June 13, 2024

Daniel Grabner
Recommender
PCI infections

Dear Dr Grabner,

I am writing to submit the revised version of our typescript now titled “Differences in specificity, development time and virulence between two acanthocephalan parasites, infecting two cryptic species of Gammarus fossarum” for consideration in *PCI infections*. We appreciate the feedback provided by yourself and two anonymous reviewers and have carefully addressed each comment in our revised draft. The suggestions received were thoughtful and helped us to make the manuscript more impactful. Below, please find our detailed responses to the reviewers comments.

We believe that the revisions have significantly improved the quality and clarity of our work and will contribute valuable insights to the field.

We kindly request that you consider our revised manuscript for publication in *PCI Journal*.

Thank you for your time and consideration. We look forward to hearing from you soon.

RESPONSES TO REVIEWERS

Comments from Daniel Grabner

- carefully revise the language

Done

- consider using GLMs for analysis of the data (optional)

Done for the analysis of natural prevalence, infection success and development duration of parasites.

- streamline the title

Done

My main concern is that the presentation of the results (table 1) is really confusing. It is not clear what is meant by the controls (with 0 uninfected for G. pulex???) Probably, it is a good idea to remove G. pulex due to the low number of individuals. Also, I suggest removing the "Suzon" population of G. pulex from the table and report the quality test only in methods.

> Partially done. We kept some of the *G. pulex* results in supplementary material (see detailed answer to reviewer 1 comments).

But you should have some sort of threshold for this to make any sense (e.g. a minimum of 60% infection with the control population must be achieved).

> We could have questioned the quality of the infestation if no infected gammarid was found in our positive controls. But since every combination of parasite/host has its specificities, it would have been a bit of a gamble to set *a priori* a specific threshold (see Franceschi 2010b for local adaptation between 6 different populations)

Review by anonymous reviewer 1

General comments

The title appears to me very long and elaborated. It is like a short abstract that summarizes almost the whole output of the research, which makes it not catchy at all.

> Title was streamlined. New title proposed: Differences in specificity, development time and virulence between two acanthocephalan parasites, infecting two cryptic species of Gammarus fossarum

In my opinion the results/aspects related to the infection with *G. pulex* should be omitted throughout the entire ms. The authors stated that they was used them "…as control for parasite "quality"…" (see Line 118), however the number of exposed and infected individuals was too low in order to make any conclusions based on comparisons.

> As required, we are now focusing on *G. fossarum.* Nevertheless, because *G. pulex* are part of our sample, we kept some of these results in supplementary material. Discussion about these samples is now restricted to a minimum in the discussion.
>
> In addition, the positive control of infection with gammarids from Suzon is indicated in the MM. Data for these individuals were removed from table 1 and the figure comparing overall infection success between the two populations was transferred to the supplementary material section.

In MM part it is not very clear how the infection of gammarids was proceed. There are no details how many individuals in total were taken for the infection experiment. The authors provide in table 1 as well as figures 2 and 3 the numbers, however, it is not clear how many of them was used as control group (this is relevant for the evaluation of the virulence/mortality). The sample size of control animals seem to me very low, on the one hand and on the other hand, the higher number of early deaths during the *P. tereticollis* exposure exceeded the number of the ones that survived (uninfected group).

This is now specified in MM line 176.

Were the early deaths also considered in the calculations of the survival rates of control group? According to the data in table 1 it is visible that the early death rates were much higher (3 to 4 times higher) in both controls and exposed groups during the infection experiment with *P. tereticollis*. Was it due to maintenance of gammarids (stress)? The authors assumed that this was not linked to the confrontation of gammarids with the parasite's eggs (lines 434-437), however such differences in early mortality could make the comparisons of survival rates between both acanthocephalan species questionable. Therefore, here is more clarification needed instead providing only a personal assumption!

Early deaths were considered only in the first parts of all analyses, both for controls and exposed individuals.

We do not know precisely the cause of this high mortality. But since the same pattern is observed for both the negative controls and the exposed animals, this is undoubtedly not linked to the exposure to parasite eggs. Since the exposure was made in two sessions, it is probable that the second round was more stressful for the gammarids. This is indicated in the discussion line 519.

It is also not clear, whether the individuals of Gf2 and Gf6 lineages were pre-sorted prior the start of infection experiment or at the end, when the gammarids were sacrificed. I guess that it was done at the end, as cutting of locomotor appendages would induce additional stress to the animals. This is also true for the identification of both acanthocephalan species. Here I assume that it was done molecularly prior the collection of the eggs based on extracted DNA of the adults, but the authors stated that they also identified them at acanthella stage (lines 206-207). Please, clarify in MM part at which stage the identification was done!

Gammarids were not pre-sorted. The whole experiment was made blind and the genotyping was made at the end, this was originally explained line 287 and is now also indicated more clearly line 177 and line 189.

Adult acanthocephalans were genotyped before preparing the eggs suspensions to determine the species of each female clutch. A RFLP was performed on these individuals to save time before infestation (added line 155).

