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**Manuscript:** “Wolbachia and host intrinsic reproductive barriers contribute additively to post-mating isolation in spider mites” (Cruz *et al.*).

Dear Dr. Jan Engelstaedter,

We would like to thank you and the referees for your constructive comments, which helped us improve the manuscript. We herewith send you a thorough revised version, along with our point-by-point replies to the questions raised by yourself and the two reviewers (see Pages 2 to 9 below, in which line numbers are given for the marked-up version of the article file). We have also updated the version of the article file and supplementary materials (without marked changes) in BioRxiv, and have added the R scripts used to carry out statistical analyses (<https://doi.org/10.1101/2020.06.29.178699>).

We hope that you will find the changes made to the manuscript useful and that you will consider its recommendation by *PCIEvolBiol*.

Yours sincerely,

Flore Zélé on behalf of all authors

Lisbon, October 31<sup>st</sup> 2020

## Recommender's report

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by Jan Engelstaedter, 2020-08-19 12:13

Manuscript: <https://doi.org/10.1101/2020.06.29.178699>

Wolbachia is a widespread maternally inherited endosymbiont of arthropods. These bacteria often induce mating incompatibilities between males and females with different infection status, which suggests that they may play a role in host speciation. In this article, Cruz et al. investigate the contribution of Wolbachia to reproductive isolation between different population of the spider mite *Tetranychus urticae*, relative to host-related incompatibilities.

Both reviewers commented positively on the experimental design of this study, and I agree that the experiments, based on a full factorial design testing all possible types of matings, are impressive in their scope and well suited to answering the questions posed. However, the reviewers also raise a number of issues that should be addressed in a revised manuscript. Most importantly, both reviewers suggest a thorough discussion of the role of pre-mating isolation in this system.

In addition, and related to this, I would also like see more context provided on the system in the introduction. The MD and FM types of CI are well explained but very little background information is given on the "biogeography" of these mites and their symbionts. E.g., do the green and red types co-occur symmetrically or only in allo- or parapatry? Is Wolbachia fixed in these populations or is there generally a polymorphism between infected and uninfected individuals? Are populations from the red and green type that are compatible infected with the same strain of Wolbachia, or are both uninfected? Given all that is known about this system, are the three populations chosen representative of the species (complex) as a whole?

**Reply 1.1 - Thank you very much for your very positive comments. We have added missing information about the system in the introduction to meet your requests (lines 91-95 and 105-112). There are several studies showing (partial) reproductive isolation among spider mite populations, which we refer in the text (lines 99-108). Obviously, we cannot state that all species pairs will be isolated with the same relative contributions of symbiont- and host-mediated isolation as those found here. It would also be humanly impossible to extend such labour-intensive experiments to more populations. Still, we believe that the ubiquity of infection with symbionts and of the variable degree of reproductive isolation observed among spider mite (and other arthropod) populations make our results highly relevant. Please see also Replies 2.2 and 2.28.**

Some other comments:

- Abstract: "However, most studies focus on closely-related populations of single species" - this sounds as if the authors are setting up the stage for a follow-up sentence like "Here, we instead do ...", but the next sentence doesn't quite live up to that expectation (partly because the study system is also a single species).

**Reply 1.2 - We agree that this was confusing and have changed this in the abstract to clarify that the majority of studies on *Wolbachia*-induced incompatibilities focus on closely-related (intrinsically incompatible) populations, whereas host-intrinsic and *Wolbachia*-induced incompatibilities have seldom been addressed together.**

- Abstract: "these two sources": not quite clear which two sources are referred to because there's a sentence in-between, perhaps be more explicit here.

**Reply 1.3 - We have changed this to "host-intrinsic and *Wolbachia*-induced sources".**

Fig.1 & 2: I was wondering why some of the crosses aren't shown here. Are those the ones that aren't as interesting for the questions asked, or is there another reason? Might be good to either add them or to mention why not in the figure legend.

**Reply 1.4 - As mentioned in the Materials & Methods (lines 203-207), crosses between infected females x uninfected males (category 5; cf. Table 1) were not performed simultaneously with the other crosses. For this reason, the results obtained with crosses of category 5 cannot be statistically compared with crosses of categories 1 to 4. This is thoroughly explained in Box S2. Furthermore, as the results obtained with crosses of category 5 recapitulate the pattern observed for crosses of categories 1 and 3, they are indeed not relevant to answer the questions asked in our study and more suited for Supplementary Materials. Following your suggestion, we have added a note in the legends of Fig. 1 and 2.**

Fig.1: I think the colour scheme used in Fig.2 is quite nice, perhaps the same scheme could be used for Fig. 1? (E.g., coloured circles above the plots instead of the numbers?)

