Peer Community In Zoology

Exploring manipulative strategies of a trophically-transmitted parasite across its ontogeny

Thierry Lefevre *based on peer reviews by* ***Adèle Mennerat*** *and 1 anonymous reviewer*

Thierry Rigaud, Aude Balourdet, Alexandre Bauer (2023) 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. bioRxiv, ver. 6, peer-reviewed and recommended by Peer Community in Zoology. https://doi.org/10.1101/2023.04.25.538244

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The intricate relationships between parasites and their hosts often involve a choreography of behavioral changes, with parasites manipulating their hosts in a way that enhances - or seemingly enhances – their transmission (Hughes et al., 2012; Moore, 2002; Poulin, 2010). Host manipulation is increasingly acknowledged as a pervasive adaptive transmission strategy employed by parasites, and as such is one of the most remarkable manifestations of the extended phenotype (Dawkins, 1982).

In this laboratory study, Rigaud et al. (2023) delved into the time course of antipredator behavioral modifications induced by the acanthocephalan P*omphorhynchus laevis* in its amphipod intermediate host *Gammarus pulex*. This system has a good foundation of prior knowledge (Bakker et al., 2017; Fayard et al., 2020; Perrot-Minnot et al., 2023), nicely drawn upon for the present work. This parasite orchestrates a switch from predation suppression, during the noninfective phase, to predation enhancement upon maturation. Specifically, *G. pulex* infected with the non-infective acanthella stage of the parasite can exhibit increased refuge use and reduced activity compared to uninfected individuals (Dianne et al., 2011, 2014), leading to decreased predation by trout (Dianne et al., 2011). In contrast, upon reaching the infective cystacanth stage, the parasite can enhance the susceptibility of its host to trout predation (Dianne et al., 2011).

The present work aimed to understand the temporal sequence of these behavioral changes across the entire ontogeny of the parasite. The results confirmed the protective role of *P. laevis* during the acanthella stage, wherein infected amphipods exhibited heightened refuge use. This protective manipulation, however,

became significant only later in the parasite's ontogeny, suggesting a delayed investment strategy, possibly influenced by the extended developmental time of *P. laevis*. The protective component wanes upon reaching the cystacanth stage, transitioning into an exposure strategy, aligning with theoretical predictions and previous empirical work (Dianne et al., 2011; Parker et al., 2009). The switch was behavior-specific. Unlike the protective behavior, a decline in the amphipod activity rate manifested early in the acanthella stage and persisted throughout development, suggesting potential benefits of reduced activity for the parasite across multiple stages. Furthermore, the findings challenge previous assumptions regarding the condition-dependency of manipulation, revealing that the parasite-induced behavioral changes predominantly occurred in the presence of cues signaling potential predators. Finally, while amphipods infected with acanthella stages displayed survival rates comparable to their uninfected counterparts, increased mortality was observed in those infected with cystacanth stages.

Understanding the temporal sequence of host behavioral changes is crucial for deciphering whether it is adaptive to the parasite or not. This study stands out for its meticulous examination of multiple behaviors over the entire ontogeny of the parasite highlighting the complexity and condition-dependent nature of manipulation. The protective-then-expose strategy emerges as a dynamic process, finely tuned to the developmental stages of the parasite and the ecological challenges faced by the host. The delayed emergence of protective behaviors suggests a strategic investment by the parasite, with implications for the host's survival and the parasite's transmission success. The differential impact of infection on refuge use and activity rate further emphasizes the need for a multidimensional approach in studying parasitic manipulation (Fayard et al., 2020). This complexity demands further exploration, particularly in deciphering how trophically-transmitted parasites shape the behavioral landscape of their intermediate hosts and its temporal dynamic (Herbison, 2017; Perrot-Minnot & Cézilly, 2013). As we discover the many subtleties of these parasitic manipulations, new avenues of research are unfolding, promising a deeper understanding of the ecology and evolution of host-parasite interactions.

