

# PCI Evol Bio submission #1

## Response to referees

### Decision

by Wolf Blanckenhorn, 2019-03-18 17:31

Manuscript: <https://doi.org/10.1101/546838>

### **The plastic effect of gut bacterial environment on life history traits in *Drosophila melanogaster***

It is well known that the rearing environment has strong effects on life history and fitness traits of organisms. Microbes are part of every environment and as such likely contribute to such environmental effects. Gut bacteria are a special type of microbe that most animals harbor, and as such they are part of the animal's environment. How much do selected gut bacteria affect the phenotype, in terms of life history and larval foraging traits, of the fruit fly *Drosophila melanogaster*, a common laboratory model species in biology? This was the main aim of the laboratory study by Guilhot et al. that was herewith assessed by two reviewers. To investigate the above question, the authors isolated 4 types/species of bacteria from the gut of their lab strain of *Drosophila*, and subsequently let *Drosophila* eggs and larvae develop in both the usual artificial laboratory medium and grapes (a natural habitat for *Drosophila* larvae) inoculated with these bacteria, singly and in combination, plus a bacteria-free control. By investigating various relevant developmental traits, the authors found that adding the bacteria DID have some visible (significant) effects on various traits, both upward (indicating improvement) and downward (being detrimental).

The study overall appears well conducted and presented. The text is largely clear, though the reviewers point out to some unclarities, which can and should be fixed. There are no major errors.

Thank you for this encouraging feed-back.

As is, the study is largely descriptive, i.e. does not test particular hypotheses or predictions in relation to the particular bacteria isolated, not least because the bacteria were chosen ad (post) hoc and there were apparently no particular prior studies from which to derive concrete predictions.

#1.

As highlighted by the editor, we had few predictions before conducting these experiments, beyond that bacterial effects on host traits would change with larval environment. Results however exceeded out expectation in that (1) environmental effects were very large (i.e. all effects of symbionts changed with the environment); (2) we discovered that in this system symbionts affected host traits by changing their developmental plasticity rather than resource acquisition.

The inclusion of a natural medium (grapes), in addition to the artificial lab medium, must be commended, because this should permit inferences and conclusions for at least one natural environment, as inferences drawn from laboratory studies for the natural situation are typically limited. Nevertheless, it remains unclear why these, and not other bacteria were chosen, further limiting general inferences.

#2.

The reason we chose to work with these four bacterial strains was indeed poorly explained in the previous version of the manuscript. We have substantially edited the relevant section of the M&M that now reads:

"Available literature did point to a number of taxa which interactions with *Drosophila* flies are described, and that we could have sourced from other laboratories. However, working with strains we could readily isolate from our fly colony meant we were certain to investigate fly-bacteria associations in their environment of origin."

"These bacteria were chosen for their ease of cultivation and our ability to discriminate them morphologically on standard microbiological medium. Our aim was not to sample the whole community of bacteria associated with our flies stock but to carry out tractable experiments using a random subset of their symbionts. "

It also remains unclear why not natural but artificial lab populations of *Drosophila* were used, if the aim is to draw inferences for the wild situation.

#3.

We agree with the editor that only working with wild strains of flies and microorganisms in realistic conditions would enable drawing inferences for the wild situation. It is, incidentally, one of our take-home messages (see last sentence of the manuscript).

Here, our aim was a little different: we meant to test how different would fly-bacteria interactions be if tested in natural fruit (and therefore in the presence of live yeast) compared to the artificial conditions they were isolated in. Given the magnitude of the differences we unveiled, we now pursue the study wild fly-symbionts interactions in the most realistic conditions we can.

But this is a good first start.

Thanks!

While the data are novel, the fact that some detectable effects of the bacterial environment on life history traits is present is not really surprising, as the reviewers point out. More interesting questions would be: 1) are these effects predictable (not (yet) at this point, or so it seems), and 2) how strong are these environmental bacterial effects relative to other, more standard effects (e.g. relative to genetic variation, population variation (cf. reviewer 2), or relative to other types of environmental effects like, say, temperature)? Although Genotype x Environment effects are invoked, they were not tested here due to lacking genetically different *Drosophila* families or populations tested. I consider this a major weakness of the study because it does

not allow comparisons to other environments or situations. So, while interesting in principle, the study appears a bit arbitrary in the end.

#4.

We understand the editor's concern. Indeed, it is not possible to compare quantitatively the magnitude of observed phenotypic to other sources of variation. Our response to this comment is two-fold and relates to the two main findings we evoked above. First, none of the bacterial effects that we observed in an environment maintained in the other one. In one case, a bacterial strain that seem neutral (i.e. commensalism) even became costly in an other environment. We think safe to conclude that, at least for the taxa we assayed, qualitative inferences made in standard laboratory conditions are unlikely to maintain in other contexts. Second, our serendipitous finding that bacteria affected host developmental plasticity seems to us at least as meaningful as dependence on environmental conditions.

The reviewers point out a number of weaknesses and unclarities, to which I add my own. Many will be fixable, and should be fixed, in a revision. But to make this study more meaningful and interesting for the community it would be best to add some additional treatment levels as suggested above (different families, populations, perhaps another environmental factor, etc.).

#5.

We utterly agree. Our take on this is that the current manuscript carries information that will be of interest to colleagues. The experimental work we now pursue is based on these first results and does explore fly genetic variation, among other things.

The questions addressed are not laid out clearly in the Introduction. What WERE the main questions/expectations/predictions?

#6.

We made substantial efforts to clarify this aspect in the introduction and through the manuscript.

