nature portfolio

Peer Review File

Ancestral neural circuits potentiate the origin of a female sexual behavior in *Drosophila*

Corresponding Author: Dr Yun Ding

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Ancestral neural circuits potentiate the origin of a female sexual behavior” by Li et al. studies the neuronal circuits for communication behavior during courtship in Drosophila santomea, a species with newly established neurogenetic accessibility that provides an interesting model for comparative studies.
The authors characterize a female specific behavior in D. santomea, wing spreading (WS) in response to specific parts of the male acoustic courtship signals.
Wing amputated females that cannot perform WS show a lower copulation rate and are courted by males with courtship songs that have shorter trains, suggesting a positive feedback effect of female WS on male courtship intensity and copulation success.
In a next step, the authors compare sex specific female descending neurons expressing doublesex in 3 species. These 2 pairs of neurons have been characterized to drive acceptance and rejection movement of the genital plates in D. melanogaster, a function that seems conserved between species. Interestingly, they additionally elicit WS in D. santomea upon optogenetic activation. Weaker WS can be elicited in D. melanogaster by modulating developmental conditions.

The paper shows an insightful example of how the evolution of behavior and its neuronal basis can be explored by combinations careful behavioral analysis across species and neurogenetic manipulation of defined neuronal classes. It is interesting for a wide audience for evolutionary biologists, neuroscientists and ethologists.
The data is presented in a clear way and the paper is written well.

Comments:
Figure 1: In Fig 1c and d, some data is shown suggesting that WS is more prevalent in receptive unmated females, whereas mated females do not display the behavior. It is mentioned that mated females are unreceptive, but no exact data is given here. Remating rate vary considerably between Drosophila species, and I think D. santomea is a species that is rather known for very little remating. It would be useful to provide some data and/or literature reference here.
How do males detect WS in females, is that by visual input? I assume no acoustic signal is connected with WS- it would be helpful if the authors made a clear statement about this somewhere in the manuscript. Fig.1b suggests that D. santomea courts in the dark. Can the authors show that in pairs where males cannot detect female WS (blind or in the dark?), copulation rates and pulse train length of males are lower?
At the end of the first result paragraph (l. 103-104), the authors conclude that WS occurs specifically in D. santomea, suggesting that it is a newly evolved behavior. Later, however (l. 220) they describe that D. melanogaster also occasionally displays this behavior spontaneously, in line what they found upon optogenetic activation. This is a bit confusing- is it now a “newly evolved” behavior or just something that is more prevalent in D. santomea? I suggest changing the narrative- why not ”reveal” right at the beginning that the behavior can be indeed seen in D. melanogaster? As elaborated in ref. 23, WS female seems rather widespread in Drosophila- the authors also state and show so further down in the manuscript (l.231 following). Therefore, it can be confusing if the readers might get initially the idea that WS is idiosyncratic to the island species in D. santomea.
In the discussion, the authors bring up the idea that the existence of a feedback/communication about receptivity might be related to the existence of a hybridization zone. That is an interesting thought - maybe it could be explored a bit more? Do D. yakuba males court D. santomea females or vice versa and could WS somehow influence the intensity/occurrence of interspecific courtship? I could also imaging that there is a relationship between the adaptiveness of a feedback signal and the remating ratio- e.g. if there is very strong block of remating and a stronger separation of receptive vs unreceptive females,

feedback is more useful for both sexes. Since remating rates have been often studied in Drosophila in the context of evolution of other traits (e.g. 10.1111/jeb.13937, 10.1016/j.jinsphys.2017.03.008), I would find this connection worth exploring a bit in the discussion.

Reviewer #2

(Remarks to the Author)
The manuscript by Li and colleagues address how new behaviors are incorporated into existing complex social contexts and neural circuitry by investigating a recently originated social behavior in the Drosophila species group, where functional comparison of neural circuits is possible. This is a truly integrative study, where they combine phylogenetic analysis, behavioral characterization, and functional manipulation of neural circuits between species to provide insight into the ultimate and proximate mechanisms underlying the origin of wing spreading behavior in females in Drosophila santomea. More specifically, the Authors argue that the origin of wing spreading in females in this species is mediated by the co-option of a descending circuit node that drives a conserved abdominal behavior and the refinement of a latent and plastic ancestral circuit. I generally found that the data support the conclusions in the paper, although my expertise is in evolutionary developmental biology and not neuroethology. Therefore, I will primarily evaluate the Author's conclusion that an ancestral developmental potential in neural circuitry potentiated the origin of wing spreading behavior.

