Dear Editors, dear Reviewers

We would like to thank you for the very constructive comments you have made regarding our manuscript, here are our responses (in red). Corrections in the manuscript have also been highlighted in red.

R1 (Olivier Duron)

Filée et al. have produced a commendable piece of work which clearly articulates the evidence that a maternally inherited bacterium, Wolbachia, could be a nutritional endosymbiont of the Rhodnius spp. vampire bugs. This is a specific topic that has been the subject of little recent research, although these bugs are vectors of diseases of medical interest (eg Chagas disease): Significant research efforts have been undertaken to understand their biology and better control them, but not recently on their nutritional endosymbionts. Symbiosis with maternally inherited bacterium is essential for the nutrition of arthropods with an obligate blood-feeding habit. In these arthropods, divergent lineages of intracellular bacteria have independently evolved functional interactions with obligate blood feeders, but all converge to an analogous biochemical feature: The provisioning of B vitamins. Similar features have been characterized in bed bugs, ticks, tsetse flies, bat flies, head lice, etc, but surprisingly not in Rhodnius bugs: Previous studies suggested that the provisioning of B vitamins in Rhodnius spp. does not depend on maternally inherited/intracellular bacteria but a rather on a extracellular gut symbiont, Rhodnius rhodnii. However, as pointed by the authors, many contradictory results tend to demonstrate that the nutritional mutualism between R. rhodnii and Rhodnius is not strictly obligatory but depends mostly on rearing condition, host bloods or symbiont strains. In the present study, the authors have done an excellent job synthesising these different lines of evidence, and together with their own data present a cohesive argument showing that nutritional symbiosis is more complex in Rhodnius bugs than previously expected. Indeed, the authors sequenced and assembled 13 novel Wolbachia genomes (all belonging to supergroup F) and present genomic evidences suggesting that Wolbachia is a B vitamins provisioning endosymbiont for some Rhodnius spp. Analyses of bug genomes further evidences of Wolbachia-to-bug gene transfers, suggesting a complex evolutionary interplay between these organisms. More specific comments are below. Overall, a great piece of work that I can recommend for publication pending some revisions.

Major comments:

- About horizontal transmission of R. rhodnii: The authors mention in their manuscript (eg at lines 71, and further) that R. rhodnii is horizontally transmitted. This is not entirely true and it should be corrected. To be exact, it has been shown that egg surfaces (and adult feces) transmit R. rhodnii to the gut epithelium of the newborn insect. It is an orally acquiring symbiont: during oviposition, females smear egg masses with symbiont-containing feces, which are ingested by newly hatched nymphs,

allowing the symbiont to pass through their digestive tract and establish in the midgut. This transmission route of nutritional gut symbiont through egg smearing is a distinctive trait in many hemipteran species as stinkbugs and others. In this context, the transmission route is vertical/maternal (although not transovarial), and not horizontal. It implies that there is a fidelity in the transmission of R. rhodnii, and thus a relative stability of the association.

We have made the requested changes in the manuscript regarding the mode of transmission of the *Rhodococcus* symbionts.

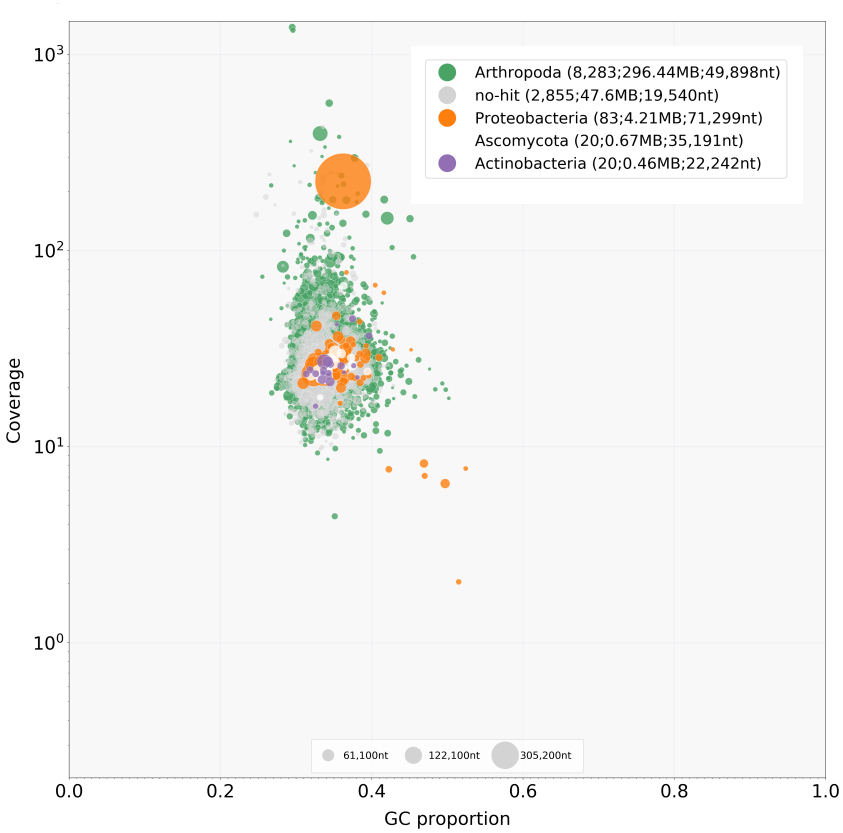
- Line 152: The detection of mtDNA introgression is interesting in the context of maternally inherited endosymbionts, but the importance of this process is not really discussed further in the text. What consequences does this process have on the interpretation of the results? In particular on the distribution and prevalence variations of Wolbachia between the different triatomine species?

