*Dr. Eva J. P. Lievens*
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Konstanz, Oct. 13th 2019

Dear Recommender,

Please find enclosed the revised version of our manuscript, MS#203, entitled 'Trait-specific trade-offs prevent niche expansion in two parasites'.

We would like to thank you for your invitation to submit a revised version of the manuscript, and the reviewers for their constructive comments. We have addressed all their concerns in this new version, resulting in a significantly improved version of the manuscript. The point-by-point details are attached. At the end of our response to the Decision letter, we have also included a list of some unprompted changes that improved the clarity of the manuscript. Finally, we added a 'Conflict of interest' section, as requested, and uploaded the data and analyses to Zenodo (https://zenodo.org/record/3476544).

All coauthors have agreed to the resubmission of the revised manuscript. We hope it will be suitable for recommendation in by PCI Evolutionary Biology.

Yours sincerely,

Eva Lievens

# Decision

*by Frédéric Guillaume, 2019-07-08 17:07*
Manuscript: https://doi.org/10.1101/621581

*Dear authors,*

*After receiving three reviews and having assessed your work, I think that your manuscript would greatly benefit from some revisions. All reviewers agree that your work is original and presents interesting results on the evolution of host specialization. I particularly like the trait-based approach that you used and find the overall analysis sound and well conducted. Most reviewers, including myself, agree with your main conclusions. I am convinced that your work will reach a broad audience interested in the role of trade-offs in the evolution of specialization.*

*The reviewers ask for some clarifications in the presentation of the study system and of the results. I join them in asking you to revise your manuscript and to carefully address the issues raised during this first round of reviews. A revised version will be suitable for a recommendation.*

*The main reviewers' comments can be summarized as follows:*
*- please comment on a possible role of inter-parasite competition during infection of the hosts, provided that multi-infections do occur?*
*- clarify what is the main mode of genetic adaptation in your experiment: from de-novo mutations or standing variation? and what it implies for the evolutionary dynamics of the traits during the experiment.*
*- better discuss alternative explanations for the apparent lack of adaptive evolution of spore production; is it a strong trade-off or a lack of genetic variation in that trait? can we interpret this lack of evolution as a negative result?*
*- enhance the presentation of the ecology of the system and the general literature on the topic.*

To those, I am adding a few of my own points:
- please clarify if A. parthenogenetica is an obligate host of A. rigaudi, as suggested by your comment on p4 lines 73-74 ("A. rigaudi has much higher fitness in A. parthenogenetica and cannot persist without this host in the field"); how does this affect A. rigaudi's survival in the lab and the results of your experiment, esp. when A. rigaudi is exposed to the alternate host only?
- similarly, if survival of A. rigaudi depends on presence of A. parthenogenetica, how does the parasite persist in the winter when A. p. is absent? (as per page 14 line 364: "A. parthenogenetica are only present from late spring to fall").
- furthermore, it seems that the two parasites don't have the same ecology, and may not co-occur in nature (p14 line 369); all these details appear late in the manuscript. Please provide a clearer and more complete picture of the ecology of the two species earlier in the manuscript, in the Methods section preferably.

We would like to thank the recommender and the reviewers for pointing out the missing information in our description of the host-parasite system. We have significantly expanded the 'Hosts and parasites > Natural system' section of the Methods. We now answer these questions, and provide more details about the evolutionary history of the system (i.e. is *E. artemiae* an invasive species?), field patterns (including seasonality and the occurrence of multiple infections), and the degree of host specialization.
To answer these specific points:
- Although *A. rigaudi* cannot be maintained on populations of the alternate host in the field, it is possible under lab conditions. Nonetheless, we lost 2 out of the 4 replicate lines in this combination

(Table 1), and were only able to revive one.
- *A. rigaudi* does indeed disappear in the winter. In the spring, epidemics are probably rekindled from some kind of spore bank, though this has not been studied.
- The parasites do co-occur often in nature, as do the hosts. We have expanded on this in the 'Natural system' section.

- what is the role of phenotypic plasticity in your experiment? can you discuss your results in terms of evolution of norms of reaction instead of only mean trait values? How plastic are the traits you measured? your results clearly present cases of evolution of reaction norms, with maybe mostly changes in intercepts (mean) than slopes (beside maybe virulence). Please comment.

