

Minor revisions

Decision:

I think the paper could easily be out with a few very minor changes proposed nicely by the reviewers.

My own comments:

In their study called "Co-obligate symbioses have repeatedly evolved across aphids, but partner identity and nutritional contributions vary across lineages" Alejandro Manzano-Marin and colleagues have sequenced the genomes of symbiotes of Aphids. While the association between aphids and their main symbiont *Buchnera* was supposed to be stable, they show multiple independent acquisition of co-symbiont. This large body of work reveals multiple interesting facets of bacterial/aphid symbiosis. Various species are recruited to be co-symbionts, some more than others relying on their lifestyle. Vitamin B2 synthesis seems to be a source of co-symbiosis as in all cases the synthesis of this vitamin is achieved by the secondary symbiont. Some co-symbionts colonise different bacteriocytes.

The analysis is done according to the state of the art and the results are nicely and thoroughly presented. All reviewers were enthusiastic about the work and suggest mostly editing and rewording issues. I have a few additional suggestions of figures.

I think that one figure showing genome and gene content against the presence of a co-symbiont could be worth having. This is an important question connecting symbiosis to genome size. The presumed age of the cosymbiosis and the genome size could be plotted to see if there are some trends.

>> In fact, while it is true that *Buchnera* lineages with co-obligate symbionts tend to be smaller and more A+T-rich, this does not hold true (see "Our study also revealed patterns [...]"). For example, *Buchnera* from Anoeciinae have a quite large genome, larger than lineages without co-obligates (*i.e.* Thelaxinae, *Chaitophorus* spp., most Calaphidinae, and Phyllaphidinae). Also, G+C content is higher or similar to many lineages without co-obligate symbionts. In Chaitophorinae, the *Buchnera* strains with no co-obligates have actually lower G+C contents than two of the lineages with co-obligates. The extreme is represented by those *Buchnera* lineages from Phyllaphidinae aphids, where they have by far the smallest genomes with the least CDSs. Finally, the genome size does not predict the G+C content, as highlighted by some Aphidinae, Eriosomatini, and Hormaphidinae, which have large genomes with lower G+C content. In summary, no discernable pattern beyond the loss of key genes that correlates with the acquisition of co-obligate symbionts. As stated, our data actually from Calaphidinae and Phyllaphidinae (smallest *Buchnera* genomes with most of them having no co-obligate associations) highlight the propensity of *Buchnera* to evolve small genomes, independently from the evolution of co-obligate associations (see "This shows that it is the inactivation [...]", p18: bottom). We are actually trying to tackle this by further sequencing genomes from the aforementioned groups and hope we can address this in the near future. We had indeed thought about encoding presence of co-symbiont in Figure 1, but it feels strange to include a result that is not yet presented in the paper, and repeating such figure later in the

manuscript looks repetitive, thus, we have included it as a supplementary figure (figure S4), and added a reference to it in the Discussion section.

“While the acquisition of co-obligate endosymbionts is often associated with a drastic gene loss (figure 7), there are also some exceptions to this pattern (figure S4, Supplementary Material online).” ≤≤

For perspectives: It could also be worth testing a model of genome size evolution with a rate of decay and to see if a two rate model is fitting the data better, the second rate occurring after cosymbiosis. (<https://academic.oup.com/sysbio/article/65/5/812/2223542?login=true>)