**Reply 1.5 - Following your suggestion, we tried to use coloured circles instead of numbers, but we felt that the Figure was even harder to understand due to the multiplication of colour codes. Nevertheless, your suggestion led us to think of another way to improve this figure (and Fig. S1 following the same logic). We hope that you will like these new versions.**

Box S1 heading: I think it should be "relating to" instead of "relative to"

**Reply 1.6 - This has been changed, thank you.**

Additional requirements of the managing board:

As indicated in the 'How does it work?' section and in the code of conduct, please make sure that:  
-Data are available to readers, either in the text or through an open data repository such as Zenodo (free), Dryad or some other institutional repository. Data must be reusable, thus metadata or accompanying text must carefully describe the data.

-Details on quantitative analyses (e.g., data treatment and statistical scripts in R, bioinformatic pipeline scripts, etc.) and details concerning simulations (scripts, codes) are available to readers in the text, as appendices, or through an open data repository, such as Zenodo, Dryad or some other institutional repository. The scripts or codes must be carefully described so that they can be reused.

-Details on experimental procedures are available to readers in the text or as appendices.

-Authors have no financial conflict of interest relating to the article. The article must contain a "Conflict of interest disclosure" paragraph before the reference section containing this sentence: "The authors of this preprint declare that they have no financial conflict of interest with the content of this article." If appropriate, this disclosure may be completed by a sentence indicating that some of the authors are PCI recommenders: "XXX is one of the PCI XXX recommenders."

**Reply 1.7 - The R scripts used to carry out the statistical analyses are now available as additional files in BioRxiv. We have also added the Data Accessibility Statement and the Conflict of Interest Disclosure in the manuscript.**

## Referee 1

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*Reviewed by anonymous reviewer, 2020-08-12 15:09*

Some comments on "Wolbachia and host intrinsic reproductive barriers contribute additively to post-

mating isolation in spider mites” by Miguel A. Cruz, Sara Magalhães, Élio Sucena, Flore Zélé (DOI <https://doi.org/10.1101/2020.06.29.178699>).

Cruz et al report here on a rather detailed and complete set of experiments aiming at assessing the respective contributions of *Wolbachia*-induced CI and other forms of incompatibilities on post-mating isolation between three populations of *Tetranychus urticae*. In fact, two of the populations are very close, and apparently not isolated at all, while the third is rather deeply divergent (over 5% divergence at the mtDNA level) and completely isolated. I do agree with the authors that teasing apart the effects of *Wolbachia* versus other causes in establishing reproductive isolation is important. I see, however, two limitations here, that the authors may want to discuss more specifically in a revised version of their manuscript. One is that the importance of pre-mating isolation is not known in this system, so that it is difficult to assess the actual contribution of post-mating isolation.

**Reply 2.1 - Thank you for these helpful comments. We agree that measuring pre-mating isolation is important to better understand the contribution of post-mating isolation in this system, and we have already highlighted this issue in the discussion (lines 711-713). Unfortunately, the lack of studies (only one to our knowledge; mentioned in lines 707-708) conducted on pre-mating isolation between the two colour forms hampers any thorough discussion concerning this issue. We have conducted another set of experiments (mate choice, mating and re-mating propensity, as well as the effect of re-mating on offspring production, which informs on sperm transfer/competition in this system), and we are currently preparing a second manuscript with these data. We chose not to include this data set because the manuscript is already dense, and to write the manuscript on post-zygotic isolation first because these data are needed to understand the patterns of pre-zygotic isolation. Moreover, we believe that the current data set stands alone, as the aim of this manuscript is to determine the relative contribution of different sources of incompatibility to post-zygotic isolation, not the contribution of post-zygotic isolation to total isolation between populations. Still, we acknowledge that without some information on pre-zygotic isolation, the reader may question the relevance of the results obtained on post-zygotic isolation. Therefore, we have added a note in the manuscript to mention that the results presented here do show that premating isolation is incomplete (otherwise no hybrids would be produced in inter-population crosses), but because we did not control for the occurrence of copulations with behavioural observations in this experiment, we cannot exclude the possibility that less copulations had occurred in crosses that resulted in an overproduction of males (lines 591-592).**

The second is that isolation without *Wolbachia* seems so high already that it is difficult to derive conclusions with regard to the importance *Wolbachia* plays in current isolation or has played in establishing speciation.

