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1 Stage-dependent behavioural changes but early castration induced by the  
2 acanthocephalan parasite *Polymorphus minutus* in its *Gammarus pulex*  
3 intermediate host.

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## 26 SUMMARY

27 Multidimensionality in parasite-induced phenotypic alterations (PIPA) has been observed in a large  
28 number of host-parasite associations, particularly in parasites with complex life cycles. However, it is  
29 still unclear whether such a syndrome is due to the successive activation of independent PIPAs, or  
30 results from the synchronous disruption of a single mechanism. The aim of the present study was to  
31 investigate the onset and progression of two PIPAs (a behavioural alteration: reversion of geotaxis,  
32 and castration) occurring in the crustacean amphipod *Gammarus pulex* infected with the  
33 acanthocephalan *Polymorphus minutus*, at different parasite developmental stages. Modifications of  
34 geotaxis in hosts differed according to the parasite developmental stage. Whereas the cystacanth  
35 stage induced a negative geotaxis (exposing the gammarid to predation by birds, the definitive  
36 hosts), the acanthella stage, not yet infective for the definitive host, induced a stronger positive  
37 geotaxis (presumably protecting gammarids from bird predation). In contrast, castration was almost  
38 total at the acanthella stage, with no significant variation in the intensity according to parasite  
39 maturation. Finally, no significant correlation was found between the intensity of behavioural  
40 changes and the intensity of castration. We discuss our results in relation with current views on the  
41 evolution of multidimensionality in PIPA.

42

43 Key words: Behavioural alterations, castration, infection syndrome, Amphipoda, Acanthocephala

44

## 45 KEY FINDINGS

- 46 • Acanthocephalans induce several phenotypic alterations in their amphipods hosts
- 47 • Alteration of host geotaxis and castration were studied simultaneously
- 48 • The two alterations were not parallel in time, nor were their intensities correlated accross
- 49 individuals
- 50 • Immature and mature parasites affected host geotaxis in opposite directions
- 51 • Although the two alterations appear to be unrelated in time, they may still depend on a
- 52 common mechanism
- 53
- 54

## 55 INTRODUCTION

56 Many parasite taxa have complex life cycles, with different host species harboring different  
57 parasite developmental stages. Adult parasites typically mate and reproduce inside a definitive host,  
58 from which eggs or larvae are released into the external environment. Parasites can then infect the  
59 intermediate host(s), where they generally grow through several developmental stages before  
60 becoming infective to the definitive host. Completion of the parasite's life cycle often occurs  
61 trophically, through predation of the intermediate host by the definitive one. Complex life-cycles are  
62 supposed to induce strong selective pressures for parasites to optimize their transmission from one  
63 host to the other. Indeed, many parasites induce various alterations in the phenotype of their  
64 intermediate hosts, some of them appearing to increase parasites' fitness at the expense of that of  
65 their hosts. Such phenomena are regularly interpreted in the framework of the 'parasite  
66 manipulation' hypothesis (Moore, 2002; Thomas *et al.* 2005; but see Cézilly *et al.* 2010). In particular,  
67 numerous parasites induce phenotypic alterations (hereafter PIPA for Parasite-Induced Phenotypic  
68 Alterations), including changes in the anti-predatory behaviour of their intermediate hosts, thereby  
69 possibly increasing trophic transmission (Moore, 2002; Thomas *et al.* 2005; Poulin, 2010; see  
70 however Perrot-Minnot *et al.* 2012).

71 Most often, numerous physiological and behavioural traits are simultaneously modified,  
72 including some with no obvious consequences in terms of trophic transmission (Thomas *et al.*, 2010),  
73 generating a parasite-induced infection syndrome (Cézilly and Perrot-Minnot, 2010; Cézilly *et al.*  
74 2013). Among the traits belonging to such syndromes is parasitic castration (Baudoin, 1975). The  
75 reduction or suppression of host reproduction by a parasitic infection is generally considered an  
76 evolutionary strategy by which the parasite increases its own fitness at the expense of that of its  
77 host (Baudoin, 1975; Jensen *et al.* 2006; Laferty and Kuris, 2009; but see Hurd, 2001 for other  
78 interpretations).

79 Both behavioural and reproductive PIPA may then be constitutive of an infection syndrome.  
80 Indeed, Cézilly and Perrot-Minnot (2010) highlighted that numerous parasite taxa inducing

81 behavioural alterations also induce castration. However, because these different PIPAs have been  
82 studied independently, it is still unclear whether such a syndrome is due to the progressive  
83 accumulation of several independent PIPAs during evolutionary time (Thomas et al. 2010), or results  
84 from a cascade of effects following disruption of a major physiological mechanism (Cézilly and  
85 Perrot-Minnot, 2010). As suggested by Cézilly and Perrot-Minnot (2010), evidence for a positive  
86 correlation between the intensities of two distinct PIPA could be suggestive of some common  
87 underlying mechanism (even if an absence of correlation would not be sufficient to conclude to the  
88 absence of such a link).

