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# Experimental infections reveal differences in specificity, development time and virulence between the acanthocephalan parasite *Pomphorhynchus tereticollis* and its sympatric counterpart *P. laevis*, in two cryptic species of *Gammarus fossarum*

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1 **Experimental infections reveal differences in**  
2 **specificity, development time and virulence**  
3 **between the acanthocephalan parasite**  
4 ***Pomphorhynchus tereticollis* and its**  
5 **sympatric counterpart *P. laevis*, in two**  
6 **cryptic species of *Gammarus fossarum***  
7

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16

17 **ABSTRACT**

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

37  
38 **Keywords:** experimental infection, *Gammarus fossarum*, *Gammarus pulex*, host specificity,  
39 infectivity, multi-host parasite, *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis*, prevalence  
40

Multi-host parasites are facing hosts of different qualities, which can contribute unequally to their transmission (Rigaud *et al.*, 2010). Key hosts, contributing much more than the others, are often characterized by high abundance, susceptibility to infection / exposure, 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, the way of how 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, the cryptic diversity 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 considered as generalists, have a narrower host spectrum at the light of the recently revealed genetic divergence (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). The reverse is true from a host perspective. Here we will focus on the effect of cryptic diversity on host-parasite relationships in European gammarids. These freshwater amphipods are now a model for studying cryptic diversity. *Gammarus fossarum*, *G. balcanicus* and *G. roeselii*, in particular, showed high numbers of cryptic lineages that are genetically divergent enough to be considered as different species while morphologically homogenous (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 at the light of these high 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* lineages. The lineage *Gf*-III (defined by Lagrue *et al.*, 2014) showed higher prevalence of *P. tereticollis* than all other lineages and *Gf*-II a higher prevalence of *P. laevis*. The *Gf*-VII host lineage 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 susceptibility to infection, thus reflecting specificity as commonly accepted (Poulin and 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); (iii) a difference in behavioural manipulation leading to a difference in predation rates. Indeed acanthocephalan parasites use gammarids as intermediate hosts, and change their antipredator behaviour in a way that increase the probability of predation, and thus the probability of transmission to the final host (Moore, 1983; Moore and 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), only experimental infections can allow us to fully disentangle the causes of the differences in prevalence observed in nature, provided the experiments are ecologically relevant. For example, Bauer & Rigaud (2015) showed with 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 (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 the parasites express either differential virulence or differential manipulation depending on the hosts they infect, but this field study does not allow disentangling these two causes of mortality.

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

## 109 Methods

### 110 Gammarids sampling

111  
112 The gammarids were collected at the beginning of January 2020 in the Albane river, between  
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), which will be referenced to as 'Suzon' below. This last population, consisting at c.a. 97% of  
116 *Gammarus pulex* (Labaude *et al.*, 2015), has repeatedly been shown to be particularly sensitive to  
117 infection by *P. laevis* (Cornet *et al.*, 2009a; b; Franceschi *et al.*, 2010a; Dianne *et al.*, 2011, 2012, 2014;  
118 Bauer and Rigaud, 2015; Labaude *et al.*, 2020). It therefore served as a control for parasite "quality" in  
119 the present experiment.

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 is made with a mix of waters from the rivers of origin of the amphipods,  
123 diluted with dechlorinated and sterile tap water (passed on activated carbon filter and a UV ramp). Only  
124 male individuals were kept, following Franceschi *et al.*, (2008). Naturally parasitized individuals were not  
125 used. The amphipod males were kept in collective tanks in batches of 500 maximum and fed *ad libitum*  
126 with conditioned dead elm leaves. Leaves were conditioned to develop a biofilm of micro-organisms,  
127 necessary for the nutrition of the gammarids (Bärlocher and Kendrick, 1975), by soaking autoclaved  
128 leaves in aerated lab water (therefore containing the micro-organisms present in the river) for about 7  
129 days before distribution. Animals were thus acclimatized to the conditions of the experiment for three  
130 weeks before the infestation procedure. This quarantine gave time for some *P. laevis* natural infections to  
131 develop since the amphipods may have ingested acanthocephalan eggs in the river before their capture.  
132 However, since *P. tereticollis* grow more slowly than *P. laevis* (data to which we did not have access  
133 before our experiment, see results), this quarantine was not long enough for this species and some  
134 *P. tereticollis* natural infections emerged during our experiment. Fortunately, these natural infections  
135 were visible much before the experimental infections, allowing us to distinguish them from experimental  
136 ones, and were analyzed separately (see results).

### 138 Sampling of parasite eggs and genotyping

139  
140 Chubs (*Squalius cephalus*), definitive hosts in which *Pomphorhynchus* reproduce, were sampled by  
141 electrofishing in the Albane River, at the same stations as the amphipods (prefectural authorization of  
142 capture no. 162, February 13, 2020). The fish were euthanized in the laboratory with eugenol (Chanseau  
143 *et al.*, 2002), dissected, and the female parasites were sampled from the digestive tract of the hosts.

144 Since no preferential host of *P. tereticollis* (such as *Barbus barbus*; Perrot-Minnot *et al.*, 2019) was  
145 collected during this campaign, the parasites of this species used in this experiment were also collected  
146 from the sampled chubs. Chubs are preferential hosts for *Pomphorhynchus laevis* but can also be infected  
147 by *P. tereticollis* (Perrot-Minnot *et al.*, 2019). It should be noted that this infestation by *P. tereticollis* is, to  
148 our knowledge, the first experimental laboratory infection attempt with this species.

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 used only batches containing  
152 at least 50 % mature eggs (where developed acanthors were clearly visible) in high density. The bodies of  
153 the parasites from which the eggs were obtained were preserved in microtubes containing absolute  
154 ethanol for genotyping (see genotyping paragraph below). Samples that failed molecular identification  
155 were not selected for further experimentation.

156

### 157 **Exposure procedure**

158

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

164 Two infestation sessions were carried out one week apart, one using *P. laevis* parasites, the other  
165 with *P. tereticollis*. Gammarids were starved during the 72 hours preceding the exposure. The exposure  
166 procedure described in Franceschi *et al.* (2008) was then followed. The individuals were placed in pairs in  
167 a 60 ml crystallizer (in pairs, the gammarids ingest the leaves better than when they are isolated,  
168 unpublished data), in the presence of a piece of elm leaf of about 1 cm<sup>2</sup> on which a volume of suspension  
169 containing an average of 300 parasite eggs were deposited. This dose, higher than those used in our  
170 previous experiments, was a compromise between guaranteeing the success of the infection and  
171 avoiding a too high rate of multiple infections (Franceschi *et al.*, 2008), especially in experimental  
172 infections involving co-evolved partners (Franceschi *et al.*, 2010b; Bauer and Rigaud, 2015). Pieces of  
173 leaves without eggs were distributed to the control batches. In each session, some *G. pulex* from Suzon  
174 were also exposed and therefore served as 'positive controls' of infection. After 48 hours, all individuals  
175 were individualized in a crystallizer (60 ml) containing lab water and a piece of healthy elm leaf.