In particular, the introduction now ends with:

"We investigated two questions. (1) We focused on the influence of environmental variation on bacterial effects analyzing each of the host's traits individually. Our aim was to unveil whether host-symbiont that occurred in the environment of origin (i.e. the laboratory) maintained in more realistic conditions. We further relate these observations to fly and microbe ecology. (2) We performed a new, simultaneous analysis of two traits in order to disentangle symbionts' effects on host developmental plasticity and resource acquisition, two non-excluding possibilities. Separating plasticity from resource acquisition is important for at least two reasons. First, long-term symbiotic associations would be more likely when symbionts provide new capabilities (i.e. resources) than when they affect quantitative traits (Fellous and Salvaudon 2009) or their plasticity (Chevin et al 2010). Second, recent literature shows that the evolution of symbiont transmission depends on which of host's traits it affects (Brown and Akçay 2019); importantly, this mathematical model is based on the plastic trade-off between survival and reproduction. Here, we based our analysis on another well-established trade-off in holometabolous insects, that between duration of larval development and adult size at emergence (Teder et al. 2014, Nunney 1996). In brief, we reasoned that bacterial effects on host developmental plasticity would move host phenotypes along the trade-off axis, while resource acquisition would allow faster development or larger size without detrimental effects on the other trait."

The results section about host developmental plasticity / resource availability also starts with:

"We expected three possible patterns when plotting average adult size in function of speed of larval development (i.e. - age at emergence): a positive relationship indicative of a similar effect of the bacteria on the two traits (i.e. bacteria modulate resource acquisition, mostly); a negative relationship indicative of bacteria affecting host position along the trade-off (i.e. bacteria modulate developmental plasticity, mostly); a lack of correlation that would have been challenging to interpret on its own as several processes could produce this result (e.g. bacterial effects on both host plasticity and resources)."

Why these and not other bacteria?

#7.

As stated above, the text now better explains why we chose to work with these taxa:

"Available literature did point to a number of taxa which interactions with *Drosophila* flies are described, and that we could have sourced from other laboratories. However, working with strains we could readily isolate from our fly colony meant we were certain to investigate fly-bacteria associations in their environment of origin."

"These bacteria were chosen for their ease of cultivation and our ability to discriminate them morphologically on standard microbiological medium. Our aim was not to sample the whole community of bacteria associated with our flies stock but to carry out tractable experiments using a random subset of their symbionts."

" [...] all the bacterial strains we isolated had already been identified as associated to *Drosophila* flies (Chandler et al. 2011; Staubach et al. 2013)"

The role of the "evolutionary" part regarding one bacterium class is not related to the rest of the study, which is not evolutionary at all but mainly physiological or developmental.

#8.

We removed the result - and associated discussion - that in one case a bacterium may have evolved during our experiment. As a result, the "Evolution" section of the discussion is now much shorter.

Hence the title is a misnomer.

#9.

The editor probably refers to the original title that contained the word "Evolution". This word has been removed. The new title is "Environmental specificity in *Drosophila*-bacteria symbiosis affects host developmental plasticity".

However, the rationale for our study was evolutionary. Symbiont-mediated adaptation of hosts to local conditions is only possible if symbiont-mediated effects are environment-specific. Similarly, as now better discussed in the manuscript, that symbionts affect host developmental plasticity more than they provide resources has evolutionary consequences.

The negative correlation between development time and body size needs to be tested properly with ANCOVA, entering ALL main factors (medium, bacteria, sex, plus interactions; cf. Table 1), such that the remaining correlation is indeed tested WITHIN treatment combinations. Fig. 3 clearly indicates that the negative regression line largely results from the two medium treatments (red vs. blue dots; lab vs. grape). I don't think this is what the authors are saying or testing.

#10.

The analyses initially presented in the manuscript were indeed insufficient, they have been improved in the new version. As pointed out by the editor, the negative relationship between bacterial effects onto development speed and adult size is largely driven by the environment. A MANOVA (see Table 2) formally demonstrates this assertion.

We believe the MANOVA is the right method for the task because it tests how factors affect several response variables simultaneously (i.e. their correlated response). By contrast, an ANCOVA enables testing whether factors affect the slope of the relationship between two quantitative traits. In other words, we did not intend testing the relationship WITHIN treatments because that would not have revealed the effect of the treatments on both larval development and adult size. Instead, a within treatment relationship between these two traits would be driven by a number of uncontrolled factors such as genetic or micro-environmental differences among replicates. The MANOVA we present tests the effect of the treatments themselves on the covariance between our two focal traits.

Statistical methods now state:

"In order to explain the factors behind the simultaneous effect of bacteria on developmental speed and adult size we carried out a multivariate analysis of variance (MANOVA) using all relevant datapoints (i.e. one datapoint per experimental unit when both estimates were available). MANOVA was chosen because it enables studying how factors affect several variables jointly, in other words it considers factors effects onto the correlation between several variables (Zar 2009, p. 319). We used a "repeated measures" personality of MANOVA and reported the tests based on the Sum response function (i.e. a M-matrix that is a single vector of 1 s; between-subject report in JMP)."

Overall, this descriptive study is worth publishing after major revision as indicated by the reviewers and myself above. Better still, adding further treatment levels would make this work more interesting and meaningful.

#11.

Thank you for this positive assessment. Your comments and those of the referees have greatly improved the manuscript, at least to our eyes. In particular, the methods and the take home message are now much clearer.

Also, we hope you'll agree with us that this work, in its new form, is not purely descriptive and presents original elements of evolutionary significance.

