

Dear Dr Nadeau,

We thank you and the reviewers for these comments that without doubt have greatly improved our manuscript. Please find here our detailed response. We attached an annotated document with changes highlighted in yellow. We hope that these improvements will make our manuscript acceptable for publication in JEB.

Best regards

for the authors
Alice Balard

Author response to Reviewers

Response to reviewer: 1

Comments to the Author This revision has greatly improved the manuscript. However, I still have a few comments that require addressing:

C1. I am concerned that the authors still do not present conceptually or theoretically-driven motivation for examining the relationship between hybridization and infection in their system. In their response to my initial critique, the authors stated that the literature is controversial and conflicted. However, the lack of a clear prediction from the wider literature and theory should be mentioned in the introduction (before introducing the system) and is in itself a superb motivation. Lines 45-51 I think mention a bit of theory and perhaps should be mentioned before introducing the system.

Following this comment, we updated our abstract (see **C5.**) and our introduction replacing lines 45-51 by the following (new lines 45-59):

“Hybrids in tension zones have reduced fitness compared to individuals with “parental” genotypes due to genetic incompatibilities revealed on parentals’ secondary contact (Barton & Hewitt, 1985). As different components of fitness can vary independently, the immune system of hybrids might either benefit from recombinant genetic heterogeneity or suffer from incompatibilities. In the case of benefit we might expect decreased parasite load in hybrid individuals; in the case of incompatibilities we might expect increased load in hybrid individuals, compared to parental hosts. Parasites are traditionally seen as decreasing their hosts’ fitness, and differences in resistance to parasites between hybrid and pure hosts were suggested to affect the dynamics of hybrid zones (Fritz, Moulia, & Newcombe, 1999). An

involvement of parasites in the maintenance or breakdown of species barriers, however, has never been clearly justified or demonstrated (Baird & Goüy de Bellocq, 2019). In the HMMZ system, there is disagreement on both the direction of effects of hybridization on parasites (see Sage et al. 1986 and Moulia et al. 1991 vs. Baird et al. 2012) and on the interpretation of these findings with regards to host fitness and hybridization (see for example Theodosopoulos, Hund & Taylor, 2018; Baird & Goüy de Bellocq, 2019).”

C2. Lines 49-51. 2007 is not recent

Indeed, we corrected that (see C1). This study (Råberg et al. 2007) is now cited only in the discussion, and not called “recent”.

C3. Line 422. What is meant by 'parasite processes'? This is not clear.

We modified as follow for clarity: (new line 454-455)

“To understand the impact of immune diversity in hybrid hosts on parasites, it is necessary to test different types of parasites.”

Response to reviewer: 2

Comments to the Author

Review of „Intensity of infection with intracellular *Eimeria* spp. and pinworms is reduced in hybrid mice compared to parental subspecies”.

The authors compared infection of hybrid vs. parental mice, as sampled from a previously unstudied transect of the European mice hybrid zone (in Brandenburg, Germany). Two types of parasites were screened: intracellular protozoans (*Eimeria* spp.) and extracellular macroparasites (pinworms). The authors assessed different measures of infection (such as prevalence and intensity) and concluded that hybrids had lower intensities of both parasites, compared to parental species. This is a well performed study; sampling design and statistical analyses are appropriate and sound.

C4. I have one major comment: it is not being mentioned at all, if “co-infection” factor was somehow

included in the analyses – I guess it was not. Maybe there were no co-infected individuals? (or there are other reasons to believe that infection by one parasite would not influence the probability of being infected by another one)? In any case, I think the authors should at least shortly mention that potential confounding factor (or its absence, if this was a case).

We agree that addressing co-infections with regards to the main goals of our study improves our manuscript. Unfortunately power issues in our likelihood analysis did not allow us to use co-infection as a grouping variable in our models: only 26 mice of 70 infected by *Eimeria* spp. were not infected by pinworms, which was a too low sample size to allow maximum likelihood estimation of the parameters. Nevertheless, we provide a statistical test showing no significant signal of interdependence of the two parasite species with regards to an assessment of the host immune system (new lines 343-348):

“Interactions between the two parasite species studied in co-infection could influence both their intensities. This would make the assessment of different parasites non-independent with regards to the host immune system. We therefore tested the influence of co-infection by one investigated parasite on the second one using Chi-square tests on a presence/absence contingency table. We found infections with one parasite to not significantly change the likelihood of infection with the other ($\chi^2_1 = 1.72$, $P = 0.18$, $N = 383$).”