176

### 177 **Gammarids maintenance and mortality survey**

178

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

186 Based on the *P. laevis* development time observed in our previous experiments (e.g. Franceschi *et al.*  
187 (2008); Labaude *et al.*, 2020), the gammarids were inspected under a binocular magnifier from 6 weeks  
188 post-exposure (Nikon SMZ 745) to check their parasitic status through the cuticle of their host.  
189 Individuals detected as infected were isolated. The growth of the parasites was then inspected twice a  
190 week. However, some parasites were visible before these 6 weeks (some of them few days after the  
191 beginning of the experiment, a development time much too short to correspond to experimental  
192 exposure, see results), corresponding to *P. tereticollis* natural infections (confirmed by genotyping). These  
193 animals were analyzed separately.

194 We ended the experiment when no new developing parasites were detected for 15 successive days.  
195 The surviving gammarids were euthanized, measured and dissected to confirm their parasitic status. The

196 developmental stage of the parasites was noted for infected individuals. These gammarids, as well as  
197 their parasites, were stored individually in absolute ethanol pending genotyping. The parasites at the  
198 acanthella stage had to be genotyped to confirm their species, while the parasites having reached the  
199 cystacanth stage were easily recognizable using a binocular magnifier (Perrot-Minnot, 2004).  
200

## 201 **Gammarids & parasites genotyping**

202

203 To genotype living gammarids, between two and four locomotor appendages were detached and  
204 placed in a tube filled with ethanol. For individuals who died during the tracking, as DNA degrades quite  
205 quickly after death, a larger amount of tissue was used (between one and three body segments). To  
206 genotype adult parasites, a piece of about two mm<sup>3</sup> of body tissue was used to extract DNA. For the  
207 parasites at the acanthella stage, the whole individual was used.

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

211 For both parasites at the acanthella stage and amphipods, the gene encoding the first subunit of  
212 cytochrome oxidase (*co1*) was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et*  
213 *al.*, 1994). To 5 µL of the solution containing the DNA were added 200 nM of each primer, 200 µM of  
214 dNTPs, 0.25 U of DNA polymerase (HotStarTaq, Qiagen Inc., Düsseldorf, Germany), 1X of buffer and  
215 supplemented with ultrapure water to obtain a total reaction volume of 20 µL. The following thermal  
216 treatment was applied: activation of the polymerase for 3 min at 95°C, then 39 cycles: denaturation for  
217 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  
218 synthesis). Amplification success was checked by electrophoresis on 2% agarose gel and visualized with  
219 ethidium bromide stain. In case of failure of the *co1* amplification for parasites, the ITS region was  
220 amplified. The primers BD1f: 5'-GTC GTA ACA AGG TTT CCG TA-3' and ACITS1r: 5'-TTG CGA GCC AAG TGA  
221 TTC AC-3' were used (Franceschi *et al.*, 2008). The reaction mixture was subjected to an initial  
222 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  
223 was performed for 5 minutes at 65°C.

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

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

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

248 All gammarid sequences were submitted to BOLD, in the 'GAMEI' project  
249 ([https://v4.boldsystems.org/](https://v4.boldsystems.org/index.php/MAS_Management_DataConsole?codes=GAMEI)  
250 [index.php/MAS\\_Management\\_DataConsole?codes=GAMEI](https://v4.boldsystems.org/index.php/MAS_Management_DataConsole?codes=GAMEI)).  
251

## 252 **Statistical analyses**

253  
254 The parasite prevalence were compared by Chi<sup>2</sup> tests, or by Fisher's exact tests, or using logistic  
255 regression with Firth correction in cases of rare events (Firth, 1993). We have considered as 'infected' all  
256 the hosts where a parasite has been detected, whatever its developmental stage.

257 Host survival was analyzed with R using the *survival* package (Therneau and Grambsch, 2000). Data  
258 were censored if animal was still alive at the end of the experiments (118 days for the individuals  
259 exposed to *P. laevis*, and 138 for those exposed to *P. tereticollis*). Cox models were constructed for  
260 comparisons. A preliminary analysis has shown that the survival data did not respect, over the entire  
261 follow-up, the condition of proportionality of hazards (checked with the *cox.zph* function from the  
262 *survival* package) necessary for the application of a Cox regression model. Indeed, an acceleration of  
263 mortality is visible after the cystacanth stage was reached (see figures 6 and 8). In addition, during the  
264 first weeks of the survey, developing acanthellae were too small to be detected with a binocular  
265 magnifier when dissecting dead gammarids, which does not make it possible to distinguish parasitized  
266 individuals from uninfected ones. Because of these two constraints, survival has been analyzed in two  
267 steps. In the first step, we distinguished only two groups: the control individuals vs. those exposed to the  
268 infection. This analysis was stopped at the date where the first experimental acanthella was detected  
269 after the dissection of a dead gammarid (44 days for *P. laevis* and 61 days for *P. tereticollis*). In the second  
270 step, between the date of first detection of acanthella and the end of monitoring, the 'uninfected' and  
271 'parasitized' groups were distinguished among the exposed gammarids. For each analysis, the condition  
272 of proportionality of risks was successfully controlled. Finally, to compare the parasites virulence in  
273 actually infected gammarids, survival data of infected amphipods were analyzed after the first detection  
274 date of *P. laevis*. Three factors were included in the Cox models: the parasite species, host strain and the  
275 intensity of infection (for which data were categorized as follows: one, two, and more than two parasites,  
276 following Franceschi *et al.*, 2008). All possible models with first order interactions were compared by  
277 Akaike information criteria, using the *dredge* function of the *MuMin* R package (v. 1.43.17 ; Bartoń, 2009),  
278 and the models minimizing the AICc were presented.  
279

280

281

## Results

282 Apart from *Gammarus roeselii*, which were not taken into account in this experiment (see Bauer &  
 283 Rigaud, 2015 for their analysis), four host lineages have been found in the gammarids of the Albane River  
 284 (table 1). Very few *Gammarus pulex* were found despite the large numbers of collected gammarids. A few  
 285 *Gf 7* individuals were detected by sequencing. Since they were genetically very close to the *Gf 6* MOTU  
 286 and belong to the same breeding group (Lagrué *et al.*, 2014), they were referred as *Gf 6* in the dataset  
 287 and the following analyses. The genotypes of all the hosts were obtained only at the end of the  
 288 experiments, which explains why it was impossible to restore any imbalances in numbers.  
 289

290 Table 1. Numbers of gammarid MOTUs found in each exposure session, according to their infection  
 291 status, including individuals with natural infections in brackets (by parasite species: *P. laevis* in red,  
 292 *P. tereticollis* in green, and undetermined in black ; & for a mixed *P. laevis* and *P. tereticollis*  
 293 infection).

