

We would like to thank the Recommenders and the Reviewers for their interest in the study and for the constructive comments that will help to improve the quality of the manuscript. We have submitted a new version of the manuscript (without annotation), another one including the track-changes, and the revised supplementary information and data (SI file). Below are details (in *italics*) of how we responded to these comments (line numbers correspond to the manuscript without annotation)..

## Reviewed by anonymous reviewer, 2020-12-24 13:47

In this study, the authors introgressed a *Wolbachia* strain into six different (previously uninfected) *Drosophila* lines to evaluate if host genotype has an influence on *Wolbachia* loads and Octomom copy numbers within *Wolbachia* genomes (octomom copy numbers were previously shown to be positively correlated with *Wolbachia* loads). Although *Wolbachia* loads significantly varied among introgression lines, the authors could not conclude that host genotype affected *Wolbachia* loads because only a single introgression line per host genotype was generated. The authors therefore generated three replicate introgression lines for two host genotypes in a second experiment, as well as reciprocal crosses between the two host genotypes. Because they found extensive load variation among replicate lines within genotypes, as well as variation over time within lines, they conclude that host genotype has no effect on *Wolbachia* load. Instead, they suggest that load variation is a consequence of drift.

### **Major comments**

1- Although I am generally strongly in favor of publishing “negative results”, I am not convinced that the study shows absence of host genotype effects on *Wolbachia* loads. The data also do not show that load variation is caused largely by drift (although I agree with the authors that the initial endosymbiont load in eggs may indeed be quite important, the data do not show this). I do agree with the authors that the variation among generations and replicate lines within the two genotypes is striking. But the authors only analyze two host genotypes with multiple replicates for introgression crosses, and based on a sample of n=2 I would not conclude absence of host genetic background effects.

→ *We agree that we observed a pattern during the first experiment, which could reflect a strong influence of the host genotype. We choose to test if the observed phenotypes were determined by the host genotype in only two host genetic backgrounds, but:*

- 1) *these genotypes exhibited extreme values and this characteristic was intended to help detect the influence of host genetic determinism (if we had compared genotypes associated with approximatively the same bacterial density, it would have been complicated to distinguish between the respective influence of host and bacterial genotypes)*
- 2) *we performed several replications of the introgression and additional reciprocal crosses to double-check the absence of host determinism. Indeed, if the extreme densities were indeed determined by a specific host genetic background, we should be able to replicate the patterns after a new introgression, and after reciprocal crosses. Instead, in these two genetic backgrounds, we rather detected an influence of the initial composition of the bacterial population in the mother, the different replicates did not exhibit the same densities, and even exhibited different densities over time.*

2- Furthermore, we do not actually know the nuclear genome composition of the introgression lines (Fig. 1 is an expectation for neutral polymorphisms...). For example, there could be selection for the retention of w1118-derived haplotypes during introgression of *Wolbachia*, or presence/absence of w1118-derived haplotypes in females could actually influence *Wolbachia* loads. Thus, different replicate lines within genotypes could fix w1118 alleles in different regions following the arrest of introgression. I don't know how likely this is, but hope these examples illustrate the different reasons why I am not convinced this study shows that host genotype is not an important driver of endosymbiont load.

→ *We agree that the introgression procedure was limited to ~90%, and that some alleles from the w1118 genetic background could remain and/or increase in frequency after several generations (we did not sequence the genome after introgression in the different lines at each timepoint). However, it seems very unlikely that the 10% of the w1118 background remaining at the first generation would explain such variation between replicates, and incomplete introgression should not affect the results of the experiment where lines with the two most extreme phenotypes were crossed. We therefore nuance this point in the discussion (I398-400).*

3- It would also be helpful if the authors could clarify sample sizes. For example, how large were the population sizes during introgression and during the maintenance of the introgression lines?

→ *As we initially mentioned in the material and method (section 'Wolbachia introgression within various host genetic backgrounds'), the controlled crosses were performed on 20 females and 10 males, and for the regular maintenance, around 80 randomly selected individuals were selected to lay eggs and start the next generation. We now included additional information concerning the rearing protocol before sampling (I111-114).*

What was the protocol for the maintenance (eg. would it be possible than only a small portion of females contributed offspring to the next generation?). This information would be useful to assess the potential for drift.

