

Peer Review File

Manuscript Title: Drosophila are hosts to the first known parasitoid wasp of adult flies

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 [no comments for authors]

Referee #2 (Remarks to the Author):

In this study, the authors report the first documented case of parasitoidism of an adult dipteran by a parasitoid wasp, providing for this comprehensive evidence both from rearing carried out in the field and under laboratory conditions. Moreover, the geographic distribution of this wasp species (eastern USA), which belongs to the family Braconidae, could be established based on specimens collected in the field and on a screening of *Drosophila* DNA sequences belonging to the barcoding locus (a fragment of the mt COI gene). The authors provide in most cases (see below) high quality digital photographs and videos showing the parasitoid behavior (host-searching, ovipositing, emergence) of this braconid wasp. This wasp represents a new species of the genus *Syntretus*, which belongs to the subfamily Euphorinae, a group of endoparasitoid wasps that is characterized by attacking adult hosts mainly of the order Coleoptera, but also Hymenoptera. This wasp species was described in this study based on specimens collected along its recorded geographic distribution.

I believe that this piece of work represents an outstanding contribution not only to the fields of insect biodiversity and systematics, but also of ecology and evolution. This extraordinary, previously unknown biology for a parasitoid wasp undoubtedly will increase the number of further rearing work of parasitoids not only in the USA but globally. Moreover, the rearing experiments, which used *D. melanogaster* as host model, were described in detail, which will help other scientist who want to replicate this kind of rearing experiments. This host-parasitoid association will therefore serve for future research in different fields of biology.

Overall, the abstract and main body of the manuscript is well-structured and concise, and the phylogenetic analyses were appropriate. Moreover, the rearing experiments that were carried out in the laboratory were well explained and its derived results were clear and easy to follow. Most of the figures also were clear and aesthetic.

The discussion section was concise and mentioned the main findings of this study. These include 1) the opportunity for future research after this discovery, since *D. melanogaster* is one of the most employed animal models in biology; 2) the implications that this host-parasitoid relationship has for the evolution of the subfamily Euphorinae and of parasitoidism of adult hosts within Hymenoptera.

Based on my above comments, I strongly suggest this manuscript for its publication in Nature after the following comments are fully addressed:

1. The figures of the new species (Figure 4; Extended Data Figure 5) are of bad quality and do not correspond to the quality of this work. I strongly suggest that the authors take high quality digital pictures of the different structures using a better camera and stereomicroscope equipment as well as an appropriate photo-compositing software.
2. I cannot see the reason for the authors to carry out a phylogenetic analysis only based on the COI marker for the different parasitoid hymenopterans that are known to attack dipterans. First, using the COI marker alone to investigate these deep relationships is clearly inappropriate due to its fast-evolving nature and because using a single locus would reveal the gene and not the species evolutionary history. Second, the taxon sampling for this phylogenetic analysis is not correct simply because it does not have all the members of Parasitica. The authors do not have to carry out a phylogenetic analysis to convince the readers that the different taxa that parasitize dipterans are not related, and that the new species of *Syntretus* that attacks adult dipterans is the only known braconid and hymenopteran with this host preference. I suggest to delete this analysis and simply emphasize this in the text.
3. Was only the COI marker available in Genbank for a high number of euphorines and species of *Syntretus*? There are three or four legacy nuclear Sanger markers that could have been sequenced for different euphorine taxa (e.g, 28S, wingless, EF-1, Opsin genes). I strongly recommend the authors to search in GenBank for nuclear markers and adding them to the phylogenetic analysis that they carried out. If there is a considerable mismatch between the COI and the nuclear marker that they could find, they could do separate analyses of with these genes and then briefly mention whether they gave similar topologies. I suggest the authors to look on the following paper for additional markers:

Stigenberg, J., Boring, A. C., Ronquist, F. 2015. Phylogeny of the parasitic wasp subfamily Euphorinae (Braconidae) and evolution of its host preference. *Systematic Entomology* 40: 570-591.

Referee #3 (Remarks to the Author):

The authors report a parasitoid wasp that uses adult *Drosophila* flies as hosts. Parasitism of adult insects is not very common and this is the first record of a parasitoid of adult flies. It is interesting because *Drosophila* are extremely commonly studied insects so it is surprising to find a new parasitoid. It is also useful to evolutionary ecologists because *Drosophila* is already a model for studying host-parasitoid interactions (larval parasitoids). The authors did a nice job collecting evidence that the association is real, and working out key aspects of its natural history. They also did a nice job taking advantage of available genomic information to search for evidence of the parasitoid in *D. melanogaster* worldwide. The manuscript is simple and clearly written and the figures clear, and the supplementary videos are fun.

