Dear Editor,
We would first like to thank you and the two reviewers for the delightfully thorough reviews of our manuscript. The comments allowed us to improve the text, at least we hope.
We are responding to all comments below in green colour.

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,

We do not understand this comment. For sure, the whole experiment was made using repeated measurements, but the statistical model nparLD used for analysis is specially designed for analysing repeated measures in a non-parametric way.

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).

We made our best to clear up doubts expressed by reviewers. We now explain more deeply why we have grouped the different uninfected categories. We are presenting, as suggested, in a supplementary material (attached in the BioRxiv deposit), figures and analyses where unexposed controls and exposed-uninfected are separated.

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).

Although the nparLD analysis is described in details in an article cited in our text, scripts are provided at the end of the supplementary file.

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?

It is not miscited, but the sentence was rather confusing, so we changed it.

- Line 108: changed instead of changes (but see one of Adèle's comment requesting a more profound rephrasing here)

Done

- Line 109: "known to be reduced as an antipredator defense in gammarids" (REF). Consider adding a ref after "gammarids".

Done

- Line 113: "P" should be italicized

Done

- Line 117 "behavioural changes". Consider recalling the two behavioral traits namely "refuge use and activity rate" in parenthesis

Done

- 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.

Done. We added the information two lines before.

- 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?

"Also" has been removed (it was referring to the date, captures were all made in February, not to the river)

- 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".

Done

- Line 164: You meant day 83 instead of 84? This sentence is confusing (see also one of Adèle's comment)

Done

- 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?

It is not the same behaviour between L. 186 (generally speaking) and L. 163 (specifically refers to activity rate), but we removed this end of sentence since it is obvious.

- Line 211: "175 individuals". Shouldn't this read 180 (see line 138).

No : 175 (in fact 176 sorry for this mistake) are the surviving individuals. It is now clearly stated, and we provide precisions in Mat&Met section about individuals that died before day 21 (L. 154). We acknowledge that these numbers were not clearly detailed. We hope it is fine now.

- 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.

This sentence was supressed following reply to reviewers 2

- Line 237 the variable “number of parasites” is a discrete numerical (and not continuous) factor.

Done

- Caption of figure 2: specify what the vertical black line represent on the figure (acanthella à cystacanth transition?)

Done, also for Figure 4

- Line 265: “but here a dichotomy must be made” unclear. Figure 2 also makes the distinction between scented and control water.

Changed

- Line 269: I assume you meant -0.11 and -0.4 (instead of 0.11 and 0.4)?

Yes, Done (at some point of the writing, the figure was up-side-down, explaining this mistake)

- Line 272: becoming negative should read “becoming positive”, right?

Yes, Done

- Line 290: “were not” instead of “were no”

Done

- Line 292: discrete numerical variable

Done

- Line 315: “general trend to decrease” instead of “increase”?

The sentence was supressed (see below)

- Line 330: “involved in predation avoidance”. Please cite a ref here.

Done

- Line 335-336: consider deleting “as seen using effect sizes” (Figure 2 also shows this).

Done

- Line 400: “was also strengthen by infection”. Please rephrase as the results indicate the contrary i.e. infected individuals are less active.

Done

Review by anonymous reviewer 1, 06 Jul 2023 09:57

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.

In an ideal world with unlimited space and resources, we would have repeated the experiment many times… but many constraints made it impossible, sorry.

It is surprising that several outcomes changed once the infection could be clearly characterized (strong example being the survival).

In fact, it is not always the case: the infection becomes visible by eye several days before the cystacanth stage is reached, stage where the switch occurs. Survival is changing after the cyctacanth stagewas reached, therefore days after the infection becomes visible.

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.

Well, this is not obvious since the sample size becomes very small at the end. The analysis including this category produced results quite similar to the analysis made with uninfected animals being all grouped, see below.

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.

Again, such a procedure has not been made here. We would have too many constraints to run it before resubmission. It would necessitate a huge experiment with ca. 5400 individuals tested only once (around 2700 per behaviour if considering the ideal sample size of 50 tested individuals per date for the 3 infection status in two water conditions). Because of the infection rate below 100%, much more for individuals have to be exposed to the infection. So it is definitely too big and too long to run this before resubmission.