The data presented by the Author's fulfill the three criteria for concluding that a trait originated and re-evolved through the induction of ancestral developmental potential: (1) they show that the trait (wing spreading) is inducible in closely-related species that have not evolved the trait; (2) that ancestral developmental potential is induced at rare frequencies in natural populations, such that wing spreading appears as rare anomalies in populations that have not evolved the trait, like in Drosophila melanogaster; and (3) The traits appears to be present in the ancestors of the group, was lost, and then re-evolved in a derived species.

This work is arresting and its implications are far reaching because it unites the fields of evo-devo and neuroethology, providing mechanistic insight to evolutionary principles. I therefore highly recommend this manuscript for publication in Nature Communications.

Here are suggestions for improvement:

This work would benefit from citing some additional works that are relevant to this manuscript:

The following 2 papers can help the authors place their findings more generally:

Foster and Baker (2019: Animal Behaviour) Loss and re-emergence of plastic ancestral behavioral traits: influences on phenotypic and evolutionary pattern
Foster (2013: Animal Behaviour). Evolution of behavioral phenotypes: influences of ancestry and expression.

West-Eberhard 2003: Developmental Plasticity in Evolution (book).

The discussion would benefit from including a few sentences on the evolutionary mechanisms through which an originally plastic trait (wing spreading) becomes genetically assimilated such that it is expressed constitutively in all individuals of the population.

The phylogeny presented in Figure 6 would benefit from better annotation on the tree itself. The authors should indicate on the tree when particular traits appear, disappear, and reappear.

General comment: Please do not use the acronym WS for wing spreading, it only adds confusion.

Line 101: why "might be",you have provided sufficient evidence to show it is a fixed species difference.

Line 114: why "suggesting", the evidence is quite strong for a signaling role.

Line 146: I had to go back and look up OE, please avoid using acronyms.

The female wing extension behavior has not been reported within the melanogaster subgroup but is reminiscent of the female WS behaviors described before copulation in species of some distantly related lineages such as the virilis group.
>> I think at this point the Authors should refer to this / ask the question of whether this is parallel or convergent evolution. Basically, they argue it is parallel evolution based on similarities of their motor pattern and the pre-copulatory context

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

All my comments/suggestions have been addressed in a satisfactory way. The changes have improved the clarity of the manuscript.

Reviewer #2

(Remarks to the Author)
The manuscript by Yun Ding and Colleagues has greatly improved. I am enthusiastic about this work and think it is almost ready for publication. Because of the important implications of this study for neuroethology, evolutionary, and developmental biology, I believe it's worth a little more effort to get the evolutionary biology right. The formal ancestral state reconstruction performed by the authors is an improvement over what was in the previous manuscript, but its needs to be revised to get the interpretation correct. First, the coding of the characters for their ancestral state reconstructions is incorrect and needs to be revised. The authors code the states of the character as VPN-only or VPN+WS. However, VPN+WS is not a real character and neither VPN-only or VPN+WS are real character states. The more appropriate way to perform the ancestral state reconstruction is to reconstruct the ancestral of the two characters (VPN and WS) seperately, each with two states. So, VPN (present or absent) and WS (present or absent). This way, these are bonafide characters and the authors can infer the confidence for each mapping on the tree. The others should perform an ancestral state reconstruction for each trait to see if WS and VPN independently reconstruct in the ancestor. Finally, parsimony is an outdated method for reconstructing ancestral states and the authors should really be using ML and Bayesian methods to get a more accurate picture of what happening. I believe these revisions will great improve the inference that the Authors can make.

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The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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We sincerely thank the reviewers for their helpful comments and insightful suggestions. Please see below where we respond point-by-point to each comment. We have improved our manuscript accordingly and the changes made were noted in the response letter and highlighted in the main text. Additionally, we modified the format to adhere to the Journal's guidelines. Given that these edits did not alter the scientific content of the manuscript, we have not detailed them here. We note that the *dsx*-GAL4 genetic reagents reported in the original manuscript have been published by a separate study of ours (submitted after the initial submission of this manuscript). We have therefore cited this study instead of describing these reagents in detail in the revised manuscript.

