

Rev #	C #	Reviewer Comment	Author Response	New Line #
1	1	Lines 116-118: “In the context of our two selection treatments, we predict that male harm and female resistance traits will be stronger in the CO populations compared to the ACO populations”: This prediction rests on the predicted increased male body size in ACO populations?	<p>This prediction is based on body size differences, as suggested by RI, but we predict stronger conflict in the larger CO* populations.</p> <p>We add a phrase to clarify this in Line 141 (Line numbers noted here refer to the clean document).</p>	141
1	2	Line 245 (and above): Dry weight is measured, i.e., not body size directly. Please add that weight is a very good proxy for body size to clarify.	Noted in Line 271.	271
1	3	Lines 287-290: Were the binomial GLMMs tested for over- and underdispersion?	Lines 309-311, 325-327. Residual dispersion from models were tested, and where appropriate, overdispersion issues were rectified using beta-binomial distributions.	309 325
1	4	References: Please check the capitalization of titles and italics for species names for consistency.	References have been updated and standardized.	
2	1	First, the premise of the study and many of the findings are not novel. Ghosh and Joshi (2012) tested a very similar premise using populations selected for accelerated development and early age reproduction vs. control populations selected for reproduction at normal age. They found clear signs of premating and postmating isolation evolving between the two sets of populations owing to their differences in body size. The current study reveals a similar isolation presumably based on body size at the premating (mate choice) stage. The extent of isolation evolved though clear is a bit underwhelming. And no postmating prezygotic isolation is detected yet in the current study. The study nonetheless merits some attention because it shows premating isolation based on size divergence is likely to emerge before the evolution of postmating isolation.	<p>We thank the reviewer for their comments on the premise for the study. As noted in the Associate Editor’s 1<sup>st</sup> comment, the manuscript does try to clarify that this literature gap has been the focus of a similar study by Ghosh and Joshi (2012). However, there are differences worth noting in the length of the selection experiments, and the patterns of assortative mating observed.</p> <p>Firstly, this study reports on populations separated for a longer period, providing a novel window into reproductive isolation under experimental divergent selection. Additionally, assortative mating in these populations were found to be homotypic in both treatments, unlike between the FEJs and JBs, where the mating pattern is assortative entirely because female flies of both</p>	

			treatments preferred (or were influenced by) the larger JB males.	
2	2	<p>Second, both individual male and female choice experiments were conducted in vials containing one pair of flies (no choice condition), from which proportion of homotypic vs. heterotypic matings and time to initiate successful matings were scored. However, an additional experiment in which a fly is housed with two potential mates from two contrasting regimes is recommended as this would clarify some of the doubts. For example, if 1 ACO male is housed with 1 ACO female and a CO female, observations over a number of replicate populations and vials would clarify if the ACO male obtains more matings with ACO females even in presence of a CO females. If this prediction turns out to be true it may indicate either preference of ACO male for homotypic matings or its ability to obtain homotypic matings more easily, either of which would point towards premating isolation.</p>	<p>Mate choice assays involved two males + one female (or vice versa). Please see the Associate Editor's 2<sup>nd</sup> comment.</p>	
2	3	<p>One problem with the size difference of the two populations though is no standard mate choice assay design will be able to detect if ACO females prefer ACO males over CO males, as the latter is likely to obtain more forced matings with ACO females outcompeting the smaller ACO males thereby overriding preference of the ACO female for its own type.</p>	<p>We agree with reviewer 2 that no mate choice assay can successfully disentangle male influence and female choice, in the presence of such marked body size differences. In fact, prior to conducting the assay, we predicted premating isolation in one direction, based on greater resistance from larger (CO) females, greater persistence of CO males, and a possible preference for large mates. As reviewer 2 notes, even if ACO females do have a homotypic preference, such a preference can only be conservatively estimated through this assay. Despite this conservative estimation, we find strong assortative mating patterns in females all 3 replicates of the ACO population.</p>	