We completely agree that the variation in traits across environments (hosts) is, by definition, a reaction norm/a GxE interaction/population-level plasticity. We do find changes in the slopes of the reaction norms when there are significant *Assay host : Treatment* interactions (Fig. 4). Specifically, the slope of the reaction norm for infectivity changes when *E. artemiae* is passaged on *A.f.* vs. *A.p.* hosts (infectivity has weaker GxE effects/is less plastic after passaging on *A.p.*), as does the slope of the reaction norm for virulence when *A. rigaudi* is passaged on *A.f.* vs. *A.p.* (virulence has stronger GxE effects/is more plastic after passaging on *A.f.*). We did not select specifically on the reaction norms, so we cannot interpret these changes as a direct response to selection. However, our interpretation - that a weak trade-off acts on infectivity and a strong trade-off acts on spore production - would also imply that infectivity can evolve to become less plastic (i.e. to have a weaker reaction norm), while spore production cannot.

*I would be grateful if you could comment on the points raised here and by the reviewers, providing arguments when you don't agree. Please provide a point-by-point answer with clear reference to the main text to speed up the assessment of your revised version. Thanks!*

Besides the answers to the reviewers' comments, we have also made a few unprompted changes:

- The reviewers suggested several changes to make the figures easier to interpret. We have taken advantage of this to further simplify the figures, by combining the two 'serial passaging' figures (previously Figs. 2&3, now Fig. 2) and the two 'final assays' figures (previously Figs. 4&5, now Fig. 3). This groups the results more obviously and declutters the manuscript. **For convenience, we have answered the reviewers' comments using the old figure numbers. The correspondence is:**
  **old Fig. 2 = new Fig. 2 (top)**
  **old Fig. 3 = new Fig. 2 (bottom)**
  **old Fig. 4 = new Fig. 3 (white panels)**
  **old Fig. 5 = new Fig. 3 (gray panels)**
  **old Fig. 6 = new Fig. 4**
- Removed "Intriguingly, the trait-specific trade-offs appear to be very similar for both parasites" from the abstract, to keep it short after responding to Reviewer 2's comment.
- Removed "Two of these were lost before we could collect a backup spore sample after P6; of the other two, only one line's P6 spores could be revived and used in the final assays", which refers to the second Results section, from the first Results section (new line 223).
- Rewrote the sentence starting with "Instead, demographic effects were the likely culprit" (new line 367).
- Corrected figure reference for the phrase "we found no evidence that the high death rates caused by E. artemiae in A. franciscana between P4 and P6 were caused by a higher virulence" (new lines 365-367).

- Removed redundant sentence “These results correspond well with previous findings (Lievens et al. 2018)” (new line 329).
- Minor changes to the sentences in new lines 391-395, to improve clarity.
- Replaced ‘parasite specialization’ with the more common term ‘host specialization’ in new lines 12 and 53.
- Updated the references in paragraph 3 of the Introduction.

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## Reviews

*Reviewed by Seth Barribeau, 2019-07-01 22:15*

*Lievens et al. present an impressive body of work. The topic is interesting and the approach is sensible. Experimental evolutions studies can be challenging, and the effort here should be commended. I do however have some issues, listed below. Among those, I think the most important involve the interpretation of the results. First, if I understand correctly, the infectious exposure came from diverse stock, it then seems likely that any adaptation was selection on standing genetic variation rather than mutations. Second, I have trouble wrapping my head around the explanation that the absence of adaptation to experimental conditions represents strong trade-off, as opposed to simply a negative result.*

**Line-by-line comments**

L66: on -> in L68: "in host use limit the evolution of specialization of two naturally occurring parasite" Previously talk about barriers to generalism. Here inverted. I get that these are two sides to the same coin, but good to be consitant to ensure readers follow. Similar around L78

Line 68 (now line 71) was adjusted as suggested, with an additional change at the end to improve clarity. It now reads “we investigated whether trade-offs in host use limit the evolution of generalism in a natural host-parasite community”. We chose to leave line 78 as is, since “the degree of specialization” encompasses both specialism and generalism.

L97: what is meant by suboptimal virulence? Too high or too low?

*A. rigaudi*’s virulence in *A. franciscana* is too high relative to its spore production. We have changed this phrase to “… and *A. rigaudi* exhibits higher-than-optimal virulence in *A. franciscana*.”

L102: Genetic diversity of parasite stocks unknown, but collected from various sites

This comment wasn’t clear to us.

parag around 115: italics for Latin names

Thank you, these had slipped our notice.

L125: "we did not control the number of spores that were transmitted from one group of hosts to the next. Thus, the size of the inoculum and of the microsporidian population in all passages after P1 were dependent on the infection dynamics that developed within each replicate line." Is this a problem? different doses selection for what then?

We don't think this is a problem for this study. By allowing infection dynamics to affect the evolutionary trajectories of our lines, we mimic evolution in field populations. In addition, we did our best to control all the environmental conditions in the experiment, so any variation in the infection dynamics between replicates of a treatment must be ascribed to stochasticity in the underlying demographic processes (e.g. variation in inoculum size at different passages). We think it is appropriate – and desirable – for an experimental evolution study to allow such effects. We have edited the end of this paragraph to clarify this (new lines 145-148).