<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>Gp D</i>	1	0	1	2	6	10
undetermined	1	4	4	0	34	43
"Suzon"	NA	NA	2	79	18	99

<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
<i>Gf 6</i>	30 (1)	12	147 (1+2)	9	372	570
<i>Gp D</i>	0	0	19	0	10	29
undetermined	3	2 (1)	22 (1&1+1)	0	22 (1)	49
"Suzon"	NA	NA	12	4	81	97

294 *Gf 2* = *Gammarus fossarum* MOTU 2 ; *Gf 6* = *Gammarus fossarum* MOTU 6 ; *Gp D* = *Gammarus pulex*  
 295 MOTU D. *Undetermined*: gammarids which could not be genotyped (and therefore removed from  
 296 statistical analyses). *Suzon*: *Gammarus pulex* from Suzon, not distinguished by MOTU. *Controls*:  
 297 gammarids not exposed to parasite eggs. *Exposed*: gammarids experimentally exposed to parasite  
 298 eggs. *Early death*: gammarids that died before the date of detection of the first acanthella. *Eni*  
 299 (*exposed not infected*): exposed gammarids that did not develop any infection.

300

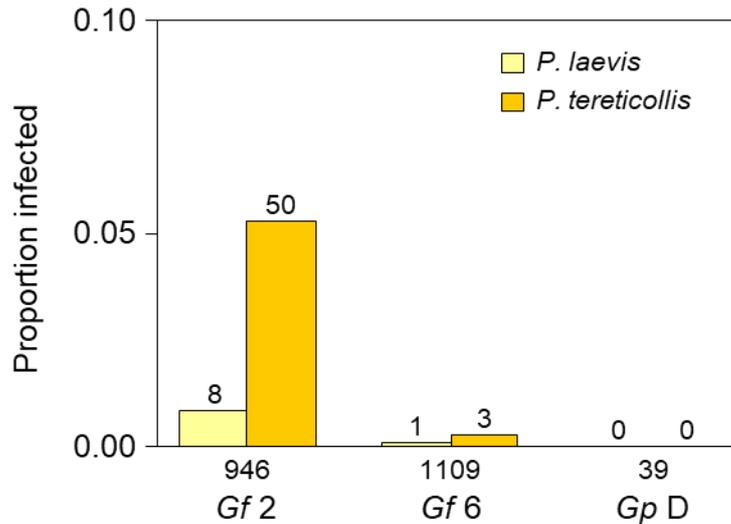
### 301 Infection rates

#### 302 Natural infections

303

304 Some acanthellae were visible 4 days after the exposure date, in exposed animals, but also in a few  
 305 unexposed animals (Table 1). Since such a short development time from exposure to acanthella stage is

306 not possible, we inferred that these infections were natural infections for which the quarantine was not  
 307 long enough for evidencing their development. Most of these infections were *P. tereticollis* parasites  
 308 (Figure 1). For the two parasite species, the prevalence were higher in *Gf* 2 than in *Gf* 6 (Fisher's exact  
 309 tests; *P. laevis*:  $p = 0.014$ ; *P. tereticollis*:  $p < 0.001$ ). No natural infection was observed for *Gp* D, for which  
 310 sample size was low (Figure 1).  
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313 **Figure 1** - Infections by *P. laevis* and *P. tereticollis* of natural origin in gammarid MOTUs from Albane  
 314 River. Numbers of infected amphipods are indicated above the bars, and total numbers of each  
 315 MOTU appear below.

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317 *Experimental infections*

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319 **Control of the infectivity of parasites using gammarids from Val Suzon**

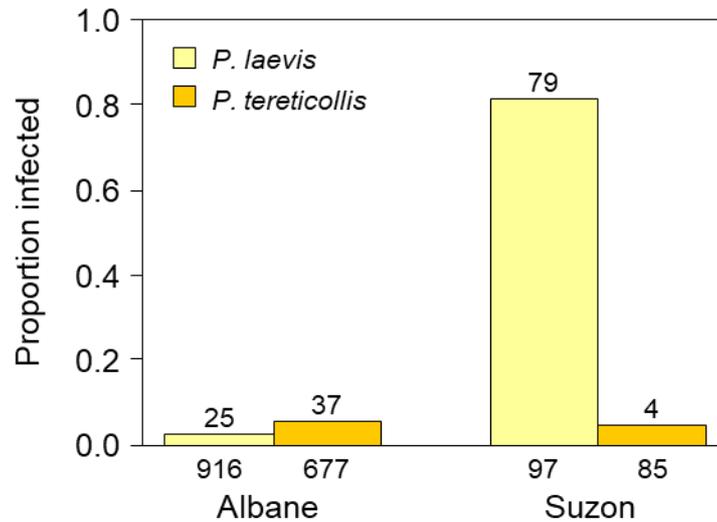
320

321 Even if the success of infection by *P. laevis* was low for gammarids from Albane (all MOTUs  
 322 combined), the high prevalence found in gammarids from the Suzon River showed that the parasites had  
 323 actually potential for infection (figure 2). In Suzon gammarids, a high *P. laevis* load was also observed,  
 324 with an average load of 4.5 parasites per infected hosts (up to 15 parasites in a single individual).

325 Only 4 individuals from Suzon developed a *P. tereticollis* infection (two with a single parasite and the  
 326 other with three). The prevalence of *P. tereticollis* was similar among gammarids from the two rivers ( $\chi^2 =$   
 327 0.001, 1 df,  $p = 0.970$ ).

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**Figure 2** - Success of laboratory infections by *P. laevis* and *P. tereticollis*, in gammarids (all MOTUs together) coming from the two rivers Albane and Suzon. Numbers of infected gammarids are given above the bars, and total sample sizes from each river below the bars.

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**Infection success in gammarids of the Albane River: prevalence and intensity**

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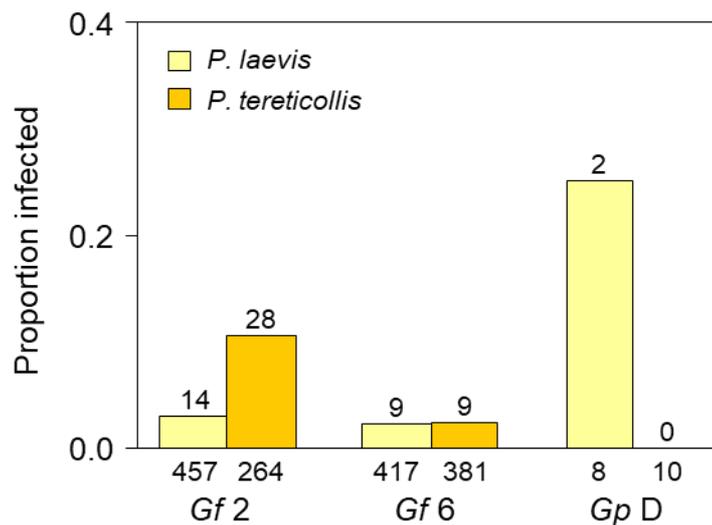
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A variation in the infection rates was observed between host lineages for the two parasite species (Fisher's exact test across all lines for *P. laevis*,  $p = 0.018$ ; for *P. tereticollis*,  $p < 0.01$ ). *Gf 2* was clearly more sensitive than *Gf 6* to *P. tereticollis* (Pearson  $\chi^2 = 18.105$ , 1 df,  $p < 0.001$ ), but was not significantly more sensitive to *P. laevis* (Pearson  $\chi^2 = 0.389$ , 1 df,  $p = 0.533$ ).