**Additional requirements of the managing board:**

As indicated in the 'How does it work?' section and in the code of conduct, please make sure that:

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



they can be reused.

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

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

## Reviews

*Reviewed by anonymous reviewer, 2019-02-18 12:04*

The aim of this manuscript is to explore whether (and how) interactions between symbionts depend on the environment where they are tested. For that the authors isolated four bacterial strains from a *Drosophila melanogaster* laboratory population and tested how they affected several larval and adult traits when tested in two different media: (ancestral) laboratory media and grape berry media with live yeast. In particular, they recorded larval size, mouthpart movement speed, number of visible larvae, survival until emergence and a proxy for adult size. Additionally, they analyzed the microbial content of recently emerged adults and tested metabolic profiles of two of the bacterial isolates and of Actinobacteria that were present in grape berry medium at the end of the experiment. In general, the authors observed that the effect of the bacteria on the several traits assayed differed between the laboratory and grape fruit environment. In fact, in several traits some of the bacterial strains change from beneficial to deleterious indicating the dangers of generalizing from laboratory studies to the natural environment.

This study tackles the impact of environmental variation in the symbiont effect on the host and how it varies between different symbionts strains. This is both interesting from the point of view of understanding how symbionts may constraint or promote adaptation to new

conditions, but it also raises an important point on whether observations done in laboratory settings can be taken as a proxy of what happens in natural conditions.

Thank you for this positive feed-back. It is rewarding you highlighted the evolutionary relevance of our work.

However, I think this manuscript needs some revision, mainly the statistical methods, results section and the discussion. I divided my comments into major and minor comments (in no particular order) and hope that these suggestions may help to improve the manuscript.

#### Major comments

I found it hard to understand what were the questions that you want to address with this study. In order to make it clearer I would suggest adding the main questions of the study at the end of the introduction (and some hypotheses for each). Then these main questions could be used in the results, material and methods and discussion section as titles to better guide the reader.

#12.

We modified the introduction accordingly. Its last paragraph now states explicitly the main questions we address in the manuscript. Besides we also changed the title of the results sections to mirror those questions. The end of the introduction now reads:

"[...] We investigated two questions. (1) We focused on the influence of environmental variation on bacterial effects analyzing each of the host's traits individually. Our aim was to unveil whether host-symbiont that occurred in the environment of origin (i.e. the laboratory) maintained in more realistic conditions. We further relate these observations to fly and microbe ecology. (2) We performed a new, simultaneous analysis of two traits in order to disentangle symbionts' effects on host developmental plasticity and resource acquisition, two non-excluding possibilities. Separating plasticity from resource acquisition is important for at least two reasons. First, long-term symbiotic associations would be more likely when symbionts provide new capabilities (i.e. resources) than when they affect

quantitative traits (Fellous and Salvaudon 2009) or their plasticity (Chevin et al 2010). Second, recent literature shows that the evolution of symbiont transmission depends on which of host's traits it affects (Brown and Akçay 2019) ; importantly, this mathematical model is based on the plastic trade-off between survival and reproduction. Here, we based our analysis on another well-established trade-off in holometabolous insects, that between duration of larval development and adult size at emergence (Teder et al. 2014, Nunney 1996). In brief, we reasoned that bacterial effects on host developmental plasticity would move host phenotypes along the trade-off axis, while resource acquisition would allow faster development or larger size without detrimental effects on the other trait"

L205:206 – Why are you profiling these two bacteria in specific and not the other ones? What does the profile mean in terms of the impact of the bacteria on the host? Would it be worth to test whether some specific metabolic profiles have a specific impact on the host? This is something that I think it's missing in the manuscript to explain why you did the profiling.

#13.

We profiled the metabolisms of the two bacteria that had the largest and most environment specific effects on the host (See text in methods copied below). This was a small addition to our study. The ecology and mode of action of the Enterobacteriaceae and the Actinobacteria are discussed in the first section of the discussion, we had however little to say of hypothesize about the other bacterial treatments. If the reviewer wishes we could remove the metabolic analyses from the manuscript so as to homogenize available data among bacterial strains.

Methods now read:

"The Enterobacteriaceae and the Actinobacteria were the main bacterial isolates that affected fly phenotypes. In order to shed light on the ecologies of these two strains and therefore on their effects on hosts, we analyzed their metabolic capabilities with Eco Microplates (Biolog) (see Text S6 for methodological details)."

Data analysis

- Instead of the post-hoc students test you should use a Tukey test, as it will correct for multiple comparisons and do the comparison considering the variance of the whole data set and not only the two levels you are comparing.

#14.

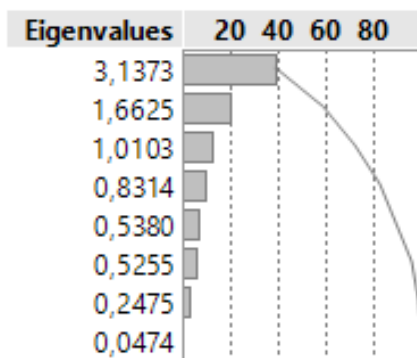
Multiple comparisons can indeed lead to spurious differences among treatments levels. We therefore removed the pairwise student's test initially reporter. Because we did not mean to test all possible differences among treatment levels, Tukey also seemed somewhat inadequate. Indeed, we were only interested in knowing whether some bacterial treatments (i.e. those that were visually remarkable) differed from the controls in the same environment. In this context, independent contrasts appeared the best option. Over the 5 phenotypic traits for which the interaction bacteria\*environment was significant we carried out 14 targeted contrasts tests. The results are presented in the result section and the figures, which legends have been edited accordingly.

