**PCIEcology #423**

Parasites make hosts more profitable but less available to predators

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## Responses to the Recommender, Luis Schiesari

- Both reviewers and myself agree that your manuscript deals with a very interesting general question in ecology, namely, the direct and indirect effects of parasites in a food web context; that the coupling of experimental and observational approaches is a positive aspect of your research; and that there is a combination of relevant response variables being measured.

  At the same time, both reviewers and myself understand that your manuscript requires revisions before it can be recommended by PCI. Please see the reviewers´ comments below.

We thank you, and the reviewers, for your interest in this study and the time that you have taken to do all these useful comments. We agree with all comments and understand that in the previous version it was difficult to get a good overview of the numerous measures on both experimentally and naturally infected *Daphnia*, and on independent and combined (MFA) analyses. We hope you will find the new version much clearer, in particular regarding the link between experimental and natural infection, with more suitable statistical analyses.

- In additipn to the comments raised by the referees, I am concerned that infection was never actually measured. The authors argue that the phenotypic consequence of infection (=iridescence) is well known, and this may be acceptable for part of the response variables (in particular for those that were derived from experimental infection). However, at least in one case the association between phenotype and the assumption of infection is problematic – this is the measure of reflectance (measure 8). That is, measuring reflection as a response to infection when infected versus non-infected individuals were classified based on reflectance is... a circular reasoning. These were wild Daphnia and there was no test for DIV-1. Can you provide reflectance of the experimentally infected Daphnia? Furthermore, the reader would be less concerned if you were able to mention that Daphnia in these particular water bodies were previously tested for DIV-1.

It is possible to test the DIV-1 infection using PCR (Toenshoff *et al.* 2018). We added in the discussion: “Clarifying this aspect would require testing if exposed individuals are infected or not, using microscopy or PCR techniques (Toenshoff et al. 2018).” (L551-553) It would be more interesting to do these measures when possible (i.e., not for predation experiments, nor for energy measures), which was not the case for our study. The motivation of our reflectance measure is not to say that the infection increases reflectance, but rather to more precisely quantify how the reflectance of white individuals is increased on a large spectrum (UV and visible domain), likely creating differences in visibility. This allows us to go beyond simple “by eye” discussion of phenotypic changes. This is also important because predators of Daphnia may use the color spectrum in ways that differ from ours. Therefore, we believe that this investigation of spectral changes is important to better understand potential changes in predator-prey interactions. We rewrote “The measure of reflectance (Fig. 2), measured in the percentage of reflected light – i.e., more the light is reflected, more the individual is colored for each wavelength/color, of naturally infected D. magna clearly shows that the white phenotype is associated with an increase of the coloration (intensity) both in the UV and visible domain, and to a lesser extent in the infrared (280 to 850 nm), underlying the higher visibility of infected individuals.” (L396-400) Based on experimental infections and on the DIV-1 and iridovirus literature, we expected the white coloration to result from infection, but we wanted to quantify this aspect. It is possible that in the “healthy” group, *Daphnia* are infected but do not show the white coloration (covert infection), and on that point it is interesting to note that there are no no-white (“healthy”) individuals with a higher UV reflectance (i.e., we cannot separate two groups of reflectance in “healthy” individuals).

- Line 53. ´concurrently in addition´ is redundant.

We modified with: “Few studies, that work on the diversity of parasite-induced phenotypic alterations, have simultaneously considered both direct and indirect effects (Cezilly et al. 2013)” (L54-56)

- Lines 200-202. Body size was measured in naturally infected individuals, but also in naturally non-infected individuals. So it would be best to use a different term (maybe ´wild Daphnia´?) in this as well as elsewhere in the manuscript (e.g. line 210)

We agree with you that the terminology was somewhat confusing. We have reworked the presentation and structure of the M&M (e.g., table 1) and result section, to make the text more readable without adding a new terminology.

- Lines 261, 265. ´proposed´ reads odd. Maybe ´offered´?

Modified, thank you.

- Line 279. Lower case i, instead of capital I

Corrected, thank you.

