This manuscript by Cavigliasso, Colinet, Poirié and co-workers, tests the relationship between the evolution of parasitic success and venom composition in L. boulardi and several Drosophila hosts. It combines a wide range of techniques to connect the evolutionary process to its underlying molecular genetics. This is a thorough and courageous approach to a difficult problem of ultimately understanding the mechanisms that “make a parasite successful”. They also explore the specificity versus generality of the evolved responses touching upon another central question in evolutionary biology. All three reviewers are generally positive about the manuscript, and concur on the originality and importance of the approach and of the question posed. However, they also suggest important clarifications, additions and extensions that would benefit the manuscript. A great deal of the proposed changes are mostly directed to improve the reading, the clarify of its data and message, and to deepen some aspects of its conclusions and discussion.

Discussion/interpretation
Several requests and recommendations have been put forward by reviewers that I summarize as:
1) Reviewer 2 expresses concern with the interpretation of mel SNasr venom composition evolution as it could derive from an experimental design artifact. Please address this concern argumentatively or experimentally;
Reply: we responded to the reviewer and made some changes in the text.

2) The potential paradox between survival at 100% (sim and mel strains) and the evolution of venom composition (reviewer #2) must be addressed;
Reply: we responded to the reviewer and made some changes in the text.

3) The relationship between the success of experimental evolution and the phylogenetic distance of the host used relative to the parasitoid strain;
Response: D. melanogaster and D. simulans are phylogenetically closer than they are to D. yakuba, a tropical species, and their immune response may therefore be more similar (doi:10.7717/peerj.226). L. boulardi is known as a specialist of the first two species, always succeeding to parasitize them to some extent (doi:10.1016/S0065-308X(09)70006-5, doi:10.1016/S0065-308X(09)70011-9). Conversely, a single population collected in Congo among the many tested (used to produce the ISy lineage) happened to be successful on some strains of D. yakuba (doi:10.1111/j.1600-0587.1999.tb00504.x). The higher success of the experimental evolution on D. melanogaster and D. simulans compared to D. yakuba was thus expected. Although interesting, we did not include the phylogenetic distance in our manuscript because it is already long and we were afraid that this could disrupt the flow.

4) Reviewer #3 has an interesting suggestion to correlate venom evolution with the two distinct strategies for parasitic success (avoidance and evasion), as well as some mention/discussion of parthenogenesis that you may consider;
Reply: we responded to the reviewer and made some changes in the text.
5) Another potential relationship to explore and discuss pertains to the success of experimental evolution (replicate extinction) vis-a-vis the phylogenetic distance of the host used relative to the parasitoid strain.
Reply: see response to comment 3) above.

Analyses and Format:
All reviewers have remarks concerning the presentation of the data that should be revised, mostly to ease the reader’s job.
1) Address the issue of infection status classification (reviewer 2#)
Reply: done
2) Reviewer #1 concerns and suggestions (shared by reviewer #2 and myself) regarding figures 1 and 3 and tables 1 and 2. In addition, please review some of the legends (tables and figures) to ensure all necessary elements are provided to fully understand the information they contain. For example, what is “estimate” on figures S2 and S3, how what is calculated and what does it mean?
Reply: done
3) Justify and explain the use of MANOVA and address the apparent lack of nesting raised by reviewer #1 and correct the minor points on statistics presentation pointed out by reviewer #2.
Reply: done
4) I am not sure about this but I wonder if figure S1 should not be part of the main text. I find it really helpful…
Reply: it was a good idea so we followed your suggestion.
5) Please consider the remaining small points included in all three reviews.
Reply: done
A final recommendation would be to revise the syntax throughout the manuscript. It need not be Shakespeare but some revision would ease the read. For example, the second term of the first two sentences of the introduction need revision: “and strong selection…success”; “but also host species”.

