JEB MS JEB-2020-00032

Dear Prof. Dr. Blanckenhorn,

with this letter we provide a revised manuscript. Below we respond to your and the reviewers' comments in detail.

Re: Reject with resubmission allowed

Dear Author,

Thank you for submitting your manuscript "Decoupling of resistance and tolerance against one of two related parasites (*Eimeria*) in mice" (JEB ms JEB-2020-00032) to the Journal of Evolutionary Biology. Your work has now been considered by two reviewers, whose comments are enclosed. Overall, and this includes my assessment, there seems to be some potential in your work being interesting for evolutionary biologists. However, as currently presented we must reject your manuscript for multiple reasons outlined by the reviewers below.

We want to thank both reviewers for the thorough reviews. We are confident we were able to substantial improve our manuscript taking into account all comments and issues raised. We changed the manuscript substantially, including the title, which now reads: "Coupling between tolerance and resistance differs between related *Eimeria* parasite species: implications for co-evolution with their mouse hosts"

C1. First and foremost, as mentioned by both reviewers, the writing, particularly the main set-up of your manuscript, leaves much to be desired and seems to be generally geared more to parasitologists than to evolutionary biologists. In particular, your work is taxonomically too focused on your system, and you fail to cite work on other species that is relevant in this context.

We now focus the manuscript on the evolutionary implications of coupling (or the absence thereof) between resistance and tolerance. We argue, these are, that host-parasite co-evolution can be expected

in the presence of coupling, much less in the absence. We then present our test for local (host subspecies) adaptation for *E. ferrisi* in this framework and conclude that our negative result for this might be explained with an absence of resistance-tolerance coupling.

C2. Second, reviewer 1 asks what the relevance or specificity of your work is in relation to the mouse hybrid zone, which of course would be very interesting to know from an evolutionary point of view but currently remains highly unclear and unargued.

We now highlight implications of our main result (resistance-tolerance coupling for one but not the other parasite) for the house mouse hybrid zone in the discussion (lines 354-366). We hope this shows the relevance of our findings within our system. This has direct implications for the evolutionary question of effects of parasites in hybrid zones. We also show that this serves an example beyond our systems (as we try to balance taxonomically focused with broad evolutionary implications as suggested by the reviewers). We argue that the framework of resistance-tolerance coupling allows to prioritize research questions to be addressed with different parasites: broad questions of relevance for the host species as a whole with parasites showing no coupling, questions of local adaptation and host-parasite co-evolution with parasites showing coupling.

C3. Third, both reviewers question your measure of tolerance. Reviewer 1 asks why you did not use the measured suggested in this context by another author that you cite, and reviewer 2 outright dismisses your index as statistically shaky if not flawed. I definitely agree with the latter assessment, which apparently is still a recurring problem in ecological and evolutionary studies.

We agree with the reviewers than our index calculated was based on measurements for host individuals. Following the advice of the reviewers, we changed it for a genotype based measurement, within each group. We described our approach in material and method as follows (lines 211-216):

> "Tolerance is usually defined as a reaction norm, i.e. the regression slope of host fitness (or health condition if that is the parameter of interest) on infection intensity per genotype (Simms, 2000; Råberg et al., 2009). Thus tolerance was assessed as the slope of maximum relative weight loss compared to day 0 on number of OPG at the day of maximal shedding, within each

mouse strain and for each parasite isolate. A steep slope indicates a low tolerance (high weight lost for a given parasite burden).”

C4. Fourth, a control group is missing.

Each animal present individual variation in weight at the time of infection. We are convinced that each individual at starting day is a better control for it’s own weight loss. The assessment of harm caused in animal infection experiments (for potentially necessary humane endpoints) does e.g. not allow comparison to a control group, but only to the starting weight of the same individual. We agree with this regulations and consider the “internal control” more relevant than uninfected control groups (which we are frankly not allowed to use in this experiment). We implement this within our analysis using a fixed a null intercept, as detailed in the previous comment.