- Lines 438-440. This hypothesis was not formulated before, nor there is a citation for such a hypothesis being proposed in the literature. Or, alternatively, frame it in a way that it makes clear that this is your interpretation, a posteriori.

We have tried to clarify our discussion of this hypothesis: "No effect on juvenile mortality was observed due to the virus exposure, which supports the previous hypothesis (Agnew et al. 1999, Marina et al. 2003, Toenshoff et al. 2018) that the virus progressively accumulates inside the host and ultimately leads to death." (L454-457)

- Lines 444-446. Unclear. Are you proposing lower speed is a cause for larger size?

Indeed, this is what we mean. We have tried to make this clearer in the new version of the manuscript: "A possible explanation would be that lower speeds (higher speeds being generally associated with larger sizes, see Dodson and Ramcharan, 1991) save part of the individual energy budget that can then be reinvested in growth." (L462-465)

- In the Figures, Legends for the colors (i.e. colored box-plots and lines) are usually missing, and in the Results please reinforce the nature of the results (that is, make sure that the reader follows whether this or that effect is based on experimental or observational evidence).

We added information on colors in the figure and/or in the legends when lacking.
We have also revised the text, and the structure of the result section, to more clearly indicate when *D. magna* were naturally or experimentally infected.

**<u>Responses to the Reviewer 1, Thierry De Meeus</u>**

- The preprint relates an interesting study on several aspect of the ecology of a complex host-parasite-predator system.

  I read the material and methods with interest, but realized that I was unfamiliar with all techniques used. I thus cannot assess the validity and quality of such experiments and measurements and trusted the authors about the reliability of those.

  There are globally too many analyses that study the same things (redundancy). Authors should get rid of the less significant ones and stick to the most important analyses and results. I also think that some statistical analyses deserves being redone.

  I believe this preprint is worth being recommended, providing the authors undertake important modifications. Authors will find below different points raised during my reading of the manuscript.

We thank you for your interest in this article. We have taken into account your numerous useful comments to improve our manuscript. We have amply restructured the text, paying a particular attention to avoid redundancy by moving the MFA in the main text (the less important analyses being moved in appendix). We also redid the statistical analysis according to your suggestions.

- 1) Lines 281-282: The levels of significance announced at 5% in the M&M section looks a little old-fashioned to me. Depending on sample sizes, on what is worse between rejecting or accepting H0, and on the number of tests handled, common researchers may adjust levels of significance. Please delete.

We agree with you on the limits of the 5% threshold. We systematically indicated the p-values and considered them for the interpretations. However, because we use this classical terminology (significativity), we feel it is important to precise its meaning in the M&M.

- 2) Line 294: Is there not a better way to analyze these data but to log transform those? Did the author checked if Kruskal Wallis provided results that proved consistent with the Anova? Could you not use a glm model with customized error? Transforming data is far from ideal.

We did the non-parametric test (Kruskal Wallis/Wilcoxon) and found results that are consistent with the ones we present here. Given the data, the choice of a non-parametric test vs a customized error glm model is not obvious to us (we used glm for other data analysis when possible, such as the total reproduction analysis with a quasi-Poisson distribution). After discussion with statisticians, we kept the parametric tests with log transformation, rather than a non-parametric test, because it is recognized that rank tests lead to a "loss of information", thus are "less efficient or less powerful"; consequently "non-parametric methods are justified when conditions are not satisfied for other methods, after variable transformations" (Dagnelie, 2006, *Statistique théorique et appliquée*, 2nd ed., de Boeck).

- 3) Line 312: I do not know about dates, but I do not think pound can be considered as a random effect. I indeed hardly believe that these two pounds share exactly the same physico-chemical, and ecological parameters. Several results confirm that there is a non-random effect of pounds.

From a statistical point of view, a random effect allows us to integrate the non-independence of data: individuals from the same pound are linked (i.e., individuals from the same pound could be more similar than individuals between pounds, due to genetic and environmental factors). Thus, adding pound as a random effect allows us to suppress the pound effect that is not part of our hypothetical framework. Similarly, adding dates as a random effect allows us to consider that samplings during close dates should be more similar than samplings with long delay.

