of species-specific primers has proved very successful for commonly examined parasitic species, typically of medical or veterinary importance for which a vast amount of molecular information is available (Terry *et al*., 2001; Hughes *et al*., 2006; Boufana *et al*., 2008). In the current study however a paucity of information was identified for the digeneans being examined. Only 18 DNA sequences for various genes were available for members of the genus *Plagiorchis* despite more than 100 species names within the genus being coined. These 18 sequences included only 10 for the ITS region, of which 5 were repeated isolates. No sequence data at all is currently available for the ITS gene region of Notocotylid species.

Consequently, both primer sets designed in the present study amplified from a non-specific source. The *Plagiorchis* primers amplified the ITS region of *Haplometra cylindracea,* a closely related Plagiorchiid species of amphibians (Lees, 1962). The *Notocotylus* primers amplified a partial region of the ITS from *Notocotylus* larval stages, collected from the wandering pond snail *Lymnaea peregra* that did not match 100% with that for *N. malhamensis* for which the primers were originally designed. Further analysis of a partial region of the 28S rRNA gene of these *Notocotylus* larval stages indicated a 99% sequence homology with an 11 nucleotide difference within a 1257bp examined sequence identified by the ClustalW2 program. These figures indicate a sequence divergence of just 0.88% (11/1257 × 100) between the *Notocotylus* larvae and the *Notocotylus malhamensis* adult specimens. The 28S rRNA gene however has a slow evolutionary rate and tends to be more highly conserved between congeneric species

(Nolan and Cribb, 2005). The non-coding regions of the ITS1 and ITS2 (including the 5.8S coding region) was furthermore analysed for sequence comparison. This gene region is more rapidly evolving and as such more likely to accumulate mutations over time that are useful for indicating interspecific variability between members of a genus (Tkach *et al*., 2000a). Analysis of a larger fragment of the ITS gene region from these *Notocotylus* larval stages using ClustalW2, indicated a 98% sequence homology with a 27 nucleotide difference within a sequence of 1235bp length. These figures indicate a larger divergence of 2.19% (27/1235 × 100) between the two samples than that demonstrated by the 28S rRNA gene.

Sequence divergence thresholds for delineating species however, are not universally agreed upon (Hebert, 2004). According to Morgan and Blair (1995) there is currently no standard for the recognition of species boundaries when using differences in sequence data. The 'Consortium for the Barcode of Life' however currently utilises a 648bp fragment of mitochondrial DNA from the cytochrome c oxidase I (COI) gene for species differentiation. The variability of DNA sequences found within this gene region average five to ten times higher than sequences typically found within nuclear genes and as such are considered to be more sensitive for distinguishing between species (Stoeckle *et al*., 2005). The use of nuclear DNA was selected for the identification of digenean species within the present study as the majority of genetic information already deposited within the NCBI database originated from these gene regions. The threshold for sequence divergence used in genetic barcoding however is 2%. In this instance more than a 2% divergence is considered enough for the identification of most species (Stoeckle *et al*., 2005) and this threshold appears to be more commonly employed for species differentiation by many researchers (Hebert *et al*., 2004; Cognato, 2006; Steinke *et al*., 2009).

Differentiation based on a single gene however should be considered cautiously. In the present study the divergence of the *Notocotylus* larval stages using the 28S rRNA gene was within a 2% threshold from *N. malhamensis* to enable this difference to be considered as intraspecific variation. Use of a second gene however indicates the larval stages to be just past this threshold which may indicate a more recent divergence between two individual species. It can be very tempting to conclude the presence of

more than one species when basing diagnosis purely on genetic data and care should be taken when the absence of other biological data such as morphology is absent (Sorensen *et al*., 1998).

Notocotylus malhamensis at Malham Tarn has been recently identified as a new species of the genus *Notocotylus* (Boyce *et al*., 2012). As such, information regarding life cycle biology including intermediate host species involved in transmission, host specificity or the morphological criteria that may be of use to identify the larval stages, remains currently unknown. Unfortunately, a lack of this information currently contributes towards the difficulty in using a classical approach to species diagnosis in this instance.

The use of molecular data therefore must be attempted in order to link up the two different morphological stages of this species. However, with a sequence divergence of 2.19% for the ITS gene region, it is difficult to determine whether these differences are actually due to intraspecific or interspecific variation and therefore it cannot be stated with any degree of certainty whether the larval stages found in *L. peregra* at Malham Tarn are in fact those of *N. malhamensis* or a different *Notocotylus* species. It must be emphasised however that no degree of intraspecific variation was observed between the nine adult specimens of *N. malhamensis* examined in chapter 5 using the same gene regions and as such it is highly likely that the identified larval stages belong to another species.

Notocotylus larvae were harboured predominantly by *L. peregra* at this location with only two specimens of *L. truncatula* being concurrently infected. Very few *Notocotylus* species have been previously reported within the UK. *N. attenuatus* is the most commonly reported UK species and the redia and cercariae have been recorded from *L. peregra* in Scotland (Williams, 1966), Lower Thames Valley, London (Morley *et al*., 2003) and Sussex (Murrills *et al*., 1985). This species was however ruled out on a molecular basis with sequence variability at 34 sites within a 1091bp sequence giving a divergence of 3.1% (34/1091 × 100), outside the boundaries of that expected for intraspecific variation (Stoeckle *et al*., 2005). A second *Notocotylus* species, *N. triserialis* which is a long disputed synonym of *N. attenuatus* (Dubois, 1951; Beverley-Burton, 1961; 1972; Pike, 1969) has also been reported from *L. peregra* found inhabiting

freshwater reens in Cardiff, Wales (Pike, 1969). No molecular data is currently available for this species to use for comparison. Pike (1969) however described the morphology of *N. triserialis* and its larval stages in detail. Although the size of the Malham cercariae overlaps with that of *N. triserialis*, Pike (1969) described this species as bearing six longitudinal rows of pigment that spread to form a network over the eyespots and have two pairs of hair like processes that are directed antero-laterally on the anterior region of the body (Pike, 1969). None of these features are found on the cercariae examined from Malham Tarn.

A traditional method for species determination of the Malham larval stages would be through experimental infection studies of definitive host species with the metacercarial stages recovered from the surface of *L. peregra* shells. Unfortunately, this was not feasible in the present study due to resource constraints. It is also a possibility that mixed infections of *L. peregra* are occurring at this site, with the larval stages of a second *Notocotylus* species in addition to *N. malhamensis* utilising the same snail host. This situation has been previously identified (Kanev *et al*., 1994). For example, the freshwater snails *Bithynia tentaculata* and *Bithynia leachi* both act as a first intermediate host for *Notocotylus imbricatus* in addition to *N. parviovatus* and *N. ponticus*. In all three *Notocotylus* species the differential characteristics are expressed by the adult digenean only thus making it impossible to identify these larval stages based on a morphological approach (Kanev *et al*., 1994). If such a scenario is occurring at Malham Tarn then it is a possibility that only the second species present has so far been subjected to DNA sequencing.

To date, only a very small sample of *Notocotylus* larvae has been examined. We can therefore only speculate at this time that the larval stages that have been molecularly defined are potential candidates to be the larval stages of *N. malhamensis.* In order to verify this situation with any certainty, a much larger sample size will need to be investigated in order to establish any degree of intraspecific variation that may be occurring at this site, if any at all.

The search for the first intermediate host species involved in the transmission of *Plagiorchis elegans* at Malham Tarn was unfortunately not successful during the present

study. No *P. elegans* larval stages were identified. It is highly likely that infection was not detected because either infection of this host is seasonal and has been overlooked, the sample size was far too small or because the host involved has been inaccessible at the relevant times. Despite such it is most probable that the species involved in transmission is a Lymnaeid.