These are but the most important criticisms of your work, and both reviewers made even more useful suggestions for change. In light of these comments and my own reading of your manuscript, I therefore cannot recommend your paper for publication and thus reject it.

We hope that you consider our revision for another round of review and that we were able to convincingly answer the reviewer’s concerns as detailed below.

However, I leave the option open for you to resubmit a substantially revised version of your article at some point in the future, if you think you can fix the main criticisms and make this work more interesting for evolutionary biologists. Any resubmitted manuscript will be treated as a new submission, and there can of course be no guarantee that the paper will ultimately be published by JEB. It is very likely that the new MS would be sent back to at least one of the original reviewers, so please think carefully about the value of resubmission (i.e. can you present new data/analyses or clarify issues in a way that is likely to satisfy the reviewer).

We added four more mouse strains and changed our analytical framework for tolerance, we hope that the revised conceptional framework satisfy the reviewers.

If you resubmit a revised version, please include a letter in which you describe how you have

responded to each of the referees' comments. Please number the comments and refer to line numbers in the original and revised paper for easy reference. A marked-up revision is also helpful. Please upload this letter with your other files so it forms part of the PDF.

We did not provide a marked-up version, as the our changes are so substantial that such document would not be useful. The text is ~70% rewritten and the remaining ~30% are reorganized.

Please submit the paper on the JEB website (http://mc.manuscriptcentral.c) as a RESUBMISSION, providing the original ms number. In your Author Center in ScholarOne Manuscripts you will find on the left a list entitled My Manuscripts. Click the Manuscripts with Decisions in this list; in the resulting list you will find this manuscript with on the right under Actions the option Create a Resubmission. By clicking this link you will be guided through the resubmission process.

Sincerely,

Wolf Blanckenhorn
Editor in Chief
Journal of Evolutionary Biology

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Reviewer: 1

Comments to the Author

In this manuscript about host-parasite interactions, resistance (parasite load) and tolerance (the maintenance of health despite infection) against Eimeria protozoan parasites of different species and strains are experimentally studied in house mice. Both resistance and tolerance should entail fitness costs, and high resistance will reduce selection on evolution of tolerance, and vice versa, so that one might expect species to exhibit either high resistance or high tolerance, but not both, predicting a negative correlation between the two. The house mice hosts were inbred strains derived from two Mus musculus musculus and two Mus musculus domesticus populations. The main result is that with infection with one Eimeria species, the two subspecies appear to show a negative correlation between resistance and tolerance, while none is evident upon infection by the second Eimeria species, which is quite interesting.

C5. I didn't understand the rationale for carrying out this experiment on Eimeria strains derived from the house mice hybrid zone – what do we learn from this that we wouldn't learn from Eimeria strains safely within the domesticus side or the musculus side of the hybrid zone? Are potential adaptations of Eimeria to mice of the hybrid zone important? Do the two Eimeria species not coincide elsewhere?

We thank the reviewer for this comment that will allow improvement of our manuscript. We now focus our conceptual framework and highlight two aims of our study: to test (1) presence/absence of resistance-tolerance trade-offs for each parasite and (2) local adaptation of *E. ferrisi*. We argue that presence or absence of coupling of resistance and tolerance allows expectation on the presence of local adaptation.

We are confident that this is now clearly pronounced in the introduction, lines 101-109:

> "We tested if coupling between resistance and tolerance differs between both parasite species and discussed the implication for parasite-host coevolution. As coevolving hosts and parasites can adapt to their local antagonist, we tested local adaptation of *E. ferrisi* to *Mus musculus*, using a parasite isolated in a *M. m. domesticus* host and one in a *M. m. musculus* host. Parasite local adaptation corresponds to a higher parasite fitness in sympatric than in allopatric host, and host local adaptation corresponds to a higher host fitness when infected with sympatric than

allopatric parasite (Schulte et al., 2011). If found, local adaptation would be indirect evidence for 109 coevolution of this parasite with *Mus musculus*."

and in the discussion, lines 385-386:

