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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.

**Keywords:** experimental infection, *Gammarus fossarum*, *Gammarus pulex*, host specificity, infectivity, multi-host parasite, *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis*, prevalence

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 and 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 and 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

75 as commonly accepted (Poulin and Keeney, 2008); (ii) a difference in parasite virulence between different  
76 hosts that can lead to an artificial deficit of some host-parasite combinations in the field and the absence  
77 of parasite accumulation in older hosts (Rousset *et al.*, 1996); and (iii) a difference in behavioural  
78 manipulation leading to a difference in predation rates. Indeed, acanthocephalan parasites use  
79 gammarids as intermediate hosts, and change their host's antipredator behaviour in a way that increases  
80 the probability of predation, and thus the parasite's chances of being transmitted to the final host  
81 (Moore, 1983; Moore and Gotelli, 1996; Lagrue *et al.*, 2007; Dianne *et al.*, 2011; Jacquin *et al.*, 2014;  
82 Fayard *et al.*, 2020). Therefore, as noticed by Poulin & Keeney (2008) and Poulin & Maure (2015),  
83 experimental infections alone can allow to fully disentangle the causes of differences in prevalence  
84 observed in nature, provided experiments are ecologically relevant. For example, Bauer & Rigaud (2015)  
85 showed in a laboratory infection experiment that, while higher prevalence in *P. laevis* are observed in the  
86 field, *G. roeselii* is less susceptible to infection by *P. laevis* than *G. fossarum* in experimental conditions.  
87 The discrepancy between field and the lab may be due to the lowest behavioural manipulation induced in  
88 *G. roeselii* (Bauer *et al.*, 2000), but the mortality induced by the parasite infection (virulence outside  
89 predation probability) was not evaluated. Galipaud *et al.* (2017) showed that there was a difference in  
90 parasite-induced mortality in the field (inferred indirectly from the absence of parasite accumulation in  
91 older hosts, see Rousset *et al.*, 1996), and that this mortality was variable between host species. This  
92 suggests that parasites express either differential virulence or differential manipulation, depending on  
93 the hosts they infect, but these two causes of mortality cannot be disentangled in a field study.

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

109

## Methods

### 110 Gammarids sampling

111

112 The gammarids were collected at the beginning of January 2020 in the Albane river, between the  
113 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,  
114 respectively). A batch was also collected in a small tributary stream of the Suzon River (47°24'12.6"N,  
115 4°52'58.2"E). This last population, consisting of c.a. 97% of *G. pulex* (Labaude *et al.*, 2015), has repeatedly  
116 been shown to be particularly sensitive to *P. laevis* in laboratory infections (Cornet *et al.*, 2009a; b;  
117 Franceschi *et al.*, 2010a; Dianne *et al.*, 2011, 2012, 2014; Bauer and Rigaud, 2015; Labaude *et al.*, 2020). It  
118 therefore served as a control for parasite 'quality' in the present experiment (Supplementary figure 1  
119 shows the results for these 'positive controls').

120 Back to the laboratory, the amphipods were placed at 15±1°C with a 12h/12h light/dark cycle in 37 ×  
121 55 × 40 cm tanks filled with water from the field, progressively replaced by 'lab' water used in the  
122 experiments. This lab water was made by mixing equal volumes of water from the rivers of origin of the  
123 amphipods and adding progressively dechlorinated and sterile tap water (passed on activated carbon  
124 filter and a UV ramp). Only male individuals were kept, following Franceschi *et al.* (2008). Naturally  
125 parasitized individuals were not used. The amphipods were kept in collective tanks in batches of  
126 maximum 500 and fed *ad libitum* with conditioned dead elm leaves. The water was partially renewed  
127 regularly. Leaves were conditioned to develop a biofilm of micro-organisms, necessary for the nutrition of  
128 the gammarids (Bärlocher and Kendrick, 1975), by soaking autoclaved leaves in aerated lab water  
129 (therefore containing the micro-organisms present in the river) for about 7 days before distribution.  
130 Animals were thus acclimatized to experimental conditions for three weeks before the infestation  
131 procedure. This quarantine gave time for some *P. laevis* natural infections to develop since the  
132 amphipods may have ingested acanthocephalan eggs before their capture. However, since *P. tereticollis*  
133 grows more slowly than *P. laevis* (a data unknown before this work was undertaken, see results), it  
134 turned out that this quarantine was not long enough and some *P. tereticollis* natural infections emerged  
135 during our experiment. Fortunately, these natural infections could be detected before the experimental  
136 infections, allowing us to distinguish them from experimental ones, and were analysed separately (see  
137 results).

138

### 139 Sampling of parasite eggs and genotyping

140

141 Chubs (*Squalius cephalus*), definitive hosts in which *Pomphorhynchus* reproduce, were sampled by  
142 electrofishing in the Albane River, at the same stations where amphipods were captured (prefectural  
143 authorization of capture no. 162, February 13, 2020). The fish were euthanised in the laboratory with

144 eugenol (Chanseau *et al.*, 2002), dissected, and the female parasites were sampled from the digestive  
145 tract of the hosts. Chubs are preferential hosts for *P. laevis* but can also be infected by *P. tereticollis*  
146 (Perrot-Minnot *et al.*, 2019). Since no preferential host of *P. tereticollis* (such as *Barbus barbus*; Perrot-  
147 Minnot *et al.*, 2019) was collected during this campaign, the *P. tereticollis* used in this experiment were  
148 also collected from the sampled chubs.

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

157

## 158 **Exposure procedure**

159

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

165 Two infestation sessions were carried out one week apart, one using *P. laevis* parasites, the other  
166 with *P. tereticollis*. Gammarids were starved during the 72 hours preceding exposure. The exposure  
167 procedure described in Franceschi *et al.* (2008) was then followed. The exposed individuals were placed  
168 in pairs in a 60 ml crystallizing dish (in pairs, the gammarids ingest the leaves better than when they are  
169 isolated, unpublished data), in the presence of a piece of elm leaf of about 1 cm<sup>2</sup> on which a volume of  
170 suspension containing an average of 300 parasite eggs were deposited. This dose, higher than those used  
171 in our previous experiments, was a compromise between guaranteeing the success of the infection and  
172 avoiding a too high rate of multiple infections (Franceschi *et al.*, 2008), especially in experimental  
173 infections involving co-evolved partners (Franceschi *et al.*, 2010b; Bauer and Rigaud, 2015). Pieces of  
174 leaves without eggs were distributed to the control batches. After 48 hours, all individuals were placed  
175 individually in a crystallizing dish (60 ml) containing lab water and a piece of parasite-free elm leaf. In the  
176 *P. laevis* exposure session, 1107 amphipods of unknown genotype were used (1012 exposed individuals,  
177 95 negative controls). All gammarids were genotyped at the end of the experiment. After the removal of  
178 *G. pulex* and uncharacterized individuals, the analysis of *P. laevis* infection included 1054 *G. fossarum*

179 (965 exposed and 89 unexposed controls; see Table 1 and Supplementary table 1). Similarly,  
180 1079 individuals were used for the *P. tereticollis* exposure and 1001 *G. fossarum* were included in the  
181 analysis of *P. tereticollis* infection (911 exposed, 90 unexposed controls; Table 1 and Supplementary  
182 table 1).