Other comments: **C5.** Line 4-5: I am not convinced by the line of argumentation in this first sentence of the abstract. Why would “range of hybrid genotypes” provide the special opportunity for studying “the control of parasite burden by their host”? One could explore the latter without any hybridization being involved, isn’t it?

Thank you for this comment, we modified the abstract as follow:

“Genetic diversity of animal immune systems is usually beneficial. In hybrid recombinants, this is less clear, as the immune system could also be impacted by genetic conflicts. In the European house mouse hybrid zone, the longstanding impression that hybrid mice are more highly parasitized and less fit than parentals persists despite the findings of recent studies. Working across a novel transect we assessed infections by intracellular protozoans, *Eimeria* spp., and infections by extracellular macroparasites, pinworms. For *Eimeria* we found lower intensities in hybrid hosts than in parental mice but no

evidence of lowered probability of infection or increased mortality in the centre of the hybrid zone. This means ecological factors are very unlikely to be responsible for the reduced load of infected hybrids. Focusing on parasite intensity (load in infected hosts) we also corroborated reduced pinworm loads reported for hybrid mice in previous studies. We conclude that intensity of diverse parasites, including the previously unstudied *Eimeria*, is reduced in hybrid mice compared to parental subspecies. We suggest caution in extrapolating this to differences in hybrid host fitness in the absence of, for example, evidence for a link between parasitemia and health.”

C6. Line 18 (keywords): “*Eimeria*” is already present in the paper title. We removed “*Eimeria*” from the keywords

C7. Line 49-51: the mentioned here 2007 paper is not that “recent”, isn’t it?

Indeed, we corrected, see **C2.** and **C1.**

C8. Line 60-63: the authors write here that Baird et al. 2012 paper is a “more recent” one, and includes “more mice” and “more suitable stats” – however, it is unclear what do they compare it with. The sentences above this fragment are describing laboratory work, whereas they referred to Baird et al. 2012 as a “field study” (?).

The previous field studies were cited at the beginning of the paragraph. For clarity, we modified as follows (new lines 68-72):

“In 2012, more than two decades after the original field studies (Moulia et al., 1991; Sage et al., 1986), Baird et al. found, (with much larger sample size, clearer sampling design and more up to date inference), reduced helminth loads in hybrid mice (Baird et al., 2012), especially for the pinworms *Aspicularis tetraptera* and *Syphacia obvelata* and the whipworm *Trichuris muris*.”

C9. Line 112-116: I suggest changing the order of sentences. First the mice were measured (what is currently reported in the very last sentence) and only then they were dissected etc., isn’t it?

Indeed, that is a good point, we modified as follow to represent the chronological order of events (new lines 121-122):

“Mice were individually isolated in cages and then euthanized by isoflurane inhalation followed by cervical dislocation within 24 hours after capture (animal experiment permit No. 2347/35/2014).

Individual mice were measured (body length from nose to anus), weighted, and dissected. Tissue samples (muscle and spleen) were transported in liquid nitrogen and stored at -80°C for subsequent host genotyping. Digestive tracts were dissected and inspected for helminth parasites (see below). Ileum, caecum and colon tissues were frozen in liquid nitrogen and then stored separately at -80°C. “

C10. Line 119: where does this big difference in a range of genotyped markers come from? (i.e. from 4 to 14). Did genotyping of some individuals fail for some reason, or is it rather the case that not all mice were genotyped with a full set of markers?

Indeed, this discrepancy is due to technical failure of some markers for some mice. For clarity, we replaced (new lines 132[...]141-142):

“The admixture of mouse genomes across the HMMZ was estimated for each mouse as a value of the hybrid index (HI) calculated as a proportion of Mmm alleles in a set of 14 diagnostic markers.[...] At least 10 loci provided information for 92% of the mice, and at least 4 loci for the remaining 8% due to technical issues.” Histograms for the number of genotyped markers, as well as their distribution across the hybrid index indicate no bias in genotyping (**Supplementary Figure S1**).

C11. Line 241: I think it should be “taxa” and not “taxons”.