"Moreover, our results did not support local adaptation of *E. ferrisi*, which might be explained by the absence of host-parasite coevolution caused by uncoupling of parasite and host fitness"

C6. I also am not sure why it is advantageous to carry out this experiment on highly inbred mice, capturing little genotypic variation, rather than on mice with natural within- population variation. I found the answer in Raberg et al. 2009, who advocate such an approach to allow an estimate of the slope of the relationship between fitness and infection intensity, which they define as tolerance, per genotype. The authors of this manuscript refer to this as the usual way to measure tolerance, but this slope is not estimated here, instead a tolerance index is used – an individual based measure, rather than a genotype based measure. There is however no explanation given as to what governed this choice, why the tolerance index is preferable to the standard way, especially given that they use "fully inbred" mice (what does fully-inbred mean?).

We revised our analysis to a "slope per genotype" based measure, as described earlier in this document (**C3**):

"Tolerance is usually defined as a reaction norm, i.e. the regression slope of host fitness (or health condition if that is the parameter of interest) on infection intensity per genotype (Simms, 2000; Råberg et al., 2009). Thus tolerance was assessed as the slope of maximum relative weight loss compared to day 0 on number of OPG at the day of maximal shedding, within each mouse strain and for each parasite isolate. A steep slope indicates a low tolerance (high weight lost for a given parasite burden)." (lines 211-216)

C7. The introduction mentions that "understanding how resistance and tolerance are coupled is necessary to conclude on health effects of parasitism". I'm not sure I understand how data on the relationship between resistance and tolerance help to understand fitness costs. The introduction implies a prediction that resistance and tolerance are negatively correlated, but reports that one study found them to be uncoupled. A quick look at the literature led me to several more examples that are relevant,

although on different host-parasite systems. The discussion is very focused on house mice and Eimeria. There needs to be broadening out of the discussion and comparison with other systems.

We now focus much less on fitness effects on the host *per se* but make (potentially more bold, we admit) conclusion on the implications of resistance-tolerance coupling. In the discussion, we developed the relevance of our results for our system. We frame this as an example to better understand the appearance of the co-evolution in host–parasite systems. We argue that co-evolution in more likely in systems presenting tolerance-resistance coupling.

C8. Does sex play a role in either resistance or tolerance? Males and females were included in this study but pooled for data analysis.

We are not aware of reasons to hypothesize higher or lower resistance or tolerance in *Eimeria* infections in one sex. For this reason, we chose to not use sex as factor in the model, but rather used a sex-balanced design within each isolate infection group to add more (meaningful) variance and obtain more conservative results.
Lines 163-164:

> "Mice were randomly allocated to experimental groups ensuring homogeneous distribution of ages and sexes between groups."

C9. The experimenters ran into some trouble during the experiment, as some mice that tested negative to Eimeria infection prior to entering the experiment, turned positive by the day the experiment started. It would be helpful to indicate what the time frame was here – how much earlier was the negative test compared to the start of the experiment? This was unfortunate, and the authors laudably make it clear that this occurred, but argue that they found no sign that it affected the outcome of the experiment, by examining the data with and without the affected trials.

We thank the reviewer to appreciate our efforts on honesty. We now make the timeframe for infections of the different batches (including the problematic batches) more clear. See lines 161 to 186:

> "All individuals were negative for *Eimeria* at the beginning of our experiment (before infection of first batch, as described in the next paragraph). In total, 168 mice were infected. Mice were randomly allocated to experimental groups ensuring homogeneous distribution of ages and

sexes between groups. Our experiments were conducted in four (partially overlapping) consecutive batches for logistical reasons. The first two batches were infected with the two *E. ferrisi* isolates (Brandenburg64 and Brandenburg139), the third and fourth by one *E. ferrisi* isolate (Brandenburg64) and one *E. falciformis* isolate (Brandenburg88). Our experimental design is summarized in **Table 1** (chronology of experimental batches can be scrutinized in **Supplementary Table 1**).