### 183 **Gammarids maintenance and mortality survey**

184  
185  
186 Throughout the duration of the experiment, the crystallizing dishes were inspected daily, the water  
187 topped up and a piece of elm leaf added when the former had been eaten. Dead gammarids were  
188 measured (height of the 4th metacoxal pereion plate) and dissected to check their infection status.  
189 Dissected tissues were stored in absolute ethanol pending genotyping at the end of the experiment. Daily  
190 monitoring made it possible to genotype dead individuals before decay. Every two weeks, one  
191 *Chironomus plumosus* larva (Europrix brand frozen fish food cubes) was distributed in each crystallizing  
192 dish as a dietary supplement, improving survival in the laboratory (Labaude *et al.*, 2015), and 24 hours  
193 later the water was completely replaced.

194 Based on the *P. laevis* development time observed in our previous experiments (e.g. Franceschi *et al.*  
195 (2008); Labaude *et al.*, 2020), the gammarids were inspected under a binocular magnifier from 6 weeks  
196 post-exposure (Nikon SMZ 745) to check their parasitic status through the cuticle of their host.  
197 Individuals detected as infected were isolated. The growth of the parasites was then inspected twice a  
198 week. However, some parasites were visible before these 6 weeks (some of them few days after the  
199 beginning of the experiment, a development time much too short to correspond to experimental  
200 exposure, see results), corresponding to *P. tereticollis* natural infections (confirmed by genotyping). These  
201 animals were analysed separately.

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

### 209 210 **Gammarids & parasites genotyping**

211  
212 To genotype gammarids still alive at the end of the experiment, between two and four locomotor  
213 appendages were detached and placed in a tube filled with ethanol. For individuals who died during the

214 tracking, a larger amount of tissue was used (between one and three body segments) to make up for the  
215 quick degradation of DNA after death. To genotype adult parasites, a piece of about two mm<sup>3</sup> of body  
216 tissue was used to extract DNA. For the parasites at the acanthella stage, the whole individual was used.

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

220 For both parasites at the acanthella stage and amphipods, the gene encoding the first subunit of  
221 cytochrome oxidase (CO1) was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.*,  
222 1994). The total reaction volume (20 µL) consisted of 200 nM of each primer, 200 µM of dNTPs,  
223 0.25 U of DNA polymerase (HotStarTaq, Qiagen Inc., Düsseldorf, Germany), 1X of buffer, 5 µL of extracted  
224 DNA, and 10,5 µL ultrapure water. The following thermal treatment was applied: activation of the  
225 polymerase for 3 min at 95°C, then 39 cycles: denaturation for 20 s at 95°C, hybridization for 45 s at 40°C  
226 and synthesis for 1 min at 65°C, then finally 2 min at 65°C (final synthesis). Amplification success was  
227 checked by electrophoresis on 2% agarose gel and visualized with ethidium bromide stain. In case of  
228 failure of the CO1 amplification for parasites, the ITS region was amplified. The primers BD1f: 5'-GTC GTA  
229 ACA AGG TTT CCG TA-3' and ACITS1r: 5'-TTG CGA GCC AAG TGA TTC AC-3' were used (Franceschi *et al.*,  
230 2008). The reaction mixture was subjected to an initial denaturation at 95°C for 3 min, then 39 cycles at  
231 95°C (20 s), 50°C (45 s) and 65°C (45 s). Final elongation was performed for 5 minutes at 65°C.

232 Adult parasite species were identified by using a RFLP procedure. We first produced our own gene  
233 sequences by Sanger sequencing by Genewiz (Leipzig, Germany). The sequences were aligned using Mega  
234 software (V10; [www.megasoftware.net](http://www.megasoftware.net)) and compared to reference sequences (see Supplementary  
235 table 2). We then used the Sequence Manipulation Suite (SMS) software ([http://www.bioinformatics.org/sms2/rest\\_map.html](http://www.bioinformatics.org/sms2/rest_map.html))  
236 to establish that the restriction enzyme VspI (Thermo Scientific™) enables  
237 distinguishing between *P. laevis* (two restriction fragments of 60 and 620 bp) and *P. tereticollis* (two  
238 fragments of 450 and 240 bp). 10 U of enzyme were added to 1 µL of associated buffer and 5 µL of PCR  
239 product and adjusted with ultrapure water to a reaction volume of 15 µL. The mixture was then placed at  
240 37°C for 16 hours and then at 65°C for 20 minutes to inactivate the enzyme. The migration of 15 µL of  
241 RFLP product on a 2% agarose gel (for 20 min at 100 V), then visualization with ethidium bromide made it  
242 possible to determine the species of the selected samples. For all parasite acanthellae obtained during  
243 the survey, PCR products were sequenced and compared to reference sequences (Supplementary table  
244 2).

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

248 For logistic reasons, 108 gammarids were also typed by using a RFLP procedure developed from the  
249 analysis of these sequences using Mega and SMS softwares. A first reaction was carried out to distinguish



250 between the *G. fossarum* and *G. pulex* groups: the *VspI* enzyme (Thermo Scientific™) cuts the DNA of  
251 *G. pulex* into two strands of approximately 200 and 450 base pairs. The *G. fossarum* amplicons were then  
252 subjected to a second RFLP with the *DraI* enzyme (Thermo Scientific™), to discriminate between the two  
253 groups *Gf* 1-2-3 and *Gf* 6-7 (species groups identified by Lagrue *et al.*, 2014). The PCR products of *Gf* 6  
254 and *Gf* 7 are cut into two strands of approximately 450 and 200 base pairs, while the enzyme does not  
255 cut the PCR products of *Gf* 1-2-3. The restriction protocol was the same as detailed before.

256 All gammarid sequences were submitted to BOLD, in the 'GAMEI' project  
257 ([https://v4.boldsystems.org/  
258 index.php/MAS\\_Management\\_DataConsole?codes=GAMEI](https://v4.boldsystems.org/index.php/MAS_Management_DataConsole?codes=GAMEI)).

