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Differences in specificity, development time and virulence between two acanthocephalan parasites, infecting two cryptic species of *Gammarus fossarum*

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Abstract

Multi-host parasites can exploit various host species that differ in abundance and susceptibility to infection, which will contribute unequally to their transmission and fitness. Several species of acanthocephalan manipulative parasites (among which *Pomphorhynchus laevis* and *P. tereticollis*) use various amphipod species of the genus *Gammarus* as intermediate hosts. Many *Gammarus pulex* and *G. fossarum* cryptic lineages are living in sympatry in European rivers, questioning the spectrum of intermediate hosts that acanthocephalans can use, and their relative contribution to their life cycles. In this work, the respective roles of parasites species (*P. laevis* and, for the first time, *P. tereticollis*) and sympatric host cryptic species (the *G. fossarum* species complex) were studied experimentally on two traits: host susceptibility to infection and parasite virulence. Differences were found, both in terms of infectivity and virulence, between the cryptic hosts and between the two parasite species. We confirm that these acanthocephalans, previously considered as generalists, show specificities among their sympatric hosts. Differences in field prevalence and susceptibility after experimental exposures were more pronounced between cryptic *G. fossarum* species for *P. tereticollis* than for *P. laevis*. The mortality of infected individuals increased significantly after several weeks of development of both parasite species. *P. tereticollis* was less virulent than *P. laevis*, perhaps due to differences in host exploitation, since we evidenced that *P. tereticollis* had a much slower growth rate.

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Introduction

Multi-host parasites are facing hosts of different qualities, which can contribute unequally to their transmission (Rigaud *et al.*, 2010). Key hosts, i.e. species that contribute much more than other potential host species to the parasite maintenance within the community, are often characterized by high abundance, high susceptibility to infection, and high number of propagules produced per infected individual (Marm Kilpatrick *et al.*, 2006; Streicker *et al.*, 2013; Manzoli *et al.*, 2021). For the management of infectious diseases, understanding the way parasites and pathogens use their multiple hosts is still challenging (Fenton *et al.*, 2015), particularly for epidemiology (Carrau *et al.*, 2021). With the advent of molecular taxonomy, cryptic diversity – represented by morphologically homogeneous lineages, genetically divergent enough to be considered as different species and thus forming "species complexes"– discovered in many animal and plants species revolutionized the study of interactions between species in general, and more particularly host–parasite relationships (Pérez-Ponce de León & Nadler, 2010; Pinacho-Pinacho *et al*., 2018). Indeed, cryptic diversity was found in most parasite groups and many of these parasites, initially presumed to be generalists, were shown in fact to have a narrower host spectrum when genetic divergence was considered (Wang *et al*., 2006; Janzen *et al*., 2009; Mlynarek *et al*., 2013; Escalante *et al*., 2016; Pérez-Ponce de León & Poulin, 2018; Zittel *et al*., 2018). Interestingly, cryptic diversity may also be found in hosts of a given parasite. Here we will focus on the effect of cryptic diversity in European gammarids host-parasite interactions. Over the years, these freshwater amphipods have become a model for studying cryptic diversity and a high number of cryptic lineages was found for *Gammarus fossarum*, *G. balcanicus* and *G. roeselii* (Müller, 2000; Lagrue *et al.*, 2014; Mamos *et al.*, 2016; Grabowski *et al.*, 2017; Copilaş-Ciocianu *et al.*, 2020; Wattier *et al.*, 2020). Parasite infection and distribution patterns have been studied in light of these highly cryptic divergences, e.g. microsporidia (Quiles *et al.*, 2019, 2020, 2021), or acanthocephalans (Westram *et al.*, 2011; Galipaud *et al.*, 2017). Westram *et al.* (2011), based on a field study in Swiss rivers, showed that two cryptic lineages of *G. fossarum (Gf* type A and *Gf* type B, lineages defined by Müller, 2000) can be infected by several species of acanthocephalans, including *Pomphorhynchus tereticollis* and *Polymorphus minutus*, with higher prevalence in *Gf* type B. Galipaud *et al.* (2017) showed in a field survey in Eastern France that infection levels by *P. tereticollis* in *G. roeselii* are lower than in two other *G. pulex*/*fossarum* lineages, but stronger for *Pomphorhynchus laevis*. They also found highly variable prevalence among *G. fossarum* molecular operational taxonomic units (MOTUs). The *Gf*-III MOTU (defined by Lagrue *et al.*, 2014) showed higher prevalence of *P. tereticollis* than all other MOTUs and *Gf*-II a higher prevalence of *P. laevis*. The *Gf*-VII host MOTU was poorly infected, suggesting resistance to acanthocephalans for this cryptic species.

These field results suggest some degrees of parasite specificity among *G. fossarum* cryptic lineages. However, the variations in prevalence observed between acanthocephalan species in the field may be due to at least three factors: (i) a difference in hosts susceptibility to infection, thus reflecting specificity as commonly accepted (Poulin & Keeney, 2008); (ii) a difference in parasite virulence between different hosts that can lead to an artificial deficit of some host-parasite combinations in the field and the absence of parasite accumulation in older hosts (Rousset *et al.*, 1996); and (iii) a difference in behavioural manipulation leading to a difference in predation rates. Indeed, acanthocephalan parasites use gammarids as intermediate hosts, and change their host's antipredator behaviour in a way that increases the probability of predation, and thus the parasite's chances of being transmitted to the final host (Moore, 1983; Moore & Gotelli, 1996; Lagrue *et al*., 2007; Dianne *et al*., 2011; Jacquin *et al*., 2014; Fayard *et al*., 2020). Therefore, as noticed by Poulin & Keeney (2008) and Poulin & Maure (2015), experimental infections alone can allow to fully disentangle the causes of differences in prevalence observed in nature, provided experiments are ecologically relevant. For example, Bauer & Rigaud (2015) showed in a laboratory infection experiment that, while higher prevalence in *P. laevis* are observed in the field, *G. roeselii* is less susceptible to infection by *P. laevis* than *G. fossarum* in experimental

conditions. The discrepancy between field and the lab may be due to the lowest behavioural manipulation induced in *G. roeselii* (Bauer *et al.*, 2000), but the mortality induced by the parasite infection (virulence outside predation probability) was not evaluated. Galipaud *et al.* (2017) showed that there was a difference in parasite-induced mortality in the field (inferred indirectly from the absence of parasite accumulation in older hosts, see Rousset *et al.*, 1996), and that this mortality was variable between host species. This suggests that parasites express either differential virulence or differential manipulation, depending on the hosts they infect, but these two causes of mortality cannot be disentangled in a field study.