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Reviews

Evaluation round #2

DOI or URL of the preprint: https://doi.org/10.1101/2023.04.25.538244
Version of the preprint: 5

Authors' reply, 09 November 2023

Dear Thierry

You'll find attached our reply to the last round of review, for which we would like to thank you and the reviewers. Our comments are underligned in yellow in the attached document "reply to reviewers R2", and the ms has been corrected according to all suggestions (a version with tracked changes is attached). The new version plus the supplementary file are on line in BioRxiv

Yours sincerely

Thierry Rigaud for all authors

Download author's reply

Download tracked changes file

Decision by Thierry Lefevre, posted 07 November 2023, validated 07 November 2023

Dear Thierry and co-authors,

The two reviewers and I are satisfied with the revisions you have made. Your efforts in addressing the concerns raised by the reviewers and conducting additional analyses are greatly appreciated. Before I proceed

to write the recommendation, could you please address the minor additional remarks raised by Adèle, as well as the following points:

- Abstract: Please consider replacing ”with or without predation threat” with ”with or without predation cues” since the risk of predation was non-existent (the predator was absent but the water was scented with the predator).
- Line 152: Consider changing ”caught in February in the Vouge River” to ”caught in February 2012 in the Vouge River.”
- Lines 166-169: The provided sentence outlines the start and end of the experiment but does not specify the frequency at which measurements for both behaviors were recorded. While this will be evident in the results section (on the x-axis in Figures 2 and 3), it would be helpful to mention the recording frequency for these two traits here or in the paragraphs describing the procedure for measuring these traits.
- Line 159 mentions ”Twenty control gammarids.” In lines 170-188 (description of the procedure for refuge use measurements), does this mean that ten of these control individuals were tested with scented water and ten with unscented control water? Consider adding this information.
- Line 200: Consider removing the second part of the sentence, ”...and the test was not made at day 83 for technical reasons.” This is already mentioned earlier that ”The experiment was stopped after 83 days post-exposure.” Additionally, it’s essential to clarify and standardize the terminology used to describe the three categories of gammarids: (i) infected individuals vs. (ii) unexposed-control individuals vs. (iii) exposed-uninfected individuals throughout the manuscript (also refer to the comment from referee 1). In this particular sentence (line 200), there is a double confusion: the term ”gammarids unexposed to parasite eggs” is not the same as the one used in line 159: ”Twenty control gammarids.” Furthermore, it contradicts the beginning of this paragraph, which states: ”The activity of infected and uninfected gammarids was recorded the day after the refuge use measurement.” At this stage in the manuscript, the ”uninfected gammarids” logically refer to the ”20 unexposed control gammarids” as it is not yet mentionned that some exposed individuals remained uninfected. Yet the end of this paragraph states (lines 200) that ”The 20 gammarids unexposed to parasite eggs were not measured for this trait”. To avoid this confusion consider replacing ”uninfected gammarids” in this sentence with ”exposed-uninfected individuals” to make the distinction with the unexposed-controls.
- Line 228: The sentence ”The effects of water type (scented vs. control), infection status (control vs. infected), and their interaction were investigated along time” is a bit confusing given that unexposed-control and exposed-uninfected specimens were pooled together. Should this sentence, therefore, read ”uninfected” instead of ”control”?

Best wishes,

Thierry

Reviewed by **Adèle Mennerat**, 06 November 2023

PCI Zoology #215

Review, round 2 – comments to authors

Dear authors,

First of all, thanks for your thorough revision and patient clarifications. The paper reads very well now, and I have no further general comments (good job clarifying the intro and discussion!) - just a few minor ones.

L45: critical to fitness of such parasites -> critical to the fitness of such parasites
L133: remove parenthesis before Franceschi, move it to before 2008
L286: whatever -> regardless of
L327: to increase clarity, add “with” before “significantly”
L328: ”increased activity according to time” could be changed to “increased activity with time”

L456: rephrase to: “Such hypotheses nevertheless explain neither why…, nor… “
L477: to avoid repetition of the word “behavior”, consider rephrasing to e.g. “the first component of the ‘protective’ behavioral manipulation”
L489, consider rephrasing to “while high and low refuge use are beneficial to the acanthella and cystacanth stages, respectively.”

Thanks again for your answers!