259

## 260 **Statistical analyses**

261

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

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

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

295  
296

## 297 Results

298 Apart from *G. roeselii*, which were not taken into account in this experiment (see Bauer and Rigaud,  
 299 2015, for their analysis), and too few *G. pulex* to be integrated in the analyses (Supplementary table 2),  
 300 three *G. fossarum* host lineages have been found in the gammarids of the Albane River. Forty-eight *Gf 7*  
 301 individuals were detected by sequencing. Since they are genetically very close to the *Gf 6* MOTU and  
 302 belong to the same breeding group (Lagrue *et al.*, 2014), they were referred as *Gf 6* in the dataset and  
 303 the following analyses. The genotypes of all hosts were obtained only at the end of the experiments,  
 304 which explains why it was impossible to restore any imbalances in numbers. The dataset is summarized in  
 305 Table 1.

306

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

<i>P. laevis</i> exposure						
MOTU	controls		exposed			total
	early death	uninfected	early death	infected	eni	
<i>Gf 2</i>	7 (1+1)	18	33	14 (7)	443 (3+23)	515
<i>Gf 6</i>	12	52	58	9 (1+1)	408	539

<i>P. tereticollis</i> exposure						
MOTU	controls		exposed			total
	early death	uninfected	early death	infected	eni	
<i>Gf 2</i>	25 (2)	23	119 (1+11+6)	28 (1)	236 (2+6)	431

Gf 6	30 (1)	12	147 (1+2)	9	372	570
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310 *Gf 2 = G. fossarum* MOTU 2 ; *Gf 6 = G. fossarum* MOTU 6. *Controls*: gammarids not exposed to  
 311 parasite eggs. *Exposed*: gammarids experimentally exposed to parasite eggs. *Early death*:  
 312 gammarids that died before the date of detection of the first acanthella. *Eni (exposed not infected)*:  
 313 exposed gammarids that did not develop any infection.

314

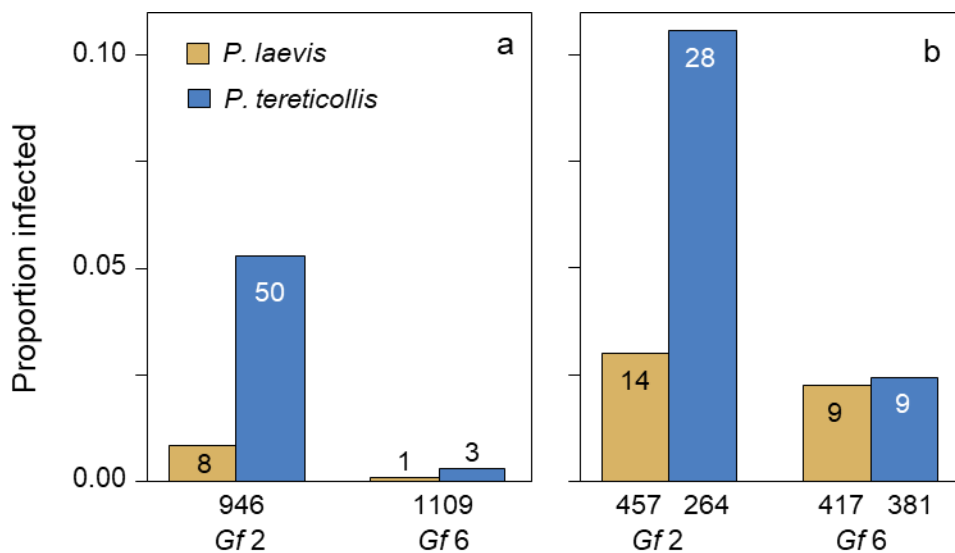
315 **Infection rates**

316 *Natural infections*

317

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

324



325

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

330

331 *Experimental infections*

332

333 **Infection success: prevalence and intensity**

334

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

339

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

348

#### 349 **Development duration of parasites**

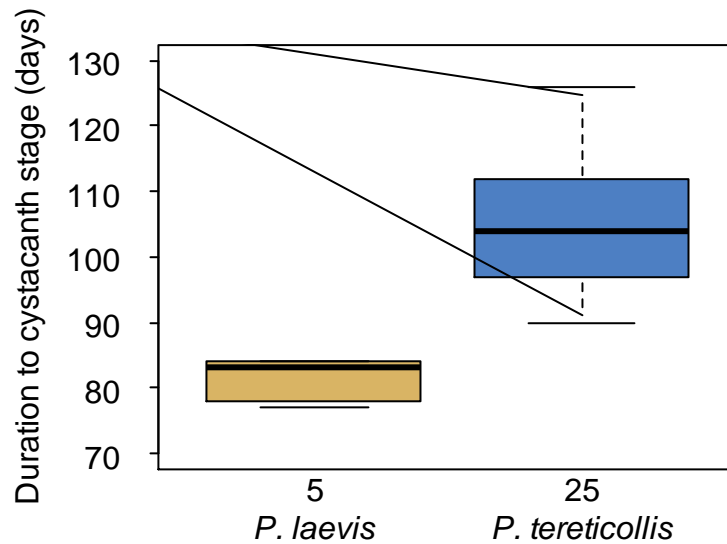
350

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

354

355 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  
356 neither found a difference between development duration according to host lineage. However, it took  
357 significantly less time for *P. laevis* to reach the cystacanth stage (81 days on average) than for  
358 *P. tereticollis* (106 days), and the variation between early and late cystacanths differentiation was found  
359 to be larger in this latter species (Figure 2).

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365

**Figure 2** - Development duration of parasites in the two species until the cystacanth stage, in days post-exposure. 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.

366

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#### Probability of natural and experimental coinfections

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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.

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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.