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 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.
-Author's 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 XXX recommenders.”
Reviews
Reviewed by anonymous reviewer, 2020-11-18 17:05

Review of the paper: Parasitic success and venom composition evolve upon specialization of parasitoid wasps to different host species

In their manuscript Cavigliasso et al performed an experimental evolutionary experiment by crossing two parasitoid lines with different host range and subsequent rearing of F2 descendants on different hosts. Parasitic success and venom composition were tested at different time points after rearing on different hosts. More specifically parasitic success was assessed as either the capacity to inhibit encapsulation of the capacity to evade capsules. The evolution of venom composition was assessed using 1-d gels and comparison of band intensities as well as statistical analysis of the results. Specific focus was on members of RhoGAPs and of the serpin family.

General comments: In general, I think this is an elegant study of the evolutionary dynamics of a host-parasitoid interaction that is expected to be under strong selection. I am wondering though whether it would be possible to correlate the set of proteins under selection with the initial observation of two different strategies (avoidance of encapsulation in the first place versus evasion from capsules). Are there any proteins that evolved in combinations that affected primarily one of these strategies? Is there any indication whether the two prime candidates (RhoGAPs and serpins) are expected to differentially affect encapsulation?

Reply: Thank you for the comment. The first question about the possibility of correlating the set of proteins being selected with the two strategies of “suppressing the encapsulation” and “escaping from the capsule” is very relevant. Unfortunately, we did not have enough statistical power to do so. It would have been possible if there were more replicas than protein bands, which was not the case in our experiment. In addition, it is possible, or even likely, that this two final events are related not only to the effect of the parasitoid itself (notably venom) but to the interaction between the host (immune response) and the parasitoid, which would end up being very complex to analyze.

For RhoGAPs and serpins, we essentially have information on the role of LbGAP and LbSPN. The function of the other RhoGAPs is still unknown as they are mutated to their catalytic site, and that of LbSPNm as well. We have already shown that the amount of LbGAP inside host lamellocytes (cells involved in the encapsulation process) of D. melanogaster R correlates with the level of morphological changes of lamellocytes. Once distorted, lamellocytes may no longer be able to encapsulate the eggs. We also demonstrated that LbSPN is involved in the inhibition of the activation of the phenoloxidase cascade (PO) of D. yakuba. The PO cascade is involved in melanization and the release of cytotoxic radicals supposed to kill the parasitoid. If we extrapolate, we could say that LbGAP would rather act on the encapsulation process itself (first strategy) whereas LbSPN would target the melanization process (second strategy). However, the encapsulation process is quite complicated, and many involved proteins and their roles are unknown, making it difficult to predict which protein would be involved in which strategy, or possibly in both.

We added information of this in lines 600-606 and 615-620.

I also think one might include mention of parthenogenetic wasps. One explanation that has been put forward for a parthenogenetic mode of reproduction in parasitoid wasps (and possible other parasites) is that it allows the fixation of optimal combinations of virulence factors and prevents
in an evolutionary landscape the drop into a suboptimal valley due to the combination of incompatibility or less compatibility between virulence factors. Even an asexual mode of reproduction will of course lose its effects in the long term in an ever-changing co-evolutionary race (which may explain the occasional switch to sexual reproduction in some species). My feeling is that some of the F2-host combinations the authors studied including some that were unstable ended up in such evolutionary troughs.

Reply: The venom is produced only in diploid females that have a venom apparatus but not in haploid males produced by parthenogenesis. The influence of parthenogenesis would be more relevant for the virulence factors produced by the developing embryo for instance (notably by teratocytes) in some parasitoid species. In Hymenoptera, passing through a haploid individual could indeed help to fix some alleles, possibly allele combinations, but this fixation would rather occur by drift than by selection (no fitness associated with venom in males). The effect of the father-provided allele combination would participate in the female fitness linked to venom. This comment is nevertheless interesting, and some F2 combinations of venom factors might indeed be unstable. Because of these reasons and the length of our manuscript, we did not include this aspect.

