Time-course of antipredator behavioral changes induced by the helminth *Pomphorhynchus laevis* in its intermediate host *Gammarus pulex*: the switch in manipulation according to parasite developmental stage differs between behaviors

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Abstract:

Many trophically transmitted parasites with complex life cycles manipulate their intermediate host's antipredatory defenses in ways facilitating their transmission to final host by predation. Some parasites also protect the intermediate host from predation when noninfective during its ontogeny. The acanthocephalan *Pomphorynchus laevis*, a fish intestinal helminth, infecting freshwater gammarid amphipods as intermediate hosts, is using such a strategy of protection- then-exposure to predation by the definitive host. The whole time-course of this sequence of behavioral switch is however not yet known, and only one antipredator behavior has been studied to date. Here we show that the protective part of this manipulation begins early during the parasite ontogeny, in accordance with theoretical predictions, suggesting that the advantages overpass the costs induced by this protective manipulation. We confirmed that the refuge use behavior is showing a switch in the few days following the stage mature for the definitive host (switching from overuse to underuse). However, such a switch was not observed for the gammarids activity rate, a behavior also known to make the host less conspicuous to predators when weak. While we predicted a low activity during early development stages, then a switch to high activity, we observed general decrease in activity during the parasite ontogeny. The possible causes for this discrepancy between behaviors are discussed. All these behavioral changes were observed mostly when animals were tested in water scented by potential predators (here brown trout), suggesting condition-dependent, adaptive, manipulation.

42

Introduction

44 Heteroxenous parasites require successive hosts to complete their life cycle (Poulin, 2007). A successful transfer from one host to another is critical to fitness of such parasites, and
46 numerous parasite species rely on tropical transmission to achieve this goal. In this process, downstream infected host(s) – the intermediate host(s) – must be consumed by upstream
48 host – the definitive host – where the parasite reproduce. Parasite-induced phenotypic alterations (PIPA) may facilitate the completion of parasite's life cycle (Moore, 2002; Poulin,
50 2010). Alterations of host behaviour are particularly frequent in trophically-transmitted parasites, with infected hosts showing behaviors increasing their chances to be predated by
52 their parasite's final host e.g. (Bethel & Holmes, 1977; Moore, 1983; Lagrue et al., 2007), reviewed, for example, in Poulin (2010). These parasites generally alter the antipredator
54 behaviors of their intermediate hosts (Svensson et al., 2022), and some parasites are even able to reverse their intermediate host's fear of predator cues (Berdoy et al., 2000; Perrot-
56 Minnot et al., 2007). These host behavioral changes favoring parasite transmission are seen as an extended phenotype of parasites and are often referred as parasitic manipulation
58 (Hughes et al., 2012).

The understanding of the adaptive significance of PIPA was reinforced by studies highlighting
60 variations in manipulations depending on the ontogeny of the parasite. (Parker et al., 2009) predicted that the trophically-transmitted parasites showing the strongest selective
62 advantage would be those able to protect from predation when not infective to the definitive host and then switch to an increase of exposure to predation when infectious. This
64 phenomenon might have been selected because trophic transmission occurring before the parasite has reached the infective stage would result in zero fitness (Parker et al., 2009).
66 Ideally, then, an optimal temporal sequence of PIPA could consist of a 'protect-then-expose' strategy against predation. Accordingly, reinforced antipredatory behaviors were observed
68 during the early stages of the ontogeny in a number of parasite species (e.g. Hammerschmidt et al., 2009; Médoc & Beisel, 2011; Bailly et al., 2018) and direct testing for
70 predation rate of uninfected versus infected hosts confirmed the existence of this switch in predation rates (Dianne et al., 2011; Weinreich et al., 2013). This phenomenon has been
72 studied in detail in a few model organisms only. An iconic model is the tapeworm

Schistocephalus solidus, where infection in its copepod first intermediate host firstly reduces the host activity and recovery time after a disturbance mimicking a predator attack (Hammerschmidt et al., 2009), resulting in a decrease in predation (Weinreich et al., 2013). This manipulation occurs rapidly after the establishment of the infection within the copepod body (Benesh, 2019). After infectivity for the next host is reached, the behavioral pattern reverses, infected copepods becoming more active and conspicuous to predators (Hammerschmidt et al., 2009). In this system, the intensity of host manipulation varied mainly according to parasite populations, and responded to selection, confirming that this trait can be a parasite extended phenotype (Hafer, 2018; Hafer-Hahmann, 2019).