Reviewer' comments in blue

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript "Ancestral neural circuits potentiate the origin of a female sexual behavior" by Li et al. studies the neuronal circuits for communication behavior during courtship in Drosophila santomea, a species with newly established neurogenetic accessibility that provides an interesting model for comparative studies.
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Wing amputated females that cannot perform WS show a lower copulation rate and are courted by males with courtship songs that have shorter trains, suggesting a positive feedback effect of female WS on male courtship intensity and copulation success.
In a next step, the authors compare sex specific female descending neurons expressing doublesex in 3 species. These 2 pairs of neurons have been characterized to drive acceptance and rejection movement of the genital plates in D. melanogaster, a function that seems conserved between species. Interestingly, they additionally elicit WS in D. santomea upon optogenetic activation. Weaker WS can be elicited in D. melanogaster by modulating developmental conditions.

The paper shows an insightful example of how the evolution of behavior and its neuronal basis can be explored by combinations of careful behavioral analysis across species and neurogenetic manipulation of defined neuronal classes. It is interesting for a wide audience for evolutionary biologists, neuroscientists and ethologists.
The data is presented in a clear way and the paper is written well.

We thank the reviewer for their enthusiasm and the very helpful suggestions, which we have addressed below.

Comments:
1) Figure 1: In Fig 1c and d, some data is shown suggesting that WS is more prevalent in receptive unmated females, whereas mated females do not display the behavior. It is mentioned that mated females are unreceptive, but no exact data is given here. Remating rate vary considerably

between Drosophila species, and I think D. santomea is a species that is rather known for very little remating. It would be useful to provide some data and/or literature reference here.

Thanks for this great point. We agree that it is important to clarify *D. santomea*'s low remating rate, and we have added a reference for (Denis et al. 2017) accordingly in the text (line 75). The authors report that 28% of unmated *D. santomea* females copulated during a 3 hr observation period, but 0% of previously mated females remated during a 2 hr observation period 24 hrs after the initial mating. Consistent with the literature, in our experiments we also observed that 0/26 mated females remated during a 1 hr recording period 24 hrs after the first mating, whereas 12/32 age-matched unmated females mated during the same period (GLM p=0.000277). Therefore, both the literature and our data show low remating rate in *D. santomea*, supporting that recently mated females are unreceptive. To maintain better flow of our main text, we referenced the published data.

2) How do males detect WS in females, is that by visual input? I assume no acoustic signal is connected with WS- it would be helpful if the authors made a clear statement about this somewhere in the manuscript. Fig.1b suggests that D. santomea courts in the dark. Can the authors show that in pairs where males cannot detect female WS (blind or in the dark?), copulation rates and pulse train length of males are lower.

We thank the reviewer for these insightful questions. *D. santomea* pairs do indeed court in the dark. Following the reviewer's suggestion, we asked if copulation rate was lower in the dark than in lighted condition, and found that copulation rate was comparable between the two groups (light: 11/18 copulated; dark: 10/15 copulated; Fisher's exact test p=1). Furthermore, while removing females' wings reduced copulation rate in lighted condition (Fig. 2a), the same manipulation no longer had an effect on copulation rate in the dark (intact female pairs: 5/24 copulated; wing-cut female pairs: 8/24 copulated; Fisher's exact test p=0.517). The lack of dependency on female wing signal for copulation success in the dark highly suggested that courting males could use compensatory mechanisms to infer females' receptivity in the absence of visual information. Indeed, this possibility is bolstered by reports in *D. melanogaster* that males employed different strategies (e.g. a "zigzag" movement pattern) and sensory modalities (e.g. pheromones) to locate females in the dark (Krstic et al. 2009; Toda et al. 2012). Recent work further shows that flies are capable of inferring the positional information of potential mates using a volatile pheromone signal (Taisz et al. 2023). Therefore, in this case, due to the multimodal nature of *D. santomea* courtship and the suggested presence of compensatory sensory cues males may use for copulation success in the dark, the dark condition does not allow a clean isolation of the visual effect to provide a clear answer of whether males detect wing spreading by vision. We think that wing spreading is likely a visual signal, because (1) wing spreading does not seem to generate an acoustic signal based on its slow wing motion and our acoustic recordings; and (2) in most cases of wing spreading, females and males are not physically touching, ruling out the possibility of wing spreading being a tactile signal or a gustatory signal. We cannot fully rule out that wing spreading might alter the pheromone field of the female or generate slight changes in the air pressure in ways that can be detected by males. Due to the inconclusive nature of the dark assay and the inability to fully exclude the alternative possibilities, we did not include this result in our manuscript.