In 2003, Norris published his work on the mollusca of Malham in which he listed the presence of 25 freshwater molluscs. From this list, four Lymnaeids were recorded including *L. stagnalis*, *L. peregra*, *L. palustris* and *L. truncatula*. Previous studies have considered *L. stagnalis* to be wide ranging throughout the Tarn's rocky shores and weed beds (Calow, 1973). During the present study however only 32 specimens of this species were examined from the rocky shore of Tarn Woods and the northeast weed beds. No samples were collected during the winter sampling period and difficulty accessing the shore during the autumn due to high water was experienced. *Lymnaea stagnalis* has been indicated as the first intermediate host for *Plagiorchis* spp. on several occasions. Bock (1984), acquired *Plagiorchis* cercariae from naturally infected *L. stagnalis* snails collected from Roggenburg fish-ponds in Germany and Obedska Bara nature reserve in Belgrade, Yugoslavia. Studies by Faltýnková *et al*. (2007) also identified *L. stagnalis* as a natural host for *P. elegans*. They examined larval trematodes of *L. stagnalis* across Central Europe and recovered *P. elegans* from 141 of 3628 snails collected from Austria, Czech Republic, Germany and Poland. During the same study *L. stagnalis* was furthermore found to host *P. laricola* and *P. maculosus* (Faltýnková *et al*., 2007). *L. Stagnalis* was also found to be the natural host for *P. elegans* at two lakes, Kuivasjärvi and Kuuhankavesi in north and central Finland at which prevalence rates of 3.7% and 17.3% were observed respectively (Väyrynen *et al*., 2000).

L. peregra is the most common and abundant species recorded within the UK and has been previously found dispersed throughout the Malham Tarn area (Cameron and Redfern, 1972). This species was the most abundant snail collected during the present study and has already been identified as the first intermediate host for *Diplostomum spathaceum* and *D. gasterostei*, parasites of fish at Malham Tarn (Kennedy and Burrough, 1978). No *Diplostomum* cercariae were however identified during the present study suggesting that snails collected from the tarns edge or immediate surroundings are not

necessarily involved in the transmission of *Diplostomum* but rather snails from within the water body of the tarn itself may be the source of infection. Snails at this particular location would have been inaccessible during sampling and it may be speculated that snails located here may also be involved in the life cycle of *P. elegans*. *L. peregra* has been recognised as a first intermediate host for *P. elegans* at other locations. *L. peregra* was found to be naturally infected from Lake Pyykösjärvi in Finland at a prevalence of 10.3% (Väyrynen *et al*., 2000) and this molluscan species was furthermore implicated as a host for *P. muris* and *P. multiglandularis* by Belyakova and Akhmetova in 1994 that identified larval stages in snails which had been collected from mountain water-bodies of Kazakstan (Gibson *et al*., 2005).

During the present study *L. palustris* was predominantly collected from the northeast reed beds however no infection with the larvae of *P. elegans* was observed at this location. As far as can be determined there have been currently no reports of *L. palustris* acting as the intermediate host for *P. elegans* in any natural situation (Gibson *et al*., 2005) although experimental susceptibility has been demonstrated in the studies of Gorman (1980) and Bock (1984). *L. truncatula* was furthermore collected from the two inflow streams and the northeast reed beds. This species has been implemented as the first intermediate host for *P. elegans* by Manga-Gonzalez *et al*. (1994) at Porma river basin located in León, northwest Spain where the digenean was recorded at a prevalence of 2.8% from 6291 collected snails. Unfortunately, none of the *L. truncatula* specimens examined during the current study were infected with the larvae of *P. elegans*.

The search for the second intermediate host involved in the transmission of *P. elegans* was also unsuccessful. This is likely due to a limited sample size, inaccessibility to various aquatic breeding sites and large deviations in the consistency of collected insects due to difficulties encountered by natural climatic changes observed during sampling.

The most commonly reported insect hosts found to be naturally infected with *Plagiorchis* are members of the order Odonata (Hong *et al*., 1998; Hong *et al*., 1999; Gibson *et al*., 2005; Hong, 2009). There was however no samples from this order collected during the present study. According to Shorrock and Sutton (2010), a

succession of poor summers and prolonged ice cover over breeding pools during the winter period of 2009/2010 resulted in a dreadful breeding year for both dragonflies and damselflies resulting in several typical species not been recorded at all during 2010. *Plagiorchis* was nonetheless recorded from *Apodemus sylvaticus* during 2010 (see chapter 4) therefore it may be speculated that other insect groups may rather be involved in transmission at Malham Tarn.

Information pertaining to the natural range of second intermediate hosts for *Plagiorchis* spp. other than those from the order Odonata appears to be limited, with the majority of reports appearing to stem from experimental infection data. To date, mosquito larvae, caddisfly larvae, dragonfly nymphs, long-horned fly larvae, beetle larvae and *Asellus aquaticus* (Macy, 1960; Bock, 1984; Webber 1987; Zakikhani and Rau, 1991) have been experimentally infected. Mosquito larvae, blackfly larvae and *Dixa* sp. have also been recorded as natural hosts for *Plagiorchis* spp. (Gibson *et al*., 2005). Unfortunately no mosquito larvae were located during the present study. *Simulium* larvae were however examined in addition to seven other commonly collected insect groups. This is however a very small representation of the insect fauna typically found at Malham Tarn. Holmes (no date), emphasised both the size and the scope of Malham's invertebrate populations. Caddis flies alone constituted 71 species that had been recorded within just a half of a mile from the tarn body, with 39 of these species being considered to breed within the tarn itself. Only five different caddis larvae were identified during the current study thus the scope of unexamined species in the search for the second intermediate host is vast.

Furthermore, as previously suggested in chapter 4, it may be speculated that *Plagiorchis* infection is related to host species that breed within the tarn body itself. Holmes (no date) discussed how several types of weed beds are rooted throughout the mud bottom of the tarn, with the most important *Chara delicatula* covering as much as a quarter to a third of the tarn and providing a dense habitat for a vast number of invertebrate fauna. Molluscs including *L. stagnalis* and *L. peregra* are thought to dominate comprising as much as 85% of the fauna, and caddis fly larvae are often very numerous (Holmes, no date). Chironomid larvae are also found throughout the tarn's weed beds as well as the bare mud bottom (Holmes, no date).

From the insects examined during the present study only three groups appear to have emergence patterns that overlap with *P. elegans* intensity and prevalence at this location including chironomid flies, caddisflies and mayflies. As pointed out by Holmes (no date) chironomid larvae are found throughout the tarn body and in close proximity to Lymnaeid snails. In the UK Chironomid species typically emerge from the water between the middle of April and late October following which they do not feed (Learner and Potter, 1974) but primarily function to mate and lay eggs after which time they die. Depending on the species involved, the adult life span of chironomid species varies from less than one day to as long as one week (Bay, 2003). Similarly, caddis flies have been found inhabiting reed beds dispersed throughout the tarn alongside both *Lymnaea* species. Following emergence, the life cycle of caddis flies involves a period of two to three days within trees and bushes within the vicinity of the water body where mating takes place either on the ground or within the trees (Hadden, 2010). It is during this period that foraging wood mice may come into contact with adult caddis flies that could have become infected during their larval stages of development. The third group, mayflies, furthermore spend a period of time in trees. Winged nymphs emerge from the water and fly into nearby bushes and trees where they shed their skin to develop into the adult stage. Adult stages are non-feeding and are relatively strong fliers that demonstrate mating swarms, an event that can occur some distance away from the aquatic habitat (Brittain, 1990). Although females return to the water for the deposition of eggs, both sexes die following mating (Hadden, 2010). The behaviour of all three insect groups place them as potential hosts involved in transmission. Further study would be required to attempt to collect both molluscs and insect larvae from the tarn body itself.

Although the initial aim of this project was to design a molecular detection assay for the identification of *N. malhamensis* and *P. elegans* larval stages within their respective intermediate host species, this study was not without its limitations, including a paucity of DNA sequence data for the digeneans under study in addition to closely related species. It is for such reasons that dissection and a classical approach to parasite identification was ultimately undertaken. The snail crushing method has been recognised as a successful approach for observing intramolluscan stage parasites (Caron *et al*., 2008) and Rees (1951) demonstrated how metacercarial cysts of *Plagiorchis*

species located within the body cavity of insect larvae can be easily observed when present. These methods proved successful for the examination of both first and second intermediate hosts although this was a far more laborious and time-consuming process.

