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ORIGINAL PAPER

Paratenic hosts as regular transmission route in the acanthocephalan *Pomphorhynchus laevis*: potential implications for food webs

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Abstract Although trophically transmitted parasites are recognized to strongly influence food-web dynamics through their ability to manipulate host phenotype, our knowledge of their host spectrum is often imperfect. This is particularly true for the facultative paratenic hosts, which receive little interest. We investigated the occurrence and significance both in terms of ecology and evolution of paratenic hosts in the life cycle of the fish acanthocephalan *Pomphorhynchus laevis*. This freshwater parasite uses amphipods as intermediate hosts and cyprinids and salmonids as definitive hosts. Within a cohort of parasite larvae, usually reported in amphipod intermediate hosts, more than 90% were actually hosted by small-sized fish. We demonstrated experimentally, using one of these fish, that they get infected through the consumption of parasitized amphipods and contribute to the parasite's transmission to a definitive host, hence confirming their paratenic host status. A better knowledge of paratenic host spectrums could help us to understand the fine tuning of transmission strategies, to better estimate parasite biomass, and could improve our perception of parasite subwebs in terms of host–parasite and predator–parasite links.

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Introduction

Incorporating parasites in food-web ecology significantly improved our perception of linkage density, chain length, energy flowing, and species diversity (Marcogliese and Cone 1997; Hudson et al. 2006; Lafferty et al. 2006, 2008; Kuris et al. 2008; Amundsen et al. 2009; Byers 2009). This is particularly true for trophically transmitted parasites and, among them, for those using several successive hosts to achieve their cycle. These heteroxenous parasites develop from one larval stage to the next in one or several intermediate host(s) and complete their life cycle after ingestion by definitive hosts where they reach adulthood and reproduce. The link between these obligatory hosts may be strengthened by the ability of numerous parasites to manipulate the phenotype of intermediate hosts, making them more vulnerable to predation by definitive hosts (Thomas et al. 2005). These parasites are thereby strongly "entangled" in food webs and increase the intensity of some links between the different trophic levels.

Next to the obligatory hosts are paratenic hosts, which typically occur before definitive hosts and where parasite larvae show no apparent growth or development (Bush et al. 2001). Paratenic hosts have received less attention than intermediate and definitive hosts, mainly because they may be facultative for the parasite. Taking paratenic hosts into account may nevertheless greatly improve our understanding of both the evolutionary biology and the ecological significance of trophically transmitted parasites. Their incorporation into parasite life cycles may indeed have a

positive fitness effect through increasing transmission success. This is the case when paratenic hosts feeding on intermediate hosts accumulate a high proportion of parasites and contribute to their transmission when being preyed upon by definitive hosts (for a compelling example, see Robert et al. 1988; Morand et al. 1995). At a larger level, paratenic hosts may be exposed to a wider range of predators than intermediate hosts, including predators that do not serve as a final host. These non-host predators may benefit from consuming the incidental food source that are parasites, in addition to the prey species hosting them. Ignoring paratenic hosts could therefore alter our perception of subwebs involving parasites regarding potential host–parasite and predator–parasite links (see Lafferty et al. 2006, 2008). Also, link intensities in food webs may be changed if paratenic hosts are not anecdotal hosts. In parasites where transmission is favored through intermediate host "manipulation", the increased level of predation could concern more the links between intermediate and paratenic hosts than the links between intermediate and definitive hosts. The occurrence of paratenicity in helminths with complex life cycles is expected to be quite variable among groups: rather low in digenean trematodes but higher in cestodes, nematodes, and acanthocephalans (Schmidt 1985; Anderson 2000; Parker et al. 2009).

Acanthocephalans are a small monophyletic group, typically displaying two-host life cycles. Adult worms reproduce sexually in the intestine of a vertebrate definitive host and eggs are released with feces in the environment. Next, they are ingested by an arthropod intermediate host where the parasite develops to a cystacanth, the infective stage for definitive hosts (Conway Morris and Crompton 1982; Schmidt 1985). Known paratenic hosts are vertebrates which usually differ from known definitive hosts, and which harbor the cystacanth larval stage in an extra-intestinal location (Bush et al. 2001; Kennedy 2006). The literature provides qualitative data on paratenic hosts in acanthocephalans, summarized in Crompton (1985) and Kennedy (2006): their occurrence varies among parasite species, they may be frequent in some groups, and they are known to create new trophic bridges. For instance, in *Corynosoma semerme*, several fish paratenic hosts feeding on amphipod intermediate hosts create a bridge with seals, the definitive hosts, which do not feed on amphipods. However, it may be hard to distinguish between "true" paratenic hosts and "accidental" non-suitable hosts where acanthocephalans partially develop to adults outside their habitat, namely definitive host's intestine (see Kennedy 2006; Düşen and Oğuz 2008). As noted by Kennedy (2006), the only way to identify a paratenic host is to determine whether the larval parasitic stage found in this host will resume its development when transferred to a suitable definitive host.