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.

We now made in a supplementary material (as suggested by the Editor) the analyses including unexposed, exposed-uninfected and exposed infected animals. The Supplementary materials are attached to the BioRxiv deposit.
First, it is not true that unexposed and exposed-uninfected animals “stop dying” at stages where acantheallae are visible through host cuticle. Figure S1 is showing that mortality rate is relatively constant for exposed-uninfected animals. However, it is true that, for unexplained reason, unexposed animals suffered a sudden drop of survival at 50 days (Figure S1), but this has nothing to see with the fact that parasites are visible or not: these individuals do not have parasites.

* 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.

We made these complete analyses in the supplementary material

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.

Such plots are provided in the supplementary material

* 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.

The reviewer is absolutely right here: we were imprudent and too strong in our conclusions (originally mainly based on values of the effect sizes instead of on confidence intervals). By being more strict and not discussing NS results, the discussion is now changed (L 378 to 395), as well as a sentence in the abstract (L 28-29). Please note that we now used the “ANOVA-type statistics” instead of the Wald test in the non-parametric analyses, because this statistics appeared to be more appropriate for small sample size after a careful rereading of Noguchi et al. (2012, p. 8 and 20). This modified somehow some detailed outcomes but not the general trends.

* 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?

See comment above, and see new discussion.

* 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?

See comment above on our changes in focusing only the significant results.

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.
See comment about repeating the experiment above

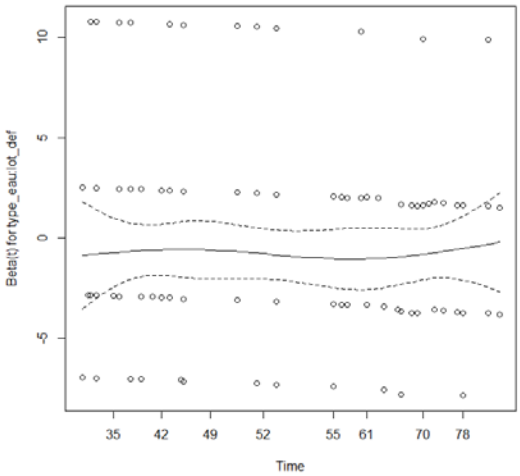
Minor comments:

* 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.
Done for figures that are the more complex to interpret (Figure 2 and Figure 4)

* 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).

See comment above on our changes in focusing only the significant results.

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).
The reviewer is right. We were first wrong because the visual inspection in cox.zph led to a drawing compatible with constant hazard ratios (see below)…



But after running the statistical test (P = 0.0001), we therefore opted for cutting the data set according to the two main developmental stages (acanthella and cystacanth), because the hazard ratios tended to reverse between these two phases (albeit not significantly during the acanthella stage). Indeed, infected individuals tended to survive better at the beginning of acanthella but clearly suffered extra mortality afterwards, making the curves crossing each other during the time-course (explaining the variation in hazard ratios).

* 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).

The complete analysis is now shown L. 249-255. Nothing significant.

* 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?

Tested (Table 3, Figure 5), but not observed.

* 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”.

There was a problem with figure size or a copy-paste to the correct size. One dot is one data. We enlarged the dots, and hope now that the problem is solved.

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).
We try to do such a presentation, but, with 20 to 60 animals, it rapidly turns to a brain teaser with crazy lines crossing themselves all over the figure. In addition, at the end of the time-course a dozen of individuals showed scores of 24 to all dates. It is here impossible to show the evolution between dates for all these ones. The figures as presented are, even imperfect, the clearest to us.

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?
Probably yes, e.g. by checking if the effect sizes are larger between dates in scented water than in control water… but the effect of water per se was not the main aim of the paper, so we prefer not to go further on this track.

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.
Time is here considered as ordinal. We add this L. 209.

* 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. herefore, it would be beneficial to include details on how the Cliff's effect size was calculated.

Informations are now added L. 214-216.

* 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.
What is surprising is that infected individuals are more inactive not only at day 69 but also at days 41 and 48 and above all that there is never any significant effect under control water (if we just retain significant effect size results, as suggested by the reviewer). If animals were "simply dying" at the end of the experiment, why aren't they also dying under control water? We are discussing this issue L. 448-461.