- An interesting analysis would be to perform a PCA using all traits analysed to see how different are the effects of bacterial strains and environments in a multivariate phenotype.

#15.

A PCA can indeed reduce dataset dimensionality and shed light on greater order source of variation than singling out the response of each trait. This is a seducing idea we had considered before writing the manuscript, but put aside as we did not see what to do with it. Below, we present the results of the PCA analysis we run as suggested by the referee 1. It could be included in the paper if you see fit.

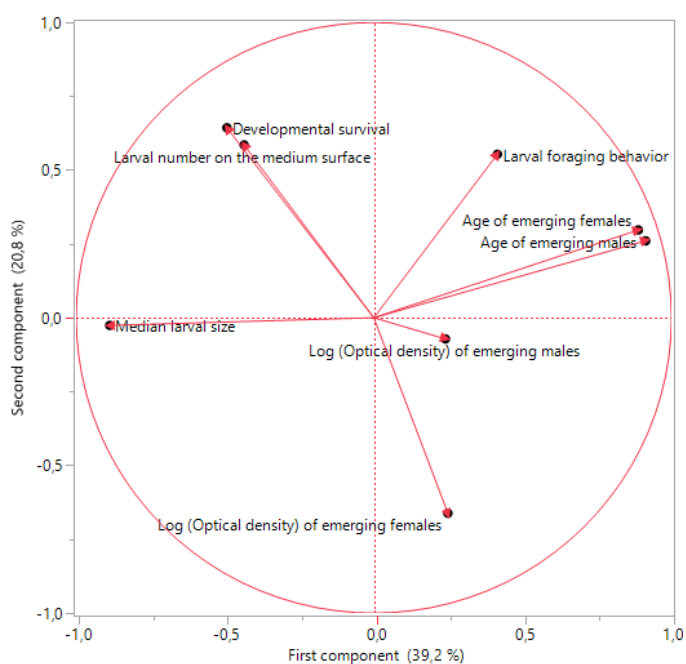
Before analysis all 8 traits (see list below) were standardized (Variance = 1, mean = 0). After running the PCA, we arbitrarily retained the first three principal components (PCs) for further study. PC1 accounted for 39.2%, PC2 for 20.8% and PC3 for 12.6% of the whole variance (Figure 1).



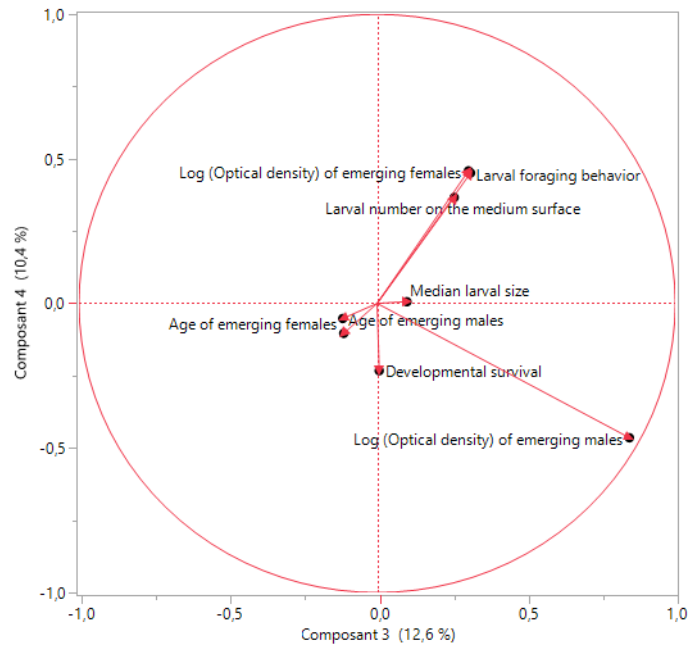
**Figure 1: Eigenvalues corresponding to each principal component in order from largest to smallest.**

### Composition of the PCs:

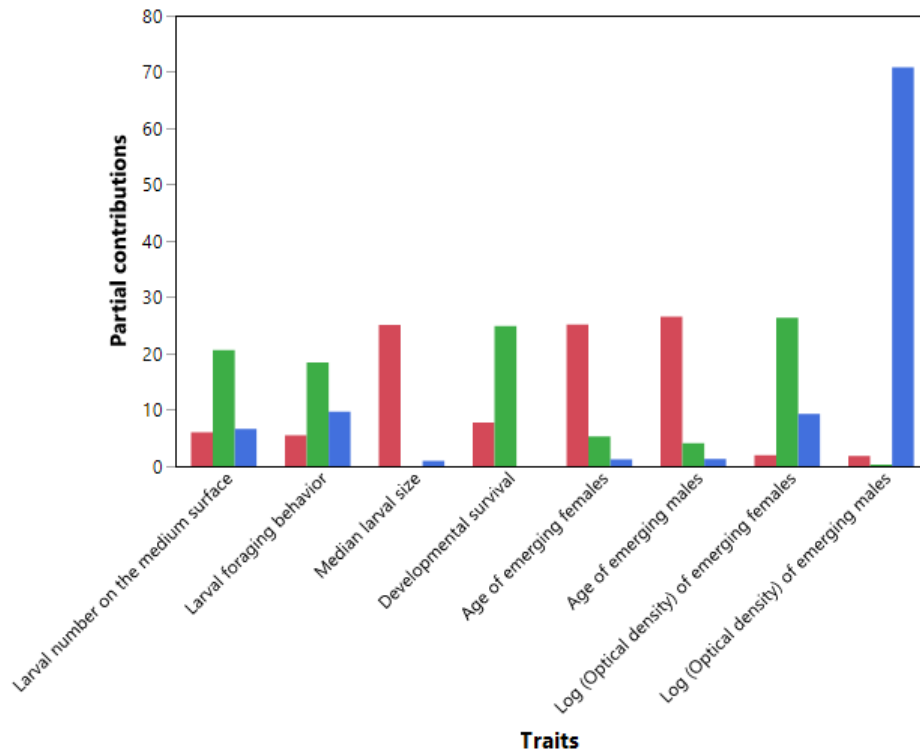
- PC1 summarized variance of developmental speed as it accounted for variation of larval size and age of emerging adults (Figures 2 and 4).
- PC 2 summarized variance of three larval traits (i.e. larval number on the medium surface, larval foraging behavior and developmental survival) and of the size of the adult females (Figures 2 and 4).
- PC 3 mostly summarized variance of the size of the adult males (Figures 3 and 4).