We investigated here the roles of two of these factors (namely, host susceptibility to infection and parasite virulence) in the interactions within a community of sympatric hosts and parasites. To this end, we experimentally infected several species of gammarids, including the *G. fossarum* cryptic species complex, with two sympatric acanthocephalan parasites, *P. laevis* and *P. tereticollis*. It should be noted that this infestation by *P. tereticollis* is, to our knowledge, the first experimental laboratory infection attempt with this species. Comparisons of the respective successes of experimental infections between the different host lineages exposed to the parasites made it possible to test whether they are differently susceptible to the parasites. Monitoring of survival during the whole parasite ontogeny was also carried out. Several costs may reduce the survival of individuals exposed to infection by acanthocephalans. First, the cost of the penetration of acanthors (early parasite larval stage) into the host digestive wall after ingesting the eggs; second the cost of resistance to infection; or third the cost of harbouring a developing parasite that grows from the acanthor to acanthella to cystacanth stages, multiplying its size by at least a factor of 10. The study of the survival of infected gammarids in the laboratory will allow assessing the virulence of the parasites during their development.

Methods

Gammarids sampling

The gammarids were collected at the beginning of January 2020 in the Albane river, between the Belleneuve and Trochères villages (47°21’25.6”N 5°15’53.6”E and 47°20’34.0”N, 5°18’22.3”E, respectively). A batch was also collected in a small tributary stream of the Suzon River (47°24’12.6”N, 4°52’58.2”E). This last population, consisting of c.a. 97% of *G. pulex* (Labaude *et al.*, 2015), has repeatedly been shown to be particularly sensitive to *P. laevis* in laboratory infections (Cornet *et al*., 2009a; b; Franceschi *et al*., 2010a; Dianne *et al*., 2011, 2012, 2014; Bauer & Rigaud, 2015; Labaude *et al*., 2020). It therefore served as a control for parasite ‘quality’ in the present experiment (Supplementary figure 1 shows the results for these ‘positive controls’).

Back to the laboratory, the amphipods were placed at 15±1°C with a 12h/12h light/dark cycle in 37 × 55 × 40 cm tanks filled with water from the field, progressively replaced by ‘lab’ water used in the experiments. This lab water was made by mixing equal volumes of water from the rivers of origin of the amphipods and adding progressivelydechlorinated and sterile tap water (passed on activated carbon filter and a UV ramp). Only male individuals were kept, following Franceschi *et al.* (2008). Naturally parasitized individuals were not used. The amphipods were kept in collective tanks in batches of maximum 500 and fed *ad libitum* with conditioned dead elm leaves. The water was partially renewed regularly. Leaves were conditioned to develop a biofilm of micro-organisms, necessary for the nutrition of the gammarids (Bärlocher & Kendrick, 1975), by soaking autoclaved leaves in aerated lab water (therefore containing the micro-organisms present in the river) for about 7 days before distribution. Animals were thus acclimatized to experimental conditions for three weeks before the infestation procedure. This quarantine gave time for some *P. laevis* natural infections to develop since the amphipods may have ingested acanthocephalan eggs before their capture. However, since *P. tereticollis* grows more slowly than *P. laevis* (a data unknown before this work was undertaken, see results), it

turned out that this quarantine was not long enough and some *P. tereticollis* natural infections emerged during our experiment. Fortunately, these natural infections could be detected before the experimental infections, allowing us to distinguish them from experimental ones, and were analysed separately (see results).

Sampling of parasite eggs and genotyping

Chubs (*Squalius cephalus*), definitive hosts in which *Pomphorhynchus* reproduce, were sampled by electrofishing in the Albane River, at the same stations where amphipods were captured (prefectural authorization of capture no. 162, February 13, 2020). The fish were euthanised in the laboratory with eugenol (Chanseau *et al.*, 2002), dissected, and the female parasites were sampled from the digestive tract of the hosts. Chubs are preferential hosts for *P. laevis* but can also be infected by *P. tereticollis* (Perrot-Minnot *et al.*, 2019). Since no preferential host of *P. tereticollis* (such as *Barbus barbus;* Perrot-Minnot *et al.*, 2019) was collected during this campaign, the *P. tereticollis* used in this experiment were also collected from the sampled chubs.

The eggs (more precisely, acanthor-stage larvae enclosed in a spindle-shaped envelope, cf. Crompton & Nickol, 1985) were extracted from the parasite body by dissection and stored in 2 ml microtubes filled with lab water. Parasite egg suspensions were checked by microscope. We manually selected, under microscope, batches containing a high density (> 50%) of mature eggs (where developed acanthors were clearly visible) to be genotyped. The bodies of the parasites from which the eggs were obtained were preserved in microtubes containing absolute ethanol for genotyping and species determination by restriction fragment length polymorphism (RFLP). Samples that failed molecular identification were not used for experimentation.

Exposure procedure

For each parasite species, a suspension was prepared by mixing 7 clutches diluted in water so as to obtain a concentration of 80 eggs per microliter, counted under an optical microscope (Nikon E600 x20). Mixing the clutches of several acanthocephalan females overcomes the possible bias towards a more or less infective (or virulent) clutch from a particular female (Franceschi *et al.*, 2010a) and is more representative of what may be encountered by gammarids in the natural environment.

Two infestation sessions were carried out one week apart, one using *P. laevis* parasites, the other with *P. tereticollis*. Gammarids were starved during the 72 hours preceding exposure. The exposure procedure described in Franceschi *et al.* (2008) was then followed. The exposed individuals were placed in pairs in a 60 ml crystallizing dish (in pairs, the gammarids ingest the leaves better than when they are isolated, unpublished data), in the presence of a piece of elm leaf of about 1 cm^2 on which a volume of suspension containing an average of 300 parasite eggs were deposited. This dose, higher than those used in our previous experiments, was a compromise between guaranteeing the success of the infection and avoiding a too high rate of multiple infections (Franceschi *et al.*, 2008), especially in experimental infections involving co-evolved partners (Franceschi *et al.*, 2010b; Bauer & Rigaud, 2015). Pieces of leaves without eggs were distributed to the control batches. After 48 hours, all individuals were placed individually in a crystallizing dish (60 ml) containing lab water and a piece of parasite-free elm leaf. In the *P. laevis* exposure session, 1107 amphipods of unknown genotype were used (1012 exposed individuals, 95 negative controls). All gammarids were genotyped at the end of the experiment. After the removal of *G. pulex* and uncharacterized individuals, the analysis of *P. laevis* infection included 1054 *G. fossarum* (965 exposed and 89 unexposed controls; see Table 1 and Supplementary table 1). Similarly, 1079 individuals were used for the *P. tereticollis* exposure and 1001 *G. fossarum* were included in the analysis of *P. tereticollis* infection (911 exposed, 90 unexposed controls; Table 1 and Supplementary table 1).

Gammarids maintenance and mortality survey

Throughout the duration of the experiment, the crystallizing dishes were inspected daily, the water topped up and a piece of elm leaf added when the former had been eaten. Dead gammarids were measured (height of the 4th metacoxal pereion plate) and dissected to check their infection status. Dissected tissues were stored in absolute ethanol pending genotyping at the end of the experiment. Daily monitoring made it possible to genotype dead individuals before decay. Every two weeks, one *Chironomus plumosus* larva (Europrix brand frozen fish food cubes) was distributed in each crystallizing dish as a dietary supplement, improving survival in the laboratory (Labaude *et al.*, 2015), and 24 hours later the water was completely replaced.