3) At the end of the first result paragraph (l. 103-104), the authors conclude that WS occurs specifically in D. santomea, suggesting that it is a newly evolved behavior. Later, however (l. 220) they describe that D. melanogaster also occasionally displays this behavior spontaneously, in line what they found upon optogenetic activation. This is a bit confusing- is it now a “newly evolved” behavior or just something that is more prevalent in D. santomea? I suggest changing the narrative- why not ”reveal” right at the beginning that the behavior can be indeed seen in D. melanogaster? As elaborated in ref. 23, WS female seems rather widespread in Drosophila- the authors also state and show so further down in the manuscript (l.231 following). Therefore, it can be confusing if the readers might get initially the idea that WS is idiosyncratic to the island species in D. santomea.

We placed *D. melanogaster* data at the end instead of at the beginning because it is contingent on the earlier results. First, the wing spreading behavior in *D. melanogaster* is extremely rare. In total, we have identified only 9 events from 7 individuals after closely scrutinizing the courtship interactions of 141 pairs of *D. melanogaster*. These observations are hard to obtain and would be easily missed by any standard analysis. If we were to specifically look for these events in any other species without wing spreading, we may also identify similar prototypic events. This unusually detailed scrutiny was motivated by the identification of latent circuit potential in *D. melanogaster* (Fig. 5b). Second, all 9 events came from the experimental group that experienced high temperature during development. This environmental perturbation was inspired by identifying the developmental temperature-based plasticity of the latent circuit (Fig. 5d-g). Finally, since the wing spreading behavior in *D. melanogaster* is extremely rare and relatively simple in its motor form, to establish its homology with *D. santomea* wing spreading behavior, we relied on its association and quantitative scaling with VPO (Fig. 3) to define it. Therefore, this result naturally follows the earlier ones, and only by placing it at the end can we provide the necessary contexts for the experiment and the interpretation.

At the conceptual level, we view the seeming contradiction (i.e., wing spreading as a species-specific new behavior originated in *D. santomea* and its occasional expression in the outgroup species *D. melanogaster*) as one of the significant points of the paper, as it reflects the continuity of evolutionary process and promote nuanced thinkings of how to define evolutionarily new behaviors in the context of latent circuit potential. In other words, if actualizing a plastic latent potential is a general mechanism underlying the evolution of new behaviors, we would expect to observe prototypic behaviors in closely related outgroup species as rare events in some individuals and under certain conditions, even though they are not a canonical or necessarily adaptive component of the outgroup species’ behavioral repertoires.

We do appreciate the noting of possible confusion and have revised our language to convey the ideas more clearly in writing. Specifically, we have taken *D. melanogaster* wildtype wing spreading data out to be its own subsection and framed it as a manifestation of latent circuit potential in the Results subsection “Expression of latent potential as rare wing spreading events in *D. melanogaster*” (line 215). Similarly, we have framed the repeated evolution of wing spreading in the same context. This way, the rare wing spreading events in the outgroup species and the repeated evolution of wing spreading across phylogeny can be viewed as two levels at which latent potential shapes animal behaviors we see in nature. Finally, we have mentioned the

implication of latent potential in defining new behaviors in the discussion "…latent potentials may broadly exist in the nervous system, serving as raw substrates that fuel the rapid evolution of new behaviors. If so, we anticipate that species-specific behaviors may commonly exist in closely related outgroup species in primitive prototypes that are occasionally expressed under certain conditions, blurring the traditional line that defines a new behavior" (line 317).

4) In the discussion, the authors bring up the idea that the existence of a feedback/communication about receptivity might be related to the existence of a hybridization zone. That is an interesting thought - maybe it could be explored a bit more? Do D. yakuba males court D. santomea females or vice versa and could WS somehow influence the intensity/occurrence of interspecific courtship?

I could also imaging that there is a relationship between the adaptiveness of a feedback signal and the remating ratio- e.g. if there is very strong block of remating and a stronger separation of receptive vs unreceptive females, feedback is more useful for both sexes. Since remating rates have been often studied in Drosophila in the context of evolution of other traits (e.g. 10.1111/jeb.13937, 10.1016/j.jinsphys.2017.03.008), I would find this connection worth exploring a bit in the discussion.