**Figure 2: Unrotated loading matrix between the traits and the principal components 1 and 2.** The closer the value is to 1, the greater the effect of the component on the variable.



**Figure 3: Unrotated loading matrix between the traits and the principal components 3 and 4.** The closer the value is to 1, the greater the effect of the component on the variable.



**Figure 4: Partial contribution of traits to each principal component.** Red corresponds to PC1, Green to PC2 and Blue to PC3.

We used linear mixed models with Restricted Maximum Estimate Likelihood (REML) to test the effects of the larval environment, the bacterial treatment and their interaction on each of the three PCs. ‘Block identity’ was defined as random factor and the others as fixed.

**Table 1: analysis of principal components in response to larva environment, bacterial treatment and their interaction.**

Principal component	1	2	3
<i>Environment (Env)</i>	$F_{1,13} = 248.27$ $p < 0.0001$	$F_{1,18} = 11.09$ $p = 0.0038$	$F_{1,10} = 0.06$ $p = 0.8185$
<i>Bacterial treatment</i> ( $\mu$ )	$F_{5,78} = 2.25$ $p = 0.057$	$F_{5,74} = 1.94$ $p = 0.098$	$F_{5,61} = 1.04$ $p = 0.4$
<i>Interaction Env*<math>\mu</math></i>	$F_{5,78} = 5.85$	$F_{5,68} = 0.93$	$F_{5,52} = 0.43$

	$p < 0.0001$	$p = 0.4667$	$p = 0.8223$
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As revealed by the linear model the Bacteria\*environment treatment was only significant for the first PC axis. The second was mostly explained by the environment.

While I see the interest of analyzing the Actinobacteria that seemed to evolve in the medium without flies, I do not think it makes sense in the context of this work (as you are studying symbiosis and how it varies across environments) but not any kind of evolution per se, so I would remove it from the manuscript. Moreover, I do not think you can take a lot of conclusions from a single replicate vial that evolved a particular kind of strain.

#16.

All reference to the possible evolution of this bacterial isolate has been removed from the manuscript.

Discussion: Symbiont-mediated evolution section – I have a problem with this section, as you don't really test evolution of the Drosophila populations with the different treatments. I don't think you can actually say whether bacteria are helpful, neutral or detrimental for adaptation per se. You can just say whether they have a different effect on grape medium or on laboratory medium in a single generation.

#17.

The "Evolution" section in the previous version of the manuscript was unnecessary long. This was in part due to lengthy discussions of the possible evolution of the Actinobacteria in the time course of our experiment. As this element has been removed, the overall section is much shorter.

However and as responded to the Editor (see response #9), the rationale for our work is evolutionary. We think that the patterns we report here (i.e. environmental specificity of



effects on larvae) have consequences for the adaptation of hosts to local conditions mediated by symbiosis with bacteria. Similarly, whether symbionts affect host resource acquisition or developmental plasticity has consequences for symbiosis evolution, an element we added to the introduction and the discussion.

Moreover, without studying the evolution of the populations you cannot disentangle whether your results stem from other sources of variation such as maternal effects or plasticity.

#18.

We are not sure we've correctly understood this question. All results we report reflect a form of phenotypic plasticity, as noted by the editor in its recommendation. When bacteria change effect in different environments, they show the phenotypic plasticity of the host-symbiont association. Regarding maternal effects, we are not sure how they could come into play. Each of the experimental blocks received fly eggs produced on a different day by various groups of females. Similarly, we used a different aliquot of each bacterium for each block.

Maybe the referee means that symbiont effects on the hosts could have been different if the flies had been reared in a different environment in previous generations (i.e. if the effects of the bacteria depended host parental effects). This is an interesting hypothesis that is in line with the numerous parental effects described in *Drosophila* flies. It would be a problem if we drew conclusion regarding this very population in a specific context; or if we claimed that bacteria from a given environment (e.g. lab) are always less beneficial in a new environment (e.g. fruit) than where they come from. However, we here test a general idea (i.e. environmental specificity) and draw conclusions (e.g. effect on plasticity). Parental effects might have affected the details of how bacteria affected some host traits, however it seems unlikely they would have suppressed the environmental specificity of symbiont effects and their reliance on host developmental plasticity we observed.

Thus, this section seems to be rather large and extended for the amount of evidence you provide.

#19.

We agree and discarded the paragraph on rapid evolution of the particular *Actinobacteria* isolate.

