

I have now gone again through the manuscript and revisions made by Filée *et. al.* following my own previous review and that of R1. I find the updated version improved in the sense of framing the data and conclusions more accordingly with their results. However, I still have some outstanding concerns as to how some of the data is portrayed in the discussion of the results and the conclusions extracted. I have detailed these concerns below retaining the original comments, the authors' responses, and my own new comments (marked with >> <<).

Major comments

My major concern with the article is the way the results are framed into a conclusion which I believe is not fully supported by the data. In my view, the current work fully supports that the *Wolbachia* identified in *Rhodnius* spp., could potentially provide a benefit to their hosts in the form of nutritional supplementation (namely biotin and riboflavin). I believe this is well supported by the presence of these intact pathways in the genomes of *Wolbachia* and its apparent widespread presence (albeit not necessarily fixed in any given species) across the *Rhodnius* genus (namely *pictipes* and *prolixus* groups). However, I do not believe there is enough evidence provided to claim (or favour) “a ménage à trois scenario rather than a dual symbiosis as conceived until now” nor to “speculate that *R. rhodnii* and wRho compose an ancient and dual association of co-symbionts, as seen in many other hemipteran”. The reasons I believe this are the following:

- It is not unexpected, at least for me, that the relationship that *Rhodnius* spp. keep with their nutritional *Rhodococcus* symbionts is not as “intimate” as that that other blood-feeders keep with theirs (e.g. ticks and *Coxiella/Francisella*, bedbugs and *Wolbachia*, tsetse flies and *Wigglesworthia*, and even *Haementeria* leeches). This comes mainly from observations that (at least some) *Rhodnius* can feed and thrive on supplementary food sources (doi: 10.1186/s13071-016-1401-0). As suggested by the authors in the aforementioned study, this additional food source might be important in the field in relation to its richer microbiota (and so, other possible sources for B vitamins).
- In addition, and as the authors of the present study remark, *Rhodnius* spp. have also been shown to develop similarly with *R. rhodnii* strains both capable and incapable of synthesising specific B vitamins (nicotinamide, thiamin, pyridoxine, riboflavin, aminobenzoic acid [pABA], or biotin). As the authors of the present study do well in pointing out, the authors of the 1976 study did not control for other bacterial symbionts. It is therefore possible that any other bacteria capable of synthesising B vitamins could be complementing the host's diet (and not necessarily *Wolbachia*). Also, as the authors also point out, B vitamin supplementation is not necessary for *Rhodnius* when feeding on certain blood diets vs. others.
- Lastly, the presence of a B vitamin operon in *Wolbachia* is not necessarily evidence of a “mutualistic” (or better said “beneficial”) relation with its host, with some examples given by the authors in the present study but also from the *Wolbachia* strains found in the spider *Oedothorax gibbosus* (doi: 10.1101/2022.05.31.494226).

Therefore, I believe there exists enough evidence to propose that while *R. rhodnii* can establish a very successful nutrition-based symbiosis with *Rhodnius* spp. (and it is very successful in infecting the new generations), its association with its host is not necessarily obligate or intimate, opening the opportunity for other symbionts to also take over the B vitamin biosynthetic role. Here is where I see that *Rhodnius*-associated *Wolbachia* strains could have been retained (due to their B vitamin biosynthetic capabilities) and co-diverged with their hosts likely given the well known capacity of *Wolbachia* spp. to be retained and both vertically and horizontally transmitted. However, I fail to see why other members of the microbiota would not similarly be providing B vitamins to their host when needed, and that *Wolbachia* has simply been more successful in spreading and being maintained, giving the impression its association is more “intimate”. Therefore, I believe even the

title “*Wolbachia* genomics support a tripartite nutritional symbiosis in blood-sucking Triatomine bugs.” communicates an incorrect message and would much better read as “*Wolbachia* genomics **reveals a potential for a nutrition-based** symbiosis in blood-sucking Triatomine bugs”, or something in that line. This would be more cautious in not overstating the potential nature of the *Rhodnius-Rhodococcus-Wolbachia* relation without any other experimental data.

We do not disagree with R2 that there are some contradictory and yet unexplained results in the historical experiments carried on in the 1950’-70’ with *Rhodococcus* and *Rhodnius*. And it’s clear that it may be worthwhile to consider redoing them with modern controls and sepsis. However, if we follow the assumption of R2 that any symbiont can provide the B vitamins instead of *Rhodococcus*, it remains to explain why *Rhodococcus* are so universally prevalent in the *Rhodnius* species ? Indeed, on a total of 36 populations (>10 species), *Rhodococcus* prevalence is 100%. We should see some symbiont losses/replacements (as observed in other hemiptera feeding on plant sap for example). Even if *Rhodnius* are able to feed on fruit juices to find vitamin-B, why are the association with *Rhodococcus* so widespread in the wild (and in lab rearing conditions) ? The symbiont system in *Rhodococcus* do not seems as dynamic as observed in other species. Maybe because the peculiar mode of inheritance of *Rhodococcus* using coprophagy do not lead to genome degradation that ultimately fueled the symbiont turn-over ? We need more data on *Rhodococcus* phylogeny and genome data.