Based on the *P. laevis* development time observed in our previous experiments (e.g. Franceschi *et al.*, 2008; Labaude *et al.*, 2020), the gammarids were inspected under a binocular magnifier from 6 weeks post-exposure (Nikon SMZ 745) to check their parasitic status through the cuticle of their host. Individuals detected as infected were isolated. The growth of the parasites was then inspected twice a week. However, some parasites were visible before these 6 weeks (some of them few days after the beginning of the experiment, a development time much too short to correspond to experimental exposure, see results), corresponding to *P. tereticollis* natural infections (confirmed by genotyping). These animals were analysed separately.

We ended the experiment when no new developing parasites were detected for 15 successive days. The surviving gammarids were euthanized, measured and dissected to confirm their parasitic status. The developmental stage of the parasites was noted for infected individuals. These gammarids, as well as their parasites, were stored individually in absolute ethanol pending genotyping. The parasites at the acanthella stage had to be genotyped to confirm their species (by sequencing, refer to the following paragraph), while the parasites having reached the cystacanth stage were easily recognizable using a binocular magnifier (Perrot-Minnot, 2004).

Gammarids & parasites genotyping

To genotype gammarids still alive at the end of the experiment, between two and four locomotor appendages were detached and placed in a tube filled with ethanol. For individuals who died during the tracking, a larger amount of tissue was used (between one and three body segments) to make up for the quick degradation of DNA after death. To genotype adult parasites, a piece of about two mm^3 of body tissue was used to extract DNA. For the parasites at the acanthella stage, the whole individual was used.

All DNA extractions were performed using an extraction kit (EZ-10 96 Well Plate Genomic DNA Isolation kit, BioBasic Inc.) following the manufacturer instructions. Elutions were made in 100 µl or 60 µl of elution buffer provided in the kits, for the gammarids and the parasites, respectively.

For both parasites at the acanthella stage and amphipods, the gene encoding the first subunit of cytochrome oxidase (CO1) was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The total reaction volume (20 µL) consisted of 200 nM of each primer, 200 µM of dNTPs, 0.25 U of DNA polymerase (HotStarTaq, Qiagen Inc., Düsseldorf, Germany), 1X of buffer, 5 µL of extracted DNA, and 10,5 µL ultrapure water. The following thermal treatment was applied: activation of the polymerase for 3 min at 95°C, then 39 cycles: denaturation for 20 s at 95°C, hybridization for 45 s at 40°C and synthesis for 1 min at 65°C, then finally 2 min at 65°C (final synthesis). Amplification success was checked by electrophoresis on 2% agarose gel and visualized with ethidium bromide stain. In case of failure of the CO1 amplification for parasites, the ITS region was amplified. The primers BD1f: 5'-GTC GTA ACA AGG TTT CCG TA-3' and ACITS1r: 5'-TTG CGA GCC AAG TGA TTC AC-3' were used (Franceschi *et al.*, 2008). The reaction mixture was subjected to an initial denaturation at 95°C for 3 min, then 39 cycles at 95°C (20 s), 50°C (45 s) and 65°C (45 s). Final elongation was performed for 5 minutes at 65°C.

Adult parasite species were identified by using a RFLP procedure. We first produced our own gene sequences by Sanger sequencing by Genewiz (Leipzig, Germany). The sequences were aligned using

Mega software (V10; www.megasoftware.net) and compared to reference sequences (see Supplementary table 2). We then used the Sequence Manipulation Suite (SMS) software (http://www.bioinformatics.org/sms2/rest_map.html) to establish that the restriction enzyme VspI (Thermo Scientific™) enables distinguishing between *P. laevis* (two restriction fragments of 60 and 620 bp) and *P. tereticollis* (two fragments of 450 and 240 bp). 10 U of enzyme were added to 1 µL of associated buffer and 5 µl of PCR product and adjusted with ultrapure water to a reaction volume of 15 µL. The mixture was then placed at 37°C for 16 hours and then at 65°C for 20 minutes to inactivate the enzyme. The migration of 15 µL of RFLP product on a 2% agarose gel (for 20 min at 100 V), then visualization with ethidium bromide made it possible to determine the species of the selected samples. For all parasite acanthellae obtained during the survey, PCR products were sequenced and compared to reference sequences (Supplementary table 2).

CO1 amplicons of most gammarid hosts were sequenced and compared to reference sequences (Supplementary table 1), using pairwise distances corrected by the Kimura two-parameters (K2P) model (see Lagrue *et al.*, 2014 and Wattier *et al.*, 2020 for details).

For logistic reasons, 108 gammarids were also typed by using a RFLP procedure developed from the analysis of these sequences using Mega and SMS softwares. A first reaction was carried out to distinguish between the *G. fossarum* and *G. pulex* groups: the VspI enzyme (Thermo Scientific™) cuts the DNA of *G. pulex* into two strands of approximately 200 and 450 base pairs. The *G. fossarum* amplicons were then subjected to a second RFLP with the DraI enzyme (Thermo Scientific™), to discriminate between the two groups *Gf* 1-2-3 and *Gf* 6-7 (species groups identified by Lagrue *et al.*, 2014). The PCR products of *Gf* 6 and *Gf* 7 are cut into two strands of approximately 450 and 200 base pairs, while the enzyme does not cut the PCR products of *Gf* 1-2-3. The restriction protocol was the same as detailed before.

All gammarid sequences were submitted to BOLD, in the 'GAMEI' project (https://v4.boldsystems.org/index.php/MAS_Management_DataConsole?codes=GAMEI).

Statistical analyses

We ran two independent logistic regressions (GLM for binomial distribution with logit transition function) for each infection type (natural and experimental) for the analysis of prevalence according to parasite species (*P. laevis* vs. *P. tereticollis*), host MOTU and their interaction. We did not run a single model with infection type as a factor because experimental infections were made with a subsample of the dataset used to estimate natural infections. Infection intensities were compared between MOTUs by Fisher's exact tests. Parasite surinfections (probability of developping an experimental infection when naturally infected) were compared by using logistic regressions with Firth correction in cases of rare events (Firth, 1993) with the *logistf* package (v1.26.0; Heinze & Schemper, 2002). We considered as 'infected' all the hosts where at least one parasite was detected, regardless of its developmental stage. Parasite development duration to the cystacanth stage was analysed using a GLM (Poisson distribution, Log transition function), with the parasite species, host MOTU, and parasites number as explaining factors, and their interactions.