Figures 1, 2, - These figures are really hard to understand. I suggest that you remove the lines between the points (as the x axis has categorical variables and the line plot are usually used for continuous variables, such as time or gradients). I think that bar plots would be easier to visualize instead of the scatter points. The letters are also really confusing. To add the information about significantly different comparisons you could use a matrix plot with all combinations of environments\*<sup>\*</sup>Treatment in both axis and color code each comparison for the different levels of significance.

#20.

Original figures were indeed cluttered with the report of the pairwise student's t tests. These tests have been removed and replaced by a few targeted contrasts. Overall, we made efforts to simplify the figures. For example, we removed sex information from the new figure 4 as it is not a significant source of variation. Regarding the lines connecting treatment levels we would like to keep them. They facilitate the visual identification of when bacteria have different effects in different environments. This is why they are common practice in phenotypic plasticity studies. Besides, articles of other fields use the same graphical representation, in particular in the evolutionary parasitology and symbiosis literature that focus on statistical interactions between host and symbiont types (e.g. Lambrechts et al. 2006 Trends in Parasitology).

#### Minor comments

L67:68 – It is not true that the most striking difference between laboratory and the wild is the substrate where the flies eat. There are a large number of other variables, such as population

size, presence of competitors and predators, high variability in weather conditions, etc. that also play a BIG role on how species live and reproduce. This sentence should be rephrased.

#21.

Yes, the introduction was revised accordingly. Similarly, in the discussion we distinguish effects due to the physical structure of the environment from those due to its nutritional composition. The introduction now states:

"Numerous variables differ between laboratory and natural environments of *D. melanogaster* flies. A substantial difference is the composition of the nutritive substrate upon which the adults feed, copulate, oviposit and within which larvae develop. Wild flies live on and in fresh or decaying fruit flesh, usually colonized by yeast, whereas laboratory flies are reared on an artificial, jellified and homogeneous diet that contains long-chained carbohydrates (e.g. starch), agar, preservatives and dead yeast cells or yeast extract."

L 143 – It would be easier/better for the reader if you had a supplementary figure explaining the experimental design.

#22.

New Figure 1 now describes the experimental design.

L 163:164 – The mixed and single bacterial isolates should have the same of bacterial density in the beginning. This way you may have an effect in the mixed treatment just because of the overall bacterial density. In fact, you observed that for the actinobacteria the inoculate size is actually important.

#23.

We fully agree with Referee 1, there was a typographical error in the previous version of the manuscript. The mixed and single bacterial inocula had the same bacterial density ( $10^4$  cells).

Statistics - In order to extract the mean value of the environment you should subtract it from the values, and not divide, as dividing will change the variance of the data, and that's what you are analyzing with your statistical analyses.

#24.

We followed the suggestion of Referee 1. Results remain unchanged.

L255 – I think it's incorrect to say that the bacteria decreased directly the movement of the mouthparts, or any other phenotype. A more correct way would be to say that in the actinobacteria treatment there was a slowing down of the movement. You should correct this kind of use of the direct causality between bacteria and phenotype in other instances of the manuscript (e.g. L245, L247, etc)

#25.

As pointed out by Referee 1, a number of intermediate processes may underlie the effect of the bacteria on host traits. We therefore changed the wording throughout the manuscript. In some instances, we wrote that bacterial treatments affected the traits as this does not assume direct causality.

L339:341 – This phrase is very hard to read. Please rephrase into a simpler form

#26.

This has been rephrased for clarity.

L341 – There are many differences between the natural and laboratory environment. Thus, I suggest you should change ALL instances of close to closer.

#27.

This has been done.

L360 – Why does the microbial growth on the surface of the vial without flies supports the hypothesis that these bacteria are eaten by the larvae? This could also happen if the larvae mix the medium while moving, and destroy the colonies and substrate, making the development of the white microbial growth impossible in their presence.

#28.

You are right, an absence of white bacterial growth in presence of larvae could be explained by the above phenomenon. We suspect this comment is due to some ambiguity in our initial text: it did not state that white growth could also be observed in presence of larvae. The mention "in absence of larvae" referred to the conditions in which the picture Figure S7 was taken. We've clarified the text to prevent further confusion.

We are grateful to Referee 1 for this comment as it prompted further thinking about our results which led to a new structure of the corresponding part of the discussion. In particular, we now explicitly discuss 2 different hypotheses regarding the mechanisms of the interactions between this bacterium and *Drosophila* larvae.

L387:390 – Any clue why would this happen? Does grape have less nutrients than in laboratory medium and so the actinobacteria are an added stress?

#29.

We added this hypothesis to the discussion as well as a full paragraph discussing the role of nutrients in the observed differences among environments.

Discussion now reads:

"[...] Why did the effect of the Enterobacteriaceae on host phenotype differ among environments? The physical nature of laboratory medium is very different from that of real fruit. In particular, the agar of laboratory medium permits the diffusion of simple nutrients and their absorption by bacteria and yeast present on surface. Besides, in grape nutrients are not free to diffuse but enclosed in cells. Surface growth is therefore more likely in artificial medium than in grape berry, leading to different effects on larval development.