≥≥ Indeed, this is a good suggestion. However, we are limited currently with our current dataset, as this misses some important subfamilies (including the speciose Greenideinae) and very shallowly covers many of the branches where smaller genome sizes have evolved (e.g. Mindarinae, Thelaxinae, Anoeciinae, Calaphidinae, Chaitophorinae, among others). We think this is a really good perspective. We would indeed like to test whether the presence of a cosymbiont impose different selective pressures on *Buchnera* (or alternatively and more likely relax some of the selective pressures on *Buchnera*) and drastically change its patterns of evolution. We were thinking that more than a model of genome size evolution (as stated above, as genome size is not always a good predictor of the presence of a co-symbiont), we could test changes in substitution model upon the establishment of cosymbiosis. But we think the dataset presented here is not sufficient to do this. As stated above we would need more genomes including more species of speciose subfamilies, that would test alternative models of evolution and also reconstruction methods for *Buchnera*. Such analyses would also need to confront phylogenies based on aphid genome data to verify more formally that they are perfectly parallel. We have a “bigger” dataset underway to conduct such analyses, but the genomes are not that well assembled and annotated to be included in the current ms. In the present paper, we tried to have complete / well annotated *Buchnera* genomes (i.e. fully curated, which is very time consuming) so we could address questions concerning the metabolic capacities of *Buchnera*. We have added a sentence in the discussion, regarding this perspective p20.

“We anticipate that analyses using a larger dataset (i.e. including more aphid subfamilies and deeper genomic investigations into each clade) will allow to formally test if *Buchnera* genome characteristics change significantly upon the acquisition of a co-symbiont. This could be achieved by, for example, testing whether substitution models vary in branches of the *Buchnera* tree where dual-symbiosis occurs.” ≤≤

I was particularly interested in the *Buchnera* strains from Hormaphidinae, which hold rather large genomes (630 and 580 kbp) with a strikingly low number of CDSs (453 and 450). Having close to 30% of non coding DNA is a very interesting feature for bacterial genomes. Could a map of these genomes be given and compared to their closest relatives. What is the source of this non-coding DNA? A zoom on some regions **could be** valuable...

≥≥ The intergenic regions from Hormaphidinae mainly correspond to genes that used to be there before the pseudogenisation and loss of them (when compared to the distantly related Aphidinae with larger genomes). This is easily detectable thanks to the highly syntenic genomes of *Buchnera*. Roughly, whenever we found a larger/smaller gene-empty region, we

observed a large/small intergenic region. We find a figure on one or two regions wouldn't be of big benefit, but we have added the following brief explanatory text.

“When compared to the larger genomes of *Buchnera* from Aphidinae, it is becomes evident these intergenic regions originated from the pseudogenisation and eventual loss of functional genes.” <<

Here are some additional thought I had on the discussion:

Regarding the proximal scenario for cosymbiosis, most arguments were associated with gene loss. But it is highly probable that through drift or antagonistic pleiotropy, some of the bacterial function may be reduced, but not inactivated. This could offer a window during which a new symbiont providing an active complementation could be selected for until it completely takes over the least functional function of *Buchnera*.

>> <<

Could there be a reason for riboflavin to be the source of switching? Is it particularly costly to produce?

>> This is definitely a key issue. And yes, there is probably a time where both symbionts co-exist which might relax some selective pressures on *Buchnera* for the synthesis of some nutrients. Problem is, we know very little about production costs and reliance on this nutrient across life stages, different diets, etc. We are not aware of any calculation for the production of riboflavin in free-living bacteria. However, it might not be much more costly than the biosynthesis of some branched chain amino acids or even biotin. And yes, *Buchnera* might be really “bad” at synthesising riboflavin or transferring it to its host, but we simply do not know. One alternative explanation would be that more riboflavin is better (but not significantly better), and thus facultative symbionts with functional riboflavin pathways would be selected for (eventually leading to one symbiont becoming reliant on the other), but then, one would expect to see more co-obligate associations based on this nutrient evolving, particularly within the Aphidinae, where associations with facultative symbionts seems to be widespread (which we do not observe). Therefore, any explanation regarding this would be at this point too speculative, and we rather stay away from it until more experimental evidence is produced favouring one or another explanation. <<

As perspective, but beyond the paper: it could be interesting to test if the acquisition of new cosymbiont is associated with a benefit for the host that would be seen at the species level, through past demographic inferences for instance, in the case of recent acquisitions.