We recently published a paper focus on the introgression events in the genus *Rhodnius* (https://doi.org/10.3389/fevo.2021.75031). We demonstrated that mtDNA introgressions occur between closely related species (for example between the sister species *R. prolixus* and *R robustus*). Thus, we do not believed that these events had a real influence on the distribution pattern of the *Wolbachia* among the *Rhodnius*. We have added a sentence in the text to state that.

- One of the most disturbing results is that Wolbachia is not fixed for all bug species. Obligate mutualistic symbionts generally have a 100% prevalence, at least in females. This is not the case in this study and this is a difficult result to explain. As I suggest just above, could this be the result of cytoplasmic introgression with a Wolbachia introduced through this way into a bug species without Wolbachia? Moreover, the Wolbachia detected here belong to supergroup F, a clade that is also often found in nematodes including filaria. In this context, how to distinguish between Wolbachia specific to bugs, and those from filaria that could infect bugs (in which case the presence of Wolbachia should be interpreted as a false positive due to cross contamination). Indeed, this could be the case for Wolbachia from Rhodnius amazonicus which presents an extremely degraded and pseudogenized biotin operon.

- We have to acknowledge that the concomitant presence of two nutritional symbiosis, one fixed and one with a patchy distribution, is puzzling. However, dual symbiosis has been reported in Hemiptera for example in Bedbugs the highly prevalent F *Wolbachia* is sometimes associated with a γ-proteobacteria BEV-like symbionts that also confers some benefices. We make the hypothesis that it might be advantageous for the host to carry two different symbiont that do not thrive in the same tissues as a rescue system if one of the symbionts is lost (L512-520).

- Regarding the nematod contamination, this point is fully legitimate, but we never find any contamination (or very few) with nematod genes. For example, here is a blobplot describing the taxonomy of each genome contigs according to the GC% and read coverage of *R. pictipes*:



This corresponds to the hybrid assembly of the same individual combining short reads used in this study and long PacBio reads. The big circle corresponds the *Wolbachia* genome in a single contig and the green ones to the bug genomes. There is no trace of nematods contigs in *pictipes* and the maximum numbers of nematod contigs found in an assembly was in *R. colombiensis* (amount of 4,5 Mb) for which *Wolbachia* is absent. We plan to submit in the next few month a genome analysis paper of these *Rhodnius* genomes that's why we are quite reluctant to publish now these data. But we have added a sentence in the text to discard the possibility of some nematod contaminations.

- Apart from phylogenomic data, the authors do not really detail the levels of divergence between bug Wolbachia: Variations in genetic composition (besides B vitamin genes), pseudogenization rate, GC%, abundance of IS, ect, between strains should be presented and discussed. This is important for understanding how similar - or divergent - these Wolbachia strains are between bug species.

This true but this is linked to the high level of *Wolbachia* genome fragmentation in our assemblies that preclude a fine-tuned analysis of their genome. What we known is that their genomes are highly similar and syntenic (Figure 2 and 3) but it's difficult to discuss gene conservation as a gene loss might eventually result from assembly artefact. We have modified the Table 2 to add some details regarding IS numbers and GC% which appears again highly stable.