In addition to physical differences between laboratory medium and fresh fruit, the nature and concentration of available nutrient are likely to differ. It is well known that lactic and acetic acid bacteria, two taxa that were not investigated in our experiment, can promote larval growth upon nutrient scarcity (Shin et al. 2011; Storelli et al. 2011, Téfrit et al. 2017). However, it is also well established that bacteria can affect *Drosophila* phenotype through signaling (Storelli et al. 2011) as well as nutrient provisioning (Brownlie et al. 2009; Bing et al. 2018; Sannino et al. 2018). In most cases, these effects which were described from laboratory flies and in laboratory medium, are condition specific (Douglas 2018). Indeed, bacteria are often only beneficial when laboratory food has a low concentration in dead yeast (i.e. amino acids) (Shin et al. 2011; Storelli et al. 2011). Along these lines, it may seem paradoxical the Enterobacteriaceae's only accelerates larval growth in rich laboratory medium rather than in grape berry (unless the bacterium synthesized a rare nutrient). Metabolic profiling (Figure S5A) further shows the Enterobacteriaceae is a generalist bacterium able to grow on a variety of substrate. However, the Actinobacteria had a narrower metabolic spectrum (Figure S5B), suggesting it is a specialist which growth largely depends on the availability of specific nutrients. The bacterium slowed down larval growth in grape (Figure 2A) for an unknown reason - maybe because it exerted additional stress onto larvae in a relatively poor medium - but had no notable effect in laboratory medium. The environment-specific effect of the Actinobacteria compares to previous reports of *Drosophila* symbionts being beneficial in some environments only (e.g. *Lactobacillus plantarum* in rich-medium), and further reveals that bacteria with little effect in an environment can become detrimental in new conditions."

L395:397 – Not really sure you can say this without a reference or showing data. So I would remove this part

#30.

We removed all reference to this observation (and the corresponding elements of discussion).

L451:452 – Grammatically this sentence is not correct. I think you should remove the “then”. I am also unsure whether the sentence scientifically correct as the genetic ability to deal with

stress of the host is not the only thing that defines bacteria to act as parasites or mutualists. For example, co-evolution in the laboratory environment led Wolbachia to change from parasite to mutualist in *D. simulans* laboratory populations (Weeks et al 2007 Plos Biol.)

#31.

We removed "then" and added "partly".

L461 – “in the” instead of “on the”

#32.

It has been done.

Conclusions – I would rephrase the last sentence with something simpler and to the point. Maybe something like “Our results demonstrate that in order to understand the ecology and evolution of symbiotic interactions in the wild it is necessary to use ecologically realistic conditions.”

#33.

Conclusions now finish with the sentence suggested by Referee 1.

L80 - recorded sounds weird, maybe “scored” would be a better term

#34.

"Recorded" was replaced with "scored" throughout.

L184:186 – Maybe a simpler of saying this is to say that for each vial the sex of the flies to measure was chosen randomly.

#35.

All this was changed.

L203:204 – I would remove this sentence altogether

#36.

Corresponding data is now in the supplementary materials, the sentence is therefore necessary.

L 270 – was influenced

#37.

This was changed.

L325 – Comparison of what?

#38.

This section was removed.

*Reviewed by Pedro Simões, 2019-02-18 12:04*

I think this is a nice study that addresses a very timely topic, the interactions between bacteria and hosts and their effects in different environments. The finding that the effects of bacteria on host are environment-specific is interesting (although not particularly surprising) and can lead to new research directly targeting the evolutionary implications of such interactions for the hosts (as well as bacteria). Additionally, it would be important to complement this study with the analysis of other populations to test if the observed effects are general or population-specific.



#39.

Thank you for the encouraging comment and highlighting our result that bacteria affected host developmental plasticity. We agree with the suggestion of extending the analysis to other host populations. This, and the inclusion of other host species will be presented in a future study.

My major concern regarding the study lies on the interpretation and discussion of the findings associated with 1) host developmental plasticity (specifically data from fig. 3) and 2) bacterial evolution (data from fig S6) – I detail these in the major comments below.

Major comments:

1) Lines 417-420 –The observed negative trend between larval development speed and adult size is expected due to a physiological trade-off, as faster development likely leads to lower adult size in *Drosophila*. In this case, bacteria that lead to an increase in larval development speed likely originate a correlated effect of lower adult size. Please explain better this part of the discussion. It might also help to clearly state the expectations for this analysis in the introduction (see comment below, lines 83-85).

#40.

Based on the referees' feed-back we have extensively revised the manuscript in order to present in more details the logic behind our analysis of how bacteria affect host-developmental plasticity. The last section of the introduction now reads:

"We performed a new, simultaneous analysis of two traits in order to disentangle symbionts' effects on host developmental plasticity and resource acquisition, two non-excluding possibilities. Separating plasticity from resource acquisition is important for at least two reasons. First, long-term symbiotic associations would be more likely when symbionts provide new capabilities (i.e. resources) than when they affect quantitative traits (Fellous and Salvaudon 2009) or their plasticity (Chevin et al 2010). Second, recent

literature shows that the evolution of symbiont transmission depends on which of host's traits it affects (Brown and Akçay 2019); importantly, this mathematical model is based on the plastic trade-off between survival and reproduction. Here, we based our analysis on another well-established trade-off in holometabolous insects, that between duration of larval development and adult size at emergence (Teder et al. 2014, Nunney 1996). In brief, we reasoned that bacterial effects on host developmental plasticity would move host phenotypes along the trade-off axis, while resource acquisition would allow faster development or larger size without detrimental effects on the other trait".

The relevant results section starts with:

"We expected three possible patterns when plotting average adult size in function of speed of larval development (i.e. - age at emergence): a positive relationship indicative of a similar effect of the bacteria on the two traits (i.e. bacteria modulate resource acquisition, mostly); a negative relationship indicative of bacteria affecting host position along the trade-off (i.e. bacteria modulate developmental plasticity, mostly); a lack of correlation that would have been challenging to interpret on its own as several processes could produce this result (e.g. bacterial effects on both host plasticity and resources)."