89         Another way to provide evidence for associations between various PIPAs could be to  
90 examine the temporal coincidence in the manifestations of various dimensions of an infection  
91 syndrome. In addition to rare cases of direct reproductive organ consumption by parasites, castration  
92 can be achieved through two main different ways, with different outcomes in their link with other  
93 PIPA. Castration can result from a large neuroendocrine disorder including the perturbation of the  
94 endocrine glands regulating gonadic development. In that case, links between parasites and  
95 neuroendocrine modulators may play a crucial role in linking behavioural alterations and castration  
96 (Hurd, 2009), and castration may coincide with behavioural alterations (Cézilly and Perrot-Minnot,  
97 2010). Alternatively, an 'energy-draining' castration can be achieved through the uptake of essential  
98 host resources by the parasite, independently of other effects of the parasite (Hall *et al.* 2007).  
99 Therefore, under the energy-drain hypothesis, castration should occur early in the course of infection  
100 (Hall *et al.* 2007) and should not coincide with behavioural modifications. However, as discussed by  
101 Hall et al (2007), the feeding rate of parasites may not be constant, as it may vary as parasites grow  
102 larger, or increase hyperbolically with food density, depending on the type of feeding functional  
103 response. In addition, recent findings on parasite behavioural manipulation showed that this  
104 phenomenon is dynamic. While parasite stages infective to the next host generally induce alterations  
105 that seem to increase trophic transmission, the reverse might be observed in intermediate hosts  
106 infected by parasites at an early developmental stage, still unable to infect the definitive host

107 (Dianne *et al.* 2011, Weinreich *et al.* 2013). This phenomenon might have been selected because  
108 trophic transmission occurring before the parasite has reached the infective stage would result in  
109 zero fitness (Parker *et al.* 2009). Ideally, then, an optimal temporal sequence of manipulations could  
110 consist of a 'protection-then-increased exposure' strategy.

111         The purpose of the present study was to test the time course and correlations between  
112 behavioural alteration and castration, two PIPAs occurring during the infection of the crustacean  
113 amphipod *Gammarus pulex* by the acanthocephalan parasite *Polymorphus minutus*. Adult *P. minutus*  
114 are intestinal parasites of various waterbird species, and rely on freshwater amphipods as  
115 intermediate hosts (Kennedy, 2006). Cystacanths (the developmental stage at which the parasite is  
116 able to infect its avian hosts) of *P. minutus* are known to alter the behaviour of *G. pulex* through  
117 reversing geotaxis (infected animals are attracted to the top of the water column, while uninfected  
118 ones tend to stay at the bottom of the water column), and increasing clinging behaviour (Cézilly *et al.*  
119 2000; Bauer *et al.* 2005). Infected animals thus spend more time at the water surface where they  
120 cling on any material available, while uninfected ones spend most of their time at the bottom of the  
121 water column. Infected hosts exhibiting such altered behaviour have been shown to be more  
122 exposed to predation by mallards, one of the *P. minutus* definitive host, than uninfected ones (Hinsbo,  
123 1972; Jacquin *et al.* 2014). Parallel to this, Bollache *et al.* (2001, 2002) evidenced a strong parasitic  
124 castration in *G. pulex* associated with the presence of *P. minutus* cystacanths. Infected males and  
125 females are almost never found in pre-copulatory mate guarding, and infected females lay no egg,  
126 following disruption of oocyte maturation. Little is known, however, on the time course of  
127 behavioural and reproductive PIPAs. Bethel and Holmes (1973) reported that *G. lacustris* infected by  
128 acanthellae (early developmental stage) of *P. paradoxus* did not express the behavioural alterations  
129 shown when they are infected by cystacanths of the same species. On the other hand, Rauque and  
130 Semenas (2009) found that infection with acanthellae of two acanthocephalan parasite species has  
131 no effect on the fecundity of their amphipod intermediate host, while Sparkes *et al.* (2006) found  
132 that behavioural reproductive suppression in freshwater isopod males infected with

133 *Acanthocephalus dirus* did not occur until the parasite had reached the cystacanth stage. Taken  
134 together, these observations suggest that reproductive and behavioural PIPAs might be synchronous,  
135 but, to the best of our knowledge, this has not yet been assessed within a single study.

136

## 137 MATERIAL AND METHODS

### 138 *Gammarid sampling and maintenance*

139 Gammarids were sampled between February and April 2016 in a tributary of the Suzon River, near  
140 Val-Suzon (Côte d'Or). Individuals were randomly collected using a hand net at all depths of the river  
141 (between ca 30 cm, the maximum depth, and the surface). Gammarids were sampled every three  
142 weeks and individuals from each sample spent the same amount of time in the laboratory. Within  
143 three days after sampling, gammarids were separated according to their infection status (uninfected,  
144 infected by an acanthellae, or infected by a cystacanth) and sex. These traits were determined  
145 through observations with a stereoscopic microscope, *P. minutus* infection and stage being easily  
146 detected through the translucent cuticle of the intermediate host. Sex of gammarids is easily  
147 distinguishable by examination of the second pair of gnathopods and, often, the presence of eggs in  
148 the female's external broodpouch (Sutcliffe 1992). Gammarids were maintained in separate tanks  
149 (30\*20\*10cm) in a mix of river water and tap water (dechlorinated and oxygenated, renewed each  
150 week). Rocks and plants were added to the tank to approximate river conditions, and conditioned  
151 elm leaves were used regularly to feed gammarids. Room temperature was  $15 \pm 1^\circ\text{C}$ , and  
152 photoperiod was 13:11h light:dark. Behavioural tests and reproductive traits were measured  
153 between day 4 and day 13 post-sampling. Each day, groups of 6 to 8 infected individuals and 6 to 8  
154 uninfected ones were tested simultaneously.