L150 missing ref

Corrected.

L171: surviving and revived lines... what does that mean? passsage 10 vs 6? Figured it out later, but could do with clarifying first use of 'revived'

Thank you for pointing this out. We now define 'revived' when it is first relevant, in line 153.

L181: only includes infected individuals in survival analysis. How many/what proportion were excluded because they were exposed but not infected?

This information was included in the results, but it would be useful here too. We have added "(see Results, Table 2 [*] for the proportion of infected hosts)" to the sentence.
*Previously Table 4, see response to Reviewer 3.

**Results**

*Pretty complicated results. Not unsurprisingly. Lots of stuff going on.*

L252: *A. rigaudi* is most virulent when assayed on *A. parthenogenetica* if passaged on other host. Suggests adaptation to host sp. and perhaps suboptimal virulence on the other. This is an important result and a figure here would be useful. This is shown in Fig4 but figure not mentioned in text here.

We have inserted the reference to the figure, and added it where it was missing in the previous and next paragraph as well.

Same for L274

Corrected.

L257: "but infected hosts did not die faster than controls" unclear here what is control. Presumably all were infected?

Corrected to "but infected hosts did not die faster than unexposed hosts".

**Discussion**

L285-6: "infectivity readily evolved towards generalism" This doesn't appear true. *E. aremiae*did evolve greater infectivity on *A.parthenogenetica* but *A. rigaudi* did not. Similarly, "virulence played a minor role" doesn't seem quite true either as *A. rigaudi* evolved reduced virulence on *A. parthenogenetica* when tested on that host. Figure 4.

In trying to provide an overview of the discussion here, we may have oversimplified. We have changed this phrase to: “infectivity showed a generalist pattern in *A. rigaudi* and readily evolved towards generalism in *E. artemiae*” (new lines 312-313). We have not changed the phrase “virulence played a minor role”, since the changes in virulence in *A. rigaudi* when tested on *A. parthenogenetica* didn’t impact the overall degree of specialization (Fig. 5 and lines 324-329), and that is what is being discussed here.

L286-288: "Our results are consistent with a strong trade-off acting on spore production and a weak trade-off on infectivity, and suggest that spore production is the key trait preventing the evolution of generalism in this system." It is pushing it a bit to interpret no adaptation to the experimental evolution treatment as strong trade-off limiting the evolution of spore production.

This is an important point, that we now discuss more thoroughly in the section ‘Discussion > Trade-offs in infectivity and spore production’. To explain our reasoning, we have copied the new lines 404-420 here:
“The second important trait for *A. rigaudi* and *E. artemiae* was spore production, which remained strongly specialized in all treatments (Fig. **Error! Reference source not found.**). This unresponsiveness to the evolutionary treatment could have three explanations. First, high stochastic variation in our experimental design (drift and measurement error) could deprive us of the power to detect any adaptive change. The increased infectivity observed for *E. artemiae* makes this explanation unconvincing for any trait under similar levels of selection. As spore production is directly related to parasite fitness, there is no reason to expect weaker selection on this trait compared to infectivity. Second, there could be a complete lack of genetic diversity in this trait – either in the initial inocula or due to *de novo* mutations. This explanation is also unlikely given the way we assembled our initial inoculum (see above) and the observation of a genetic response in other traits (virulence in *A. rigaudi* and infectivity in *E. artemiae*). The populations we used were not generally devoid of genetic variation, and there is no reason to expect that mutation rates are inherently lower for spore production. We also observed phenotypic variation for spore production trait among lines (Supp. Fig. **Error! Reference source not found.**), which reinforces this point. The third explanation, which we decidedly favor, is that there is a strong trade-off between spore production in *A. franciscana* and spore production in *A. parthenogenetica*. Such a trade-off would allow small improvements in the direction of increased specialization (black arrows in Fig. **Error! Reference source not found.**), but make improvements on the novel host much more difficult to achieve (red arrows in Fig. **Error! Reference source not found.**), thereby preventing the emergence of more generalist phenotypes.”
In short, a lack of adaptation to the experimental evolution treatment is the expected consequence of a strong trade-off, and the observation of changes for other traits make interpretations based on a lack of power or a lack of variation less compelling.

Paragraph starting at L319: As genetic diversity of the initial infectious dose is unknown but taken from multiple source populations (L106) I would have thought that the most parsimonious explanation in the absence of genetic evidence is that there was selection upon standing genetic variation rather than accumulation of mutations of any flavour.