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.



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**Figure 3** - 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.

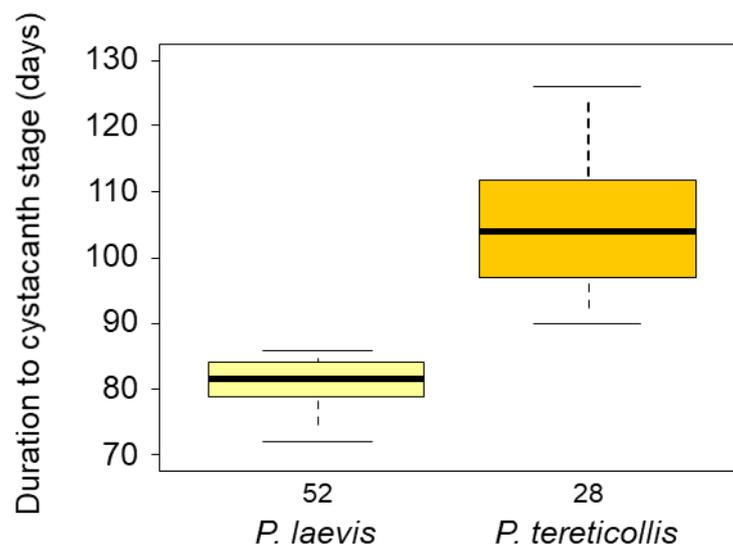
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The average load of *P. laevis* in Albane gammarids was lower than in those of the Suzon river (Wilcoxon test,  $W = 1347.5$ ,  $p < 0.01$ ). Among the Albane gammarids, the average parasite intensity was 2.07 parasites in *Gf 2*, half of the individuals being mono-infected, and the other half hosting between 2 and 7 parasites. The average intensity was 4 parasites 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 only two infected *Gp D* hosted 1 and 2 parasites respectively. Albane's *Gf 2* and *Gf 6* 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* only after 61 days. The first *P. laevis* reached the cystacanth stage at 72 days and the first *P. tereticollis* at 92 days.

We did not observe any difference in *P. laevis* development duration between Albane and Suzon gammarids (t test,  $t = 0$ ,  $p = 1$ ). Since we observed only 3 Suzon gammarids in which developing *P. tereticollis* reached the cystacanth stage, we could not compare the duration between populations for this parasite. No correlation between the number of parasites and their development duration was observed (*P. laevis*: Kendall's rank correlation,  $\tau = 0.137$ ,  $z = 1.210$ ,  $p = 0.226$ ; *P. tereticollis*:  $\tau = -0.010$ ,  $z = -0.0609$ ,  $p = 0.951$ ). We neither found a difference between development duration according to host lineage, at least between *Gf 2* and *Gf 6* (Wilcoxon tests, *P. laevis*:  $W = 4.5$ ,  $p = 0.554$ ; *P. tereticollis*:  $W = 54$ ,  $p = 0.809$ ). Therefore, to estimate the parasites development duration to the cystacanth stage, we cumulated data from all infected individuals, whatever their intensity of infection, to maximize sample size. It took significantly less time for *P. laevis* to reach the cystacanth stage (81 days on average) than for *P. tereticollis* (106 days) (Mann-Whitney U test:  $W = 0$ ,  $p < 0.001$ ), and the variation between early and late cystacanths differentiation was found to be larger in this latter species (figure 4).



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Figure 4 - 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.

385 **Probability of natural and experimental coinfections**

386

387 Natural infections by *P. laevis* did not influence significantly subsequent experimental infection,  
 388 whether by *P. laevis* (2.63 % probability of infection when not firstly infected (24/912) and 25.0 % in  
 389 gammarids already infected (1/4), Logistic regression with Firth correction,  $\chi^2 = 2.853$ ,  $p = 0.091$ ) nor by  
 390 *P. tereticollis* (5.34 % (36/674) and 2.77 % (1/3) respectively, Logistic regression with Firth correction:  $\chi^2 =$   
 391 2.216,  $p = 0.136$ ), but since numbers were very low for some categories, the relevance of the statistics  
 392 are questionable.

393 Natural infection by *P. tereticollis* strongly influenced an experimental coinfection by *P. laevis*: while  
 394 the probability of *P. laevis* lab infection in gammarids not previously infected was 1.92 % (17/885), it  
 395 reached 25.81 % (8/31) in gammarids already naturally infected by *P. tereticollis* (Logistic regression with  
 396 Firth correction:  $\chi^2 = 25.899$ ,  $p < 0.0001$ ). For lab infections by *P. tereticollis*, the previous effect of  
 397 *P. tereticollis* natural infection was non-significant (Logistic regression with Firth correction:  $\chi^2 = 0.00$ ,  
 398  $p = 1$ ), the prevalence reaching 5.52% (37/633) in animals uninfected naturally and 0.0% (0/7) in animals  
 399 already infected.

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401 **Parasite virulence**

402 *P. laevis*-exposed individuals

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404 The results for the *Gp D* host lineage are not presented because the numbers are too low to be  
 405 analyzed. In the first part of the tracking, *Gf 2* gammarids survive slightly better than *Gf 6*, and the  
 406 exposed individuals survived better than the control gammarids (figure 5, table 2).