Host survival was analysed with R using the *survival* package (Therneau & Grambsch, 2000). Data were censored if animal was still alive at the end of the experiments (118 days for the individuals exposed to *P. laevis*, and 138 for those exposed to *P. tereticollis*). Cox models were constructed for comparisons. A preliminary analysis showed that survival data did not respect, over the entire follow-up, the condition of proportionality of hazards (checked with the *cox.zph* function from the *survival* package) necessary for the application of a Cox regression model. Indeed, an acceleration of mortality was visible when the cystacanth stage had been reached (see figures 3 and 4). In addition, during the first weeks of the survey, developing acanthellae were too small to be detected with a binocular magnifier when dissecting dead gammarids, making it impossible to distinguish parasitized individuals from uninfected ones. Because of these two constraints, survival was analysed in two steps. In the first step, we distinguished only two

groups: the control individuals vs. those exposed to the infection. This analysis was stopped on the day at which the first experimental acanthella was detected after the dissection of a dead gammarid (44 days for *P. laevis* and 61 days for *P. tereticollis*). In the second step, between the day of the first detection and the end of monitoring, the 'uninfected' and 'parasitized' groups were distinguished among exposed gammarids. For each analysis, the condition of proportionality of risks was successfully controlled. Finally, to compare the parasites virulence in actually infected gammarids, survival data of infected amphipods were analysed after the first detection date of *P. laevis*. Three factors were included in the Cox models: parasite species, host lineage, and intensity of infection (for which data were categorized as follows: one, two and more than two parasites). All possible models with first order interactions were compared by Akaike information criteria, using the *dredge* function of the *MuMin* R package (v. 1.43.17 ; Bartoń, 2009), and the models minimizing the AICc were presented.

Results

Apart from *G. roeselii*, which were not taken into account in this experiment (see Bauer & Rigaud, 2015, for their analysis), and too few *G. pulex* to be integrated in the analyses (Supplementary table 2), three *G. fossarum* host lineages have been found in the gammarids of the Albane River. Forty-eight *Gf* 7 individuals were detected by sequencing. Since they are genetically very close to the *Gf* 6 MOTU and belong to the same breeding group (Lagrue *et al.*, 2014), they were referred as *Gf* 6 in the dataset and the following analyses. The genotypes of all hosts were obtained only at the end of the experiments, which explains why it was impossible to restore any imbalances in numbers. The dataset is summarized in Table 1.

Table 1 - Numbers of *G. fossarum* MOTUs analysed, according to their infection status after experimental exposures. They include individuals with natural infections, indicated in brackets by parasite species (*P. laevis* are in light brown, *P. tereticollis* are in blue, and undetermined in black).

	MOTU	controls		exposed			total
		early death	uninfected	early death	infected	eni	
***P. laevis* exposure**	*Gf* 2	7 (1+1)	18	33	14 (7)	443 (3+23)	515
	Gf 6	12	52	58	9 (1+1)	408	539
***P. tereticollis* exposure**	*Gf* 2	25 (2)	23	119 (1+11+6)	28 (1)	236 (2+6)	431
	Gf 6	30 (1)	12	147 (1+2)	9	372	570

Gf 2 = *G. fossarum* MOTU 2 ; *Gf* 6 = *G. fossarum* MOTU 6. *Controls*: gammarids not exposed to parasite eggs. *Exposed*: gammarids experimentally exposed to parasite eggs. *Early death*: gammarids that died before the date of detection of the first acanthella. *Eni (exposed not infected)*: exposed gammarids that did not develop any infection.

Infection rates

Natural infections

Some acanthellae were visible 4 days after the exposure date, in both exposed and unexposed animals. Since such a short development time from exposure to acanthella stage is not possible, we inferred that these infections were natural infections for which the quarantine was not long enough for evidencing their development. Most of these infections were *P. tereticollis* parasites (Table 1, Figure 1a). For the two parasite species, the prevalence were higher in *Gf* 2 than in *Gf* 6, and the prevalence of *P. tereticollis* was overall higher than the one of *P. laevis* (GLM analysis is in Supplementary table 3).

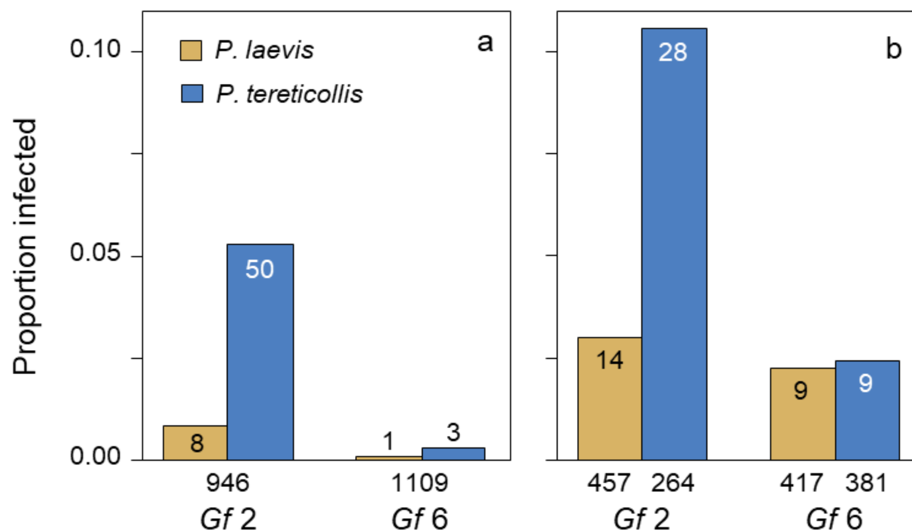

Figure 1 - Infections by *P. laevis* and *P. tereticollis* of natural origin (a) and for experimental infections (b) in *G. fossarum* MOTUs from Albane River. Numbers of infected amphipods are indicated at the top of the bars, and total numbers of each MOTU appear below. Early deaths were not considered in (b) since it is not possible to assertain the success of experimental infection.

Experimental infections

Infection success: prevalence and intensity

There was no overall significant difference in infection levels between the two gammarid MOTUs, but they were not infected by the two parasites in the same way, as indicated by the significative interaction in the GLM model: *Gf* 2 was significantly more sensitive to *P. tereticollis* than *Gf* 6, while the two host MOTUs were equally sensitive to *P. laevis* (GLM analysis in Supplementary table 3).

The average *P. laevis* infection intensity was 2.07 parasites per host in *Gf* 2 (half of the individuals were infected by one parasite, and the other half hosted between 2 and 7 parasites). The average infection intensity was 4 parasites per host in *Gf* 6 (six were mono-infected, one harbored 3 parasites, but two individuals presented a very high load of 12 and 15 parasites, which skews the distribution). This difference between *Gf* 2 and *Gf* 6 was not significant (Fisher's exact test, p = 0.254). The amphipods were mostly mono-infected by *P. tereticollis* and the maximum load was 3 (the difference in parasite intensity was not significant: Fisher's exact test, p = 1 ; the average intensities being 1.28 for *Gf* 2 and 1.22 for *Gf* 6).

Development duration of parasites

The dissections of gammarids that died during the tracking allowed detecting the first experimental *P. laevis* acanthellae 44 days post-exposure and those of *P. tereticollis* after 61 days. The first *P. laevis* reached the cystacanth stage at 77 days and the first *P. tereticollis* at 90 days.