Nematode infection is common in breeding facilities (Baker, 1998) and could interact with *Eimeria* (Clerc et al., 2019). Nematode eggs were observed in flotated feces of mice belonging to all genotypes before the experiment. Despite treatment of the first infection batch of mice (B1, 22 mice) with anthelminthics (Profender®, Bayer AG, Levekusen, Germany) following the protocole of Mehlhorn et al. (2005), nematodes were still detected with PCR (following the protocole of Floyd, Rogers, Lambshead, & Smith, 2005) in randomly sampled fecal samples a week later. We therefore decided not to treat mice of the following infection batches. Moreover, we observed *Eimeria* oocysts in the feces of 28 mice belonging to the last experimental batch (batch B4) at the day of infection, likely due to cross-contamination between batches. For following statistical analyses, we considered along with the full data set (N=168) a conservative data set in which cross-contaminated animals and animals treated by anthelminthic were removed (N=118). Results obtained on the conservative data set can be found in **Supplementary Material S2**. Despite differences in significance due to a lower statistical power, the main conclusions of our analyses were consistent with those obtained on the main data set."

C10. I think this is an error on page 10 – "SHUNT (Mmd subspecies) shed more OPG at the peak of shedding than both Mmm subspecies, PWD and BUSNA". It looks like it should be "less" not "more".

Thank you for spotting this mistake! We reshaped the document (following other comments), and therefore removed this sentence completely.

C11. Tables 2 until 6 duplicate some information from the figures – the upper diagonal of the tables shows estimate contrasts and SE, while estimates and CI are plotted in the figures. The lower diagonal shows statistics and p values. It would help the reader to indicate the difference between the upper and lower diagonals in the table headings. I would also suggest moving these tables to the Supplement, but keeping the figures in the main document.

We thank the reviewer for this comment. As we reshaped the document (following other comments), we do not discuss in details the differences between each strains any longer.

C12. It seems clear that the controversy about degree of parasite resistance in house mice in the hybrid zones interests the authors – but the links to this study seem fuzzy, as the house mice were not from the house mice hybrid zone, only the parasites were.

As developed earlier (**C2.**), we revised our focus and we now highlight implications of our main result (resistance-tolerance coupling for one but not the other parasite) for the house mouse hybrid zone in the discussion (lines 358-366). We hope this shows the relevance of our findings within our system:

> "In our host system, the house mice, for example, it has been shown that hybrids between *M. m. domesticus* and *M. m. musculus* are more resistant to parasites (Baird et al., 2012), including *Eimeria*, but tolerance could not be measured under natural conditions (Balard et al., 2020). The effect of parasites on host fitness in the evolution of the house mouse hybrid zone is thus still rather ambiguous (Baird & Goüy de Bellocq, 2019). We show that careful distinction between parasite species is necessary when analysing parasite host interaction (see also Jarquin et al 2019) and that it is indispensable to measure both resistance and tolerance in *Eimeria* infections of house mice. "

C13. Were the data analysed twice (once looking at subspecies/species differences and once looking at strain differences) because there wasn't enough data to do one model with strain nested within species, fitting all the variables?

> We previously tried to summarize results at the parasite species and mouse subspecies level. This comment made clear that this was confusing. We thus only report results at the detailed mouse strain level and parasite isolate level. The other "summary analyses" were redundant.

Reviewer: 2

Comments to the Author

C14. In this paper, the authors explore the effect of experimental parasitism by two closely related Eimeria parasites on four widl-derived strains of inbred mice to infections in terms of body mass loss and parasitism load (density of oocysts in feces). They claim to have estimated the level of host defences against experimental infection in terms of resistance and tolerance response as the inverse of maximum density of oocysts and the ratio between body mass loss and maximum density of oocysts,

respectively. I do not think the approach used to estimate tolerance is suitable, for two reasons: first, by using ratios (Instead of reaction norms) tolerance might by confounded with vigour; and second, resistance values are used in the denominator of the equation to estimate tolerance, which by itself could explain the claimed negative association found for one of the parasites.