155

### 156 *Behavioural tests*



157 *Geotaxis*

158           Based on the procedure described in Bauer *et al.* (2005), we tested the vertical distribution of  
159 infected and uninfected gammarids. To that end, animals were placed individually in transparent  
160 vertical glass columns (35cm height, 6cm diameter) filled with stock water and covered by a net to  
161 allow gammarids to grasp at the water surface. This setup was placed in a large box, black inside,  
162 with one side open for observation. Columns were vertically divided in six equal parts, numbered  
163 from 0 (lower part) to 5 (upper part). Following an acclimatization period of 10 minutes, a score was  
164 attributed to the individual depending on its vertical position in the column, every 30 seconds during  
165 the next 10 minutes. The overall score for each individual was then calculated as the sum of each  
166 score, such that a gammarid staying at the bottom of the column for 10 minutes scored 0 (strong  
167 positive geotaxis), whereas one staying at the top for the same amount of time scored 100 (strong  
168 negative geotaxis). The water column was emptied and filled again after each test. Data were  
169 analyzed using non-parametric Wilcoxon or Kruskal-Wallis tests, followed by a Steel-Dwass non-  
170 parametric post-hoc test.

171 *Inclination of males to pair*

172           During precopulatory mate guarding (or amplexus), male gammarids carry the females  
173 underneath their own ventral surface, for several days before egg laying. We used a protocol similar  
174 to that of Bollache *et al.* (2001) to assess the motivation of males to pair. After having been tested  
175 for geotaxis, control and infected males were individually placed in small cups (3 cm height, 6 cm  
176 diameter), filled with stock water, during 30 minutes of acclimatization. During this time, uninfected  
177 females receptive to mating (i.e. paired female taken from the stock tank, carrying eggs at similar and  
178 advanced development), were separated from their males and isolated. At the end of the  
179 acclimatization time, a single receptive and uninfected female was added to each cup containing a  
180 focal male. Cups were checked every 5 minutes over one hour, to see if individuals had entered  
181 in amplexus. The experiment ended if amplexus did not occur after one hour, since, after such a time

182 period, males rarely engage in pairing (Bollache *et al.* 2001). Data were analyzed with a Generalized  
183 Linear Model (GLM), using a binomial distribution and a Logit link function, and using Firth Bias-  
184 adjusted Estimates, allowing the analysis of rare events.

185 At the end of the behavioural tests, each individual was kept separately in a small cup (3 cm height, 6  
186 cm diameter) until dissection and measurement of reproductive parameters.

#### 187 *Reproductive traits*

188 Dissections were made in Ringer, using a stereoscopic microscope (Nikon SMZ-10A) and a video  
189 measurement system (Nikon NIS – Elements D, v4.10). Gammarids were first anesthetized in  
190 carbonated water and their size determined by the distance from the fourth coxal plate basis to the  
191 dorsal limit of the individual (Bollache *et al.* 2002). They were then dissected, and the parasite  
192 developmental stage was confirmed in infected animals.

#### 193 *Reproductive traits in males*

194 The second pair of gnathopods is used by males to grasp females during amplexus (Conlan,  
195 1991). We therefore investigated whether parasitism affected this morphological trait associated  
196 with reproduction. The maximum length and maximum width of the right second gnathopod were  
197 measured to the nearest 0.01 mm, and their product was used as a proxy of gnathopod size. Since  
198 gnathopod size shows an allometric relationship with body size (see Cothran and Jeyasingh 2010), a  
199 linear regression was calculated between gnathopod size and the size of the fourth coxal plate, after  
200 log transformation. The residuals of the regression were used to compare infection groups using an  
201 analysis of variance. Sperm reserves were estimated by a method modified from Lemaître *et al.*  
202 (2009). One intact testis (including seminal vesicle) was isolated, and placed into 1000 µl of  
203 demineralized water. The gonad was fragmented in small pieces, and creation of sperm heaps was  
204 avoided by gently stirring the solution with forceps, to obtain a homogenous dilution of sperm in  
205 1000 µl. Four sample of 10 µl were placed on a slide, and dried during 10 to 20 minutes at 37°C.  
206 Using a microscope (“Nikon Eclipse E400”, objective x100), the total number of spermatozooids for

207 each sample was counted, to obtain a total number of spermatozooids in 40  $\mu$ l, after having verified  
208 the repeatability between samples. Since spermatozoid number may depend on male body size, we  
209 used a GLM using a Poisson distribution and a Log link function, corrected for overdispersion, to  
210 estimate the link between spermatozoid count and the size of the fourth coxal plate after its log  
211 transformation. The residuals of this model were used to compare infection groups using an analysis  
212 of variance.

### 213 *Reproductive traits in females*

214 The total number of eggs or embryos present in the ventral brood pouch was counted to estimate  
215 female fecundity. To determine the effect of infection on oocyte maturation, gonads were dissected,  
216 and the number of developed oocytes and white (non-developing) oocytes were counted in one  
217 ovary, following Bollache *et al.* (2002). The number of 'blue oocytes' (developing but altered), which  
218 only occurred in *P. minutus*-infected individuals, was also counted (see Bollache *et al.* 2002). The  
219 proportion of females carrying eggs or embryos were analyzed with a GLM, using a binomial  
220 distribution and a Logit link function, and using Firth Bias-adjusted Estimates. Oocyte counts were  
221 analyzed using non-parametric Kruskal-Wallis tests, followed by a Steel-Dwass post-hoc test.