Indeed, selection on standing genetic variation is more likely (though the population sizes were large enough that *de novo* mutations should not be ruled out). This paragraph was not well thought-out, and has now been rewritten (new lines 345-363).

"It is worth noting that our conclusions would have been very different if we had not measured the parasites’ traits separately. Based on the overall fitness (Fig. 5), we would have concluded that A. rigaudi was unable to adapt to its mismatched host, while E. artemiae was able to evolve towards

generalism after exposure to A. parthenogenetica. This would have suggested that the two parasites had asymmetrical fitness trade-offs between hosts: a strong trade-off for A. rigaudi, and a weaker trade-off for E. artemiae." I certainly agree that measuring different components of parasite biology is informative. But I may have missed something here. This sounds precisely like the argument given by figure 5. That *E. artemiae* has weaker trade-offs, hence the blue arrow.

This is indeed the conclusion that can be drawn from Fig. 5, if it were the only data available. However, we argue that taking the trait results into account (Fig. 4) shows that this conclusion is incorrect. The trade-offs acting on *E. artemiae* are not weaker than those acting on *A. rigaudi*. Instead, the same kind of trade-offs (weak for infectivity, strong for spore production) appear to act on both microsporidians, with *E. artemiae* simply 'lagging behind' in its evolution of generalist infectivity.

Fig4: gray is hard to make out. Also not obvious the star system. eg. stars over curly brackets, does this say that the values within this group are sig different from one another, or from teh other group in curly brackets. Looking at the figure I would have said within group but the values look too similar to be different from one another. I would recommend add square brackets to clarify that the stars refer to between host species.

Please see our response to Reviewer 3's comments about Fig. 4.

Based on supplemental materials, there should be a strongly significant difference between virulence of *E. artemiae* in the different hosts ($X=58.1$, $p<0.0001$), but is not given in figure. Nor do the virulence values look very different in the different hosts.

The analyses for virulence include unexposed control hosts to account for background differences in survival, and they drive the significant *Assay host* effect: *A. parthenogenetica* had a higher background mortality. In Fig. 4, however, survival is presented relative to that of the unexposed controls (since the background mortality is not interesting for our purposes). This was pointed out in the legends of Supp. Table 2 and Fig. 4 but may not have been clear enough; we have now changed the phrase in the former to read "For virulence, *Treatment* also included a 'Control' category of unexposed hosts, so that in these analyses the effect of *Assay host* reflects the difference in background mortality (note that in Fig. 4, the effect of background mortality is removed by representing virulence as the survival of infected hosts compared to that of controls of the same *Assay host*).". The figure legend now reads "The significant experimental variables are noted for each microsporidian × treatment combination (except that of *Assay host* for virulence, see Supp. Table 2)."

**Suppl**

fig1: why so few evolved lines (dashed, hollow points, '+' signs?) - only three of the plots have one of those.

Oops, this was a typo. It has now been corrected to 'revived' lines.

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*Reviewed by Anne Duplouy, 2019-06-14 15:02*

*Lievens et al. provide here a study testing and discussing the evolution of niche specialization in a parasite, using an experimental evolution approach. After 10 generations of passaging separately*

*two parasites on either their preferred host/occasional host/or both(alternatively between passages), they collect data on parasite spore production, infectivity of parasite (define as % of hosts infected during assay) and parasite virulence (defined as ratio of time until host death compared to control hosts) of the evolved lines on the two types of hosts.*

*The hosts species are A. franciscana and A. parthenogenetica - The parasites are microsporidia E. artemiae and A. rigaudi. The study shows that all 6 evolved lines maintained a higher fitness on their preferred host species. This results is mostly driven by a strong variation in spore production between parasite-host associations. Despite 10 generations of habituation to their occasional hosts A. franciscana , the A. rigaudi parasites evolved lines still produced as much spores as the A. rigaudi lines that have evolved on A. parthenogenetica, when both lines were put in contact with A. franciscana in a last essay. (And the other way around for E. artemiae evolved lines). The authors thus suggest that spore production is the barrier preventing the evolution of generalist strategy in these host/parasite systems. Additionally, E. artemiae evolved lines show variations in their fitness when put in contact to A. parthenogenetica in the last essay, while the three evolved lines show similar fitness. The authors here suggest that E. artemiae can evolve a more generalist strategy, while A. rigaudi can not.*

*In brief, the authors argue that a weak trade off between the ability of a parasite to successfully infect host1 and its ability to infect host2 could evolve a more generalist strategy. In contrast, if the trade offs are strong, the parasite is most likely to remain a specialist of host1. I believe such results might have important implications for the study of parasitism, highlighting potential constraints to their spread and evolution in natural environments.*

I have little comments on this research article. I would simply ask the authors to more rapidly describe the type of trade-offs they are considering. I am an Ecologist and Evolutionary Biologist. My first idea of a trade-off is between two different traits pulling on opposite direction, here the trade-offs refer to one unique trait but pulled to it's opposite extremes in the two host environment. It might help if this is made clear from the abstract.