6.5 Summary

In conclusion, the intermediate host species for *Notocotylus malhamensis* and *Plagiorchis elegans* at Malham Tarn have not yet been identified, although the identity of the *Notocotylus* larval stages found within *L. peregra* may be questioned with the sequence divergences of these samples being within borderline threshold for that expected using current standards. The first intermediate host for *P. elegans* is likely to be a Lymnaeid fitting with the current literature. Failure to detect intramolluscan stages or metacercariae of *P. elegans* is possibly due to the small sample size and inaccessibility of molluscs at various locations within the tarn area. It may be speculated that intermediate hosts within close proximity to one another within the tarn body itself are propagating the life cycle of this digenean at Malham Tarn. Furthermore, although the high specificity and sensitivity of the PCR assay has been well documented for use in ecological studies, the use of molecular techniques on many understudied parasitic species is not without its limitations. A lack of DNA sequence data available for use during the current study was illustrated by the non-specific amplification of closely related digenean species present within field-collected samples.

CHAPTER 7:

General discussion

The analysis of digenetic trematodes at Malham Tarn Nature Reserve in North Yorkshire, UK was the focus of the current research. This location within the UK was selected for study following the report of a rare occurrence of *Plagiorchis muris* from the intestinal tract of the wood mouse *Apodemus sylvaticus* (Rogan *et al*., 2007). This digenean typically has a complex transmission strategy that utilises a molluscan first intermediate host and an arthropod second intermediate host, although little is known regarding the exact life cycle of this species. The primary aim of this research was to attempt to dissect the life cycle of this digenean at Malham Tarn and indicate intermediate host species involved in transmission, by the use of molecular probes designed specifically for this task. The consistently high prevalence recorded at this site since 1993 (Rogan *et al*., 2007) is very likely a representation of important invertebrate fauna present at the reserve, however to date no intermediate host species have been successfully implicated in transmission at this site (chapter 6) despite a continual recovery of adult digeneans from wood mice dispersed throughout the reserve (chapter 4).

In total 2021 molluscs of 11 different species and 1472 aquatic insect larval stages belonging to six major groups were examined. The methods employed were successful for the identification of other larval digeneans thus deeming the techniques used to be an appropriate means for detection (chapter 6). Failure to identify *Plagiorchis* larval stages therefore may be due to several reasons including the inaccessibility to invertebrate species breeding within specific niches as discussed in chapter 4, although the possibility that precocious development is occurring at this site cannot be ruled out. Precocious development in *Plagiorchis* spp. has been observed by McMullen (1938) who observed the precocious development of metacercariae inside sporocysts within the molluscan host. Furthermore according to Lefebvre and Poulin (2004), aquatic arthropods tend to be removed from the life cycle of most progenetic trematodes thus suggesting that infected snails could be the infection source if consumed directly by the definitive host. No infected arthropods were identified during the current study but until infected molluscs are identified and examined, the idea that truncation is occurring

cannot be investigated further at this locality. The sample sizes examined during the current study however are relatively small when considering the size of the Malham Tarn area thus future work would benefit from increasing the sampling effort to acquire a greater number of snails from an increased sampling range.

Examination of the literature does suggest *P. muris* to have a more limited distribution than *Plagiorchis elegans* which was identified as the species present at Malham Tarn (chapter 4). It makes sense that *P. elegans* would have a much more extensive range which has been identified to encompass most of the Palaearctic and the Nearctic regions, with this species typically being considered an avian digenean. As discussed by Poulin *et al*. (2011), hot spots for biodiversity are also typical hot spots for parasite diversity and the distribution of the parasite is generally coupled with the spatial ecology of the host. As such the maximum geographical distribution of any helminth species is therefore expected to equate the combined range of the hosts involved in the lifecycle (Poulin *et al*., 2011). The use of migratory hosts therefore, in particular for those, in which flight is involved, creates a successful dispersal mechanism that enables a regular exchange of helminth infectious stages between locations (Gorman, 1980). For *P. muris* on the other hand that is typically considered to utilise rodent hosts, limited infection foci would be expected. Although the majority of *P. muris* infection appears to occur in Southeast Asia, this species nonetheless has been identified from regions further afield. This information therefore suggests that in order for this digenean to be dispersed outside of its typical expected range, at some point it must incorporate host species capable of a wider spatial distribution (Granovitch, 1999).

Interestingly however, Blankspoor (1974) commented that there may in fact be no difference between certain Plagiorchiids known to occur in both avian and mammalian hosts, which could possibly be the case for *P. muris* and *P. elegans* that according to the current literature have both been described from a range of birds and mammals. What is evident from the present study however is that there appears to be much ambiguity in the literature regarding the important morphological characteristics that can be used to differentiate the two species in question. Chapter four discusses the possibility of previous misidentification with regards to these two digeneans, however here I suggest that it could furthermore be possible that these two species are actually synonyms, and

that in fact previous cases of identification regarding *P. muris* and *P. elegans* have been influenced by the host species from which the specimen was recovered. If so, the question should be raised as to just what extent has the effect of host-induced intraspecific variation been previously overlooked in this group of digeneans.

There is then the question of the 304bp 28S DNA sequence presented for *P. muris* in the NCBI database (Lee *et al*., 2004). According to the methods and materials section, Lee *et al*. (2004) used metacercariae and adult specimens of *P. muris* for sequence analysis that they respectively obtained from dragonflies and infected mice supplied by Hong *et al*. (1999). In the paper of Hong *et al*. (1999) however, the focus was metacercariae derived from dragonflies with there being no mention of adult material. Lee *et al*. (2004) did not appear to conduct species identification prior to their analysis, at least there was no mention of this presented in their paper, thus the reliability of the parasite material used for generating this sequence data may be questionable. Further research is therefore required and this is a situation in which molecular differentiation could strongly assist.

This idea of synonymisation between *P. muris* and *P. elegans* has been incorporated as a fleeting comment at several points within the literature (Blankespoor, 1974; Gorman, 1980) however as far as can be determined, there have been no attempts to reconcile this notion. Investigation into this idea of possible synonymisation therefore could be suggested for future work. Similarly to the investigations carried out by Tkach *et al*. (2000a), type material for the two disputed species could be compared, re-described and both morphologically and molecularly defined in order to establish nomenclatural stability, although the acquisition of type material from various sources would be the greatest challenge to implement. Creation of a dichotomous key and generation of appropriate DNA sequences would nonetheless be extremely useful in assisting future identifications of either species should they be determined to be distinct or not.

Similarly, the concept of referring to members of the genus *Notocotylus* as 'bird' or 'rodent' species may also be considered precarious. The assumption that infection with *Notocotylus malhamensis* at Tarn Fen is restricted to the rodent host should be considered cautiously as it is highly likely that this species is furthermore capable of infecting waterfowl. Other *Notocotylus* species such as *Notocotylus imbricatus* Looss,

1893 (Cribb, 1991) and *Notocotylus ephemera* Nitzsch, 1817 (Gibson *et al*., 2005) have been recorded infecting both bird and mammalian hosts. Furthermore, according to Simon-Vicente *et al*. (1985a), when Ablasov and Iksanov identified *Notocotylus noyeri* Joyeux, 1922 typically considered to be a rodent species, from the caeca of a piscivorous bird in1958, they commented that this species might in fact be an avian digenean. It is highly likely therefore that the original dispersal unit for *N. malhamensis* to the Malham Tarn area has been a bird and that the parasite has undergone a host-shift in order to maintain a continual infection cycle at this location. According to Poullain and Nuismer (2012), there are three steps involved in the establishment of a host shift. The first step requires contact to be made between the parasite and the novel host species. The second involves the ability of the parasite to infect this host and thirdly the parasite must be capable of generating a self-sustaining infection within the novel host population (Poullain and Nuismer, 2012). *N. malhamensis* was recorded from the bank vole *Myodes glareolus* over a two-year period suggesting that its transmission ecology is relatively stable within this particular rodent population.