222 Correlations between geotaxis scores and reproductive traits were analyzed using non-parametric  
223 Spearman's rho, followed by a Bonferroni correction. All statistical tests were made using JMP v.  
224 10.0.0 (©SAS Institute 2012).

225

## 226 RESULTS

227 During the three field sampling periods, the prevalence of infection with acanthellae progressively  
228 decreased from 63.0% in February to 0% in April (Figure 1). From this observation, as well as  
229 considering results from other longitudinal field studies of acanthocephalans (e.g. Sparkes *et al.*  
230 2006, Kennedy 2006), we inferred that parasites were maturing progressively from acanthella to

231 cystacanth stages in the population. For measurements of geotaxis, we therefore compared similar  
232 parasite developmental stages among sampling dates.

233

#### 234 *Geotaxis measurements*

235 At any date and within each infection status, geotaxis scores did not differ significantly between *G.*  
236 *pulex* males and females (Wilcoxon tests, all  $P > 0.09$ ). Data were therefore pooled for further  
237 analyses. There was no significant variation in geotaxis score between sampling dates for both  
238 uninfected individuals (three sampling dates,  $n = 60, 49, \text{ and } 13$ , respectively, Kruskal-Wallis:  $\chi^2 =$   
239  $2.29; 2 \text{ d.f.}, P = 0.32$ ) and individuals infected by acanthellae (two sampling dates,  $n = 34 \text{ and } 16$ ,  
240 respectively, Wilcoxon:  $\chi^2 = 2.07; 1 \text{ d.f.}, P = 0.15$ ). Samples from different dates were therefore  
241 pooled for further analyses. However, geotaxis score varied according to sampling date for  
242 individuals infected with a cystacanth (three sampling dates,  $n = 20, 25 \text{ and } 23$ , respectively, Kruskal-  
243 Wallis:  $\chi^2 = 8.31; 2 \text{ d.f.}, P = 0.016$ ). Infection with a cystacanth was associated with a lower score in  
244 February than in March and April (Steel-Dwass post-hoc test,  $P = 0.04 \text{ and } P = 0.03$ , respectively),  
245 while the scores did not differ significantly between March and April ( $P = 0.98$ ). Owing to the  
246 variation in cystacanth prevalence through time reported above, we distinguished two cystacanth  
247 categories, depending on whether they had been sampled in February or in March-April, for further  
248 analyses.

249 The geotaxis score of *G. pulex* varied significantly according to their infection status (Figure 2,  
250 Kruskal-Wallis:  $\chi^2 = 30.19; 3 \text{ d.f.}, P < 0.0001$ ). Infections with cystacanths sampled in March-April  
251 were associated with geotaxis scores higher than those of uninfected gammarids, while infections by  
252 acanthellae were associated with geotaxis scores lower than those of uninfected individuals (Figure  
253 2). Scores of individuals infected by cystacanths sampled in February were significantly lower than  
254 those recorded in March-April, but not significantly different from those of individuals infected by  
255 acanthellae and those of uninfected ones (Figure 2).

256

257 *Male reproductive traits*

258 About 40% of uninfected males initiated pairing with receptive females. This proportion significantly  
259 differed from the proportions of infected males initiating a pair (maximum 10%), irrespective of the  
260 parasite developmental stage (GLM:  $\chi^2 = 25.73$ , 3 d.f.,  $P < 0.0001$ ) (Figure 3a).

261 Sperm count, corrected for gammarid body size, was different among groups (ANOVA  $F_{3,105} = 3.00$ ,  $P$   
262  $= 0.03$ ). Males infected with cyctacanthos from the March-April sample had more sperm reserves than  
263 uninfected males, but not males of other infection categories (Figure 3b).

264 The size of the second gnathopod, corrected for gammarid body size, did not differ according to the  
265 infection status (ANOVA  $F_{3,131} = 1.12$ ,  $P = 0.34$ ; Figure 3c).

266

267 *Female reproductive traits*

268 The proportion of females carrying eggs or embryos in their ventral incubating pouch (i.e.  
269 reproductive females) was 89.29 % in uninfected individuals. This proportion significantly differed  
270 from that of reproductive infected females (maximum 13.6 %), irrespective of the parasite  
271 developmental stage (GLM:  $\chi^2 = 82.08$ , 3 d.f.,  $P < 0.0001$ ) (Figure 4a).

272 Oocyte count was not analyzed in females infected with cystacanthos sampled in February, because of  
273 the small sample size of successful dissections ( $n = 4$ ). In other categories, the number of oocytes per  
274 ovary did not differ significantly among uninfected and infected females (Kruskal-Wallis:  $\chi^2 = 3.74$ , 2  
275 d.f.,  $P = 0.15$ ) (Figure 4b). However, the proportion of developing oocytes was much higher in  
276 uninfected females than in infected ones, irrespective of the developmental stage of parasites, in  
277 which this proportion was virtually zero, with one single exception (Kruskal-Wallis:  $\chi^2 = 54.44$ , 2 d.f.,  
278  $P < 0.0001$ ; Figure 4c). Indeed, the proportion of blue oocytes (i.e. showing abnormal development),  
279 as well as the proportion of white oocytes (i.e. not developing oocytes) were higher in infected

280 females than in uninfected ones, irrespective of the developing stage of the parasite (Kruskal-Wallis:  
281  $\chi^2 = 37.15$ , 2 d.f.,  $P < 0.0001$ , Figure 4d, and  $\chi^2 = 34.75$ , 2 d.f.,  $P < 0.0001$ , Figure 4e, respectively).