As suggested, we have revised the first mention of trade-offs in the abstract to “The origin of this specialization was not infectivity, which readily evolved and traded off weakly between the host species for both parasites. Instead, the overall specialization was caused by spore production, which did not evolve in any treatment. This suggests the existence of a strong trade-off between spore production in *A. franciscana* and spore production in *A. parthenogenetica*, making this trait a barrier to the evolution of generalism in this system.” (new lines 18-23).
Note that this usage of the word ‘trade-off’, meaning that different trait values are optimal in different environments, is very frequent in evolutionary biology. See for instance Fry 1996 *Am. Nat.* (“The evolution of host specialization: Are trade-offs overrated?”) and Ravigné et al 2009 *Am. Nat.* (“Live where you thrive:…”), which make extensive surveys on respectively the empirical and theoretical literature on ecological specialization theory.

L146-150: This is not 100% informative. I would have prefer to have a clear explanation of when these tests were necessary and used; rather than have to guess when you actually used them.

We understand this comment, but have chosen to keep this paragraph as is. It would make the text much longer to repeat that we did likelihood ratio tests and Tukey HSD for every analysis. However, we have added the “The specific models for each analysis are described below” after “Unless stated otherwise, we built full models with the relevant experimental factors, and tested for the significance of effects using the likelihood ratio test”, which should help clarify this paragraph.

Figure 2: I wonder if you could add information on which host species are each passage of the 'Alternating host' line.

In fact, we started half our 'Alternating hosts' lines on *A. franciscana*, and half on *A. parthenogenetica*. Some lines therefore alternated *franciscana–parthenogenetica–franciscana–…*, while others alternated *parthenogenetica–franciscana–parthenogenetica-….*
For these analyses, we had to split our data into two: survival of *franciscana* hosts and survival of *parthenogenetica* hosts. In Fig. 2, the former are represented in the left subplot, and the later in the right subplot. Lines that were passaged only on *franciscana* appear only in the left subplot (triangles); lines that were passaged only on *parthenogenetica* appear only in the right subplot (circles). For alternating lines (crosses), the data is shown in the left plot when passaging was on *franciscana*; the same line's data is included in the right plot when passaging was on *parthenogenetica*.

Figure 4&5: to make it clear maybe, and stand alone figure; potentially add 'Evolved line of A.rigaudi' and 'evolved line of E. artemiae' at the top of figures. And to keep consistent with your text maybe 'Final assay host' at the bottom of your graph.

These are good suggestions, thank you! We have also changed the legend's label to 'Passaging treatment'.

On L.248-249: You write: 'No infection was detected for the line---Replicate 1, so it was excluded from further analyses. This particular replicate is however included in Table 3, and is not counted as 'lost', Did I read this table wrongly?

You read Table 3* correctly – this line was not lost. However, during the 2$^{nd}$ final assay, exposure to spores from this line didn't result in any infections in the experimental hosts. Therefore, it had to be excluded from analyses of virulence and spore production, though it was included in the analyses of fitness (with fitness set to 0).
*Now Table 1, see response to Reviewer 3.

Could you also discuss the fact that in the field, those parasites are not occurring on their own on those hosts, and competition between parasite is likely to influence each of the traits you have been studying here. Is there any data on whether one or the other parasite is more competitive? Or is there anything on other potential hosts or 'reservoirs' for these Microsporidians in their natural environments/regions where you collected them?

Co-infections are indeed very common in the field, something we have expanded upon in the new 'Methods > Hosts and parasites > Natural system' section (see also our response to the recommender's comments). Unfortunately, not much is known about them. There is circumstantial evidence that established infections can exclude a newcomer (Lievens et al. 2019), but this hasn't been properly studied. In general, we hypothesize that the parasites compete with each other for resources and have a competitive advantage in their 'matched' hosts. Although this could be expected to reinforce selection for specialism through niche partitioning,, so little is known about the effects of coinfection that it is difficult to make predictions. We have, however, added a sentence mentioning this additional complexity to the discussion of evolution in the field (new lines 401-403): "Of course, other factors than demography could affect the evolution of infectivity in the field, including trade-offs with other traits (Alizon and Michalakis 2015) and competition between parasites (Mideo 2009)".