The other parasite-host well-studied system for switching in behavioral parasitic manipulation is the acanthocephalan *Pomphorhynchus laevis* infecting the amphipod *Gammarus pulex*. Here, the infection by the non-infective stage (the acanthella) protects the host from predation by increasing the rate of refuge use in the amphipod, a trait known to protect gammarids from predation by fish (Abjornsson et al., 2004). A switch then occurs few days after the parasite reached the cyctacanth stage (infective for the next host), exposing the infected host to fish predation (Dianne et al., 2011). In this system, the intensity of behavioral changes depends on parasite strains (Franceschi, Bollache, et al., 2010) or populations (Franceschi, Cornet, et al., 2010; Labaude et al., 2015), suggesting that manipulation is a parasite trait. However, here, a number of data are still lacking to further understand the 'protect-then-expose' strategy. For example, no data are available on the course of behavioral changes during the whole duration of the acanthellae stage. Indeed, measures of refuge use or predation rates were made at the end of this acanthella stage, few days before the parasite reached the infective stage (Dianne et al., 2011, 2014). Therefore, it is not known if *P. laevis* invests in host protection early in its ontogeny, knowing that the development of the acanthella stage necessitates quite a long time, i.e. between 2 and 3 months depending on temperature (Labaude et al., 2020). Parker et al. (2009) suggested that protective manipulation should have evolved more easily if parasite's development time is long. However, a long time spent in a host would necessitate a long investment in protection. Such a trait is perhaps too costly to be maintained during the whole ontogeny (see Dianne et al., 2014) for an example of cost induced by the protective manipulation). The life-cycle of *Schistocephalus solidus*, much shorter (around two weeks

before being infective in the copepod host), probably allows early investment in the protective manipulation (Hammerschmidt et al., 2009). In addition, while it is known that exposure manipulation of gammarids by *P. laevis* is multidimentional, i.e. that multiple traits are changed in the hosts (Perrot-Minnot et al., 2014; Fayard et al., 2020), we still do not know if multiple antipredatory traits are changes during protective manipulation. The activity rate, for example, known to be reduced as an antipredator defense in gammarids should therefore be reduced during the acanthella stage, and then switch to an increase at the cystacanth stage, but this has never been tested. Finally, while Dianne et al. (2011) found that acanthellae forced their hosts to use refuges more intensively only in the presence of predator cues (fish scent), Dianne et al. (2014) found that this protective manipulation in P. *laevis* was observed both in presence or absence of predatory cues, suggesting that the protective manipulation is not condition-dependent. However, again, no data is available on the entire ontogeny of the acanthella.

In this study, we analyzed the behavioral changes induced by *P. laevis* in *G. pulex*, for the first time during the whole ontogeny of parasites. Three main questions were addressed: (i) is the protective manipulation present during early acanthella stages of parasite's larval development? (ii) is this manipulation condition(predator)-dependent during the whole larval ontogeny? (iii) are these two different anti-predatory behaviors affected in the same way by *P. laevis* during its ontogeny? Both the intensity of host refuge use and the host activity were measured after experimental infections and under two conditions: with or without predation threat.

Material and Methods

Animals and experimental infections

Collection, maintenance and infection procedure were as described in (Franceschi et al., 2008). In brief, uninfected *G. pulex* amphipods were collected in February 2012 from a small tributary of the Suzon River, Burgundy, eastern France (47°23'56.19''N, 4°50'31.13''E). Only male gammarids were kept for the experiment. They were acclimated in the laboratory for 3 weeks before experimental infections under a 12:12 light:dark cycle and fed during the experiment with conditioned elm leaves (*Ulmus laevis*). Adult *P. laevis* parasites were

134 removed from the intestine of naturally infected chub (*Leuciscus cephalus*) also caught in
February in the Vouge River, France (47°09'34.36''N, 5°09'02.50''E) and characterized
136 molecularly as described in Franceschi et al. (2008). Parasite eggs were collected from six
females of *P. laevis* sampled from four different fish. Male gammarids were allowed to feed
138 for 48 h on a 1 cm^2 piece of elm leaf, on which about 200 eggs were deposited. A total of 180
G. pulex males were exposed to parasite eggs. After exposure, gammarids were maintained
140 in isolation in cups (6 cm diameter) filled with c.a. 50 mL of aerated water. Twenty control
gammarids were handled and maintained under the same conditions as the exposed
142 gammarids.

144 *Behavioral measurements and survival*

Survival was controlled every day post-exposure, and dead gammarids were dissected within
146 the 24 hours post-death in order to control for parasite presence. Due to small size of
acanthella, we were unable to detect this presence before 20 days (personal observations),
148 so comparison of refuge use was measured every week after the 20th day post-infection.

To measure the rate of refuge use, gammarids were introduced individually into a 10.5 × 16
150 cm rectangular box filled with 330 mL of either control or fish-scented water, with each
gammarid kept in the same water type during the whole experiment. Individuals were
152 randomly assigned to these water treatments. An opaque refuge, consisting of half a
terracotta saucer (8.5 diameter) in which a 1 cm^2 opening was made, was placed at one end
154 of each aquarium, covering approximately 18% of the total aquarium area. Refuge use was
recorded after an acclimation period of 30 min. The gammarid's position in the box was then
156 checked every 5 min over a period of 120 min. A score of 1 was given if the gammarid was
inside the refuge and a score of 0 if it was found outside. For each gammarid, the total score
158 of refuge use therefore ranged between 0 (gammarid always outside the refuge) and 24
(gammarid always inside the refuge). After behavioral measure, the gammarid returned to
160 its cup, where new aerated water (without fish scent) and food were provided.