282

### 283 *Correlations between geotaxis and reproductive traits*

284 We considered categories of infections in two different ways. First, we distinguished acanthellae and  
285 the two cystacanths categories, as described above. Second, we grouped all the developmental  
286 stages into a single, "infected" category. Such a grouping was made to take into account a potential  
287 correlation between the time course of behavioural changes (as described before) and parasite  
288 castration. Only one of these correlations was found to be significant (Table 1): there was a positive  
289 correlation between geotaxis score and sperm number in infected males, after pooling data from all  
290 developmental stages (Figure 5).

291

## 292 DISCUSSION

293 We found that both the direction and extent of modification of geotaxis in *G. pulex* were  
294 highly dependent on the developmental stage of *P. minutus*, whereas castration was not.

295 Whereas the cystacanth stage induced a reversion of geotaxis (negative geotaxis, compared  
296 to positive geotaxis in uninfected animals), confirming previous observations (e.g. Cézilly *et al.* 2000;  
297 Bauer *et al.* 2005; Jacquin *et al.* 2014), the acanthella stage, not infective yet for the definitive host  
298 (Bethel and Holmes 1974), induced a stronger, positive geotaxis than that observed in uninfected  
299 gammarids. A previous study on the congeneric acanthocephalan *P. paradoxus* concluded that  
300 acanthellae induce no change in behaviour in gammarids (Bethel and Holmes, 1974). However, that  
301 study examined evasive behaviour and not geotaxis, such that further investigations are necessary to  
302 have a better appreciation of the time course of multidimensionality in manipulation in relation to  
303 parasite development. The present observations are however consistent with others showing that

304 immature stages of parasites may induce behavioural alterations opposite to those induced by the  
305 parasite stages infective for the next host (Hammerschmidt *et al.* 2009; Dianne *et al.* 2011, Gopko *et*  
306 *al.* 2015), or, at a finer scale, that parasites may alter host behaviour in a time-specific manner  
307 throughout the day, resulting in a protection from predation by non-suitable definitive hosts (Levri  
308 1998). Such a phenomenon has been predicted by Parker *et al.* (2009) as an optimal ‘protection-  
309 then-increased exposure to predation’ strategy for trophically-transmitted parasites, and  
310 experiments have indeed revealed that such opposite behavioural alterations are associated with  
311 lower predation rates (Dianne *et al.* 2011; Weinreich *et al.* 2013). Whether infection with acanthellas  
312 of *P. minutus* is associated with lower vulnerability to predation by definitive hosts remains to be  
313 tested in the present case.

314         Our results further suggest that the transition between positive and negative geotaxis is  
315 progressive. Indeed, cystacanths sampled in February were associated with geotaxis scores  
316 intermediate between those of acanthellae and those of cystacanths that were sampled one month  
317 later. February cystacanths being on average younger than March-April cystacanths, we evidenced  
318 here a progressive shift in a parasite-induced behavioural change – ‘progressive’ meaning here that  
319 the change occurred over several days. This observation is consistent with the results obtained by  
320 Bethel and Holmes (1974) on *P. paradoxus*, for which 17 days, on average, separate the invagination  
321 of the acanthellae (defining the cystacanth stage) from the onset of altered behaviour in the  
322 intermediate host. This confirms that the intensity of behavioural changes increases with the course  
323 of acanthocephalan maturation after cystacanth stage (Franceschi *et al.* 2008).

324         Contrasting with what was observed for behavioural changes, we observed no variation in  
325 the intensity of castration as a function of parasite maturation. In both males and females the extent  
326 of castration at the acanthellae stage, did not differ from that observed at the cystacanth stage of  
327 infection. Even if acanthellae in this study were rather old (they were colored and visible through the  
328 host's cuticle), this result contrasts with those of Sparkes *et al.* (2006) and Rauque and Semenas

329 (2009) who observed, in three other acanthocephalan species inducing total or partial castration,  
330 that castration did not occur before parasites had reached the cystacanth stage. Therefore,  
331 castration induced by *P. minutus* seems to be triggered earlier during parasite development than in  
332 other castrating acanthocephalans.

333 As reported in Bollache *et al.* (2001), infected males paired at lower rates compared to  
334 uninfected ones. We further explored this phenomenon by considering whether this lower pairing  
335 success was due to gonadal dysfunction or morphological alteration. We found no evidence for an  
336 alteration of sperm reserves associated with infection by *P. minutus*, nor did we observe any  
337 alteration of gonadal structure. This is consistent with another study on acanthocephalans  
338 (Bierbower and Sparkes, 2007). Actually, *G. pulex* males infected with older cystacanth had higher  
339 sperm reserves. Since sperm tends to accumulate if males do not inseminate females (Lemaître *et al.*  
340 2009), sperm accumulation after a longer period of sexual abstinence, due to a longer infection time,  
341 could explain this result. This is corroborated by the positive correlation between geotaxis score and  
342 sperm reserve when taking into account all stages of the *P. minutus* development (Figure 5): the  
343 older the parasites, the more they altered their host's geotaxis and the more the hosts accumulated  
344 sperm, probably as a consequence of behavioural castration. Due to our experimental procedure, we  
345 were unable to assess whether the sperm accumulated in infected males was functional or not. Such  
346 an assessment would not be straightforward, however, because sperm is not motile in gammarids,  
347 such that a comparison of insemination success between infected and uninfected males would be  
348 necessary to address the question. However, during our dissections, we did not observe any  
349 abnormal sperm structure. In addition, infection status had no influence on the size of the second  
350 gnathopod, the appendage used to grasp females during mate guarding. Therefore, alterations of  
351 reproductive function or morphology are unlikely to explain reduced male mating success in *G. pulex*  
352 infected with *P. minutus*. One possibility, then, is that infection with *P. minutus* has an effect on the  
353 nervous system of male *G. pulex* that disrupts their normal reaction towards sexually receptive