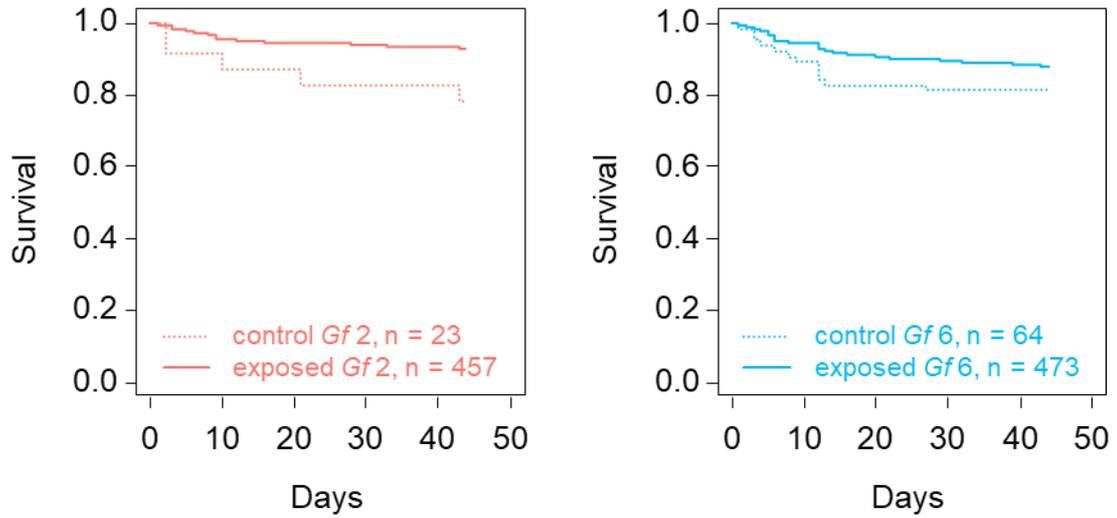
407

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

Development stage	Comparison	$\beta$	odd ratio	$\pm$ s.e.	z	df	p
1st part	MOTU: <i>Gf 2</i> vs. <i>Gf 6</i>	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: <i>Gf 2</i> vs. <i>Gf 6</i>	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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Figure 5 - Survival of *P. laevis* exposed gammarid MOTUs (plain lines) and controls (dotted lines), before the first acanthella detection date.

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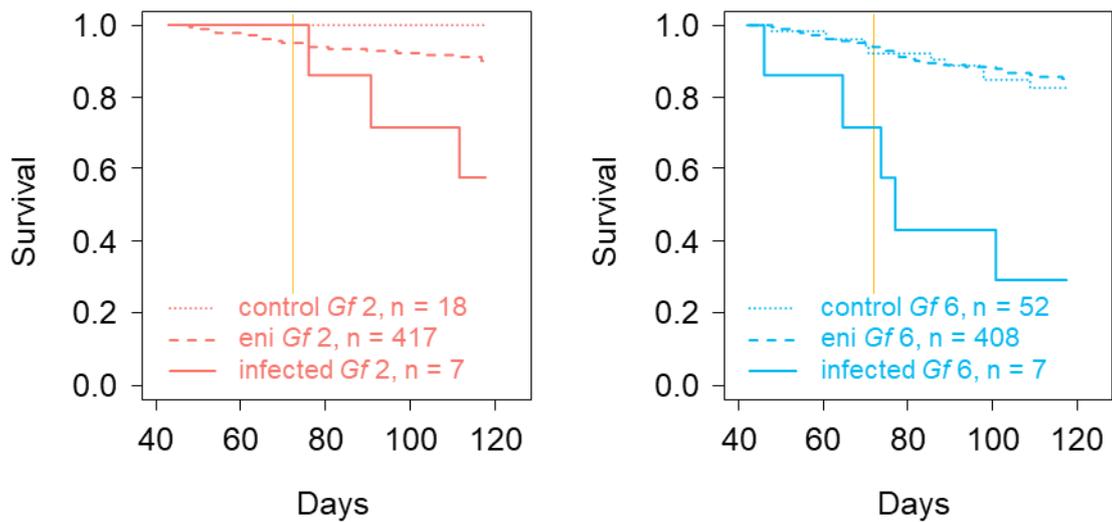
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In the second part of the tracking, increased mortality of parasitized individuals appeared as soon as the first acanthellae were detected by dissection in *Gf 6*, but it occurred later, from the date of differentiation of the first cystacanths, in *Gf 2* (figure 6, table 2). The survival of uninfected individuals - 'eni' and 'controls' - were similar. Globally, survival was better in *Gf 2* than in *Gf 6*.

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Figure 6 - Survival of *P. laevis* exposed gammarid MOTUs and controls (dotted lines), after the first acanthella detection date. 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.

431

432 *P. tereticollis*-exposed individuals

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434 High mortality of Albane *Gammarus* exposed to *P. tereticollis* was observed at the beginning of the  
 435 survey (compared to *P. laevis* exposed ones, see Figure 5 and figure 7), but it did not seem to be linked  
 436 with the confrontation to parasite eggs, since the same trend was observed in both exposed and control  
 437 gammarids (figure 7, table 3). No significant overall difference in survival was observed between *Gf 2* and  
 438 *Gf 6* MOTUs. However, when testing only *Gf 6* individuals, exposed animals survived better than control  
 439 ones ( $\beta = -0.575$ ,  $z = -2.296$ ,  $p = 0.022$ ).