- About Wolbachia nomemclature: The authors nammed Wolbachia wRho all Wolbachia that they have sequenced from several bug species. However these Wolbachia strains have genomic differences and should be named differently. This is quite confusing, even though these strains are phylogenetically close. The basic rule for Wolbachia is to name each strain differently: for example for the Wolbachia of bed bugs, wClec, the first letter, w, stands for Wolbachia, the second, C, is for Cimex, and the next, le, are for lecturalis (only Drosophila Wolbachia has different rules for historical reasons). The same logic should be applied here for Wolbachia of bugs.

We have systematically modified the text to avoid "wRho" for all *Rhodnius* species. In the figure, we take the option to name the corresponding *Wolbachia* with "w" following the name of strains, for example "wINCP". This choice is guided by caution because that the name of the species is uncertain due to introgression as discussed earlier, and because the nomenclature of the different species appears unstable, especially in the *prolixus* group.

- About genomic insertions in bug genomes: There are several Wolbachia genes inserted in bug genomes, but does this have any impact on PCR survey for estimaning Wolbachia prevalence? For example, if the target genes of the screen (coxA and FtsZ) are present in bug genomes, it will completely bias the prevalence results. Another related question: What percentage of these inserted genes are pseudogenized and therefore non-functional? Conversely, do any of the inserted Wolbachia genes seems functional (based on their sequences and orf prediction) and could they have a (nutritional) function for the bugs?

This is a valid point. As we find 4 different complete or nearly complete ftsZ and 1 CoxA gene inserted, we added a sentence to indicate that such insertions might eventually biased the PCR survey for estimating *Wolbachia* prevalence.

These insertions are highly conserved due to the detection threshold (>95% similarity with the corresponding *Wolbachia* genomes). But it's clear that the future availability of high-quality genome of both host and *Wolbachia* genomes will be helpful to better characterized these insertions.

- About the biotin operon: I fully agree that this operon is rare in Wolbachia and moves through lateral gene transfers from Wolbachia-to-Wolbachia, but the transfer capabilities of this biotin operon are not limited to Wolbachia. Accumulating genomic sequences confirm that lateral transfer of this compact, streamlined biotin operon is rampant in nutritional symbioses of obligate blood feeders: related biotin operons (i.e., that diverged recently from the same operon ancestor) have been detected in diverse B vitamin-provisioning symbionts, including Midichloria and Rickettsia in ticks (https://doi.org/10.7554/eLife.72747) and Legionella in rat lice. Its extensive spread across bacterial lineages is definitely a key driver of the emergence of novel nutritional symbioses with obligate blood feeders (reviewed here: https://doi.org/10.1016/j.pt.2020.07.007).

Thanks, we have added these references.

- Line 339: The authors estimated the divergence between wCle and wRho around 5My. I would be careful about this estimate: the evolutionary rate they used was based on a different biological interaction (facultative Wolbachia in bees), so my feeling is that the latter did not evolve at the same rate as expected for a nutritional endosymbiosis.

We have removed this argument.

Minor comments:

- Lines 52-54: Perhaps this sentence is a little bit too speculative: The observed results do not really allow to be conclusive on this point. I would recommend removing it from the abstract.

Done

- I am surprised that there is no mention of the use of R. rhodnii as a potential method of control for triatomine bugs. Over the last 20 years, several studies have focused on R. rhodnii genetic transformation (paratransgenesis) to eliminate pathogens from vector populations. The strategy was to engineer R. rhodnii to express proteins such as Cecropin A that are toxic to Trypanosoma cruzi or that block the transmission of T. cruzi. The success of this strategy mainly depends on the positive fitness effect of R. rhodnii. This should be at least discussed in the discussion.

This point is now inserted in the conclusion

- Worth to mention somewhere that these genomic results will have to be proven experimentally. All previous studies on bugs have been done by cleaning the eggs (to remove the R. rhodnii deposited from egg smearing) but never with antibiotic treatments which are needed to remove Wolbachia.

This have been already stated in conclusion, but we added a sentence dealing with that in the discussion section (see also our response to R2).