*Thanks*

*Reviewed by Cindy Gidoin, 2019-06-17 10:24*

**Global appreciation**

*The study produces interesting results and I encourage to publish it in PCI Evolutionary Biology if the authors give the clarification asked below.*

**Main issues**

1) The main issue I see is the apparent lack of coherence between the question and the choice of the study system. In the introduction, the authors explain that there are potentially two factors that may prevent the evolution from a specialist parasite to a generalist one: 1) strong fitness trade-off between hosts' exploitation and 2) the low encounter rates with alternative hosts (in other word, the alternative host availability). To disentangle the effect of these two factors on the evolution of specialization, the authors propose to study two microsporidian parasites (Anostracospora rigaudi and Enterocytospora artemia) and their sympatric hosts (Artemia parthenogenetica and Artemia franciscana). In the Methods (Hosts and parasites > Natural System), the authors explain that the host A franciscana is highly prevalent, the two species coexist most of the year and can be found in the same microhabitats (l.87-89). Moreover, the author continue by explaining that the two studied parasites are the most prominent parasite infecting Artemia with a very high prevalence for both hosts (l.90-91). Consequently, it seems unlikely that the low encounter rates of the alternative hosts (A. parthenogenetica for E. artemia and A. franciscana for A. rigaudi) is a plausible explanation of the specialization of the two parasites for their "matched" hosts.

It is true that *A. rigaudi* and *E. artemiae* encounter both hosts regularly in the field, but host availability is not the only factor in play. Host quality (whether it is a source or a sink environment) is also important to the evolutionary outcome, as are the gene flow and details of density-dependence (Ravigne et al. 2009). *A. rigaudi* and *E. artemiae*'s partial specialization means that the quality of the two hosts is very different, and source-sink dynamics are likely to occur. Indeed, we know that they occur for *A. rigaudi*: in the field, it cannot persist if *A. parthenogenetica* is absent, indicating that the *A. franciscana* hosts are insufficient to maintain this parasite (Lievens et al. 2019, Int. J. Parasitol.). It is likely that the reverse is true for *E. artemiae.*
When a mismatched host is a sink, a small proportion of the spores in the parasite population has 'encountered' that host. This is why we manipulated host availability. We now motivate this more clearly in new lines 76-81.

Finally, the reader has to wait for the end of the discussion to learn that the parasite E. artemia is present year-round while its mismatched host A. parthenogenetica is only present from late spring to fall and so consequently, E. artemia mainly infect and evolve on A. franciscana in their natural system (l.363-365). We also learn too late that A. rigaudi is less specialist than E. artemia (l.394-395). These information must clearly appear in the Methods to justify the choice of this system to respond to the objective of this study.

We have indeed expanded on the seasonal patterns of the *Artemia* and parasites in the improved 'Methods > Hosts and parasites > Natural system' section (see also our response to the recommender's comments). We also mention there that *E. artemiae* is a poor infector of *A. parthenogenetica*.

2) In the introduction, the state-of-art focusing on the objective of this study is not enough develop in my point of view. In particular:

- I would like to know if other studies have try to disentangle the effect of host availability and strong trade-off on the evolution of specialization. And of course, what were the results ? You write (l.59) : "studies that take the natural context of parasite populations into account are rare". Is it what you do by exploring the potential effect of host availability and strong trade-off on specialization evolution in your study system ? Please, clarify this point. You can potentially explain here why your studied system is interesting to explore the question...(the question motivates the choice of the studied system). Or why you need to disentangle the effects of these two factors because they may have a role on specialization of the parasites you study (the studied system motivates the choice of the question).

As far as we are aware, only two other experimental evolution studies of host specialization have taken the natural context into account. Jaenike & Dombeck (1998) interpreted the stubborn generalism of their nematodes to the extremely fluctuating availabilities of their drosophila hosts (i.e. the ancestral population is supposed to be extremely selected for generalism). In contrast, Fellous et al. (2014) showed that the broad host range and suboptimal performance of mites on certain host plants could be attributed to pleiotropy, and was likely not caused by host availability selecting for intermediate generalists.
We have expanded this point in the text (new lines 59-62): "However, very few experimental evolution studies take the natural context of parasite populations into account. Doing so can help disentangle the consequences of genetic constraints from the effects of host availability on the response to selection (see Jaenike and Dombeck 1998, Fellous et al. 2014)."

- l.56-57, you write that fluctuations in environmental conditions (which kind?, be more precise please) may influence evolution toward more generalist phenotypes but you say nothing about the potential effect of fluctuations on the evolution of specialization in your system, neither in introduction nor in discussion. Please explain why you consider that fluctuations have a minor influence on your system compared to host availability or strong trade-off. It could be also interesting to discuss about the relative contribution of fluctuations and strong trade-off on the evolution of generalist-specialist. l. 65-66, you write "only strong trade-offs may prevent the evolution of a generalist parasite on the long term". I guess you should add "in stable environmental conditions".