Reviewed by anonymous reviewer 1, 21 October 2023

I have meticulously reviewed the revised version of the manuscript, the supplementary material, and the authors’ response to the reviewers. I genuinely appreciate the effort with which the authors addressed and integrated our feedback. This is a very nice study.

In my previous review, I had suggested rerunning the experiment to validate the results. I recognize the inherent challenges and constraints in doing so. My initial recommendation stemmed from the ambiguity and some over-interpretations present in the first version. In this revised manuscript the authors prudently focused on their more robust findings. I am now more confident about the replicability of the strong effects observed in this study.

That said, I’d like to point out some lingering inconsistencies, notably with the terms “not infected” and “uninfected.” For instance, I think that it can be observed in figure 1 and possibly on line 228. It would be good to go through the manuscript for that, since this detail created a fair amount of confusion for me in the earlier version.

To address further things that I pointed out before, and to go in the sense of the authors. The data, now in supplementary material, unambiguously indicates that both the exposed-non infected and non-exposed groups exhibit the same mortality rate. It is also reasonable to conclude that the behaviour of exposed-non infected individuals doesn’t significantly differ from their non-exposed counterparts in a meaningful way. Therefore, it is justified to combined them in the analyses.

Lastly, I found the supplementary material to be a valuable complement to the manuscript. The French annotations in the code are, I believe, acceptable but it may be a decision of the journal.

Evaluation round #1

DOI or URL of the preprint: https://doi.org/10.1101/2023.04.25.538244
Version of the preprint: 2

Authors’ reply, 04 October 2023

Download author’s reply
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Decision by Thierry Lefevre, posted 13 July 2023, validated 13 July 2023

Dear Dr. Rigaud,

You preprint has now been read and assessed by two independent peers who both point out a lot of merit and value in the work. I also enjoyed reading the manuscript and thought these experiments provide a neat novel body of work. However, both reviewers suggest a number of changes that would make this work stronger and clearer to the scientific community. These two reviews provide valuable insights and are complementary: while the anonymous reviewer provides a list of key points regarding the material & methods, and the results

sections, Adèle (who signed her review) suggests a number of concrete improvements for the introduction and discussion sections.

Specifically, the anonymous reviewer questions the validity of the results because of putative problems with the experimental setup and analyses, including the temporal pseudoreplications with repeated observations on the same individuals, the lack of clear distinction between “uninfected controls” and “exposed-uninfected” individuals, and concerns regarding the ability to effectively distinguish the parasite and accurately categorize/assign individuals during the early stages of parasite development). I agree with her/him that the distinction between ”exposed-uninfected” and ”unexposed controls” individuals can sometimes be misleading (e.g., line 192, see also one of Adèle’s comment). I suggest that the authors give further consideration to this distinction (unexposed controls vs. exposed-uninfected) throughout the text, in the statistical analyses, and in the figures (the authors may choose to present this distinction in the main figures or produce additional supplementary figures illustrating the distinction between the 3 groups: exposed-infected vs. exposed-uninfected vs. unexposed controls). Similarly, more details about the statistical analysis would be required (an R script would be great along the already available raw data on the repository).

I also found the improvement recommendations put forward by Adèle regarding the introduction and discussion sections to be highly relevant. I would therefore encourage you to revise and resubmit your preprint to PCI Zoology together with point-by-point replies to each of the reviewers’ comments. Please indicate in each reply where changes were made in the manuscript. Once your revision is received, we will contact both reviewers for their views on whether their concerns have been adequately addressed.

Finally, below are some minor comments of my own.