383

#### Parasite virulence

384

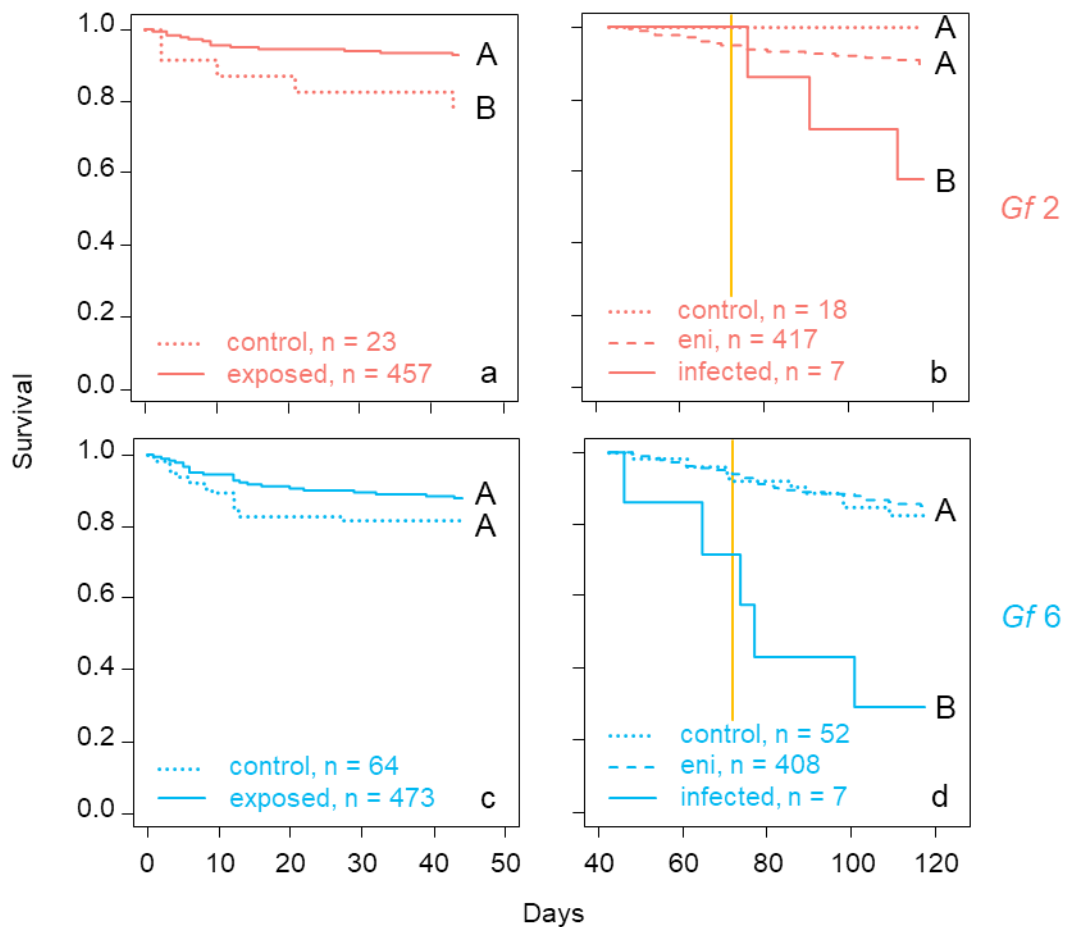
*P. laevis*-exposed individuals

385

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

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

395  
 396



397

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

404

405 *P. tereticollis*-exposed individuals

406

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

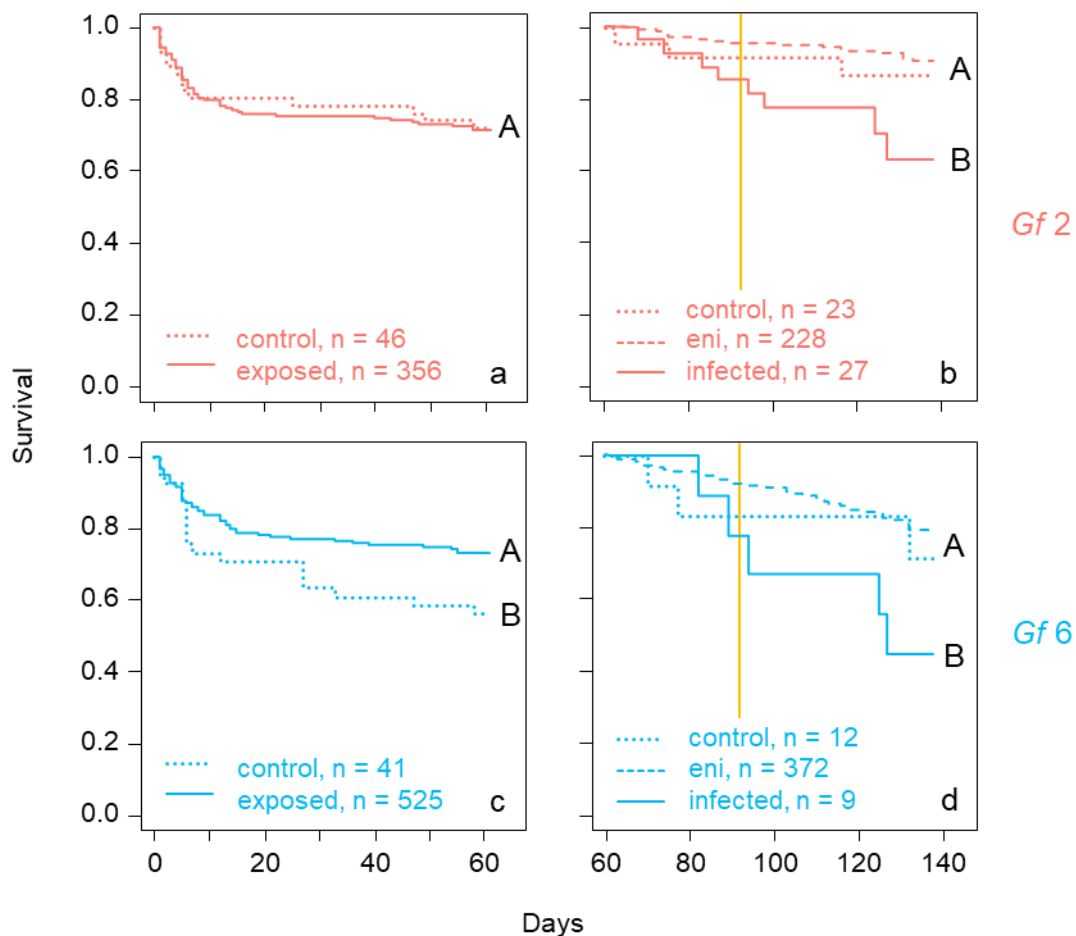
412

413

414

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

418



419

420 **Figure 4** - Survival of *P. tereticollis* exposed gammarid MOTUs (plain lines) and controls (dotted  
421 lines), before the first acanthella detection date (a,b). Survival of *P. tereticollis* exposed gammarid  
422 MOTUs and controls (dotted lines), after the first acanthella detection date (c,d). Infected  
423 individuals (plain lines) can now be distinguished from exposed but not infected ones ('eni', in

424 dashed lines). Letters on the right of the graphs indicate the statistical groups. The vertical orange  
425 lines denote the first cystacanth detection date.