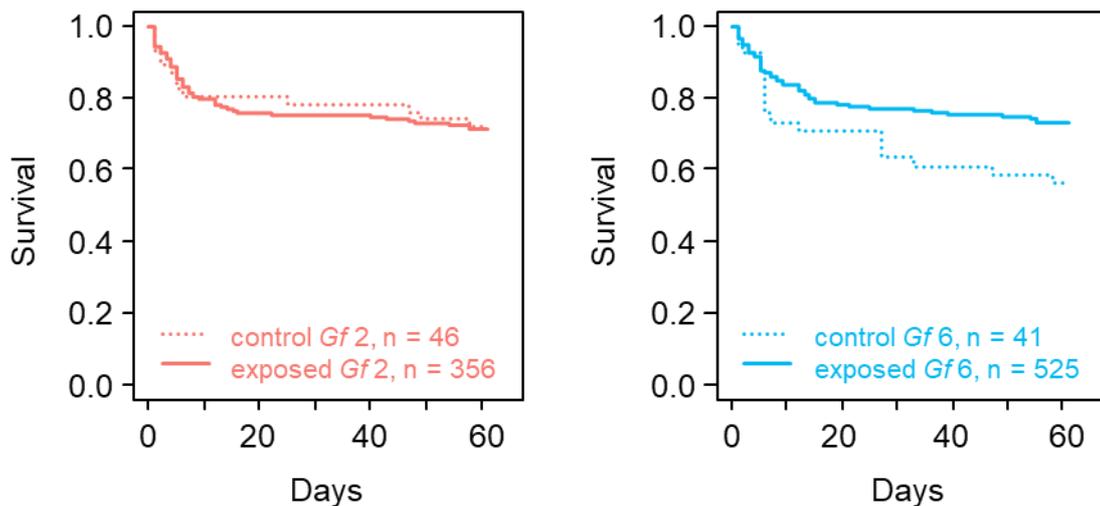
440

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

Development stage	Comparison	$\beta$	odd ratio	$\pm$ s.e.	z	df	p
1st part	MOTU: <i>Gf 2</i> vs. <i>Gf 6</i>	-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: <i>Gf 2</i> vs. <i>Gf 6</i>	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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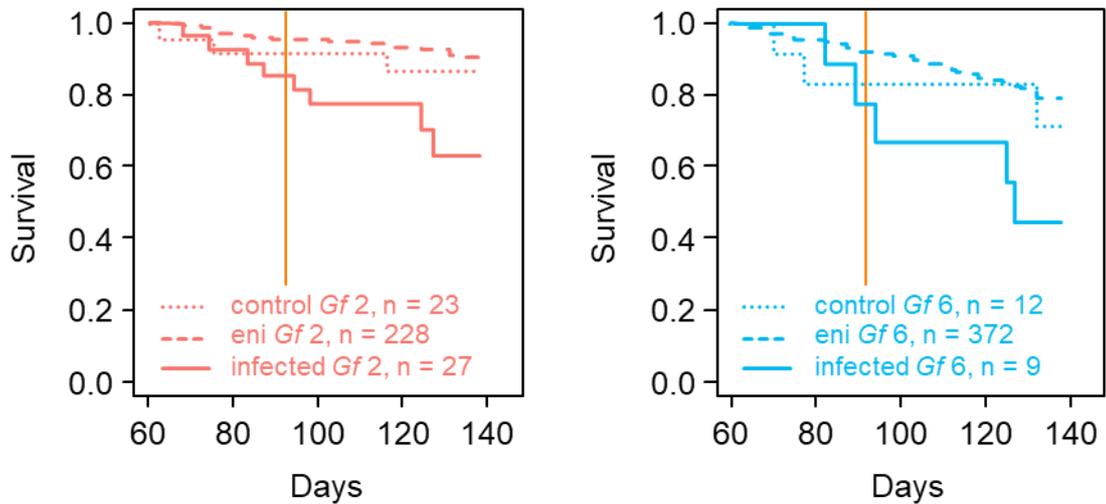
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450 Figure 7 - Survival of *P. tereticollis* exposed gammarid MOTUs (plain lines) and controls (dotted  
 451 lines), before the first acanthella detection date.

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453 In the second part of the follow-up and for both MOTUs, parasitized individuals showed increased  
 454 mortality compared to uninfected, whether these latter are exposed but not infected or controls  
 455 (figure 8, table 3).

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458 Figure 8 - Survival of *P. tereticollis* exposed gammarid MOTUs and controls (dotted lines), after the  
 459 first acanthella detection date. Infected individuals (plain lines) can now be distinguished from  
 460 exposed but not infected ones ('eni', in dashed lines). Letters on the right of the graphs indicate the  
 461 statistical groups. The vertical orange lines denote the first cystacanth detection date.

462

463 *Survival in infected gammarids*

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465 Survival analysis in infected gammarids began after the date acanthellae became visible. Interactions  
 466 were not significant and therefore removed from the presented model (see Supplementary table 2 for  
 467 the detailed comparison of possible models). We found only a significant effect of parasite species (whole  
 468 model:  $\chi^2 = 10.69$ , 3 df,  $p = 0.01$ ; table 4). Globally, *P. laevis*-infected gammarids died nearly twice than  
 469 those infected by *P. tereticollis* (figure 9a). The observed difference in survival between infected *Gf 2* and  
 470 *Gf 6* (whatever the parasite species, figure 9b) was not significant, nor the number of hosted parasites  
 471 (figure 9c).

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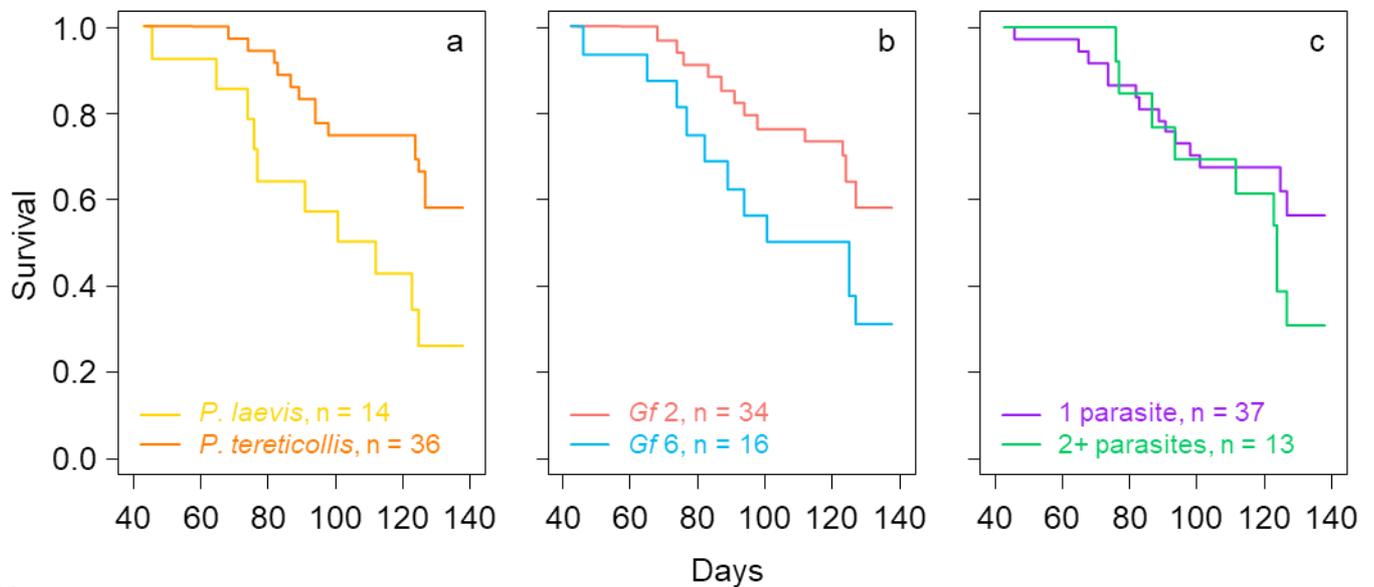
473

474 Table 4. Results of the Cox model analysing the survival of infected gammarids as a function of  
 475 parasite species, gammarid MOTUs and intensity of infection, after the first *P. laevis* acanthella  
 476 detection date. The interaction between factors were not significant and removed from the model.  
 477 Global 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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Figure 9 - 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.

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## Discussion

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

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We investigated by experimental infections two important factors explaining natural patterns of acanthocephalans infection in the wild: infection success and virulence. Behavioural manipulation, an important factor also explaining differences in prevalence in the field, has not been investigated 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 complete prospect 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 *Pomphorhynchus tereticollis*. The experimental infestations with *P. tereticollis* led to a relatively high infection rate in *Gf 2* MOTU of *Gammarus fossarum*, and to a lesser extent in *Gf 6* and *Gammarus pulex*. They did not generate any infection in the few *Gp D* from the Albane River, but produced a few in *G. pulex* from Val-Suzon River. 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 observation that the *G. pulex* from Val-Suzon are not particularly susceptible to *P. tereticollis* (in any case much less than to *P. laevis*) contradicts the hypothesis proposed by Franceschi *et al.* (2010b) which stated that amphipods of this site are more sensitive to infections by *Pomphorhynchus* parasites because this population is naïve to these parasites. Harris (2020) found similar results in England about *P. tereticollis* infections: high prevalences were found in *G. fossarum* (up to 90%), but no parasites (cystacanth stages) in *G. pulex*. We could therefore propose a general lower susceptibility of *G. pulex* to *P. tereticollis* compared to *G. fossarum*, but that the MOTU of *G. fossarum* matters. Our finding that *Gf 2* is more sensitive to infection than *Gf 6* sharply contrasts with field data from Galipaud *et al.* (2017) where the prevalence in *P. tereticollis* was found similar between these MOTUs, and highlights the usefulness of experimental infections in deciphering host-parasite relationships (Poulin and 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 and Rigaud, 2015). One of these processes is parasite virulence that we will discuss thereafter.