>> Yes. This is indeed a very interesting perspective. Thinking about exploring the sign of demographic expansion through population genomic studies, It would be quite interesting but challenging with aphids, given the aphid's particular population genetics (due to cyclical parthenogenesis and long term asexuality for some lineages/populations). Classical demographic models do not apply well to aphids. Regardless, in one of the most studied aphid species (*Acyrtosiphon pisum*), there is no formal test of a demographic expansion hypothesis though the idea has been put forward. <<

There are few sentences that were not clear:

-Our 16S rRNA amplicon survey revealed that Fukatsuaia-related symbionts are found in populations of aphid species from different subfamilies, but much frequently than *Se. symbiotica*. do you mean "much less frequently"

>> Corrected. <<

-Given the multiple benefits conferred by this endosymbiont, the potential for vertical transmission through the host plant of the aphid host. I am not sure here that transmission through the host could be said to be vertical transmission

>> Corrected. This was an error and we indeed meant to write "horizontal transmission" <<

Reviews

Reviewed by anonymous reviewer, 13 Feb 2023 16:25

In the manuscript entitled "Co-obligate symbioses have repeatedly evolved across aphids, but partner identity and nutritional contributions vary across lineages", Manzano-Marín and collaborators have assembled and investigated a large metagenomic database generated from 25 aphid species, as well as an important metabarcoding database generated from 223 individual aphids from 147 species. In their study, the authors aimed at identifying and characterizing the insects' obligate symbionts and their evolution, particularly focusing on the occurrence of co-obligatory symbioses, as aphid symbioses are mostly known for the insect association with their main obligate symbiont *Buchnera*. With their approach, the authors were able to convincingly show that co-obligatory symbioses are not a rare event, as they independently and repeatedly appear within different lineages of aphids. The authors are also microscopically describing two co-obligatory symbioses that were poorly studied, which, despite feeling a little offbeat at first, validates the authors' approach in showing that their inferences of co-obligatory symbioses can be validated and describes convergent patterns in the evolution of symbiotic associations in aphids.

Overall, the manuscript is nicely written and quite readable. The methods are sound and nicely detailed so it would be easy to reproduce what the authors did. I commend the authors for the nice job they have done in summarizing so relevantly the large amount of data that they have brought, processed and analyzed. The figures are particularly helpful to understand this study's approach. The authors show a great knowledge over the literature surrounding their work, but maybe sometimes too specifically, to the point that there is fewer discussion out of the aphid and hemipteran world. It is not deleterious at all but the scope of their findings could have been broadened on some points (for instance on symbiont replacements, or on the discussion on the potential and interest to investigate co-obligatory symbionts in radically different insects or even other arthropods). This said, the discussion does not need to be expanded as it is already long enough, but surprisingly quick to read (it could be maybe shortened on some points, for instance on the metabolic paragraphs as this is the least "original" part of this study).

In conclusion, the present work is a relevant read to better understand the evolution of symbiotic associations in insects (especially aphids in this case). The conclusions of this study are quite convincing and combined with the easy-to-understand methods and approaches described here, could make this work a nice reference for further studies of the same nature.

Following are some rather picky comments and questions that could maybe be used to improve the manuscript.

Abstract

"Lastly, patterns ..." this sentence could be rephrased to be easier to understand. If I got it correctly, the authors want to highlight that it's not the number of genes lost over genome erosion that can promote the rise of co-obligate symbioses but rather the specificity of a few genes. The sentence could be streamlined.

>> We have rephrased as

"Lastly, patterns of *Buchnera* genome evolution reveal that small losses affecting a few key genes can be the onset of these dual systems, while large gene losses can occur without any co-obligate symbiont acquisition." <<

"often thought of as exclusive, appears fragile" I disagree on such a strong statement to qualify the association between *Buchnera* and aphids. Granted that associations are not set in stones, but it does not mean they are "fragile" as most of them remain stable for millions of years. Maybe the authors could shift for something fitting their results but slightly less assertive. Maybe, something like "appears adjustable/movable/more flexible than initially thought".