This is a misunderstanding – the 'environment' here is the host, so 'fluctuating environmental conditions' refers to fluctuating host availability. We have corrected this (new line 57) to "selection for generalism when host availabilities fluctuate".

- Is the study of several components of fitness original in the context of specialist-generalist parasite evolution ? Or it was already done ? L.60-61, you write " few studies look for the traits underlying fitness trade-offs". You say nothing more about these studies. Do they also study evolution of parasitism ? Do they show that it is important to consider fitness components instead of "direct fitness" ? Please, develop a bit.

We now say a bit more about these studies, which are all experimental evolution studies of host specialization (new lines 62-67): "In addition, few studies look for the traits underlying fitness trade-offs. Parasite fitness is a composite of successful infection, host exploitation, and transmission. Some host specialization studies have shown that these traits can respond differently to selection on novel hosts (Magalhães et al. 2009, Bedhomme et al. 2012, Messina and Durham 2015), but their causal

effects on parasite evolution have been largely unexplored (Hall et al. 2017).".
More precisely, Magalhães et al. showed that only 2 of 4 mite traits responded to selection on novel hosts, and that in different ways, Messina & Durham showed that seed beetle traits may evolve differently across replicate lines, and Bedhomme et al. showed that viral traits evolved differently across host species.

**Important changes to help the understanding of the readers**

*In overall, I have noticed some confusing and potentially contradictory elements in the Table 1 to 3. I have also found the Figure 2 to 5 difficult to understand and with important information missing.*

1) The Table 1, 2 and 3 are not clear for me. If I well understand, there are two events that may lead to lose a parasitic line : 1) parasite population collapses (they potentially can be revived using P6 backup) and 2) host population collapses (they potentially can be revived by adding 5 hosts at the beginning of the incubation period). Only 4 lines have not been saved by these two processes (Table 1).
In Table 1, may you add the reason of the lost ? I guess the reason for the lost of the two first lines is that host populations collapse (because these two lines are in Table 2) and the reason for the two last lines is that parasite populations collapse (because these lines are not in the Table 2). BUT, the line A. rigaudi × A. f. host – Repl. 2 is present in the Table 1, 2, 3. In the Table 1, I understand that this line is lost. In the Table 2, I understand that the loss is because the host population collapses. And in Table 3, I learn that it is because parasite population collapses ("revived from P6 backup"). Is it possible that for this line, the two populations (parasite & host) collapsed ? Anyway, you say that the line is lost in Table 1 and that it is revived in Table 3, so something is unclear.

Thank you for pointing out how difficult these tables were to parse. We have now condensed all the information into a single table (the new Table 1). The new table is more complex, but it provides a clearer overview and avoids the confusion of the previous, partially redundant, tables.
In fact, we only lost parasite lines due to a collapse of the parasite population; this should be clear in the new table. We have also clarified this in the text (new lines 222, 226-227).

2) Figure 2-5: the significant differences of Treatment, Passage Number or Assay Host have to be presented on the main Figures and not only in the Table 1 or 2 of the supplementary material. With an adapted y-scale (in Figure 3 from 10-3 to 10-6 for example instead of from 0 to 10-6), you should have more place to add letters for the significant effect of treatments. Please, see below potential solutions.
Fig. 4, It looks that some significant tests are illustrated and some other not (for no apparent reason). According to Supplementary Table 2, Assay host is significant for the infectivity of E. artemiae, virulence of both species and spore production of both species. But you illustrate this significant difference only for spore production. Consequently, we don't know which is right, the figure or the table ?
You write l.307-309 "E. artemiae's fitness did evolve in some treatments. E. artemiae lines whose passaging history included A. parthenogenetica had a higher fitness on this host, while their fitness in A. franciscana was not detectably changed (compare cross & circle to triangle in Fig. 5)." But it is not clearly shown in the figure. We need to see the test significativity (with letters for example that are the same when the difference is not significant, and different otherwise). For example, see the illustration below.

We had chosen not to represent the type III sum of squares test results (the significances 'below' a significant interaction effect, see Supp. Table 2 legend) in Fig. 4 and Fig. 5, because we thought the

figures would be too cluttered, but your suggestions are very useful. We now represent all the significant results in the figures.
Regarding the significance of *Assay host* for virulence and its representation in Fig. 4, please see our response to Reviewer 1's 2nd-to-last comment.