Specific comments: Line 40: in* not on Line 41: subject agreement, is* not are Line 57: here was* Line 67: introduce LbGAP? why did you look into GAP proteins in relation to venom/success? Line 63, 67: delete “indeed,” becoming redundant and distracting Line 88: had* Line 102: Originated* Line 130: never allowed to oviposit (parasitize) instead of parasite? Line 147: fix tense Line 168: global analysis: perhaps specify Line 184: redundant citations Line 190: tense Line 212: In* one host, in* another. Line 410: what previous mass spec data? Line 417: The protein GMC oxidoreductase would indeed be relevant for this analysis, but we do not yet have a good specific antibody for it. It was therefore impossible to confirm/disprove this result. Nor do we know the role of this protein in virulence. So we focused on the proteins for which we had available antibodies. It is true that from the results of this study, the role of the GMC oxidoreductase in parasitic success should be studied. From line 77, we have simplified the last paragraph of the introduction based on what we did in the discussion part.

Line 450-601: discussion is perhaps too redundant with results. Perhaps trim down.

Reply: we tried to reduce the discussion

Line 503, 522,548: Indeed used quite often. Delete “indeed”. Means nothing and is confusing.

Reply: Done

Line 517: more information about they there is a cost for virulence factors in one fly over another would be very interesting, see also general points.

Reply: we added more information on the cost hypothesis (lines 501-506)
Line 544-549: Is there more information about the kind of immune response mounted by drosophila and how the prophenoloxidase cascade may differ in different species and how this might be affect by geography?

Reply: In this paper (https://doi.org/10.1186/s12915-015-0193-6), the authors describe the presence of three PPO genes in the three species we tested (D. melanogaster, D. simulans and D. yakuba) while many other species only have two. However, the gene sequences differ from one species to the other, suggesting that the function of the protein could somewhat differ.

We added some information in the main text (lines 546-548)

Furthermore, their level of basal expression and after parasitism may also differ. To our knowledge, there is no study on intraspecific variation of PPO in terms of sequence and function. In some lab experiments, we observed no melanization of the collected hemolymph in at least one third of D. melanogaster larvae, a weak melanization in another third and a strong one in the last third. This suggests occurrence of intraspecific variation in their phenoloxidase hemolymph content (knowing that these components are mainly stored in circulating specific cells called crystal cells).

Although there is little data on these aspects, it has been shown that the virulence of ISy on D. yakuba is associated with an inhibition of the PO cascade activation in this species, which was not observed after parasitism with ISm (doi:10.1016/S0065-308X(09)70006-5).

Line 578: information about Rho and GAPs and SPN and their involvement and function in parasitization would be nice. As would information about why these proteins are favourable for acting upon in an evolutionary manner.

Reply: We added this information in lines 546-548 and 600-606 and 615-620.

Line 585: lbSPN misspelled

Reply: Changed

Reviewed by alexandre leitão, 2020-11-29 10:57

This study tackles a fascinating question in evolutionary biology, what makes a parasite successful? The biological system studied, Drosophila species as hosts for the parasitoid wasp L.boulardi, is well established and its ecological relevance has been demonstrated. Interestingly, the authors use a similar approach used to demonstrate how Drosophila hosts evolve resistance to parasitoid wasps. This can make future studies very complementary and the further dissection of the system will be aided by recent sequencing of the L.boulardi genome. The article is well written and clear. The predictions are well established and carefully tested.