Malham Tarn however does boast a vast population of birds of which as many as 200 species have been recorded, including regular summer visitors, occasional visitors and vagrant species (Sutton and Shorrock, 2008). The description of *N. malhamensis* from Tarn Fen was published in the Journal of Parasitology in 2012 and has since been recorded by Professor Jerzy Behnke of the University of Nottingham, from the island of Anglesey, UK (personal communication, unpublished data). This new find by Behnke does suggest that birds may in fact be important in the dispersal of this helminth and furthermore that they may be an original source of infection.

It is suspected that there are three likely scenarios therefore occurring at Tarn Fen. One, that birds are seasonally carrying the digenean into the area and due to low definitive host specificity, infection can over-spill into the vole population. Two, that the parasite cycle is completely maintained by the vole population and is propagated by the ecology of the Tarn Fen area and three, both situations are contributing to the high prevalence rates observed. In the life cycle of *Notocotylus neyrai* Gonzalez-Castro, 1945, in Spain, it was noted that the southern water vole *Arvicola sapidus* deposits its excrement along the border of the water's edge (Simon-Vicente *et al.,* 1985b). If this is also occurring for

voles at Tarn Fen, then the eggs of *N. malhamensis* would be readily returned to the water body and within the vicinity of freshwater molluscs to continue the life cycle independently of other definitive host species.

What is interesting is that Behnke furthermore found *N. malhamensis* from the bank vole as was the case from Tarn Fen. Many parasites are known to exhibit strict host specificity and as such may be geographically limited in relation to their host's distribution. The vast majority of *Notocotylus* species however do predominantly utilise birds as their definitive host and as pointed out by Granovitch (1999), a single parasitic system might be interspersed by the migration of host species that are capable of a wide spatial distribution. It could therefore be the case that *N. malhamensis* from Tarn Fen and those identified by Behnke in Anglesey belong to the same parasitic system and that there may be further foci dispersed throughout the British Isles that have not yet been recorded. During the current study, *N. malhamensis* was recovered from the caecum of voles only; no infection of the wood mouse *A. sylvaticus* was identified despite careful observations. Within the UK, *A. sylvaticus* does tend to be the rodent of choice in the examination of helminth fauna (Elton *et al*., 1931; Lewis, 1968a; Lewis & Twigg, 1972; Behnke *et al.,* 1999; Abu-Madi *et al*., 2000) with studies involving voles being far more limited (Elton *et al*., 1931; Lewis, 1968b), and as such it is highly likely that this could simply be the reason why *N. malhamensis* has not been previously described from UK rodents.

The work carried out during this study has categorically contributed to the current knowledge in the field of helminthology. Not only has a new species of digenean been described (chapter 5), but several limitations associated with studying wildlife parasitology have been highlighted and several questions have been raised. This study has specifically demonstrated the need for the integration of modern molecular tools and classical species identification methods in order to ascertain species identifications. This however in itself is a complex task to achieve. As demonstrated by the present study, species identification can be simplified by the combination of these two disciplines and the identity of the *Plagiorchis* species at this location was confirmed by undertaking a molecular approach. Furthermore, the combined use of classical identification with molecular verification was proved useful for the elimination of

Notocotylus attenuatus from the list of potential *Notocotylus* species that could be present at Tarn Fen. This was a far simpler approach than that utilised to eliminate all other *Notocotylus* species for which there was no molecular data available, in particular when the differences in adult morphology were minute.

At the time of analysis however DNA sequence data for the digeneans under study was extremely lacking. For example, there was a paucity of sequence information available for the genus *Notocotylus* with as little as five sequences available for various genes. To add to matters, from these five sequences, three sequences had only been matched to the genus. Poulin and Leung (2010) reported that there has been a considerable decrease in taxonomic resolution of helminth species since the year 2000. They explained this phenomenon to be due to the loss of expertise in parasite taxonomy worldwide with new researchers focusing their funding priorities on scientific approaches that are likely to have a greater impact factor and therefore tending to focus on molecular approaches for diagnosis and means of identification. Although these approaches may be commonplace and completely relevant for parasite species that have been very well studied due to them being of medical, veterinary or ecological importance there appears to be an insufficient amount of data and therefore a gap in the present knowledge relating to those species that have not been well studied and therefore as yet have not been molecularly defined. Therefore despite attempts to utilise both approaches for species identification, this limitation in the field may ultimately require identification to be based upon attempting to acquire original species reports that are potentially irretrievable, lost or damaged.

There is therefore a need to refocus our approach to species differentiation by attempting to connect these two methods before all 'taxonomic expertise' is completely lost (Poulin and Leung, 2010). Particularly for species reports whereby only a morphological description is presented yet the species is known to demonstrate intraspecific variations or for reports that base diagnosis upon molecular data and morphologically identify the parasite to genus level only. In this respect, reports that include a full morphological description, the extent of intraspecific variation if known in addition to the DNA sequence of a common gene will be beneficial for future work.

Currently, Vasyl Tkach of the University of North Dakota, USA appears to be foremost in pushing these approaches forward having described as many as 33 new digenean species and emphasising the importance of using both molecular and traditional systematics for analysis. This is highly important in terms of enabling molecular diagnostic techniques to be developed. The subclass digenea is estimated to contain as many as 18000 nominal species (Bray, 2008) and new species are continually described on a daily basis (Boyce *et al*., 2012; Flores *et al*., 2012; Tkach *et al*., 2012). As such, this study demonstrated the inability to design species-specific molecular detection assays for the digenean species under study due to a limited access to molecular data. Design of such techniques requires a vast amount of information for a representative number of species within a genus. At the time of primer design, the NCBI nucleotide database held 116,401 DNA sequences for the digenea (13.01.11) which furthermore included several isolate sequences for many species. From this figure only 2224 sequences based on the Internal Transcribed Spacer regions of digenean trematodes had been deposited. This figure has now increased to 3525 sequences being present (analysis date: 31.01.13) indicating that this is an ongoing challenge and a considerable work-in progress for molecular taxonomists.

For those digenean species whose morphology, life history and genetic material have been well established, molecular techniques can prove to be highly beneficial. For example, Hamburger *et al*. (1988) demonstrated the successful use of PCR as a source of mass screening and control for Schistosomiasis by the detection of *Schistosoma mansoni* within its snail intermediate host. The high sensitivity of the PCR assay enabled the detection of a single infected snail when pooled with material extracted from 100 uninfected snails and a positive PCR result was detected just one day after penetration of the snail by the cercariae. Other studies have furthermore demonstrated the high sensitivity of the PCR assay in detecting a single cercariae or metacercariae within an intermediate host (Maleewong *et al*., 2003).

Wongsawad *et al.* (2006) analysed the DNA content of various life cycle stages of six trematode species (*Haplorchis taichui*, *Stellantchasmus falcatus*, *Opisthorchis viverrini*, *Ganeo tigrinus*, *Paramphistomum epiclitum* and *Fischoederius elongatus*). The quantity of DNA typically held within a single trematode egg ranged from 3.57 to 17.53ng and

trematode metacercariae from 40.98 to 50.70ng (Wongsawad *et al.,* 2006). The primers designed within this thesis were capable of detecting parasite DNA within host DNA at a concentration as low as 5×10^{-3}ng/µl for *N. malhamensis* and 0.5ng/µl for *P. elegans* with an optimal yield occurring at a DNA concentration of 50ng/µl, equating to the range of DNA expected to be present within a single digenean metacercariae (Wongsawad *et al*., 2006). Nonetheless, despite the successful development of PCR probes as originally specified at the beginning of this study, their use in the field was unsuitable due to the lack of sequence data as previously discussed.