- 4) Line 314: Please rewrite "analyze" into "analysis".

Corrected, thank you.

- 5) Line 348: We expect that exposed (or infected) individuals produce less offspring, and hence tests should be one-sided. Was this the case? If not then the test may be significant after all. This applies to all comparisons between uninfected, exposed and infected Daphnia.

While we initially used two-sided tests we rather agree with your argument.. We now use one-sided tests for fecundity and fitness results, and also for predation results (when we have clear predictions). It seems not technically possible to do a one-sided test for survival analysis (and it should not affect our conclusions). Two tests are now significants (at the 5% threshold), without modifications of our interpretations.

- 6) Lines 387-392: I think Figure C3 is interesting, very meaningful, and should be in the main text. Too many obscure quantities are given here. Cannot you just say that, according to the figure, it is obvious that, between 300 and 800 nm, infected Daphnia reflect much more light than healthy ones for most of the spectrum, except for two very narrow wavelengths intervals (p-value<0.001)?

We have followed the referee's advice and moved the figure to the main text. We modified the text to better highlight the main effect and detailed the less important effects on the peaks shift: "The measure of reflectance (Fig. 2), measured in the percentage of reflected light – i.e., more the light is reflected, more the individual is colored for each wavelength/color, of naturally infected D. magna clearly shows that the white phenotype is associated with an increase of the coloration (intensity) both in the UV and visible domain, and to a lesser extent in the infrared (280 to 850 nm), underlying the higher visibility of infected individuals." (L396-400) and "Furthermore, few differences were observed on the position of the peaks of reflectance." (L402-403)

- 7) Lines 395-410: The legend indicating the identity of different colors should be represented in the Figure 4. I am surprised that search times difference was not significant. I would redo this test with a one sided signed rank Wilcoxon test for paired data on a single table combining 1st, 2nd and 3rd preys.

We added the color code in the figure.
As proposed in a following comment (point 12) "We expect that search time is smaller for infected prey". Consequently expectations from a one-sided test would be opposite to the differences visible in the figure (an higher search time for the infected) and p-values of a one-

sided test would be inflated (but note that a one-sided test for the opposite prediction are also not significant - p-values>0.1, either for separate or combined prey).
We now use one-sided tests for all predation results: no difference for search time (no effect) or handling time (effect), which leads to a significant preference (p-value = 0.03) for the infected prey.

- 8) Lines 424-434: I do not understand this. Infected hosts produced significantly less clutches of similar size as compared to healthy ones, so fecundity is negatively affected by the virus. Authors should avoid multiple and contradictory sentences in the discussion and just go straight to the major points.

We apologize for the lack of clarity. Fitness is here distinct from fecundity, also containing survival aspects (i.e., variations in mortality). We have clarified our definition of fitness and fecundity in the M&M “The experimental infection allowed us to clearly distinguish between the effects on fecundity and survival. We do not consider offspring production along lifetime as a proxy of fecundity, but rather as a proxy of fitness, because it encapsulates both fecundity parameters (clutch size, clutch frequency, and age at maturity) and survival (lifespan).” (L153-157) Following this distinction, there is no contradiction between the sentences pointed out by the reviewer: the reduction of lifetime clutches number is due to a survival reduction, not to a fecundity reduction.

- 9) Lines 443-444: Same as point 8. Fecundity is the natural capability to produce offspring. To this respect, this virus has a negative effect on the fecundity of Daphnia. Alternatively, maybe I missed something and this needs being rephrased.

See our answer to point 8, above.

- 10) Line 496: I do not understand why a decrease in Daphnia populations should necessarily yields a decrease in the fitness of notonects or fishes, unless Daphnia are the only prey for such predators, which I seriously doubt.

We agree the sentence is speculative and dropped it in the new version.

- 11) References list: Please harmonize the format. Many article titles display a capital for the first letter of each word, which is not standard.

Corrected, thank you.