The Palearctic acanthocephalan *Pomphorhynchus laevis* exploits several cyprinids and salmonids as definitive hosts and several amphipod crustaceans as intermediate hosts (Kennedy 2006). Crompton (1985) and Kennedy (2006) reported no paratenic host for *P. laevis*, while Düşen and Oğuz (2008) qualified the frog *Rana ridibunda* as a paratenic host (with a possible confusion with an accidental host, see before). However, it is known that *P. laevis* can exhibit post-cyclic transmission from one definitive host to another, for instance when a fish feeding on a smaller infected fish gets infected by the adult worms harbored by the latter (Kennedy 1999). This was interpreted as an adaptation to escape the rigidity of a strict two-host life cycle, especially when no paratenic hosts are available (Kennedy 2006). During previous preliminary investigations, some of us identified individuals of a few fish species of small size harboring extra-intestinal parasites at the cystacanth larval stage (Bollache and Perrot-Minnot, unpublished data). These species, especially the minnow *Phoxinus phoxinus* and the gudgeon *Gobio gobio*, are known to be definitive hosts for *P. laevis*, but where the proportion of female worms completing their development until sexual maturity is low (e.g., Kennedy 1999). These small fish are therefore good candidates for being paratenic hosts. Knowing the paratenic hosts used by *P. laevis* is of importance since it is a biological model frequently used to understand the ecology, evolution, and mechanisms of "parasitic manipulation". Several lines of evidence are available on its ability to (1) change some amphipod behaviors (e.g., Cézilly et al. 2000; Baldauf et al. 2007; Kaldonski et al. 2007; Franceschi et al. 2008), (2) increase the trophic links between amphipods and suitable host predators such as chub or trout (e.g., Lagrue et al. 2007), but also (3) increase the trophic links between amphipods and some non-suitable host predators (Kaldonski et al. 2008). Until today, only obligatory hosts and the classical route of transmission from amphipods to fish are considered. However, if paratenic hosts exist for *P. laevis*, two predictions can be proposed. First, they should not be anecdotal hosts in fish known to feed on aquatic invertebrates. This is because since *P. laevis* manipulates amphipods in a way that increases their susceptibility to fish predation, paratenic hosts should be at least as sensitive to this facilitation than definitive hosts. Second, they should create alternative routes of transmission if the extra-intestinal larval stages (cystacanths) found in paratenic hosts are able to infect definitive hosts after their consumption.

To test these predictions, we conducted field and laboratory investigations and answered the following questions. (1) What is the quantitative estimate of extra-intestinal cystacanths within a fish assemblage? The fish assemblages of three French rivers were examined in search

of extra-intestinal *P. laevis* larvae. (2) How were fish infected with cystacanths? We investigated experimentally the two possible infection pathways: through the ingestion of free-living eggs or already-developed cystacanths using one of the species found to harbor numerous extra-intestinal cystacanths in nature. The ingestion of free-living eggs is an improbable pathway owing to the known acanthocephalan life cycle (Kennedy 2006), but nevertheless necessary to test given the high prevalence and intensity found during the field census. (3) Do the cystacanths hosted by fish successfully resume development once transferred to definitive hosts? Predation tests were performed between fish harboring cystacanths and a known definitive host. Overall, these experiments provide estimates for the importance of fish in the transmission of *P. laevis* cystacanths and allow to better infer the trajectory of this parasite in the food web.

Materials and methods

Host and parasite collections

In spring 2005 and 2009, sampling campaigns were carried out in tributaries of the river Saône (Burgundy, Eastern France): the Ouche (47°17′ N, 05°02′ E), the Vingeanne (47°20′ N, 05°27′ E), and the Vouge (47°08′ N, 05°10′ E). The presence of *P. laevis* at these sites was confirmed by previous investigations (Franceschi et al. 2008) and sampling was performed on a representative section whose length was 10 times the river width.

Gammarus pulex, the usual intermediate host of *P. laevis*, was collected with a Surber net (500 μm mesh) from the three main natural microhabitats (i.e., stones, gravel, and macrophytes), each being sampled 12 times. The samples were preserved with 70% ethanol and sorted in the laboratory. Amphipods were counted and dissected under a binocular microscope to record their infection status and the number of parasites.

Fish were sampled using a battery-powered portable electrofishing gear (Dream Électronique Society, France) and maintained in aerated 40-L plastic tanks until their arrival to the laboratory. Within 24 h after collection, fish were identified to the species level according to Keith and Allardi (2001), weighed (to the nearest 0.01 g), and measured (fork length to the nearest 0.1 cm, Anderson and Neumann 1996). They were then anaesthetized with clove oil (90% eugenol), sacrificed, and dissected. During dissections, mature fish were sexed based on gonadal structure and sexual dimorphism. The body cavity was examined under a binocular microscope in search of extra-intestinal acanthocephalans. We recorded the number of parasites and their developmental stage (cystacanth or adult). Two genetically distinct but closely related *Pomphorhynchus* species co-occur in rivers of Eastern France (*Pomphorhynchus tereticollis* and *P. laevis*, Perrot-Minnot 2004; Bombarová et al. 2007). Since they can hardly be distinguished at the cystacanth stage and are often still considered as the single species *P. laevis* (Amin et al. 2003), field prevalences were considered without distinction for genotype. The viability of parasites was assessed by an activation test in chub bile. We randomly collected 30 cystacanths among those found in fish taken from the river Vouge in 2009 and placed them individually in a microtube filled with 1 mL of chub bile (diluted 1:10 in water). After 2 h spent in the dark, everted cystacanths showing movements were counted and those unable to evert were considered dead.