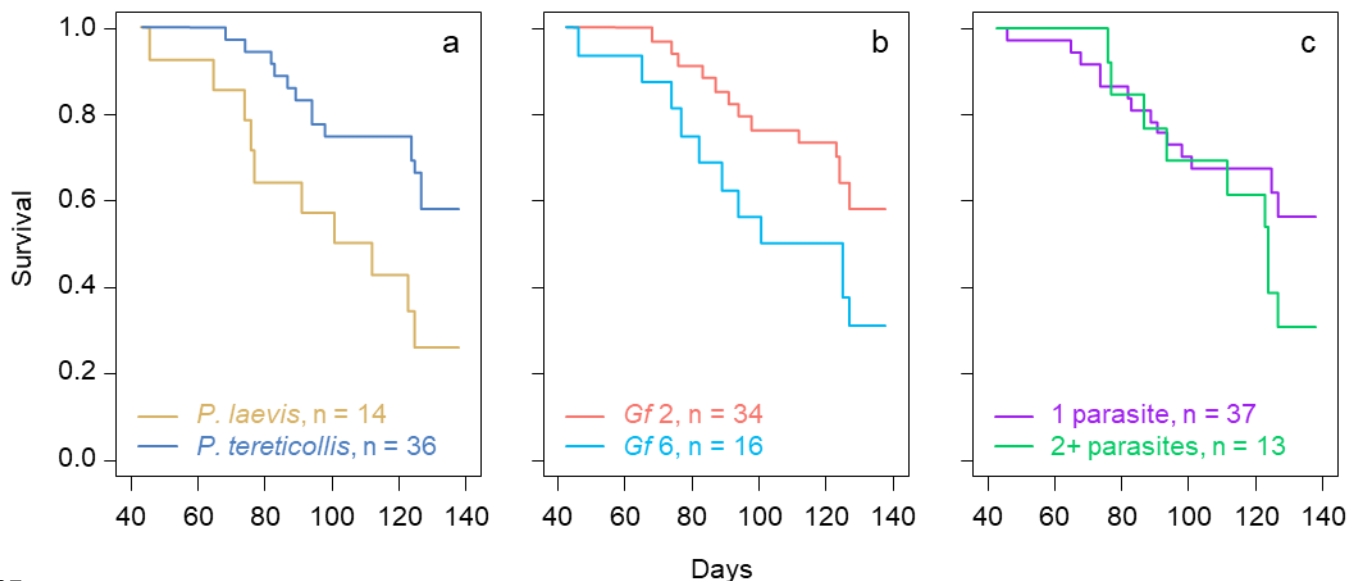
426

### 427 *Survival in infected gammarids*

428

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

436



437

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

440

441

## 442 Discussion

443

### 444 Susceptibility to infections by different parasite species in different host lineages

445



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

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

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

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

469 Despite low numbers that limited statistical analyses, it is worth noting that experimental infections  
470 also suggest that *G. pulex* from Albane River were particularly sensitive to *P. laevis* compared to *Gf 2* and  
471 *Gf 6*, but not to *P. tereticollis* (Supplementary table 1 and Supplementary Figure 2). The *G. pulex* of the  
472 Suzon population also showed the same pattern (Supplementary figure 1), confirming the field  
473 observations of Harris (2020). Because they appear to be contradicting observations by Galipaud *et al.*  
474 (2017), who found low *in natura* prevalence of *P. laevis* cystacanths in *G. pulex*, our results need  
475 experimental confirmation with a larger number of individuals. However, as showed by Bauer & Rigaud  
476 (2015), a low prevalence of cystacanths found after direct collection in nature could simply mean that  
477 either parasite virulence is high, or that the parasite increased so much the predation rate of its host that  
478 parasitized individuals disappear rapidly from populations. Here, our measures of prevalence were made  
479 after quarantine or after experimental infection, i.e. in conditions where predation was absent or the  
480 survival controlled, highlighting the usefulness of experimental infections in deciphering host-parasite  
481 relationships (Poulin and Maure, 2015). This is particularly true for acanthocephalans, where the

482 prevalence *in natura* reflects a combination of several processes due to the long intra-host development  
483 time (Bauer and Rigaud, 2015). One of these processes is parasite virulence that we will discuss  
484 thereafter.

#### 485 **Natural and experimental coinfections**

486

487 Natural infection by *P. tereticollis* favours a further interspecific experimental infection by *P. laevis*.  
488 Contrastingly, natural infection by *P. tereticollis* did not influence intraspecific experimental  
489 superinfections by *P. tereticollis*, and natural infection by *P. laevis* did not influence either a secondary  
490 infection by another acanthocephalan. However, sample size were sometimes so low that the statistical  
491 power of these latter analyses are questionable. Cornet *et al.* (2009) showed that the level of immune  
492 defences of *G. pulex* is strongly depressed by the acanthocephalans *P. laevis* and *P. tereticollis*, and that  
493 acanthocephalan infection favors superinfection by *Escherichia coli* bacteria. However, Dianne *et al.*  
494 (2010), using successive experimental infections by *P. laevis*, did not observed any increase of  
495 acanthocephalan intraspecific superinfection. It is therefore possible that the immune depression only  
496 favours interspecific parasite/pathogens superinfections but not intraspecific ones, suggesting that this  
497 immune depression is a costly by-product of the acanthocephalan infections. Since multiple infections are  
498 the rule in nature, further experimental investigations are welcome, particularly for the study of the  
499 evolution of virulence (Alizon *et al.*, 2013).

500

#### 501 **Development duration of parasites**

502

503 Acanthocephalans development time in their intermediate hosts is strongly influenced by the  
504 environment (Awachie, 1966; Lackie, 1972; Tokeson and Holmes, 1982; Bratney, 1986; Labaude *et al.*,  
505 2020), highlighting the necessity of studying this life-history trait under controlled conditions. The  
506 development duration of *Polymorphus minutus* and *P. laevis* under laboratory conditions have already  
507 been studied (Hynes and Nicholas, 1957; Butterworth, 1969; Franceschi *et al.*, 2008; Labaude *et al.*, 2015,  
508 2020), but *P. tereticollis* development was not documented before this study. The development time in  
509 *P. laevis* observed here was similar to the one found by Labaude *et al.* (2020) at a similar temperature.  
510 The development of *P. tereticollis* took 23 % longer than *P. laevis* under the same conditions. The among-  
511 host variation in maturation time (time between the appearance of the first and the last cystacanths) was  
512 also larger and more variable than that of *P. laevis*. This longer development time explains *a posteriori*  
513 why we observed so many natural *P. tereticollis* infections after the quarantine we imposed to  
514 gammarids, since the quarantine time was estimated from our previous experience on *P. laevis*  
515 development.