>> Agreed. We have rephrased as "seems more flexible" <<

Introduction

Spherical cell shape of endosymbiont: the statement is misleading as it suggests that it is a general feature of obligate endosymbionts with reduced genomes, while in total opposition, some endosymbionts with reduced genomes present rod-shape gigantism (e.g. *Wigglesworthia*) or intense elongation (e.g. *Nardonella*). The authors could rephrase the sentence in a less general context. For example, "is a characteristic shared among several aphid-associated endosymbionts."

>> Wholeheartedly agree. This is not how the phrase was intended to sound. It has now been reworded as "characteristic of many obligate symbionts of aphids and adelgids with drastically reduced genomes". This should keep it confined here and not extended to other symbionts such as the ones pointed out or even *Sulcia* or *Baumannia* in Auchenorrhyncha. <<

Results

Microscopy on new co-obligate symbionts: How do the authors confidently assess the shape of the symbionts with their pictures? Especially for *Fukatsia*, the bacteriocytes are so packed with endosymbionts that it is very hard to conclude that the bacterium is spherical. Could the

authors display a close-up picture? If relying solely on the finding from Michalik et al. 2014, maybe it could be more accurate to state that there is no observation of an obvious pattern that could be contradictory.

≥≥ Indeed the shape can be difficult to discern in these FISH images due to resolution and magnification. The apparent spherical shape can be somewhat seen when closing up on the FISH images (particularly looking at the supplementary full-resolution tif images). But we do indeed corroborate this when looking at the much more detailed work of Michalik *et. al.* 2014. Therefore, we have rephrased as

“Taken together, these observations support that the *Fukatsuia* symbiont of *An. corni* resides in separate bacteriocytes to those of *Buchnera* and shows a spherical shape.” ≤≤

Discussion

If the authors want to broaden their point and extend generalization to holometabolous insects, they could cite that symbiont replacement has also been described in weevils (Lefèvre et al., 2004; Conord et al., 2008 in Mol. Biol. Evol).

≥≥ Agreed, and thanks for pointing out this issue. We have accordingly adapted the text “There are now many descriptions of symbiont replacement and complementation (Sudakaran et al., 2017), in sap feeding insects, blood-feeding ticks and lice (Buisse et al., 2021; Říhová et al., 2021), and weevils (Conord et al., 2008; Lefèvre et al., 2004; Toju et al., 2013).” ≤≤

"the latter being published just months before the preparation of this manuscript". What is the relevance of this comment? Do the authors want to highlight a coincidental interest for the same subject? Do they want to support the importance of the finding by convergent studies? I don't see any point risen after this comment, which could be interpreted as a frustration of not being the first to describe the stated findings.

≥≥ Deleted. This was a remnant from a previous revision from a submission to a journal where “novelty and being the first one” was important but had a scooping policy. Thus, it was important to point out the (very) recent nature of those studies. ≤≤

Figure 7: I find this figure very nice, but not as informative as it could be due to the format. For instance, the blue color chosen for *Se. symbiotica* is very hard to discriminate from the black as it is surrounded by grey. It could be improved as this is in my opinion one of the most relevant parts presented in this figure, as it shows clearly the repeated acquisitions of this symbiont as co-obligate. Also, maybe the co-obligate genera could be represented when they are facultative symbionts as well. It could emphasize that they regularly interact with the different aphids. As such, I was under the impression that it was rather a rare event to observe the rise of co-obligation and that these symbionts are not that present among the different aphids, while it's the opposite as I assume that the grey highlight represents that all these aphids' taxa are interacting with facultative symbionts.

≥≥ We have now adapted the image following suggestions. We had originally chosen the colour scheme to be consistent with some of our own previous publications. The grey displays the aphid “backbone” and black *Buchnera*. We have now also made the grey less intense. It is

complicated to encode however the presence of facultative lineages across the different branches (as they are abundant and widely distributed). This is why we have limited to list them at the bottom left of the figure and make it easy to compare with the list to the right of co-obligate taxa. There was a confusion in the “grey” colour code that could be interpreted as these endosymbionts being present as facultative in all the grey branches. We have now adapted the colours and the figure to avoid misunderstandings. The distribution of these secondary across subfamilies can be seen in figure 4. <<

Reviewed by Alex C. C. Wilson, 01 Mar 2023 19:34

Review of Manzano-Marín et al., “Co-obligate symbioses have repeatedly evolved across aphids, but partner identity and nutritional contributions vary across lineages”.

