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The life cycle of the parasite *Pomphorhynchus tereticollis* in reference to 0+ cyprinids and the intermediate host *Gammarus* spp in the UK.

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Abstract

Pomphorhynchus tereticollis is a recently resurrected parasite species that spans the UK and continental Europe. The parasite is the only *Pomphorhynchus* spp in the UK and has been researched since the early 1970's. The species has an indirect life cycle which uses a *Gammarus* spp as an intermediate host and cyprinids and salmonids as final hosts although the main hosts are *Squalis cephalus* (*S. cephalus*) and *Barbus barbus* (*B. barbus*). Research on *P. tereticollis* has primarily focused on mature fish while there is limited research on 0+ fish and the intermediate host in the UK. Previous studies have shown that paratenic and post-cyclic transmission may occur in the life cycle of *P. tereticollis* although there has been little field research to demonstrate how much of an impact this may have on the parasite. As well as this, little data exists for what may influence infection within 0+ fish species. Therefore, a field study was designed to understand the relationship between *P. tereticollis* and 0+ fish as well as to explore the potential for post-cyclic or paratenic transmission of *P. tereticollis*. *B. barbus*, *Leuciscus leuciscus* (*L. leuciscus*), *S. cephalus* and *Phoxinus phoxinus* (*P. phoxinus*) were sampled from the River Teme (UK) in the summer and early autumn of 2015, 2016 and 2019. Data was collected on fish length, fish gape, gut fullness, temperature and date. A binary logistic regression was conducted to find variables that predicted infection with the parasite. Fish length, gut fullness and species where all factors that predict infection with *P. tereticollis* in the 0+ fish. Each species had different variables that predicted infection with fish length the only variable that predicts infection in *B. barbus* and only gut fullness predicting *S. cephalus* infection. Infection in *L. leuciscus* was predicted by temperature, date and gut fullness while infection in *P. phoxinus* was predicted by fish length, gut fullness and date. *P. phoxinus* is likely to aid transmission of *P. tereticollis* in a paratenic and post-cyclic manner due to the discovery of extra-intestinal infections and limited attachment to the alimentary tract when infections where found. A low prevalence was found in 0+ *S. cephalus* which suggests *Gammarus* spp are an insignificant part of the diet for the species. Diet is likely to be the driver for differences in infection of the different species. Gut fullness was higher in infected individuals compared to uninfected individuals in all species except *S. cephalus* which suggests an energy deficit caused by the parasite. This may cause a higher probability of predation in 0+ fish and facilitate the potential paratenic and post-cyclic transmission of *P. terticollis*.

As well as this, Recent research in Switzerland and France has shown that *P. tereticollis* displays a preference for *Gammarus fossarum* (*G. fossarum*) compared to *Gammarus pulex* (*G. pulex*). Until recently, it was thought that *G. pulex* was the only fresh water *Gammarus* spp in southern England until *G. fossarum* was discovered with eDNA, morphological and standard DNA extraction techniques. Therefore, research was conducted to examine the intermediate host use of *P. tereticollis. Gammarus* spp were collected from four rivers in the south of England and dissected to find the prevalence. Species was observed morphologically using the location of Plumose setae. *G. fossarum* was present in all four rivers tested and was the most dominant species in eastern flowing rivers. Only *G. fossarum* carried cycstacanth infections of *P. tereticollis* in all sites tested. Rivers which contained the highest proportion of *G. fossarum* to *G. pulex* had the highest prevalence of *P. tereticollis*. The distribution of *G. fossarum* in the UK is likely to explain the discontinuous distribution of *P. tereticollis* described in previous research. The preference is likely to be due to overlap in the distributions of *P. tereticollis* and *G. fossarum* before and during previous ice ages. The misidentification of *G. fossarum* in the UK and the strong preference of *P. tereticollis* for one gammarid over another highlights the difficulties in conservation of habitats regarding cryptic species.

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Chapter 1: The Factors predicting prevalence of *Pomphorhynchus tereticollis* in 0+ Cyprinids in the River Teme (UK)

1. Introduction

Pomphorhynchus tereticollis is an acanthocephalan parasite which is abundant in many areas of Europe (Perrot-Minnot et al, 2018) and capable of infecting 16 species of fish, many of which belong to the Cyprinidae and Salmonidae families (Kennedy, 2006 p.22). Similar to all species in the Acanthocephalan phyla, the parasite has an indirect life cycle which requires at least a crustacean (*Gammarus* spp for *P. tereticollis*) as an intermediate host and a vertebrate as a final host to successfully complete its life cycle (Kennedy, 2006 p.12). The life cycle of *P. tereticollis* beings when a shelled acanthor is ingested by a feeding *Gammarus* spp (Crustacea: Arthropoda) in the benthos (fig 1). The acanthor develops within the *Gammarus* spp and transitions through three life stages from acanthor to late acanthella to the cystacanth (Kennedy, 2006 p. 14). Its development within the intermediate host takes between 40-65 days (Rumpus, 1973) and is only infective to final hosts when the cystacanth stage is reached (Kennedy, 2006 p.14). If the *Gammarus* spp is predated by a fish during this period, the cystacanth will evert its proboscis within the alimentary tract of the fish and attach to the wall using hooks on the proboscis (Kennedy, 2006 p.13). Successful establishment is dependent on a variety of factors including temperature (Kennedy, 1972) and species (Hine and Kennedy, 1974a). Established individuals will seek members of the opposite sex in order to reproduce. Specifically, *P. tereticollis* males will progress down the alimentary tract mating with females until they are expelled from the vertebrate host (Kennedy, 2006, p.13), while the females mature and produce shelled acanthors which are then expelled from the fish to be consumed by gammarids (Kennedy, 2006 p.17).

P. tereticollis has previously been considered a generalist at the definitive host stage as the parasite is prevalent in many species of fish (Hine and Kennedy, 1974a). However, there are clear definitive hosts for *P. tereticollis* as evidenced by different maturation rates in different fish species. For example, 60-70% of female *P. tereticollis* in *Squalius cephalus* (*S. cephalus*) and *Barbus barbus* (*B. barbus*) are gravid and produce acanthors (Kennedy, 2006 p.67) while *P. tereticollis* never matures in *Phoxinus phoxinus* (*P. phoxinus*) in the UK (Kennedy, 1999). Other fish species such as *Leuciscus leuciscus* (*L.*

leuciscus) can act as a definitive host for the parasite but less females mature and become gravid than in *S. cephalus* and *B. barbus* (Hine and Kennedy, 1974a). Like *L. leuciscus* salmonids can also act as a definitive host to a lesser extent (Hine and Kennedy, 1974a).

Recent research completed by Medoc et al, (2011) demonstrated that *P. tereticollis* may use paratenic hosts. A paratenic host is defined as a host where no maturation occurs and parasites may be found in the body cavity (instead of the alimentary canal) with their proboscis partially everted (Kennedy, 2006 p.20). Medoc et al, (2011) showed that successful transmission of *Pomphorhynchus* spp was 18% when mature *P. phoxinus* naturally infected with extra-intestinal infections were predated by *S. cephalus.* As well as the possibility of paratenic hosts, Kennedy, (1999) has shown the possibility of post-cyclic transmission in *P. tereticollis* which is defined as the transfer of a parasite from one host to another through predation (Kennedy, 1999) although it is usually defined as transfer of the parasite from definitive host to a predator (Kennedy, 2006 p.24). This is different from the use of paratenic hosts as the parasite does not need to be present extra-intestinally (Kennedy, 2006 p.20). Both these mechanisms have the potential to play a significant role in transmission and life cycle of *P. tereticollis.* Despite their importance, there is still limited research regarding which species may contribute to the life cycle of *P. tereticollis* in a paratenic or post-cyclic manner in UK field studies.

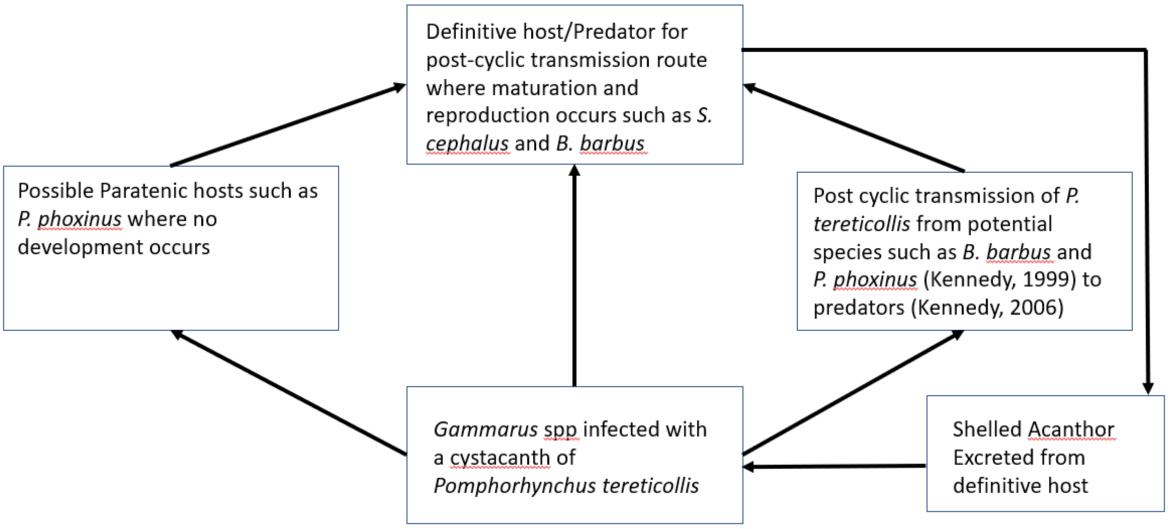


Figure 1. life cycle of *Pomphorhynchus tereticollis* with potential paratenic and post-cyclic transmission routes.

Gammarus spp are a highly ubiquitous organism across the whole of Europe and North America and are considered a keystone species (Labude et al, 2016). The genus is usually found in the benthos of rivers and regularly feed on detritus such as leaf litter and algae. They have also been known to be cannibalistic and predators of smaller invertebrates in certain circumstances. The genus is considered an integral part of most European freshwater food webs and could be a contributing factor to the wide distribution of *Pomphorhynchus* spp across America and Europe (Perrot-Minnot et al, 2017).

Although *P. tereticollis* is considered a generalist parasite in both the intermediate and definitive stage, this could be due to considerable confusion between *P. tereticollis* and *Pomphorhynchus laevis* two cryptic species in the genus. The parasites are closely related and until recently were considered the same species. Spakulova et al, (2011) resurrected *P. tereticollis* as a species using morphological and molecular techniques which demonstrated that there are two distinct species. As *P. tereticollis* is the only species in the UK (Perrot-Minnot et al, 2017) all previous studies conducted on *Pomphorhynchus* spp in the UK will be considered *P. tereticollis* in this chapter.

Since the resurrection of *P. tereticollis*, there have been several differences observed between *P. tereticollis* and *P. laevis*. For example, *P. laevis* is more manipulative to the gammarid host compared to *P. tereticollis* (Perrot-Minnot, 2004). *P. laevis* manipulates *Gammarus* spp by altering the brain serotonin levels which results in the *Gammarus* spp displaying an increase in photophilic behaviour (Tain et al, 2007). This results in gammarids infected with *P. laevis* residing higher in the water column compared to *P. tereticollis*. The parasites also occur in different fish species as *P. tereticollis* occurs mainly in benthic feeders such as *B. barbus* whereas *P. laevis* mainly occurs in bentho-pelagic feeding fish such as *S. cephalus* and *P. phoxinus* possibly due to the difference in manipulation displayed by the parasites (Perrot-Minnot et al, 2019). The parasites also have different phylogeography. *P. laevis* likely orginiated in the Mediterranean region of Europe and has strong genetic structuring whereas, *P. tereticollis* likely diverged in central and Ponto Caspian regions of Europe and has weak genetic structuring (Perrot-Minnot et al, 2017).

Although there are no studies linking *P. tereticollis* with fish mortalities or highly adverse effects on fish health, the parasite could play an important role in food web links due the manipulative behaviour it displays such as in the nematomorph parasites (Sato et al, 2011). There is a lack of data on 0+ fish in the UK due to past publications from the UK focusing primarily on adult fish (Hine and Kennedy, 1974a; Hine and Kennedy, 1974b; Kennedy et al, 1989). Little attention has been paid to 0+ fish by researchers in many locations. The lack of data collected on 0+ fish could hinder understanding of the life cycle of *P. tereticollis* especially because 0+ fish are regularly predated by larger fish and so may be acting as paratenic hosts or facilitating the post-cyclic transmission of *P. tereticollis*. Furthermore, factors affecting the prevalence of *P. tereticollis* in 0+ fish in the UK are poorly understood. Correspondingly, the aims of the study were to (a) determine the prevalence of *P. tereticollis* in 0+ fish, (b) determine key abiotic and biotic parameters driving this prevalence and (c) investigate the potential of 0+ fish acting as paratenic hosts or being key to post-cyclic transmission in the life cycle of *P. tereticollis.*

Considering the lack of information on the relationship between 0+ fish and *P. tereticollis* as well as potential for paratenic hosts use in the life cycle, research on 0+ fish interaction with the parasite is justified. Specifically, to try and record factors affecting the infection of 0+ fish as well as to better understand the life cycle of *P. tereticollis*.

2. Methods

2.1 Fish Species and variable Justification

B. barbus, L. leuciscus, P. phoxinus and *S.cephalus* were the species used in this study for a variety of reasons. Firstly, all these fish are cyprinids which are the most frequent hosts of *P. tereticollis* in the UK (Hine and Kennedy, 1974b) as well as *B. barbus* and *S. cephalus* being the main definitive hosts for the parasite in Europe (Kennedy, 2006 p.41). *L. leuciscus* also becomes frequently infected by the parasite (Hine and Kennedy, 1974b) with some females becoming gravid (Kennedy, 2006 p.67) while *P. phoxinus* could be considered a paratenic host (Medoc et al, 2011). This means that these fish have the most research affiliated with them in their mature adult stages and so will be suitable for comparison with other studies. Additionally, these fishes all have different life strategies compared to each other for example, *B. barbus* is a highly benthic feeder (Perrot-Minnot et al, 2019) while *S.cephalus* and *P. phoxinus* are bentho-pelagic feeders with the latter regularly taking food items such as aerial insects from the surface of the water (Frost, 1943; Michel, 1995) as well as eating predominately algae. *L. leuciscus* will feed on the most abundant food source depending on the time of year (Helawell, 1974) and can include plant matter in the winter and invertebrates in the summer (Hine and Kennedy, 1974a). This means that there are sufficient diet differences between the chosen fish species that could result to difference in the prevalence of *P. tereticollis* amongst them. Finally, these fish species also represent the most common cyprinid spp in the river studied, allowing for temporal samples to be collected.

As well as species, Fish length, temperature, date and gut fullness were used as variables in the study. Fish length was observed as it is unknown what size classes of 0+ fish become infected by *P. tereticollis*. Understanding the size classes of the different species infected with *P. tereticollis* is essential to understanding both the life cycle of the parasite and food web dynamics.

Temperature data was also used in the study as Kennedy, (1972) showed that successful colonisation of the parasite was dependent on temperature in laboratory experiments. This however has never been researched in field studies and so this variable may be important for explaining the prevalence in different periods of the year. As well as temperature, date was used as previous research by Frost, (1943) and Hine and Kennedy, (1974a) suggests that *P. phoxinus* and *L. leuciscus* diet changes with date. This means infection may occur at a different prevalence in the different species at different dates. This information is not only useful to understanding the life cycle of *P. tereticollis* but will also be useful for collecting future samples of *P. tereticollis* from rivers.

Gut fullness was used to explore the possibility of negative impacts to the fish hosts. This has never been researched regarding *P. tereticollis* and current understanding suggests that the parasite causes no higher risk of mortality due to similar sizes and weights of infected and uninfected adult fish (Hine and Kennedy, 1974b). However, if increased or decreased feeding is present in infected individuals this would imply some negative effects on the individual such as irritation to the alimentary tract in decreased feeding or an energy deficit regarding an increase in gut fullness.

2.2 Fish Collection

All fish used for this study were taken from the same site near Powick in the River Teme (52.169870, -2.242785) as this site had the highest prevalence of *P. tereticollis* during all samples in July, August, September and October 2015 and June, July, August and September 2016. Fish collected in July, August and September 2019 were collected using a seine net with a mesh size of 2.5mm and 25M in length was hauled over an area of slack water of the main flow of the river. This also kept the sampling consistent with fish caught and data collected in previous years.

The net was hauled between 1 and 3 times depending on how many fish were caught per haul with the aim to catch 20 fish per species although this was not possible for all species in all months. Fish that were caught were removed from the net and euthanised with an overdose of MS 222 and stored in 99% ethanol. If too many of a fish species were caught, then a subsample of fish was selected at random and the rest returned to the river. Samples were taken in July, August and September 2019. Samples were then organised into species (Pinder, 2001) in the lab and then placed into the relevant sample pot according to date and species and stored at 5°C until dissected.

Fish from 2015 and 2016 where collected in July, August, September, October in 2015 and June, July, August and September in 2016 by a post graduate researcher from the same site using the same method (Guttmann-Roberts, 2018) and where kept in 70% IMS under 5°C until they were needed for this project. Some previously unpublished data from this location in July, August, September and October 2015 and June, July, August and September 2016 (Guttmann-Roberts, 2018) was used as the presence and absence of *P. tereticollis* was recorded but never analysed for any study.

2.3 *Gammarus* collection and dissection.