>> I believe the authors did not get the point of my major concern. That being, that given what we know (from published work) of the nature and association of the *Rhodnius-Rhodococcus* symbiosis, it is reasonable to believe that additional bacterial species with the capacity to supplement nutritional deficiencies can have an easier path to being maintained vs. what is observed in symbiotic associations which display more intimate characteristics. I do not believe the mode of transmission is an explanation for the lack of genome reduction or other typical characteristics of obligate nutritional symbionts. For example, you have *Ishikawaella capsulata*, that displays extensive genome reduction and an A+T-biased genome while being extracellular and being inherited through symbiont capsules deposited on the external surface of the eggs. What is most important is whether the vertical transmission is "leaky", in a sense of the possibility of closely related bacteria recombining with the "true" symbiont or even taking over the infection of the developing host. Now, the prevalence of *Rhodococcus* is certainly good evidence for some sort of true symbiotic role, which in my opinion certainly includes B-vitamin provision. However, other roles could also fall onto this symbiont, given its extensive gene set. Also, one can imagine that the finely-tuned regulation on gene expression that exist, in say *Wigglesworthia-Glossina* (<https://doi.org/10.1128/AEM.02052-12>), might not exist in the *Rhodnius-Rhodococcus* symbiosis, and this is what might leave the door opened for other symbionts, such as *Wolbachia* or others, to overtake or supplement some of these essential functions. So, my initial comment and concern still stands. I do not believe there is enough evidence to “speculate that *R. rhodnii* and *Wolbachia* compose an ancient and dual association of co-symbionts, as seen in many other hemipteran”. Rather, I believe what the author's data show is the potential for a nutritional complementation/supplementation by *Wolbachia*. Therefore, I strongly the aforementioned "speculation" need to be remove and/or nuanced in a similar way as I suggest.<<

Minor comments

>> Here I would like to start by stressing one of the points raised by Reviewer 1 on contamination. It is a good starting sign that indeed the authors do not find contamination with nematode nuclear DNA. As I believe this is an important point and due to the lack of specific FISH microscopy analyses, that the authors also make sure that no nematode mitochondria are recovered. The reasoning behind this is that, despite these contaminations generally being low (or of low coverage unless high infection is present), the mitochondria, as do the endosymbionts, tend to have much higher coverage than nuclear data (sometimes several hundred times higher). So, despite not finding

many nematode hits, one can find even complete mitochondria (I seldom whole mitochondrial genomes of parasitoid wasps in my aphid data or nematode in my leech data while no nuclear DNA is found of these contaminations), which would then raise the question about the origin of the *Wolbachia* contigs. <<

Line 328: The authors refer to conservation of synteny. however, when looking at the assembled files and table 2, I cannot but notice these assemblies are highly fragmented, which makes me wonder, exactly how can the authors speculate anything more than conservation of synteny at very small scale (AKA micro-synteny)? With such sort of data claiming synteny conservation across the genomes (as it is shown in Figure 3b).

Blocs of synteny have been computed after the reordering the contigs with respect to wCle. That means that we will see a recombination if the breakpoint is located inside a given contig. As the N50 of the assemblies are rather low, the sensitivity of this approach is limited but it's striking that almost all of the *Wolbachia* genomes display very similar patterns with very few recombinations/inversions. That strengthen our vision that these genomes display few rearrangements, at least at local scales.

>> I would reiterate, synteny cannot be evaluated with such fragmented genomes. For example, in a genome with an N50 of 2 to 7Kbp, one cannot evaluate synteny beyond a string of about 2-7 genes on average. In addition, I would remind the authors that, as a general rule, these mobile-element-rich genomes tend to break synteny in repeat elements, such as those that break an assembly of an organism such as these *Wolbachia* done with only short reads. As examples of works analysing this phenomenon see

<https://doi.org/10.1101/2022.05.31.494226>

and

<https://doi.org/10.1093/gbe/evu133> <<

Line 217-219: I understand the logic behind searching for flanking regions of *Wolbachia* insertions. But I see that many of the claimed *Wolbachia* HGTs have very small distances to the end of the "host" contigs. In my opinion, this cannot assure these are *bona-fide* HGTs, as these regions can well originate from chimeric sequences artefact from the sequencing technology. Moreover, if they were *bonna-fide* Horizontally-transferred regions, why would they very often (30% of the times) land in contig ends (as repeats do)?