354 females. Such a possibility has been observed in an insect infected by another acanthocephalan  
355 species, where males cannot perceive normally sex pheromones (Carmichael *et al.* 1993).

356         No significant correlation was found between the intensity of behavioural changes and the  
357 intensity of castration, either when considering acanthellae- and cystacanth-induced behavioural  
358 changes as separated phenomena (i.e. correlations analyzed separately for the different  
359 developmental stages), or as two components of the same phenomenon (analyses made after  
360 grouping all parasite stages). This lack of correlation, as well as the differences in temporal dynamics  
361 between castration and geotaxis changes, does not argue in favour of a mechanistic link between  
362 these two PIPAs (Cézilly and Perrot-Minnot, 2010; Thomas *et al.* 2010). However, this result is, of  
363 course, not sufficient to conclude to the absence of such a link. Over the course of infection,  
364 products excreted by the parasite may accumulate in its host. In the same time, several by-products  
365 of the immune response of the host may accumulate too. However, the dose-response of castration  
366 and that of reversed geotaxis in relation to the concentration of a single or several key substances  
367 may not be the same. For instance, a behavioural or physiological dose-response may not be linear  
368 but sigmoid (Lau *et al.* 2000; Byers, 2013). It has recently been proposed that reversed geotaxis could  
369 be a behavioural response to hypoxia signaling, resulting either from the sustained hypoxic-like  
370 conditions induced by *P. minutus* cystacanths, or the release, from the excretion/secretion products  
371 of parasites, of lactate and succinate in the host brain (Perrot-Minnot *et al.* 2016). The problem  
372 remains that the two opposite geotaxis disruptions induced by the two parasite developmental  
373 stages may hardly been explained by such a phenomenon. Interestingly, however, some dose-  
374 responses can show bi-phasic shape, corresponding to a hormetic-like effect. In such cases, the  
375 exposition to a low dose of a substance may induce in an organism the opposite response to a very  
376 high dose (see for instance Rabhi *et al.* 2014). This could explain, from a mechanistic point of view,  
377 the contrasted effect of the developmental stages of *P. minutus* on the geotaxis of *G. pulex*.  
378 Independently, some studies have showed that natural or experimentally-induced hypoxia down-  
379 regulates the expression of vitellogenin, the major egg-yolk protein, in a number of crustacean and

380 fish (Brown-Peterson *et al.* 2011; Li and Brouwer, 2013; Lai *et al.* 2016 and references therein).  
381 Therefore, under the hypothesis that hypoxia has an early and strong effect on reproduction but that  
382 phototaxis behaviour shows a bi-phasic response to hypoxia, these two traits might actually be  
383 synchronous. Now that the timing of expression of castration and geotaxis changes has been clarified  
384 in *G. pulex* infected by *P. minutus*, unraveling the shape of hypoxia signaling dose-response on both  
385 geotaxis and castration would be a promising avenue for testing the link between these different  
386 PIPAs.

387

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391

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511

512

513

514 **Figure captions**

515 Figure 1: Proportion of acanthella developmental stage of *Polymorphus minutus* in their infected *G.*  
516 *pulex* hosts, according to sampling date. Other developmental stages were cystacanths.

517

518 Figure 2: Scores of geotaxis in *Gammarus pulex* according to their infection status, with categories as  
519 defined in the text. U: uninfected animals, A: infected with acanthellae, Cfeb: infected with  
520 cyctacanths sampled in February, CMar-Apr: infected with cyctacanths sampled in March and April  
521 (see text). Sample size are provided. Box-plots are median, interquartile and interdecile (and  
522 outliers). Boxed labelled with different letters denotes scores significantly different after a Steel-  
523 Dwass non parametric post-hoc test ( $\alpha = 0.05$ ).

524

525 Figure 3: Reproductive traits in *Gammarus pulex* males, according to their infection status (U:  
526 uninfected animals, A: infected with acanthellae, Cfeb: infected with cyctacanths sampled in  
527 February, CMar-Apr: infected with cyctacanths sampled in March and April). (a) Proportion of males  
528 initiating pairing in one hour. Bars surrounded by different letters denotes proportions significantly  
529 different after Odd-ratios tests; (b) Sperm reserve in testis. Dots are means, bars are standard errors  
530 on the mean; Bar surrounded by a star denotes a mean significantly different for the uninfected  
531 control group after Dunnett post-hoc test ( $P = 0.03$ ) (c) Size of second pair of gnathopods as a  
532 function of gammarid size and infection status. Sample size are provided within brackets below the x  
533 axes.