Interactions between explaining factors were not significant and therefore removed from the model (Supplementary table 4). There was no effect of parasites number on development duration and we neither found a difference between development duration according to host lineage. However, it took significantly less time for *P. laevis* to reach the cystacanth stage (81 days on average) than for *P. tereticollis* (106 days), and the variation between early and late cystacanths differentiation was found to be larger in this latter species (Figure 2).

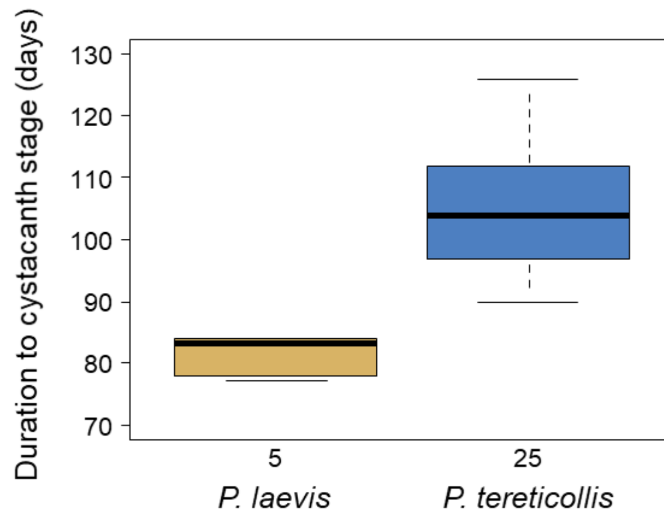

Figure 2 - Development duration of parasites in the two species until the cystacanth stage, in days post-expos ure. Thick black bars indicate medians, boxes are the upper and lower quartiles, and whiskers represent the upper and lower values. Numbers are sample size. Sample size are lower than in Table 1, because several gammarid hosts died before the cystacanth stage was reached.

Probability of natural and experimental coinfections

Natural infections by *P. laevis* influenced significantly subsequent experimental infection by *P. laevis* (2.53 % probability of infection when not firstly infected (22/870) and 25.0 % in gammarids already infected (1/4), logistic regression with Firth correction, $\chi^2 = 4.959$, $p = 0.026$) but not those by *P. tereticollis* (5.61 % (36/642) and 2.77 % (1/3) respectively, logistic regression with Firth correction: $\chi^2 = 3.56$, $p = 0.059$), but since numbers were very low for some categories, the relevance of the statistics are questionable.

Natural infections by *P. tereticollis* strongly influenced an experimental coinfection by *P. laevis*: while the probability of *P. laevis* lab infection in gammarids not previously infected was 1.78 % (15/843), it reached 25.81 % (8/31) in gammarids already naturally infected by *P. tereticollis* (logistic regression with Firth correction: $\chi^2 = 28.330$, $p < 0.0001$). For lab infections by *P. tereticollis*, the previous effect of *P. tereticollis* natural infection was non-significant (logistic regression with Firth correction: $\chi^2 = 0.019$, $p = 0.889$), the prevalence reaching 5.79% (37/639) in animals uninfected naturally and 0.0 % (0/6) in animals already infected.

Parasite virulence

P. laevis-exposed individuals

In the first part of the tracking, *Gf* 2 gammarids survived slightly better than *Gf* 6, and, overall, exposed individuals survived better than the control gammarids (figure 3a,c; Supplementary table 5). However, when testing only *Gf* 6 individuals, the survival of exposed and control individuals was not significantly different (ß = -0.481, z = -1.516, p = 0.129).

In the second part of the tracking, increased mortality of parasitized individuals appeared as soon as the first acanthellae became detectable by dissection in *Gf* 6, but it occurred later, from the date of differentiation of the first cystacanths, in *Gf* 2 (figure 3b,d; Supplementary table 5). The survival of uninfected individuals - 'exposed but not infected (eni)' and 'controls' – was similar. Globally, survival was better in *Gf* 2 than in *Gf* 6.

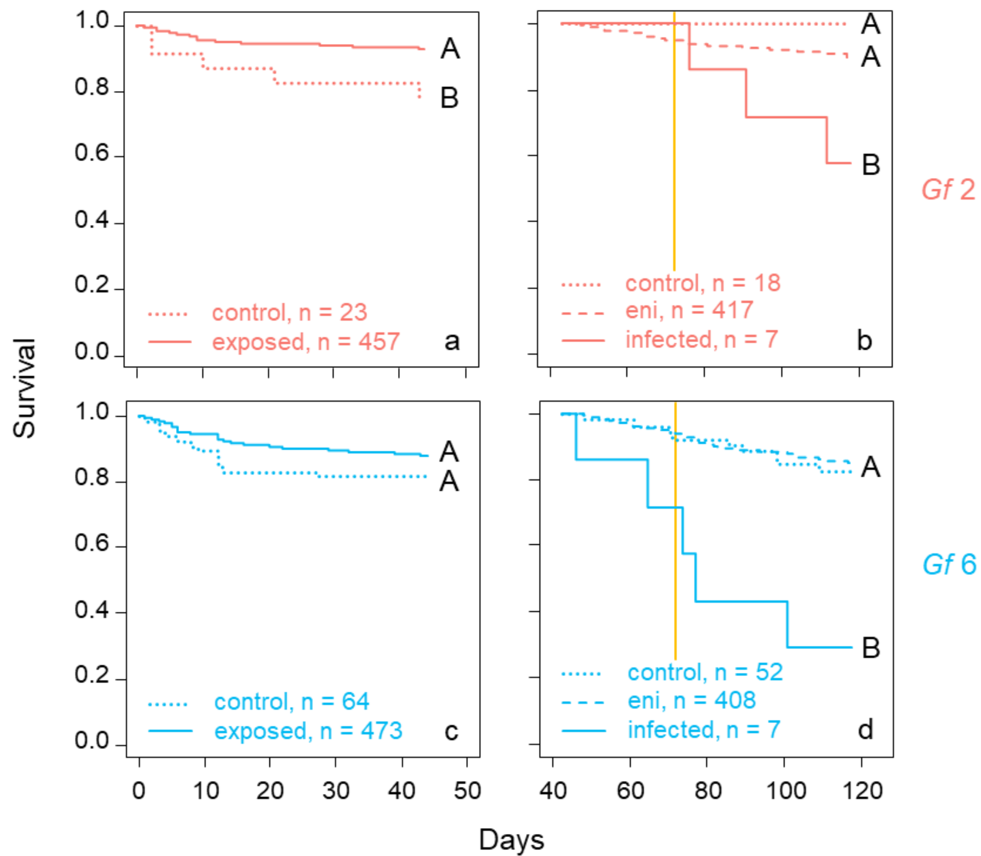

Figure 3 - Survival of *P. laevis* exposed gammarid MOTUs (plain lines) and controls (dotted lines), before the first acanthella detection date (a,c). Survival of *P. laevis* exposed gammarid MOTUs and controls (dotted lines), after the first acanthella detection date (b,d). Infected individuals (plain lines) can now be distinguished from exposed but not infected ones ('eni', in dashed lines). Letters on the right of the graphs indicate the statistical groups. The vertical orange lines denote the first cystacanth detection date.