To ensure that changes in the prevalence of *P. tereticollis* were due to variables tested rather than a change in the prevalence of cystacanths in the *Gammarus* spp population, *Gammarus* spp were collected from the site using a kick sample technique where the gravel was disturbed 20 times. A hand net with mesh size 250μm was placed downstream of the site disturbed to catch potential *Gammarus* spp. If low numbers of *Gammarus* spp were caught the technique was repeated until approximately 15 individual *Gammarus* spp over 5mm in length were caught. Only individuals that were over this length were collected and dissected due to the extremely low prevalence of cystacanths in *Gammarus* spp smaller than this length (Hine and Kennedy, 1974a; Harris et al, unpublished). *Gammarus* were stored in 99% ethanol until dissection.

Gammarus spp were dissected using a standard Brunel dissection kit. *Gammarus* spp were removed from the ethanol and their length to the nearest millimetre (mm) was measured. Fine point tweezers were then used to remove the head of the *Gammarus* spp. The *Gammarus* spp was then opened ventrally and any potential *P. terticollis* infections were removed from the *Gammarus.* The cystacanth was confirmed morphologically as *P. tereticollis* if there was a presence of ventral bands round the entire parasite which are more easily detectable on *P. tereticollis* compared to *Polymorphous minutus* (*P. minutus*). It was also differentiated from by the size of the cystacanth as *P. tereticollis* is larger than *P. minutus.* As well as this, a previous sample collected in August 2016 was also used.

2.4 Fish Dissections

Fish were removed from either IMS or 99% ethanol and placed onto a plastic petri dish. The fish standard length was then measured using digital callipers to 2d.p in millimetres before being dissected. Fish standard length was used rather than ontogenetical stage to assess the maturity of the fish as fish length is less subjective.

Fish were then dissected using a Brunel dissection kit. Blunt tip forceps were used to hold down the fish and scissors were placed into the anus of the fish and used to cut from the anus to level with the gills. Fine point tweezers were then used to remove the digestive tract. The tract was pulled out of the fish slowly from the bottom until it was stretched out and the tweezers where placed into the fish and the top of the tract removed as close to the moth as possible.

Gut fullness was assessed once the tract had been removed from the fish. The tract was deemed 100% full if it was completely full and extended diameter wise. If the tract was full but not completely extended it was given a value of 90% fullness and if the gut was partially full so that some regions were empty and some regions were full, a relevant percentage of full to not full was given (for example, if half the gut was half full and half empty it was given a value of 50%) until the tract was completely empty and 0% fullness was given. The tract was then opened laterally, and the contents removed to find potential infections of *P. tereticollis*.

Table 1. Total number of fish dissected per month per species in 2015, 2016 and 2019. Mean fish length in millimetres (mm) of each species per sample is presented in Parentheses.

	Barbus barbus	*Leuciscus leuciscus*	*Phoxinus phoxinus*	*Squalius cephalus*
Jul-15	62 (17.67)	20 (28.27)	11 (15.04)	4 (15.93)
Aug-15	60 (23.69)	39 (37.92)	20 (18.87)	25 (19.96)
Sep-15	15 (28.67)	32 (41.02)	26 (23.55)	22 (21.93)
Oct-15	0 (N/A)	22 (42.16)	20 (18.70)	20 (22.74)
Jun-16	0 (N/A)	11 (21.90)	10 (25.27)	0 (N/A)
Jul-16	0 (N/A)	20 (28.15)	21 (29.47)	0 (N/A)
Aug-16	18 (23.06)	13 (32.68)	22 (31.33)	0 (N/A)
Sep-16	21 (24.81)	18 (37.58)	10 (28.44)	0 (N/A)
Jul-19	0 (N/A)	5 (50.66)	20 (31.21)	15 (29.07)
Aug-19	0 (N/A)	0 (N/A)	11 (36.82)	6 (25.00)
Sep-19	0 (N/A)	0 (N/A)	11 (35.27)	0 (N/A)

2.5 Statistical analysis

All statistical analysis was completed in IBM SPSS 26 in windows 10 except for length-gape regression analysis which was completed in Rx64 3.6.1. Autocorrelation in the data was tested by regressing fish length, fish gape, temperature, gut fullness and sampling date. If two variables had an $r^2>0.8$ only one of the two variables were used in further analysis.

A binary logistic regression was chosen to find the predictor variables of infection. This test was chosen as the independent variables were a mixture of continuous and categorical with the dependant variable being categorical with two levels where the individual was either infected (1) or uninfected (0). The binary logistic regression was then executed with all variables that were not autocorrelated these were fish length, temperature, gut fullness and sampling date. A model was first built using all individual fish samples (n=631). However, if data was missing in one column for example in gut fullness the individual was removed listwise. To remove factors that were clearly not linked to infection, both forward and backward stepwise selection were used for the model.

As binary logistic regression has the possibility to overfit the model to the data, both Akaike information criterion (AIC) and Bayesian information criterion (BIC) were calculated for each possible model subset. For example, an AIC and BIC calculation would be tested for all predictors of fish length, gape, gut fullness, temperature and date then one of these variables would be removed and so on until all possible combinations of models had been performed. If forward and backward selection produced different results from each other, then the model with the lowest AIC and BIC value was used. The same process was then repeated for each individual fish species (*B. barbus L. leuciscus, P. phoxinus* and *S. cephalus*) so the individual predictors for each species could be identified.

Once the overall predictor variables had been identified, each variable that was important to infection was explored individually to observe what the relationship to infection was. This was then tested with the relevant statistical test.

When fish length was found to be a factor that contributed to infection, size categories of 2 millimetres (mm) where constructed and the prevalence for the size category calculated and plotted as the dependent variable. This was done so the data could be plotted with no covariates such as date. The relationship of fish length and *P. tereticollis* prevalence was tested using a spearman’s correlation as this statistical test can be used with a categorical independent variable.

When fish gut fullness was an important factor, the gut fullness data was plotted with the covariate of month as there are very large differences in the gut fullness between early summer and late summer/early autumn for both infected and uninfected fish. As well as this, most infections occurred in later months where gut fullness is lower for all fish which would skew the data. For comparison between fish species, only the data collected in 2015 was used as this year contained the most data and was the only year where all fish were found regularly in samples. Medians were plotted for each month in 2015 for infected and uninfected individuals as the gut fullness histograms revealed that data was not normally distributed. A Mann-Whitney U test was therefore used to compare the medians.

When temperature was an important factor, temperature data collected from the closest Meteorological Office weather station to the River Teme was used (Ross-On-Wye). Temperature data was collected for all sampling periods when the fish species was found and an average for the month was constructed by taking an average of the average highest and average lowest temperature as this was the only data publicly available. Graphs were then constructed using the temperature data as the independent variable and the prevalence of *P. tereticollis* as the dependant variable. The data was then checked for heteroskedasticity by plotting the ZPRED with the ZRESID in IBM SPSS 26. If heteroskedasticity was found a Spearman's correlation was used to test significance and if not, a regression analysis was performed.

Fish length, gut fullness, temperature and species were selected as variables for several reasons. Fish length was selected as

2.6 Identification of *P. tereticollis*

Identification of *P. tereticollis* was completed using morphological techniques. As although *P. tereticollis* is thought to be the only species within its genus in the UK (Perrot-Minnot et al, 2019), it is still possible that *P. laevis* may be present undetected. Therefore, parasites were confirmed *P. tereticollis* by the clear indication of ventral bands round the parasites body cavity (Perrot-Minnot, 2004). As well as this, *P. tereticollis* was differentiated from *Polymorphus minutus* (*P. minutus*) by the colour of the parasites as *P. minutus* has a stronger orange/red colour compared to *P. tereticollis* which by comparison has a duller orange colour.

3. Results

3.1 Prevalence of cystacanths within *Gammarus* spp

The prevalence of cystacanth infections within *Gammarus* spp remained relatively constant with infections of 90% for both July and August and 60% for September 2019. The mean length of *Gammarus* spp used in the study was 8.73mm ± 1.28. As well as this, prevalence from previously collected data was 90% in August 2016.

3.2 Infection of 0+ fish

Pooling all fish together, there were a total of 629 fish used in this study. All species of fish were found in several months in at least 2 years with *P. phoxinus* being present in all months and all years. *S. cephalus* was present in July, August, September and October 2015 and July, August 2019. *L. leuciscus* was present July, August, September and October 2015, June, July, August and Septmeber 2016 and July 2019 and *B. barbus* present in July, August, Septemeber 2015 and July, August 2016.

Table 2. Prevalence of *Pomporhynchus tereticollis* within *Barbus barbus, Leuciscus leuciscus, Phoxinus phoxinus* and *Squalius cephalus* in each month where samples were collected. N/A represents when no fish of the species were caught during the sampling period. Mean Abundance is presented in parentheses

	Barbus barbus	*Leuciscus leuciscus*	*Phoxinus phoxinus*	*Squalius cephalus*
Jul-15	0 (0)	0 (0)	0 (0)	0 (0)
Aug-15	15.0 (0.25)	7.7 (0.18)	0 (0)	4.0 (0.04)
Sep-15	53.3 (1.27)	21.9 (0.30)	14.3 (0.14)	0 (0)
Oct-15	N/A (N/A)	36.3 (0.73)	20.0 (0.30)	15.0 (0.15)
Jun-16	N/A (N/A)	9.1 (0.09)	0 (0)	N/A (N/A)

Jul-16	N/A (N/A)	5.0 (0.05)	19.0 (0.4)	N/A (N/A)
Aug-16	16.7 (0.02)	15.4 (0.02)	40.9 (0.57)	N/A (N/A)
Sep-16	23.8 (0.28)	35.3 (0.78)	50.0 (0.60)	N/A (N/A)
Jul-19	N/A (N/A)	20.0 (0.20)	0 (0)	0 (0)
Aug-19	N/A (N/A)	N/A (N/A)	9.1 (0.09)	0 (0)
Sep-19	N/A (N/A)	N/A (N/A)	16.7 (0.17)	N/A (N/A)

Prevalence of *P. tereticollis* within *B. barbus, L leuciscus, S. cephalus* and *P. phoxinus* varied between months (fig 2). There was an increase in the prevalence of *P. terticollis* towards the last sample of the year for all species. *S. cephalus* had the lowest overal prevalence compared to the other species and in 2015 *S. cephalus* had the lowest prevalence with prevalence peaking in Ocotber 2015 at a value of 15% (1d.p) compared to 36.3% (1d.p) for *L. leuciscus* and 20% (1d.p) for *P. phoxinus. B. barbus* had the highest prevalence of *P. tereticollis* in 2015 of 53.3% (1d.p) occuring in September. In 2016, *P. phoxinus* had the highest prevalence of *P. tereticollis* reaching 50% (1d.p) in September 2016 compared to 23.8% (1d.p) for *B. barbus* and 35.3% (1d.p) for *L. leuciscus*. No *S. cephalus* was caught in 2016. In 2019 no *B. barbus* were caught and only *P. phoxinus* was caught in all three samples. In 2019 the prevalence of *P. tereticollis* in *P. phoxinus* was similar to 2015 and 2016 with the prevalence of the parasite increasing towards the autumn samples. Both *L. leuciscus* and *P. phoxinus* carried infections of *P. tereticollis* extra-intestinally with 20% (1d.p)of *P. tereticollis* found within *P. phoxinus* and 6.7% (1d.p) found extra-intestinally within *L. leuciscus.*

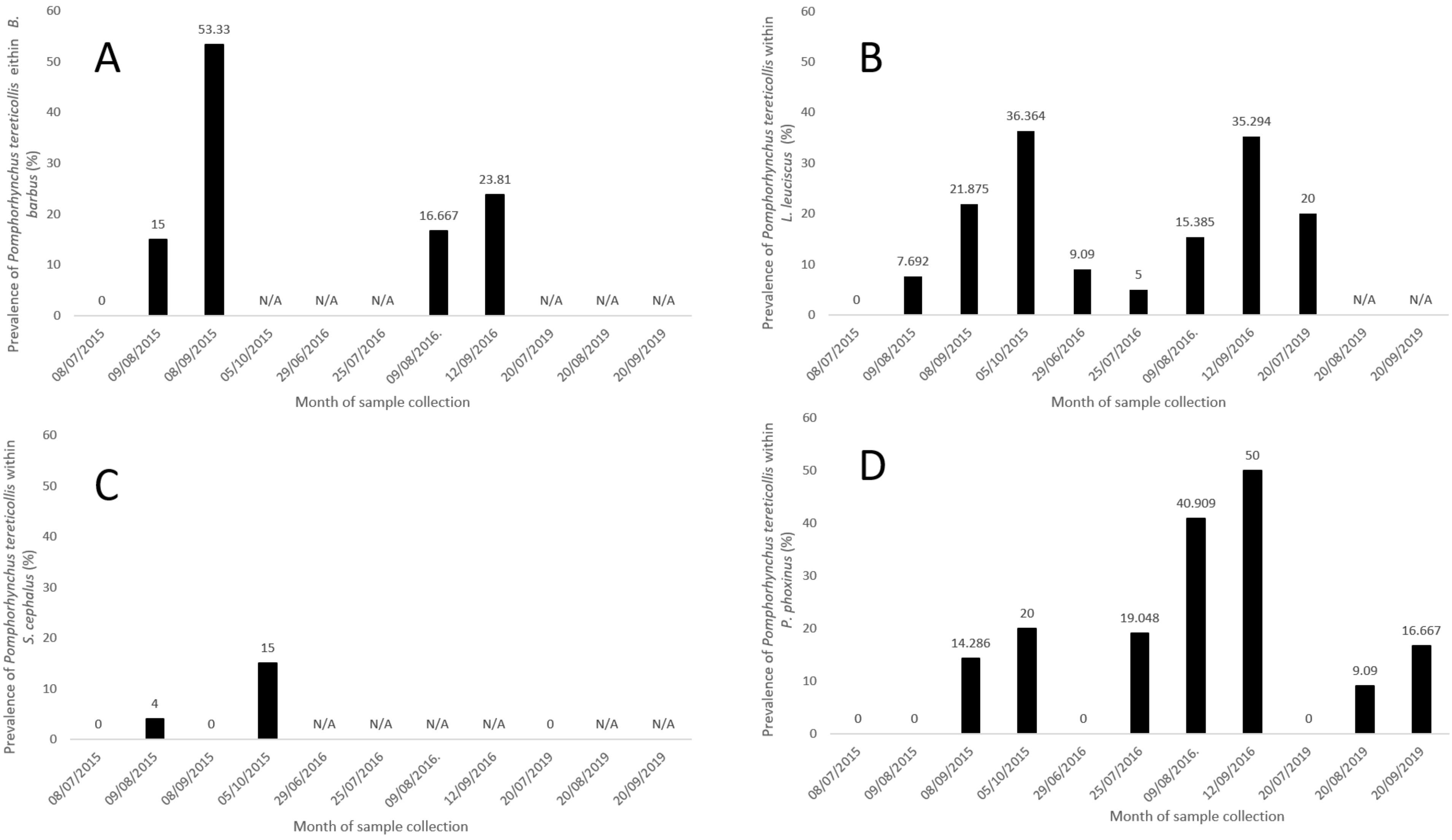

A
Prevalence of *Pomphorhynchus tereticollis* eithin *B. barbus* (%)
60
50
40
30
20
10
0
0
15
53.33
N/A
N/A
N/A
16.667
23.81
N/A
N/A
N/A
08/07/2015
09/08/2015
08/09/2015
05/10/2015
29/06/2016
25/07/2016
09/08/2016.
12/09/2016
20/07/2019
20/08/2019
20/09/2019
Month of sample collection
B
Prevalence of *Pomphorhynchus tereticollis* within *L. leuciscus* (%)
0
7.692
21.875
36.364
9.09
5
15.385
35.294
20
N/A
N/A
Month of sample collection
C
Prevalence of *Pomphorhynchus tereticollis* within *S. cephalus* (%)
0
4
0
15
N/A
N/A
N/A
N/A
0
N/A
N/A
Month of sample collection
D
Prevalence of *Pomphorhynchus tereticollis* within *P. phoxinus* (%)
0
0
14.286
20
0
19.048
40.909
50
0
9.09
16.667
Month of sample collection

Figure 2. Prevalence of *Pomphorhynchus tereticollis* within *Barbus barbus* (A), *Leuciscus leuciscus* (B), *Squalis cephalus* (C) and *Phoxinus phoxinus* (D). N/A represents an absence of the species in sample period. *L. leuciscus* and *P. phoxinus* were caught in most samples.

3.3 Binary logistic regression using the combined data across all fish species and sampling dates

Fish length and gape were strongly correlated with a $r^2>0.8$ therefore only fish length was used for further statistical analysis. Table 1 shows the r^2 values for all the other variables with the largest being between temperature and date which was still below 0.8; therefore, fish length, date and gut fullness were included in further statistical analysis.

Table 3. Regression analysis of all variables used in the binary logistic regression for all fish species and sampling samples.

Variables	regression output
fish length and temperature	Regression F=3.426, d.f=1 and 601, p=0.65, r2=0.006
Fish length and date	Regression F=40.804, d.f=1 and 601, p<0.001, r2=0.064
Fish length and gut fullness	Regression F=2.292, d.f=1 and 498, p=0.131, r2=0.005
date and temperature	Regression F=156.599, d.f=1 and 604, p<0.001, r2=0.206
temperature and gut fullness	Regression F=82.685, d.f=1 and 501, p<0.001, r2=0.142
Gut fullness and date	Regression F=9.271, d.f=1 and 501, p=0.002, r2=0.018

Output from the binary logistic regression for all the fish in the study showed that infection was affected by fish length, gut fullness and species. Species appears to have the biggest impact on infection with *P. tereticollis* with *B. barbus* being the strongest positive predictor of infection although this was not significant (table 4). *L. leuciscus* was the strongest negative predictor of infection followed by *S. cephalus*. Temperature and date do not appear to predict infection when all species are used in the binary logistic regression.