Some information and discussion was missing related to the likely host range of the wasp species. It was found first in *D. affinis* and with further sampling was found at a low rate (1% in males). This would be a very low rate for a parasitoid that didn't have other primary hosts. This makes me wonder if there are other main *Drosophila* hosts around the Eastern US. The authors searched in available genomic material worldwide for evidence of the wasp in *D. melanogaster*. It was found in samples for the original study area. Notably it was not found in samples from Africa which is the origin of *melanogaster* and the

success rate in reared melanogaster was low. Thus, it seems like melanogaster also would be an unlikely regular host of the wasp. The author should discuss this, as well as provide some background information about *Drosophila* diversity and distribution, and also some background about parasitoid host range.

Smaller issues

In the summary I suggest putting the lines “Despite a deep history of research attention and stunning biodiversity, no wasp that attacks and develops inside the adult stage of a fly host has ever been described. Here we report the discovery of a new wasp species that infects the adult stage of fruit flies in the genus *Drosophila*, including one of the most deeply studied model organisms in biology, *Drosophila melanogaster*” before the more specific info about *Drosophila*, just above.

Line 46: the first line of the intro doesn’t see relevant to the story

Line 51: leave out marvelous.

Lines 76-70: It is notable and strange that parasitism of adult insects is rare! But text about why is misleading because parasitoids of any life stages may have novel venom components, and many have RNA virus that manipulate the host in various way. So these things in themselves are not special to parasitism of adults

Line 78- consider adding “collected as adults.” To the end of the line to explain why host isn’t known.

Line 156- Beneath the surface of what?

Figure 1b- label the y axis

Figure 1d include the total number of samples parasitized and unparasitized for each state

Line 347: in the intro or the discussion you might want to mention the cool work that has been done on ecology and evolution with *drosophila* parasitoids, and how this new parasitoid can now we used in this research.

Quicray, M., Wilhelm, L., Enriquez, T., He, S., Scheifler, M., & Visser, B. (2023). The *Drosophila*-parasitizing wasp *Leptopilina heterotoma*: A comprehensive model system in ecology and evolution. *Ecology and Evolution*, 13(1), e9625.

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Author Rebuttals to Initial Comments:

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The discussion section was concise and mentioned the main findings of this study. These include 1) the opportunity for future research after this discovery, since *D. melanogaster* is one of the most employed animal models in biology; 2) the implications that this host-parasitoid relationship has for the evolution of the subfamily Euphorinae and of parasitoidism of adult hosts within Hymenoptera.

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We are grateful for the reviewer's careful reading of our manuscript and for the suggested changes outlined here and below. Based on this comment, we critically reviewed all panels in Fig 4 and Extended Data Fig 5 for opportunities to improve the quality of each panel with special attention on clarity of detail and lighting. The original images were taken on a Leica model M205C microscope equipped with a Leica model DFC295 camera and processed using Leica Application Suite X version 5.1.0.25446 auto-montage image processing software. In the revised manuscript, almost every panel has been reshot and reprocessed with the goal of improving lighting and clarity of detail. In addition to the revised figures within the manuscript document, we have also included full resolution .TIF format images within the "Data for revision review.zip" supplement. These represent the quality and resolution of the files that will be submitted for final publication. Our goal is to ensure high quality photographs of this species accompany our description. If these revisions do not meet that standard, we would be most grateful for specific comments detailing shortcomings.

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We appreciate the reviewer's feedback on the utility of this analysis. As suggested, we have removed the original Extended Data Figure 6 and references to it from the revised manuscript but retained emphasis of the divergence between the new and previously known parasitoids of drosophilids.

3. Was only the COI marker available in Genbank for a high number of euphorines and species of *Syntretus*? There are three or four legacy nuclear Sanger markers that could have been sequenced for different euphorine taxa (e.g, 28S, wingless, EF-1, Opsin genes). I strongly recommend the authors to search in GenBank for nuclear markers and adding them to the phylogenetic analysis that they carried out. If there is a considerable mismatch between the COI and the nuclear marker that they could found, they could do separate analyses of with these genes and then briefly mention whether they gave similar topologies. I suggest the authors to look on the following paper for additional markers:

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Euphorinae (Braconidae) and evolution of its host preference. Systematic Entomology 40: 570-591.