P. tereticollis-exposed individuals

High mortality of gammarids exposed to *P. tereticollis* was observed at the beginning of the survey (compared to *P. laevis* exposed ones), the same trend was observed in both exposed and control gammarids (figure 4a,c; Supplementary table 6). No significant overall difference in survival was observed between *Gf* 2 and *Gf* 6 MOTUs. However, when testing only *Gf* 6 individuals, exposed animals survived better than control ones (ß = -0.575, z = -2.296, p = 0.022).

In the second part of the follow-up and for both MOTUs, parasitized individuals showed increased mortality compared to uninfected, whether the latter were eni or controls (figure 4b,d; Supplementary table 6).

Survival in infected gammarids

Survival analysis in infected gammarids began on the day acanthellae became visible. Interactions were not significant and therefore removed from the model (see Supplementary table 7 for the detailed comparison of possible models). We found only a significant effect of parasite species (whole model: $\chi^2 = 10.69$, 3 df, p = 0.01; Supplementary table 8). Globally, *P. laevis*-infected gammarids died nearly twice faster than those infected by *P. tereticollis* (Figure 5a). The observed difference in survival between infected *Gf* 2 and *Gf* 6 (whatever the parasite species, Figure 5b) was not significant, nor the number of hosted parasites (Figure 5c).

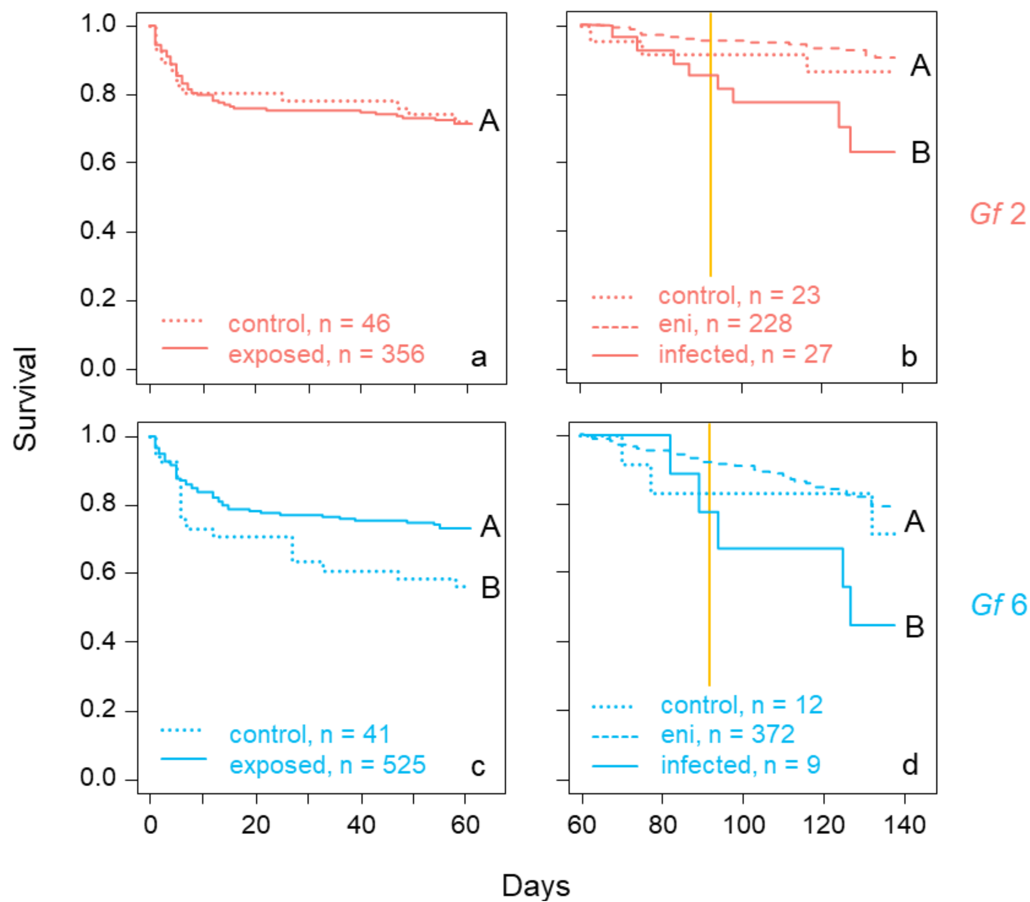

Figure 4 - Survival of *P. tereticollis* exposed gammarid MOTUs (plain lines) and controls (dotted lines), before the first acanthella detection date (a,b). Survival of *P. tereticollis* exposed gammarid MOTUs and controls (dotted lines), after the first acanthella detection date (c,d). Infected individuals (plain lines) can now be distinguished from exposed but not infected ones ('eni', in dashed lines). Letters on the right of the graphs indicate the statistical groups. The vertical orange lines denote the first cystacanth detection date.

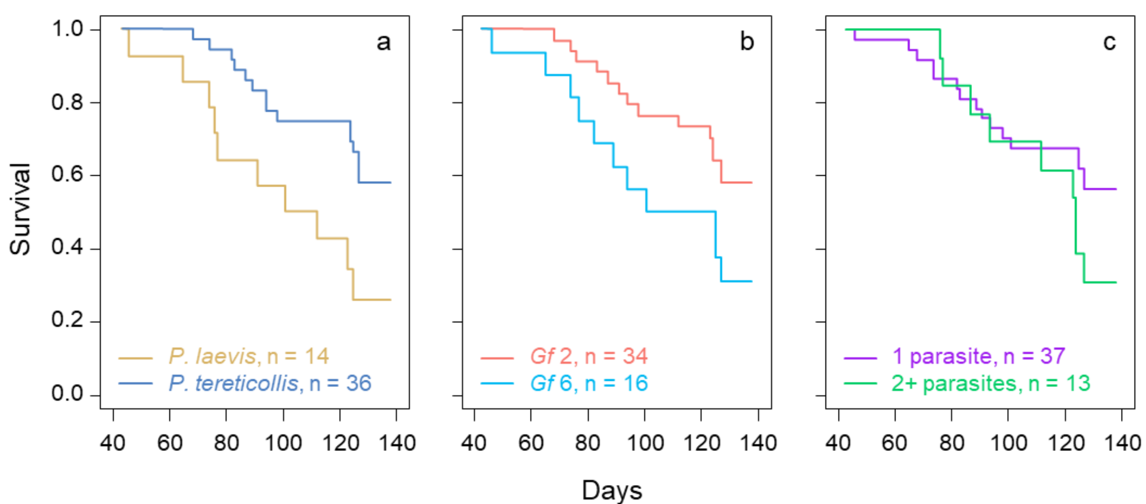

Figure 5 - Comparison of survival of infected gammarids after the first *P. laevis* acanthella detection date: a) effect of parasite species; b) effect of host MOTU; c) effect of the intensity of infection.