For a long time it has been understood that the endosymbiont of aphids is *Buchnera*. While there have been a few known exceptions to this rule, including two examples of *Buchnera* being replaced entirely, and others where *Buchnera* is supported by a second co-obligate symbiont, the extent to which aphid lineages are supported by co-obligate symbionts, and the nature and evolution of such systems across a diversity of aphids has been unknown. In this manuscript, using metagenomics on 25 aphid species representative of nine subfamilies, and 16S rRNA amplicon sequencing from 147 species representative of 12 subfamilies, the authors explore the prevalence of aphid species hosting co-obligate endosymbionts. The results of this work solidly shift the paradigm. No longer can we generalize that aphids have a primary symbiont *Buchnera*. The evolution of endosymbiosis in aphids is much more dynamic and variable than had previously been appreciated. This paper will become a well-cited piece of the story of endosymbiosis in aphids.

I really enjoyed reading this paper. It is well-written, mostly in an accessible, conversational style. The figures are excellent - they are clear and accessible, containing just the right amount of detail to communicate the data/results. The figure legends and keys within the figures are well-planned supporting the figures so that they stand alone from the text. Figures 2, 3 and 5 are real standouts (especially figure 3)! While the results are mostly unsurprising, they are interesting and make an important contribution to the field.

>> We thank the reviewer for her positive comments! You can find below a point-by-point response to your reviews. <<

Things I will cite this paper for:

*The evolution of co-symbiosis is common in aphids outside the Aphidinae.

*Two distantly related *Buchnera* lineages have gained a gene by HGT!

*The evolution of co-obligate symbiosis in aphids commonly involves just a handful of nutritional pathways, these include tryptophan biosynthesis (twice), histidine biosynthesis, riboflavin biosynthesis (six times), biotin biosynthesis (five times)

*The evolution of co-obligate symbiosis in aphids appears to commonly expands the metabolic provisioning of the endosymbiosis to include thiamin.

Suggestions for improving the manuscript:

*I suggest revising the abstract so that it reflects the fact that there were 25 newly sequenced genomes, AND 20 re-assembled and/or reannotated genomes – as this almost doubles the number of Buchnera genomes that were included in the analysis.

>> We have now adapted the abstract to read:

“Here, using metagenomics on 25 aphid species from nine subfamilies, re-assembly and re-annotation of 20 aphid symbionts previously sequenced, and 16S rRNA amplicon sequencing on 223 aphid samples (147 species from 12 subfamilies), we show that dual symbioses have evolved anew at least six times.” <<

*I suggest rewording “falling along this extremes”. The point you are trying to make is not clear as it could be. I also suggest here rewording talking about GC content because 27.02% GC is still “low” GC content but the current wording of this section suggest that it is not. Maybe low GC and lower GC or lowest GC content?? With respect to Figure 1 – are there dots in between the 18.37 and 27.02% GC, or are they just the two sizes? Maybe reduce the size of the dots in the figure so that there is less overlap among the dots ... so that they can appear as discrete data points?

>> We have rephrased as:

“With our expanded dataset, we have also observed that many genomes have intermediate values between those of Aphidinae and Lachninae, namely those of Chaitophorinae, Thelaxinae, Neophyllaphidinae, Anoeciinae, Mindarinae, and one Eriosomatini.”

We have reworded “low” to “lower”, as suggested.

We have reworked Figure 1 to reduce the dot overlap while keeping the G+C content coding with the dot size clear. We have also added the triangle next to the G+C content dots to give the visual impression that this is a size key (and amended the figure caption to reflect this). We have kept the colour transparency to also include visual information on the amount of genomes represented per family. <<

*Page 5, 2nd sentence, there is a word missing “As in previous work...”