516

517 **Virulence**

518

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

524 Ingestion of parasites (both *P. laevis* and *P. tereticollis*) and the early beginning of larval development  
525 did not negatively affect the host survival. Therefore, the wounds inflicted when the acanthors pierce the  
526 digestive tract of the arthropod to pass into the general cavity, and/or a possible early resistance to  
527 infection (cellular response, humoral and immune encapsulation (Crompton and Nickol, 1985) were not  
528 costly enough to increase mortality. The literature is contradictory about the host survival in early stages  
529 of infection by acanthocephalans. Increased mortality shortly after acanthocephalan experimental  
530 exposure was observed by Crompton & Nickol (1985) and Hynes & Nicholas (1957), but these studies  
531 were most likely carried out with massive doses of eggs. Although the dose used in these works is not  
532 mentioned, the high prevalence reported and the known dose-dependant prevalence relation  
533 (Franceschi *et al.*, 2008), the high dose exposure hypothesis is reasonable to consider. In such cases, the  
534 damages inflicted by many acanthors crossing the digestive tract wall may have been deleterious. On the  
535 other hand, Uznanski & Nickol (1980) did not find any increased mortality in the first 24 hours post-  
536 exposure in *Hyalella azteca* exposed to *Leptorhynchoides thecatus*. Yet, here again, the high infection  
537 success suggested the usage of high doses of eggs. Even more intriguing, experimental infections of  
538 *Asellus* by *Acanthocephalus lucii* studied by Bratley (1986) and Benesh & Valtonen (2007) induced early  
539 increased mortalities, whereas Hasu *et al.* (2006) found a better survival of infected individuals on the  
540 same biological models. This latter and somewhat intriguing result is what we observed in the present  
541 study: gammarids exposed to parasite eggs showed improved survival in the early days post-exposure,  
542 i.e. before the infection can be detected. Only speculative discussions on these observations can be  
543 proposed because it was not possible to ensure whether individuals dying early in the survey had been  
544 infected or not. At least, we can exclude a selective mortality due to exposure. One of the possible  
545 explanations is that the observed improved survival of exposed gammarids could be part of 'parasite  
546 manipulation', where the parasite would protect its host from predation, before becoming infective to  
547 the next host (Parker *et al.*, 2009). Such a 'protection' was described for *P. laevis* in the late acanthella  
548 stage (Dianne *et al.*, 2011; Rigaud *et al.*, 2023), but this was a protection against mortality due to  
549 predation, which is not the case here. A mechanistic explanation of such a phenomenon could be that the  
550 early stages of the parasite divert some of the host energy in such a way as to improve the host survival.  
551 Castrating parasites are known to divert energy normally allocated to host reproduction to their own  
552 purpose, in particular for the survival of the host they infect (Benesh and Valtonen, 2007). Gammarid

553 acanthocephalans are known to castrate their hosts, partially (Bollache *et al.*, 2002) or totally (Bollache *et*  
554 *al.*, 2002; Bailly *et al.*, 2018). It is not known precisely at what stage of parasite growth castration occurs  
555 in *Pomphorhynchus*, but it happens early during *Polymorphus* ontogeny, at least before the acanthellae  
556 are visible in their host (Bailly *et al.*, 2018). Alternatively, egg parasites eaten by gammarids, but unable  
557 to develop, can themselves become a form of diet supplementation for the hosts that 'prey' on them  
558 (Goedknecht *et al.*, 2012), improving host survival. Finally, perhaps the presence of eggs in the water  
559 triggers protective mechanisms (e.g., heat shock proteins, mechanisms against oxidative stress) that help  
560 the host sustain stress in the lab.

561 Parasite virulence was expressed in our study when the acanthellae enter their exponential phase of  
562 growth. It is interesting to note that *Gf6* was more sensitive to *Pomphorhynchus* infections than *Gf2*,  
563 whatever the parasite species. In *Hyalella azteca* infected by *Corynosoma constrictum*, a higher mortality  
564 was observed at the time of rapid development of the parasite larvae, only in heavily infected hosts  
565 (Duclos *et al.*, 2006). This increased mortality associated with acanthocephalan infections is interpreted  
566 as being due to displacement of the host's organs (Bentley and Hurd, 1993; Dezfuli *et al.*, 2008), or effects  
567 on metabolism (Rumpus and Kennedy, 1974). In our study, the energy requirements of the two parasites  
568 are probably different, since *P. tereticollis* larvae are larger than *P. laevis* (Perrot-Minnot, 2004).  
569 However, the virulence of *P. laevis* was higher than that of *P. tereticollis*. An effect on metabolism would  
570 therefore only be possible if one considers that *P. laevis* grows faster than *P. tereticollis*, which could  
571 exhaust the host's metabolism at higher intensity. If so, this would be in line with the 'boom-bust' and  
572 'slow and steady' strategies proposed by Le Clec'h *et al.* (2019) for schistosome infections. Studies  
573 looking at metabolism of infected gammarids have only been carried out with parasites at cystacanth  
574 stages (Plaistow *et al.*, 2001; Gismondi *et al.*, 2012; Korkofigas *et al.*, 2016). All these studies showed that  
575 parasitized amphipods are deprived of lipid and protein reserves, while they have higher glycogen rates.  
576 This shows high demand for immediate energy (glycogen) in infected amphipods, which are forced to  
577 draw on their reserves (lipids), probably at the benefit of the parasite metabolism. Such a strategy is  
578 common in parasites. In schistosomes, such effects on the host fitness and energy diversion occur both  
579 early and in the late development of the parasite (De Jong-Brink *et al.*, 2001). In *Plasmodium*, the intra-  
580 host competition in cases of multiple infections is leading to increased energetic demand due to host  
581 overexploitation by the faster-developing parasites (De Roode *et al.*, 2005). Differences in survival  
582 between *Gf2* and *Gf6* hosts may be due to differences in metabolism, a hypothesis that necessitate  
583 further investigations. Another possible explanation for a differential in survival between host lineages  
584 could be differences in the ability of acanthocephalans to reduce the intensity of the immune responses  
585 of their hosts (Cornet *et al.*, 2009a). On the one hand, this partial immunosuppression might be  
586 advantageous for the host, owing the cost of mounting an immune response, either in terms of  
587 physiology (Moret and Schmid-Hempel, 2000) or because of immune cytotoxicity of enzymes used for the  
588 invertebrate immune responses (Sadd and Siva-Jothy, 2006). On the other hand, as discussed before, this

589 immunosuppression can make hosts more susceptible to other infections, such as bacterial infections  
590 (Cornet *et al.*, 2009a; Cornet and Sorci, 2010). This immune modification is not observed in all  
591 gammarids: infections of various populations of *G. roeselii* by *P. laevis* do not show immunosuppression,  
592 with some populations even showing an immune response to the infection (Moret *et al.*, 2007). In  
593 addition, the level of immune defenses are variable among gammarid populations (Cornet *et al.*, 2009b).  
594 Therefore, *Gf 2* and *Gf 6* could be immunosuppressed differently, a hypothesis that remains to be tested.

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

## 598 **General conclusion**

599

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

610

611

612

## Appendices

613

### 614 Supplementary table 1 – numbers of all gammarid MOTUs and not genotyped individuals sampled

615 Numbers of gammarids sampled in the Albane River, according to their infection status, including  
 616 individuals with natural infections in brackets (by parasite species: *P. laevis* in light brown,  
 617 *P. tereticollis* in blue, and undetermined in black ; & for a mixed *P. laevis* and *P. tereticollis*  
 618 infection). Undetermined gammarids could not be genotyped.