Yours sincerely, Thierry Lefèvre

———————————————–

- Line 86. Ref Abjornsson et al. 2004 is miscited here (although interesting it has nothing to do with parasites protecting their hosts), shouldn’t it be one of Diane et al. paper instead?
- Line 108: changed instead of changes (but see one of Adèle’s comment requesting a more profound rephrasing here)
- Line 109: “known to be reduced as an antipredator defense in gammarids” (REF). Consider adding a ref after “gammarids”.
- Line 113: “P” should be italicized
- Line 117 “behavioural changes”. Consider recalling the two behavioral traits namely “refuge use and activity rate” in parenthesis
- Line 121: “are these two different anti-predatory…”. I assume that the word ”these” refers to ”activity rate and shelter use”, but ”activity rate” has not yet been mentioned in this paragraph so it’s not clear what ”these” refers to here.
- Line 134 The use of the word ”also” here implies that the parasite was collected from the same river as the gammarids. This would indeed be relevant, as it would indicate the use in the laboratory of a sympatric combination that has coevolved in the field. However, line 130 states that the gammarids were collected from a branch of the Suzon, while line 134 states that the parasites were collected from the Vouge. Is the Vouge the name of the branch of the Suzon mentioned in line 130?
- Line 148: “every week”. Consider rephrasing to make it clear how many times this trait was measured e.g. “…measured once a week from day 21 to day 83”.
- Line 164: You meant day 83 instead of 84? This sentence is confusing (see also one of Adèle’s comment)
- Line 186: This is a bit confusing as you wrote at lines 163 that the activity rate of the 20 unexposed gammarids were not measured?
- Line 211: “175 individuals”. Shouldn’t this read 180 (see line 138).
- Line 227: “under scented water”. Could it possibly be “under control water” instead? Please double check and consider rewording this sentence, I found this sentence confusing.
- Line 237 the variable “number of parasites” is a discrete numerical (and not continuous) factor.

- Caption of figure 2: specify what the vertical black line represent on the figure (acanthella à cystacanth transition?)
- Line 265: “but here a dichotomy must be made” unclear. Figure 2 also makes the distinction between scented and control water.
- Line 269: I assume you meant -0.11 and -0.4 (instead of 0.11 and 0.4)?
- Line 272: becoming negative should read “becoming positive”, right?
- Line 290: “were not” instead of “were no”
- Line 292: discrete numerical variable
- Line 315: “general trend to decrease” instead of “increase”?
- Line 330: “involved in predation avoidance”. Please cite a ref here.
- Line 335-336: consider deleting “as seen using effect sizes” (Figure 2 also shows this).
- Line 400: “was also strengthen by infection”. Please rephrase as the results indicate the contrary i.e. infected individuals are less active.

Reviewed by **Adèle Mennerat**, 12 July 2023

Download the review

Reviewed by anonymous reviewer 1, 06 July 2023

In this study, Rigaud et al. are investigating the manipulation of a host (a shrimp) by a parasite (a helminth) throughout the parasite’s development. This parasite has a complex life cycle and manipulates its intermediate host to increase its chances of being consumed by its final host, a fish. This behavior is well-documented and is employed by many parasites as a strategy. The current hypothesis is that the parasite initially protects its host from predators before exposing them to continue its life cycle. However, the timing of this shift in behavior is not known and is the subject of this study. Additionally, the study explores the effect of predator scent in the water, which personally, I found very intriguing and it brings an interesting perspective to the research.

I enjoyed reading the introduction, but I am not up to date with the literature on the topic. I will entrust the author, the editor, and possibly other reviewers to better judge if something is missing.

Overall, I would conclude that it is a nice and interesting study. However, to ensure the validity of the conclusions, I would recommend repeating the experiment once more, ideally with an unexposed group for comparison. It is surprising that several outcomes changed once the infection could be clearly characterized (strong example being the survival). I hope my concern is evident in the comments I provided below. I wish the study had compared individuals exposed to the pathogen with individuals who were non-exposed (possibly discarding or analyzing individuals who resolved the infections in a separate category). I am confident that the effect would be clearer when compared to the unexposed group. The authors acknowledged that multiple handling could have influenced their conclusions. In a repeated experiment, they could use a new group for each day of experiments, ensuring that each individual is handled only once. Without such a repeat, I would exercise caution, as most confidence intervals largely overlap for many of the claims, and refrain from delving too deeply into the details, such as the effect observed in the early days.