R2 (Alejandro Manzano Marín)

In the current work, Filée et. al. explore the presence and putative beneficial role of Wolbachia bacteria in the blood-feeding Rhodnius genus (Hemiptera: Triatominae). These blood-feeding insects are generally thought to be dependent on B vitamin supplementation by their Rhodoccocus symbionts. However, there exists conflicting evidence regarding their obligate dependence on the aforementioned symbiont, as under certain blood types, no dependence is observed. The authors first used diagnostic PCR reactions in a diverse set of samples representing 17 out of 24 species currently recognised in the genus. Through the use of this diagnostic PCR, they found that, in addition to the widespread nutritional symbiotic Rhodoccocus bacteria, a number of the samples were also found to be infected with Wolbachia bacteria. In addition, these Wolbachia symbionts seem to be closely related to the nutritional Wolbachia symbiont of Cimex lectularius (wCle) and, those within the prolixus group, encode for a complete biotin biosynthetic gene cluster. The hypothesis the authors put forward is that ”Wolbachia may also act as a nutritional mutualist in triatomines, as observed in bedbugs, in complementation (or in rescue) to the R. rhodnii gut symbionts”. I find the work well done and the methods generally adequate for analysing the data. However, I have one major conceptual concern the way the current article and the conclusions are framed.

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 Rhodoccocus 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 vitmain supplementation is not necessary for Rhodnius when feeding on certain blood diets vs. others.

• Lastly, the presence of a B vitmain 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 vitmain biosynthetic role. Here is where I see that Rhodnius-associated Wolbachia strains could have been retained (due to their B vitmain biosynthetic capabilities) and co-diverged with their hosts liekly 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 mantained, giving the impression its association is more “intimate”.

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 widepreads 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.

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-Rhodoccocus-Wolbachia relation without any other experimental data.

Agree, we have modified the title.

Minor comments

Line 328: The authors refer to conservation of synteny. however, when looking at the assembled files and table 2, I cannot but notice these assembles 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 stricking that allmost 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.

Line 38-39: The authors talk about complete and almost complete genomes. I do not see how was this assesed. Where assembly graphs inspected for completeness of sequences belonging to an isolated Wolbachia graph? Otherwise, I would stay away from categorising these genomes as "complete" or "nearly complete" In the methods, it is unclear to me if the authors performed mapping and reassembly following the extraction of bins of Wolbachia contigs/scaffolds. Did the authors do that? I was surprised by the number of contigs from each assembly (sometimes well over a thousand), especially so when I myself have performed assemblies with this sort of sequencing data and rarely results in these large number of contigs. This might also help get more contiguous assemblies to better assess synteny. I would suggest to explicitly group Table 1 species by group, as it makes it more comparable and easier to read by a non-Triatominae expert.

We have remove all the mentions of "complete" genomes, we agreed that we do not have any convincing argument to evaluate the completeness of the genome assemblies (outside their size compared to the size of the wCle genome).

The binning method combines the coverage and the similarities with known *Wolbachia* sequences. We assume that these methods are somewhat rough but the fragmentation of the metagenomic assemblies complicates the task. This fragmentation is mainly due to the use of short reads with relatively low coverage.

Table 1 have been modified according to R2.

Line 175-178: […] BLAST hit and sequence identity >90% for Wolbachia and >99% for R. rhodnii.. How were the percentage thresholds calculated

This threshold have been arbitrary fixed as we do not have at the beginning of this work any idea of the *Wolbachia* kinship in *Rhodnius* whereas a complete *Rhodococcus rhodnii* genome was available. This explain the difference between the two thresholds : a relaxed one for *Wolbachia* and a more stringent

one for *Rhodoccocus*. We don't think that it's necessary here to introduce a more sophisticated method to assign the taxonomy of PCR products obtained with standard *coxA* and *ftsZ* primers. We have added this explanation in the Material & Methods section.

Line 191-194: If authors note the difference in coverage between host and Wolbachia contigs, why was this not also used when binning? It might have helped them retrieve more Wolbachia contigs. At least I myself often use this hriteria to complement BLAST-based (and graph-based) binning, as it can help also retrieve extrachromosomal sequences not easily identified by BLAST-based binning.

Yes we used these criteria, we have made some improvements to better explain the methodology.

Line 217-219: I understand the logic behind searching for flaning 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.