The activity of infected and uninfected gammarids was recorded the day after the refuge use
162 measurement, using a ViewPoint device and software (©Viewpoint Life Sciences, Inc. –
2010, France). The 20 gammarids unexposed to parasite eggs were not measured for this

trait, and the test was not made at 84 days for technical reasons. Gammarids were placed individually in Petri dishes (diameter 8.5 cm, height 1 cm) filled with either control water or scented water, this water treatment being the same as for refuge use measurements for each gammarid. After 2 min of acclimation, gammarid activity was recorded with an infrared camera for 5 min (continuous recording), as the proportion of time spent inactive. Inactivity was defined as movements at a speed below 10 mm s^{-1}. This speed threshold was determined based on preliminary tests, showing that below 10 mm s^{-1} discrimination is not possible between gammarids moving at very low speed (crawling) and gammarids moving their pleopods for respiration.

To produce predator cues, six young brown trouts (*Salmo trutta*), each ~ 20 cm long, were kept in the laboratory at 15 ± 1 °C under a 12:12 light:dark cycle, in a tank filled with 80 L of tap water previously dechlorinated, oxygenated and UV-treated. Trouts were acclimated for 2 weeks prior to the experiments and fed with a mix of frozen chironomid larvae and alive gammarids in order to strengthen the predation signal (Wudkevich et al., 1997). The water from a similar tank, treated as previously described but without trout scent, was used as a control water for behavioral experiments.

The infection procedure allowed synchronized development of acanthellae. Around 55 days post-exposure, the presence of *P. laevis* acanthellae was unambiguously detectable through the host cuticle (translucent light-orange and shapeless larval stages). Therefore, at that time, all gammarids were assigned to infected or uninfected categories (either dead gammarids showed an infection after dissection, or the infection became detectable in living gammarids). It means that behavioral tests were made in blind for the infection status before 55 days post-exposure, except for the 20 unexposed (uninfected) gammarids. Cystacanth stage was reached ten days later (appearing through the cuticle as small bright orange opaque balls).

Statistical analysis

The survival of gammarids was analyzed using Cox regressions, where the effects of infection status (control vs infected), water type (scented vs control) and their interaction were analyzed.

194 Scores for refuge use and activity rate were analyzed as repeated measures using the
'nparLD' R software package (V 2.1), with the F2 LD F1 model. This function enables
196 nonparametric analyses of right-censured longitudinal data, allowing the decrease in sample
size along time, due to individuals' death (Noguchi et al., 2012). The effects of water type
198 (scented vs control), infection status (control vs infected) and their interaction were
investigated along time. Wald statistics were performed, the statistical analysis of time-
200 series with nparLD being based on rank-order of the observed data (see Noguchi et al., 2012)
for details). We also report Cliff's δ with their 95% confidence intervals (Cliff, 1993) as
202 measures of non parametric effect size for the difference in refuge scores or activity rate
between infected vs uninfected gammarids at each time step, for each water type.

204 All statistical analyses were performed using R version 3.1.1 software (R Foundation for
Statistical Computing, Vienna, Austria) except the Cox analysis made using JMP version
206 10.0.0 software (SAS Institute, Cary, NC, USA).

208 **Results**

We were able to detect unambiguously infections through the cuticle of the hosts at the late
210 acanthella stage around the 55th day post-exposure. Before this stage, parasite infections
were only detectable after dissection of dead individuals. Out of the 175 individuals exposed
212 to parasite eggs, 132 were finally infected (75.4%). Among them, 60 (45.5%) were infected
by only one parasite, 39 (29.5%) were infected by two parasites and 33 (25.0%) were
214 infected by more than two parasites.

The experiment was stopped after 83 days post-infection and all surviving animals were
216 dissected to confirm their infection status and count the parasites. Since exposed-uninfected
animals did not differ significantly from unexposed gammarids for their survival (LR $\chi 2$ =
218 1.2121, 1 d.f., p = 0.2709), they were cumulated to create the uninfected category. Only the
effect of the infection on the survival was statistically supported (Figure 1, Table 1).

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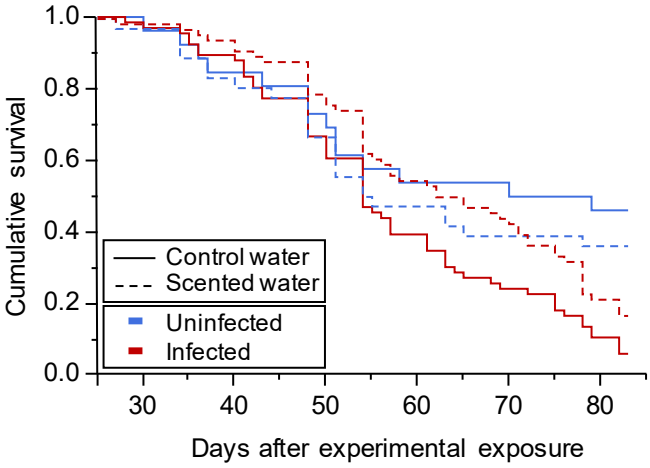

222 **Figure 1:** Cumulative survival of *Gammarus pulex* after experimental exposure to *Pomphorhynchus laevis*, maintained in water that was (or not) signed with fish scent.

224

The uninfected individuals survived better than the infected ones, mainly after the parasites

226 reached the cystacanth stages (Figure 1). The tendency observed on Figure 1 that this survival trend is more pronounced under scented water was almost supported statistically

228 (Table 1).

230 **Table 1:** Effect of water type, infection status by *Pomphorhynchus laevis*, and their interaction on the survival of *Gammarus pulex* individuals.