We revised our analysis to a "slope per genotype" based measure, as described earlier in this document (**C3**):

> "Tolerance is usually defined as a reaction norm, i.e. the regression slope of host fitness (or health condition if that is the parameter of interest) on infection intensity per genotype (Simms, 2000; Råberg et al., 2009). Thus tolerance was assessed as the slope of maximum relative weight loss compared to day 0 on number of OPG at the day of maximal shedding, within each mouse strain and for each parasite isolate. A steep slope indicates a low tolerance (high weight lost for a given parasite burden)." (lines 211-216)

C15. The paper is quite difficult to follow, and the importance of the study and results in evolutionary scenarios is vaguely introduced. In fact, most results merely describe the effects of experimental infection. The part of the manuscript that in my opinion is more interesting for evolutionary biologist is the study of associations between defensive tolerance and defensive resistance, and the manuscript should therefore be focused in this matter. However, as mentioned before, I think the estimate of tolerance is not appropriate and thus I do not think the conclusions on this experiment are strong enough.

We rewrote it almost entirely, and changed our estimate of tolerance. We are curious how the reviewers perceive this new focus.

C16. There are no line numbers, which make difficult pointing at the sentences to comment. In any case, with the hope of explaining further my point of view, I offer some comments below.

We apologize for this mistake and added line numbers in the current (we previously had the experience that the JEB submission system adds a – then confusing – own set of line numbers).

C17. Abstract has to be completely rewritten with direct and clear messages introducing the importance of the study, methodology employed, results and inferences of result. By now, from the abstract, it is difficult understand the performed work, whereas readers should be able to figure out what has been done. Some examples:

Abstract, 4th line: what do you mean with "hybrid hosts"?

Abstract, 5th line: what kind of fitness effects? What do you mean with modulatory effect?

Abstract: brief explanation of how resistance and tolerance responses were estimated is necessary here, before exposing the results.

Abstract, the two last lines: why findings of resistance in …. have to be interpreted carefully?

We re-wrote the abstract following this advice but also to reflect the revised conceptual focus of our manuscript.

C18. Introduction:

"In natural populations, costs of the two lines of defences against parasites predict that resistance and tolerance are negatively correlated". I do not agree! Tolerance and resistance can be positively, negatively or nor related to each other. It is just that two different lines of resistance defences, which both are costly, can be positively, negatively or nor related to each other.

We agree and modified the introduction highlighting the different possible scenarios of a) negative, b) positive or c) absence of correlation (see lines 55 to 83).

C19. The Introduction is devoted to (i) explain that both resistance and tolerance defensive responses have associated costs, (ii) introduce mouse subspecies immunological characteristics, the two protozoa Eimeria parasites, their prevalence, and their effects in their mouse hots. The authors finish the introduction mentioning that because of differences in prevalence and pathogenicity, associations between resistance and tolerance against the two parasites might differ. I think authors should pay more effort to explain the importance of the study in evolutionary scenarios by, for instance, make clearer the

importance of detecting different defensive strategies of hosts against different parasites in scenarios of host-parasites interactions and evolution.

We entirely rewrote our introduction following this advice, please see in revised manuscript. We focus on the implication of coupling between resistance and tolerance.

C20. Material and methods

Please, try not to use acronyms, or reduce them to a minimum. It makes it very difficult to follow the text.

We replaced all Mmm and Mmd acronyms by species names for clarity.

I am not an expert on the described lab methodologies, and thus I cannot comment on them. Experimental approach:

C21. Mice were orally infected with 150 sporulated oocysts, weight recorded and faeces collected daily.

All 108 used mice we infected and no control group was followed. In my opinion, control groups are necessary given that cross-contamination between batches can occur, and because experimental mice might be infected by nematodes or others kinds of parasites (last paragraph of “experimental infection).