513 This study confirms, under controlled conditions, that all MOTUs present in the Albane River can be  
514 infected by *P. laevis*. The infection success in *Gf2* and *Gf6* is of the same order of magnitude.  
515 Experimental infections tend to show that *GpD* seems to be particularly sensitive to this parasite  
516 compared to *Gf2* and *Gf6*. These results are in contradiction with those of Galipaud *et al.* (2017) who  
517 found low *in natura* prevalence of *P. laevis* cystacanths in *G. pulex*. However, as showed by Bauer &  
518 Rigaud (2015), a low prevalence of cystacanths found after direct collection in nature could simply mean  
519 that either parasite virulence is high, or that the parasite increased so much the predation rate of its host  
520 that parasitized individuals disappear rapidly from populations. Here, our measures of prevalence were  
521 made after quarantine or after experimental infection, i.e. in conditions where predation was absent or  
522 the survival controlled. In addition, the Suzon population, composed of *G. pulex* (Labaude *et al.*, 2015), is  
523 very sensitive to infection, mirroring the few *G. pulex* from the Albane River. Alternatively, despite our  
524 precautions to standardize the infections, we cannot completely rule out an artefact linked to the  
525 experimental conditions. Indeed, even if we controlled the dose of parasite eggs provided to the  
526 gammarids, we are not able to control how many eggs have actually been consumed. *G. pulex* is usually  
527 larger than *G. fossarum* (Nijssen, 1963), and because food consumption is positively correlated to size in  
528 *Gammarus* (Sutcliffe *et al.*, 1981), this species may have consumed more eggs than the other MOTUs.  
529 However, if this hypothesis may possibly explain the differences in infection success obtained for  
530 *P. laevis*, it certainly cannot explain the results of the infestation with *P. tereticollis*, for which *G. pulex*  
531 appeared to be poorly sensitive. We therefore propose that *P. laevis* is a parasite better adapted to  
532 *G. pulex* than to *G. fossarum* (whatever the genetic lineage).

533 Our results therefore highlight the need for exposing each of the MOTUs of the *G. pulex / fossarum*  
534 complex species to experimental infections to understand their relative sensitivity to the different species  
535 of acanthocephalans.  
536

### 537 **Natural and experimental coinfections**

538  
539 Natural infection by *P. tereticollis* favours a further interspecific experimental infection by *P. laevis*.  
540 Contrastingly, natural infection by *P. tereticollis* did not influence intraspecific experimental  
541 superinfections by *P. tereticollis*, and natural infection by *P. laevis* did not influence neither a secondary  
542 infection by another acanthocephalan. However, sample size are sometimes so low that the statistical  
543 power of these later analyses are questionable. Cornet *et al.* (2009) showed that the level of immune  
544 defences of *G. pulex* is strongly depressed by the acanthocephalans *P. laevis*, *P. tereticollis*, and that  
545 acanthocephalan infection favors further superinfection by *Escherichia coli* bacteria. However, Dianne *et*  
546 *al.* (2010), using successive experimental infections by *P. laevis*, did not observed any increase of  
547 acanthocephalan intraspecific superinfection. It is therefore possible that the immune depression only  
548 favours interspecific parasite/pathogens superinfections but not intraspecific ones, suggesting that this  
549 immune depression is a costly by-product of the acanthocephalan infections. Since multiple infections are  
550 the rule in nature, further experimental investigations are welcome, particularly for the study of the  
551 evolution of virulence (Alizon *et al.*, 2013).  
552

### 553 **Development duration of parasites**

554  
555 Acanthocephalans development time in their intermediate hosts is strongly influenced by the  
556 environment (Awachie, 1966; Lackie, 1972; Tokeson and Holmes, 1982; Bratney, 1986; Labaude *et al.*,  
557 2020), highlighting the necessity of studying this life-history trait under controlled conditions. The  
558 development duration of *Polymorphus minutus* and *Pomphorhynchus laevis* under laboratory conditions  
559 have already been studied (Hynes and Nicholas, 1957; Butterworth, 1969; Franceschi *et al.*, 2008;  
560 Labaude *et al.*, 2015, 2020), but *Pomphorhynchus tereticollis* development was not documented before  
561 this study. The development time in *P. laevis* observed here was similar to the one found by Labaude *et*  
562 *al.* (2020) at a similar temperature. The development of *P. tereticollis* took 23 % longer than *P. laevis*  
563 under the same conditions. The among-host variation in maturation time (time between the appearance  
564 of the first and the last cystacanths) was also larger and more variable than that of *P. laevis*. This longer

565 development time explains *a posteriori* why we observed so many natural *P. tereticollis* infections after  
566 the quarantine we imposed to gammarids, since the quarantine time was estimated from our previous  
567 experience on *P. laevis* development.  
568