→ *It is unlikely that a small portion of females contributed offspring to the next generation, as about 40 females were transferred to new tubes 4 to 5 days after hatching, giving the ovaries time to develop and the females time to become fertile. We clarified this point in the revised manuscript (I112).*

Also, why waiting 8 generations after the introgression protocol to measure *Wolbachia* load?

→ *We are sorry for the confusion, in fact we measured *Wolbachia* load after 2 generations of random sib-mating, but waited for 8 generations before doing the second and third experiments (indeed we did not expect such infection pattern in the first experiment and decided after a couple of months to dig into the reasons that could explain the pattern). We corrected the number of generations in the manuscript (I138-156).*

How many individuals per strain were used to quantify *Wolbachia* loads and octomom copies? etc.

→ This information was indicated under the respective graphs. It is now added in the material and method section (l159-160).

L140 and following (Quantification of wMelPop density and Octomom copy number): I suppose this was done on female flies only? (please clarify)

→ Corrected. wMelPop density and Octomom copy number were indeed quantified in females only (the same DNA extract was used for both measurements)

#### **Minor comments.**

- I am not sure what to make from the positive correlation between load and octomom copy number. What does it mean exactly? Do the authors find anything new here with respect to previous studies? Or is it an independent confirmation of the same pattern (also useful) – would be helpful if this could be clarified.

→ The wMelPop / *D. melanogaster* model system presents several advantages from the literature so far: a high variability in the bacterial population and the fact that one genomic region, called Octomom, is one of the bacterial mechanisms controlling Wolbachia density. When we observe a variation in Wolbachia density, we can thus determine if most of the variation is controlled by the number of Octomom copies in the bacteria (if the correlation between the density observed and the number of copies in the population is significant) or controlled by another mechanism (if the correlation is not significant). We clarified this point throughout the manuscript.

- I suggest revising the statement L240 “...These results suggest that different host genetic backgrounds selected specific variants of the symbiotic community. However, other factors, like genetic drift through a founder effect during the vertical transmission of symbionts from the donor line and / or from one host generation to the other, could also explain this pattern.” The results are “consistent with”, but since the authors later revise their conclusion, better not first suggest the results are caused by host genetic effects.

→ We agree and modified the sentence as follows (l259-263): “These results are consistent with the selection of specific variants by different host genetic backgrounds. However, other factors, like genetic drift by founder effect during the vertical transmission of symbionts from the donor line and / or from one host generation to another, could explain this pattern. To disentangle these hypotheses, we thus performed two sets of experiments using the two lines that exhibited the most extreme patterns of infection in the preliminary experiment (i.e., Bolivia and USA).”

- Some explanation for the positive correlation of octomom copies and bacterial density as a consequence of drift would be useful in the abstract

→ The abstract was corrected accordingly

- Figure 1, second panel doesn't really depict a reciprocal cross (but a unidirectional introgression cross as in the first panel).

→ the second panel has been modified to better reflect the reciprocal crosses

- Spelling and grammar errors:

L11 density of endosymbiont density

→ corrected

L17 I would say “between host individuals”

→ corrected

L82 flies “with endosymbionts” harboring more copies of Octomom (note that throughout the text, it is often not clear, from the formulation, whether the flies or Wolbachia have octomom copies).

→ corrected

L89 symbionts (plural)

→ corrected

L130 explained (past)

→ corrected

L211-212 reformulate to “differ significantly among introgression lines” – the statement “are significantly influenced by the host genotype” goes against the author’s own interpretations and would in fact require replicate introgression experiments per genotype.

→ corrected

L230 remove double citation of the same paper

→ corrected

Reviewed by Simon Fellous, 2021-01-20 14:49

In this manuscript Bénard et al. describe the results of a series of experiments exploring the genetic determinants of intra-cellular symbiont regulation in insect hosts. Using the *Drosophila melanogaster-Wolbachia* system, the authors tested whether host genotype controls symbiont cell density. This work builds upon previous studies from these and other authors that established a link between the number of copies of the Octomom gene in *Wolbachia* and symbiont density. The present study did not observe stable host control of symbiont numbers over time, suggesting drift largely determines how many symbionts may be found in hosts.

The experiments and analysis appear sound and the writing is clear. It is a well thought study. I only have comments on the discussion/interpretation of the data and would welcome a couple of additional analyses and figures.

Once these points are addressed, the manuscript should make an excellent contribution to PCI Evol Bio and the field in general.