Differences in prevalence between host species were tested using Pearson's chi-square tests. We used Mann–Whitney *U* tests for the other parameters since data did not meet normality assumptions. The distribution of parasites was investigated in minnows (*Phoxinus phoxinus*), one of the most infected fish species from the river Vouge. We performed an ANOVA on Box–Cox transformed data to test the effects of fish size, fish gender, and their interaction on parasite abundance (i.e., the number of parasites per fish). The normality of transformed data was assessed using a Shapiro–Wilk test. To test whether or not infection influences fish host condition, we calculated the condition coefficient $K=W/L^3$, with W and L being the weight and the length of minnows, respectively (Le Cren 1951). An ANOVA was conducted to test the effects of fish gender, parasite intensity (i.e., the number of parasites per infected fish), and their interaction on the condition coefficient K. To detect potential parasite-induced mortality (Anderson and Gordon 1982; Thomas et al. 1995; Rousset et al. 1996), we investigated the relationship between host size and mean parasite abundance (MPA) in distinct minnow populations.

Experimental infections

Origin of hosts and housing conditions

Minnows serving as hosts were collected by electrofishing in spring 2009, from the River Suzon (Burgundy, 47°24′ N, 4°52′ E), where *Pomphorhynchus* parasites have never been reported (L. Bollache, unpublished data). Medium-sized fish (55 to 65 mm) were selected and returned to the laboratory in 40-L plastic tanks. Thirty randomly chosen minnows were anaesthetized and dissected to control for the absence of parasites. As expected, no parasite was found in this subsample. The others were placed in 60-L plastic tanks (46×34×38 cm height) filled with dechlorinated, UV-treated, and aerated tap water at a temperature of 15±1°C, under a 12:12 h light/dark cycle. Fish

density did not exceed 20 individuals per tank. Each tank was equipped with a 5-cm layer of gravel serving as a substratum and a hollow brick to provide shelter. An air-driven under-gravel filter was used to ensure oxygenation and to maintain water quality. Fish were fed every 2 days using commercial flake food, and the water was changed every 2 weeks. The tanks were checked daily to remove dead individuals. At the end of a 1-month acclimatization period, fish were distributed in three groups: two groups in which fish were infected with either parasite eggs or cystacanths and one control group in which fish were handled but not infected. The control group provided a comparison for treatment-induced mortality and absence of infection.

Origin of eggs and cystacanths

To avoid any confounding effect due to the two possible *Pomphorhynchus* genotypes, we matched the parasite genotype used during controlled infections with that of naturally infected minnows. For this, 124 cystacanths found in minnows from the Vouge population were fixed in ethanol after dissections. A diagnostic PCR test based on the length of the ITS amplification products was performed as described in Franceschi et al. (2008). All these parasites were assigned to the genotype of *P. laevis sensu stricto* (Perrot-Minnot 2004).

Parasite eggs were taken from female parasites infecting chubs (*Leuciscus cephalus*), one of the main definitive host of *P. laevis* (Kennedy 2006), from the River Vouge following the procedure described in Franceschi et al. (2008). Parasite eggs were obtained by dissecting six *P. laevis s.s.* females (genotype confirmed by the diagnostic PCR test). Their clutches were mixed in microtubes filled with 400 μL of water and the concentration of the resulting egg suspension was estimated by averaging the counts made under a microscope in 10 samples of 1 μL. After dilution with water, we obtained an egg suspension with 10 mature eggs (Crompton 1985) per microliter. Experimental infections were carried out within 3 days.

Cystacanths were obtained from amphipods infected experimentally using the egg suspension described above (procedure of Franceschi et al. 2008). *Gammarus pulex* were sampled in a small tributary of the river Suzon, where *P. laevis* is absent, and fed with 1-cm² dry elm leaves on which the egg suspension was deposited (≈100 eggs per amphipod). After a 48-h exposure to eggs, amphipods were placed in housing tanks. From the sixth week, amphipods were inspected under a binocular microscope to detect parasite infection, cystacanths being visible through their cuticle. Ten weeks post-exposure, cystacanths were taken from infected amphipods, and controlled infections in fish were immediately performed. Prior to this experiment, activation tests were performed on 30 randomly chosen cystacanths to assess their viability (see above).

Fish infection procedure

Fifty-nine minnows were infected each with 50 mature eggs (5 μL of egg suspension), and 28 others received five cystacanths each (placed in 10 μL of water). Parasites were inoculated orally using a 5-cm-long plastic tube (Ø= 0.8 mm for eggs, 1 mm for cystacanths) fixed on the tip of a Gilson micropipette. Parasites were therefore released directly into the stomach. Minnows from the control group (N=10) were inoculated with 10 μL of water. Handling time did not exceed 30 s per fish so as to minimize stress, and all the minnows of a same treatment were processed within a day.