Another factor further impacting the suitability of the designed molecular probes for species-specific use was a lack of information regarding potential host species. The DNA of both *Lymnaea peregra* and *Lymnaea stagnalis* snails were included in the specificity testing panel as potential first intermediate host species that were likely to be collected from the field. Both specimens were first-generation laboratory reared animals and as such could be regarded to be parasite free with some degree of certainty. Although, no cross-reactivity was observed between either of these hosts and the molecular probes for either digenean species, there is no certainty that other molluscan hosts would follow suit. Additionally, due to both resource and time constraints, it was not feasible to include the DNA from potential second intermediate host species within the testing panel. In addition to not knowing the full scope of insect species that would be ultimately collected from the sampling locations, there also remains a paucity of sequence data within the NCBI database for a considerable number of insect species. Ideally, DNA would be extracted from insect tissue that is not infected with parasites however the acquisition of laboratory reared insect samples as a means of ensuring the absence of parasite infection appeared beyond the scope of this study.

As also highlighted by the current study, other limitations can stem from the collection of wildlife samples. Often attaining samples can be unreliable and the numbers collected can be inconsistent resulting in limitation by sample size. For example, prior to commencement of the sampling period, adverse weather conditions observed during the winter of 2009, resulted in no wood mice or bank voles being trapped for the first three months of 2010 by the Malham Tarn Field Studies Centre. Population numbers were furthermore observed to remain low until some time in August (Shorrock and Sutton,

2009), which had detrimental effects on the number of samples collected during the first half of 2010. Desirably, statistical packages such as R statistics would have been applied to the data for analysis. Although attempts were made to analyse factors contributing to the helminth communities of rodents at this location using R (version 2.14.0) this was unsuccessful. Analysis required the use of a negative binomial zero-inflated model due to the number of excess zeros in the data set, however in several instances difficulties were encountered during the analysis as the data set was deemed too small for use in the programme.

Finally, it should be taken into consideration that the Malham Tarn area is an enormous sampling location in which there are many representative habitat types included. The area may be considered too large to be covered by the sampling efforts of a single individual and therefore this in itself is an extremely limiting factor. The likelihood of acquiring a larger number of hosts and covering a greater sampling area would be much more successful should a team rather be involved in collection. This would enable a larger scale screening of intermediate host species and an increased likelihood of identifying the hosts involved in transmission of digenean species at this location.

General conclusion

In conclusion, this study has been highly successful in contributing new information to the field of helminthology. The complexity associated with understanding parasite-host interactions has been demonstrated with the greatest challenge of all being the need for reliable, consistent and accurate identification tools for all parasite life cycle stages, in addition to the ability to be able to link these life cycle stages together. These are issues that can be significantly enhanced by the use of molecular tools however as indicated in the present study these tools are not without limitation. Although the use of DNA sequencing was proved to be highly useful at times for species identification during this research, attempts to create a robust molecular detection assay were deemed to be unsuccessful. This study has therefore highlighted the lack of DNA sequence data within databases for many understudied parasites and furthermore, emphasises the difficulty in stating with any degree of certainty that a designed molecular-based test is 'specific'. In hindsight, if this study was to be repeated, it would be highly recommended to initially screen the Malham Tarn area prior to attempting to design a molecular

detection assay for specific parasites. This screening approach would allow identification of potential closely related parasites, which themselves could be characterised at the level of the ITS region and the rRNA gene. The resulting DNA sequence data could then be exploited to assist the design of 'species-specific' primers for the parasites central to this study. As such, this investigation has clearly identified that the greatest obstacle to employing molecular probes for parasite screening is the acquisition of the necessary molecular data, which is not always feasible and remains an ongoing challenge for molecular taxonomists. This gap in the field therefore requires the collaborative efforts of individual researchers to continue to expand the relevant databases in order to maximise the potential use of available molecular techniques. The DNA sequences in combination with the morphological descriptions presented in this thesis for *Notocotylus malhamensis* and *Plagiorchis elegans* will be important resources for use by future researchers.

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Appendix A

1. The percentage of each sex of *Apodemus sylvaticus, Myodes glareolus and Microtus agrestis* trapped from each location during the present study. **Key = SP**: Spiggot Hill; **HM**: Ha Mire; **TF**: Tarn Fen; **TW**: Tarn Woods; **F**: female; **M**: male; **C**: combined.

Rodent species	Location	Sex	Percentage caught (%)	95% Confidence Limits	
				Lower	Upper
A. sylvaticus					
(n = 52)	**TW**	**F**	30.8	19.85	44.34
		M	69.2	55.66	80.15
		C	**44.4**	35.76	53.48
(n = 21)	**TF**	**F**	52.4	32.37	71.66
		M	47.6	28.34	67.63
		C	**18.0**	11.98	25.96
(n = 17)	**SP**	**F**	47.1	26.16	69.04
		M	52.9	30.96	73.84
		C	**14.5**	09.18	22.14
(n = 27)	**HM**	**F**	40.7	24.48	59.31
		M	59.3	40.69	75.52
		C	**23.1**	16.33	31.54
My. glareolus					
(n = 20)	**TW**	**F**	65.0	43.16	82.01
		M	35.0	17.99	56.84
		C	**37.0**	25.40	50.40
(n = 9)	**TF**	**F**	33.3	11.73	64.91
		M	66.7	35.09	88.27
		C	**16.7**	08.79	28.97
(n = 8)	**SP**	**F**	87.5	50.78	99.89
		M	12.5	00.11	49.22
		C	**14.8**	07.44	26.87
(n = 17)	**HM**	**F**	64.7	41.16	82.83
		M	35.3	17.17	58.84
		C	**31.5**	20.62	44.80
Mi. agrestis					
(n = 3)	**TW**	**F**	33.3	05.63	79.76
		M	66.7	20.24	94.37
		C	**33.3**	11.73	64.91
(n = 6)	**TF**	**F**	83.3	41.78	98.86
		M	16.7	01.14	58.22
		C	**66.7**	35.09	88.27

2. Trapping rates of *Apodemus sylvaticus, Myodes glareolus and Microtus agrestis* each season in the present study. **Key = Wi**: winter; **Sp**: spring; **Su**: summer; **Au**: autumn; **F**: female; **M**: male; **C**: combined.

Rodent species	Season	Sex	Percentage caught (%)	95% Confidence Limits	
				Lower	Upper
A. sylvaticus					
(n = 8)	**Wi**	**F**	25.0	40.09	93.69
		M	75.0	06.31	59.91
		C	**06.8**	03.32	13.10
(n = 8)	**Sp**	**F**	50.0	21.52	78.48
		M	50.0	21.52	78.48
		C	**06.8**	03.32	13.10
(n = 22)	**Su**	**F**	18.2	60.88	93.29
		M	81.8	06.71	39.12
		C	**18.8**	12.69	26.90
(n =79)	**Au**	**F**	45.6	43.50	64.95
		M	54.4	35.05	56.50
		C	**67.5**	58.58	75.35
My. glareolus					
(n = 10)	**Wi**	**F**	90.0	57.40	>99.99
		M	10.0	<0.01	42.60
		C	**18.5**	10.19	31.03
(n = 8)	**Sp**	**F**	25.0	06.31	59.91
		M	75.0	40.09	93.69
		C	**14.8**	07.44	26.87
(n = 16)	**Su**	**F**	68.7	44.15	86.09
		M	31.3	13.91	55.85
		C	**29.6**	19.07	42.90
(n = 20)	**Au**	**F**	60.0	38.60	78.18
		M	40.0	21.83	61.40
		C	**37.0**	25.40	50.40
Mi. agrestis					
(n = 4)	**Su**	**F**	50.0	15.00	85.00
		M	50.0	15.00	85.00
		C	**44.4**	18.84	73.37
(n = 5)	**Au**	**F**	80.0	35.96	97.97
		M	20.0	02.03	64.04
		C	**55.6**	26.63	81.16

1: *Apodemus sylvaticus*. Key = SP: Spiggot Hill; **HM**: Ha Mire Plantation; **TF**: Tarn Fen; **TW**: Tarn Woods.