Thanks for finding this mistake, we corrected. (new line 255)

C12. Line 295: differences between which “subspecies” (were allowed)? We mean that we didn’t fix the parental load but allowed the model to possibly chose two different ones. We modified for clarity: “Differences between the subspecies were allowed.” to “Differences between the loads of the pure parental subspecies on each side of the hybrid zone were allowed.” (new lines 309-310)

C13. Line 344: “more or less uninfected” – maybe better (i.e. more intuitive): “more or less infected”?

Indeed it is more intuitive and we corrected as advised. (new line 370)

C14. Line 405: “or mortality” – shouldn’t it be “in mortality” instead?

Indeed this is a typo, thank you for mentioning it, we modified. (new line 437)

C15. Line 422: it is unclear what is meant here by “parasite processes”.

This concern was shared by reviewer 1, we modified, see **C3**.

C16. Line 423: “types for parasites”, shouldn’t it be “types of parasites”?

Sorry this is a typo, thank you for mentioning it, we modified. (new line 455)

C17. Line 437-438: The sentence “Power to detect distinct male/female load is expected to be reduced by lower prevalence and abundance.” ends somehow abruptly, without relating (such as: “(...) and abundance, thus (...)”) to the above described findings.

Thank you for pointing this out. We noticed that our power argument was unclear and misleading, and simplified as follows: (new lines 469-471)

“We found differences between the Brandenburg and Czech-Bavarian transects in pinworm infection such as distinct loads between males and females and lower prevalence (52.5%) and abundance (18.7) in the former compared to the latter (no significant difference between sexes; prevalence 70.9%, abundance 39.18; Baird et al., 2012). Geographical locations of the HMHZ likely present different ecological conditions underlying such differences. Despite this fact, the direction (lower intensity in hybrids) and strength of the hybridization effect were very similar in the two study areas. This similarity reinforces our confidence that reduced parasite load in mouse hybrids is a general phenomenon, intrinsic to the individual host or host-parasite interplay rather than a by-product of ecology.”

C18. Line 440: what do the authors mean with the “direction” of hybridization effect?

We added for precision “direction (lower intensity in hybrids)”. (new line 471)

General comments from the editor:

C19. In particular, I agree with reviewer 1 that more broad background and theory in relation to the role of parasites in hybrid fitness and speciation is needed in the introduction and the abstract.

We corrected our introduction and abstract following this suggestion, see **C1.** and **C5.**

C20. Also, while I appreciate the point made in response to the previous reviewers' comments, that studying co-infection is beyond the scope of the current study, I think reviewer 2 is correct to say that co-infection should at least be mentioned, even if it is just to say that this was not investigated with a brief explanation of why.

We corrected following these comments, see **C4**.

C21. Please pay careful attention to the formatting of tables, figures and references, as well as the style used for reporting the results of statistical tests (see Instructions for Authors, <http://onlinelibrary.wiley.com>). Please make sure any in-line statistics conform to the Instructions for authors ("In-line statistical results should be presented as Test-statistics: degrees of freedom as subscript(s) to test-statistics (e.g. $F_{1,12} = \dots$ or $t_8 = \dots$), followed by P-value., e.g. ($F_{1,12} = 4.931$, $P = 0.0464$). Statistical results in tables should be comprehensive, allowing future meta-analyses. Depending on the details of the analyses, results reported may include parameter estimates, test-statistics, degrees of freedom, significance levels and err/residual model information (e.g. error MS's and df's in ANOVA or regression models). Since exact P-values can be useful for meta-analyses, we recommend that these are quoted even when non-significant, e.g. $t_{23}=0.25$, $P=0.34$, or $F_{2,32}=1.12$, $P=0.55$. However, non significant tests (i.e. $P > 0.05$) should always be interpreted as such.")

We made sure to provide comprehensive in-line information on our statistics and completed in the following (new) lines:

lines 340-342; 343-348; 357-370; 375-378; 384-388; 393-396; 402-406; 415-417; 426-431.

Finally, upon revision, we noticed that 3 sentences placed in results were more appropriate for the results section. Hence we moved them from lines 323-325, 331-332, and 337 to new lines 125 to 129.

“A median of 2 mice per locality were captured. A table of individual mouse data including hybrid indices, georeferences and parasite loads is available in **Supplementary Table S3**. To investigate *Eimeria* infections we checked 384 mice sampled in 2016 and 2017 for the presence and intensity of tissue stages (**Figure 2a**). Between 2014 and 2017, 585 mice were investigated for helminths (**Figure 3a**).”