**Reply 2.2 - We agree that it would have been great if the green- and red-form populations used here were not fully isolated. Unfortunately, we were unaware of this when we designed these experiments. We have highlighted the need to study population pairs with variable degrees of post-mating isolation (*i.e.* incomplete isolation) in the discussion (lines 664-672). In light of your comments (see also Reply 2.28), we also opted to remove the discussion concerning the role of *Wolbachia* on ongoing processes of speciation in spider mites, which was rather speculative. However, we would like to point out that, even though *Wolbachia* does not ultimately contribute to full reproductive isolation between the populations used here, our study is, to our knowledge, the first to provide a meticulous methodology to disentangle the relative effects of HI and wCI on offspring production. By doing so, we show that they act independently, through different mechanisms, which we consider to be a strong point and a novelty in the study. Furthermore, as discussed in lines 673-713, even with the populations we used, and which are fully post-zygotically isolated, CI induction by *Wolbachia* in intra-population crosses may have important ecological consequences at the between-population level.**

Another general comment is that I found the Results section rather difficult to follow. It may help to guide the reader more gently through the results, possibly by asking / answering questions one at a time? In relation with this, it seems to me that useful take home numbers would be estimations of how much gene flow would be increased if one or the other causes of post-mating isolation were removed (assuming no premating isolation). I suggest trying to derive such numbers from this analysis.

**Reply 2.3 - Thank you for your suggestions. Indeed, these results are complex and finding the most straightforward way to present them was challenging. We organized the Results section to present each analysed variable, one by one, because we did not know, *a priori*, what would be the consequences of wCI and HI. For instance, wCI also leads to MD-type incompatibilities in some *T. urticae* populations (mentioned in lines 78-82). Following your suggestion, we have clarified the structure of this section by explicitly asking questions at the end of the first paragraph (lines 373-379), and we improved guidance of the reader by adding a brief summary of the results at the end of each paragraph (lines 396-408, 420-423, 450-453, 488-491, and 527-529). We also added the extent (percentage) to which each cause of post-mating isolation reduces hybrid production in the abstract and in the discussion (lines 535-536). We do not explicitly refer to gene flow because there is no gene flow in any interpopulation crosses in this study. We could do so only if assuming at least partial F1 fertility and F2 viability (note that premating isolation is incomplete; cf. Reply 2.1).**

Below are some more detailed comments, that I hope will be found helpful.

L28: “polymorphs” => polymorphism?

**Reply 2.4 - This has been changed, thank you (line 28).**

L30: “Moreover, whereas speciation induced by wCI...”; I think this idea could be made clearer by adding a few words: “Moreover, whereas speciation SOLELY induced by wCI may require very specific conditions, WOLBACHIA could STILL play a significant role in host speciation by interacting with other (intrinsic) isolation mechanisms.”

**Reply 2.5 – Thank you for this suggestion, changes have been made accordingly (lines 30-32).**

L42: “However, in the latter scenarios...”; I think “however” could be removed.

**Reply 2.6 - We feel this “However” is necessary because it highlights the knowledge gap that we addressed in our study.**

L44: “additive or interacting effects”; but the words above “either a complementarity or a synergy” seem to indicate additivity in the first case and positive interaction in the second? So, in what sense have interaction’s not been studied?

**Reply 2.7 - Thank you for pointing this out. In fact, in both cases, we were not referring to additive effects versus interactions, but to the direction of crosses. We agree that it was not obvious, that the sentence was overall confusing, and that the word ‘synergy’ was inappropriate. We have changed this sentence to explicitly state what we mean (lines 37-45).**

L68: “variable levels of wCI”; it seems the abbreviation “CI” would be better suited in this context.

**Reply 2.8 - As we reorganized an important part of the introduction (cf. Reply 2.9), the context of this sentence has changed and we think that using wCI is now appropriate (line 82-83).**

L69: “In spider mites, as in some other haplodiploid species...”: is this part of the paragraph at the best possible place? The paragraph starts with specific statements on the study system, and we thus have these general statements on CI in haplo-diploids, without any specific information on *T. urticae*. Here we would expect some statements on whether CI leads for male development or female mortality in this species?