Source	d.f.	LR $\chi 2$	p-value
Water type	1	0.27949	0.8602
Infection	**1**	**7.27815**	**0.0026**
Water type*infection	1	2.27592	0.0666

232 Whole model: LR $\chi 2$ = 13.3663, 3 d.f., p = 0.0039

234 Among the infected individuals, the infection intensity (i.e. the number of parasites), did not influence significantly the survival (LR $\chi 2$ = 1.1039, 1 d.f., p = 0.2934, if the number of

236 parasites are categorized as “1 parasite” or “more than 1 parasite” ; LR $\chi 2$ = 0.4871, 1 d.f., p = 0.4852 if the number of parasites are considered as continuous factor).

238 Exposed-uninfected animals did not differ significantly from unexposed gammarids for their refuge use at any date (Wilcoxon tests, 0.990 > all p ≥ 0.077). They were therefore

240 cumulated to create the "uninfected" category for further analyses. The refuge use by gammarids varied considerably with time (Figure 2, Table 2), being most of the time
242 moderate at the beginning of our survey, becoming progressively more intensive until reaching high values around 30 to 40 days.

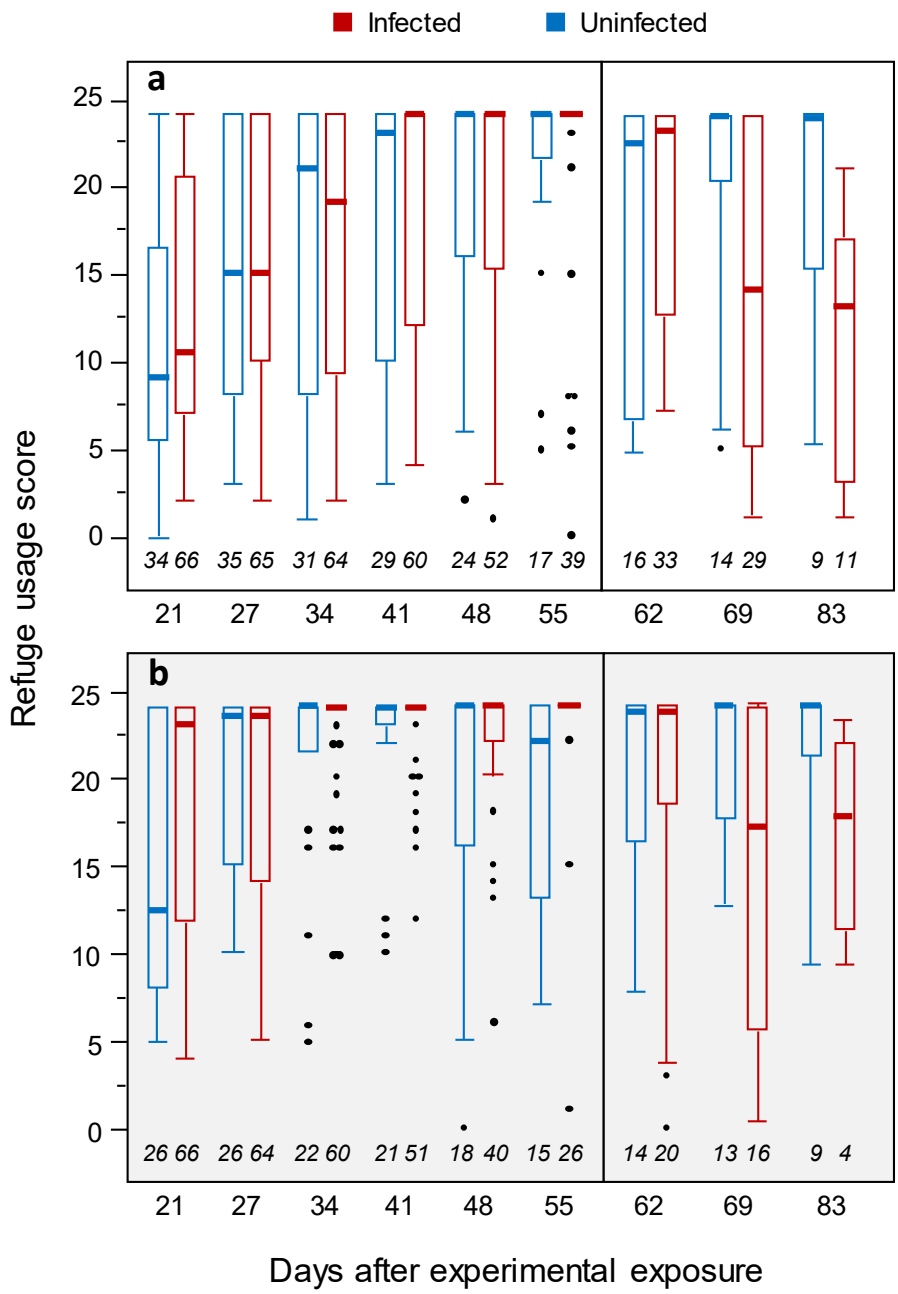

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Figure 2: Scores of refuge use by gammarids, according to their infection status by *Pomphorhynchus laevis*,
246 between day 21 and day 83 post-experimental exposure to parasite eggs. Gammarids were tested in control water (a), or water signed with fish scent (b). Scores range from 0 (individuals always outside the refuge) to 24
248 (individuals always inside the refuge). Thick lines are the medians, boxes are the upper and lower quartiles, whiskers are the upper and lower 1.5*interquartile range, and dots represent outliers. Sample sizes are given in
250 italics below each plot.