We thank the reviewer for suggesting these directions to explore in the discussion. To explore the role of wing spreading in heterospecific mating, we tested (1) if *D. yakuba* males courted and copulated with *D. santomea* females, (2) if *D. santomea* females responded to *D. yakuba* male courtship with wing spreading, (3) if *D. yakuba* males, in turn, extended their pulse singing in response to *D. santomea* female wing spreading, and (4) if the social feedback loop was orchestrated by *D. santomea* female wing spreading by removing females' wings. We recorded courtship between either intact or wing-cut *D. santomea* females and *D. yakuba* males. None of the 24 intact female pairs or 28 wing-cut female pairs copulated within the observation window, posing a barrier to further assess whether wing spreading could affect copulation success in this heterospecific context. *D. yakuba* males courted *D. santomea* females, albeit less intensely (measured in pulse trains per minute) than *D. santomea* males did in conspecific pairings (ED Fig. 2f). Similar to conspecific courtship (Extended Data Fig. 2a), removing females' wings had no effect on male courtship intensity (ED Fig. 2f), suggesting that wing spreading did not influence the intensity of heterospecific courtship. *D.santomea* females also responded to *D. yakuba* pulse songs with wing spreading less frequently (ED Fig. 2g). These results were not surprising, as sensory modalities other than vision and audition are involved in *Drosophila* courtship to reduce heterospecific courtship. We also asked if *D. yakuba* males could respond to *D. santomea* female wing spreading. We found that wing spreading was not associated with longer pulse trains, and removing *D. santomea* females' wings had no effect on *D. yakuba* male pulse train length (ED Fig. 2h,i). To summarize, *D. yakuba* males' courtship and *D. santomea* females' corresponding wing spreading response were both less intense than among *D. santomea* pairs, and the social feedback loop centered around wing spreading breaks down in heterospecific courtships. This suggests that wing spreading could be one of the premating mechanisms preventing hybridization between *D. santomea* and *D. yakuba*. We added a paragraph in the Results subsection "Function of wing spreading as a receptive female signal" and corresponding figures in the Extended Data to describe these results.

Because sensory modalities and sensory cues aside from wing spreading are also involved in the premating reproductive isolation between *D. santomea* and *D. yakuba*, heterospecific courtship (and copulation) rate is lower than the conspecific courtship rate of either species. Remating is rare in both *D. santomea* and *D. yakuba* (Chang 2004) due to the transfer of long-lasting seminal fluid proteins such as Sex Peptide, and after a female has mated with a conspecific male, remating with heterospecific males is exceedingly rare. Since wing spreading is specific to *D. santomea* females and low remating rate is a common feature for both *D. santomea* and *D. yakuba*, it is unlikely that wing spreading has evolved as a signal for remating receptivity in heterospecific matings.

We also thank the reviewer for the insight to consider low remating rate as a possible driver for the origin of wing spreading, where more effective social feedback on female receptivity will avoid unwanted and unfruitful courting. Thanks to the reviewer's suggestion, we explored the publications on the remating rate in *D. santomea* and *D. yakuba*. Fig. 1 and 2 from (Denis et al. 2017) showed that both species exhibit strong post-mating inhibition, and that *D. santomea* might have slightly lower remating rate than *D. yakuba*. On the other hand, Fig. 2 from (Macartney and Bonduriansky 2022) summarized that *D. yakuba* has similar, if not slightly lower, remating rate than *D. santomea*. Considering the subtlety of the reported difference, the inconsistency between the two reports, as well as the noted strain variation of the remating rate in these species (Chang 2004; Matute 2010), overall we could not find strong support for a lower remating rate in *D. santomea* than in *D. yakuba*, precluding us from further establishing the relevance of remating rate in the origin of wing spreading.

Reviewer #2 (Remarks to the Author):

The manuscript by Li and colleagues address how new behaviors are incorporated into existing complex social contexts and neural circuitry by investigating a recently originated social behavior in the Drosophila species group, where functional comparison of neural circuits is possible. This is a truly integrative study, where they combine phylogenetic analysis, behavioral characterization, and functional manipulation of neural circuits between species to provide insight into the ultimate and proximate mechanisms underlying the origin of wing spreading behavior in females in Drosophila santomea. More specifically, the Authors argue that the origin of wing spreading in females in this species is mediated by the co-option of a descending circuit node that drives a conserved abdominal behavior and the refinement of a latent and plastic ancestral circuit. I generally found that the data support the conclusions in the paper, although my expertise is in evolutionary developmental biology and not neuroethology. Therefore, I will primarily evaluate the Author's conclusion that an ancestral developmental potential in neural circuitry potentiated the origin of wing spreading behavior.