**Reply 2.9 - We agree that MD- and FM-type CI are not specific to spider mites, as they concern the expression of CI in any haplodiploid species. However, we do not see how to mention this before introducing the study system, which is haplodiploid. Up to this point the introduction aimed at placing our study in a much broader context. Nevertheless, we tried to improve the flow of this part of the introduction by moving up this entire paragraph. We hope that, by doing so, the link between the first mention of haplodiploidy as a specificity of spider mites and MD- and FM-type incompatibilities is clearer. Following your suggestion, we also highlighted that the information provided pertains to the spider mite system in particular, and provided more information regarding wCI in spider mites (lines 58-86).**

L79: “Wolbachia-induced CI (wCI)”; the abbreviation was defined earlier

**Reply 2.10 - We have removed the definition here (line 113).**

L91: “COI mtDNA”; the CO1 difference is very large (over 5%) and falls largely over standards for conspecific populations. It would be of interest to know how much this translates into effective isolation. Is information available on how much the nuclear compartments differ?

**Reply 2.11: Unfortunately, we do not possess information on nuclear divergence aside from the nuclear ribosomal DNA *ITS2* (cf. Box S1), which is very conserved among spider mite species, and shows very low levels of divergence between the red and green form populations studied here. Conversely, the CO1 difference is indeed very large, but this is quite common in spider mites, especially for populations with low *ITS2* divergence (e.g. Navajas et al. 1998. *Heredity* 80: 742–752). Full genome sequencing of two of the populations used here (Ru1 and Gu) is currently in progress, and we hope to shed light on this in the future.**

L130: I suppose “quiescent females” in this context means “young and thus virgin females”, that will produce only males? Please provide explanations for the non-specialist.

**Reply 2.12 - Thank you for pointing this out. An explanation has been added (lines 188-191).**

L152: could it be that female cryptic choices introduces some errors in this estimation, for example, a trend to fertilise fewer eggs in crosses between U females and I males would lead to more males being produced even though diploid zygotes are killed by CI?

**Reply 2.13 - Females were exposed to a single male in this experiment (lines 192-193). Moreover, if the females had fertilized fewer eggs in crosses between U females x I males (i.e. crosses affected by wCI), we would see an increase in the proportion of sons even before computing the MDcorr index, which is not the case (cf. Fig. 1). Nevertheless, the overproduction of males in crosses between green-form females and red-form males can indeed result from incompatibilities affecting any stage of the reproductive process in the females’ reproductive tract (and not only sperm-egg incompatibilities). We have added this possibility in lines 583-588.**

L157: I would add something like the following sentence: “MDcorr is thus a measure of the excess in

male production in comparisons with controls, taking a value close to 1 when this excess is maximal”.

**Reply 2.14 - The meaning of the values taken by *MDcorr* has been added (lines 218-220).**

L197: “P-values were defined as...”; I don’t understand this sentence and the meaning of these P-values. Could the authors clarify the rationale behind this test?

**Reply 2.15 - We have changed the text to clarify the method and the meaning of this test (lines 255-264).**

L230: “As F1 female fertility corresponds to their ability to lay at least one egg, and/or a normal number of eggs”; I don’t understand this sentence very well; is it meant that the term fertility can mean both of these things (being fertile or sterile, versus being very much fertile or not?). This should be clarified; same comment on L257.

**Reply 2.16 - We define fertility as the ability to lay a 'normal' number of eggs (thus both laying no eggs and laying fewer eggs are cases of infertility). We have changed this sentence to avoid confusion (line 298; and line 325 for male fertility). We also reworded some sentences in the Results section accordingly (lines 461-463).**

L245: “a single maternal chromosome”; so, these animals have only one pair of chromosomes? Was that mentioned earlier?

**Reply 2.17 - Thank you for pointing this out, it has been corrected (lines 313-314).**

L253: “for males to mate”; isn’t that a strange wording, considering that females do mate as well?

**Reply 2.18 - This has been changed for “the individuals to mate” (line 321).**

L274: “R statistical package”; I think “program” should be used rather than “package” (R includes many packages).

**Reply 2.19 - This has been corrected (line 342).**

L312 “Overproduction of males (MD-type incompatibility)”; at the end of this section, at least after a 1st read, I must say it is difficult to have a clear view of what is causing this excess of males. I suggest adding some interpretations / hypotheses, rather than sticking to results only.

**Reply 2.20 – We have already mentioned, in the previous paragraph, that MD-type incompatibilities correspond to an overproduction of males resulting from fertilization failure and/or paternal genome elimination (lines 375-376). We then discuss this thoroughly in the Discussion (lines 567-592).**

L35: again, “wCI” is used when it seems that the usual “CI” abbreviation would seem sufficient.