- 12) Appendix A: I understand that the t-test was paired by individual hosts, but I have several concerned. Why did authors log transform the data? I would instead advise undertaking a Wilcoxon signed rank test for paired data on untransformed measures. We expect that search time is smaller for infected prey, so I would expect one-sided tests. Was it the case? I could not find p-values. I would redo all these tests with a one-sided Wilcoxon test for paired data, and would analyze a single table with all preys to get a single test (much more powerful).

As detailed in a previous answer, we believe that a non-parametric test should be considered if parametric tests are not possible, even on transformed data. However, we did a Wilcoxon test and found the results to be qualitatively equivalent. We now use one-sided tests. The search time for the first prey is now significatively (at 5%) lower, but there is no other difference (no effect for the three prey together). We updated the text and the figure with these new statistical analyses.

- 13) Appendix B is too hard to read, especially to those not familiar with MFA. If first and 2nd axes are explained by a few variables, these should be identified in the graph with the direction of increase. I personally could not interpret these graphics the same as the authors did in the text of this appendix. I noticed several counter-intuitive observations: Life span and size are negatively correlated; NbEgg is negatively correlated with size; mobility is positively correlated with clutch size. Regarding the virus, if I understand well, infected hosts display a larger body size, a smaller life span, and larger clutch sizes (why?); Control are less mobile than exposed hosts (why?). The why needs beuing discussed more clearly for each of these observations.

To increase clarity and limit redundancy, we moved the MFA in the main text, and isolated analyses (survival, fecundity, size and mobility for experimentally and naturally infected individuals) in the appendix.

We have reworked the results and the discussion based on the various points you mention and hope that you will find these changes satisfactory. Note however that Fig 1a indicates a negative correlation between mobility and clutch size, no larger clutch size for infected (we discuss about no-fecundity effect) and that exposed individuals are less mobile (as we discuss in the article). We now clearly write which axes are linked to each measure, and that “The first axis is therefore sufficient to separate Infected individuals from the others, although both the first and second axes are necessary to separate Control and Exposed individuals.” (L383-385)

- 14) Figure 1C: There is an obvious superimposed effect of infection on survival of hosts, which interfere with the results presented here. A better comparison would be the absolute total numbers of eggs produced by healthy and infected hosts.

In this part of the analysis, we consider only the proportion of reproductive adults, consequently we did not take into account survival effects. With these naturally-infected *D. magna* we cannot obtain a total number of eggs produced (as reproduction events likely have happened before sampling in the field). If the problem is about our definition of fitness, fecundity and survival, we added in the M&M: “The experimental infection allowed us to clearly distinguish between the effects on fecundity and survival. We do not consider offspring production along lifetime as a proxy of fecundity, but rather as a proxy of fitness, because it encapsulates both fecundity parameters (clutch size, clutch frequency, and age at maturity) and survival (lifespan).” (L153-157)

- 15) Raw data sets: There are no legends in the datasets. Legends should be added at least in excel files.

We added legends in the readme.txt file.

- 16) I am not sure that Notonecta usually use D. magna as preys, as they may prefer larger preys, so maybe this would require at least a little sentence of discussion.

In the literature *Notonecta* consumes a high variety of prey, depending on their size (varying with stage and species). We added: “a common generalist predator (Giller, 1986; van der Lee et al., 2021)” (L114)

- 17) There is an important question that is not discussed in the manuscript. Is the DIV1 virus staying in or infecting the Notonecta? If yes, then the increased predation of D. magna and other crustacean (amphipods, isopods), will increase the concentration of viruses in the aquatic environment and maybe the infection of Daphnia.