The data presented by the Author's fulfill the three criteria for concluding that a trait originated and re-evolved through the induction of ancestral developmental potential: (1) they show that the trait (wing spreading) is inducible in closely-related species that have not evolved the trait; (2) that ancestral developmental potential is induced at rare frequencies in natural populations, such that wing spreading appears as rare anomalies in populations that have not evolved the trait, like in

Drosophila melanogaster; and (3) The traits appears to be present in the ancestors of the group, was lost, and then re-evolved in a derived species.

This work is arresting and its implications are far reaching because it unites the fields of evo-devo and neuroethology, providing mechanistic insight to evolutionary principles. I therefore highly recommend this manuscript for publication in Nature Communications.

We thank the reviewer's enthusiastic support for our work and the recognition that this work unites the fields of evo-devo and neuroethology. We are also grateful for the reviewer's insightful suggestions for improvement, which we have addressed below.

Here are suggestions for improvement:

1) This work would benefit from citing some additional works that are relevant to this manuscript: The following 2 papers can help the authors place their findings more generally: Foster and Baker (2019: Animal Behaviour) Loss and re-emergence of plastic ancestral behavioral traits: influences on phenotypic and evolutionary pattern
Foster (2013: Animal Behaviour). Evolution of behavioral phenotypes: influences of ancestry and expression.
West-Eberhard 2003: Developmental Plasticity in Evolution (book).

We now have incorporated the two citations and highlighted that re-emergence of lost ancestral behaviors is an important mode of behavioral evolution (line 318). As noted below in detail, our phylogenetic inferences do suggest the possibility of wing spreading in *D. santomea* as a behavioral novelty without an ancestral homolog. However, considering the caveats of phylogenetic inferences, we acknowledged re-emergence of a lost ancestral behavior as a possibility and cited the reference in the context of mentioning this possibility.

West-Eberhard 2003 has been cited in the context of the plasticity of prototypic phenotypes (line 348). Here we take this opportunity to communicate that we deeply value West-Eberhard's major contributions and appreciate how our work aligns with her insights. We also recognize that the related theoretical framework places a great emphasis on adaptive plasticity (*versus* non-adaptive plasticity) and the contingency of evolution on a pre-existing plasticity, which cannot be directly interpreted from our data. A future review or opinion paper may be a more suitable place for a comprehensive discussion to attend these conceptual subtleties.

2) The discussion would benefit from including a few sentences on the evolutionary mechanisms through which an originally plastic trait (wing spreading) becomes genetically assimilated such that it is expressed constitutively in all individuals of the population.

We now have incorporated the concept of "genetic assimilation" in the discussion (line 339).

3) The phylogeny presented in Figure 6 would benefit from better annotation on the tree itself. The authors should indicate on the tree when particular traits appear, disappear, and reappear.

Guided by the reviewer's feedback, we explored inferring the origins and losses of wing spreading across the phylogeny. To increase the accuracy of phylogenetic inference, we included data from (Spieth 1952) to achieve a denser sampling. We performed two commonly used methods, maximum parsimony and maximum likelihood (both assuming equal transition rates), to enhance the robustness of our conclusion. The maximum parsimony method using data of 50 species supports the absence of wing spreading in all ancestral nodes of *D. santomea* over its ~47 million years' phylogeny since the divergence between *Sophophora* and *Drosophila* subgenera. On the other hand, the maximum likelihood method using data of 46 species supports equal probability of wing spreading being absent *versus* present in most ancestral nodes of *D. santomea*. Noting that ancestral state reconstructions are limited in their ability to accurately reflect evolutionary histories (Cunningham et al. 1998) and that our inferences reported uncertainty, we (1) acknowledged the possibility of wing spreading being a re-emergence of a lost ancestral behavior, and (2) pointed out that based on the phylogenetic inferences, it is equally possible, if not more likely, that wing spreading in *D. santomea* is a novelty without an ancestral behavioral homolog. We have added a new Results subsection (titled "Ancestral state reconstruction of wing spreading in the *Drosophila* genus") and a new figure (ED. Fig 6) to include the new result. We have also slightly modified the relevant discussion to best reflect the results (lines 320-323).