* 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.

We changed our description of results accordingly.

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

Reviewed by Adèle Mennerat, 12 Jul 2023 09:39

PCI Zoology #215
Comments to authors
This is a neat study showing adaptive manipulation of host behaviour by a parasite, by looking at changes following infection of the gammarid intermediate host G. pulex by the acanthocephalan helminth P. laevis (whose final host is a fish predator). More specifically the authors investigate how host behavioural changes vary according to the parasite's developmental stage (i.e., whether it is pre-infective or infective) and how they are affected by the presence of predator cues.
The main findings are:
1) early on in the infection and as long as the parasite P. laevis hasn't reached the (cystacanth) infective stage, G. pulex hosts hide in refuges to a greater extent than uninfected hosts - especially so in predator-scented water.
2) that a clear switch in behaviour occurs when the parasite is ready for transmission to the final host: infected gammarids start spending less time than uninfected ones in refuges, while

increasing the proportion of time spent inactive (not swimming) – thus being more exposed to predation than uninfected gammarids.

Taken together, these results provide evidence for adaptive host manipulation in this study system – more than phenotypic changes in the host simply being byproducts of infection (although some of the increase in time spent inactive at the end of the infection period could in part be due to energy depletion in the host, too).

This is a valuable study that I enjoyed reading, but I do have a couple of comments and suggestions, see below.

General comments

My main general comment is that this paper does not introduce or discuss clearly enough the alternative explanation for phenotypic changes in infected hosts, namely that some of the changes following infection – including activity rates or predator exposure – might, at least in part, also be a byproduct of infection and not only adaptive manipulation by the parasite. This can easily be addressed by adding these aspects to the introduction, and then discussing your results in the light of this. It would make your paper stronger, as your results anyway build a convincing case for adaptive manipulation of host behaviour in this study system; acknowledging that some of the changes could also partly be byproducts would make your conclusion stronger, not weaker, as the two need not be mutually exclusive.

More specifically, you could in the introduction beQer explain what the difference is between PIPA (parasite-induced phenotypic alterations) and host manipulation – and how “true” manipulation can be recognized. Similarly in your discussion, your paper would be strengthened if you acknowledged the alternative explanation for phenotypic changes in the host. As it is now, you only first refer to it L408 as “a non-adaptive pathogenic byproduct of the infection, as sometimes suggested”. The difference between by-product changes and adaptive manipulation deserves more aQention earlier in your paper, precisely because your study provides evidence for host manipulation – so it is important to clarify to the broader readership what the alternative explanation could have been, and that some byproduct effects may also occur at the same time without invalidating the conclusion.

You might want to use these references:

Håkonsrud Jensen et al. (2023) Adaptive host responses to infection can resemble parasitic manipulation. Ecology & Evolution, in press, doi:10.1002/ece3.10318

Lefèvre et al. (2008) Exploiting host compensatory responses: the ‘must’ of manipulation? Trends in Parasitology 24, 435-439

Poulin & Maure (2015) Host manipulation by parasites: a look back before moving forward. Trends in Parasitology 31, 563-570.

Helluy (2013). Parasite-induced alterations of sensorimotor pathways in gammarids: collateral damage of neuroinflammation? Journal of Experimental Biology 216, 67.

Detailed comments

Introduction:

L46: tropical transmission -> trophical transmission

Done (shame… tropical could have been fun !)

L47:by upstream host -> by the upstream host

Done

L49: parasite’s life cycle -> the parasite’s life cycle

Done

L48: what is the difference between PIPA and adaptive manipulation of hosts by parasites? Please clarify and expand on this (see my general comment above).