Since it is not possible to determine easily the acanthellae species on morphological criteria, they were preserved in alcohol after dissection of their hosts and genotyped later by sequencing (line 206).

The molecular method that was used to distinguish between both acanthocephalan species is based on the size of PCR products, however, it is not applicable for proper identification of other close related *Pomphorhynchus* species like *P. bosniacus*, as recently published by Reier et al. 2019. In this regard, did the authors also sequenced a larger sample size using the procedure of Reier et al. in order to exclude any incidental infections with *P. bosniacus*, which might also exhibit completely different host specificity and thus blur the results?

Only the adults were determined by RFLP (see answer to the previous comment).

All acanthellae were sequenced and we found only *P. laevis* or *P. tereticollis*. Thus far, *P. bosniacus* has been described in France only in round gobies. Moreover, a two-year survey on gammarids and parasites of the Albane river population was performed before this experiment, during which *P. bosniacus* was never detected (unpublished data).

Another issue that deserves clarification is related to the egg suspensions that were used for the infection. In lines 151-152 the authors stated that batches with at least 50% mature eggs were used. How this percentage was determined/quantified? Additionally, inconsistent percentage of mature eggs in the suspensions (variation between 50% and 100 %) could potentially bias the infection success, in my opinion. My question here is: did the authors quantified also the upper range (%) of mature eggs? If yes, please provide it.

The inspection of clutches was made by eye under microscope just after their removal from females to select those which were worth being genotyped. To prepare the eggs suspensions for infestations, only mature eggs were counted. This is now specified more clearly line 151. We are not sure we found an appropriate terminology: is ‘manually’ clear enough?

Specific comments

In addition of the comments listed above, in my opinion, this ms needs in depth linguistic improvement. Even if I’m not a native English speaker, I’ve detected various wording/phrasing issues as well as wrong prepositions and partly improperly used grammar. As there are many of them, I’ll not address every single one. Therefore, I recommend a proof reading by a native speaker, if it is possible.

Done

Further, the genus names should be consistently abbreviated once they were introduced (e.g. *P. laevis, P. tereticollis, G. pulex, G. fossarum*).

Done

I also recommend to combine figures 5 and 6 as well as figures 7 and 8. In this case there won't be a confusing overlap in the ranges of x-axis and different resolutions (e.g. figures 5&6), whereas the line for the infected group will start at day 40 and 60 for *P. laevis* and *P. tereticollis*, respectively.

Done

In my opinion, the tables (e.g. 2, 3 and 4) could be taken out of the main script and placed in the supplementary data.

Done

Review by anonymous reviewer 2

[…] The manuscript could benefit from a check on English grammar and syntax, and more detail on the methodology (see suggestions below).

Done

[…] I would have employed a different approach to statistics, particularly on the evaluation of the difference between the parasite species in terms of the development duration until the cystacanth stage, and the difference among lineages in terms of the susceptibility of infection. Perhaps a general(ized) linear model would have been more suitable, especially since the data was skewed in some cases.

We applied GLMs to analyse natural infections levels, experimental infections success and the parasites development duration.

Line 43: "contributing much more than the others", specify what you mean by "others"

Done, line 41.

Line 54: "The reverse is true from a host perspective." Please explain. It is not clear what is the reverse situation.

Done, line 55.

Line 117: “has repeatedly been shown to be particularly sensitive to infection by P. laevis.” In laboratory infections? Please specify.

Done, line 116.

Line 122: Was the water mixed in equal volumes? Please specify.

Yes, this is now specified line 122.

Line 129-130: Was the water changed during the three weeks of acclimation? What happened to the gammarids during this time?

Yes, the water was regularly changed (specified line 126). Some individuals have undoubtedly died, but we could not follow the survival in the collective tanks.

Line 144: I assume the initial distinction between the acanthocephalan species was done morphologically? Perhaps you could cite Špakulová et al (2011) 10.2478/s11687-011-0038-y, if you use the suggested morphological features in that study to distinguish between the species.

The determination of species in adult parasites was made by genotyping. This is explained at the end of the *Sampling of parasite eggs and genotyping* section, and detailed in the *Gammarids & parasites genotyping* section. Specified line 155; see also the answer to Ref#1 comment.

Line 161: Instead of “weakness”, I would rather say “bias towards a more or less infective (or virulent) clutch” since it could go both ways.

Integrated line 162.

Line 167: Is crystallizer the right word here? Do you mean a glass crystallizing dish?

Sorry, we anglicized the French name without checking the accurate denomination… Modified.

Line 175: “parasite-free” or “non-inoculated” instead of “healthy”

Modified

Line 181: “infection status” instead of “parasitic status”

Modified

Line 213: Rephrase to “The total reaction volume (20 µL) consisted of 200 nM of each primer, 200 µM of 213 dNTPs, 0.25 U of DNA polymerase (HotStarTaq, Qiagen Inc., Düsseldorf, Germany), 1X of buffer, 5 µL of extracted DNA, and X µL ultrapure water”.