Following inoculation, minnows were replaced in six housing tanks so as to not exceed the maximum density of 20 individuals per tank (one, three, and two tanks for control, egg-inoculated, and cystacanth-inoculated fish, respectively) and maintained under the laboratory conditions described above. The tanks were checked daily to remove dead individuals. The time needed to reach the cystacanth stage in the intermediate host *G. pulex* was about 8 weeks. We waited 12 weeks before investigating infection in egg-inoculated minnows. Concerning cystacanth-inoculated minnows, the post-exposure period was set at 4 weeks based on previous reports in chubs (Siddall and Sures 1998). At the end of the experiment, fish were anaesthetized, sacrificed, sexed, and dissected. Parasites were recorded and we performed activation tests to determine the viability of 30 randomly chosen cystacanths.

Differences in prevalence between minnows infected with eggs and minnows infected with cystacanths were tested using Fisher's exact tests. We conducted a logistic regression to analyze the effect of treatment, host size, and host gender on the presence or absence of *P. laevis* in fish. Among infected minnows, some adult parasites were found. The difference in establishment success between cystacanths and adult parasites was tested using a Wilcoxon's signed rank test.

Predation tests

To test whether or not cystacanths coming from fish can successfully establish and resume development when ingested by definitive hosts, we performed predation experiments between naturally infected minnows and chubs. Chubs (25 to 35 cm) were collected from the river Ouche and randomly distributed in two groups of 10 individuals: one control group and one experimental group, each being placed in a 150-L tank equipped as described above. After a 5-day acclimatization period during which

chubs were deprived of food, 60 minnows (60 to 65 mm) from the river Vouge were introduced into the experimental tank while the control group was fed using commercial trout pellets. After a 15-day exposure period during which the experimental group was allowed to feed on minnows only, seven living minnows were removed from the tank. The absence of dead bodies suggested that the 53 others had been consumed by chubs. During a post-infection period of 6 weeks, chubs were fed every 3 days with trout pellets and the tanks were checked daily to remove dead individuals. At the end of the experiment, all the chubs were anaesthetized, sacrificed, and dissected in search of parasites. Parasites were measured to the nearest 0.01 mm using a digital caliper. Since most of the chubs taken in the wild harbored *P. laevis* adults, experimental infections were distinguished from natural infections based on parasite size and color, young adults being smaller and paler than older ones (Crompton 1985). We therefore predicted that, in case of successful infections, chubs of the control group should host only large and orange parasites, while those exposed to minnows should host additional small and pale parasites.

Between-group differences in prevalence and parasite intensity were tested using Fisher's exact test and Wilcoxon's test respectively. The size–frequency distributions of parasites were determined for the two groups and compared using a Kolmogorov–Smirnov's two-sample test. All analyses were performed with JMP software v. 5.01 (SAS Institute Inc., Cary, NC, USA).

Results

Occurrence of extra-intestinal parasites in fish

We captured 665 individuals from 16 fish species (Table 1). Extra-intestinal parasites were recorded in nine of these species, including seven cyprinids, the gasterosteid fish *Gasterosteus aculeatus*, and the ictalurid catfish *Ameiurus melas*. All these *Pomphorhynchus* parasites were at the cystacanth stage and the 30 randomly selected individuals were all found to be alive (they everted in chub bile). Prevalence commonly exceeded 30% and reached the maximum value of 83% in minnow and catfish, the sample size being only six for the latter. Depending on the fish species, prevalence varied with location and sampling date. For instance, in the river Ouche, minnows were significantly more parasitized in 2005 than in 2009 (Pearson's $\chi^2=30.13$, $P<0.001$), but they were also larger (Mann–Whitney U test—$Z=6.50$, $P<0.001$). For different dates but with fish of equivalent sizes ($Z=1.01$, $P=0.31$), prevalence in minnows was significantly higher in the river Vouge in 2009 than in the river Ouche in 2005 (Pearson's $\chi^2=12.18$, $P<0.001$). Conversely, and considering the same fishing campaigns, there was no difference in prevalence for gudgeon ($\chi^2=0.04$, $P=0.84$) even though they were larger in the river Ouche than in the river Vouge (Mann–Whitney U test—$Z=6.50$, $P<0.001$). In 2005, chubs of equivalent size ($Z=0.52$, $P=0.60$) were found harboring cystacanths in the river Vingeanne but not in the river Ouche (Table 1). Chubs are known to be common definitive hosts for *Pomphorhynchus* parasites and, as such, we also found intra-intestinal adults in the fish collected (Perrot-Minnot and Bollache, unpublished data). Parasite abundance and intensity varied greatly both between and within fish species but, in general, the higher prevalence, the higher parasite abundance and intensity. Nevertheless, this was not verified for chubs of the river Vingeanne with high intensities but a low number of infected fish (Table 1). The highest intensities were found in minnow and gudgeon with, respectively, 49 and 51 parasites in the same individual host. Individual fish harboring more than 10 parasites were common in minnow, gudgeon, chub, and catfish.

Of the 6,602 *G. pulex* collected, only 39 were found infected with *Pomphorhynchus* parasites so that the prevalence was less than 1% in all rivers (Table 1). Multiple infections were uncommon and maximum parasite intensity was three.