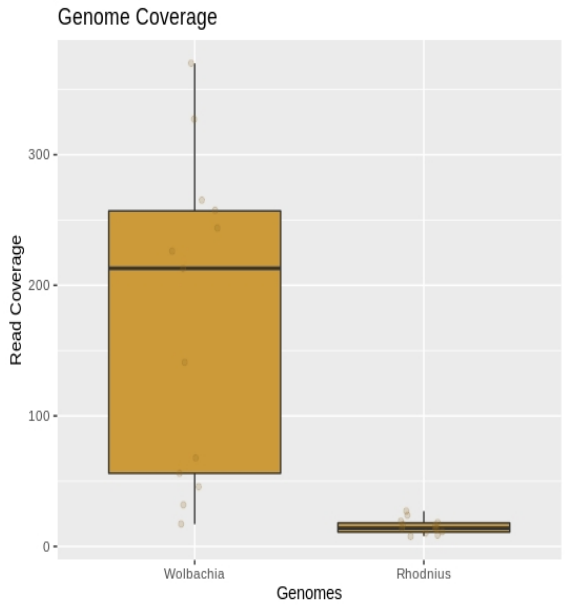
We do not have any clear explanation for the presence of the *Wolbachia* genes in the boundaries of the contig. We believed that better genome assemblies with longer N50 may help resolving this question.

Line 217-219: The authors referred to "masked" genomes. Masked for what? repeats? low complexity regions? Is table 2 missing a caption? I did not see the explanation of what * stood for.

"Masked genomes" means that the integrated *Wolbachia* sequences have been replace by "N", precision have been added. The asterisk indicates that the RobQ assembly has been excluded for the estimation of the mean (we have modified this table).

Line 301: I would suggest displaying the coverages in a box plot format with semi-transparent colouring of dots on top of it. This would make it much more readable and easier to interpret.

Sure, this is clearly better, we now provide a boxplot as Sup. Figure 1 :



Line 336-338: Do not see how having only a very small draft genome (likely missing most of the gneome) 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 encoded 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.

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.

Line 496: Didn’t Mesquita et al. (2015) reported 25 HGTs, not 21?

True, correction done.

Line 502: I do not understand the statement that “most all the Rhodnius samples have been infected by wRho at one time”. Do the authors mean the common ancestor of Rhodnius spp.? It now reads as if all the samples, rather than the species, have been infected at one time, which is not necessarily not true.

This sentence has been clarified.

Line 522-523: I would stay away form doing such divergence estimations with these sort of data, especially when comparing infections across distantly related hosts.

Deleted.

Line 540-543: I would stay away, with current evidence, from making any sort of suggestion regarding “ a direct Wolbachia transfer between an ancestor of bedbugs and an ancestor of the Rhodnius triatomine”, as as the authors rightly point, there is simply not enough genetic nor genomic data from the Wolbachia F supergroup.

To be more neutral, we have mentioned the alternative scenario : direct host switch between Rhodnius and Cimex.

Line 544-549: Has cleptohaematophagy been observed between Rhodnius and bedbugs? Otherwise, it would seem unlikely, right? I guess the fact that they feed on similar hosts is much better evidence for a possible transmission route of their microbiota. Are there any studies revealing Wolbachia can be found in sterile blood after a Rhodnius or bedbug has fed on it?

We have no data about this.

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.

Edits

Particularly in the abstract, it reads strange when using the past tense when referring to what is shown in the article. For example “In this study, we showed that Wolbachia symbionts were also widely

distributed in the Rhodnius genus". As it stands, it reads that the symbiont were (and thus, not any more) widely distributed. I would suggest to change to the present tense. For example "In this study, we show that Wolbachia symbionts are also widely distributed in the Rhodnius genus" When referring to nutritional mutualism, better to refer to it as a phenomenon rather than a "process". I can the the process of "genome reduction", but not the process of "nutritional mutualism" It would be good to offer a general genome/assembly characteristics table early in the manuscript. I believe it would make the paper easier to read.

All of these corrections have been done.

As a general comment on the figures, please provide better quality ones. Using PDF figures is much better than pre-rendered ones. It was often difficult to read small text or images due to pixelation. I suggest to change the black vs. grey triangle differentiation to a filled/unfilled one. It makes it easier to read especially with such small triangles. I would suggest the authors to go through the manuscript one last time to correct some typos and strange phrases across the manuscript such as (not an extensive list): • In most case(s), Wolbachia […] • A subsample of 36 specimens including Wolbachia-free and contaminated insects were used f. replace for "infected" • biotine (remove trailing e) • We cannot rule(d) out a whole Wolbachia lateral transfer/replacement.

All of these corrections have been done.

I believe that after addressing these comments and making necessary clarifications, corrections, and changes, the article would be a very interesting addition to both the Wolbachia- and the bloodfeeding symbiosis- literature. Sincerely, Alejandro Manzano Marín