The virus does not seem to infect Notonecta (we have written "non-trophic-transmitted parasite"), nor other crustaceans. The general idea is that DIV1 is quite specialized on Daphnia as the many DNA exchanges between the virus and the daphnia suggest tight coevolution (Toenshoff et al. 2018). However, whether the predation effect actually lowers transmission rates is, we feel, quite interesting and we added "Such experiments would also offer a way to understand how predation on host affects parasite dynamic, the conditions under which it reduces infection (healthy herd hypothesis, Packer et al., 2003) or when it favors the dispersal of a non trophically-transmitted parasite, as Chaoborus do for the spores of a Daphnia's fungal parasite (Cáceres et al., 2009)." (L569-573)

**<u>Responses to the Reviewer 2, Eglantine Mathieu-Bégné</u>**

- In the article entitle "Parasites make hosts more profitable but less available to predators" the authors assess in a design combining experimental measurements and natural observations different facets of phenotype alterations caused by the Daphnia iridescent virus 1 on its Daphnia magna host, along with potential indirect effects on predation. The authors in particular manage to identify effects in terms of mortality, appearance, mobility, and profitability. They also strongly suggest that those effects could contribute to make those Daphnia preferred prey although this effect might be balanced in natural system by infected Daphnia abundances that decrease over the course of infection.

  I personally found the present study very valuable as it contributes to increase our knowledge about indirect effects of parasitism notably at the food chain/ food web level. The combination of experimental and descriptive measure is of interest to distinguish some effects and the range of measures taken on Daphnia is quite complete. However, I think that three main aspects of the current manuscript should be addressed prior to further consideration for publication, namely control of confounding effects between size and infection status on predation rate, co-variation of phenotypic traits that are considered separately and quality of expression plus synthesis.

We thank you for your interest in our work. Based on your valuable comments we have reworked the text. We hope you will find the new version clearer. In particular, we moved the MFA in the main text, and put the separated analyses in the appendix. Thus, the redundant measures, with naturally-infected individuals, allow us to support our experimental infection's results.

- One of the issues I have with the conclusion that are made upon predation rate (handling time) is that in the experimental design both infection status and size of the Daphnia are varying. Since infected Daphnia are also bigger compared to healthy one (L374) to me it is not straightforward to conclude that infected preys are preferred. In particular the iridescence virus is not castrating Daphnia which is known to usually cause gigantism due to resources reallocation. Without either a clear mechanism to explain how parasitism can cause Daphnia bigger size or an experimental design testing the effect of infection status on predation while controlling for host size I think that conclusions drawn on indirect effect of parasitism on predation through size modulation should be nuanced. Alternatively, is there any evidence that bigger Daphnia are more often parasitized (ie independent effect of size on parasitism and on predation)?

We understand that the bigger size of infected *D. magna* in a predation experiment could be considered a problem, bigger prey being more attractive. However, our experimental infection showed that the virus leads to a larger size of the host (for a same age) so we consider that it is here part of the disease syndrome. We added in the discussion: "A possible explanation would be that lower speeds (higher speeds being generally associated with larger sizes, see Dodson and Ramcharan, 1991) save part of the individual energy budget that can then be reinvested in growth." (L462-465). You are right that we did not link size and handling time in our discussion, and we agree that it is an important point. We added: "Consequently, in spite of a higher handling time, possibly due to the fact that the prey are bigger, the large increase in energy content leads to a higher profitability for the infected individuals." (L515-519)

We identified three phenotypic host modifications through coloration, speed, and size. Our aim was to test whether the phenotype of infected individuals modulates predation risk compared to normal phenotypes, and not to identify which of the three alterations was the cause of this modulation. Disentangling correlations among the different phenotypic alterations is an interesting perspective that goes beyond the scope of our study. We added in the discussion: "It would be interesting to determine the relative importance of the various phenotypic changes observed in infected individuals. That is, whether predators prefer infected individuals because they are larger, slower, more visible, or due to changes in the energetic contents.” (L485-489)

- Secondly, in the manuscript the authors present two ways of analyzing their data, one being a Multiple Factor Analysis and the other being separated analyses for each trait considered. Since some phenotypic traits are co-varying as shown in the MFA, it is likely that the test power is biased by the number of tests done on the same individuals, also a significant effect on one trait could be misleading if this trait is covarying with another one already associated with parasitism. In this regard, the MFA might be more indicated than the individual tests ran on each trait although less informative in appearance.

You are right that the MFA is more suitable for the experimental infection’s results. Consequently, we moved it to the main text. We moved the results on the other measures (survival, fecundity, size, and mobility) from naturally-infected individuals to the appendices and discussed there how they confirm or infirm the previous results. We also moved to the appendix the isolated analysis of experimental infection’s results to allow direct data comparison between natural and experimental infections.