534

535 Figure 4: Reproductive traits in *Gammarus pulex* females, according to their infection status (U:  
536 uninfected animals, A: infected with acanthellae, Cfeb: infected with cyctacanths sampled in  
537 February, CMar-Apr: infected with cyctacanths sampled in March and April). Sample size are  
538 provided below the x axes. (a) Proportion of females with eggs in the brood pouch. Bars surrounded  
539 by different letters denotes proportions significantly different after Odd-ratios tests; (b) Number of  
540 oocytes counted in one ovary; (c) Proportion of developing oocytes (d) Proportion of blue oocytes,  
541 (e) Proportion of white oocytes. From (b) to (e), box-plots are median, interquartile and interdecile  
542 (and outliers). Bars surrounded by different letters denotes significant differences after a Steel-Dwass  
543 non parametric post-hoc test ( $\alpha = 0.05$ ).

544

545 Figure 5: Correlation between geotaxis scores and sperm number in *Gammarus pulex* males infected  
546 with different stages of *Polymorphus minutus*. White dots: infected with acanthellae; grey dots  
547 infected with cyctacanths sampled in February; black dots; infected with cyctacanths sample in  
548 March-April.

Table 1: Correlations (Spearman's rho) between geotaxis scores and major reproductive traits of *Gammarus pulex* males and females, according to their infection status.

Sex	Trait correlated with geotaxis	Infection category	N	Rho	P
Males	Gnathopod size*	U	64	-0.14	0.28
		A	28	0.001	0.97
		C <sub>Feb</sub>	13	0.47	0.11
		C <sub>Mar-Apr</sub>	27	-0.20	0.31
		I	68	-0.001	0.99
	Sperm number**	U	53	-0.07	0.63
		A	22	0.40	0.07
		C <sub>Feb</sub>	10	0.30	0.39
		C <sub>Mar-Apr</sub>	21	-0.07	0.75
		<b>I</b>	<b>53</b>	<b>0.35</b>	<b>0.001</b>
Females	% white oocytes	U	49	-0.05	0.70
		A	12	-0.16	0.61
		C <sub>Feb</sub>	n.a.		
		C <sub>Mar-Apr</sub>	16	-0.15	0.55
		I	33	-0.18	0.29
	% blue oocytes	U	n.v.		
		A	12	-0.02	0.94
		C <sub>Feb</sub>	n.a.		
		C <sub>Mar-Apr</sub>	16	0.007	0.98
		I	33	0.12	0.50

U: uninfected animals; A: infected with acanthellae; C<sub>Feb</sub>: infected with cyctacanthus sampled in February; C<sub>Mar-Apr</sub>: infected with cyctacanthus sampled in March and April; C: infected with cyctacanthus (all date grouped); I: infected animals (all developmental stages grouped).

\*corrected for body size (residuals of the regression)

\*\*corrected for body size (residuals of the Quasi-Poisson GLM)

