

Article Review:

Experimental infections reveal differences in specificity, development time and virulence between the acanthocephalan parasite *Pomphorhynchus tereticollis* and its sympatric counterpart *P. laevis*, in two cryptic species of *Gammarus fossarum*

This manuscript evaluates the susceptibility of two cryptic amphipod species to infections by two acanthocephalan parasites, *Pomphorhynchus laevis*, and *P. tereticollis*, as well as the parasites' virulence. The manuscript is well structured, the experiments were meticulously designed and the results carefully discussed. The manuscript could benefit from a check on English grammar and syntax, and more detail on the methodology (see suggestions below). However, the results derived from this study are fascinating and represent a significant and novel contribution to the body of knowledge on Parasitology since it provides important information on the degree of specificity of an ecologically important parasite and opens a venue for further research on the transmission dynamics of parasites to cryptic species. Most importantly, it provides essential biological information on a highly understudied parasite species, *P. tereticollis*. 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. Below are some suggestions which are mostly minor comments.

Line 43: “*contributing much more than the others*”, specify what you mean by “others”

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

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

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

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

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.

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.

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

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

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

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”.

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>

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

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

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.

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

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

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.

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.

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

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

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

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...

Line 682: Link to data is broken

Suggested Questionnaire:

- **Title and abstract**
 - Does the title clearly reflect the content of the article? [x] Yes, [] No (please explain), [] I don't know
 - Does the abstract present the main findings of the study? [x] Yes, [] No (please explain), [] I don't know
- **Introduction**
 - Are the research questions/hypotheses/predictions clearly presented? [x] Yes, [] No (please explain), [] I don't know
 - Does the introduction build on relevant research in the field? [x] Yes, [] No (please explain), [] I don't know
- **Materials and methods**
 - Are the methods and analyses sufficiently detailed to allow replication by other researchers? [x] Yes, [] No (please explain), [] I don't know
 - 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**
 - 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.
 - Are the results described and interpreted correctly? [x] Yes, [] No (please explain), [] I don't know
- **Discussion**
 - Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? [x] Yes, [] No (please explain), [] I don't know
 - Are the conclusions adequately supported by the results (without overstating the implications of the findings)? [x] Yes, [] No (please explain), [] I don't know