Discussion

Susceptibility to infections by different parasite species in different host lineages

We investigated by experimental infections two important factors explaining natural patterns of acanthocephalan infections in the wild: infection success and virulence. Behavioural manipulation is a third important factor, because subsequent predation by definitive hosts lowers the overall cystacanths prevalence observed on the field. Unfortunately, we could not investigate this latter aspect of the host-parasite interaction in this study because of the globally low success of infection. It will be necessary to further evaluate differences in manipulation among cryptic hosts to get a more complete picture of the link between host genotype and efficient exploitation by *Pomphorhynchus* spp. acanthocephalans.

To our knowledge, this work presents the first results of an experimental infestation of *Gammarus* spp. with *P. tereticollis*. The experimental infestations with *P. tereticollis* led to a relatively high infection rate in *Gf* 2 and to a lesser extent in *Gf* 6. High prevalence of *P. tereticollis* in *G. fossarum* has been already found in previous field works (Westram *et al.*, 2011; Galipaud *et al.*, 2017; Harris, 2020). Our finding that *Gf* 2 is more sensitive to infection than *Gf* 6 sharply contrasts with field data from Galipaud *et al.* (2017) in which the prevalence in *P. tereticollis* was found to be similar between these MOTUs.

This study also confirms, under controlled conditions, that all *G. fossarum* MOTUs present in the Albane River can be infected by *P. laevis*. The experimental infection success by *P. laevis* was similar in *Gf* 2 and *Gf* 6, and was significantly lower than that by *P. tereticollis* in *Gf* 2. This suggests a lower susceptibility to *P. laevis* of this latter host MOTU. Alternatively, despite our precautions to standardize the infections, we cannot completely rule out another phenomenon. Indeed, even if we controlled the dose of parasite eggs provided to the gammarids, it was not possible to control the number of eggs that were actually consumed.

Our results nevertheless highlight the need for subjecting each of the MOTUs of the *G. pulex* / *fossarum* complex species to experimental infections to understand their relative sensitivity to the different species of acanthocephalans, as suggested by Poulin & Maure (2015).

Despite low numbers that limited statistical analyses, it is worth noting that experimental infections also suggest that *G. pulex* from Albane River were particularly sensitive to *P. laevis* compared to *Gf* 2 and *Gf* 6, but not to *P. tereticollis* (Supplementary table 1 and Supplementary Figure 2). The *G. pulex* of the Suzon population also showed the same pattern (Supplementary figure 1), confirming the field observations of Harris (2020). Because they appear to be contradicting observations by Galipaud *et al.* (2017), who found low *in natura* prevalence of *P. laevis* cystacanths in *G. pulex*, our results need experimental confirmation with a larger number of individuals. However, as showed by Bauer & Rigaud (2015), a low prevalence of cystacanths found after direct collection in nature could simply mean that either parasite virulence is high, or that the parasite increased so much the predation rate of its host that parasitized individuals disappear rapidly from populations. Here, our measures of prevalence were made after quarantine or after experimental infection, i.e. in conditions where predation was absent or the survival controlled, highlighting the usefulness of experimental infections in deciphering host-parasite relationships (Poulin & Maure, 2015). This is particularly true for acanthocephalans, where the prevalence *in natura* reflects a combination of several processes due to the long intra-host development time (Bauer & Rigaud, 2015). One of these processes is parasite virulence that we will discuss thereafter.

Natural and experimental coinfections

Natural infection by *P. tereticollis* favours a further interspecific experimental infection by *P. laevis*. Contrastingly, natural infection by *P. tereticollis* did not influence intraspecific experimental superinfections by *P. tereticollis*, and natural infection by *P. laevis* did not influence either a secondary infection by another acanthocephalan. However, sample size were sometimes so low that the statistical power of these latter analyses are questionable. Cornet *et al.* (2009a) showed that the level of immune

defences of *G. pulex* is strongly depressed by the acanthocephalans *P. laevis* and *P. tereticollis*, and that acanthocephalan infection favors superinfection by *Escherichia coli* bacteria. However, Dianne *et al.* (2010), using successive experimental infections by *P. laevis*, did not observed any increase of acanthocephalan intraspecific superinfection. It is therefore possible that the immune depression only favours interspecific parasite/pathogens superinfections but not intraspecific ones, suggesting that this immune depression is a costly by-product of the acanthocephalan infections. Since multiple infections are the rule in nature, further experimental investigations are welcome, particularly for the study of the evolution of virulence (Alizon *et al.*, 2013).

Development duration of parasites

Acanthocephalans development time in their intermediate hosts is strongly influenced by the environment (Awachie, 1966; Lackie, 1972; Tokeson & Holmes, 1982; Brattey, 1986; Labaude *et al*., 2020), highlighting the necessity of studying this life-history trait under controlled conditions. The development duration of *Polymorphus minutus* and *P. laevis* under laboratory conditions have already been studied (Hynes & Nicholas, 1957; Butterworth, 1969; Franceschi *et al*., 2008; Labaude *et al*., 2015, 2020), but *P. tereticollis* development was not documented before this study. The development time in *P. laevis* observed here was similar to the one found by Labaude *et al.* (2020) at a similar temperature. The development of *P. tereticollis* took 23 % longer than *P. laevis* under the same conditions. The among-host variation in maturation time (time between the appearance of the first and the last cystacanths) was also larger and more variable than that of *P. laevis*. This longer development time explains *a posteriori* why we observed so many natural *P. tereticollis* infections after the quarantine we imposed to gammarids, since the quarantine time was estimated from our previous experience on *P. laevis* development.

Virulence

We first observed higher mortality in the *P. tereticollis* exposure experiment, at the beginning of the survey, compared to the *P. laevis* exposure. Since this pattern was found both in exposed and control unexposed gammarids, the higher mortality was not linked to parasite eggs exposure. It is therefore likely that conditions of the *P. tereticollis* experiment were more stressfull than those of the *P. laevis* experiment, even if it was made in the very same room only a few days later.