If we consider all the cystacanths recorded in this study, 96.9% were hosted by fish (more than 90% whatever the river). We do not know the relative densities of fish and amphipods in our systems and the fish/amphipods ratios of our samples probably underestimate the proportion of amphipods (1:10 in the river Ouche, 1:50 in the river Vingeanne, and only 1:5 in the river Vouge), so it is difficult to know precisely where most of the cystacanths are. However, if we consider the more realistic ratios of 1:100 or 1:1,000, then the proportion of cystacanths harbored by fish should be roughly of 81% and 30%, respectively.

Parasite distributions in minnow

In the minnow population of the river Vouge, there was no significant difference in prevalence between male and female fish (83.3% and 90.3%, respectively, Pearson's $\chi^2=0.011$, $P=0.92$). Parasite abundance was positively influenced by host size (ANOVA, global model—$F_{3,125}=42.99$, $P<0.001$; size effect—$F_{1,105}=77.50$, $P<0.001$), but not influenced by host gender ($F_{1,125}=1.03$, $P=0.31$) or by the size–gender interaction ($F_{1,125}=0.007$, $P=0.93$). A gender effect was not estimated for the minnow population of the river Ouche because of the high proportion of fish for which gender could not be determined (37% and 77% in 2005 and 2009, respectively). Regardless of the origin of minnows (the river Ouche—Fig. 1a, b, the river Vouge—Fig. 1c), MPA increased with fish host size.

Table 1 Record of extra-intestinal *Pomphorhynchus* parasites in fish from the rivers Ouche, Vingeanne, and Vouge

Host species	*N*	Host size (cm, mean±SD)	Prevalence (%)	Abundance (mean±SD)	Intensity (mean±SD)	Min.–max.
The river Ouche (2005)						
Fish						
Minnow (*Phoxinus phoxinus*)	54	6.20±1.21	57.4	3.89±7.73	6.77±9.24	1–49
Gudgeon (*Gobio gobio*)	44	12.47±1.72	34.1	1.45±3.22	4.27±4.37	1–17
Soufie (*Leuciscus souffia)*	24	13.32±2.44	8.3	0.29±1.23	3.50±3.53	1–6
Three-spined stickleback (*Gasterosteus aculeatus*)	65	5.06±0.39	6.1	0.11±0.47	1.75±0.96	1–3
Stone loach (*Nemacheilus barbatula*)	52	8.31±1.06	3.8	0.04±0.19	1.00±0.00	1–1
Chub (*Leuciscus cephalus*)	49	17.19±8.47	0	0	0	0
Barbel (*Barbus barbus*)	21	27.42±9.50	0	0	0	0
Bullhead (*Cottus gobio*)	8	9.92±1.25	0	0	0	0
Bleak (*Alburnus alburnus*)	4	9.47±3.70	0	0	0	0
Roach (*Rutilus rutilus*)	4	21.15±7.72	0	0	0	0
Perch (*Perca fluviatilis*)	2	19.00±2.83	0	0	0	0
Tench (*Tinca tinca*)	2	18.00±1.41	0	0	0	0
Bitterling (*Rhodeus sericeus*)	1	4.70	0	0	0	0
Crustaceans						
Gammarus pulex	2631	Not measured	0.84	0.01±0.13	1.32±0.57	1–2
The river Ouche (2009)						
Fish						
Minnow (*Phoxinus phoxinus*)	92	4.23±1.57	13	0.27±0.85	1.83±1.47	1–6
Three-spined stickleback (*Gasterosteus aculeatus*)	4	5.00±0.42	0	0	0	0
The river Vingeanne (2005)						
Fish						
Catfish (*Ameiurus melas*)	6	14.22±1.65	83.3	11.50±13.18	13.80±13.31	2–32
Nase (*Chondrostoma nasus*)	15	20.71±4.26	46.7	3.87±7.46	8.28±9.34	1–25
Roach (*Rutilus rutilus*)	12	13.90±1.62	41.7	1.92±3.80	4.60±4.93	1–13
Chub (*Leuciscus cephalus*)	27	17.63±3.15	11.1	1.52±7.30	13.67±21.08	1–38
Barbel (*Barbus barbus*)	9	13.17±6.55	0	0	0	0
Stone loach (*Nemacheilus barbatula*)	3	6.77±0.25	0	0	0	0
Gudgeon (*Gobio gobio*)	2	9.20±0.28	0	0	0	0
Bream (*Abramis brama*)	1	23.00	0	0	0	0
Bullhead (*Cottus gobio*)	1	10.80	0	0	0	0
Crustaceans						
Gammarus pulex	3,174	Not measured	0.42	0.005±0.08	1.23±0.44	1–3
The river Vouge (2009)						
Fish						
Minnow (*Phoxinus phoxinus*)	125	5.88±0.86	83.2	7.82±9.12	9.40±9.23	1–46
Gudgeon (*Gobio gobio*)	28	8.25±1.80	39.3	2.79±9.66	7.09±14.77	1–51
Barbel (*Barbus barbus*)	4	8.32±5.66	0	0	0	0
Chub (*Leuciscus cephalus*)	3	10.20±6.10	0	0	0	0
Stone loach (*Nemacheilus barbatula*)	3	8.43±0.60	0	0	0	0
Crustaceans						
Gammarus pulex	797	Not measured	0.5	0.005±0.08	1.00±0.00	1–1