Site	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
SP	**F**	17.25±4.03	**17.41±5.36**	11-22	7.51±0.58	**7.61±0.90**	7.0-8.5
	M	17.56±6.58		08-29	7.70±1.15		6.3-9.2
HM	**F**	15.18±2.71	**17.30±4.37**	12-20	7.46±0.56	**7.84±0.76**	6.6-8.5
	M	18.75±4.75		10-26	8.09±0.79		7.2-9.3
TF	**F**	13.55±6.39	**16.43±6.82**	04-25	7.18±1.65	**7.61±1.36**	4.5-9.8
	M	19.60±6.04		11-26	8.09±0.77		7.2-9.1
TW	**F**	17.88±4.21	**16.88±6.14**	09-26	7.86±0.80	**8.12±0.69**	5.9-9.0
	M	18.50±3.68		12-28	8.23±062		7.0-9.5

2: *Myodes glareolus*. The sample set from Spiggot Hill contained a single male* with a weight of 24.0g and a length of 8.5cm. **Key = SP**: Spiggot Hill; **HM**: Ha Mire Plantation; **TF**: Tarn Fen; **TW**: Tarn Woods.

Site	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
SP	**F**	13.85±1.83	**15.12±3.98**	11-16	7.77±0.68	**7.86±0.68**	7.0–8.7
	M	24.00±.000*		-24	8.50±.0.00*		-8.5
HM	**F**	16.04±4.31	**17.91±5.5**	12-27	7.48±1.06	**7.85±1.13**	6.3-9.5
	M	21.36±6.13		13-27	8.52±0.99		7.2-9.3
TF	**F**	19.97±4.40	**20.20±5.17**	16-25	8.10±1.15	**8.58±0.82**	6.8-9.0
	M	20.31±5.92		13-27	8.82±0.58		8.1-9.4
TW	**F**	13.05±5.54	**13.64±5.03**	10-26	7.75±0.85	**7.81±0.82**	7.0-8.9
	M	14.73±4.09		12-23	7.93±0.80		6.5-8.7

3: *Microtus agrestis*. The data set from Tarn Fen contained only a single male* with a weight of 35.25g and a length of 11.5cm and the sample set from Tarn Woods contained only a single female* with a weight of 21.00g and a length of 10.00cm. **Key = SP**: Spiggot Hill; **HM**: Ha Mire Plantation; **TF**: Tarn Fen; **TW**: Tarn Woods.

Site	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
TF	**F**	21.60±5.18	**24.33±6.78**	16-28	8.90±0.82	**9.30±1.29**	7.5-09.5
	M	35.25±0.00*		-35	11.50±0.00*		-11.5
TW	**F**	21.00±0.00*	**18.67±5.86**	-21	10.00±0.00*	**9.03±1.27**	-10.0
	M	17.50±7.78		12-23	8.55±1.34		7.6-09.5

4: ***Apodemus sylvaticus*****. Key = Wi:** winter**; Sp:** spring**; Su:** summer**; Au:** autumn**.**

Season	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
Wi	**F**	16.50±6.36	**20.88±4.79**	12-21	7.50±1.13	**8.08±0.79**	6.7-8.3
	M	22.33±3.72		19-29	8.27±0.66		7.6-9.2
Sp	**F**	21.50±4.20	**21.75±3.85**	17-26	7.53±0.75	**7.89±0.82**	7.0-8.6
	M	22.00±4.08		16-25	8.25±0.80		7.3-9.2
Su	**F**	18.75±6.13	**19.64±4.56**	11-25	8.50±1.15	**8.36±0.81**	7.0-9.8
	M	19.83±4.34		14-28	8.33±0.75		7.2-9.5
Au	**F**	15.17±4.21	**15.35±5.61**	06-22	7.44±0.99	**7.74±0.92**	4.5-9.0
	M	17.10±4.50		08-25	7.99±0.77		5.8-9.3

5: ***Myodes glareolus*****.**The sample set from winter contained a single male***** with a weight of 10.11g and a length of 6.5cm. **Key = Wi:** winter**; Sp:** spring**; Su:** summer**; Au:** autumn**.**

Season	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
Wi	**F**	15.69±1.44	**15.13±2.23**	15-19	8.14±0.73	**7.98±0.86**	7.4-8.9
	M	10.11±0.00*****		-10	6.50±0.00*****		-6.5
Sp	**F**	19.62±4.93	**23.24±3.51**	15-25	8.10±1.27	**8.69±0.73**	7.2-9.0
	M	24.45±0.74		24-26	8.88±0.48		8.1-9.3
Su	**F**	14.03±4.69	**16.29±6.20**	11-26	7.55±0.87	**7.83±0.93**	6.3-8.8
	M	21.27±6.64		13-28	8.44±0.81		7.2-9.2
Au	**F**	14.01±6.06	**14.11±4.71**	12-27	7.43±0.95	**7.76±0.95**	6.3-9.5
	M	14.25±1.58		12-17	8.25±0.77		7.3-9.4

6: ***Microtus agrestis*****.** The sample set from summer contained only two females both of which measured 9.5cm in length ***** . The sample set from autumn contained a single male***** with a weight of 12g and a length of 7.6cm. **Key = Su:** summer**; Au:** autumn**.**

Season	Sex	Mean weight (g) ± SD	Combined mean weight (g) ± SD	Range (g)	Mean length (cm) ± SD	Combined mean length (cm) ± SD	Range (cm)
Su	**F**	25.71±2.99	**27.42±5.65**	24-28	09.5±0.00	**10.0±1.00**	-09.5*
	M	29.13±8.66		23-35	10.5±1.41		9.5-11.5
Au	**F**	17.50±2.12	**15.67±3.51**	16-19	8.88±1.03	**8.62±1.06**	7.5-10.0
	M	12.00±0.00*		-12	7.60±0.00*		-7.6

1. Age structure of *Apodemus sylvaticus, Myodes glareolus and Microtus agrestis* examined each season during the present study. **Key = Wi**: winter; **Sp**: spring; **Su**: summer; **Au**: autumn.

Rodent species	Season	Age cohort	Percentage caught (%)	95% Confidence Limits	
				Lower	Upper
A. sylvaticus					
(n = 8)	**Wi**	**Juvenile**	12.5	00.11	49.22
		Young adult	12.5	00.11	49.22
		Adult	75.0	40.09	93.69
(n = 8)	**Sp**	**Juvenile**	0	0	0
		Young adult	37.5	13.49	69.62
		Adult	62.5	30.38	86.51
(n = 22)	**Su**	**Juvenile**	04.5	<00.01	23.51
		Young adult	45.5	26.91	65.35
		Adult	50.0	30.72	69.28
(n = 79)	**Au**	**Juvenile**	19.0	11.74	29.11
		Young adult	53.2	42.27	63.77
		Adult	27.8	19.12	38.63
My. glareolus					
(n = 10)	**Wi**	**Juvenile**	10.0	<00.01	42.60
		Young adult	90.0	57.40	>99.99
		Adult	0	0	0
(n = 8)	**Sp**	**Juvenile**	0	0	0
		Young adult	12.5	00.11	49.22
		Adult	87.5	50.78	99.89
(n = 16)	**Su**	**Juvenile**	37.4	18.37	61.47
		Young adult	31.3	13.91	55.85
		Adult	31.3	13.91	55.85
(n = 20)	**Au**	**Juvenile**	15.0	04.39	36.88
		Young adult	80.0	57.82	92.51
		Adult	5.00	<00.01	25.41
Mi. agrestis					
(n = 4)	**Su**	**Juvenile**	0	0	0
		Young adult	0	0	0
		Adult	100	45.41	100
(n = 5)	**Au**	**Juvenile**	20.0	02.03	64.04
		Young adult	20.0	02.03	64.04
		Adult	60.0	22.91	88.40

2. Age structure of *Apodemus sylvaticus, Myodes glareolus and Microtus agrestis* examined from each location during the present study. **Key = SP**: Spiggot Hill; **HM**: Ha Mire; **TF**: Tarn Fen; **TW**: Tarn Woods.