<i>P. laevis</i> exposure						
MOTU	controls		exposed			total
	early death	uninfected	early death	infected	eni	
<i>Gf</i> 2	7 (1+1)	18	33	14 (7)	443 (3+23)	515
<i>Gf</i> 6	12	52	58	9 (1+1)	408	539
<i>Gp</i> D	1	0	1	2	6	10
undetermined	1	4	4	0	34	43

<i>P. tereticollis</i> exposure						
MOTU	controls		exposed			total
	early death	uninfected	early death	infected	eni	
<i>Gf</i> 2	25 (2)	23	119 (1+11+6)	28 (1)	236 (2+6)	431
<i>Gf</i> 6	30 (1)	12	147 (1+2)	9	372	570
<i>Gp</i> D	0	0	19	0	10	29
undetermined	3	2 (1)	22 (1&1+1)	0	22 (1)	49

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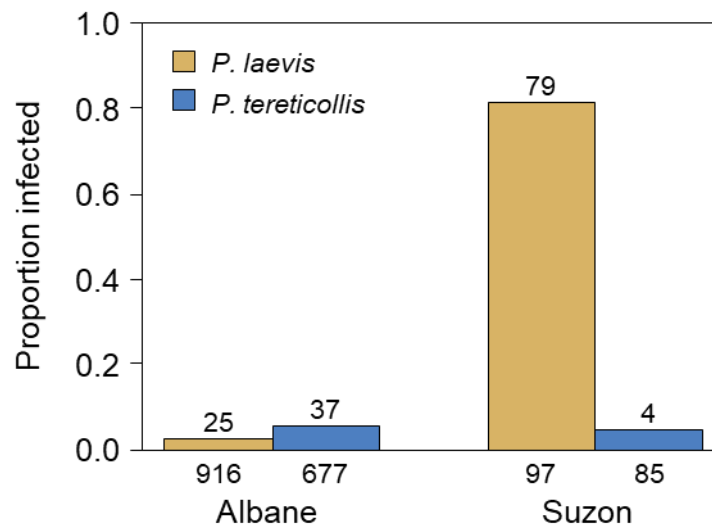
### 622 Supplementary table 2

623 Accession numbers of the reference sequences used to assign the gammarid MOTUs and the  
 624 parasite species

gammarids		parasites	
MOTU	GenBank	species	GenBank
<i>G. fossarum</i> 1	MT411346.1	<i>P. minutus</i> CO1	EF467865.1
<i>G. fossarum</i> 2	MT978910.1	<i>P. minutus</i> ITS	JF803287.1
<i>G. fossarum</i> 3	MT411291.1	<i>P. minutus</i> ITS	AY532067.1
<i>G. fossarum</i> 4	MT411024.1	<i>P. laevis</i> CO1	EF051071.1
<i>G. fossarum</i> 5	MT411035.1	<i>P. laevis</i> ITS	AY135417.1
<i>G. fossarum</i> 6	MT980277.1	<i>P. laevis</i> ITS	AY135415.1
<i>G. fossarum</i> 7	MT411467.1	<i>P. tereticollis</i> CO1	AY423351.1
<i>G. pulex</i> A	Wattier, pers. com.	<i>P. tereticollis</i> ITS	KY075817.1
<i>G. pulex</i> B	Wattier, pers. com.	<i>P. tereticollis</i> ITS	LN995058.1
<i>G. pulex</i> C	Wattier, pers. com.		
<i>G. pulex</i> D	Wattier, pers. com.		

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627

**Supplementary figure 1 - Control of the infectivity of parasites using gammarids from Val Suzon**



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629 Success of laboratory infections by *P. laevis* and *P. tereticollis*, in gammarids (all MOTUs together)  
630 coming from the two rivers Albane and Suzon. Numbers of infected gammarids are given above the  
631 bars, and total sample sizes from each river below the bars.

632 Even if the success of infection by *P. laevis* was low for gammarids from Albane (all MOTUs  
633 combined), the high prevalence found in gammarids from the Suzon River showed that the  
634 parasites had actually potential for infection. In Suzon gammarids, a high *P. laevis* load was also  
635 observed, with an average load of 4.5 parasites per infected hosts (up to 15 parasites in a single  
636 individual).

637 Only 4 individuals from Suzon developed a *P. tereticollis* infection (two with a single parasite and  
638 the other with three). The prevalence of *P. tereticollis* was similar among gammarids from the two  
639 rivers ( $\chi^2 = 0.001$ , 1 df,  $p = 0.970$ ).

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642 **Supplementary table 3 - Analysis of parasite prevalence**

643 Results of the GLM analysing the prevalence of natural prevalence and the infection success of  
644 parasites after experimental exposure, as a function of gammarid MOTUs, parasite species and  
645 their interactions.

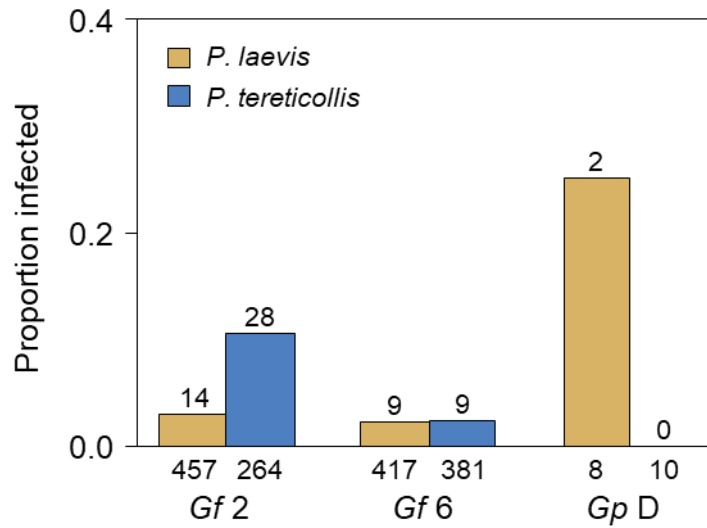
	Source	estimate	± s.e.	z	df	p
Natural prevalence	MOTU: <i>Gf</i> 2 vs. <i>Gf</i> 6	-2.251	1.061	-2.120	1	0.034
	Parasite species: <i>P. laevis</i> vs <i>P. tereticollis</i>	1.879	0.384	4.897	1	<0.001
	Interaction MOTU × parasite species	-0.778	1.217	-0.639	1	0.523
Infection success	MOTU: <i>Gf</i> 2 vs. <i>Gf</i> 6	-0.36	0.433	-0.831	1	0.406
	Parasite species: <i>P. laevis</i> vs <i>P. tereticollis</i>	1.323	0.337	3.924	1	<0.001
	Interaction MOTU × parasite species	-1.230	0.584	-2.107	1	0.035

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**Supplementary figure 2 - Success of experimental infections in Gp D**



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Success of experimental infections by the two parasite species according to the host lineages present in the Albane River. Numbers of infected gammarids are given above the bars, and total sample sizes for each MOTU are below the bars. Despite the low sample size, Gp D was found significantly more susceptible than both *G. fossarum* lineages to infection by *P. laevis* (Fisher's exact test between Gf 2 and Gp D:  $p = 0.028$ ; Gf 6 and Gp D:  $p = 0.016$ ), whereas no experimental infection by *P. tereticollis* was detected.