Parasite abundance and intensity refer to the number of parasites per fish and per infected fish, respectively

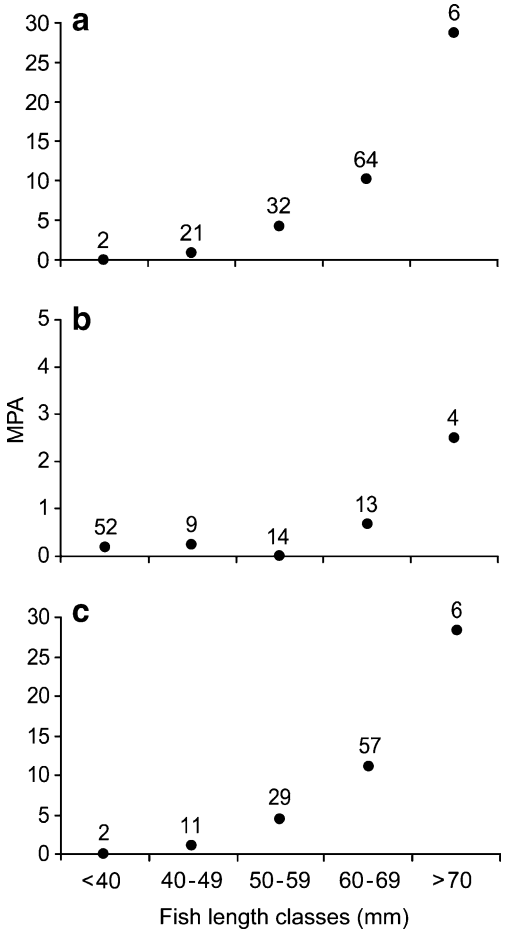

Fig. 1 Mean parasite abundance (*MPA*) of extra-intestinal *Pomphorhynchus* parasites in relation to host size in *Phoxinus phoxinus* from the river Ouche in 2005 (**a**) and 2009 (**b**), and from the river Vouge in 2009 (**c**). Values above *dots* indicate the number of hosts analyzed for each length class

Neither fish gender nor parasite intensity or their interaction had a significant effect on the condition coefficient *K* used to reflect fish host condition (ANOVA, global model—$F_{3,125}=1.57$, $P=0.20$).

Origin of cystacanths in fish

Of the 97 minnows used for experimental infections, six died during the post-treatment period and mortality did not significantly differ between groups (0%, 6.8%, and 3.6% for control, egg-infected, and cystacanth-infected fish, respectively; Fisher's exact test—$P=0.82$).

The presence of cystacanths in experimentally exposed minnows was explained by the treatment, but not by host characteristics regarding size and gender (Table 2). Neither cystacanths nor adult parasites were found in control and egg-exposed fish. Conversely, 23 of the 27 cystacanth-infected fish were found infected with no significant difference in the proportion of infected individuals between the two housing tanks (Fisher's exact test—$P=0.59$, data pooled in Table 3). We recorded the two parasite development stages and the prevalence of infection with cystacanths was significantly higher than with adults (Fisher's exact test—$P<0.001$, Table 3). Parasite abundance did not differ between genders (Mann–Whitney *U* tests, for cystacanths—$Z=1.28$, $P=0.21$, for adults—$Z=-0.90$, $P=0.38$), and maximum parasite intensity was one for adults and three for cystacanths.

Table 2 Logistic regression for *Pomphorhynchus laevis* infection in experimentally exposed minnows (*Phoxinus phoxinus*) as a function of host size, host gender (males, females, and undifferentiated), and treatment (infection with eggs or cystacanths)

Source	*df*	Wald chi square	*P* value
Size	1	0.085	0.770
Gender	2	0.006	0.997
Treatment	1	21.554	<0.001

Overall, 36.2% of the cystacanths inoculated to the 27 surviving fish were recovered at the end of the experiment: 33.3% as cystacanths inside the abdomen and 2.9% as adults attached to the intestine. Establishment success was thus significantly higher as cystacanths than as adults (Wilcoxon's signed rank test—$Z=3.81$, $P<0.001$). Among cystacanths, 7.8% were considered dead following activation tests in chub bile.

Transmissibility to definitive hosts

Of the 20 chubs used for the experiment, one individual from the experimental group died at the beginning of the post-treatment period and thus was excluded from the analysis. Overall, we recorded and measured 273 intra-intestinal adult parasites. The prevalence was higher in chubs fed with minnows (100%) than in control chubs (60%), but the difference was not significant (Fisher's exact test—$P=0.087$). Similarly, parasite intensity was higher in exposed chubs (median=16) than for controls (median=4), but this was not significant (Wilcoxon's test—$W=41$, $P=0.11$).

The size–frequency distribution of parasites significantly differed between groups (Kolmogorov–Smirnov's two-sample test—$D=0.384$, $P<0.001$, Fig. 2). Among parasites, those measuring less than 4 mm were recorded only in chubs exposed to naturally infected minnows (Fig. 2b). Their prevalence was significantly higher in chubs fed with minnows than in control chubs (Fisher's exact test—$P<0.001$). All these small parasites also differed from larger ones by their coloration: they were pale yellow instead of bright yellow or orange.