**Minor issues**

1) In overall, I would appreciate more details about the study system that the authors seem to know very well. About the two parasites: Is E. artemia an invasive species arriving with its invasive "matched" host A. franciscana ? About the interaction host-parasite: What are the consequences of the parasitism for the hosts (because there is no control, we cannot distinguish between parasite-induced and background host mortality) ? Does the parasitism occur in a specific period of host life cycle (phenology of hostparasite interaction) ? Is there competition between the two parasites for the exploitation of the two hosts ? Does a co-infection lead to a higher host mortality than an infection by only one parasite ?

We have addressed these and other points in the improved 'Methods > Hosts and parasites > Natural system' section (see also our response to the recommender's comments). Briefly:
- We do not know whether *E. artemiae* is co-invasive with *A. franciscana* or native to the Old World; both are possible given the available data (Rode et al. 2013, Int. J. Parasitol.).
- The parasites cause both reproductive and survival virulence; we have added this to the improved Methods. It's true that there were no controls for background mortality during the serial passages, but we were most interested in the parasite-induced mortality during the final assays. In those, we did have unexposed hosts to control for background mortality.
- Parasitism does not occur in a particular life cycle step. To the best of our knowledge, it is possible as soon as juvenile *Artemia* develop a digestive tract.
- We know very little about coinfections, either about their competitive outcomes or their virulence. We hypothesize that there is competition but have only circumstantial evidence for it (see also our response to Reviewer 2).

2) If the results are not presenting in a Figure or a Table in the main text, they should not be explain in the main text. For example, l.212-222 refer to results in the Supp. Table 1 and not clearly visible in the Figure 1 presented in the main text. Why not having a Supplementary Results document where you explain these results and refer to them in the main text if it is useful (not sure it is useful for the example)?

We don't quite understand this comment. The supplementary tables just provide a nicely organized summary of the statistical tests and significance; it is quite standard to refer to them when we present the results.

3) l.66. Suppress the "a" : "such key traits occur is a largely unexplored".

Here the 'a' referred to 'a largely unexplored question'. This confusing phrase has disappeared in the rewrite of this paragraph.

4) l.69 you write "naturally occurring parasites", should be "naturally co-occurring parasites", right ?

Indeed this line was unclear, we have revised it to "whether trade-offs in host use limit the evolution of generalism in a natural host-parasite community".

5) l.91 “with infections of either reaching prevalences of up to 100% in both host species”. Does it mean that the infection of each parasite may reach 100% in both host species ? Or only in their “matched” host species ? Please, clarify. Here, you could explain that A. rigaudi is less specialized than E. artemia and give an idea of the host-parasite phenology.

We have recorded prevalences of 100% in all host-parasite combinations except in the mismatched *A. parthenogenetica* and *E. artemiae*, but the maximum recorded prevalence in that combination still exceeds 80%. We have clarified this, and provided more details about the degree of specialization (see also our response to the recommender’s comments).

6) l.101-105, you use the term “stock” for both parasite stocks and host stocks. It is unclear. Please, every time you use “stock”, precise if you mean parasite or host.

We have improved this as suggested.

7) l.103, you use lab-bred hosts for the experiment to be sure that only one parasite infest them. These hosts could have evolve a higher tolerance to lab conditions and a potentially higher vulnerability to parasites as they are parasite free. How this potential evolution of lab-host could influence your results ? You don’t discuss about this point but it could be interesting to discuss it.

This is possible, though the lab-bred hosts typically have only been in the lab for several generations. However, we don’t expect this to influence our conclusions, because we are interested in the differences in traits between evolutionary treatments, not in the exact traits themselves.

8) l.132, replace (see Results) by (Table 3).

Changed to (see Results and Table 3*). This way the reader can find the information in the precise table, but also knows that the information is explained in the Results section.
*Now Table 1, see response to previous comment.

9) l.161, replace (see Results) by (Table 1).

Changed to (see Results and Table 1), as for the previous comment.

10) l.283 & 391. Clarify the term “semi-specialized”. If it means that only a part of the traits related to host exploitation is involved in the specialization, please explain it clearly. If the term is previously used for describing this phenomenon, please cite the reference. If the term is chosen by you to describe this original result, please make it clear.

Thank you for pointing out this inconsistency – in previous papers (and elsewhere in this manuscript), we have used the term ‘partially specialized’. We have corrected this throughout, and given an explanation and citation in the new ‘Methods > Hosts and parasites > Natural system’ section.

11) l.285, “infectivity readily evolved towards generalism”. Right but for E. artemiae only. Be more precise here.

We have corrected this (see also our response to Reviewer 1).

12) l.293-294, “This result is consistent […] with the general host-conservative behavior of microsporidians”. Please, develop this idea or remove it. What is the general conservative behavior of microsporidians ?

This refers to a phylogenetic study of microsporidians and their host, which found that they are tightly linked. We have removed this idea and reference.