Not all PIPA are obligatorily adaptive. Some words have been added L. 56-58.
L57: referred as -> referred to as
Done
L79-81: here it sounds as if you are saying that the fact that a trait responds to selection makes it part of an extended phenotype. This can lead to confusion; please clarify, e.g. adding 'rather than a trait of the host', or something similar, at the end of the sentence
Done
L82 rephrase -> Another well-studied parasite-host system etc.
Done
L88-93: here you seem to interchangeably use behavioural changes and manipulation, but these are two different things; please clarify. Also, L91-93 are not needed, you could go straighter to the point by removing these two sentences
We kept these two sentences (former L. 91-95), to make the parallel with Shistocephalus (precision for these traits being parasite traits).
L97-102: these two points would work beQer if they were moved further up, e.g. before the sentence starting L96.
Done
L105-108: a bit unclear, please try to rephrase.
Done
L109-111 is the main prediction of your study, this should appear more clearly
Done
L112: replace 'forced' with another word – that acanthellae induce – or cause – an increased used of refuges, for example
Sentence changed
L114: increased use of refuges was found in both the presence and absence of predator cues: how does this fit with your hypothesis (see L97-102) of costs / investment into 'protective manipulation'?
In fact our two previous studies gave contradictory results for predatory cues. So the present study will provide a third "replicate".
L122-124: move up to L118
Done

Methods
L130-136: why did you only use males?
Justified L. 135-137.
L146-148: how many hosts died prior to d20 post-infection?
It is now noted L. 154. 14 exposed and 2 unexposed died. But since we were unable to know who is who (infected or not), the analysis began at day 21.
L150-160: how olen was the water changed between tests of individual gammarid behaviour?
Once a week, noted L. 147-148
I may not know the study species well enough, but gammarids are rather social; could successive gammarids have lel odor cues in the water that could have affected their behaviour? Do you know if their rank in the testing sequence has any effect on their use of refuges?
We are not sure to understand the question. Each individual gammarid was tested in one water type only and all individuals were tested at the same time, so we do not understand the question about rank in testing sequence.
L163-164: a bit confusing here, I'd suggest moving this bit of information somewhere else, for

example at the end of the paragraph

Done

L166: at this point I wondered how the water was scented and how much, but it is only described further down; this can be avoided by moving L173-179 up in the methods description

Done

L177: I do not understand how feeding trouts with live gammarids would reinforce their predator signal; can you explain this briefly? Do they take the scent of the prey they eat?

No. In fact, the water include the scent of "crushed" preys (partially consumed by fish). Scent of dead gammarids apparently is a signal for other gammarids around (see reference cited in the text).

L180-188: move this up to the start of the 'behavioral measurements' paragraph

Not Done. We feel the proposal of the reviewer is not logical because the way dissected animals were counted as infected would be explained afterward…

L192: control vs infected, or was it uninfected vs infected?

Uninfected, sorry. Changed

Results

L264-272: make a new paragraph for this, as this is a different line of results

Done

L265: interpretation -> observation

Done

L288 : rephrase, using neither / nor

Done

L313: hypothesis -> observation / trend

The sentence has been changed

Figure 5: in the labels, change 'non-signed' and 'signed water' to 'non-scented' and 'scented'

Done

Discussion

L406-430 is the main conclusion of your study, the discussion should gradually lead to it: keeping the same structure to your discussion, you could turn it into a more explicit step-by-step explanation why your study gives evidence for manipulation, as opposed to only representing byproducts effects of infection.

We have to recall that this point (if these behavioural changes are adaptive or not) was not the aim of this study. It turns that some result strengthen the feeling that it is indeed adaptive for parasites.

Here are a few suggestions.

L332-341: at this point, this result alone could be either adaptive manipulation or byproduct – you could state this more explicitly

It seems that the reviewer considered that the "protection" is adaptive in itself, separated from "exposure". However, this is not the case: both components are linked to consider the adaptive "manipulation".

L347-353: what would you have expected to find instead, if there was no manipulation? More specifically, would you observe the same paQerns of increased refuge use with time as a byproduct, for example if the parasites become increasingly costly and the host increasingly depleted with energy over time?

This section has been changed when addressing some questions of reviewer 1.

L353, 356: products -> chemicals, molecules, factors

Done

L358: oscillate -> fluctuate

Done

L350-360: could this be caused by energy depletion? What did they eat during the experiment?

Dead elm leaves

L381-398: this is the step where adaptive manipulation becomes the main conclusion, because only manipulation allowd to explain the full range of your observations, and especially such a shil in host behaviour, synchronised with the parasite reaching infective stage – you could consider making this a distinct paragraph and presenting it as your final piece of evidence for adaptive host manipulation.

The end of discussion has been modified…