>> Corrected. <<

*Page 6 is “deserted” the best choice of word? Also, did you look in these intergenic regions for the presence of repetitive elements or the small RNAs reported by Hansen, A. K. & Degnan, P. H. Widespread expression of conserved small RNAs in small symbiont genomes. *Isme Journal* 8, 1–13 (2014)??

>> We agree, “deserted” is definitely not the best choice of word, as transcriptional promoters, terminators, and other DNA/RNA structural features can be in intergenic regions. We have reworded as

“revealing that large portions of their genomes are devoid of any detectable gene trace (28.9% and 25.3%).”

As to the RNA transcribed features. Regarding the 25 conserved putative sRNAs in table S6 (the most trustable as true sRNAs) from Hansen *et al.* (2014), it becomes complicated to determine their presence/absence without a co-variance model for Infernal. This is due to the largely divergent nucleotide identity across distant *Buchnera* (particularly with those *Buchnera* from non-Aphidinae, such as those from Hormaphidinae). Since the authors do not provide sequences, we cannot build models for these. In addition, no particularly conserved repeats (tandem, inverted, or other) are found in those gene-devoid DNA stretches. These mainly correspond to genes that used to be there before the pseudogenisation and loss of them. Roughly, whenever we found a larger/smaller gene-empty region, we observed a large/small intergenic region. ≤≤

*Page 6 “afore-mentioned” – check spelling/hyphenation

≥≥ Corrected. ≤≤

*Page 8 – “symptoms of long-term associations” I like this word choice, I also like your use of “auxotrophies” and “complementarities”.

≥≥ Thanks! ≤≤

*The discussion of transcriptional frameshifting only cites Tamas et al 2008, I am pretty sure that there is an earlier paper by Wernegreen’s group in ants that should also be cited here.

≥≥ We were not familiar with the other work by Wernegreen, but after doing some digging, we found the citation. It is a following work to that of Tamas *et al.* (2008, which includes Wernegreen), where they show that these poly(A) tracks actually show conservation and are maintained by natural selection in *Blochmannia* (<https://doi.org/10.1093/molbev/msp290>), which is great!. We have added the reference to this work too as following:

“Due to the conserved nature of this region, we considered it as likely rescued by transcriptional frameshifting, as this rescue mechanism has been experimentally demonstrated in other *Buchnera* and *Blochmannia* strains (Tamas *et al.*, 2008) and shown to be maintained by natural selection in the latter (Wernegreen *et al.*, 2010).” ≤≤

*Figure 4 – can you revise this figure to present the data in a way that corrects for sampling depth? If not, I suggest removing this figure because I think that it can lead to misinterpretation of what the situation is.

≥≥ We agree the display of the numbers could definitely lead to an erroneous interpretation of the results. We have now scaled the secondary symbiont count to the number of sequenced samples within each subfamily and included the “none” category. This way, the visual representation of symbiont frequency within the absolute sample count is much clearly represented and reflects better the actual situation. We have accordingly modified the figure caption and added a sentence in the “Materials and Methods” section as follows.

“Presence/absence was codified as 0 and 1, respectively. These 0/1. These numbers were then scaled to the total number of samples per subfamily to provide a more accurate visual representation of secondary symbiont abundance across the samples in figure 4.” ≤≤

*Page 10 – discussion of a Bacteroidota symbiont in Fordini that was not found in this work – did you find Buchnera in those samples instead? If you did not find Bacteroidota, what did you find?

≥≥ Here, we did not sample any *Geopemphigus*, which are the aphids shown to have a *Bacteroidota* symbiont replacing *Buchnera*. What we meant to express is that we did not find a *Bacteroidota* symbiont as an additional symbiont in any of our sampled Fordini (which did not include any *Geopemphigus* spp.). To make this clearer in the text, we have rephrased to

“[...] we did not recover any secondary bacterial 16S rRNA sequence belonging to this bacterial taxon in the Fordini sampled in this study (*Baizongia pistaciae*, *Forda* sp., and *Geoica* sp.).” ≤≤

**Sodalis* – I had missed the earlier papers! I find it exciting to learn that *Sodalis* is also found in aphids.

≥≥ Indeed! And several times with varying genome characteristics for the symbionts! ≤≤

*Page 12 – “tissue trophism” can you define or reword. It wasn’t clear to me exactly what was meant here.