As discussed earlier (**C4.**) each animal present individual variation in weight at the time of infection. We are convinced that each individual at starting day is a better control for it’s own weight loss. The assessment of harm caused in animal infection experiments (for potentially necessary humane endpoints) does e.g. not allow comparison to a control group, but only to the starting weight of the same individual. We agree with this regulations and consider the “internal control” more relevant than uninfected control groups (which we are frankly not allowed to use in this experiment). We implement this within our analysis using a fixed a null intercept, as detailed in earlier comments.

Measures of resistance and tolerance: I am not sure the approach is appropriate or correct to test the idea.

C22. - Resistance: “number of oocysts per gram of feces (OPG) at the day of maximal shedding”. Do you mean the inverse of number of oocysts …. ?

We corrected this point, see lines 192-193:

> "Therefore, as a proxy of (inverse of) resistance we used the number of oocysts per gram of feces (OPG) at the day of maximal shedding. "

C23. - Tolerance: "We defined a tolerance index for each individual, describing how its health varied with infection intensity, between day 0 of infection (weight = 100%, parasite intensity = 0 oocyst per mouse gram) and highest impact (weight = maximum weight loss relative to day 0, parasite intensity = maximum parasite number per gram of feces)."

Two points: (i) But you first mentioned that tolerance is defined as the slope (reaction norm) of the relationship between parasite burden and fitness related variable (… ??). Why didn't you estimate the reaction norms, then?

(ii) Ratios between fitness related variables (or phenotypic quality) and intensity of parasitism are not a good approach to characterise tolerance because it might be confound with individual vigour (i.e., better quality individuals will experience smaller negative effect of a target parasitism burden, but it does not mean they are more tolerant than individuals of lower quality). See discussion on Raberg et al. 2009 and Fry 1993 (Fry JD. 1993. The "general vigor" problem: can antagonistic pleiotropy be detected when genetic covariances are positive? Evolution. 47:327–333.) for the exposition of the namely vigor problem.

(iii) In any case, tolerance is estimated by using the value of resistance in the denominator of the ratio, implying additional problems of interpretation of results, mainly when the aim of the manuscript is to explore association between estimates of tolerance and of resistance tolerance = (maximum relative weight loss … / maximum number of oocysts …), As the maximum number of oocysts is used as a measure of resistance, tolerance is defined on the base of the level of resistance. Thus, it is not unexpected that estimates of resistance and tolerance were negatively related, it is just the mathematical consequence of the methods used to estimate them.

We agree with the reviewers than our index calculated was based on measurements for host individuals. Following the advice of the reviewers, we changed it for a genotype based measurement, within each group. We described our approach in material and method as follows (lines 211-216):

> "Tolerance is usually defined as a reaction norm, i.e. the regression slope of host fitness (or health condition if that is the parameter of interest) on infection intensity per genotype (Simms,

> 2000; Råberg et al., 2009). Thus tolerance was assessed as the slope of maximum relative weight loss compared to day 0 on number of OPG at the day of maximal shedding, within each mouse strain and for each parasite isolate. A steep slope indicates a low tolerance (high weight lost for a given parasite burden)."

We also discuss possible statistical artifacts that could emerge as well from a linear regression between tolerance (measured now as reaction norm) and resistance (lines 254-265):

> "Of note, tolerance (in absolute value) is measured as the slope α of the linear regression of parasite load (x) on maximum relative weight loss (y) of equation $y = \alpha x + \beta$ (α being the slope and β the intercept, 0 in our case). Therefore, tolerance is expressed as $\alpha = y/x - \beta$. As x and y/x are by definition not independent, testing the correlation between resistance and tolerance can lead to spurious correlation (Brett, 2004). To alleviate the dangers of this statistical artifact, we additionally tested differences in resistance, impact on health and tolerance between mouse strains separately and also the underlying correlation between mean parasite load (x) and mean relative weight loss (y). We use the terminology "coupling" (between resistance and tolerance) to describe genotype-level correlation between tolerance and resistance additionally supported by the absence of positive correlation between health-effect and resistance."

We thank the reviewers for these comments that without doubt have greatly improved our manuscript. We hope that these improvements will make our manuscript acceptable for publication in JEB.

Yours sincerely,
The authors

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