Major comments:

* There is a need for improved clarity regarding the distinction between uninfected individuals who were exposed but did not contract the infection. While the authors have been cautious in their terminology by using ”uninfected” and ”infected,” it is commonly assumed that uninfected individuals represent control subjects who were not exposed at all. To address this confusion, it is advisable to explicitly state this distinction in the text legends of the figures. For example, using labels such as ”exposed, uninfected” versus ”exposed, infected” (as done on line 238 for behavioural assays) would help clarify the terminology. Additionally, including a control

group in the survival graphic would be beneficial to enhance clarity. This inclusion would help explain why ”uninfected” individuals exhibit similar mortality rates to ”infected” individuals early in the experiment.

Furthermore, there is a concern regarding the certainty of categorizing individuals as uninfected during the early stages of infection, especially when the acanthellae are still small and not easily detectable. Although it is mentioned that Gammarus specimens were dissected upon death (line 184), it remains unclear why all treatment groups exhibit comparable mortality rates until the acanthellae become detectable through the cuticula. At that point, the uninfected individuals stop dying. Is it possible that many infected individuals died early, but their acanthellae went undetected? This uncertainty emphasizes the importance of including an unexposed control group in the figure. While it may be regrettable that only 20 individuals were included in the unexposed control group, it would still provide valuable insights, especially if there were no natural deaths throughout the course of the experiment. Please note that I agree that the non-exposed group should not be included in the survival analysis.

* It is a bit tricky to use a non-parametric test to justify the fact that exposed-uninfected individuals are not statistically different from unexposed gammarids in terms of their refuge use. If the result had been significant, it would have been more convincing. However, the opposite outcome could indicate a lack of statistical power. It is difficult to assess this because the data supporting these findings are not shown. I wonder why the parameter was not included in the model. By including variables for ”infected” versus ”uninfected” and ”exposed” versus ”non-exposed,” the analysis would be more accurate and not necessarily more complicated. I may be mistaken in my assessment, but I feel that this could have very important consequences on the whole study and I am afraid that it is hiding some of the effect/biology.

Furthermore, I strongly recommend making Figure 2 and 4 wider and displaying the data points within the box plots. There are several arguments to support the inclusion of the data points. One simple reason is that a box plot with only four individuals does not provide much information (see Figure 2, day 83, infected). Additionally, this would be an opportunity to color-code the data points based on whether the individuals were exposed or not.

* Throughout the manuscript, it should be acknowledged by using more cautious wording that the major results concerning the difference between infected and uninfected individuals in scented versus control water are based on trends or tendencies. Despite the substantial overlap in the 95% confidence intervals, they have been disregarded.

* Lines 333- 336: “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”.

This is part of the discussion, and I do not see where the conclusion on refuge use comes from. It is important to note that there is no significant statistical interaction between infection status and water type (Figure 2), and Figures 3 and 5 do not provide evidence supporting this conclusion. Table 2 could support the idea that the strategy regarding the use of refuge over time depends on the infection status and water type since both the interactions ”date x infection status” and ”date x water type” are significant. However, the triple interaction is clearly not significant, which suggests that the strategy of using the refuge over time is not different between infected and uninfected individuals for different water types. If we disregard the statistics and focus on Figure 3 (including the 95% confidence intervals, which serve as graphical statistics), we can observe a tendency at day 55 regarding the use of refuge, with infected individuals showing increased refuge use. However, this is strangely the time point at which the parasite can be reliably detected. Was the difference hidden before because the detection was not reliable?

* line 347: “The second information is that this protective manipulation was observed – in scented water – early during parasite ontogeny (as soon as 20 days post-infection).”

Then, line 357: “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.”

The author acknowledges the inconsistency in their results but only attempts to explain it through biology. While I appreciate the amount of work done, it is important to recognize that the experiment has only been conducted once. Therefore, it should not be ignored that the reason for the variation may be that the effect is minimal or not present. Although there is a trend at 21 days, the confidence intervals largely overlap, and the effect disappears at 27 days. Why would day 21 be the correct day while 27 might be an artifact due to handling?

Note, that I could agree with this “artifact” explanation, but it is one among several possibilities. Overall, most confidence intervals overlap significantly with zero (indicating no effect) and even more so with the other condition used for comparison (i.e., the water type). Therefore, I would recommend repeating the experiment to confirm the finding or to exercise caution in interpreting the results.