Table 4. Results of binary logistic regression testing the significance of variables affecting the infection of fish with *Pomphornhynchus tereticollis.* The model was selected using forward and backward stepwise selection and was confirmed to be the best model using both Akaike information criterion (AIC) and Bayesian information criterion (BIC)

Variable	B	S.E.	Wald	*P*
fish length	0.174	0.025	46.959	<0.001
gut fullness	-0.11	0.004	6.051	0.14
P. phoxinus			18.113	<0.001
B. barbus	0.6	0.368	2.657	0.103
S. cephalus	-0.316	0.601	0.276	0.599
L. leuciscus	-1.435	0.499	10.19	0.001
Constant	-5.81	0.796	53.329	<0.001

3.4 Fish length and infection with *P. tereticollis*

The output from the binary logistic regression was affected positively by fish length B=0.176, p<0.001. An increase in fish length increased the prevalence of *P. tereticollis* within all species apart from *S. cephalus*. The relationship between fish length and prevalence was only significant for *B. barbus* and *P. phoxinus* (fig 2). In *B. babrus* there is a clear break point where infection with *P. tereticollis* begins between 18mm and 22mm and increases sharply to the largest of the 0+ fish which carried the highest infection with size groups of 30-32mm and 32mm+ carrying prevalence’s of *P. tereticollis* of 75.00% (2d.p) and 71.43% (2 d.p) respectively. The relationship between *B. barbus* length and prevalence of *P. tereticollis* was highly significant Spearman’s rho=0.913, p<0.001, N=11. *L. leuciscus* fish length was not a good predictor of infection with *P. tereticollis* (table 4). There was a general positive trend of prevalence increasing with fish length but this was not significant Spearmans rho=0.454, p=0.103, n=14 (fig 2).

S. cephalus length was shown to be a bad predictor of infection with *P. tereticollis* (table 5). No overall positive or negative trend for infection with *P. tereticollis* was found and the prevalence of *P. tereticollis* appears sporadically at a low prevalences in the middle fish size lengths and then returns to 0%. The trend was also not significant Spearmans rho=-0.109, p=0.797, n=8.

Although date was considered to be an important factor predicting infection in *P. phoxinus* (table 6), length was independent of month as several spawns happen in a year and different lengthed fish were found mixed in all the months meaning the relationship between length of *P. phoxinus* and infection with *P. tereticollis* is not affected by date. There is a positive relationship betweeen the two variables where infcetion begins when fish are 15-20mm in size and then increases linearly where the highest levels of infection occur in the largest *P. phoxinus* with prevalences of over 40% in the largest two sizes classes of *P. phoxinus*. The relationship was highly significant Spearman’s rho=0.923, n=13, p<0.001.

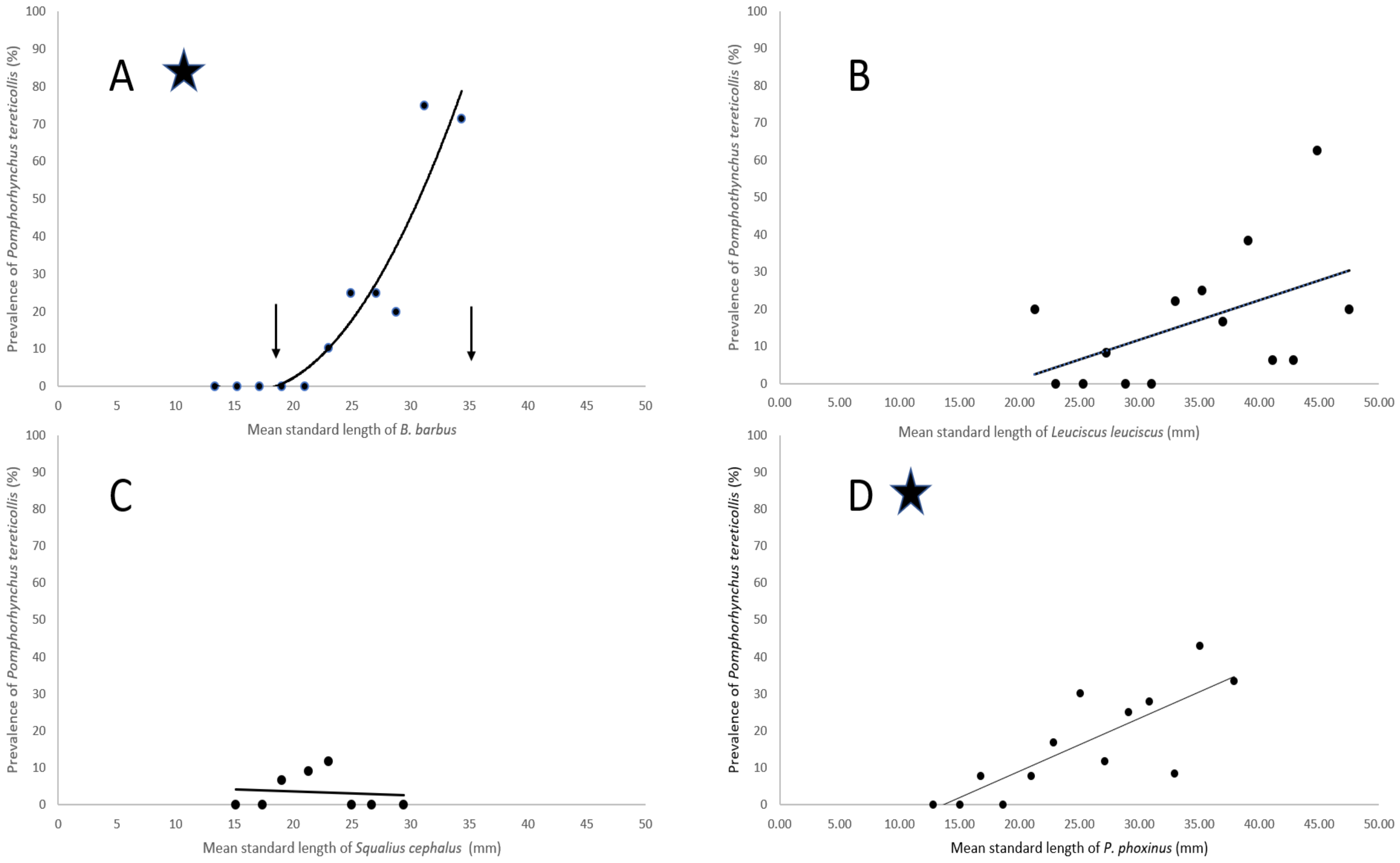

A
B
C
D
Prevalence of *Pomphorhynchus tereticollis* (%)
Prevalence of *Pomphothynchus tereticollis* (%)
Mean standard length of *B. barbus*
Mean standard length of *Leuciscus leuciscus* (mm)
Mean standard length of *Squalius cephalus* (mm)
Mean standard length of *P. phoxinus* (mm)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
40
45
50
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00

Figure 3: Mean standard length of *Barbus barbus* placed into size groups with an increment of 2mm plotted against the prevalence of *Pomphorhynchus tereticollis* (%) within the group. Data for fish was pooled from all periods when they were caught. Although *Phoxinus phoxinus* were caught in 2019, they were exluded from the production of the graph due to low prevalence of *P. tereticollis* for the year which would have skewed the top of the data as all fish caught were large. Black stars represent significant relationships

3.5 Gut fullness and prevalence of *P. tereticollis*

Infection with *P. tereticollis* was predicted by a lower gut fullness although this was not significant B=-0.11, p=0.14 (table 4). When month is a covariate, *B. barbus*, *L. leuciscus* and *P. phoxinus* had higher median gut fullness when infected with *P. tererticollis* in Septemeber and October 2015 and similar gut fullness values to uninfected fish in August 2015. *S. cepahalus* had a lower median gut fullness when infected with *P. tereticollis* although sample size for this speceies was low (fig 4). Infected *P. phoxinus* gut fullness was significantly different from uninfected *P. phoxinus* whereas the difference was not significant for the other species.

Gut fullness (%) of *B. barbus* collected over July 2015, August 2015 and September 2015 for all fish (infected and uninfected pooled for each month) was significantly different Kruskal-Wallis, chi squared=28.838, d.f=2, p<0.001. Infected individuals were only found in August and September (fig 4). Infected and uninfected individuals in August showed similar gut fullness median=80±15 for uninfected individuals and median=75±35 for infected individuals. In September, uninfected individuals had lower gut fullness compared to infected individual's median=30±30 for uninfected individuals and 47.50±51 for infected individuals. The difference between infected and uninfected individuals in both months was not significant Mann-Whitney U= 171.000, n= 60, p=0.216 and Mann-Whitney U=16.500, n=15, p=0.182 for August and September respectively. Infected individuals were larger than uninfected individuals 28.30±0.99 and 22.87±0.51 respectively in August 2015. In September 2015 infected individuals mean standard length was 31.21±1.83 (2d.p) and uninfected mean standard length was 25.77±0.90 (2d.p) although larger fish did not have a higher gut fullness (See appendix).

Median gut fullness of *L. leuciscus* was obtained for July 2015, August 2015, September 2015 and october 2015. However, individuals that were infected with *P. tereticollis* were only found in August, September and October 2015. The overall gut fullness of fish (infected and uninfected grouped together) was statistically significant between months Kruskal-Wallis, chi squarred=45.200, d.f=3, p<0.001. Infected and unifected individuals showed similar median gut fullness in August median= 90±3.125 for unifected and median=85±5 for infected individuals and then in September and October the uninfected individuals had lower median gut fullness compared to infected individuals median=40±22.5 for uninfected and 60±22.5 for infected for September and median=35±27.5 for uninfected and 55±30.625 for October. The difference in median gut fullness between months was not significant Mann-Whitney U=23.00, n=22, p=0.235, Mann-Whitney U=22.000, n=20, p=0.173, Mann-Whitney U=31.000, n=20, p=0.188 for August, September and October respectively. Infected and uninfected individuals did not differ in size in the months of 2015.

There were not enough *S. cephalus* individuals infected with *P. tereticollis* to produce a graph for all months in 2015 for gut fullness as the total number of individuals infected with *P. tereticollis* was 4. Although there were 3 individuals infected in Ocober and the median=2.00 whereas, the median for unifected individuals was median=40.00±60 (fig 12) which is the reciprocal of all the other fish gut fullness trends. The difference in gut fullness for October 2015 was not significant between infected and uninfected individuals Mann-Whitney U=7.500, n=17, p=0.54.

Infected *P. phoxinus* individuals only occurred in September (n=4) and October (n=4) where the median gut fullness was 80.00% (2d.p) and 15.00% (2d.p) for infected individuals compared to 50.00% (2d.p) and 5.00% (2d.p) for uninfected in September and October respectively (fig 4). The difference in gut fullness for all fish (infected and uninfected grouped together) differed between months Kruskal-Wallis, chi squared=26.491, d.f=3, p<0.001. The difference between infected and uninfected gut fullness was significant for both September, Mann-Whitney U=5.500, n=18, p=0.014 and October, Mann-Whitney U=2.000, n=23, p=0.003. The infected fish where larger than uninfected fish Mean infected=25.04 (2d.p), mean uninfected=21.26mm (2d.p) for September (n=4) and mean infected= 23.35 (2d.p), mean uninfected=17.96 (2d.p) for September and October respectively.

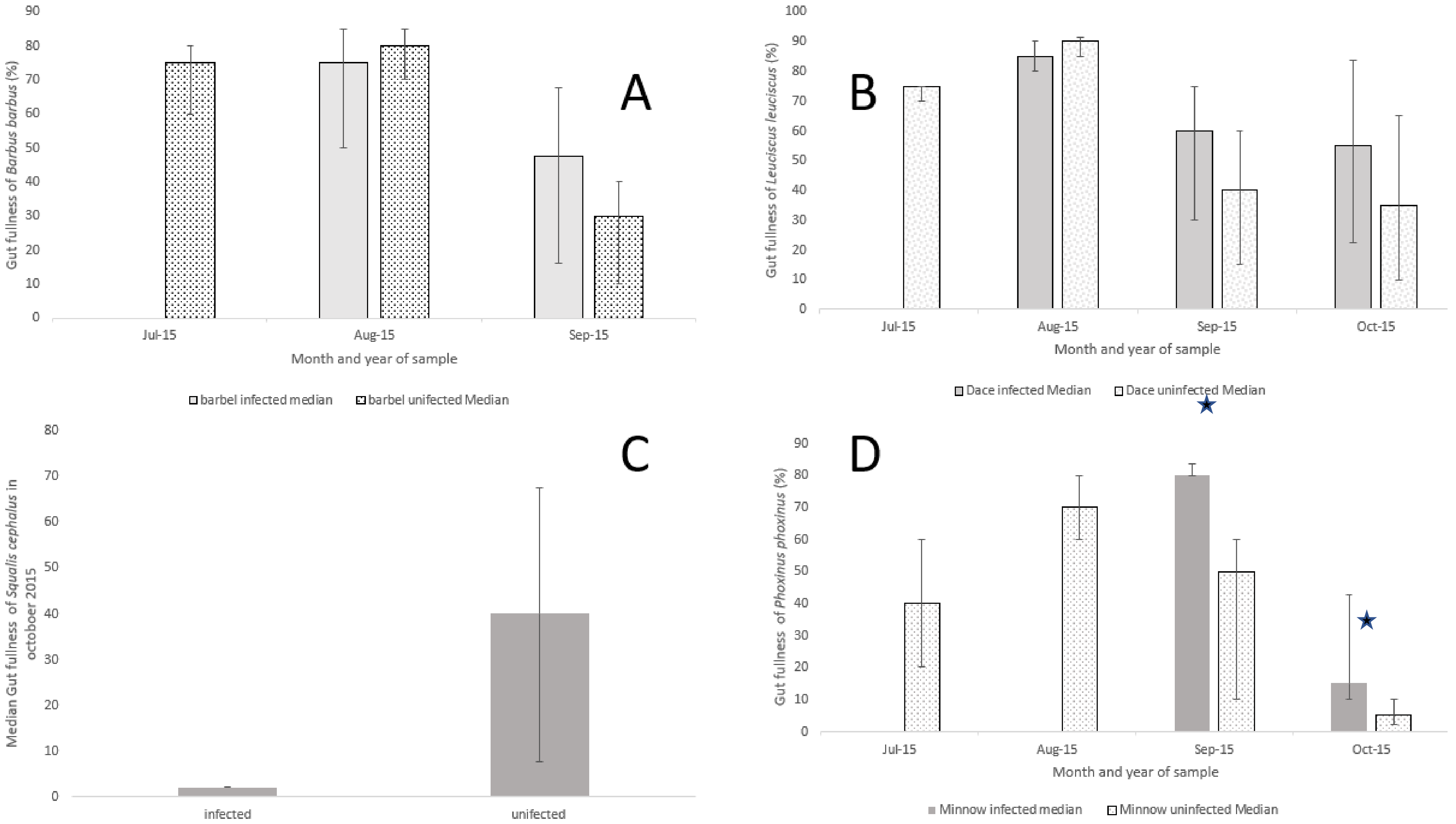

A
Gut fullness of Barbus barbus (%)
0
10
20
30
40
50
60
70
80
90
Jul-15
Aug-15
Sep-15
Month and year of sample
barbel infected median
barbel unifected Median
B
Gut fullness of Leuciscus leuciscus (%)
0
10
20
30
40
50
60
70
80
90
100
Jul-15
Aug-15
Sep-15
Oct-15
Month and year of sample
Dace infected Median
Dace uninfected Median
★
C
Median Gut fullness of Squalis cephalus in octoboer 2015
0
10
20
30
40
50
60
70
80
infected
unifected
D
Gut fullness of Phoxinus phoxinus (%)
0
10
20
30
40
50
60
70
80
90
Jul-15
Aug-15
Sep-15
Oct-15
Month and year of sample
★
Minnow infected median
Minnow uninfected Median

Figure 4: Median gut fullness (%) of infected and uninfected *Barbus barbus* in July 2015, August 2015 and September 2015. Infected individuals were only found in August (n=9) and September (n=8). (A). Median gut fullness (%) of infected and uninfected *Leuciscus leuciscus* in July 2015, August 2015, September 2015 and October 2015. Infected idividuals where found in August (n=4), September (n=5) and October (n=8). (B). Median gut fullness of *Squalius cephalus* infected (n=3) and unifected (n=18) with *P. tereticollis* in October 2015. Median gut fullness (%) of *Phoxinus phoxinus* infected with *P. tereticollis* and not infected with *Pomphorhynchus tereticollis* plotted with month the fish were sampled. Infected individuals only occurred in September (n=4) and October (n=4). Significant relationships are represented with black stars. Error bars on the figure represent the interquartile range of the median.

3.5 Binary logistic regression of *B. barbus*

B. barbus infection with *P. tereticollis* was predicted by fish length and gut fullness although only fish length was significant (Table 3). Fish length was the best predictor of infection with *P. tereticollis* and was also highly significant B=0.412, p<0.001 whereas, gut fullness had a very small impact and was also not significant B=-0.005, p=0.662.

Table 5. Results of binary logistic regression testing the significance of variables effecting the infection of *B. barbus* with *Pomphornhynchus tereticollis.* The model was selected using the lowest value produced by both Akaike information criterion (AIC) and Bayesian information criterion (BIC)

Variable	B	S.E.	Wald	*P*
Fish length	0.412	0.08	26.562	<0.001
Gut fullness	-0.005	0.011	0.191	0.662
Constant	-11.743	2.296	26.157	<0.001

3.6 Binary Logistic regression of *Leuciscus leuciscus*

L. leuciscus infection was predicted by gut fullness, temperature and Date (Table 4) although only temperature and date where significant. Temperature was the best predictor and had a negative relationship to infection B=-0.475, p<0.001. Wheras gut fullness had only a very slight postive affect on probabillity of infection although this was not significant B=0.017, p=0.083.

Table 6. Results of binary logistic regression for the variables affecting infection of *Pomphorhynchus tereticollis* within *Leuciscus leuciscus*. The model was selected using the lowest value produced by both Akaike information criterion (AIC) and Bayesian information criterion (BIC). Gut fullness, temperature and date where all variables affecting infection although temperature and date where the only significant factors.