Done, line 222.

Table 1: I suggest changing colors to another combination so it is inclusive to color blind people or using bold vs normal font. For help choosing colors that are colorblind safe, you can use https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3

Done, thank you for the tips!

Figure 1: Do these include the infections encountered after the quarantine period?

Yes, these are only infections encountered after the quarantine.

Line 352: “2.07 parasites”, decimals not relevant for infection intensity measurements.

We agree that we cannot have a 0.07 parasite, but this is an average value.

Line 352: “mono-infected” to my understanding means infected by one species of parasite (as opposed to “co-infected” for instance), not necessarily hosting only one parasite individual. Please clarify.

Clarified: “half of the individuals being infected by one parasite”, line 341.

Line 435: “but it did not seem to be linked with the confrontation to parasite eggs” This belongs in the discussion

Removed from the results and added in the discussion, line 508.

Line 489: Replace “acanthocephalans infection” with “acanthocephalan infections”

Done

Line 490: “has not be investigated in this study because of the globally low success of infection.” Not sure what is meant here by the globally low success of infection and why this impeded behaviour evaluation in this study.

Clarified line 447.

Line 526: You placed gammarids in pairs because this makes them eat more (which is understandable). However, gammarids are known to be cannibalistic, especially when they are together in a small space. Did you see this in your experiment? How did you deal with it in the data analysis? It would also be important to highlight that the samples (each gammarid) were not completely independent from each other since the presence of the other gammarid might have influenced the chances of the other to become infected. I do not think this affects the relevance of the results but is a weakness that should be acknowledged.

> Gammarids were in pairs only during the 48 h exposure period. The survival analysis began after the exposure, when gammarids where individualized. This is explained in the methods at the end of the MM **Exposure procedure** paragraph, line 174.

Line 544: “P. laevis, P. tereticollis,” Place “and” between the two.

> Done

Line 578-579: Also gammarids are well adapted to sustain microinjuries in the wild by melanizing immediately the injury site.

> We agree, but we also know that melanization is a costly process, therefore it is even more intriguing to find a better survival in exposed animals…

Line 596: "A mechanistic explanation of such a phenomenon would be that the early stages…” Use "could" instead of "would"

> Done

Line 595-604: Before the first acanthella detection date, P. laevis-exposed gammarids, and P. tereticollis-exposed Gf6 faired better than unexposed ones. I know that acanthocephalans immunosuppress the host. However, could other biochemical parameters be activated by the initial infection that helps mitigate stressful conditions in the lab as a side effect? Despite that you tried to make the gammarids as comfortable as possible, it is still an unnatural setting representing a stressful situation for the host. Perhaps, the exposure of infection triggers protective mechanisms (e.g., heat shock proteins, mechanisms against oxidative stress) that help the host sustain stress in the lab. Something worth thinking about...

> Yes indeed. We acknowledge this possibility line 558.

Line 682: Link to data is broken

> Sorry for that, this is now corrected.

Suggested Questionnaire:

• Title and abstract

o Does the title clearly reflect the content of the article?

[x] Yes, [ ] No (please explain), [ ] I don't know

o Does the abstract present the main findings of the study?

[x] Yes, [ ] No (please explain), [ ] I don’t know

• Introduction

o Are the research questions/hypotheses/predictions clearly presented?

[x] Yes, [ ] No (please explain), [ ] I don’t know

o Does the introduction build on relevant research in the field?

[x] Yes, [ ] No (please explain), [ ] I don’t know

• Materials and methods

o Are the methods and analyses sufficiently detailed to allow replication by other researchers?

[x] Yes, [ ] No (please explain), [ ] I don’t know

o Are the methods and statistical analyses appropriate and well described?

[ ] Yes, [x] No (please explain)

▪ Please add more details in methodology (see suggestions above). I do not consider this cause for concern, but I would have employed a different approach to statistics, particularly on the evaluation of the difference between the parasite species in terms of the development duration until the cystacanth stage, and the difference among lineages in terms of the susceptibility of infection. Perhaps a general(ized) linear model would have been more suitable, especially since the data was skewed in some cases. However, the results are clear and the authors discuss the limitations of their statistical analyses in the discussion.

• Results

o In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)?

[ ] Yes, [x] No

▪ In the case of the facilitation of secondary infections, sample size was low and therefore the lack of significance might be related to low sample size. However, this is acknowledged by the authors in the discussion. Although a power analysis might have been helpful, this (the susceptibility to second infections) was not the focus of this study and, therefore, I think it does not represent a real weakness in this case.

o Are the results described and interpreted correctly?

[x] Yes, [ ] No (please explain), [ ] I don't know

• Discussion

o Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument?

[x] Yes, [ ] No (please explain), [ ] I don't know

o Are the conclusions adequately supported by the results (without overstating the implications of the findings)?

[x] Yes, [ ] No (please explain), [ ] I don't know