Based on the assumption that the small and pale-yellow adult parasites were those coming from lab predation on minnows, we could estimate the success of this infection

Table 3 Record of *Pomphorhynchus laevis* infection in minnows (*Phoxinus phoxinus*) infected experimentally with eggs or cystacanths

	Control fish (*N*=10)		Egg-infected fish (*N*=54)		Cystacanth-infected fish (*N*=27)	
	Cys.	Ad.	Cys.	Ad.	Cys.	Ad.
Prevalence (%)	0	0	0	0	77.8	14.8
Abundance (mean±SD)	0	0	0	0	1.48±1.16	0.15±0.36
Intensity (mean±SD)	0	0	0	0	1.90±0.94	0.19±0.40
Min.–max.	0	0	0	0	1–3	1–1

Ad. adults, *Cys*. cystacanths

pathway. Considering that 53 minnows were preyed upon by 10 chubs, and given that field-collected minnows measuring 60 to 65 mm harbored on average 8.3 cystacanths (95% CI=6.6 to 10.1), the mean number of cystacanths experimentally consumed by chubs can be calculated to be equal to 442 (95% CI=348.8 to 535.3). Given the fact that 80 small parasites were recorded in chubs, the infection success could be estimated at 18.1% (95% CI=14.9 to 22.9).

Discussion

The present study combined field data with laboratory experiments to assess the ecological and evolutionary significance of fish harboring *Pomphorhynchus* cystacanths in an extra-intestinal location. Of the three aquatic webs we examined, four cyprinids—minnow (*Phoxinus phoxinus*), gudgeon (*Gobio gobio*), nase (*Chondrostoma nasus*), and roach (*Rutilus rutilus*)—were found heavily infected with cystacanths at both the population and individual levels regarding prevalence (from 34% to 83%) and parasite intensity (in means from 4.3 to 9.4 parasites per fish), respectively. Chub (*Leuciscus cephalus*), soufie (*Leuciscus soufia*), stickleback (*Gasterosteus aculeatus*), and stone loach (*Nemacheilus barbatula*) were also found infected but to a lesser extent (Table 1). Prevalence and intensity in amphipods were similar to those reported by Lagrue et al. (2007) and very low compared to what we found in fish. Based on the hypothetical fish/amphipods ratios of 1:100 and 1:1,000, we estimated the proportion of cystacanths harbored by fish as being roughly of 81% and 30%, respectively. This suggests that the presence of *P. laevis* cystacanths in fish is far from trivial, even considering the lowest estimate (30%), and that fish paratenic hosts may represent a potential reservoir for transmission.

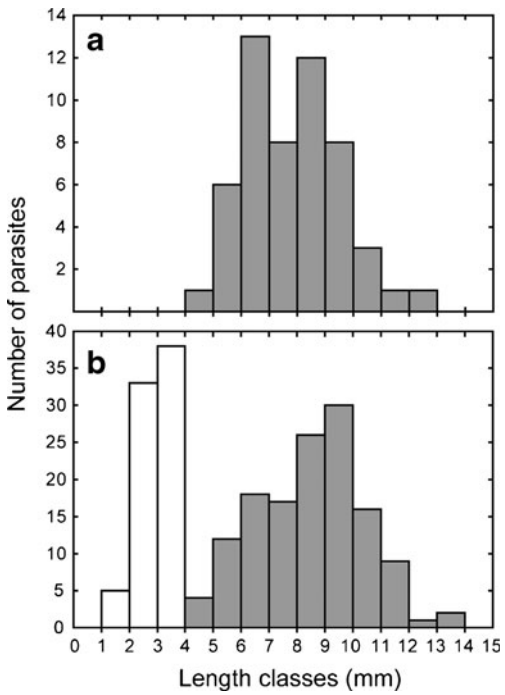

Fig. 2 Size–frequency distribution of intra-intestinal *Pomphorhynchus* parasites in control (**a**) and experimentally infected (**b**) *Leuciscus cephalus*

P. laevis cystacanths were recorded in minnows inoculated with cystacanths but not in those inoculated with eggs. This means that, in nature, fish get infected by eating infected amphipods, and that parasites stay at the same developmental stage, fitting the definition of a paratenic host (Bush et al. 2001). In addition, minnows represent a transmission pathway for the parasite. Indeed, cystacanths transferred from minnows to chubs via predation successfully developed in the definitive host. Our experiment was not long enough to show that these parasites can reach sexual maturity, but our study is strongly suggestive that minnows serve as paratenic hosts for *P. laevis*. Similarly, the other heavily infected fish species whose size allows ingestion by definitive hosts are likely to be paratenic hosts because the cystacanths they hosted were alive (e.g., able to actively evert in chub bile), and thus probably able to infect a new host. Additional studies are needed to confirm the host status of these species and to estimate the establishment success of the larva they harbor. The establishment success from minnows to chubs (the most common