**Reply 2.21 - It has been removed (line 412).**

L425: “males mated with (and sons of) green females”; the bracket is not clear to me and does not read very well. Same comment L430 & 441 & 442.

**Reply 2.22 - We agree that this is not the most elegant wording, but because the maternal**

population of the males and the population of the females they mated with are the same, it is not possible to disentangle their effects. We added an explicit note concerning this and reworded the sentence in which this explanation was provided to make it clearer (lines 496-498).

L443: “cannot necessarily be explained”; I don’t really understand this sentence, may be “necessarily” is superfluous?

**Reply 2.23 – We agree that the whole paragraph (including this sentence) was not particularly well written. We have corrected it (lines 516-526).**

One background issue that is not discussed is how much these different types of crosses could occur in nature, and thus what is the actual impact of the various post-zygotic problems seen here? Could it be that some of the post-zygotic barriers are simply not barriers because gene flow is prevented by other sources?

**Reply 2.24 - This is an interesting question, which we highlight in our discussion (lines 711-713). Please see Reply 2.1.**

L453: “up to 2 times more”; some additional explanation of what this number means would help. Something like “how much gene flow would be increased if one or the other factor was removed” would be helpful.

**Reply 2.25 - Please see Reply 2.3.**

L463: can you clarify what “unidirectional” means here?

**Reply 2.26 - An explanation has been added (lines 561-562).**

L487: I don’t think “Nevertheless” is useful here.

**Reply 2.27 - It has been removed (line 591).**

L524: “The combined effects...”; I am not at ease with the content of this paragraph. It is argued that one should try and quantify the respective contributions of CI and HI in restricting gene flow. If I understood well, it is seen that isolation would be complete even without *Wolbachia*. But still, it is argued that *Wolbachia* could play a critical role in isolation. The problem is that this Discussion faces a usual problem of interpretations of speciation processes: once several factors are at play, it is difficult to know which one actually mattered initially...

**Reply 2.28 - Even though *Wolbachia* does not ultimately contribute to full reproductive isolation between the populations used here, our methodology allows disentangling the relative effects of HI and wCI on F1 hybrid production (see also Reply 2.2). What we say in our discussion is that our results suggest that *Wolbachia* could play a critical role in isolation between populations that are not yet fully isolated (*i.e.* among which gene flow occur), which is common in spider mites as mentioned in the introduction (lines 99-101). To make it clearer, we have re-written the end of this section (lines 657-672). Also, we acknowledge that the next section of the discussion ('The role of *Wolbachia* on ongoing processes of speciation in spider mites') was quite speculative, and we removed it entirely.**

**Referee 2**

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*Reviewed by Wolfgang Miller, 2020-08-17 14:27*

This is a carefully planned and painstakingly performed study aimed to estimate the contributions of Wolbachia-induced (wCI) and host-associated (HI) incompatibilities in the haplodiploid spider mite *Tetranychus urticae* at the post-mating level between sympatric populations differing genetically such as in coloration. By multi-factorial crosses within and between populations at different infection states they found that in this incipient speciation system HI dominates over wCI in effectivity (1.5 – 2), where wCI seems to affect mortality of female embryos but HI triggers symmetric F1 sterility and F2 hybrid breakdown in backcrosses. In addition, HI is causing the overproduction of sons in these incompatible crosses and not the Wolbachia endosymbiont. They further show that both mechanisms can act together synergistically and that possibly such cyto-nuclear incompatibilities might predate nuclear ones.

Hence, this elegant study has managed to dissect - for the first time to my knowledge - the different degrees of contributions of the two postmating isolation mechanisms acting in this system, i.e., cyto-nuclear vs nuclear-nuclear BDMIs.

**Reply 3.1 - Thank you very much for these very positive comments.**

Minor comments:

Line 130: please explain to non spider mite experts what is meant with “quiescent females”.

**Reply 3.2 - We have now added this information (line 188-191).**

This last part is not meant as a critique: Although discussed later on, the design of this current study cannot rule out the possibility that not all females were inseminated by males in their crossing experiments and thereby the role of premating isolation is not yet fully determined. As nicely mentioned in the discussion, future cytological studies on early hybrid embryos will uncover additional mechanistic clues. In summary, however, this is a very important work that will further deepen our understanding in the short- and long-term dynamics of symbionts and their potential role in speciation, a highly exciting but still controversial subject in evolutionary and ecological biology.

**Reply 3.3 - We agree with the importance of studying premating isolation in this system. We are currently preparing a manuscript with these data (see reply 2.1).**