Essential revisions:

1) The classification of the infection status is confusing. Parasitic success is partitioned in two phenotypes, one where the parasitoid larva is found with no signs of capsule and another one where the wasp larva is found with signs of capsule formation. These phenotypes should be complementary. But, in certain cases, the authors report 100% of both phenotypes (for example, figure 1 B at generation F3). How is this possible? It may be my misunderstanding but really struggle to make sense of those numbers and I think any reader would have the same problem.
Reply: The misunderstanding comes from the fact that the escape ability was measured among the hosts who made a capsule, not among all host larvae. Therefore, the proportion of parasitic success is not equal to the sum of the proportion of inhibition of capsule formation and the escape ability. For this reason, in Figure 2B, the parasitoid's ability to inhibit encapsulation is close to 100% for *D. melanogaster* SNasr. We observed a capsule in one or two larvae from which the parasitoid has escaped. Therefore, if there is a parasitoid larva escaped from a capsule in a single Drosophila larva, the proportion of the escape capacity will be 1. This may also explain the very large error bar for this setting in some hosts. We have clarified how we calculated each parameter in 292-297 lines and changed the presentation of Figure 2A and its corresponding caption.

2) It is somehow surprising to see that the venom composition is selected when wasps are maintained in a very susceptible host like *D. melanogaster* SNasr. The selective pressure should very reduce to change venom composition. Certainly, the authors put forward an interesting hypothesis to explain this observation, that a cost may be associated with certain venom proteins. However, this change may be an artefact of the crossing scheme used in this study. It is known that crosses with certain strains of *L. boulardi* results in variable levels of female fertility, in F0 and F1 (Allemand, R. et al., 2002 Ann Soc Entomol, 38(4), pp. 319–332.). If this phenomena occurs asymmetrically between the two cross directions, then at F2 the representation of each genotype can already be biased, given that we are dealing with a haplodiploid system. To exclude this hypothesis, the authors should test female fertility (measuring the sex ratio of the offspring) in the F1 of both cross directions.

Reply: In a previous experiment in which we crossed the same lines in one direction, we observed a venom evolution in response to a susceptible line of *D. melanogaster* (same SNasr strain as here). However, what you mentioned is an interesting argument and it would be worth measuring female F1 fertility from the two cross directions in a future study. In another experiment, we crossed a female of one line with a male of the other line in both directions. Crosses between female ISm and male ISy yielded more offspring (or at least more pairs yielded offspring) than opposite cross. This suggests a possible bias in F2, but crosses were only individual crosses without much repetition.

Whether or not it exists, such a bias should decrease after several generations. We observed that the composition of the venom also evolved between F7 and F11 in response to *D. melanogaster* SNasr (p < 0.001, Table S4 ) suggesting that even if there was a bias due to differential fertility at the beginning of experimental evolution, this does not explain all the observed changes. In addition, the bias would be similar for both susceptible strains and therefore, without the evolution of the venom, we would have observed a similar venom composition in response to these hosts. Instead, we observed a differential evolution of the venom (Figure 4F) between these two hosts suggesting a real evolution of the venom. For these reasons we did not include this hypothesis in our manuscript.

Minor points:
1) When possible, report actual p values, do not report things like p>0.05 for a single p value.
Reply: Done
2) When possible, report the statistical comparisons being made. For example: line 303 “This increase seemed to result solely from the increased capacity to escape from the capsule (Figure 1B, Table S2, GLMM, p = 0.001”. What is the comparison reported in here?
Reply: Changes have been made

3) I would suggest changing the nomenclature of generation to reflect the start of selection. F3, is one generation of selection which can be easily be mixed and interpreted as having 3 generations of selection.
Reply: We kept our nomenclature to be consistent with the results of our previous paper. We have followed an advice from the recommender by adding Figure S1 (about the experimental design) to the main part. We hope it will help understanding the numbering of the generations and reducing the confusion.

4) The legend for figure 1 C is incorrect. Line 845 “The host listed to the right of each bar plot is the “selection host”, those listed down below are the “tested hosts”, used for parasitism assays.” It should be the other way around.
Reply: You are right, we made a mistake in the legend, thank you for noticing. We have modified the figure to help the reading according to a comment from another reviewer and therefore we have changed the legend as well.