definitive host) was estimated between 15% and 23% parasites established after 12 weeks. These values have to be compared with those of the "classical" intermediate to definitive host route of transmission. Such a comparison is not straightforward, mainly because the success of acanthocephalan establishment in fish intestine is generally subject to either regulatory processes and/or density dependence mortality (Kennedy 2006). We therefore have to compare similar densities of cystacanths provided and similar post-exposure time lags for the same species. The study of Brown (1986) fits these criteria: after 12 weeks post-exposure, the establishment success of 50 *P. laevis* cystacanths taken from intermediate hosts was estimated to be 30% in a salmonid fish, a suitable definitive host. Considering that this experiment was made using cystacanths extracted from the hosts, while in our experiment they came from the body of ingested fish (probably limiting the easiness of establishment), we can therefore suppose that their establishment success when coming from fish paratenic hosts is only slightly lower than when coming from amphipod intermediate hosts. Further investigations are needed to test whether laboratory values for establishment success differ from those observed in the field. For instance, using standardized conditions to determine establishment success (e.g., absence of other food items in definitive host's diet) may provide overestimated values.

The trophic link between amphipods and fish paratenic hosts is not surprising based on the feeding ecology of these fish but seems very intense regarding the high prevalences and intensities observed (notably in minnow, gudgeon, nase, and roach). Such an intense link is likely to be a consequence of the ability of *P. laevis* to manipulate amphipods' behavior. *P. laevis*-infected amphipods indeed show an altered reaction to light (Bauer et al. 2000; Cézilly et al. 2000; Tain et al. 2006), to fish scent (Baldauf et al. 2007; Kaldonski et al. 2007), and an increased drift (McCahon et al. 1991; Maynard et al. 1998; Wellnitz et al. 2003). As a result, the proportion of infected *G. pulex* in the stomach of bullheads (*Cottus gobio*), one of the definitive hosts of *P. laevis*, was found to be 26.3 to 28.3 times higher than the prevalence in the benthos (Lagrue et al. 2007). In the present study, the relative prevalence and intensities of *P. laevis* cystacanths in *Gammarus* and in paratenic fish are similar and coherent with the hypothesis that manipulating infected amphipods increases transmission to paratenic fish hosts, and not just to final fish hosts.

This finding brings arguments to the debate that parasite-increased trophic transmission does not need to specifically target definitive hosts to be considered adaptive. Seppälä and Jokela (2008) showed, using a theoretical model in a simple intermediate-definitive hosts system, that even a slight increase in transmission due to a higher vulnerability to definitive hosts is sufficient to positively influence parasite's fitness at the population level. Our results suggest that, in addition, paratenic hosts may be an important component of the life cycle: we estimated the transmission success between paratenic (minnow) and definitive (chub) hosts to be 15–23%. Another, hypothetical, question can be raised about transmission between paratenic and definitive hosts: are parasites able to increase their transmission as they do between intermediate and definitive hosts? This would mean that they could be able to manipulate paratenic host phenotype (e.g., behavior) in ways favoring predation. While our experiments were not designed to address this question, our data are suggestive for an absence of this phenomenon. The mean parasite abundance (MPA) increased with paratenic fish size. This suggests no parasite-induced mortality (Rousset et al. 1996); otherwise, we should have observed a drop in MPA among larger hosts. A decrease in MPA with age is typical of manipulative parasites (as observed in Lagrue et al. 2007 for *P. laevis*). This field observation has to be confirmed with experiments, but is in agreement with the literature reporting on the absence of manipulation in paratenic hosts (Parker et al. 2009). It is also consistent with the fact that parasite intensity did not influence fish condition in our study.

Beyond the consequences at the level of *P. laevis*'s life cycle, the link between paratenic hosts and upper trophic level predators may influence energy flow when considering the parasite as a potential source of food (Johnson et al. 2010). This depends on the role the predator of paratenic hosts plays in parasite transmission. It can be a definitive host or a non-suitable host, leading to host–parasite or predator–prey links, respectively (Lafferty et al. 2006, 2008). Even when the predator is a definitive host, the infection success is never complete. In our experiment, we already noted that about 18% of the cystacanths ingested by chubs successfully attached to host's intestine, therefore acting as parasites. It means that around 80% did not and are a potential source of food for the predator. The extent to which parasites serve as a source of food nevertheless depends on other factors, most of them being beyond the scope of this study. First, it depends on the ability of the predator preying on the paratenic host to digest parasite larvae. This could be assessed by recording parasites in feces during controlled infections. Second, it depends on where the paratenic host occurs along the food chain. For instance, most of the catfish *Ameiurus melas* we collected harbored *Pomphorhynchus* cystacanths. Native to North America, this catfish has become widespread in Europe (Wheeler 1978) and experiences few predators in its invasive range. Catfish may thus be a dead-end for parasites (using the term "paratenic host" is here questionable), but also may disrupt the involvement of parasites in the food chain.

To conclude, our results suggest that looking beyond obligatory hosts offers promising perspectives when it comes to incorporate parasites to food-web theory. A better knowledge of paratenic host spectrums is of importance to understand the fine tuning of transmission strategies, which in turn helps us to predict and identify the trophic links strengthened by favorization processes at the community level.

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