Rodent species	Location	Age cohort	Percentage caught (%)	95% Confidence Limits	
				Lower	Upper
A. sylvaticus					
(n = 52)	**TW**	**Juvenile**	05.8	01.38	16.25
		Young adult	55.8	42.33	68.41
		Adult	38.4	26.45	52.06
(n = 21)	**TF**	**Juvenile**	33.3	17.05	54.78
		Young adult	33.3	17.05	54.78
		Adult	33.3	17.05	54.78
(n = 17)	**SP**	**Juvenile**	17.6	05.38	41.84
		Young adult	35.3	17.17	58.84
		Adult	47.1	26.16	69.04
(n = 27)	**HM**	**Juvenile**	14.8	05.30	33.10
		Young adult	51.9	33.98	69.26
		Adult	33.3	18.53	52.29
My. glareolus					
(n = 20)	**TW**	**Juvenile**	36.8	19.05	59.06
		Young adult	52.6	31.70	72.67
		Adult	10.5	01.70	32.63
(n = 9)	**TF**	**Juvenile**	0	0	0
		Young adult	44.4	18.84	73.37
		Adult	55.6	26.63	81.16
(n = 8)	**SP**	**Juvenile**	25.0	06.31	59.91
		Young adult	62.5	30.38	86.51
		Adult	12.5	00.11	49.22
(n = 17)	**HM**	**Juvenile**	11.8	02.03	35.59
		Young adult	58.8	35.95	78.44
		Adult	29.4	12.99	53.43
Mi. agrestis					
(n = 3)	**TW**	**Juvenile**	33.3	05.63	79.76
		Young adult	0	0	0
		Adult	66.6	20.24	94.37
(n = 6)	**TF**	**Juvenile**	0	0	0
		Young adult	50.0	18.76	81.24
		Adult	50.0	18.76	81.24

Appendix D

1. Overall prevalence and intensity of helminths recorded during the present study. **Key: As** = *Apodemus sylvaticus* (n=117); **Mg** = *Myodes glareolus* (n=54); **Ma** = *Microtus agrestis* (n=9).

Helminth	Host	Host sex	Prevalence (%)	95% Confidence limits		Intensity range	Mean intensity ± SD
				Lower	Upper		
P. elegans	As	M	32.4	22.62	43.98	0-275	30.0±66.1
		F	08.7	02.90	20.86	0-26	7.3±12.51
		C	23.0	16.33	31.54	-	26.6±61.51
N. malhamensis	Mg	M	20.0	07.49	42.18	0-175	67.8±82.1
		F	05.9	00.65	20.07	0-294	148±205.8
		C	11.1	04.83	22.55	-	94.7±119.4
	Ma	M	00.0	00.00	61.75	-	-
		F	50.0	18.76	81.24	0-6	4.3±2.9
		C	33.3	11.73	64.91	-	-
B. recurvum	As	M	02.8	00.19	10.29	0-3	2.0±1.4
		F	00.0	00.00	09.20	-	-
		C	-	-	-	-	-
H. polygyrus	As	M	71.8	60.40	81.02	0–76	11.3±14.9
		F	60.9	46.43	73.63	0-25	4.9±5.2
		C	67.5	58.58	75.35	-	9.2±12.7
H. glareoli	Mg	M	05.0	<00.01	25.41	-5	-
		F	00.0	00.00	12.07	-	-
		C	-	-	-	-	-
	Ma	M	33.3	05.63	79.76	-83	-
		F	00.0	00.00	44.28	-	-
		C	-	-	-	-	-
S. stroma	As	M	23.9	13.76	38.09	0-117	32.2±38.9
		F	32.4	22.62	43.98	0-108	33.1±35.4
		C	29.1	21.58	37.87	-	32.5±37.2
***Syphacia* sp.**	Mg	M	0.00	00.00	18.98	-	-
		F	17.7	07.97	33.89	0-37	15.2±13.2
		C	11.1	04.83	22.55	-	-
A. murissylvatici	As	M	07.0	02.68	15.81	0-8	4.2±2.7
		F	06.5	01.59	18.15	0-1	1.0±0.0
		C	06.8	03.32	13.10	-	3.0±2.7
	Mg	M	05.0	<00.01	24.41	-6	-
		F	11.8	04.07	27.22	0-26	10.3±10.6
		C	09.3	03.60	20.33	-	9.4±9.4
H. diminuta	Mg	M	05.0	<00.01	25.41	0-1	-
		F	8.82	02.29	23.72	0-1	1.0±0
		C	7.40	02.42	18.05	-	-
	Ma	M	-	-	-	-	-
		F	16.7	01.14	58.22	0-13	-
		C	-	-	-	-	-

2. Common helminth infections of male (**M**) and female (**F**) rodents of each species.

Helminth species	Host sex	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
			Lower	Upper	Lower	Upper	
P. elegans	M	32.4	22.62	43.98	0	275	30.0±66.09
	F	08.7	02.90	20.86	0	26	7.0±12.5
N. malhamensis	M	57.1	24.98	84.25	0	175	67.8±82.1
	F	62.5	30.38	86.51	0	294	62.0±129.7
H. polygyrus	M	71.8	60.40	81.02	0	76	11.54±14.89
	F	60.9	46.43	73.63	0	25	4.29±5.18
S. stroma	M	32.4	22.62	43.98	0	117	32.2±38.9
	F	23.9	13.76	38.09	0	108	33.1±35.3
***Syphacia* sp.**	M	00.0	00.00	18.98	-	-	-
	F	17.7	07.97	33.89	0	37	15.2±13.2

3. Common helminth infections of adult (**A**) young adult (**Y**) and juvenile (**J**) rodents of each species.

Helminth species	Host age	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
			Lower	Upper	Lower	Upper	
P. elegans	A	43.0	29.67	57.79	0	275	36.0±71.72
	Y	14.3	07.16	26.00	0	24	5.0±7.86
	J	00.0	00.00	21.63	0	0	-
N. malhamensis	A	62.5	30.38	86.51	0	294	95.8±133.4
	Y	57.1	24.98	84.25	0	89	25.5±42.4
	J	-	-	-	-	-	-
H. polygyrus	A	84.1	70.32	92.39	0	76	12.4 ± 14.1
	Y	66.1	52.96	77.12	0	35	5.4 ± 6.9
	J	29.4	12.99	53.43	0	8	2.8 ± 3.0
S. stroma	A	29.5	18.06	44.32	0	108	21.0±37.1
	Y	33.9	22.88	47.04	0	117	36.7±35.4
	J	11.8	02.03	35.59	0	100	67.5±45.9
***Syphacia* sp.**	A	07.7	<00.01	35.42	0	4	-
	Y	03.2	<00.01	17.58	0	21	-
	J	40.0	16.71	68.84	0	37	16.5±15.37

4. Common helminth infections by season (**Wi**) winter (**Sp**) spring (**Su**) summer (**Au**) autumn.

Helminth species	Season	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
			Lower	Upper	Lower	Upper	
P. elegans	Wi	00.0	-	-	-	-	-
	Sp	12.5	00.11	49.22	0	179	-
	Su	27.3	12.88	48.43	0	8	3±2.94
	Au	25.3	16.96	35.96	0	275	26±61.46
N. malhamensis	Wi	-	-	-	-	-	-
	Sp	100	38.25	100.0	0	294	156.7±147.4
	Su	33.3	09.25	70.43	0	6	4.5±2.1
	Au	80.0	35.96	97.97	0	89	25.5±42.4
H. polygyrus	Wi	100	62.78	100.0	0	18	8.3±6.7
	Sp	87.5	50.78	99.89	0	35	12.0±12.1
	Su	81.8	60.88	93.29	0	76	9.2±17.2
	Au	58.2	47.21	68.48	0	65	8.9±11.9
S. stroma	Wi	37.5	13.49	69.62	0	13	8.0±5.0
	Sp	37.5	13.49	69.62	0	100	36.3±55.1
	Su	40.9	23.21	61.31	0	32	14.4±11.1
	Au	24.1	15.90	34.61	0	117	44.3±41.6
***Syphacia* sp.**	Wi	00.0	00.00	32.09	0	-	-
	Sp	00.0	00.00	37.22	0	-	-
	Su	20.0	01.57	31.32	0	19	13.5±7.8
	Au	16.0	05.79	35.27	0	37	16±16.4