Dace	B	S.E	Wald	*P*
Gut fullness	0.017	0.1	2.998	0.083
Temperature	-0.475	0.13	13.478	<0.001
Date	0	0	9.3	0.002
Constant	-604.579	198.885	9.241	0.002

3.7 Temperature and infection of *Leuciscus leuciscus* with *P. tereticollis*

The prevalence of *P. tereticollis* is negativly correlated with temperature with the largest prevalence of infection occuring when the temperature is 11.4°C (2d.p) with a prevalence of 36.36% (2.d.p). when temperatures are warmer, the the prevalence is lower and at the warmest temperatures prevalence usually remains below 10% (fig 5). The relationship between temperature and the prevalence of *P. tereticollis* was significant Spearmans rho=-0.706, n=9, p=0.034.

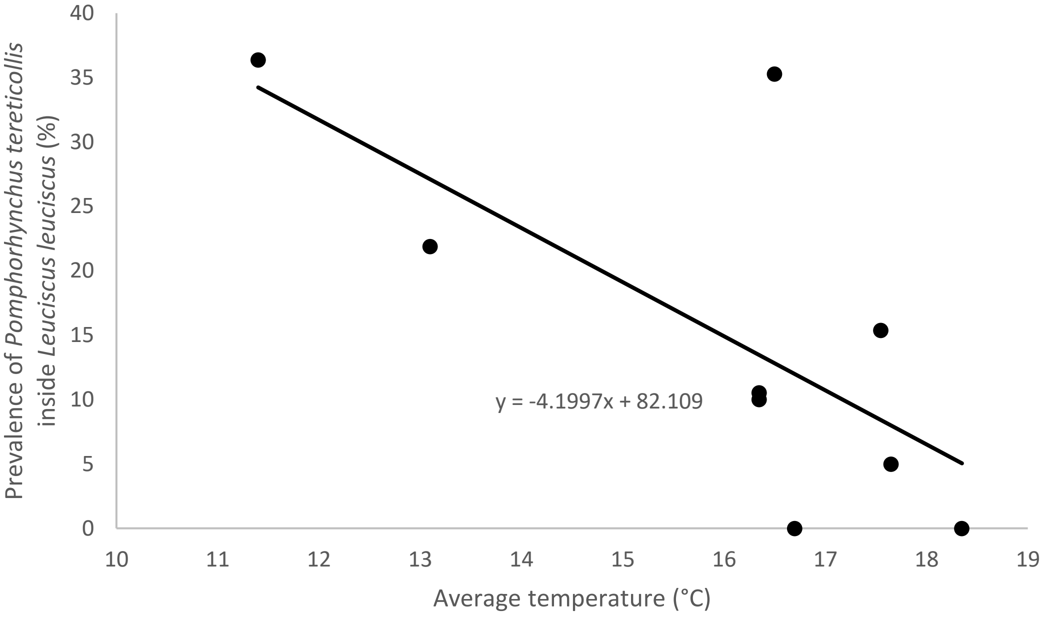


Figure 5. Average temperature of the month where a sample of *Leuciscus leuciscus* was taken plotted with the prevalence of *Pomphorhynchus tereticollis* (%) within the fish. The trend between the two varaibles is negative as the temperature decreases, the prevalence of *P. tereticollis* increases. The relationship between temperature and the prevalence of *P. tereticollis* was significant Spearmans rho=-0.706, n=9, p=0.034.

3.8 Infection of *Leuciscus leuciscus* with *P. tereticollis* and the Date of samples collected

The prevalence of *P. tereticollis* increases with month with the highest prevalences occuring in the latest months of sample collections in October 2015 and September 2016. Size has little effect on the prevalence in the month and so date can be ruled out as a proxy for length as prevalence increases in both months even when the fish are smaller in 2016 with higher prevalences than 2015 (fig 3). This suppots fig 9 which shows that the relationship between fish length and prevalence is not significant. The prevalence in each month was significantly different from each other Chi squarred=20.355, d.f=7, p=0.0049.

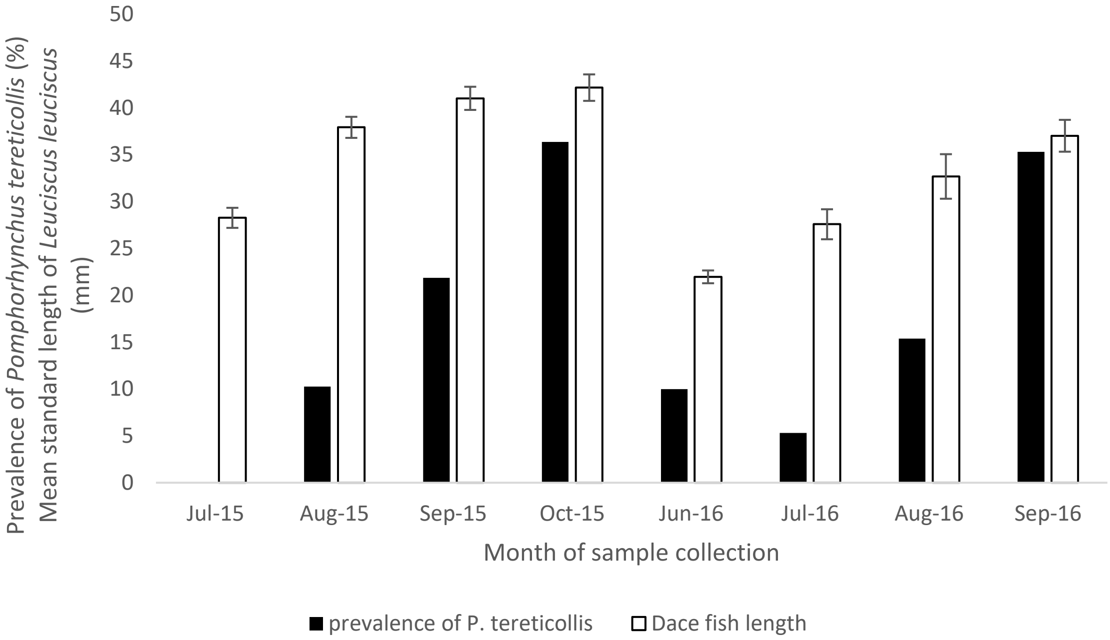


Figure 6. Months where *Leuciscus leuciscus* was sampled plotted with prevalence of *Pomphorhynchus tererticollis* in the intestine. As the date increases towards the end of both 2015 and 2016, the prevalence increases. Size has little effect on the prevalence in these months as can be seen by infcection being higher in August 2016 compared to August 2015 despite the larger size of the fish in 2015. As well as this, infection is much higher in September 2016 compared to september 2015 despite the larger. The difference in prevalence in the different months was significant Chi squarred=20.355, d.f=7, p=0.0049

3.9 Binary logistic regression for *Squalius cephalus*

S. cephalus infection was predicted by a lower gut fullness. This was not significant B=-0.4, p=0.34. 3 out of 4 of the parasites found within *S. cephalus* occurred in October 2015 were gut fullness was low for both infected and unifected fish (fig 4) and this the reason why infection is predicted by gut fullness.

Table 7. Binary logistic regression output for the variable affecting infection of *Squalis cephalus* with *Pomphorhynchus tereticollis*. The model was selected using the lowest

value produced by both Akaike information criterion (AIC) and Bayesian information criterion (BIC). Gut fullness was the only variables that significantly predicted infection with *P. tereticollis* all other variables (fish length, temperature and date) significantly increased the AIC and BIC value.

Chub	B	S.E	Wald	*P*
Gut fullness	-0.4	0.19	4.494	0.34
Constant	-1.28	0.707	3.3275	0.07

3.10 Binary logistic regression of *Phoxinus phoxinus*

Infection of *P. phoxinus* was predicted by three variables, fish length, gut fullness and date. Fish length had the strongest positive effect of the three variables and was also highly significant B=0.253, p<0.001, while a negative gut fullness predicted infection of *P. phoxinus* with *P. tereticollis* B=-0.18, p=0.03.

Table 8. Binary logistic regression output for the variables affecting infection of *Phoxinus phoxinus* with *Pomphorhynchus tereticollis*. The model was selected using the lowest value produced by both Akaike information criterion (AIC) and Bayesian information criterion (BIC). Fish length, gut fullness and date were the variables that significantly predicted infection with *P. tereticollis.* Temperature significantly increased the AIC and BIC value and so was excluded from the model.

P. phoxinus	B	S.E	Wald	*P*
fish length	0.253	0.058	19.33	<0.001
gut fullness	-0.18	0.008	4.727	0.030
Date	0	0	4.548	0.033
Constant	378.204	179.987	4.415	0.036

3.11 Date of sample collection and prevalence of *P. tereticollis* within *Phoxinus phoxinus.*

The prevalence of *P. tereticollis* was significantly different between months chi squared=31.315, d.f=10, p=0.0005. The prevalence increases towards the last sample of the year. 2016 had the highest prevalence of the three years.

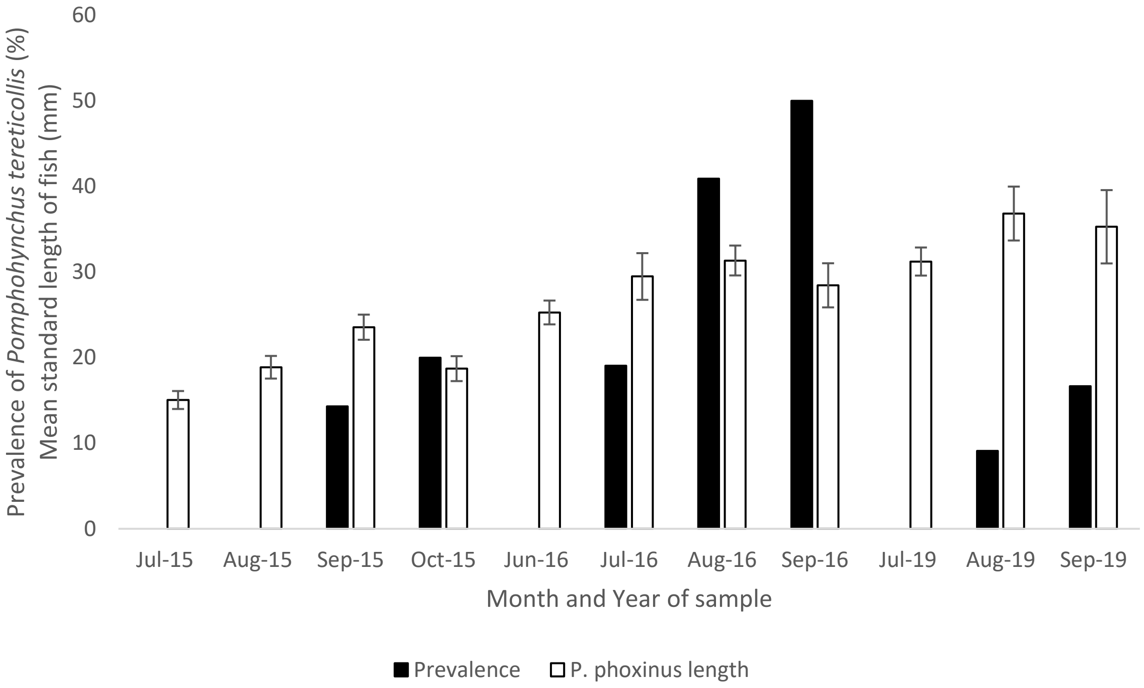


Figure 7. Months where *Phoxinus phoxinus* was sampled plotted with prevalence of *Pomphorhynchus tererticollis* in the alimentary tract. As the date increases towards the end of 2015, 2016 and 2019, the prevalence generally increases. The prevalence of *P. tereticollis* in 2019 was low compared to 2016 but higher than 2015 and likely to have a high prevalence in October if sampling continued. The largest prevalence of *P. tereticollis* occurred in septmeber 2016 with a prevalence of 50%. The difference in prevalence across the months was significant chi squared=31.315, d.f=10, p=0.0005.

4. Discussion

A variety of variables which predict the presence of *P. tereticollis* in the river Teme were tested these were species including *B. barbus*, *L. leuciscus*, *S. cephalus* and *P. phoxinus*, fish length, gut fullness, temperature, date. The variables which predict infection within each species of cyprinid were then tested. As well as this, the presence of cystacanths in *Gammarus* spp was observed in 2019 and combined with previous data in August 2016. The presence of cystacanths within *Gammarus* spp remained at a high prevalence throughout 2019 as well as in the sample collected in 2016 (see section 3.1). It is therefore unlikely that the prevalence of *P. tereticollis* in the river was limited by the presence of cystacanths within *Gammarus* spp.

The factors for predicting infection when all fish species were included in the binary logistic regression were fish length, species and gut fullness although gut fullness was not significant (Table 4). Fish length was a positive indicator of infection which could be explained by two factors:

1. A fish's length is a proxy for its age meaning larger fish have had more time to predate a cystacanth infected *Gammarus* spp
2. *Gammarus* are only ever infected with cystacanths when they are over 5mm in length (Hine and Kennedy, 1974a; Harris, unpublished data). Larger fish have larger gape and so are more likely to consume a *Gammarus* spp individual of this size compared to a smaller fish.

4.1 Infection with *P. tereticollis* across species

Each fish species had a different effect on the binary logistic regressions ability to predict *P. tereticollis* infection (table 4). *B. barbus* had a positive relationship with infection. *S. cepahlus* and *L. leuciscus* had a negative effect on infection. The different relationships between *P. tereticollis* and the different species is explained by their differing life strategies.

The relationship between standard length of *B. barbus* and infection with *P. tereticollis* was highly significant and had a high Spearmans rho indicating a good fit. Standard length explains most of the variation in infection in the species (fig 3). There is a large increase in the prevalence of *P. tereticollis* between 20mm and 32mm where infection increases from 0% to 75% (fig 3). This can be explained by the feeding ecology of the fish as it is a highly benthic feeder (Perrot-Minnot et al, 2019) and so is likely to regulary encounter *Gammarus* spp whilst feeding. As *B. barbus* increases in length, the infection increases as the fish is very likely to consume *Gammarus* spp infected over 5mm due to the high encounter rate between the species and increasing gape of the fish. The high infection in *B. barbus* is supported by Kennedy, (2006 p.67) and Perrot-Minnot et al, (2019) who both show that prevalence and abundance is high in *B. barbus* adults.

It is likely that the main source of *P. tereticollis* infections occur through the eating of infected *Gammarus* spp. This is due to *P. tereticollis* being less manipulative than *P. leavis* (Perrot-Minnot, 2004) meaning that *Gammarus* infected with *P. tereticollis* remain low in the water column causing a high encounter rate between *B. barbus* and infected *Gammarus*. This could explain why although *B. barbus* are smaller than most of the *L. leuciscus* sampled, they often have a higher prevalence of *P. tereticollis.*

Prevalence of *P. tereticollis* in 0+ *S. cephalus* is the lowest of all species and is not dependent on size (fig 3). At similar sizes, *B. barbus* and *P. phoxinus* show much higher prevalence's (fig 3). This suggests that *Gammarus* spp are not an important part of 0+ *S. cephalus* diet. Mann (1975), looked at the diet of *S. cephalus* when they were juveniles and adults. He found that *Gammarus* were also rarely a part of *S. cephalus* diet with an occurrence of 1.9% for juveniles, 0% in medium sizes and 4% for adults. The prevalence and abundance of *P. tereticollis* infections within *S. cephalus* is regularly high in the UK for example in the River Avon and River Culm mean abundance is 68 and 74.1 respectively with both having a prevalence of 100% (Kennedy, 2006 p.67). The high prevalence in adult *S. cephalus* and low prevalence in 0+ fish suggests *P. tereticollis* may be exploiting post-cyclic transmission or a paratenic host in its life cycle due to irregular contact between *S. cephalus* and *Gammarus* but a high prevalence overall in adults. Medoc et al, (2011) showed that paratenic transmission of *Pomphorhynchus* spp from *P. phoxinus* to *S. cephalus* was possible and that 18% of the *Pomphorhynchus* spp established within *S. cephalus* after predation of *P. phoxinus.* This could explain how a fish that rarely feeds benthically on *Gammarus* could have such a low prevalence when 0+ (fig 3) but as adults have high prevalence's, as 0+ *S. cephalus* are unable to predate smaller infected fish and carry low infection (fig 3) but when the species is larger are able to predate fish (Mann, 1975) and carry a high prevalence of *P. tereticollis* (Kennedy, 2006 p.67).

It is unlikely that a high prevalence of *P. tereticollis* is due to adult *S. cephalus* consuming more infected *Gammarus* spp than uninfected *Gammarus* spp as *P. tereticollis* is less manipulative than *P. laevis* and so both infected and uninfected *Gammarus* spp reside lower in the water column (Perrot-Minnot, 2004). As *S. cephalus* feed bentho-pelagically and both infected and uninfected *Gammarus* spp remain low in the water column the encounter rate should be low between *S. cephalus* and infected and uninfected *Gammarus* spp. This has been confirmed in field studies where the infection of *S. cephalus* with *P. laevis* is much higher than *P. tereticollis* (Perrot-Minnot et al, 2019) and they concluded that this was because *Gammarus* infected with *P. laevis* would have a much higher encounter rate with *S. cephalus* due to being higher in the water column.