252 This phenomenon was found whatever the infection status and the water type, but was found to be more rapid under scented water than control water (Figure 2), the interaction
254 between water type and date being statistically significant (Table 2). The interaction between date and infection was also highly significant (Table 2).

256

Table 2: Results of the model from the nparLD analysis, testing for the effects of water type (scented vs.
258 control), infection status (infected with *P. laevis* vs. uninfected) and date of measurement on the scores of refuge use by *G. pulex* individuals.

	Wald statistics	df	P-value
Water type	**7.8568**	**1**	**0.0051**
Infection	0.9645	1	0.3261
date	**107.5206**	**8**	**< 0.00001**
Water type * Infection	0.4299	1	0.5120
Water type * date	**25.4258**	**8**	**0.0013**
Infection * date	**57.1303**	**8**	**< 0.00001**
Water type * Infection * date	7.1682	8	0.6030

260

262 This reflects the observation that, after the cystacanth stage being reached, the refuge score became weak for infected individuals, while during the acanthella stage, the tendency was
264 that infected individuals used more refuges than uninfected ones (Figure 2). This interpretation is reinforced by the analysis of the effect sizes, but here a dichotomy must be
266 made between scented and control water. Indeed, at acanthella stage, under non-scented water, the difference in refuge scores between infected and uninfected animals was always
268 close to zero, indicating negligible effect of the infection (Figure 3). Under scented water, however, the values of δ were often between 0.11 and 0.4, i.e. a range where effect sizes are
270 considered as small to medium (Vargha & Delaney, 2000; Kraemer & Kupfer, 2006). After the cystacanth stage was reached, and here whatever the type of water, the effect size reversed,
272 becoming negative, and can be considered as large, with values of δ exceeding -0.4 (Figure 3).

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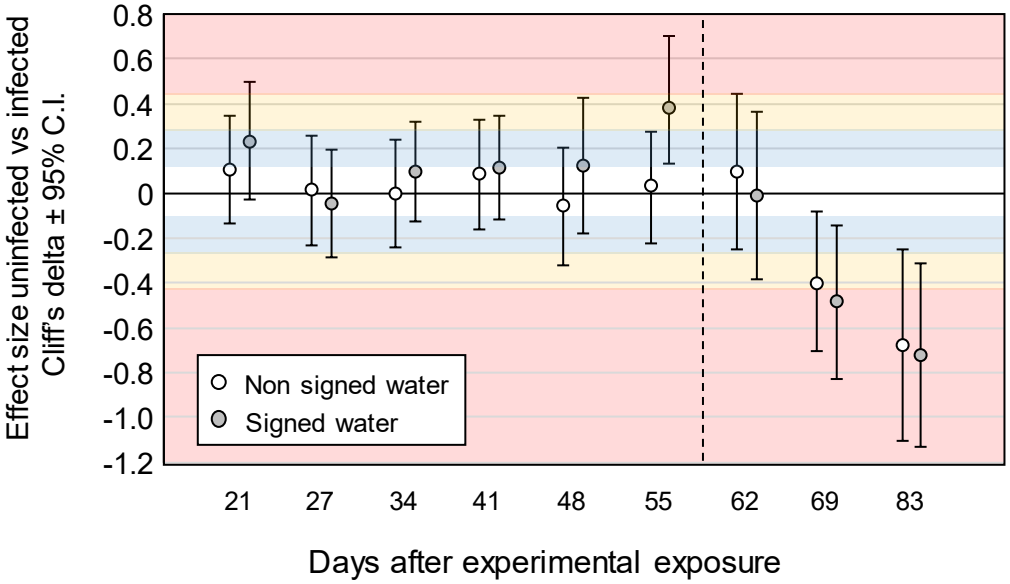

Figure 3 : Effect sizes of refuge use of *Gammarus pulex* infected with *Pomphorhynchus laevis* compared to uninfected individuals, according to the date when the tests were made, separated by the water type in which they were tested. Points represent Cliff 's δ and error bars represent 95% confidence intervals. Positive values mean that the uninfected individuals used the refuge more than infected individuals, and negative values mean a lower use. The white, blue, yellow and red colors corresponded to areas where the effect size are considered as trivial, small, medium and large, respectively, using the comparisons between Cliff 's δ and Cohen's d effect size made by (Kraemer & Kupfer, 2006). These areas must be seen as indicative. The dotted line denotes the transition between acanthella and cystacanth stages.

The effect of infection intensity was explored in infected animals only for the two dates where the analysis above suggested reversed effects of the infection, and where sample size was high enough to guarantee reasonable power, i.e. at days 55 and 69 post-exposure. At day 55, no effect of parasite number was found on refuge use, neither at day 69. Indeed, after categorization as “1 parasite” and “more than 1 parasite”, Wilcoxon tests were no significant ($z = -0.8144$, $p = 0.4097$ and $z = -1.3688$, $p = 0.1675$, respectively). The same result was found if parasite number was treated as a continuous factor since the correlations between parasite number and refuge use scores were non-significant for the two dates (Spearman’s $\rho = 0.0595$, $p = 0.6379$, $n = 65$ and Spearman’s $\rho = 0.2333$, $p = 0.1229$, $n = 45$, respectively).