5) The discussion would benefit with the inclusion of a similar artificial selection study done with another parasitoid wasp species (Kraaijeveld, A. R. et al. (2010), Evolution, 55(9), pp. 1815–1821.)
Reply: You are right so we added it in lines 510-512

Reviewed by Simon Fellous, 2020-12-03 11:41
A. Description
This rich manuscript describes the results of an experimental evolution project on the ability of parasitoid to infect different strains of hosts. Analysis is largely rooted in venom composition evolution, in line with previous work by the same research group. The methods are sound and the results prolific. The figures are sometimes complex and hard to grasp, but the writing is very clear.

B. Overall assessment regarding impact and suitability for PCI Evol Biol
This is work is at the intercept between evolutionary biology and mechanistic approaches. Indeed, the authors made a great deal of efforts at identifying venom molecules that underlay parasitoid success. This enabled investigating whether venom component had general or specialized effects relative to host strains. The discussion mostly compares current results to those of previous studies with the same species. Implications for general evolutionary theory are therefore scarce in the discussion - a little too scarce in my opinion as an evolutionary biologist. The manuscript may benefit from a more general rooting in the literature, beyond the specific cases of the species (sometimes even strains) of insects used. However, the obvious quality of the work justifies its recommendation once a couple of comments (listed below) have been addressed.
C. Interpretations and discussion

There are two elements about which I would welcome a deeper interpretation. 1. About selection on host strains (namely Dmel S and Dsim) that both ancestral parasitoid could infect (and on which the cross had 100% success from the first generation): there is nonetheless evidence for directional evolution of venom composition. This may seem a paradox (isn’t venom here to overcome host defenses?), that the authors solve by evoking unspecified “costs” (e.g. line 489-490). If parasitoids have 100% infection success form the first generation, that means selection occurred at the adult stage rather than the egg/larval stages. Moreover, the fact that there is differential evolution of the venoms in different susceptible hosts suggests the costs of production of venoms components were host specific. I think these elements should be clarified and argued.

Reply: We developed and clarified our arguments in lines 501-506. In addition to the cost, another argument would simply be the production of new combinations of venom factors from F2. Indeed, although we observed 100% parasitoid success in F2, a small difference in fitness may be enough to end up selecting new factor combinations.

2. L472-479: interesting bit of discussion on survival (and later extinction) of the parasitoids on a host strain the two ancestors could not parasitize. This striking line of reasoning suggests ability to infect relied on codominance rather than the combination of alleles at different loci. This hypothesis may be further explored with the analysis of venom composition at F3 in the 1907 line (before the extinction of the parasitoid lines). Do you have the data? This is particularly important as the final sentence of the MS is about this phenomenon that is otherwise little discussed.

Reply: Unfortunately, we do not have the venom data available for F3 from 1907. Although some parasitoids have been successful, we did not have enough individuals available for the next generation, the parasitic tests, and the venom analysis. We found the result on success of this line really interesting and the experiment will be worth reproducing in a next future. In the meantime, since we do not have venom data on successful individuals, we prefer not to further discuss on the possible protein combinations involved.

The authors selected the parasitoids on standing genetic variation. This is different from de novo mutation as the phenotypic space and the nature of the trade-offs that can be revealed may differ. These are concepts the MS would probably benefit from citing and making clear. It would help comparing this study to the numerous others on the experimental evolution of parasite specialization (many of which using crosses and standing genetic variation).

Reply: we have specified that it was on standing variation (lines 464-466). However, we did not develop this idea because we were afraid it could disrupt the flow and our manuscript is already quite long.