As all the genomes have been sequenced with the same technology, chimeric sequences would have been generated with the same ratio. So, we should expect a similar level of (artifact) HGT between all the genomes. This is clearly not the case as the amount of HGTs vary greatly between genomes (from 4 kb to 350kb). This observation indicates that the HGTs observed here are not the result of sequencing artifacts but are *bona-fide* true insertions.

We do not have any clear explanation for the presence of the *Wolbachia* genes in the boundaries of the contig. We believed that better genome assemblies with longer N50 may help resolving this question.

>> I do not believe the statement that Chimeric sequences would have been generated at the same ratio due to the use of the same sequencing (and I imagine library prep) technology. The generation of Chimeric sequences depend on many factors, one of which would be the DNA ratios of the different genomic molecules in there. For example, if the amount of *Wolbachia* relative to the host DNA is variable, one can expect different amount and type chimeric sequences to be generated. Similar expectations would be true if the genomes vary, which judging from Fig 2., this seems to be true for *Wolbachia*. These chimeric regions would of course cause breaks in the assembly, and

would thus leave them in contig ends. Chimeric regions can especially be generated with low coverage data, which is something to be especially aware of. So, I would still suggest that the claim, especially for putative HGTs located close to contig ends, to be presented with the nuance that is required and highlighting the limitations of the data in hand. <<

Line 336-338: Do not see how having only a very small draft genome (likely missing most of the genome) would make wRobQ cluster with the *pictipes* group. Only thing I can think of is a lot of missing data in that genome making it cluster “erroneously” with the *pictipes* group. Is this correct? Did the authors encode a lot of missing data for this genome in the alignment? Otherwise, I would probably think that its clustering is correct.

Only parts of gene alignments without missing data have been retained for the phylogeny, this lead to a subset of the alignment of Comandatore et al. (23500nt instead of 34 000nt ; 80 genes instead of 90) . So, there is no missing data on the RobQ alignment.

>> OK, but then, if no missing data was retained in the alignments, why thinking the clustering is erroneous? I would think that the case of RobQ suggests that *Wolbachia* can also horizontally get transferred and likely even replace that present in the receiving lineage, right? I would also say that the grouping of RobQ is not "aberrant", but unexpected given the *a priori* assumptions. <<

Line 387-389: Couldn't the erratic distribution of B vitamin genes might simply come from the highly fragmented (and likely incomplete) *Wolbachia* assemblies making it hard to detect these genes?

If so, why the Biotin, Riboflavin and half of the Flavin operons appears so well conserved compared to the other pathways ? Incompleteness would lead to many random losses. By contrast, our data indicate specific gene absences in some pathway, not in all of them. Our genome assemblies are fragmented but, by many aspects, their completeness, judged as their size and comparison with the genome of wCle, are good enough to make the predictions we made.

>> When assembling a genome from such a metagenome, the coverage, especially of such a lowly abundant genome in the mix, is not (at all) normally distributed, which means that while some regions might be 10x, some might be closer to zero. Thus, this is why some parts can be better and more universally represented than others. If coverage is sufficiently high, one usually has no such problem (and can easily extract all interconnected scaffolds/contigs from an assembly graph and thus insure to a high certainty completeness). For the lowly covered genomes, I would think this is an important issue. So, I'm not saying all (or even most) of the gene losses that you observed are due to this, but it is definitely an important caveat to mention and be taken into account to nuance conclusions based on your presented data. <<

Line 556-558: I would not say that just because two *Wolbachia* strains belong to the same supergroup they are both necessarily sharing the phenotype of being beneficial nutritional symbionts, especially so in distantly-related hosts. This is just not good evidence for a specific type of symbiotic relationship

We agree but we said that these genomic similarities “legitimate the hypothesis of a nutritional mutualism”, at any moment we claimed that *Wolbachia* effectively provide B-Vitamins.

>> I reiterate, the close phylogenetic relation and genomic similarities between two *Wolbachia* strains present in two distantly related hosts with different biology is not evidence of a nutritional mutualism as a shared phenotype. Therefore, I do not believe that the aforementioned data provides any support whatsoever to the nature of the *Wolbachia* symbiotic association. <<

I believe that after addressing these comments and making necessary changes, the work can make a great addition to the peer-reviewed literature on *Wolbachia* symbioses.

Sincerely,

Alejandro Manzano Marín