The analysis of levels of activity revealed more time spent inactive in infected gammarids compared to uninfected ones, but also an increased inactivity according to time (Figure 4, Table 3). However, this trait was stronger in fish-scented water, as revealed by the significant interaction between water type and date (Table 3, Figure 4).

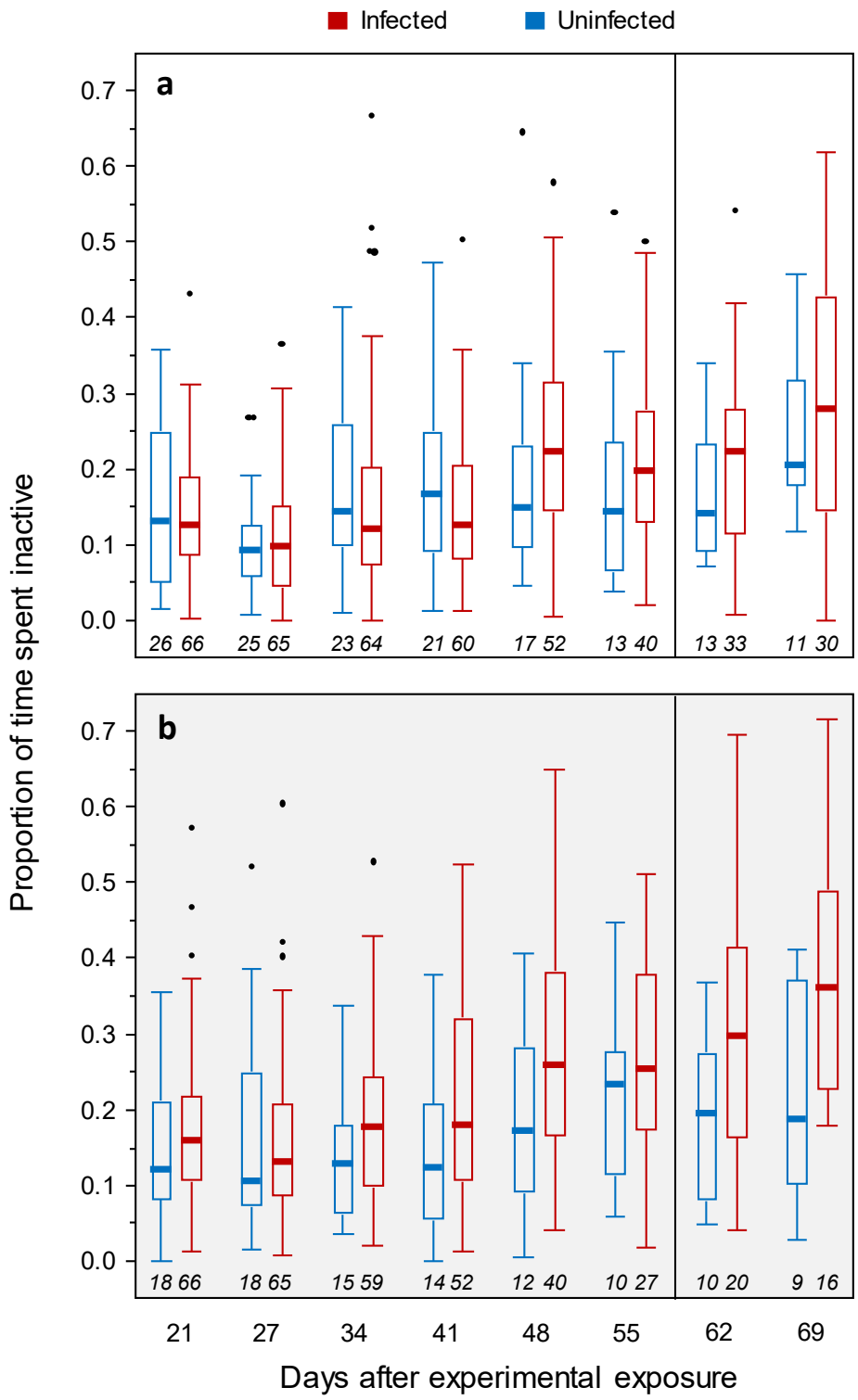

Figure 4: Proportion of time spent inactive in gammarids, according to their infection status by *Pomphorhynchus laevis*, between day 21 and day 69 post experimental exposure to parasite eggs. Gammarids were tested for 5 minutes in control water (a), or water signed with fish scent (b). Thick lines are the medians, boxes are the upper and lower quartiles, whiskers are the upper and lower 1.5*interquartile range, and dots represent outliers. Sample sizes are given in italics below each plot.

Table 3: Results of the model from the nparLD analysis, testing for the effects of water type (scented vs. control), infection status (infected with *P. laevis* vs. uninfected) and date of measurement on the activity rate of *G. pulex* individuals.