- Finally, I strongly encourage the authors the review the language throughout their manuscript. In the minor comments I pointed at some mistakes or language misuse but this is not exhaustive. Also, I think the overall clarity of the manuscript could be really improved by synthetizing more the information and structuring better between the main text content and the supplementary content (eg., about the two types of predators used, or the MFA and single test approach).

Thank you for highlighting these mistakes. The manuscript had also been corrected by a native speaker. We now moved all redundancies (single test, naturally infected individuals, second predator) in the appendix, and they are now only used as a point of comparison for the results in the main text. In the main text, to link with experimental infection’s results, we added: “Results are similar for natural populations (Appendix B), with no effect on fecundity, lower mobility and higher body size for infected individuals.” (L369-370). We also hope that many other modifications (e.g., in response to the other reviewers' comments) improve the overall clarity of the manuscript.

- L96 Write Daphnia full name, not just D when put at the beginning of a sentence.

Corrected, thank you.

- L108 Precise effect of the parasite on the host.

We have written "a reduction in host survival and fecundity"

- L133 Before the beginning (not start) of the experiment.

Modified, thank you.

- L133 Rephrase or just remove the sentence starting with "in the main body of the article". It might not be necessary to precise in the method section what content is in the MS and in the supplementary, it can be done while presenting the results.

We removed it.

- L. 137 In this study, not this article.

Modified, thank you.

- L137-143 Please renumber the different measure in appearance order.

We modified the paragraph and added the table 1: "In this study, we performed an experimental infection to determine the effects of DIV-1 on fecundity (Measure 1), mortality (Measure 2), mobility (Measure 3), and size (Measure 4). We also used naturally-infected individuals to measure fecundity (Measure 1), mobility (Measure 3), size (Measure 4), energy content (Measure 5), coloration (Measure 6), vulnerability to predation (Measure 8), and predator preference (Measure 9). Table 1 summarises the measures performed on each collected *Daphnia*." (L143-149)

- L165 and along the MS Please write numbers under ten in letters.

We agree with you, however our English language reviewer has written some numbers under ten in number, likely when it was linked with a unity or a date/time.

- L185 five minutes (then provide abbreviation)

Thanks for your comments, however, because min is a unity abbreviation accepted by the SU, it is not mandatory to provide the complete word (as h, kg, cm …)

- L 209 Would you say that a concentration has been measured?

We would prefer to keep the current terminology as we feel concentration would be more suitable in a context of quantities of molecules within liquids (solutions).

- L 257 why a different brand of water has been used?

We used spring water with Notonecta because (1) it was useless to use mineral water for short-term experiment, and (2) to have similar conditions between fish and Notonecta experiment (and for fish experiment in larger water volume, using Volvic water would be too costly)

- L 295 replace by “with a quasi-poisson error term and a logarithmic link function”

Modified, thank you. Similarly, we modified “with a Gamma error term and an inverse link function”

- L 342 Exposed individuals were not

Corrected, thank you.

- L 403 different from

Corrected, thank you.

- L 404, precise infection effect.

At this step the statistical analysis only informs us that there is a significant effect of infection without any detail on the directionality. At the end of the paragraph, we have written “the profitability of infected *D. magna* is significantly higher than the profitability of healthy ones” (L424-425).

- L 424 No changes of

Corrected, thank you.

- L 441 the meaning of this sentence is not clear, please rephrase.

We modified by: “Many phenotypic alterations, such as body size, mobility, and coloration, could lead to indirect effects affecting trophic interactions.” (L458-459)

- L 554 loan is not correct, prefer for lending material and providing help.

Corrected, thank you.

- L 555 Repeat for providing

Modified, thank you.

- L 758 How much of the total variation is capture by each axe?

We added in the text: “(30% and 21.3% of the total variation)” (L371) and “– while the third axis, 16% of the total variation, does not separate Control and Exposed *D. magna*” (L372-373)