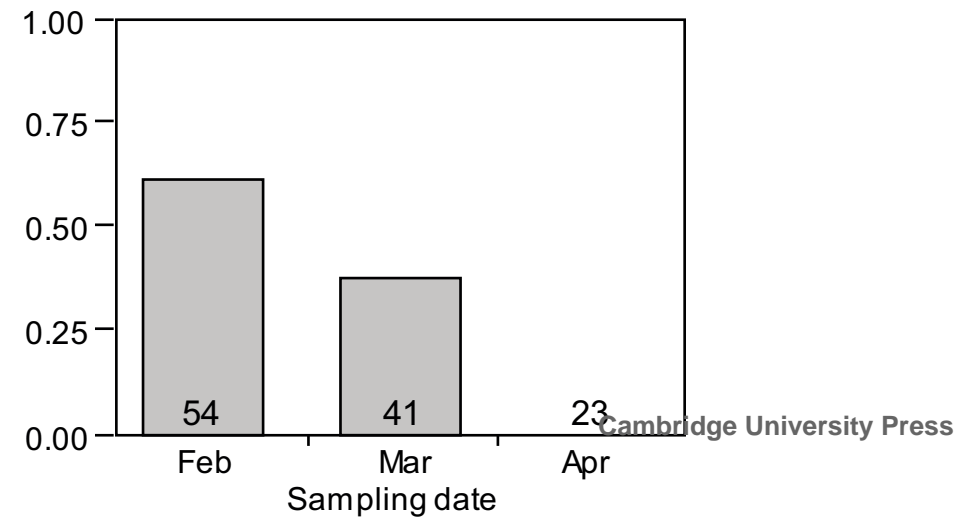
n.a. sample size too low to be analyzed

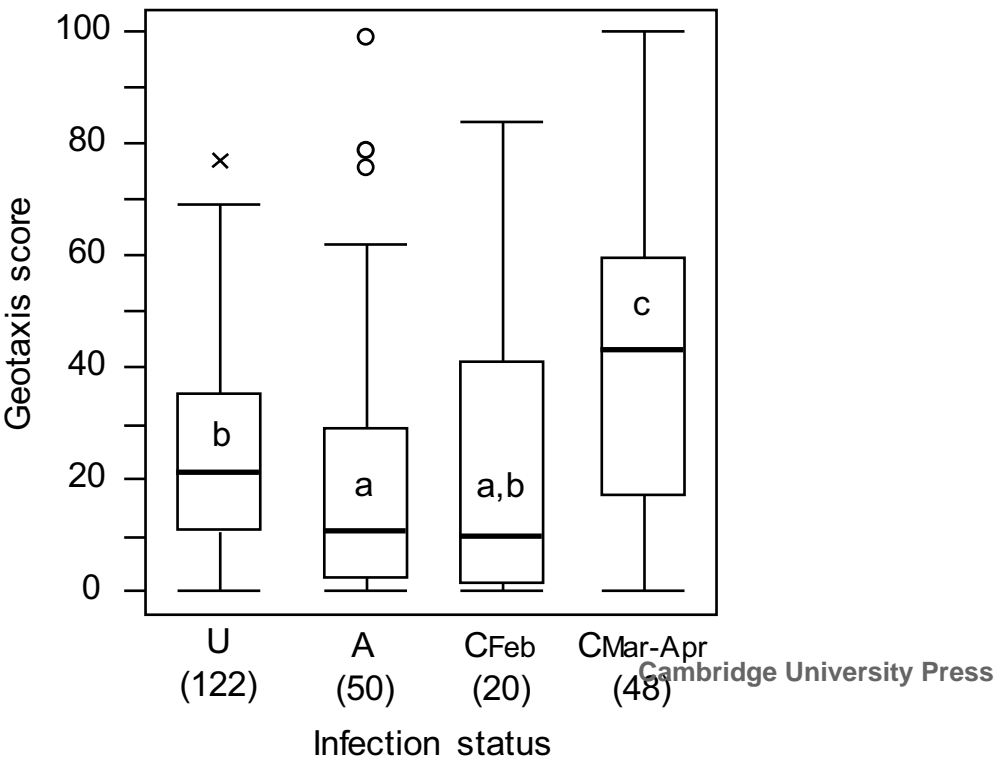
n.v. no variation on this trait

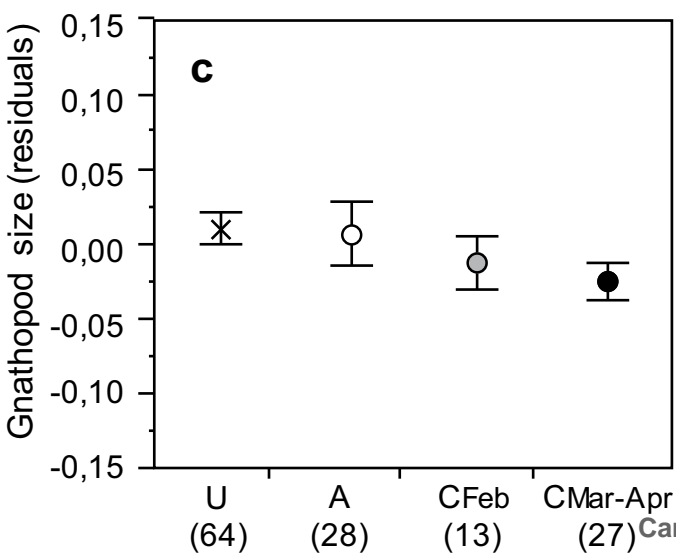
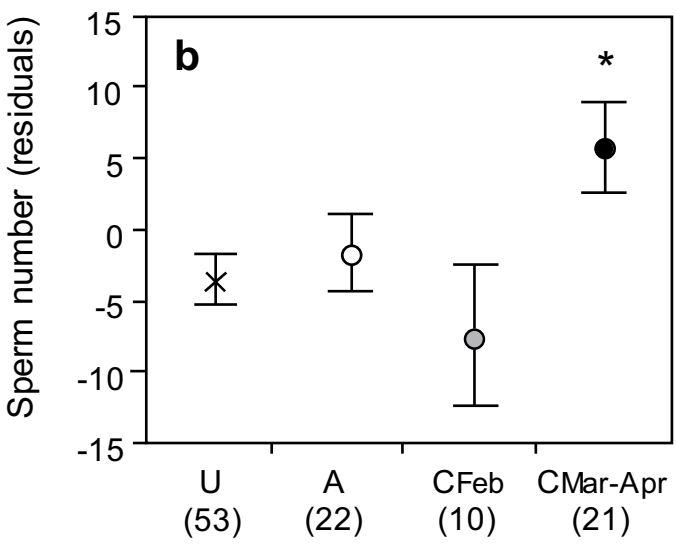
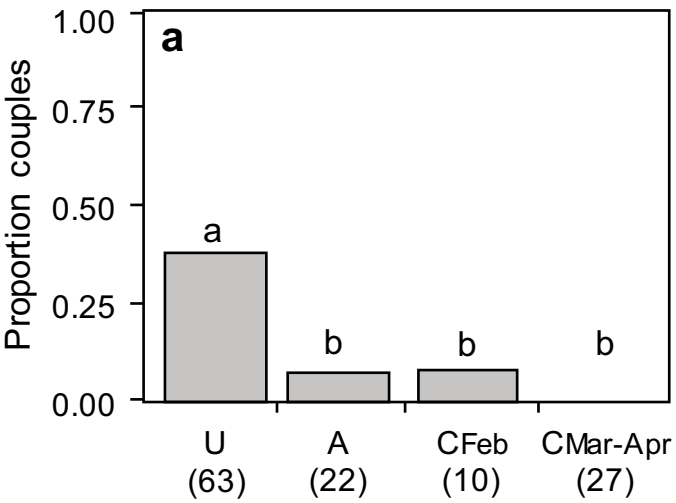
Correlation in bold was significant even after Bonferroni correction.



Proportion acanthella among infected







Infection Status