The most likely post-cyclic or paratenic transmission route for *P. tereticollis* is from *P. phoxinus* to *S. cephalus* as *P. phoxinus* carry the second highest prevalence of all the 0+ fish. Paratenic transmission from *P. phoxinus* is possible as 20% of *P. tereticollis* infection were harbouring extra-intestinally (own observations). As well as this, infections found within the intestine of *P. phoxinus* displayed limited attachment of the proboscis to the intestine and where easily removed from the species compared to the other species in the study. This is supported by Kennedy, (1999) who showed that post-cyclic transmission was unsuccessful when *S. cephalus* was predated by *Oncorhynchus mykiss* compared to when *Cottus gobio* was predated by *O. mykiss*. Kennedy, (1999) said the difference was due to a difference in the host encapsulation effect between the two species with *P. tereticollis* within *S. cephalus* displaying a larger host encapsulation effect and an unsuccessful establishment after predation compared to *C. gobio* where host encapsulation was limited and establishment was successful following predation. Due to both the extra-intestinal infections and the limited attachment of proboscis within the intestine makes it likely that *P. phoxinus* is contributing to the life cycle of *P. tereticollis* in a paratenic or post-cyclic manner.

Maitland, (1965) found the occurrence (%) of *Gammarus* in the digestive tract of *P. phoxinus* to be 12%. Although this is the 7th most common food consumed by *P. phoxinus* in the study, other food sources were consumed more often for example, the occurrence of algae species was found in 28% of fish and *orthocladius* spp found in 49% of stomaches. As well as this, most of the *Gammarus* spp by bulk occurred in the winter months and there was a decrease in the bulk of *Gammarus* spp in summer months. Diptera represented the most abundant food source by bulk in the summer months indicating that for large periods of time *P. phoxinus* fed on the surface of the water. This agrees with the results of Frost, (1943) who found that *P. phoxinus* midwater and surface feeding increased in summer months and then feeding increased in the benthos during winter. This could explain why length increases prevalence of *P. tereticollis* but the prevalence remains lower than *B. barbus* of the same length (fig 3). Fish length increases the probability that a *P. phoxinus* will predate a *Gammarus* spp (fig 3) but as *Gammarus* spp are a relatively small part of the diet of *P. phoxinus* in the summer months when the samples were collected, the limiting factor is not just length of *P. phoxinus* but also the diet of the fish as they could be predating *Gammarus* spp as more of a chance food compared to *B. barbus* which are regualy eating *Gammarus* spp as their length increases (fig 3).

Although there was a slight positive relationship between standard length of *L. leuciscus* and infection with *P. tereticollis* ($y=1.0507x+19.542$) standard length was neither significant (fig 3) or a predictor of infection (table 4). This is due to other factors such as temperature and date playing a more significant role on infection as the size groups show a sporadic plot.

4.2 Date and infection of *P. phoxinus* with *P. tereticollis*

The diet of *P. phoxinus* could explain why date is an important predictor of infection for the species. Prevalence is regulary higher in September compared to earlier months in the same year (fig 2). The midwater and surface water feeding decreases with month and so they may predate on more *Gammarus* spp in september and october due to increased benthic feeding (Frost, 1943). This would support the results of Perrot-Minnot et al, (2019) which found that there were almost no infections of *P. tereticollis* in *P. phoxinus* as a proportion of *P. laevis* to *P. tereticollis.* This agrees with the diet hypothesis described here as samples for Perrot-Minnot et al, (2019) where taken in spring and summer when the benthic feeding of *P. phoxinus* is reduced (Maitland, 1965; Frost, 1943). *Gammarus* spp infected with *P. tereticollis* remain lower in the water column compared to *Gammarus* spp that are infected with *P. laevis* so infection with *P. laevis* would be higher for *P. phoxinus* as the encounter rate for spring and early summer would be higher.

However, the prevalence may also be lower due to less successful establishment in the species compared to *B. barbus*. This is due to the host encapsulation that occurs in some species but not in others (Kennedy, 1999). This process did not occur in any of the *P. phoxinus* studied and parasites were discovered in many areas of the digestive tract suggesting that parasites may be more readily lost from the fish compared to *B. barbus* where host encapsulation does occur.

4.3 Infection of *L. leuciscus* with *P. tereticollis* with Temperature and Date

L. leuciscus infection with *P. tereticollis* was predicted by both temperature and date (table 4). As temperature decreases, the prevalence of *P. tereticollis* increases (fig 5) and as the month increases towards the end of the year, so does the prevalence (fig 6). This could be explained by several factors. Firstly, as the temperature decreases, the fish may reside lower in the water column and so encounter and predate more *Gammarus* spp increasing prevalence. As well as this, warmer temperatures decrease the probability of successful establishment compared to lower tempertures (Kennedy, 1972). Unsuccessful establishment is unlikely as *P. phoxinus* infection for July and August 2016 would be lower than September and October 2015 if temperature was having a large effect on fish (fig 20) therefore it is more likely due to temperature related differences in feeding within the species.

Food availability is likely to explain why infection can be predicted by date as *L. leuciscus* food preferences are linked to whatever is available (Helawell, 1974) and so will likely consume whatever can be found as long as the prey item is in high abundance. As *Gammarus* spp can be found in every month (Hine and Kennedy, 1974a; Harris et al, unpublished data) and there is a decrease in the number of different prey items consumed by *L. leuciscus* as the month heads towards October (own observations), it may be possible that they are shifting towards predating *Gammarus* spp more regualary. This is supported by Hine and Kennedy, (1974a) which showed that *Gammarus* spp where consumed by *L. leuciscus* all year at a low occurrence then predation of *Gammarus* spp increases in September and March (roughly 9% and 11% respectively). They also noted the increase in March was linked to a change in diet from plant to animal matter with the amount of animal matter being highest in summer and the plant matter becoming the main food source in winter. *L. leuciscus* infection with *P. tereticollis* could therefore be explained by *L. leuciscus* predating *Gammarus* spp when other animal matter in low inabundance in september and October before switching to plant matter in the winter months.

Although prevalence is reasonably high towards the end of the year, it is unlikely that *P. tereticollis* will have success in this species due to the inability of *P. tereticollis* to mature in this species over winter (Hine and Kennedy, 1974b). This means that even if there is both male and female *P. tereticollis* present in the alimentary tract of individual *L. leuciscus,* it is unlikely that they will mature in time to reproduce before being expelled as in hosts that are not preferred such as *Carassius auratus*, they are expelled within 70 days when food availability is low (Kennedy, 1972). *L. leuciscus* can be considered a host that is not preferred as the production of gravid females in this species is lower than *B. barbus* and *S. cephalus* as well as the parasites achieving a smaller average weight (Kennedy, 2006 p.67).

This means that 0+ *L. leuciscus* is likely to be a dead end host for *P. tereticollis* unless *L. leuciscus* can be classed as a paratenic host or facilitate the post-cyclic transmission of the parasite. Although there is little data, it can be tentatively suggested that post-cyclic transmission is possible from *L. leuciscus* to larger fish as the proboscis do not penetrate deeply or cause an extreme host encapsulation response (Hine and Kennedy, 1974b). As well as this, 6.7% (1.dp) of *P. tereticollis* infections were found extra-intestinally in the study potentially making paratenic transmission possible.

4.4 Gut fullness

There is a negative B value for gut fullness suggesting that infection occurs when gut fullness is lower (table 4). This can be explained by most infected fish occurring when food availability was lower for compared to fish caught in early summer samples (fig 4). This means that a randomly selected uninfected fish will have a higher gut fullness as the uninfected fish are also frequently found in periods when food availability is high. When month is plotted as a covariate, infected fish consume more food than uninfected fish in the same month (fig 4). Rather than being a predictor of infection it is more likely to be a consequence of infection as although infected individuals are larger, there is no significant relationship between size and gut fullness (table 3; see appendix) indicating that larger fish are consuming more food to maintain their body size due to an energy deficit or behaviour change caused by *P. tereticollis*. This is supported by the *L. leuciscus* data as length was not significant to explain prevalence of *P. tereticollis* and there was also no size difference between uninfected and infected gut fullness groups.

Behaviour changes caused by Acanthocephalan in vertabrate hosts are thought to not occur (Kennedy, 2006 p. 85) and only one study has shown this (see Kennedy, 2006 p.85). Gut fullness was significantly higher in infected *P. phoxinus* compared to uninfected *P. phoxinus* (fig 4) which could contradict current understanding of Acanthocephalan interactions with vertebrate hosts. Adult fish infected and uninfected with *P. tereticollis* do not differ in size or growth rate (Hine and Kennedy, 1974b) and as there was no positive trend between size of *P. phoxinus* and gut fullness (see appendix) it can be conclueded with relative certainty that fish infected with *P. tereticollis* are increasing their feeding activity in order to maintain size and growth rate. If Kennedy, (2006 p.85) is correct, then this is likely to be due to an energy deficit in infected individuals either by higher immune activity (Harris, 1972) or by *P. tereticollis* feeding in the alimentary tract therefore stealing resources from the fish as oppose to direct manipulation by the parasite.

The increased feeding of *P. phoxinus* may correspond to a higher probability of predation as the higher energetic cost will have to be adjusted by increased feeding which could cause fish to spend more time under the risk of predation. This has been show in (Giles, 1987) where *Gasterosteus aculeatus* (three-spined sticklebacks) which were infected with *Schistocephalus solidus* returned to feeding faster after a predation risk. As well as this, infected individuals consumed more food than uninfected individuals although these affects may be less severe regarding *P. phoxinus* and *P. tereticollis* as no growth of *P. tereticollis* occurs within *P. phoxinus* (Kennedy, 2006) so the energy stolen from *P. phoxinus* by *P. tereticollis* may be less than in a host where the parasite is actively growing. There is still a large increase in feeding between infected and uninfected fish as in September and October, gut fullness was 60% and 200% higher in infected fish compared to uninfected fish (fig 4). Although, this should be treated with caution due to low numbers of infected individuals (n=4 for both months). If further work shows the trend of higher food consumption in infected individuals to be correct, then this behaviour change has the possibility to impact the transmission of the parasite through post cyclic and paratenic transmission. As well as this, the parasite may strengthen the link between infected *P. phoxinus* and predators compared to uninfected individuals which has been shown in other parasites (Sato et al, 2011). Regardless of whether this change in host behaviour is a side effect of parasitism (Lafferty, 1999) or direct manipulation of *P. phoxinus* by *P. tereticollis*, there would seem to be an advantage to the parasite as increased foraging may increase predation and so increase the chance of transmission to a host were the parasite can mature and produce offspring.

As with *P. phoxinus* both infected *B. barbus* and *L. leuciscus* display an increase in gut fullness compared to uninfected fish in September and October (Fig 4) although neither species infected gut fullness was significantly different from the uninfected in any month. This could still have implications for the individuals infected though. For example, the increase in predation as mentioned above. However, post cyclic transmission is less likely in *B. barbus* compared to *P. phoxinus* and *L. leuciscus* due to the difference in host encapsulation observed between preffered host to unpreferred hosts (Hine and Kennedy, 1974b). As well as this, 0+ *B. barbus* harboured no extra-intestinal parasites and so can not be regardeded as a partenic host.

Infected fish may come under the risk of starvation during winter if they require more food than uninfected individuals. Over winter, food availabillity declines and so the parasitised fish may be more likely to starve compared to uninfected fish. As well as this, under 10 C the fishes ability to metabolise food is reduced. If this were the case and the infected individuals were eating to much food, this could result in food not be metabolised quickly enough and may increase the risk of bacterial infections. This is demonstrable in Hine and Kennedy, (1974b) where two *Thymallus thymallus* individuals died in the laboratory probably as a result of half digested food found in the body cavity with a large build up of bacteria due to the fact the food could not be removed similar to what could happen in the alimentary tract when metabolic activity is lower. Although this is merely a hypothesis and would require much more further work.

5. Conclusion

The prevalence of *P. tereticollis* across 0+ fish differed between species with *B. barbus* having the closest relationship with *P. tereticollis* in terms of fish length. Considering that *S. cephalus* and *B. barbus* are considered the main hosts for the parasite in adult fish, it is surprising that such a low prevalence was found in the 0+ *S. cephalus* compared to all other species examined. No relationship to any variable apart from gut fullness was found and gut fullness was not significant. This is likely due to larger *S. cephalus* predating infected smaller fish and obtaining infections through post-cyclic transmission. The difference in prevalence of 0+ *B. barbus* and 0+ *S. cephalus* highlight the possibility of more complex interactions in the life cycle of *P. tereticollis*. Further work will be needed to comfirm this proposed mode of transmission where smaller possible paratenic hosts would have to be present and absent from experiments with other food sources avaliable so that *S. cepahalus* are not forced to eat *Gammarus* or *P. phoxinus* due to the absence of other food sources which could have been the case in Medoc et al, (2011).

The 0+ fish are not important for the success of *P.tereticollis* without the possibillity of paratenic or post-cyclic transmission due to the low abundance and timing of infections. The infections of *P. phoxinus* and *L. leuciscus* are possibly dead ends for the parasite as *P. tereticollis* never matures in *P. phoxinus* in the UK and most infections for 0+ *L. leuciscus* occur too late in the year for the parasites to mature (Hine and Kennedy, 1974b). Only *P. tereticollis* that infects *B. barbus* have the possibillity of successful reproduction as it is a preffered host and some hosts contain multiple parasites although this is unlikey to have a large impact on the population of *P. tereticollis* due to egg production being potentially linked to host size (Perrot-Minnot et al, 2019). *P. tereticollis* within 0+ *S. cephalus* do not have the possibillity to reproduce due to low abundance and prevalence. These parasites will likely die before reproduction can occur as it is unlikely that post cyclic transmission is possible due to the host encapsulation process undertaken in *S. cephalus*.

P. tereticollis may be having a negative impact on 0+ fish survival compared to adults as increased gut fullness was observed in all infected individual species apart from infected *S. cephalus* (fig 4). The potential energy deficit caused by *P. tereticollis* has the potential to increase stravation compared to uninfected fish although further laboratory experiments will be needed to determine if there is a higher feeding activity in infected fish as well as the impact this could have on overwinter survival compared to non-infected fish. Further work should also be conducted to determine if infected 0+ are more likely predated due to increased feeding activity.

Chapter 2: Intermediate Host Use By *Pomphorhynchus tereticollis* in Southern English Rivers

1. Introduction

Pomphorhynchus tereticollis is an Acanthocephalan parasite of fish. Like all Acanthocephalans, the species has an indirect life cycle and requires an invertebrate intermediate host to complete its life cycle (Kenedy, 2006 p.12). Regarding *Pomphorhynchus* spp, the intermediate host is always a Gammarid species. The life cycle of *P. tereticollis* begins when a shelled acanthor is expelled from the alimentary tract of the fish by a mature female parasite that has successfully infected a fish and reproduced with a male. Once the shelled acanthor is expelled it will lie dormant in the benthos until it is ingested by a *Gammarus* spp. If the host is a viable species, the acanthor will develop through three life periods from acanthor to late acanthella to cystacanth with the process usually lasting 40-65 days (Rumpus, 1973). The cystacanth stage is infectious to fish and is more manipulative to the gammarid host in this stage of its development compared to earlier stages (Franceschi et al, 2008).

P. tereticollis is a generalist parasite at the definitive host stage (Kennedy, 2006 p.67) and has been found in over 16 species in both salmonids and cyprinids (Hine and Kennedy, 1974b). The main hosts for the parasite in the south of England are *Barbus barbus* and *Squalius cephalus* (Hine and Kennedy, 1974b). Evidence for this can be seen in the high percentage of gravid females (71.0% for *B. barbus* and 67.2% for *S. cephalus*) compared to other species such as *Leusiscus leusiscus* where on average gravid females are half as common in the south of England (Kennedy, 2006 p.67). However, local adaptation of the parasite has been observed in rivers where preferred hosts are less common with the parasite adapting to *L. leusiscus* in the absence of *B. barbus* in the River Culm and to *Salmo trutta* in the River Otter when both *B. barbus* and *S. cephalus* are absent (Kennedy, 2006 P. 66). The generalist nature of the parasite has likely enabled the widespread distribution across the entire of the UK and most of central, western and Ponto-Caspian Europe (fig 3) (Perrot-Minnot et al, 2017). *Gammarus* spp are a ubiquitous freshwater crustacean present in most European rivers (Hine and Kennedy, 1974a; Weiss et al 2011; Blackmann et al, 2017; Perrot-Minnot et al, 2019) (fig 2) and also contribute to the widespread distribution of the parasite. *P. tereticollis* is considered a generalist at the intermediate stage capable of infecting many species of *Gammarus* spp (Kennedy, 2006 P. 66). However, recent research has highlighted there may be a preference in intermediate host use for the parasite. Both Westram et al, (2011) in Switzerland and Galipaud et al, (2017) in France showed that infection of *P. tereticollis* occur most often in *Gammarus fossarum* (*G. fossarum*), compared to *Gammarus pulex* (*G. pulex*) as well as other Gammarid species.

G. fossarum and *G. pulex* have similar distributions in Europe (Pockl et al, 2003; Westram et al, 2011; Blackman et al, 2017; Galipaud et al, 2017) and are considered cryptic species as highlighted by the grouping of the two species in to the *Gammarus pulex/fossarum* complex in France (Perrot-Minnot et al, 2019). There are however distinct differences between the species for example, *G. pulex* regularly obtain a larger size than *G. fossarum* Harris et al, (unpublished) and usually prefers slower flow rates and lower stretches of rivers compared to *G. fossarum* (Pockl et al, 2003). There are also morphological differences such as the absence of plumose setae on the outer edge of Uropod III in *G. fossarum* (fig 1) (Blackmann et al, 2017). Genetic structuring also separates the two species as *G. pulex* has strong genetic structuring in the Mediterranean region (Muller, 1999) and weaker genetic structuring in Northern and Western Europe (Scheepmaker, and Dalfsen, 1989) while *G. fossarum* has stronger genetic structuring in the central and Ponto-Caspian regions of Europe (Muller 1999).