	Wald statistics	df	P-value
Water type	3.3850	1	0.0658
Infection	**6.1807**	**1**	**0.0129**
date	**85.7227**	**7**	**< 0.00001**
Water type * Infection	2.5109	1	0.1131
Water type * date	**14.5811**	**7**	**0.0418**
Infection * date	6.3569	7	0.4987
Water type * Infection * date	13.3344	7	0.0644

In addition, the triple interaction (water type*infection*date) was close to significance (Table 3), suggesting that this increase with time is not the same between infected and uninfected animals. This hypothesis is reinforced by the analysis of the effect sizes (Figure 5), where the effect sizes of the infection on gammarids activity were stronger in scented water, with a general trend to increase with time.

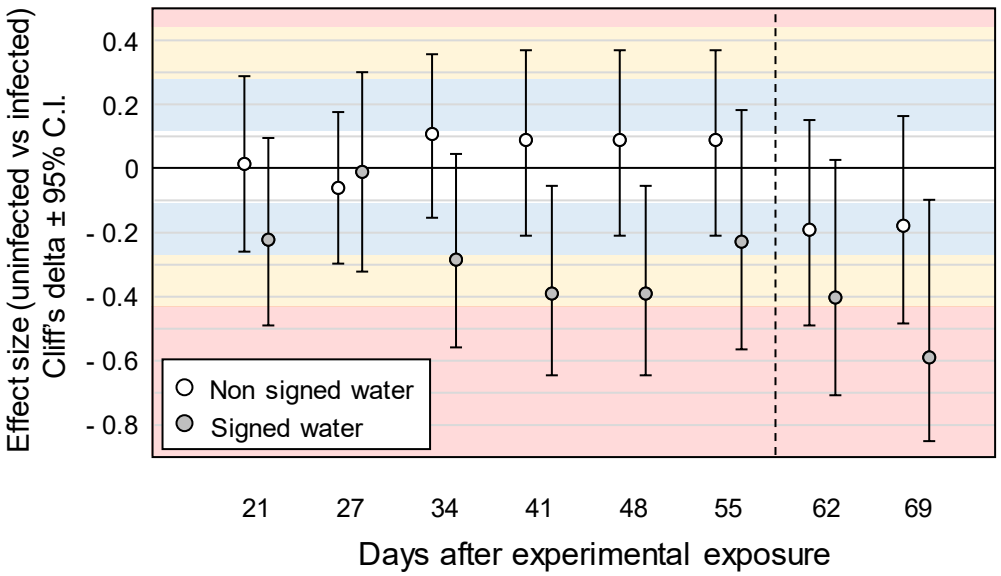

Figure 5 : Effect sizes of time spent inactive in *Gammarus pulex* infected with *Pomphorhynchus laevis* compared to uninfected individuals, according to the dates when the tests were madeand the water type. Points are Cliff 's δ and error bars represent 95% confidence intervals. Negative values mean that the infected individuals are more inactive than uninfected animals. The white, blue, yellow and red colors corresponded to areas where the effect size are considered as trivial, small, medium and large, respectively (see Figure 3). The dotted line denotes the transition between acanthella and cystacanth stages.

Discussion

Our longitudinal survey of antipredatory behaviors in *Gammarus pulex* infected by *Pomphorhynchus laevis*, made in repeated measures, first confirmed previous observations that a shift occurred in refuge use between acanthella and cystacanth stages, i.e. between parasite stages non-infective and infective for the next host in the cycle (Dianne et al., 2011, 2014). This shift was nevertheless not observed for the activity rate, another behavior involved in predation avoidance. In addition, our study brings new information on this anti-predatory behavior.

First, confirming observations of Dianne et al. (2011), we found a difference in the 'protective' behavioral patterns of refuge use and activity rate according to the presence or absence of predatory cues. Indeed, while almost no difference between infected and uninfected animals was found when tests were made in control water, as seen using effect sizes, *G. pulex* infected by acanthellae use more the refuges and are more inactive than uninfected ones in fish-scented water. Fish scent is definitely a source of stress for gammarids: they generally increase their anti-predatory behavior when they perceive predatory cues (e.g. they are repulsed by the chemical cues originating from fish (Perrot-Minnot et al., 2007) and reduce their activity (Andersson et al., 1986; Bollache et al., 2006). The infection by acanthellae would therefore strengthen this behavior. In another acanthocephalan parasite, *Polymorphus minutus*, the protective behavior has been observed in absence of predatory cues (Bailly et al., 2018). This parasite nevertheless shows a different nature of behavioral changes, probably in link with the difference in life cycle (*P. minutus* is using birds as definitive hosts), with probable different mechanisms involved (Tain et al., 2006; Perrot-Minnot et al., 2016).