4) General comment: Please do not use the acronym WS for wing spreading, it only adds confusion.

We have made the suggested edits.

5) Line 101: why "might be",you have provided sufficient evidence to show it is a fixed species difference.

We now use "is" instead of "might be".

6) Line 114: why "suggesting", the evidence is quite strong for a signaling role.

We now use "supporting" instead of "suggesting".

7) Line 146: I had to go back and look up OE, please avoid using acronyms.

We have made the suggested edits.

8) The female wing extension behavior has not been reported within the melanogaster subgroup but is reminiscent of the female WS behaviors described before copulation in species of some distantly related lineages such as the virilis group.
>> I think at this point the Authors should refer to this / ask the question of whether this is parallel or convergent evolution. Basically, they argue it is parallel evolution based on similarities of their motor pattern and the pre-copulatory context

We appreciate the reviewer's suggestion. We agree with this interpretation and that this idea aligns with the classical definition of parallel evolution. Therefore, we revised our manuscript to deliver this idea more directly in lines 250-253. Here we did not use a dichotomy framework of parallel *versus* convergent evolution for both technical challenges and historical complexities

associated with the terms. The distinction between parallel and convergent evolution relies on the inference of ancestral state and typically operates on the assumption that related species vary in similar directions. Our study raises the possibility of “a shared neural mechanism” (e.g., through the co-option of vpoDN) but does not yet address the precise causal events to make a strong statement. Further, there are nuances and major debates surrounding the classical definitions of these terms (e.g., (Arendt and Reznick 2008; Gompel and Prud’homme 2009; Losos 2011; Ord and Summers 2015)). These debates highlight possible conceptual challenges of dichotomizing parallel *versus* convergent evolution. For instance, Arendt and Reznick (2008) argue that parallel and convergent evolution should be viewed as ends of a continuum; and Losos (2011) argues that conclusions may vary depending on the level of biological processes analyzed. Therefore, we use the more inclusive term “repeated evolution” to describe the evolutionary mode of wing spreading elsewhere in the manuscript.

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Reviewer' comments in blue

Reviewer #1 (Remarks to the Author):

All my comments/suggestions have been addressed in a satisfactory way. The changes have improved the clarity of the manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript by Yun Ding and Colleagues has greatly improved. I am enthusiastic about this work and think it is almost ready for publication. Because of the important implications of this study for neuroethology, evolutionary, and developmental biology, I believe it's worth a little more effort to get the evolutionary biology right. The formal ancestral state reconstruction performed by the authors is an improvement over what was in the previous manuscript, but its needs to be revised to get the interpretation correct. First, the coding of the characters for their ancestral state reconstructions is incorrect and needs to be revised. The authors code the states of the character as VPN-only or VPN+WS. However, VPN+WS is not a real character and neither VPN-only or VPN+WS are real character states. The more appropriate way to perform the ancestral state reconstruction is to reconstruct the ancestral of the two characters (VPN and WS) seperately, each with two states. So, VPN (present or absent) and WS (present or absent). This way, these are bonafide characters and the authors can infer the confidence for each mapping on the tree. The others should perform an ancestral state reconstruction for each trait to see if WS and VPN independently reconstruct in the ancestor. Finally, parsimony is an outdated method for reconstructing ancestral states and the authors should really be using ML and Bayesian methods to get a more accurate picture of what happening. I believe these revisions will great improve the inference that the Authors can make.

We sincerely thank the reviewer for pointing out this important issue and providing valuable suggestions. We agree completely and have revised the manuscript as suggested. We now use a single character with two states (presence *versus* absence) for the ancestral state reconstruction; we have also removed the results of maximal parsimony and added the inference using a Bayesian method. Note that VPO is present in all 46 species used here for the reconstruction, so an ancestral state reconstruction of this trait would not be particularly meaningful. Therefore, we only include the results for wing spreading–the relevant subject of this investigation and the focal trait of the study. The inference from the newly added Bayesian method is highly congruent with that of the updated ML: both methods estimated overall higher probability of wing spreading being absent *versus* being present across ancestral nodes of *D. santomea* and *D. melanogaster* in this phylogeny (the revised ED Fig. 6). Therefore, our major conclusion remains unchanged from the previous revision. We have modified the language regarding the interpretation to only reflect the results based on the ML and Bayesian methods.