Thank you for this excellent suggestion. Despite the relatively limited availability of sequences for members of this subfamily, we agree with the reviewer that this analysis could be improved by including information from nuclear loci. In the revised manuscript, we followed Stigenberg et al. 2015's approach and reconstructed the phylogeny displayed in Figure 1 using an alignment of concatenated nuclear loci (CAD, 18S rRNA, and 28S rRNA) and the mitochondrial COI locus. We present our results on L115-122 of the revised manuscript. Like Stigenberg et al., we found this tree strongly supports the monophyly of individual euphorine tribes but does not resolve all intertribal relationships within the subfamily. We also include in the revised Data for Revision Review supplement two individual phylogenies of the subfamily, one built from concatenated nuclear loci and the other from COI only. There are differences in intertribal relationships between the two trees as noted by Stigenberg et al. 2015, and our phylogenetic analyses were consistent with those observations. For example, neither the nuclear data nor the mitochondrial locus individually resolve with strong support the relationships between syntretines and sister tribes, while the concatenated dataset places the tribe Myiocephalini as sister to Syntretini with strong support in both our analysis and that of Stigenberg et al., 2015.

Within the genus *Syntretus*, we found the topology to be similar between nuclear and mitochondrial gene trees. Both trees individually and the concatenated tree support the placement of *Syntretus perlmani* within the genus *Syntretus*, sister to *Syntretus* sp. JS10-00152, *Syntretus idalius*, and *Syntretus taegeri*. These phylogenies are now included in the manuscript in the revised Extended Data Fig. 1.

In summary, in response to this reviewer suggestion we have sequenced the CAD, 18S rRNA, and 28S rRNA loci of *S. perlmani* and *S. trigonaphagus*, reconstructed the phylogeny shown in Fig 1 using Stigenberg's dataset of 3 nuclear and 1 mitochondrial marker, noted in the revised text (L115 forward) the similarity between this tree and the one presented by Stigenberg et al., 2015, and also noted in the text that nuclear and mitochondrial markers agree as to the specific position and sister taxa of *Syntretus perlmani* within the tribe Syntretini. Finally, we have added relevant information to the Methods section and updated Supplementary Data Table 1 and the Data Availability Statement.

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We appreciate the reviewer's thorough, constructive suggestions for improving the manuscript and we have addressed all of them as outlined below. First, with regard to observed infection frequency and host preferences, we have added further discussion points starting at L404 of the revised manuscript, as suggested. We agree that 1% is a low infection rate for a parasitoid that does not have additional hosts. We also agree the wasp's apparent absence in African populations of *D. melanogaster* suggests this species has not historically been the primary host of *S. perlmani*, but it may indeed be a regular host in parts of its expanded global range. The use of both *D. affinis* and *D. melanogaster*, two fly species separated by an estimated 17-31 million years of evolutionary divergence¹, suggests that this wasp may be able to use a wide variety of *Drosophila* species as hosts. In the original manuscript, we referenced this indirectly by making connections to the broad host range of some other euphorine wasps. In the revised manuscript, we provide more direct support and discussion around the possibility that *S. perlmani* is a generalist by reporting another species of *Drosophila* that successfully hosts *S. perlmani* in the lab, *D. acutilabella*. We chose this species in part because it belongs to a different subgenus than *D. affinis* and *D. melanogaster*, it has a different diet and ecology, as a mushroom-breeding species, and its range has very limited overlap with that of *D. affinis* (it is a Neotropical species with a limited North American distribution within peninsular Florida). Parasitism data from 102 exposures of *D. acutilabella* to *S. perlmani* are included in the revised Supplementary Data Table 6 and reported on L231 of the revised manuscript. Similar to *D. affinis* and *D.*

melanogaster hosts, *D. acutilabella* infections yielded adult wasps in ~21% of cases. We believe confirming a third and highly divergent species that supports *S. perlmani* development provides valuable context to the infection frequencies we have observed so far in wild flies, i.e. by further supporting that *S. perlmani* may be a generalist parasitoid of *Drosophila*. However, in trials with a fourth potential host species, *Drosophila immigrans*, we found evidence that some species can mount a robust and effective immune response against *S. perlmani* infection. This result is presented alongside the *D. acutilabella* results, and we have added a figure showing melanotic encapsulation of *S. perlmani* eggs in parasitized *D. immigrans* (Extended Data Fig. 5).

We have also added references to an excellent online resource for drosophilid species diversity and distribution² (L411), and briefly added a little more background on parasitoid host range in general on L402-3 of the revised manuscript.

Smaller issues

In the summary I suggest putting the lines “Despite a deep history of research attention and stunning biodiversity, no wasp that attacks and develops inside the adult stage of a fly host has ever been described. Here we report the discovery of a new wasp species that infects the adult stage of fruit flies in the genus *Drosophila*, including one of the most deeply studied model organisms in biology, *Drosophila melanogaster*” before the more specific info about *Drosophila*, just above.

We made this change, but ultimately felt that a little background about the known diversity of *Drosophila* parasitoids more effectively introduces the statement about our discovery. We are open to considering this further with editor feedback.