5. Common helminth infections by site (**TW**) Tarn Woods (**TF**) Tarn Fen (**SP**) Spiggot Hill (**HM**) Ha Mire.

Helminth species	Site	Prevalence (%)	95% Confidence limits		Intensity range		Mean intensity ± SD
			Lower	Upper	Lower	Upper	
P. elegans	TW	23.1	13.58	36.28	0	179	21±50.33
	TF	19.1	07.08	40.59	0	6	2±2.5
	SP	5.90	<00.01	28.92	0	275	-
	HM	37.1	21.47	55.84	0	66	19±24.2
N. malhamensis	TW	-	00.00	16.91	-	-	-
	TF	60.0	35.67	80.25	0	294	94.7±119.4
	SP	-	00.00	37.22	-	-	-
	HM	-	00.00	21.63	-	-	-
H. polygyrus	TW	73.1	59.65	83.33	0	76	10.3±16.5
	TF	71.4	49.79	86.44	0	21	7.5±6.3
	SP	64.7	41.16	82.83	0	35	11.9±10.6
	HM	55.6	37.30	72.43	0	22	6.1±5.9
S. stroma	TW	38.5	26.45	52.06	0	117	30.4±38.6
	TF	33.3	17.05	54.78	0	100	26.9±37.3
	SP	29.4	12.99	53.43	0	108	53±39.3
	HM	07.4	00.96	24.47	-	35	22.5±17.7
***Syphacia* sp.**	TW	10.0	01.57	31.32	0	37	14.3±19.65
	TF	00.0	00.00	34.46	0	0	-
	SP	12.5	00.11	49.22	-	-	-
	HM	17.6	05.38	41.84	0	19	13.5±7.78

APPENDIX E: Key morphological criteria used to differentiate each *Notocotylus* species (n = 68) identified under the *Notocotylus* Diesing group, 1839 against the Malham Tarn specimens. Black shading indicates a paucity of information available for a morphological comparison to be made. Only criteria used to differentiate against the Tarn Fen specimens has been included in the table. Full species descriptions from the literature can be accessed via the reference section.

Species	Morphological description from Literature										Reference
	Length (mm)	Ventral gland composition (lateral / median / lateral)	Metratterm: cirrus sac ratio (%)	Cirrus	Cirrus sac length (µm)	No. uterine loops	No. uterine loops ahead vitelline glands	Uterine loops confined/ overflow	Caecum	Position of genital pore to bifurcation	
N. attenuatus			55-70					Confined			Beverley-Burton, 1961
N. ephemera		9-10 lateral									Dubois, 1951
N. linearis			33								Dubois, 1951
N. vespertilionis											
N. triserialis			33-50	Papillated							Pike, 1969
N. gibbus		11 / 4-5 / 11									Beverley-Burton, 1961
N. imbricatus			56					Confined			Beverley-Burton, 1961
N. aegyptiacus			<50								Dubois, 1951
N. urbanensis	<3.5	13-14 /13-14 / 13-14	50				10				Harrah, 1922
N. filamentis	<3.30	12-13 / 12-13 / 12-13	100	Papillated				Confined			Barker, 1915
N. seineti	2.00	12 /12-14 / 12	66								Lal, 1935a; Dubois, 1951
N. noyeri	<4.16	15 /15 / 15		Smooth							Simon-Vicente *et al.*, 1985a
N. chionis						16 - 26					Dubois, 1951
N. tachyeretis	>5.2				2500 - 2800	21 - 26				Ventral	Duthoit, 1931
N. intestinalis				Smooth	600 - 810						Besprozvannykh, 2010
N. naviformis	1.80	10 lateral									Dubois, 1951
N. pacifera		11 /4 / 11									Guillen and Morales, 2003
N. thienemanni		10 / 15 / 10	50								Lal, 1935a
N. magniovatus			25 - 50								Dubois, 1951
N. parviovatus						21+					Lal, 1935a
N. babai			25 - 50				10 - 11				Bhalerao, 1935
N. indicus	2.18				<580			Confined			Lal, 1935a
N. lucknowensis	2.63		33							Ventral	Lal, 1935a
N. ralli						27 - 34					Baylis, 1936
N. anatis			25			22 - 27					Ku, 1937
N. orientalis		23 / 23 / 23				>20					Ku, 1937
N. dafilae							6 - 8				Dubois, 1951
N. micropalmae							9				Dubois, 1951
N. regis		10 median									Dubois, 1951
N. porzanae							0	Confined			Dubois, 1951
N. stagnicolae	3.40							Confined			Herber, 1942
N. neyrai	<3.85		<30								Simon-Vicente *et al.*, 1985a

N. skrjabini		5 / 5 / 5									Bisset, 1977
N. lopezneyrai		11-12/13/11-12									Dubois & Vigueras, 1953
N. mamii	<2.4	11-12 / 11-13 / 11-12	+100					Confined		Ventral	Hsu, 1954
N. solitaria	2.00	15 / 14 / 15		Tuberculated	<704			Confined			Singh, 1954.
N. ratti	<2.57	10-11 / 5-6 / 10-11	40								Lea *et al*., 1956
N. marinus	1.84		66		2 x uterus						Ginetsinskaya, 1958
N. minutus	<1.50	16 / 16/ 16	50		<560						Stunkard, 1960
N. duboisianus		10-13 / 9-11 / 10-13									Cribb, 1991
N. nathipandei		18 / 14 / 18	40			20	7	Confined		Ventral	Nath and Pande, 1963
N. ponticus											
N. wetlugensis									Diverticular		Tenora *et al*., 1983.
N. kiangsuensis	<2.17	10 / 5 /10							Diverticular		I-Ping, 1965
N. atlanticus			<50		600 - 840						Stunkard, 1966
N. duboisi	Synonym of *N. imbricatus* Looss, 1893										Stunkard, 1966
N. breviserialis		4 / 5 / 4									Stunkard, 1967
N. zduni			50	Eggs possess a tuft of short and thin filaments at one end							Vassilev and Kanev, 1984
N. barmerensis		11 / 4-5 / 11									Gupta, 1970
N. panjnadensis		12 /12 /12	60			20					Bhutta and Khan, 1975
N. casarcai		6-10/6-10/6-10									Gupta & Jehan, 1977
N. kanpurensis		23 / 19 / 23									Deshmukh, 1985.
N. poecilorhynchai		12/11/12								Ventral	Gupta & Jehan, 1977
N. schmidti		4 / 3 / 4									Brooks and Heard, 1977
N. anseri		23 / 20 / 23									Deshmukh, 1985.
N. gallinulae		10-13 / 9-11 / 10-13									Cribb, 1991
N. gonzalezi	<4.57	15 / 15 / 15	25-30	smooth							Simon-Vicente *et al*., 1985a
N. paithanensis		11 / 5 / 11									Deshmukh, 1985
N. vinodae						17-21		Confined		Ventral	Gupta & Singh, 1983
N. mcdonaldi			<50			20-22					Gupta & Singh, 1983
N. guptai			<50			15-20			Diverticular	Prebifurcal	Gupta & Singh, 1983
N. ajgaini						20		Confined	Diverticular	Prebifurcal	Gupta & Singh, 1983
N. lianhuaensis	<1.35	4-6 / 6-7 / 4-6				10-11					Qiongzhang, 1988
N. johnstoni		10-13 / 9-11 / 10-13		Smooth						Prebifurcal	Cribb, 1991
N . polylecithus	<3.05	27-28 / 24-25 / 27-28									Qiongzhang, 1992
N. biomphalariae		11 / 4 / 11									Flores & Brugni, 2005
N. fosteri		10-13 / 10-13 / 10-13				15 - 18	5 - 7			Prebifurcal	Kinsella and Tkach, 2005
N. loeiensis			100							Prebifurcal	Chaisiri *et al*., 2011