**Major comments:**

1- The conclusion that drift during maternal transmission is the main factor at play is based on the lack of effect of host genetics on *Wolbachia* density. However, you did not discuss the possibility that unidentified selective pressures drove these variations. External selective factors could indeed vary over time and among fly lines/fly vials. For example, Rohrscheib et al. (2016) argue an abiotic factor such as temperature can drive *Wolbachia* density variations. Similarly, these bacteria being involved in anti-viral defences, outbreaks of viruses in fly vials could have run unnoticed. Incidentally, it would explain why symbiont densities reached peaks

and dropped simultaneously in some line replicates. Do you have reasons to exclude selection by external factors as a determinant of *Wolbachia* density?

→ *We exclude the possibility that unidentified selective pressures drove variations in Wolbachia density, as we performed the experiments in the same controlled conditions of temperature (25°C), humidity (60%) and day/night cycle (12h/12h) for all samples, using one climate chamber for all the samples. In addition, the experiment was performed in a climate chamber where no DCV experiment was ever performed and the presence of DCV was not detected. Finally, we performed 3 replicates for each experiment, which should limit the effect of potential unidentified selective pressures.*

2- Along these lines, do you exclude the possibility that a gene or set of *Wolbachia* genes different from *Octomom* determined symbiont density? In that case maybe did these factors evolved quickly.

→ *We cannot exclude that another gene or set of genes different from *Octomom* could determine symbiont density. However, the strong correlation between the density observed and the number of *Octomom* repeats in all the experiments (generally >80% when the density was high) suggests that *Octomom* is the main determinant of bacterial density. We discuss this point l415-419.*

3- The initial relationship between *Octomom* copy number and *Wolbachia* density is useful to formulate your hypotheses (Fig 3). It would be equally useful to present this correlation, or lack of, in the final sets of data (from Fig 4 and 5). Btw, do you have individual data? This would give an idea of within-host line variation? Does the correlation stand at the scale of individuals as well as populations?

→ *We do have individual data where we have measured on the same extract the bacterial density and the number of *octomom* repeats. We have mentioned these results l302-304 and l359-361, and have included in the supplementary information file additional figures indicating the correlations between (fig s1 and s3) and within lines (fig s2 and s4).*

4- L 401-404: association between mean symbiont numbers and variance is reported but not statistically tested nor illustrated. Can you please provide support to this element of discussion? Also, does the relationship stand when data is log-transformed? ... simply because greater variance is to be expected when mean increases.

→ *We have reanalyzed and replot the data after log-transformation (fig 3, fig 4, fig 5), and noticed that we were previously biased by the representation of the data concerning the variation observed in high-density lines. In addition, we have calculated coefficients of variation in lines which exhibited high density or on the contrary low density and we do not find any significant increase in variation over generation in high density lines. We thus have modified the corresponding sentences in the discussion (l442-450).*

5- L 410-414: is it possible to distinguish transmission drift from host population drift? One is necessarily nested into the other. A naïve expectation (my naïve expectation to be fair) would be that even if transmission drift was high, a lack of pop drift should maintain mean symbiont

density due to a population scale averaging effect. In other words, transmission drift may only drive trait variation if associated with subsequent host population bottlenecks, that is with population drift.

→ *We agree that transmission drift is nested into population drift, and that transmission drift may only drive trait variation if associated population drift. We added this point in the discussion (l439-441).*

**Minor suggestions:**

L 19: "between partners" please be more specific

→ *the sentence was modified as follows (l19-21): 'Such genetic heterogeneity within the host could promote conflicts between bacteria themselves and with the host, notably by increasing within-host competition between symbiont genotypes through a process analogous to the tragedy of the commons'*

L 42-43: this description better matches horizontally transmitted parasites that vertically transmitted ones. Maybe talk about reproductive output? Baby numbers...

L 44: rather than density, maybe use reproduction rate or resource exploitation rate. These terms are compatible with macroparasites which numbers don't change even though they extract more or less resources from their host.

→ *this paragraph has been clarified to mention specificities of vertically transmitted symbioses (l45-48)*

L96: please give equivalent location precision for all pops

→ *Sorry, all these populations were trapped by colleagues around 1998 (<https://doi.org/10.1093/oxfordjournals.molbev.a026215>), except for USA (Seattle) in 2005 and France (Sainte-Foy-les-Lyon) in 2010. Unfortunately, no specific locations were recorded for Arabia and Bolivia. We have added the article citing the initial trapping (l102)*