Line 46: the first line of the intro doesn't see relevant to the story

We appreciate this perspective. Given that we are introducing a new parasitoid species that uses an uncommon strategy (adult parasitism), we find it in an unexpected host (first record in dipterans), and we find a significant host shift from hymenopterans underlies this host range, we believe that anchoring the introduction very briefly with a statement that parasitism is fertile ground for evolutionary innovation does add contextual value, especially for non-biologist readers.

Line 51: leave out marvelous.

We have made this change on L51 of the revised manuscript.

Lines 76-70: It is notable and strange that parasitism of adult insects is rare! But text about why is misleading because parasitoids of any life stages may have novel venom components, and many have RNA virus that manipulate the host in various way. So these things in themselves are not special to parasitism of adults

We agree. The statement has been revised to avoid this confusion on L67-69 of the revised manuscript. The revised wording is (edited line underlined):

“This may be due to increased mobility of potential hosts, thickness of the chitinous exoskeleton, and development of defensive responses not available to all juvenile stages, e.g. kicking appendages, powerful mandibles, and potent chemicals. Even so, imagobionts have thrived in this unique niche, as evidenced by the effectiveness with which they parasitize and manipulate host biology and the peculiar mechanisms sometimes involved. For example, an RNA virus and potentially other venom components of the euphorine, *Dinocampus coccinellae*, paralyze adult spotted lady beetles to elicit a cocoon-guarding behavior following larval emergence from the host body.”

Line 78- consider adding “collected as adults.” To the end of the line to explain why host isn’t known.

We have made this change on L79 of the revised manuscript.

Line 156- Beneath the surface of what?

Beneath the soil surface in the wild, but in the laboratory environment, beneath the surface of a variety of loose substrates. We revised the wording on L161 of the revised manuscript to “As reported for other *Syntretus* spp., larvae prefer to burrow into a loose substrate and cocoon beneath the surface.”

Figure 1b- label the y axis

Figure 1 does not have a panel b, we assumed Figure 3 may have been intended here. We modified the legend of Figure 3b into an axis label for this figure panel in the revised manuscript.

Figure 1d include the total number of samples parasitized and unparasitized for each state

For states in which field collections were performed (Mississippi, Alabama, and North Carolina), we added the total number of male flies collected and screened, and the number infected to Figure 3d. For states in which DNA sequence read sets were screened, we added the total number of read sets screened and the number containing *S. perlmani* reads. We have also updated the figure description to explain these labels.

Line 347: in the intro or the discussion you might want to mention the cool work that has been done on ecology and evolution with drosophila parasitoids, and how this new parasitoid can now we used in this research.

Quicray, M., Wilhelm, L., Enriquez, T., He, S., Scheifler, M., & Visser, B. (2023). The *Drosophila*-parasitizing wasp *Leptopilina heterotoma*: A comprehensive model system in ecology and evolution. *Ecology and Evolution*, 13(1), e9625.

Wertheim, Bregje. "Adaptations and counter-adaptations in *Drosophila* host–parasitoid interactions: advances in the molecular mechanisms." *Current opinion in insect science* 51 (2022): 100896.

We have revised the final paragraph of the discussion to include specific examples of important research advances supported by *Drosophila*-parasitoid systems and examples of areas in which this new parasitoid can be used to extend research (L429 forward). We have cited Quicray et al. 2023 in the original manuscript and have added a citation to Wertheim 2022 on L49 of the revised manuscript.

Line 359: I think the section on attempts to rear could be reduced.

We have made this change.

References for authors' responses

1. Obbard, D. J. *et al.* Estimating divergence dates and substitution rates in the *Drosophila*

phylogeny. *Mol. Biol. Evol.* **29**, 3459–3473 (2012).

2. Bächli, G. The Database on Taxonomy of Drosophilidae. (2015).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have addressed all the comments and corrections that were suggested by the two reviewers. In particular, they paid special attention about the use of both nuclear and mitochondrial markers to reconstruct the phylogenetic relationships among the species of *Syntretus*. They also improved the digital pictures of the new species and addressed well the comment about the infection rate. I therefore recommend the manuscript for its publication. I believe that this piece of work will represent an outstanding contribution for a better understanding of the biology and evolution of parasitoid wasps. I also find remarkable the rearing experiments that were conducted in order to discover the biology of the new species. This protocol will serve for future studies that seek to investigate the host preferences of other parasitoid taxa.

Referee #3 (Remarks to the Author):

The original ms was interesting and well structured. The authors made an excellent revision. All of the points that I made were very well addressed, and it looks like the others were very well addressed too, including much better phylogenetic analysis and figure photos. this is a very nice manuscript.