**Supplementary table 4 – Parasite development duration to the cystacanth stage**

662 Results of the GLM analysing the development duration of parasites as a function of parasite  
663 species, gammarid MOTUs and number of hosted parasites. The interaction between factors were  
664 not significant and removed from the model.

Source	estimate	± s.e.	z	df	p
Parasite species: <i>P. laevis</i> vs. <i>P. tereticollis</i>	0.253	0.057	4.462	1	<0.001
MOTU: <i>Gf 2</i> vs. <i>Gf 6</i>	-0.036	0.046	-0.779	1	0.436
Parasite number: 1 vs. 2	-0.038	0.051	-0.734	1	0.463

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668 **Supplementary table 5 - Statistics of the survival analysis for gammarids exposed to *P. laevis***

669 Results of the Cox models analysing the survival of gammarids exposed to *P. laevis* as a function of  
 670 gammarid lineages and parasite treatment, divided according to the parasite development stage  
 671 (before the first acanthella detection date [1st part] and after this date [2nd part]). The interaction  
 672 between factors was not significant, and was removed from the model. Global risks proportionality:  
 673  $\chi^2 = 0.821$ , 2 df,  $p = 0.66$  for the first part;  $\chi^2 = 1.558$ , 3 df,  $p = 0.67$  for the second part.

Development stage	Comparison	$\beta$	odd ratio	$\pm$ s.e.	z	df	p
1st part	MOTU: Gf 2 vs. Gf 6	0.463	1.589	0.204	2.273	1	0.023
	Treatment: exposed vs. control	-0.655	0.519	0.267	-2.453	1	0.014
2nd part	MOTU: Gf 2 vs. Gf 6	0.528	1.695	0.184	2.869	1	0.004
	Treatment: global						<0.001
	- control vs. 'eni'	0.103	1.108	0.349	0.294	1	0.768
	- control vs. infected	-1.912	0.148	0.489	-3.906	1	<0.001
	- 'eni' vs. infected	-1.809	0.164	0.368	-4.917	1	<0.001

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677 **Supplementary table 6 - Statistics of the survival analysis for gammarids exposed to *P. tereticollis***

678 Results of the Cox model analysing the survival of gammarids exposed to *P. tereticollis* as a function of  
 679 gammarid MOTUs and parasite treatment, divided according to the parasite development stage  
 680 (before the first acanthella detection date [1st part] and after this date [2nd part]). The interaction  
 681 between factors was not significant, and was removed from the model. Global risks proportionality:  
 682  $\chi^2 = 4.47$ , 2 df,  $p = 0.107$  for the first part;  $\chi^2 = 1.717$ , 3 df,  $p = 0.63$  for the second part.

Development stage	Comparison	$\beta$	odd ratio	$\pm$ s.e.	z	df	p
1st part	MOTU: Gf 2 vs. Gf 6	-0.017	0.983	0.123	-0.138	1	0.809
	Treatment: exposed vs. control	-0.297	0.743	0.192	-1.552	1	0.121
2nd part	MOTU: Gf 2 vs. Gf 6	0.777	2.175	0.209	3.712	1	<0.001
	Treatment: global						<0.001
	- control vs. 'eni'	-0.383	0.682	0.424	-0.904	1	0.366
	- control vs. infected	0.982	2.669	0.484	2.029	1	0.042
	- 'eni' vs. infected	-1.365	0.255	0.288	-4.738	1	<0.001

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687 **Supplementary table 7 - Model selection table for the survival analysis of infected gammarids**

688 Model selection table for the survival analysis of infected gammarids (MuMin R package). Factors  
 689 compared : intensity of infection (nb), host MOTU (motu), parasite species (para) and their first  
 690 order interactions.

Factors						Analysis parameters					
nb	motu	para	nb × motu	nb × para	motu × para	adj R <sup>2</sup>	df	logLik	AICc	delta	weight
+	+	+				0.348	3	-84.9	179.9	0.00	0.163
	+	+				0.267	2	-86.3	177.2	0.31	0.140
		+				0.194	1	-87.5	177.2	0.33	0.139
+		+				0.256	2	-86.5	177.5	0.69	0.116
+	+	+		+		0.367	4	-84.5	179.0	2.13	0.056
+	+					0.212	2	-87.2	179.0	2.15	0.056
	+					0.131	1	-88.4	179.1	2.43	0.049
+	+	+			+	0.359	4	-86.2	179.6	2.71	0.042
	+	+			+	0.273	3	-84.8	179.7	2.80	0.040
+	+	+	+			0.349	4	-84.8	179.7	2.80	0.040
+		+		+		0.265	3	-86.4	179.8	3.00	0.036
						0.000	0	-90.2	180.4	3.54	0.028
+						0.067	1	-89.3	180.8	3.99	0.022
+	+	+		+	+	0.389	5	-84.0	181.2	4.39	0.018
+	+		+			0.212	3	-87.2	181.6	4.74	0.015
+	+	+	+	+		0.368	5	-84.5	181.1	5.23	0.012
+	+	+	+		+	0.362	5	-84.6	182.3	5.48	0.011
+	+	+	+	+	+	0.390	6	-84.0	184.7	7.87	0.003

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694 **Supplementary table 8 - Statistics of the survival analysis for infected gammarids**

695 Results of the Cox model analysing the survival of infected gammarids as a function of parasite  
 696 species, gammarid MOTUs and intensity of infection, after the first *P. laevis acanthella* detection  
 697 date. The interaction between factors were not significant and removed from the model. Global  
 698 risks proportionality:  $\chi^2 = 3.085$ , 3 df,  $p = 0.38$ .

Source	$\beta$	odd ratio	$\pm$ s.e.	z	df	p
Parasite species	-0.958	0.384	0.422	-2.268	1	0.023
MOTU: Gf 2 vs. Gf 6	0.773	2.166	0.416	1.856	1	0.063
Parasite number: 1, $\geq 2$	0.767	2.125	0.433	1.769	1	0.077

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### **Authors contributions**

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704 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  
705 the experiments. A.B., N.D. and M.T. contributed to sample preparation. A.B. analyzed the data. A.B. and  
706 T.R. contributed to the interpretation of the results. A.B. took the lead in writing the manuscript. All  
707 authors provided critical feedback and helped shape the research, analysis and manuscript.

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716 The dataset (in French) and data description file explaining the variables are available on the  
717 data.InDoRES repository: <https://data.indores.fr/dataset.xhtml?persistentId=doi:10.48579/PRO/X9ZRVB>.

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### **Conflict of interest disclosure**

721 The authors declare that they comply with the PCI rule of having no financial conflicts of interest in  
722 relation to the content of the article.

723 TR is recommender for PCI infections.

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