D. Presentation of the results:

- Fig IC: if technically correct, the data could be presented in reader-friendlier way. I’d recommend having a table where host of origin and assayed host are on each axis, but not repeated as is on X axis. The three generations may be put next to each other, in the same table cell, so one could visualize the evolution of the phenotype in time.
Specialization/local adaptation is often studied in an axis/off-axis framework, even if this is not studied as such statistically, using the same graphical representation would help. Also, please put the legend for the axes on the figure, not only in the legend. (this later comment is valid for several figures)

- Fig 3: the figure is gorgeous but frankly it is challenging to recognize what is described in the results. Treatments overlap too much for that.
- Table 1: is it necessary to keep all bands for which no significant change is reported? (i.e. blank lines). Maybe can you save space here.
- Table 2: an optional suggestion. Maybe have a colon for each host strain, just as in Table 1. Readers could see at a glance how bands varied together, or not on each host.

Reply: We have followed your advices and changed the Tables and Figures. For Figure 3 (Figure 4 now), we moved the complicated and complete figure in supplementary and replaced it by a simpler figure in the main text.

E. Stats and clarifications:
- Please clarify why a MANOVA is an appropriate framework to study the evolution of venom composition (i.e. multiple phenotypes). In particular, reader should know how this may reveal qualitative changes in venom composition (one component increases, the other decreases), when MANOVA is often used to study the correlated variation of several responses. Interaction terms can be used - within and between subject interactions – but this is not very clear in the manuscript. Maybe a response to the referee would suffice.

Reply: To be more accurate, we did not use MANOVAs but PERMANOVAs (permutational MANOVAs; https://doi.org/10.1002/9781118445112.stat07841). These non-parametric versions of MANOVAs do not analyze the correlation among the variables, but only the multivariate distance among groups. To take your example up again, if you consider the case of only two variables (x and y) and two groups, which differ for the two variables, but opposite in the direction, the difference for each variable contributes to the overall distance among groups, regardless the direction of the difference. The main difference with MANOVAs is that the correlations among variables within each groups are not analyzed. We clarified these analyses in the manuscript, lines 190-206.

- Unless I missed it, I see no form of nesting of the populations (i.e. replicates) within host strain (or use of a random effect) in the MANOVAS and LDAs. If I am not mistaken, this is necessary to test among host variation taking into account inter-replicate variation, instead of inter-sample variation. How is this taken into account?

Reply: For PERMANOVAs, we included the population as a fixed factor (a population = a replicate on a specific host). I agree, it would be better to have it as a random effect, but the random effects are not implemented for PERMANOVAs. However, including it as a fixed factor allows to account for this source of variability.

We used LDA not to test the difference among groups (this was done by PERMANOVAs), but to characterize the differences detected by PERMANOVAs. In this analysis, we ran the discriminant analysis not on a PCA, as often done, but on a “within class analysis (wca)” to centered replicates (all replicates have the same mean for each variable). So, the variation we observed mainly comes from variation resulting from the generation and the host, not from replicates. We tried to better explain this in lines 190-225.
I should also state that I don’t have the statistical knowledge to evaluate each of the numerous statistical methods used, some are fairly complex.

F. minor comments:

- Strain availability. You refer to Gif strain numbers. Are these strains available to the community, deposited in stock centers?
  
  Reply: Regarding the availability of parasitoid and *Drosophila* strains/species used in the experimental evolution, they are kept in the laboratory and can be sent on request. For parasitoid strains, including mainly *Drosophila* parasitoids, an initiative was recently taken that brought together different laboratories to produce a database called DROP that aggregates descriptions of strains and species, the location of available strains, and transcriptomic and genomic information. An article has been submitted entitled "DOP: Molecular voucher database for identification of *Drosophila* parasitoids." Information about our parasitoid strains and their availability is listed here. The preprint 10.1101/2021.02.09.430471 has been posted on bioRxiv: https://biorxiv.org/cgi/content/short/2021.02.09.430471v1

- L130: I guess you mean “parasitize”, or “oviposit”.
  
  Reply: Yes, we changed it by “oviposit”

- L503: please be more specific. This trade-off only occurred when comparing yakuba 307 and melanogaster R strains. A reference to the figure would help too.
  
  Reply: You are right and the sentence was modified accordingly (line 523-525)