The second section of the discussion is dedicated to this analysis, it starts with:

"In holometabolous insects, the duration of larval development and adult size are often negatively correlated due to a physiological trade-off: faster development reduces the duration of food intake and leads to smaller adult size (Teder et al. 2014, Nunney 1996). We propose to exploit this trade-off to separate symbionts' effects on host developmental plasticity and resource availability. As discussed above, symbionts of *Drosophila* flies can modify host's signaling (e.g. Shin et al. 2011, Storelli et al. 2011) as well as provide rare resources (e.g. Brownlie et al. 2009, Sannino et al. 2018). These two mechanisms are expected to have different effects on the trade-off between speed of development and size. Signaling (i.e. plasticity) should move hosts along the trade-off, while the provisioning of greater resources should enable faster growth and/or larger size without sacrificing the other trait. In order to test this idea we extracted bacterial effects on host phenotype by subtracting control trait values to those of each of the bacterial treatments in each environment. The resulting plot of symbionts effects on developmental speed and adult

size (Figure 5, S5) reveals the influence of the bacteria on the host independent of the general effects of the environment (i.e. those not due to the bacteria). "

Overall, this data is a bit fragile as it is based on the combined data across environments (and there are no analyses testing for differences between environments in this relationship).

#41.

With only ten bacterial\*environment treatments statistical power is too low to test whether the relationship stands "within an environment". However - thanks to the comments of the referees - we carried out an additional analysis, a MANOVA, that aimed at disentangling the relative influence of the bacteria and the environment on the correlated response of larval developmental speed and adult size. The analysis revealed the environment was the strongest driving factor behind the observed correlation (Table 2). It could hence be argued that combining data from two environments enabled this discovery.

2) Lines 488-493 - I do not see evidence for the referred adaptation to grape. From Fig S6 I conclude that in general actinobacteria reduce in number with increasing time in grape. Please explain better your interpretation. Also there is no figure S6.1.

#42.

Given the unanimous feed-back from the referees and the editor we removed this result from the study. We now consider this observation as a preliminary result based on which we will design further research.

3) Lines 472-502 - I would reduce this paragraph in size so to downplay its overall importance in the manuscript. I think it is a bit too speculative considering the strength of the data obtained. (see comment above about fig S6).

#43.

This is another comment shared by the referees and the editor. We followed the suggestion and largely shortened it. As stated above, we nonetheless wish to keep several elements of discussion on the evolutionary relevance of our work.

Specific Comments:

Lines 83-85 - expectations for this analysis should be clearly stated in the introduction.

#44.

As described in the response #39, we now provide a lot more detail on the rationale behind the analysis and expected results.

Lines 90-92 - some more info here would be helpful (e.g. number of founders, generations in the new laboratory).

#45.

We accordingly added available information. M&M now read:

"Insects were from the Oregon-R *Drosophila melanogaster* strain that was founded in 1927 and has since been maintained in numerous laboratories. Our sub-strain was founded  $\pm 2$  years earlier from a few dozen individuals provided by colleagues."

Lines 97-99 - is it possible to have an idea of the relative abundance of the bacteria chosen in the whole bacterial community of this *D. melanogaster* strain?

#46.

This is an interesting question for which we have partial data. As now detailed in the M&M, the relative abundance of these bacterial strains was variable among fly vials (i.e. sub-populations). Each of the bacteria was not always visible in every sample (they may have been present though, but in low numbers).

M&M now reads:

"Four bacterial morphotypes of variable frequency were chosen based on visible and repeatable differences in size, color, general shape and transparency during repeated sub-culturing on fresh media (Figure S2)."

Lines 223-224 – Was a correction for multiple testing applied? It might be adequate as several comparisons were made.

#47.

This comment is shared with referee 1, see response #14 for a comprehensive argument. Using pairwise student's tests indeed lead to the testing of numerous differences. In order to the deleterious effects of multiple testing in the post hoc analysis of significant effects we now use independent contrasts.

Lines 305-307 - was this tested statistically? if so please refer test statistics. It might be relevant to do so for both males and females, as in both cases heterogeneity is observable between environments.

#48.

The MANOVA we now present (Table 2) is a formal test of the factors behind the observed influence of the environment on the correlated effect of the bacteria on larval development and adult size. This analysis shows the environment and its interaction with bacterial treatment both affected the correlation; however bacteria had no significantly repeatable effects across environments.

Line 333 – I think you mean figure S5.2A.

Also I would state here the major finding of the study: that the effects of different symbionts on host phenotype are environment-dependent.

#49.

This was added as suggested.

Lines 399-401 - I don't see how this conclusion can result from figure 4. Actually I don't think this can be concluded at all (based on the data from fig S6) – see also comments 2) and 3).

#50.

All reference to the conclusion that one bacterium sub-strain evolved during the course of our experiment was removed.

Line 15: replace “adaptation” by “conditions”.

Line 33 - replace “participate” by “contribute”.

Line 34 - replace “to” by “for”.

Lines 266-267 - I would say "means not connected by at least one shared letter are significantly different."

Line 335 – I think you mean figure S5.2B. Also fig S5.1 should be referred in this section.

Lines 339-341 - I had trouble understanding this sentence, please rephrase it. Lines 411-413 - Remove or move to methods.

Line 470 - replace “participate” by “contribute”.

#51.

We proceeded to all the minor changes suggested by Referee 2.