Minor comments:

* Generally speaking, I would recommend including a concise conclusion in every figure legend. Many readers tend to focus primarily on the figures rather than reading the paper thoroughly, so it is important to ensure that the key message you want to convey is understood from there.

* The interaction between water type and infection status shows borderline significance in the survival analysis, but the tendency reveals a clear reversal that should not be ignored. This could be attributed to the relatively low sample size for the control group (175 - 132 = 43 exposed uninfected and 20 non-exposed).

Moreover, the analysis itself is likely a contributing factor. It appears that the current Cox regression performed may not be entirely appropriate for this situation. Cox models assume that the hazard ratio (HR) remains constant over time, which is not the case here (a condition that can be tested using cox.zph in R). It seems that there are two distinct phases in the survival pattern, which may warrant a slightly different analysis approach (such as using stratification or a time-dependent covariate). While the Cox regression was conducted in JMP, it might be worthwhile to perform it in R as well. This could provide additional options and improve the repeatability of the analysis, but I don’t know enough JMP.

Note that this analysis may be affected by my concerned that some dead infected were categorized as uninfected just because the parasite was not found during dissection.

* I would also plot the HR ratio as a second figure associated with the survival, this allow to give the 95 % CI, and the sample size under each treatment. Providing HR and 95% CI also prevents sentences like “was almost supported statistically” based on pvalue.

* In the analysis section, I would provide the exact models used to analyse the data and the package when R was used (which is recommended for reproducibility).

* There are no details about the analysis of the impact of the parasite intensity on survival (beside that the analysis was done as either infected with 1 parasite versus infected with more than one, or as a continuous variable). I guess that the individuals from the treatment “Scented water” and “Control water” were included but how? Was treatment as a covariate? (I would have done that, and it might be worth testing if the treatment affects the outcome).

* Line 329: “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.” Was not observed or not tested?

* Figure 2: Why do some outliers appear larger than others? As I see dots side by side, I guess it does not mean “more dots”.

If the objective is to track behavioral changes over time, it might be more effective to plot the individual’s behavioral variations on a continuous x-axis and connect the dots from a same individual. The lines could terminate when individuals die, but this representation may better capture the data points and align more

closely with the analysis (it currently suggests that data point are independent across time points). Such an approach would likely provide a stronger visual representation of the statement, 'The refuge use by gammarids varied considerably with time (Figure 2, Table 2), being moderate at the beginning of our survey and progressively intensifying until reaching high values around 30 to 40 days' (lines 240-243).

Additionally, it is mentioned that '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), with the interaction between water type and date being statistically significant' (lines 252-254). However, the table only presents a Wald statistic and lacks an estimate of the effect. Without additional context, it is difficult to discern the specific influence of water type on the rate of refuge usage. Can't the method used in Figure 3 be used to show this effect between water types?

Regarding the analysis, it is not explicitly specified in the methods whether time is treated as a continuous or ordinal variable. It would be helpful to provide clarification in this regard.

* The Cliff's effect size estimation is appropriate; however, a description of what it entails should be included in the method section. For instance, it should be explained that the Cliff's delta measures the frequency of values in one distribution being larger than the values in another distribution. Additionally, it is important to clarify whether a value of 0.2 is considered a small or medium effect size and provide the corresponding scale. Currently, the discussion provides the only indication of this information, which requires careful reading. Therefore, it would be beneficial to include details on how the Cliff's effect size was calculated.

* Infected individuals are using less refuge and are more inactive than non-infected individuals when at Day 69. Aren't they simply dying from the infection on the ground? I would address that with in the manuscript because this would be the most expected for most infections although it is here (including in the abstract) presented as something surprising.

* Figure 3 & 5: I like it very much, but the fact that 95% CI are largely overlapping is often ignored. I am aware that part of the conclusion are based on the model (Table 3) but looking at the data, it is likely that the effect is not linear and I wonder how the model deals with that.

* Figure 4: Most of the comments given for Figure 2 are relevant here too.