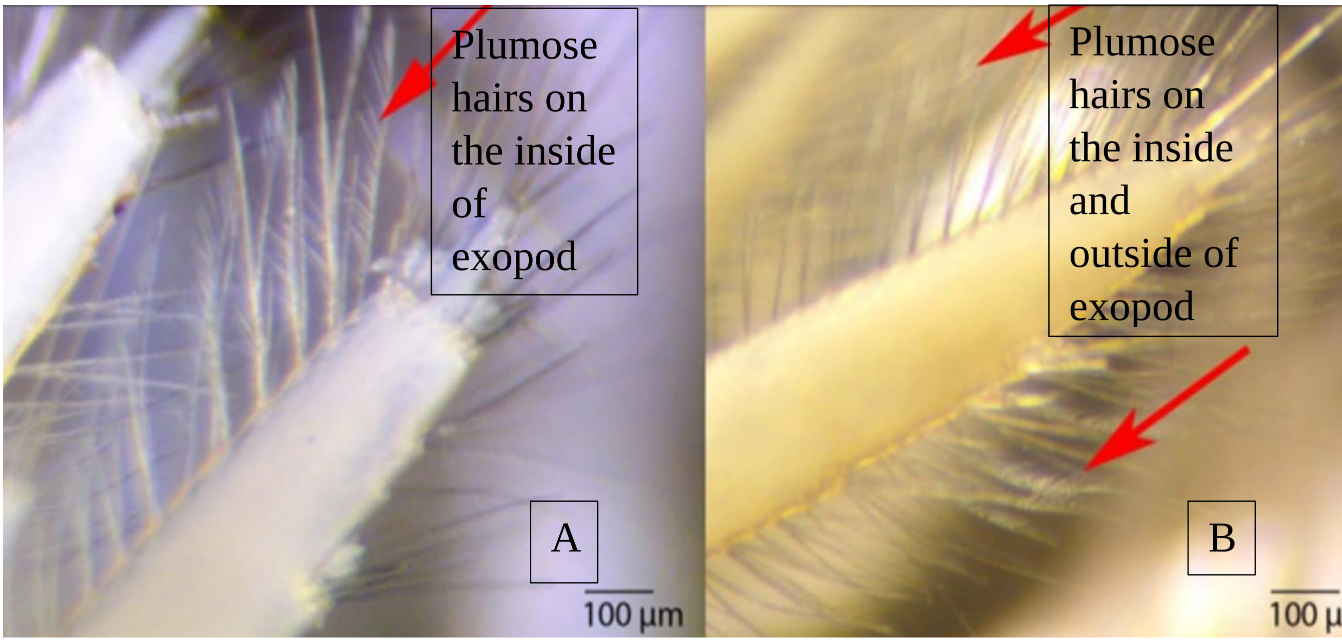
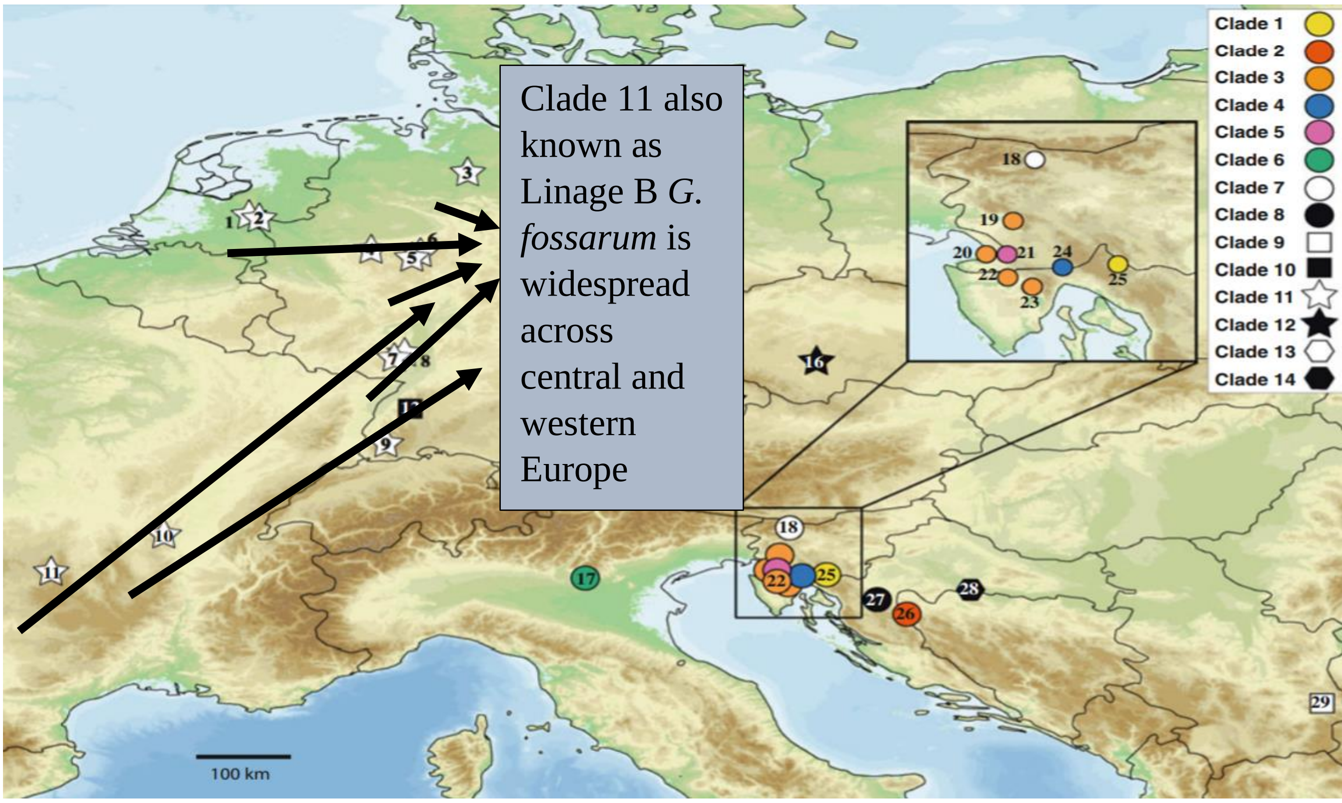


Figure 1. Presence of plumose hairs on the Exopod of Uropod III. *Gammarus fossarum* have plumose hairs on the inside of the exopod only (A) while *Gammarus pulex* have

plumose hairs on the inner and outer edge of the exopod (B). Image adapted from Blackman et al, (2017).



Clade 11 also known as Linage B *G. fossarum* is widespread across central and western Europe

Figure 2. Distribution of *Gammarus fossarum* across Europe. Adapted from Weiss et al, (2014)

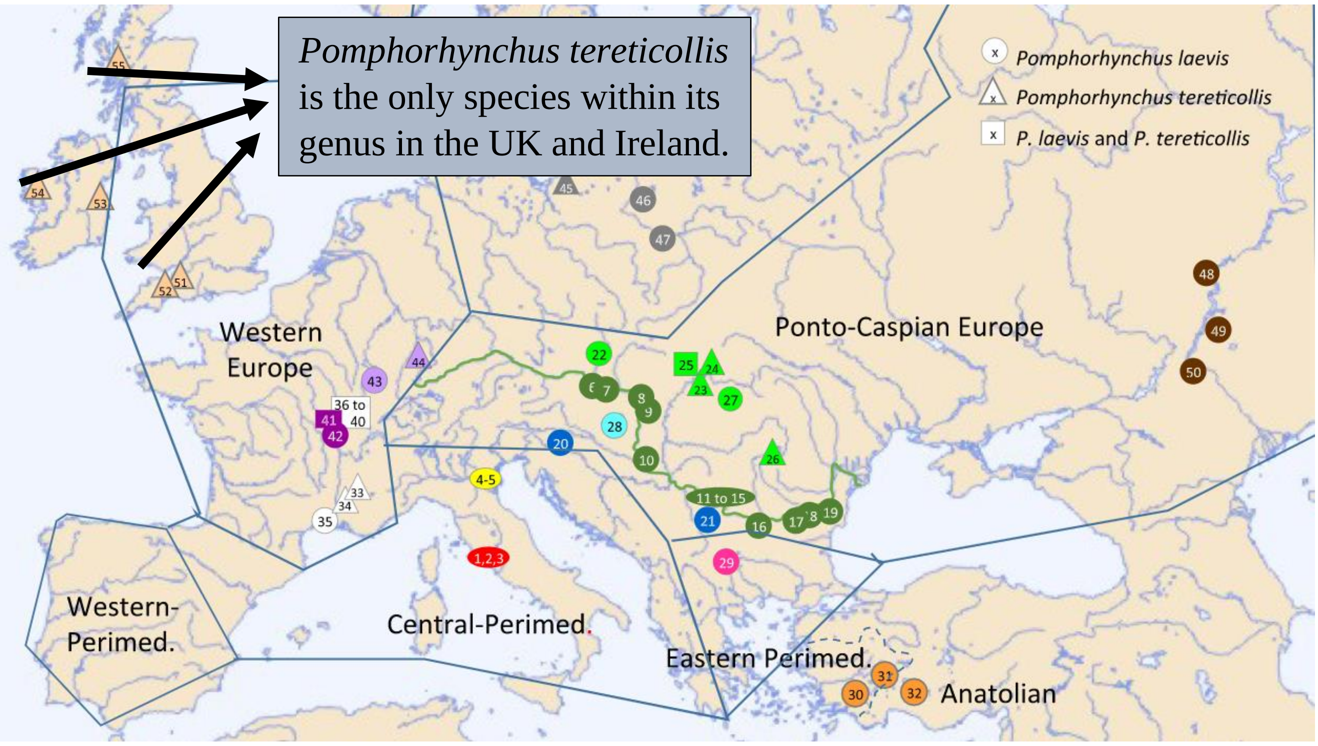


Figure 3. Distribution of *Pomphorhynchus tereticollis* and *Pomphorhynchus laevis* within Europe. Adapted from Perrot-Minnot et al, (2017).

It was thought that the only freshwater gammarid species in the UK was *G. pulex* until Blackmann et al, (2017) recently discovered *G. fossarum* with eDNA, morphology and standard DNA extraction techniques in several widespread rivers in the UK. When *Gammarus* spp samples from the Natural history museum were examined, it was found that the species had been present in the UK since at least the 1960's. There has never been a study produced in the UK that confirmed the species that *P. tereticollis* uses at the intermediate host stage and previous researchers based host usage on the assumption that *G. pulex* was the only species found in the UK. Considering the strong preference of *P. tereticollis* to *G. fossarum* in Westram et al, (2011) and Gaulipaud et al, (2017) and the recent discovery of *G. fossarum* in the UK a study into the host use of the parasite is needed.

Therefore, the research objectives of the study were to (a) investigate the degree of generalism displayed by *P. tereticollis* in its intermediate host in southern England, (b) observe the prevalence of *P. tereticollis* within different rivers in the south of England, (c) observe the proportions of *G. pulex* and *G. fossarum* in different rivers in Southern England

2. Method

2.1 *Gammarus* sample collection

Gammarus spp were collected from the River Teme (near Knightwick, Powick and Tenbury), the River Kennet (near Padworth), the River Avon (near Ibsley) and the River Loddon (near Dinton Pastures, Stanford end Statfield, Swallowfield, longriver and Arborfield) (fig 1). These rivers where chosen as the river Kennet and river Loddon are western flowing rivers which would have been connected to mainland Europe in the last glacial maxima meaning that *Gammarus* spp and the parasite were likely to colonise the rivers naturally (Perrot-Minnot et al, 2017) compared to the river Avon and the River Teme which are eastern flowing and so likely to have been colonised by introductions during the translocation of cyprinids. *Gammarus* spp were collected using a kick sample technique where the gravel was disturbed 20 times with the foot. A kick net with mesh size 250µm was placed downstream of the site disturbed to catch potential *Gammarus* spp. If low numbers were caught, then several kick samples were performed until at least 20 *Gammarus* spp which were large enough to speciate were caught. The *Gammarus* spp were then preserved in 99% ethanol until dissection.

All the locations were collected in different months ranging from July to September 2019 (Table 1). This however does not matter as previous research (Harris et al, unpublished) showed that both species of *Gammarus* remained in the relative proportions throughout the year and both show similar ecologies (Grabowski et al, 2007)

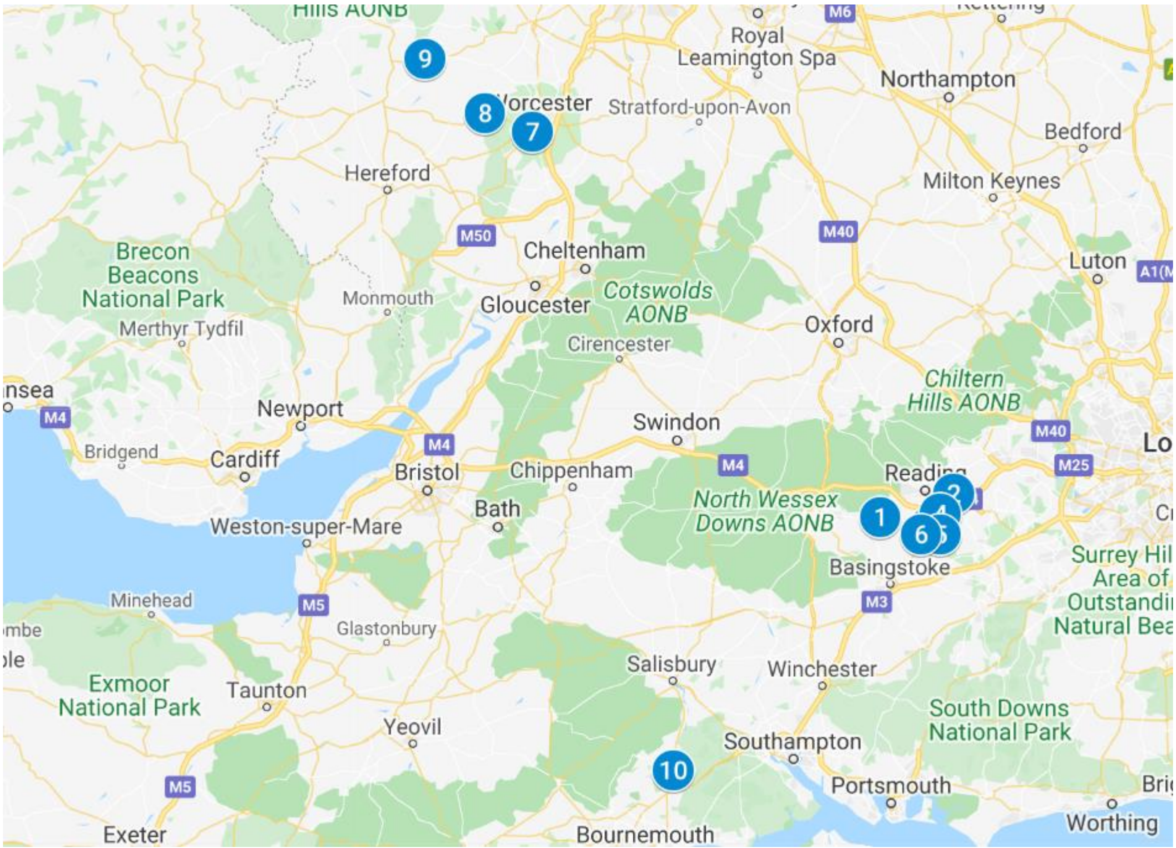


Figure 4. Sampling sites for the collection of *Gammarus* spp, 1=Padworth (River Kennet), 2=Dinton Pastures (River Loddon), 3 (between 4, 5 and 6)= Swallowfield (River Loddon), 4=Arborfield (Blackwater river), 5=Longriver (long river), 6= Stanford end (River Loddon), 7=Powick (River Teme), 8=Knightwick (River Teme), 9= Tenbury (River Teme), 10=Ibsley (River Avon). Map constructed using Google maps.

2.2 *Gammarus* Dissection

Gammarus spp were dissected using a standard Brunel dissection kit. *Gammarus* spp were removed from the ethanol and their length to the nearest millimetre (mm) was measured. Fine point tweezers were then used to remove the head of the *Gammarus* spp. The *Gammarus* spp was then opened ventrally and any potential *P. terticollis* infections were removed from the *Gammarus.* The pleon of the individual was then used for species identification. While the rest of the *Gammarus* spp was stored in 99% ethanol for DNA extraction. Ten individual *Gammarus* spp were dissected per site due to low sample sizes at some locations and the time required to morphologically speciate the gammarids.

2.3 *Gammarus* identification

Gammarus spp were identified to the species level using the methodology by Blackmann et al, (2017). Specifically, the species was determined by the location of plumose setae. When the plumose hairs were present on the inner and outer edge of the exopod of Uropod III, the gammarid was identified as *G. pulex* and when plumose hairs where found only on the inner edge of the same exopod, the gammarid was identified as *G. fossarum.* Individuals were only considered for species identification if the individual was mature enough to have sufficient numbers of plumose hairs as they are not present on immature specimens and sufficient numbers of the plumose hairs would make the identification more reliable. For this reason, individuals smaller than 6.5mm (1d.p) were not used for species identification and a mean length of all individuals speciated was 8.8mm (1d.p). The species was also checked by the ratio of the endopod to the exopod where if the ratio was below 0.5 the species was confirmed *G. fossarum* whereas, the species was confirmed *G. pulex* if the ratio was over 0.5 assuming the location of plumose setae agree with this confirmation. After dissection and species identification, the *Gammarus* spp were placed into individual microcentrifuge tubes containing 99% ethanol for molecular analysis.

2.4 Statistical analysis

All statistical analysis was completed in SPSS using Windows 10. A Pearson's correlation was performed between the proportion of *G. fossarum* and the prevalence of *P. tereticollis* due to heteroscedasticity in the data. All other data was analysed using a chi squared test to compare prevalence between groups such as prevalence of *P. tereticollis* between rivers and between *G. fossarum* and *G. pulex.*

3. Results

Both *G. pulex* and *G. fossarum* were found in the study. In the River Teme, only one site had *G. pulex* in the sample, which was Knightwick in August with a low prevalence of 10%. The River Loddon had a more mixed prevalence of *G. pulex* and *G. fossarum* compared to the River Teme where only the site at Long River had a prevalence of 100% *G. pulex* and 0% *G. fossarum* with the rest consisting of relatively mixed samples (Table 1).