The second information is that this protective manipulation was observed – in scented water – early during parasite ontogeny (as soon as 20 days post-infection). This means that reinforcing the host's antipredatory behavior is important for the life cycle of the parasite and outweighs the potential physiological cost of inducing this behavior, as predicted by (Parker et al., 2009). For refuge use, this protective manipulation tend to increase as acanthellae are developing: the effect size was larger at 55 days than during the preceding days of test. Parasites often secrete products that have the potential to be involved in host behavioral change (Berger et al., 2021, and references therein). Therefore, the increase of

protective manipulation with time observed in our study may suggest that the cause for this behavioral change could be the accumulation during ontogeny of products excreted from *P. laevis*. However, this protective manipulation was not constant with time since we observed variation (in scented water) on the differences between infected and uninfected gammarids: the effect size oscillate between negligible values to medium values. This variation may be due to the fact that uninfected gammarids did not use refuges constantly through time, a phenomenon that may interfere with the parasite's manipulation. Indeed, from day 21 to day 55, there was a general trend of *G. pulex* using refuge more intensively as time is passing, and this independently from their infection status. After four behavioral measurements, all gammarids spent most of their time under the refuge, and this phenomenon is more intense in scented water. Gammarids, like other crustaceans are sensitive to stress, and one response to stress is to increase the intensity of anti-predatory behaviors (Fossat et al., 2014; Perrot-Minnot et al., 2017). It has been observed in shrimps that regular handling increase the stress and modify the animal's behavior (Takahashi, 2022). Therefore, we may infer that our repeated measurements are sources of stress for gammarids, which is not surprising owing the experimental procedure used here. Indeed, individuals were isolated for several weeks (gammarids are gregarious animals and isolation have consequences on their behavior, e.g. (Labaude et al., 2017) and were regularly handled to take them out of their isolation box and transfer them to a new environment (the aquarium or petri dish for behavioral tests). The observation that this phenomenon is more rapid under predatory cues is not surprising since predators are a major, additional, source of stress. Whatever its mechanistic basis, this general increase refuge use interferes with our ability to observe behavioral differences between gammarids infected or not by acanthellae, because it becomes less easy to find a difference in refuge use when this usage is already intense. Indeed, the difference between infected and uninfected animals was stronger in the first trial (21 days) where stress was probably lower than in following trials (27 to 48 days). After the parasite reached the cystacanth stage, we observed the shift in refuge use, and infected *G. pulex* are using the refuge less intensively than uninfected animals, as repeatedly observed in cases of infection with *P. laevis* (Franceschi, Bollache, et al., 2010; Dianne et al., 2011; Bauer & Rigaud, 2015; Fayard et al., 2020). The effect size of this exposure manipulation appeared higher than the one of protective manipulation in the present experiment, which was not the case in Dianne et al. (2011). However, in this later study, only

one time point at acanthella and cystacanth stages each were measured, and the acanthella time-point was the one closest to cystacanth stage. If we compare only the absolute values of effect size at 55 and 69 days in our study, in the scented water, we will find no clear difference. However, by considering the whole ontogeny, it seems that the effect size of manipulation is greater at cystacanth stage than acanthella stage. Poulin (2010) noted that the relationship between the intensity of behavioral manipulation and the increased of predation rate (supposed to be due to manipulation) was not always straightforward, so we cannot really conclude about the efficiency of protection and exposure in our parasite-host system. However, since increase in mortality was mainly observed around the cystacanth stage being reached (see also Labaude et al., 2020), it would make sense that the exposure manipulation is stronger than the protective manipulation because the selective pressure of reaching the definitive host is strong at that stage.

The third information of our study is that, as predicted, a second antipredatory behavior, namely the activity rate, was also strengthened by infection during acanthella stage. Here again the differences in activity rate between infected and uninfected hosts were found mostly under predatory threat. In control water, such a difference was found only after cystacanth stage was reached. However, and contrary to predictions, it is noticeable that no shift in behavioral modification was observed for this behavior between acanthella and cystacanth stages, as the one observed for refuge use. On the contrary, the infected gammarids were less active at cystacanth stages than at acanthella stages. Two alternative and exclusive hypotheses may be proposed for this observation. The first one is that the decreased activity rate in infected animals is a non-adaptive pathogenic byproduct of the infection, as sometimes suggested for some behavioral changes induced by parasites (Levri, 1999; Cézilly et al., 2010). Under this hypothesis, the progressive decrease according to time could just be interpreted as the long time spent by parasite in the host, decreasing progressively its mobility capacity. Such a hypothesis nevertheless does not explain why such a byproduct of the infection is not observed during acanthella stage in control water. The second hypothesis would be to consider that decreasing activity is adaptive for the parasite at both stages of development. During acanthella stage, this low activity rate would make the gammarid less conspicuous towards predators, as classically interpreted (Williams & Moore, 1985; Bollache et al., 2006). Notably, high rate of activity allows gammarids to

initiate swimming and consequently enter the river current (drift) where predators are present (Williams & Moore, 1985; Schaffer et al., 2013). During the cystacanth stage, the animal's tendency to search light (Franceschi et al., 2008) and avoid refuges (this study, Dianne et al., 2011) would already bring them into the river drift (McCahon et al., 1991; Lagrue et al., 2007). A low level of activity would therefore accentuate the predation exposure by reducing the swimming capacity of gammarids, which could be easily transported passively by the current of the river. Indeed, Lagrue et al. (2007) observed that the presence of *G. pulex* in the river drift is increased by 25-30 times when infected by *P. laevis*, the very same proportion of gammarids being found in the stomach of Bullhead predators. We could have here an example of parasite's manipulation of a single host behavior that could be beneficial at all parasite stages, a characteristic that could have facilitated its selection.

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