## 569 **Virulence**

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571 Ingestion of parasites and the early beginning of larval development did not negatively affect the host  
572 survival. Therefore, the wounds inflicted when the acanthors pierce the digestive tract of the arthropod  
573 to pass into the general cavity, and/or a possible early resistance to infection (cellular response, humoral  
574 and immune encapsulation (Crompton and Nickol, 1985) were not costly enough to increase mortality.  
575 The literature is contradictory about the host survival in early stages of infection by acanthocephalans.  
576 Increased mortality shortly after acanthocephalan experimental exposure was observed by Crompton &  
577 Nickol (1985) and Hynes & Nicholas (1957), but these studies were probably carried out with massive  
578 doses of eggs. This dose was not specified in these studies, but due to the dose-dependant prevalence  
579 (Franceschi *et al.*, 2008), and due to the high prevalence observed in these studies, this hypothesis is  
580 reasonable to consider. In these cases, the damages inflicted by many acanthors crossing the digestive  
581 tract wall may have been deleterious. On the other hand, Uznanski & Nickol (1980) did not find any  
582 increased mortality in the first 24 hours post-exposure in *Hyalella azteca* exposed to *Leptorhynchoides*  
583 *thecatus*. Yet, here again, the high infection success suggested the usage of high doses of eggs. Even  
584 more intriguing, experimental infections of *Asellus* by *Acanthocephalus lucii* studied by Bratley (1986)  
585 and Benesh & Valtonen (2007) induced early increased mortalities, whereas Hasu *et al.* (2006) found a  
586 better survival of infected individuals on the same biological models. This later, intriguing, result is what  
587 we observed in the present study: gammarids exposed to parasite eggs showed improved survival in the  
588 early days post-exposure, i.e. before the infection can be detected. Only speculative discussions on these  
589 observations can be proposed because it was not possible to ensure whether individuals dying at the  
590 beginning of the survey were infected or not. At least can we exclude a selective mortality due to the  
591 exposure. One of the speculative explanation is that the observed improved survival of exposed  
592 gammarids could be part of "parasite manipulation", where the parasite would protect its host from  
593 mortality, before becoming infective to the next host (Parker *et al.*, 2009). Such a protection was  
594 described for *P. laevis* in the late acanthella stage (Dianne *et al.*, 2011; Rigaud *et al.*, 2023), but this was a  
595 protection against mortality due to predation, which is not the case here. A mechanistic explanation of  
596 such a phenomenon would be that the early stages of the parasite divert some energy of the host for  
597 improving its survival. Castrating parasites are known to divert the energy normally allocated to host  
598 reproduction to their own purpose, in particular for the survival of the host they infect (Benesh and  
599 Valtonen, 2007). Gammarid acanthocephalans are known to castrate their hosts, partially (Bollache *et al.*,  
600 2002) or totally (Bollache *et al.*, 2002; Bailly *et al.*, 2018). It is not known precisely at what stage of  
601 parasite growth castration occurs in *Pomphorhynchus*, but it happens early during *Polymorphus*  
602 ontogeny, at least before the acanthellae are visible in their host (Bailly *et al.*, 2018). Alternatively, egg  
603 parasites eaten by gammarids, but unable to develop, can themselves become a form of diet  
604 supplementation for the hosts that 'prey' on them (Goedknecht *et al.*, 2012), improving host survival.

605 Parasite virulence was expressed in our study when the acanthellae enter their exponential phase of  
606 growth. It is interesting to note, even if the differences observed are not always supported statistically,  
607 that *Gf 6* was more sensitive to *Pomphorhynchus* infections than *Gf 2*, whatever the parasite species. In  
608 *Hyalella azteca* infected by *Corynosoma constrictum*, a higher mortality was observed at the time of rapid  
609 development of the parasite larvae, only in heavily infected hosts (Duclos *et al.*, 2006). This increased  
610 mortality due to acanthocephalan infections is interpreted as being due to displacement of the host's  
611 organs (Bentley and Hurd, 1993; Dezfuli *et al.*, 2008), or effects on metabolism (Rumpus and Kennedy,  
612 1974). In our study, the energy requirements of the two parasites are probably different, since  
613 *P. tereticollis* larvae are larger than *P. laevis* (Perrot-Minnot, 2004). However, the virulence of  
614 *P. tereticollis* was weaker than that of *P. laevis*. An effect on metabolism would therefore only be possible  
615 if one considers that *P. laevis* grows faster than *P. tereticollis*, which could exhaust the host's metabolism  
616 at higher intensity. If so, this would be in line with the "boom-bust" and "slow and steady" strategies  
617 proposed by Le Clec'h *et al.* (2019) for schistosome infections. Studies looking at metabolism of infected

618 gammarids have only been carried out at parasite cystacanth stages (Plaistow *et al.*, 2001; Gismondi *et al.*, 2012; Korkofigas *et al.*, 2016). All these studies showed that parasitized amphipods are deprived of  
619 lipid and protein reserves, while they have higher glycogen rates. This shows high demand for immediate  
620 energy (glycogen) in infected amphipods, which are forced to draw on their reserves (lipids), probably at  
621 the benefit of the parasite metabolism. Such a strategy is common in parasites. In schistosomes, such  
622 effects on the host fitness and energy diversion occur both early and in the late development of the  
623 parasite (De Jong-Brink *et al.*, 2001). In *Plasmodium*, the intra-host competition in cases of multiple  
624 infections is leading to increase energetic demand due to host overexploitation by the faster-developing  
625 parasites (De Roode *et al.*, 2005). Differences in survival between *Gf 2* and *Gf 6* hosts may be due to  
626 differences in metabolism, a hypothesis that necessitate further investigations. Another possible  
627 explanation for a differential in survival between host lineages could be differences in the ability of  
628 acanthocephalans to reduce the intensity of the immune responses of their hosts (Cornet *et al.*, 2009a).  
629 On the one hand, this partial immunosuppression might be advantageous for the host, owing the cost of  
630 mounting an immune response, either in terms of physiology (Moret and Schmid-Hempel, 2000) or  
631 because of immune cytotoxicity of enzymes used for the invertebrate immune responses (Sadd and Siva-  
632 Jothy, 2006). On the other hand, as discussed before, this immunosuppression can make hosts more  
633 susceptible to other infections, such as bacterial infections (Cornet *et al.*, 2009a; Cornet and Sorci, 2010).  
634 This immune modification is not observed in all gammarids: infections of various populations of  
635 *G. roeselii* by *P. laevis* do not show immunosuppression, with some populations even showing an immune  
636 response to the infection (Moret *et al.*, 2007). In addition, the level of immune defenses are variable  
637 among gammarid populations (Cornet *et al.*, 2009b). Therefore, *Gf 2* and *Gf 6* could be  
638 immunosuppressed differently, a hypothesis that remains to be tested.  
639  
640

#### 641 **General conclusion**

642  
643 Our results complement those of a previous study on Acanthocephala, carried out *in natura* by  
644 counting infections by cystacanths (Galipaud *et al.*, 2017). *G. pulex* might be a more important host than  
645 *G. fossarum* for the persistence of *P. laevis* in the rivers. However, differences in susceptibility to  
646 *P. tereticollis* were more pronounced between different host genotypes within the *G. fossarum* species  
647 complex than between *G. fossarum* and *G. pulex*. Our data therefore confirm that the specificity to a  
648 given parasite does not necessarily depend on the phylogenetic distance between the hosts, but indicate  
649 that we can no longer ignore cryptic hosts' genetic information to determine the level of specialization of  
650 the parasites. In addition to differences in susceptibility to infection, we also showed that a strong  
651 virulence is expressed when *P. laevis* or *P. tereticollis* reached the cystacanth stage, but that the different  
652 genotypes of *G. fossarum* were not affected with the same intensity.  
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## Appendices

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Supplementary table 1 - Accession numbers of the reference sequences used to assign the gammarid MOTUs and the parasite species

657

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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Supplementary table 2 - Model selection table for the survival analysis of infected gammarids (MuMin R package). Factors compared : intensity of infection (nb), host MOTU (motu), parasite species (para) and their first order interactions.

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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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### **Authors contributions**

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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**

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The dataset (in French) and data description file explaining the variables are available on the [data.indores.fr](https://data.indores.fr) data repository.

685

### **Conflict of interest disclosure**

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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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