Table 1. Proportion of *Gammarus pulex* and *Gammarus fossarum* in samples collected from the River Loddon, River Teme, River Avon and the River Kennet. Several locations along the rivers were sampled for the River Loddon and River Teme while only one sample site was used for the River Avon and River Kennet. *G. fossarum* was the most predominate species in the River Teme while the River Loddon had mixed samples apart from the site at long river where 100% of the *Gammarus* were *G. pulex.*

River	Date	Location	Proportion of *G. pulex* (%)	Prevalence of *P. tereticollis* within *G. pulex* (%)	Proportion of *G. fossarum* (%)	Prevalence of *P. tereticollis* within *G. fossarum* (%)
Loddon	24/07/2019	Swallowfield	13	0	88	25
Loddon	24/07/2019	Arborfield	57	0	43	14
Loddon	21/08/2019	Longriver	100	0	0	0
Loddon	25/09/2019	Dinton Pastures	60	0	40	10
Loddon	25/09/2019	Stanford End	60	0	40	30
Teme	23/07/2019	Powick	0	0	100	88
Teme	20/08/2019	Powick	0	0	100	88
Teme	24/09/2019	Powick	0	0	100	50
Teme	23/07/2019	Knightwick	0	0	100	0
Teme	20/08/2019	Knightwick	10	0	90	90
Teme	23/07/2019	Tenbury	0	0	100	20
Avon	20/09/2017	Ibsley	20	0	80	40
Avon	20/07/2017	Ibsley	20	0	80	17
Kennet	23/07/2019	Padworth	80	0	20	0

The prevalence of *P. tereticollis* within samples collected in the River Teme, the River Loddon, the River Kennet and the River Avon shows heteroscedasticity as the proportion of *G. fossarum* increases. The relationship between *G. fossarum* and *P. tereticollis* was not significant Pearson Correlation=0.935, p=0.65, n=4.

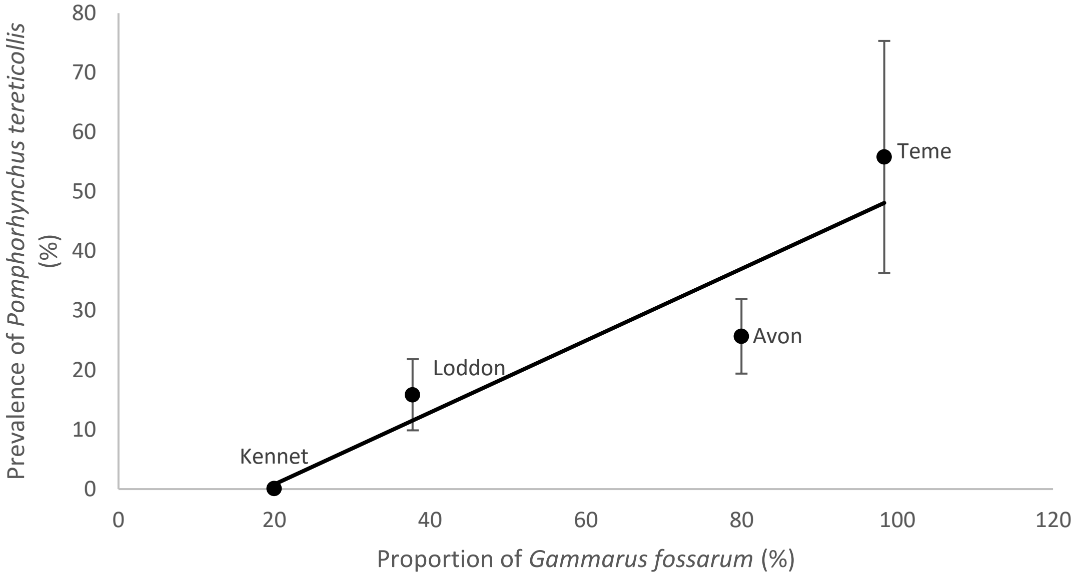


Figure 5. Proportion of *Gammarus fossarum* in sample (%) of *Gammarus* spp collected from the River Teme, the River Loddon, the River Kennet and the River Avon plotted with the prevalence of *Pomphorhynchus tereticollis* (%). The graph shows heteroscedasticity and this relationship was not significant Pearson Correlation=0.935, p=0.65, n=4.

The prevalence of *P. tereticollis* increased with the proportion of *G. fossarum* in the river and decreased with the proportion of *G. pulex* (fig 2). The River Teme had the highest prevalence of *P. tereticollis* in the sample as well as the highest proportion of *G. fossarum* to *G. pulex*. The River Kennet sample had the lowest prevalence of *P. tereticollis* and the highest proportion of *G. pulex*. The difference in the prevalence of *P. tereticollis* in each river was not significant Pearson Correlation=0.935, p=0.65, n=4.

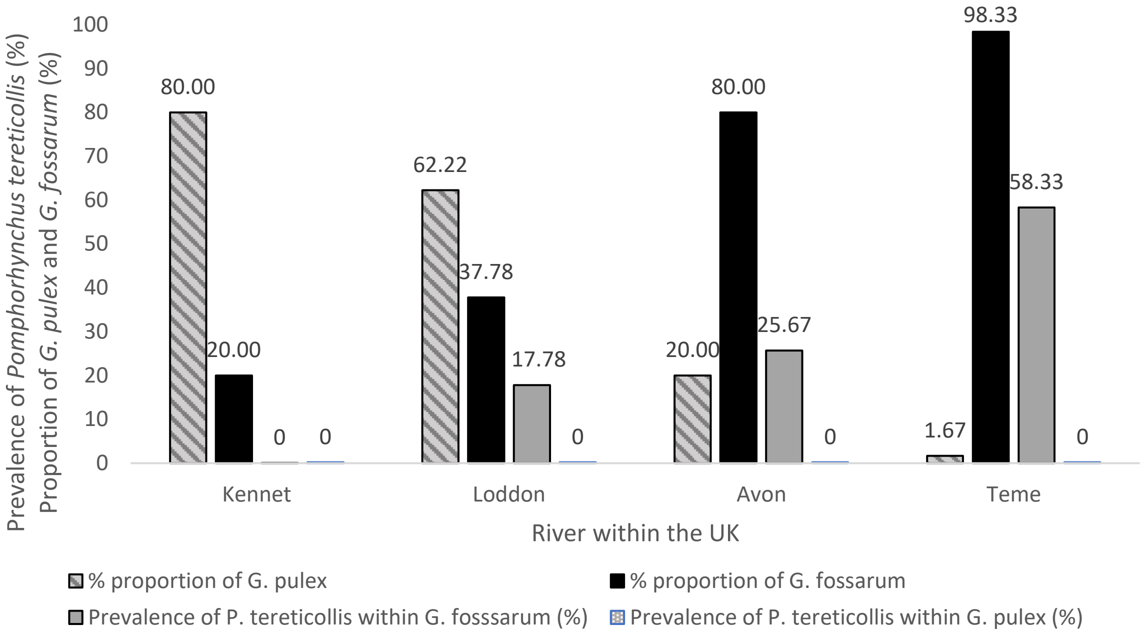


Figure 6. Proportion of *Gammarus pulex* and *Gammarus fossarum* (%) within each river in the UK plotted with the prevalence of *Pomphorhynchus tereticollis* (%). Samples were collected from the River Teme and the River Loddon in July, August and September 2019 and from the River Kennet in July 2019 and form the River Avon in July and September 2017. When samples are pooled across all months and placed together for whole rivers the difference in prevalence was not significant Pearson Correlation=0.935, p=0.65, n=4.

The difference in prevalence of *P. tereticollis* across the two species was significant chi squared=35.000, d.f=1, p<0.0001. The prevalence in *G. pulex* was 0% and the prevalence in *G. fossarum* was 61.02% (2 d.p). *Gammarus* spp were pooled from all sample sites including the River Teme, the River Avon, the River Loddon and the River Kennet.

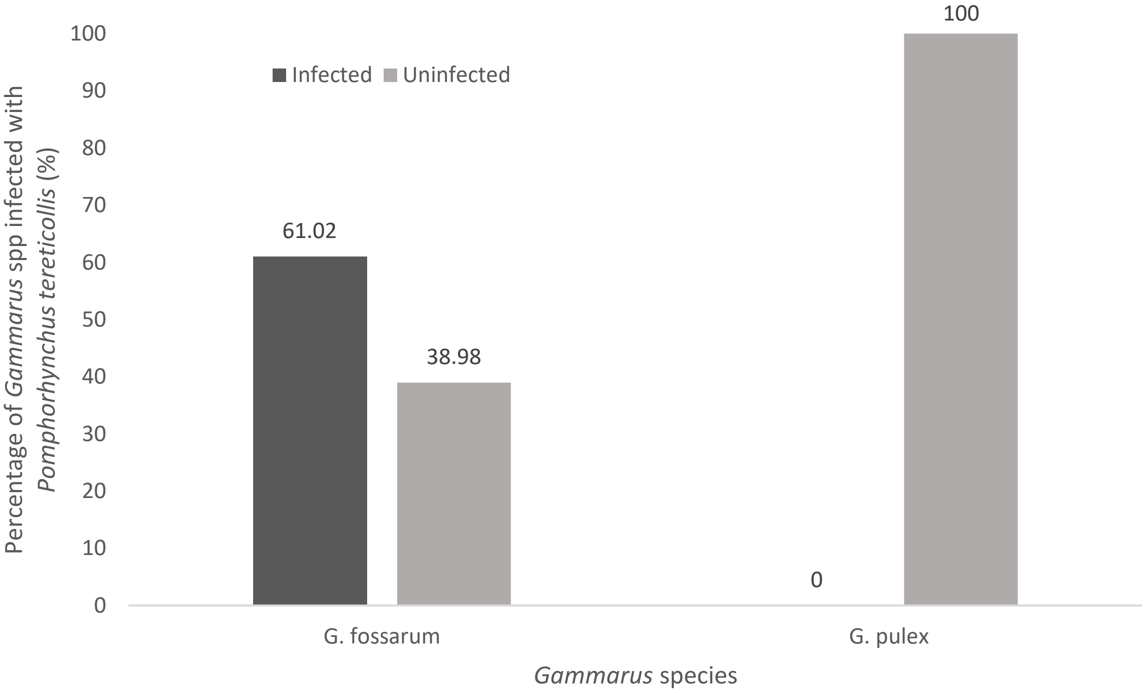


Figure 7. *Gammarus* species plotted with the percentage of *Pomphorhynchus tereticollis* within the species. The prevalence of *P. tereticollis* across the two species was significant chi squared=35.000, d.f=1, p<0.0001. The prevalence in *G. pulex* was 0% and the prevalence in *G. fossarum* was 61.02% (2d.p). *Gammarus* spp were pooled from all months and sample sites within the rivers including the River Teme, the River Avon, the River Loddon and the River Kennet. The prevalence of prevalence of *P. tereticollis* was significant chi squared=35.000, d.f=1, p<0.0001.

4. Discussion

Gammarus Fossarum was present in all four rivers tested in southern England and only *G. fossarum* was infected by *P. tereticollis* (fig 7). The prevalence of the parasite had a linear relationship with the proportion of *G. fossarum.* Heteroscedasticity in the data was present as the proportion of *G. fossarum* increased (fig 5). The high variation in the prevalence of *P. tereticollis* when *G. fossarum* was in high proportions was due to random crashes in the population of *P. tereticollis* cystacanths (Table 1). Crashes in prevalence of the parasite also occurred in Hine and Kennedy, (1974a) and in previous research (Harris et al, unpublished). This means variability in the prevalence of *P. tereticollis* will be higher when *G. fossarum* is in abundance as they can carry high and low prevalence's of *P. tereticollis* compared to when *G. pulex* is in abundance as the species cannot become infected by *P. tereticollis* in the UK resulting in a constant low prevalence of the parasite.

When whole rivers and several months are combined, the rivers with the largest proportion of *G. fossarum* also have the highest prevalence's of *P. tereticollis* (fig 6). *G. fossarum* therefore plays an essential role in the life cycle of *P. tereticollis* as the parasite cannot infect *G. pulex* in the UK and rivers with the large proportions of *G. fossarum* carry more cystacanths (fig 6). The difference in prevalence of *P. tereticollis* in English rivers is likely due to the distribution of *G. fossarum.* For example, the River Teme and the River Avon had the highest prevalence of *P. tereticollis* and the highest proportion of *G. fossarum* (fig 6). Which supports Kennedy, (1989) which also showed that the Avon and the Severn (the Teme being a tributary) had high prevalence's of *P. tereticollis.*

Specificity of the parasite for *G. fossarum* has also been shown on mainland Europe in both Switzerland (Westram et al, 2011) and France (Galipaud et al, 2017). Westram et al, (2011) showed with DNA techniques that *P. tereticollis* preferred lineage B *G. fossarum* to *G. pulex* and prevalence within *G. fossarum* was five times higher than in *G. pulex.* Galipaud et al, (2017) showed similar results to Westram et al, (2011). However, in both Westram et al, (2011) and Galipaud et al, (2017) *G. pulex* can still become infected with *P. tereticollis* although this may be due to a lack of Host-Parasite coevolutionary time in the UK.

P. tereticollis likely diverged in western and central Europe then moved into Germany where it diverged again and finally into the UK which has the youngest haplotype of the parasite (Perrot-Minnot et al, 2017). It is likely that *P. tereticollis* colonised the UK through a connection between the Rhine and the Thames during the last glacial maxima around 0.35 million years ago (Perrot-Minnot et al, 2017). Considering the specificity of *P. tereticollis* to *G. fossarum* it is likely that *G. fossarum* colonised the UK at the same time as *P. tereticollis* as oppose to a later colonisation as suggested by Blackman et al, (2017). This is supported by *G. fossarum* in the UK grouping more closely with *G. fossarum* from Germany compared to France and other European countries (Blackman et al, 2017). As well as this, the lack of strong genetic structuring or a clear UK haplotype of *G. fossarum* in the UK suggests several colonisation events similar to *P. tereticollis* (Perrot-Minnot et al, 2017). Pervious climatic conditions also make this route into the UK possible as glaciers went no further south than northern England and so populations of *G. fossarum* could have survived once in the UK as long as water temperatures were over 7.5°C (Pockl et al, 2003). As well as this, a lack of adaptation to *G. pulex* makes it unlikely that *G. fossarum* is a recent introduction as some adaptation to the gammarid would be expected if *G. pulex* was the host of *P. tereticollis* in the UK for nearly 0.35 million years. This is supported by Westram et al, (2014) who show that *G. pulex* can become infected with *P. tereticollis* where the parasite and *G. pulex* have occurred in sympatry since pre-glacial times (Scheepmaker and Dalfsen, 1989).

Considering the similarities between *G. pulex* and *G. fossarum*, the difference in host use is unlikely to be caused by differences in life history. For example, both species have similar minimum temperature tolerances with a range of 10-20°C optimum for *G. pulex* (Maazouzi ey al, 2011) and 7.5°C degrees minimum for *G. fossarum* to be present in a river (Pockl et al, 2003). As well as this, both species can occur in sympatry without competing (Pockl et al, 2003) and breed at similar times. The difference in host preference is therefore more likely due to past overlap in the parasite and *G. fossarum* as the preferred host, lineage B *G. fossarum* (Westram et al, 2011) diverged from A and C lineages between 9 and 19 million years ago and had refugia west of the Alps in more central/Ponto Caspian areas of Europe (Muller, 1999). This coincides with the divergence of *P. tereticollis* which was also present in central areas of Europe (Perrot-Minnot et al, 2019). Whereas, *G. pulex* likely originated in the Med region due to high genetic structuring in the location (Muller, 1999) and weak genetic structuring in northern western groups suggesting a more recent origin. However, *G. pulex* has been in Lowland central Europe since pre-glacial times and this could be the reason why

Westram et al, (2011) found some *G. pulex* infected with *P. tereticollis* as the parasite has had a longer time to adapt to *G. pulex* in this region.

It is likely that the difference in host use by *P. tereticollis* has occurred since the main research of the parasite began in the UK. Hine and Kennedy, (1974a) studied the parasite within *Gammarus* spp in the River Avon and observed that the largest *Gammarus* spp were all uninfected. This result was likely due to mixed samples of both *G. fossarum* and *G. pulex* as both are present in the river (Table 1) and adult *G. pulex* grow to a larger size compared to adult *G. fossarum*. The misidentification of the two cryptic gammarid species and the high specificity of *P. tereticollis* highlights the importance of understanding cryptic species for conservation purposes as cryptic species may spread non-native diseases to new areas at different rates especially in regard to translocation of different species through human activities. For example, Emde et al (2012) shows *Dikerogammarus villosus* (killer shrimp) becomes infected by *Pomphorhynchus* spp at a low prevalence (0.4%) while other species such as *G. fossarum* become infected at much higher prevalence's while *G. pulex* becomes infected at an intermediate prevalence in Switzerland (Westram et al, 2011) and are not able to be infected in the UK (fig 7). The cryptic nature of *Gammarus* and other freshwater invertebrates make it complex to study Host parasite relationships and caution should be displayed when translocating both fish and *Gammarus* spp to new areas. The work here provides further questions into the range of *G. fossarum* and the possible cryptic nature of other Acanthocephalan-host relationships in the UK such as with the parasites *Acanthocephalus anguillae* and *Polymorhus miniutus.*

5. Conclusion

Research in other European countries such as Germany is still needed to determine if *G. fossarum* is the main host over the entire distribution of the parasite. However, it seems highly probable based on evidence from three wide geographical areas (fig 4), (Westram et al, 2011; Galipaud et al, 2017). The research here highlights the potential for cryptic species to carry different prevalence's of parasites and highlights the potential that other acanthocephalan may have preferences for different gammarid species creating scope for research into *P. miniutus* and *A. anguillae* host use in the UK. As well as Acanthocephalans, other parasitic groups such as the microsporidians which infected *Gammarus* spp should also be studied in this context. It is also highly likely that there have been many misidentifications in host use when invertebrates are used as a hosts by parasites considering the confusion surrounding *Gammarus* spp.