≥≥ We agree. Tissue tropism is a very general term. We have rephrased as

“In order to investigate the distribution of the newly identified co-obligate endosymbionts inside of their aphid hosts” ≤≤

*The bacteriocytes occupied by *Fukatsuia* are very large!

≥≥ They indeed are! They are similar (also still bigger) to what can be observed with the *Sodalis*-like symbionts in embryos of *Eulachnus* spp., *Cinara* (*Schizolachnus*), and *Essigella*. However, this is still in embryos, and would be exciting to see how their distribution and relative size changes across development of the aphid. ≤≤

*Page 14, first line, there is a space missing at the beginning of the sentence that starts “Along the same...”. Second line replace “relay” with “rely”?? Second last line of first paragraph – isn’t it three Eriosomatini?? Second paragraph, third last line “and Auchenorrhyncha...”

≥≥ Corrected. For Eriosomatini, there are only two where the biotin auxotrophy has evolved. The *Buchnera* from *Eriosoma lanigerum* does keep a functionally intact biotin pathway (Figure S1), meaning the auxotrophy has evolved twice: Once in *Tetraneura ulmi* and once in (at least) *Eriosoma grossulariae*. ≤≤

*Page 16, 5th line word missing “ but much more frequently...”. Later: replace “This mirrors” with “These patterns mirror...” Second paragraph “56% of samples were”.

≥≥ Corrected (much less frequently). ≤≤

*Page 16 last paragraph through page 17 – I find this part of the discussion weak, overly speculative, and not very helpful. I suggest either dropping this section or thinking more about it, and then revise the section strengthening the arguments.

≥≥ We agree that at this point, the only evidence available is the upregulation of *ribA*, *ribD*, and *ribH* (all essential enzymes in riboflavin biosynthesis) in early and intermediate life stages of *A. pisum*'s embryos (Bermingham *et. al.*, 2009) when compared to the adult tissue. However, we still lack evidence throughout more developmental life stages of aphids and also direct measure of their dependence of this nutrient. Logic was that, if riboflavin biosynthesis is only needed in early and intermediate embryonic life stages, then, deleterious mutations can accumulate in these genes as *Buchnera* populations continue to grow and make more genomic copies throughout later life stages of the aphid (as this nutrient is no longer needed). An evolved auxotrophy for riboflavin in *Buchnera* would then be tolerated and subsequently fixed if a secondary endosymbiont capable of synthesising this nutrient is present. However, as we agree that with a lack of more compelling evidence this remains speculative, we have removed this part and rephrased as following:

“A common pattern in the dual nutritional symbioses revealed here is the riboflavin biosynthetic-role takeover by the new co-obligate symbiont. Biotin was the second most common nutrient predicted to be supplied by the secondary co-obligate endosymbionts” ≤≤

*Page 19 Gil et al, Van Ham et al ... about pLeu. Shouldn't Chong and Moran also be cited here?

≥≥ It is correct, we have now accordingly cited Chong and Moran (2019) ≤≤

*The HGTs – did you figure out their origins?

≥≥ Short answer is no. The closest matches in the databases are simply too distant to build reliable phylogenies. We have also found similar resolvase/invertase proteins in plasmids from other lineages not included in this study, but they are also distant enough to think that they have originated from independent HGT events. In the case referred to from *Erwinia haradaeae* (Manzano Marin *et. al.* 2020), we could also not determine the origin from them given their phylogenetic distance to other members of the Tn3 family, but we could definitely determine these were most closely related to Tn3 resolvase/invertases found in *Hamiltonella* symbionts (which are also their top BLAST hits). ≤≤