Ingestion of parasites (both *P. laevis* and *P. tereticollis*) and the early beginning of larval development did not negatively affect the host survival. Therefore, the wounds inflicted when the acanthors pierce the digestive tract of the arthropod to pass into the general cavity, and/or a possible early resistance to infection (cellular response, humoral and immune encapsulation (Crompton & Nickol, 1985) were not costly enough to increase mortality. The literature is contradictory about the host survival in early stages of infection by acanthocephalans. Increased mortality shortly after acanthocephalan experimental exposure was observed by Crompton & Nickol (1985) and Hynes & Nicholas (1957), but these studies were most likely carried out with massive doses of eggs. Although the dose used in these works is not mentioned, the high prevalence reported and the known dose-dependant prevalence relation (Franceschi *et al.*, 2008), the high dose exposure hypothesis is reasonable to consider. In such cases, the damages inflicted by many acanthors crossing the digestive tract wall may have been deleterious. On the other hand, Uznanski & Nickol (1980) did not find any increased mortality in the first 24 hours post-exposure in *Hyalella azteca* exposed to *Leptorhynchoides thecatus*. Yet, here again, the high infection success suggested the usage of high doses of eggs. Even more intriguing, experimental infections of *Asellus* by *Acanthocephalus lucii* studied by Brattey (1986) and Benesh & Valtonen (2007) induced early increased mortalities, whereas Hasu *et al.* (2006) found a better survival of infected individuals on the same biological models. This latter and somewhat intriguing result is what we observed in the present study: gammarids exposed to parasite eggs showed improved survival in the early days post-exposure,

i.e. before the infection can be detected. Only speculative discussions on these observations can be proposed because it was not possible to ensure whether individuals dying early in the survey had been infected or not. At least, we can exclude a selective mortality due to exposure. One of the possible explanations is that the observed improved survival of exposed gammarids could be part of 'parasite manipulation', where the parasite would protect its host from predation, before becoming infective to the next host (Parker *et al.*, 2009). Such a 'protection' was described for *P. laevis* in the late acanthella stage (Dianne *et al.*, 2011; Rigaud *et al.*, 2023), but this was a protection against mortality due to predation, which is not the case here. A mechanistic explanation of such a phenomenon could be that the early stages of the parasite divert some of the host energy in such a way as to improve the host survival. Castrating parasites are known to divert energy normally allocated to host reproduction to their own purpose, in particular for the survival of the host they infect (Benesh & Valtonen, 2007). Gammarid acanthocephalans are known to castrate their hosts, partially (Bollache *et al.*, 2002) or totally (Bollache *et al.*, 2002; Bailly *et al.*, 2018). It is not known precisely at what stage of parasite growth castration occurs in *Pomphorhynchus*, but it happens early during *Polymorphus* ontogeny, at least before the acanthellae are visible in their host (Bailly *et al.*, 2018). Alternatively, egg parasites eaten by gammarids, but unable to develop, can themselves become a form of diet supplementation for the hosts that 'prey' on them (Goedknegt *et al.*, 2012), improving host survival. Finally, perhaps the presence of eggs in the water triggers protective mechanisms (e.g., heat shock proteins, mechanisms against oxidative stress) that help the host sustain stress in the lab.

Parasite virulence was expressed in our study when the acanthellae enter their exponential phase of growth. It is interesting to note that *Gf* 6 was more sensitive to *Pomphorhynchus* infections than *Gf* 2, whatever the parasite species. In *Hyalella azteca* infected by *Corynosoma constrictum*, a higher mortality was observed at the time of rapid development of the parasite larvae, only in heavily infected hosts (Duclos *et al.*, 2006). This increased mortality associated with acanthocephalan infections is interpreted as being due to displacement of the host's organs (Bentley & Hurd, 1993; Dezfuli *et al.*, 2008), or effects on metabolism (Rumpus & Kennedy, 1974). In our study, the energy requirements of the two parasites are probably different, since *P. tereticollis* larvae are larger than *P. laevis* (Perrot-Minnot, 2004). However, the virulence of *P. laevis* was higher than that of *P. tereticollis*. An effect on metabolism would therefore only be possible if one considers that *P. laevis* grows faster than *P. tereticollis*, which could exhaust the host's metabolism at higher intensity. If so, this would be in line with the 'boom-bust' and 'slow and steady' strategies proposed by Le Clec'h *et al.* (2019) for schistosome infections. Studies looking at metabolism of infected gammarids have only been carried out with parasites at cystacanth stages (Plaistow *et al.*, 2001; Gismondi *et al.*, 2012; Korkofigas *et al.*, 2016). All these studies showed that parasitized amphipods are deprived of lipid and protein reserves, while they have higher glycogen rates. This shows high demand for immediate energy (glycogen) in infected amphipods, which are forced to draw on their reserves (lipids), probably at the benefit of the parasite metabolism. Such a strategy is common in parasites. In schistosomes, such effects on the host fitness and energy diversion occur both early and in the late development of the parasite (De Jong-Brink *et al.*, 2001). In *Plasmodium*, the intra-host competition in cases of multiple infections is leading to increased energetic demand due to host overexploitation by the faster-developing parasites (De Roode *et al.*, 2005). Differences in survival between *Gf* 2 and *Gf* 6 hosts may be due to differences in metabolism, a hypothesis that necessitate further investigations. Another possible explanation for a differential in survival between host lineages could be differences in the ability of acanthocephalans to reduce the intensity of the immune responses of their hosts (Cornet *et al.*, 2009a). On the one hand, this partial immunosuppression might be advantageous for the host, owing the cost of mounting an immune response, either in terms of physiology (Moret & Schmid-Hempel, 2000) or because of immune cytotoxicity of enzymes used for the invertebrate immune responses (Sadd & Siva-Jothy, 2006). On the other hand, as discussed before, this immunosuppression can make hosts more susceptible to other infections, such as bacterial infections (Cornet *et al*., 2009a; Cornet & Sorci, 2010). This immune modification is not observed in all gammarids:

infections of various populations of *G. roeselii* by *P. laevis* do not show immunosuppression, with some populations even showing an immune response to the infection (Moret *et al.*, 2007). In addition, the level of immune defenses are variable among gammarid populations (Cornet *et al.*, 2009b). Therefore, *Gf* 2 and *Gf* 6 could be immunosuppressed differently, a hypothesis that remains to be tested.

Whatever the underlying mechanism, the greater sensitivity of *Gf* 6 to acanthocephalans could explain, at least in part, why the prevalences were lower in nature in this MOTU than in *Gf* 2, compared to experimental infections.

General conclusion

Our results complement those of a previous study on Acanthocephala, carried out *in natura* by counting infections by cystacanths (Galipaud *et al.*, 2017). Different host genotypes within the *G. fossarum* species complex, experimentally exposed to parasites, are significantly different in their susceptibility to *P. tereticollis*, but not to *P. laevis*. These results explain the overall differences in prevalence in the field. Our data therefore indicate that we can no longer ignore cryptic hosts' genetic information to determine the level of specialization of the parasites. In addition to differences in susceptibility to infection, we also showed that a strong virulence is expressed when *P. laevis* or *P. tereticollis* reached the cystacanth stage, but that the different genotypes of *G. fossarum* were not affected with the same intensity, bringing refinement to explain the pattern of prevalence observed in the field.

Authors contributions

A.B. and T.R. conceived and planned the experiments. A.B., L.D., S.M., M.T., N.D. and T.R. carried out the experiments. A.B., N.D. and M.T. contributed to sample preparation. A.B. analyzed the data. A.B. and T.R. contributed to the interpretation of the results. A.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Data, scripts, code, and supplementary information availability

The supplementary information, dataset (in French) data description file explaining the variables and scripts are available on the data.InDoRES repository (https://doi.org/10.48579/PRO/X9ZRVB; Bauer, 2024).

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article. TR is recommender for PCI infections.

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