The intermediate host likely restricts the distribution of *P. tereticollis* more than previously thought as without the presence of *G. fossarum* in the UK, *P. tereticollis* was unable to be present in the location. This could be a good method to prevent the spread of *P. tereticollis* to new locations as the parasite is not a generalist at the intermediate host level in the UK. Further work is needed to find the entire distribution of *G. fossarum.*

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Appendix

Size of *B. barbus* and gut fullness.

In August and September 2015, there was no significant relationship between the size of the fish and the gut fullness Regression F=1.288, d.f.=1 and 57, p=0.261, r^2=0.022 and Regression F=3.129, d.f= 1 and 13, p=0.100, r^2=0.194 respectively. The trend was slightly negative in August (fig A8) and positive in September (fig A9). The positive trend in September can be explained by the difference in size between infected and uninfected individuals as the infected are predominantly larger than uninfected fish and have a larger median gut fullness (fig 4) and so are mostly on the right hand side of the graph whereas uninfected appear on the left and so appear as a trend of positivity. However, when individual trendlines are drawn the trend for uninfected with size is slightly negative (fig A10)

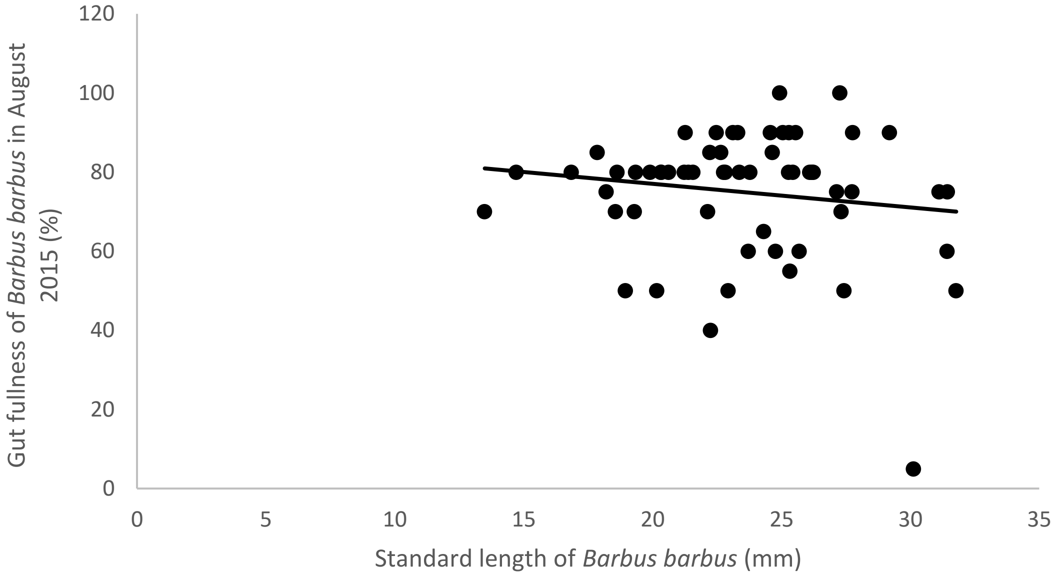

Figure A8. Standard length of infected and uninfected *Barbus barbus* (mm) plotted with Gut fullness in August 2015 (%). There is a slight negative trend between the size of *B. barbus* and gut fullness although this is not significant Regression F=1.288, d.f.=1 and 57, p=0.261, r^2=0.022

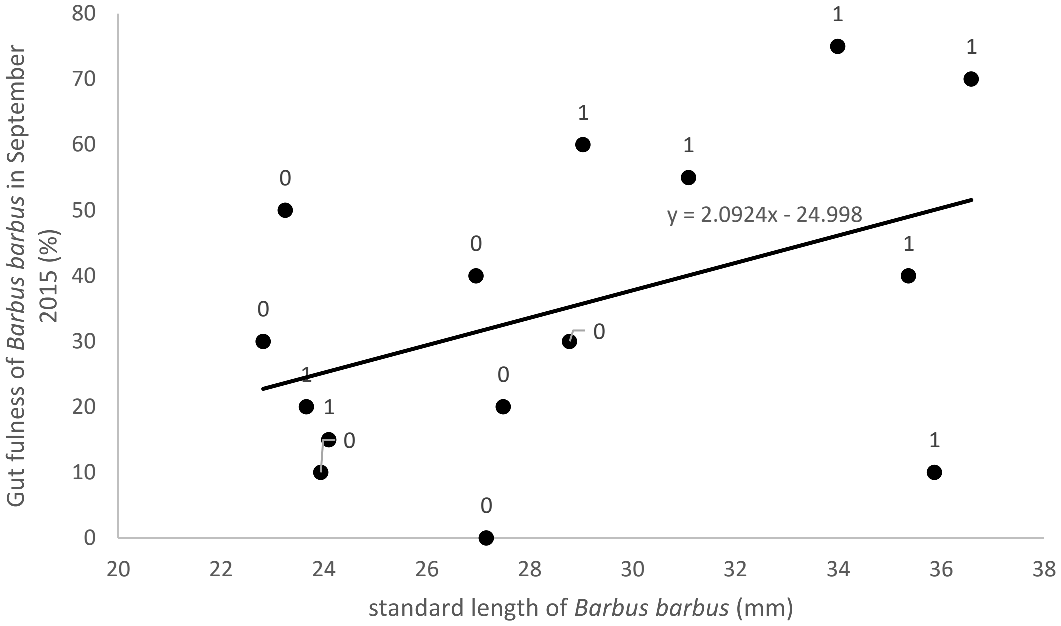

Figure A9. Standard length of *Barbus barbus* (mm) plotted with the gut fullness in septmeber 2015. There is a positive relationship between size of fish and gut fullness although, can be explained by the fact that infected (1) individuals predominatly have a gut fullness higher than uninfected individuals and are also larger and so appear mostly on the right hand side of the graph whereas unifected (0) smaller individuals appear on the left. The relationship between standard length and gut fullness was not significant Regression F=3.129, d.f= 1 and 13, p=0.100, r^2=0.194.

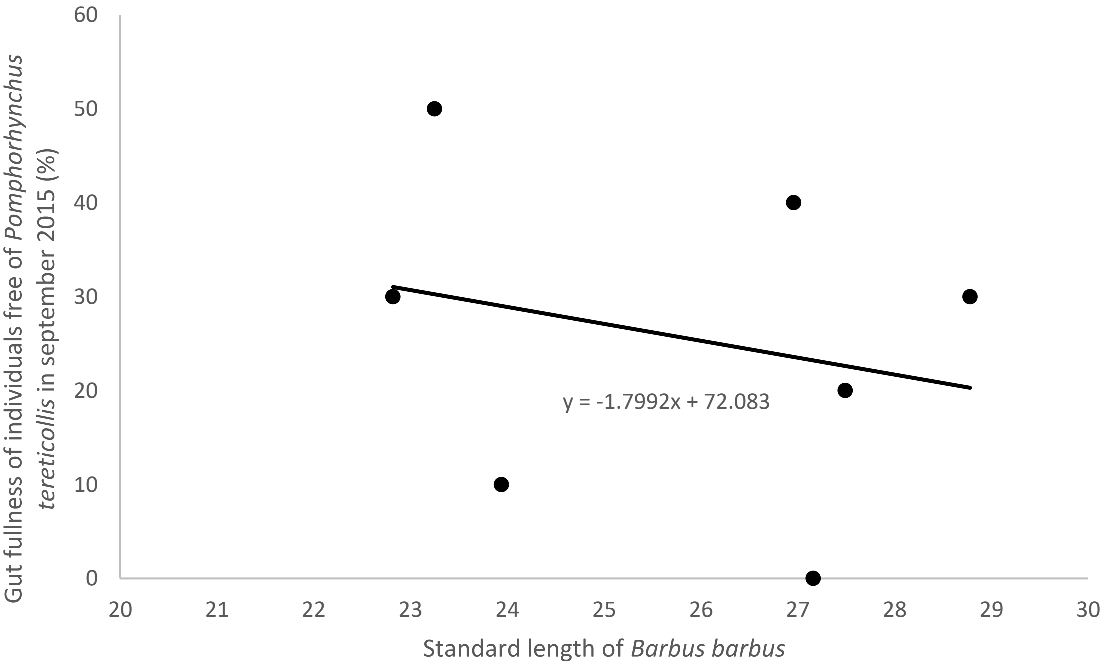

Figure A10. Standard length of *Barbus barbus* (mm) not infected with *Pomphorhynchus tereticollis* plotted with gut fullness in september 2015. There is a slight negative trend although this was not significant Regression F=1.643, d.f= 1 and 6, p= 0.256, r^2= 0.247.

Size of *P. phoxinus* and gut fullness.

In September and October 2015, there was no significant relationship between the size of the fish and the gut fullness Spearman's rho=0.119, n=22, p=0.597and Spearman's rho=0.062, n=18, p=0.808 respectively. The trend was slightly positive in September (fig A11) and also slightly positive in October (fig A12) although, this reversed when infected individuals are removed from the graph (fig A13). The positive trend in October 2015 can be explained by the difference in size between infected and uninfected individuals as the infected are predominantly larger than uninfected fish and have a larger median gut fullness (fig 4) and so are mostly on the right hand side of the graph whereas uninfected appear on the left and so appear as a positive trend. However, when individual trendlines are drawn the trend for uninfected with size is slightly negative (fig A13).

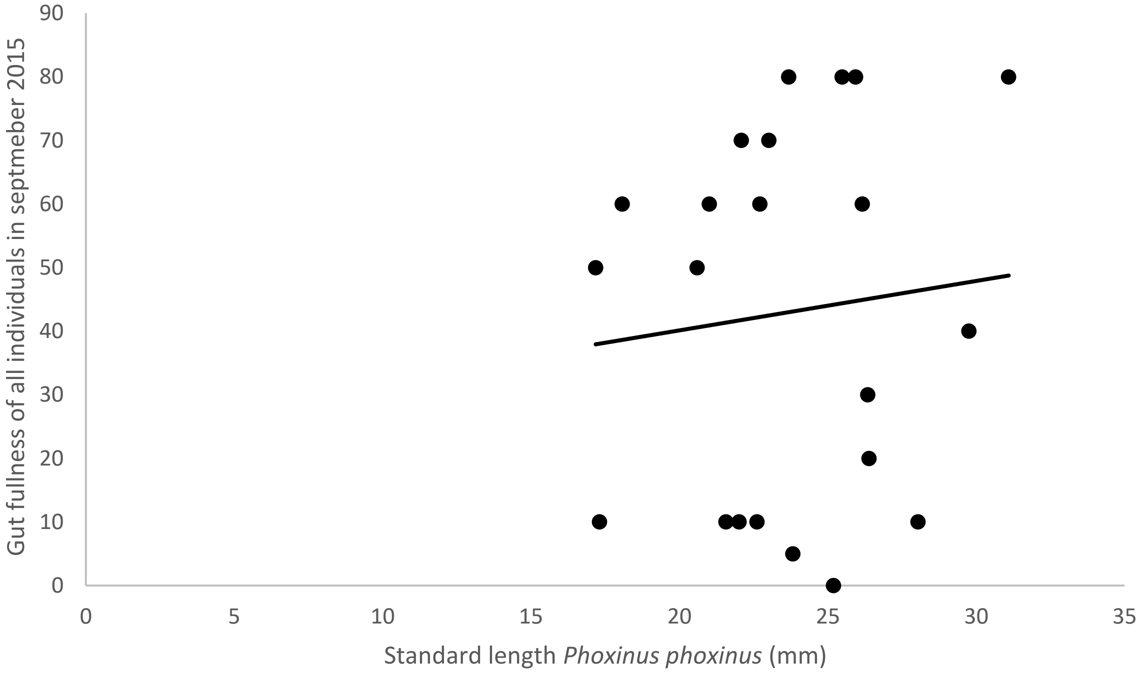

Figure A11. Standard length of *Phoxinus phoxinus* plotted with gut fullness of both infected and uninfected individuals in September 2015. There is a slight positive trend in the in the relationship between the standard length and Gut fullness although there are large amounts of variability in the trend and the relationship is not significant Spearman's rho=0.119, n=22, p=0.597.

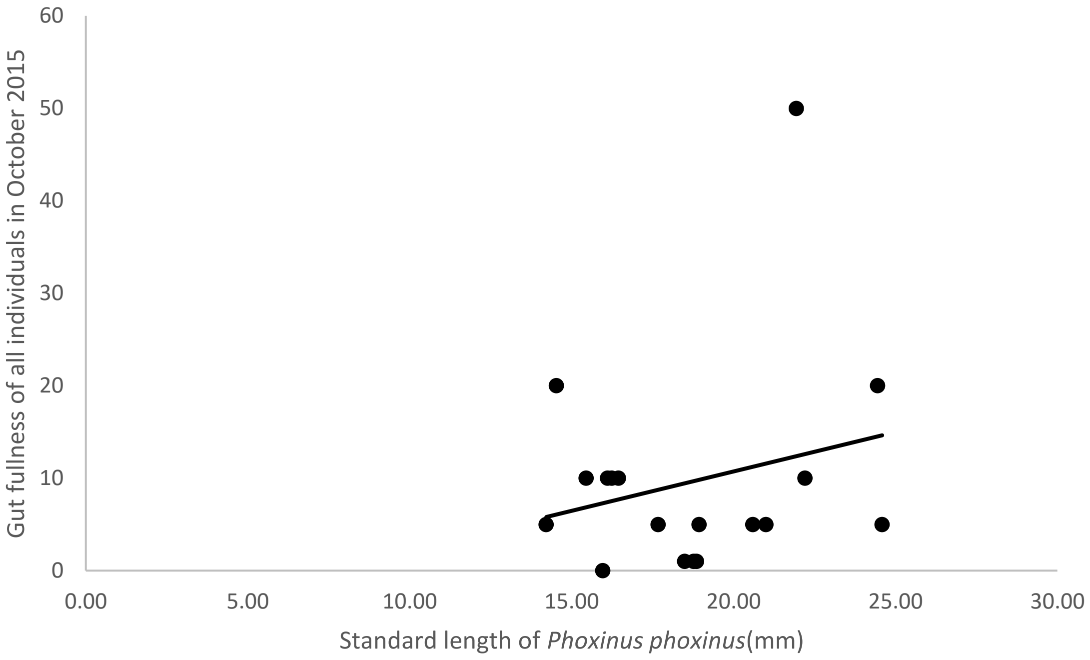

Figure A12. Standard length of *Phoxinus phoxinus* plotted with gut fullness of individuals in October 2015. There is a slightly positive trend in the data although there is large variability in the data and is probably skewed by the high data point of gut fullness at 50 (this was not found to be an outlier). The positive trend was not significant Spearman’s rho=0.062, n=18, p=0.808.

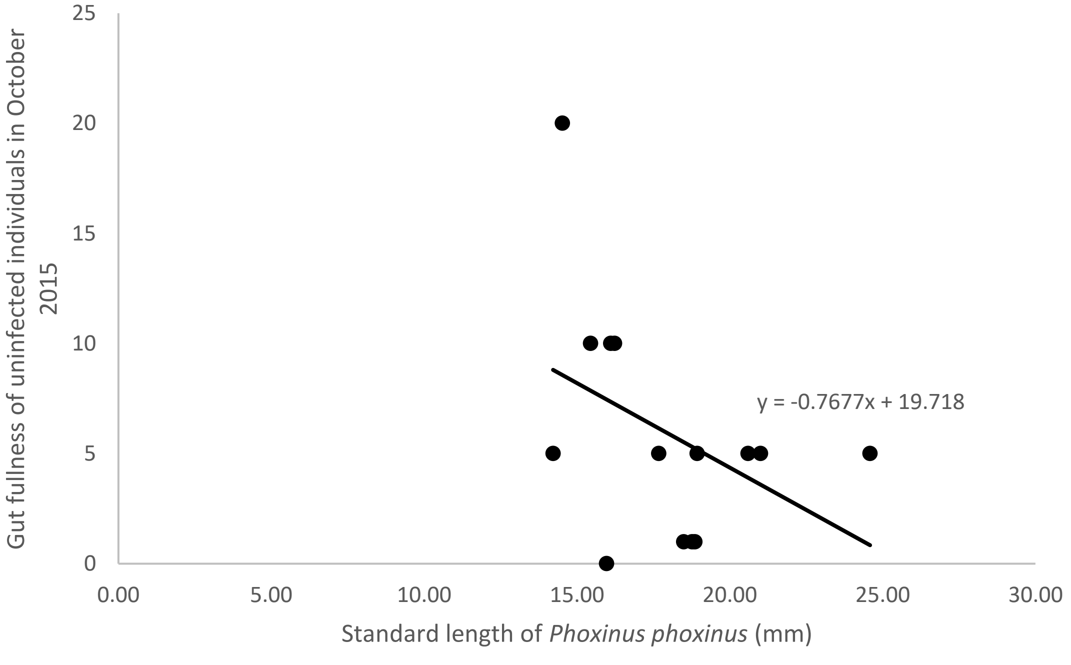

Figure A13. Standard length of *Phoxinus phoxinus* plotted with gut fullness of uninfected individuals in October 2015. There is a slightly negative trend in the data although there is high variability in the data. The relationship was not significant Spearman’s rho=-0.330, n=14, p=0.249.