2	4	Third, I have concerns about the design of the group mating assay. In this study, the group matings appear to involve 12 males of one population and 10 females of one population. A clearer pattern is likely to be found if the authors use a large glass petri dish (described in Ghosh & Joshi 2012), or a small transparent cage/box in which they house flies of all combinations (ACO males & females, CO males & females). The proportion of homotypic vs. heterotypic matings would show the choice/compatibility of the populations under study when mixed together.	Noted in Line 612	612
2	5	The authors mention from the mate choice assays data about the mating latency and the identity of mates were obtained. What is not clear from the manuscript is how these data were used to calculate the proportion of homotypic vs. heterotypic matings. If only mate identity was used to calculate the said proportions, then what was use of the mating latency data? Did it support the observed homotypic/heterotypic ratios?	<p>Mating latency and duration data did align with our interpretation of the mating proportions data. Briefly, mating latency in female choice assays depended on the male identity (C males achieved lower mating latency) and did not differ in male choice assays. Mating duration depended on female identity and the interaction between female and male identity in the female choice assay; and only on the female identity in the male choice assay.</p> <p>Please see below for changes in the manuscript describing how this data was analyzed and how we interpreted it.</p> <p>Changes:</p> <p>1. Analysis: Lines 312-318</p> <p>2. Results:</p> <ul style="list-style-type: none"> <li>• Lines 359-376 for latency and duration data from the female choice assay;</li> <li>• Lines 395-409 for latency and duration data from the male choice assay.</li> </ul> <p>3. Discussion:</p>	312 359 395 405 537 549

			<ul style="list-style-type: none"> <li>• Lines 505-511 for a general summary and</li> <li>• Additional mentions in lines 537-538, 549-550</li> </ul>	
2	6	My concerns are focused mostly around the premating assays, their designs and interpretations as discussed above. I have no major concerns about the postzygotic stage assays as some of those experiments (fertility) have their own limitations and the authors clearly mention it. Other experiments testing postzygotic isolation that are clearly executed but they do not lead to any crucial findings.		
2	7	Line 66: "intra-population" assortment?	Here we use the phrase to refer to assortative mating taking place within a single population on the basis of variation in a character.	
AE	1	Reviewer 2's first comment is about the novelty of the study, given that a similarly selected (i.e. for faster pre-adult development and early reproduction) population had shown roughly similar responses related to reproductive isolation. I too have the same concern. I note that the authors have already done an honest effort in terms of placing their findings in the context of the earlier study. Therefore, at least in my opinion, there is not much more to be done along that axis. I also note that the focus of the paper is very Drosophila-centric. Therefore, one possibility might be to cast a slightly wider net and connect these findings to what is known about incipient reproductive isolation in other species, particularly wrt selection on life-history traits. That would give a wider context to the paper and would perhaps be a good value addition.	<p>Broader context in introduction, in lines 24-33, 56-64.</p> <p>Studies focussed on speciation through life history traits, in lines 93-100</p>	24 56 93
AE	2	Reviewer 2's second comment is about the mate choice experiments being conducted under "no choice" conditions. My reading of the manuscript (lines 165-167) suggested that this was not the case, and actually there were two males + one female (or vice versa) in the set up. When contacted, the reviewer agreed that there is a possibility that they have misunderstood this part. I would like the authors to confirm that my reading is correct. If yes, nothing needs to be	The Associate Editor is correct, mate choice assays involved two males + one female (or vice versa).	

		done. If I am wrong and the reviewer is indeed correct, then obviously that concern will have to be addressed in discussion.		
AE	3	Reviewer 2's third comment is about the conduct of the group mating assay in vials which limits the amount of space available for remating. This comment is in line with the authors' own admission that "In the group mating assays, vials were more crowded (32 individuals vs. 3) - potentially creating circumstances where resisting forcible mating-attempts is more difficult for females." Therefore, a quick mention of the possibility that the reviewer brings up (using a bigger arena like a glass petri plate or a small box would have reversed this result) seems to be a good idea. This is also consistent with how the authors themselves have speculated about the result, and therefore, I do not foresee any major issues here.	Please see the response to reviewer 2's 4 <sup>th</sup> comment.	
AE	4	Here there was a potential misunderstanding on my part. The authors have written (Line 199-200) that "we evaluated female mate choice in vials containing 10 females and 12 males from each regime.". I interpreted this to mean there were $(10+12) \times 2 = 44$ flies, and therefore was confused how there could only be 10 possible matings (line 203). After some re-reading, I realized that perhaps there were $10 + 12 \times 2 = 34$ flies per vial, which is consistent with 10 matings. Some rephrasing will be helpful here. Also, if my understanding is correct, should it not be 34 flies in line 500 as opposed to 32?	We thank the Associate editor for pointing this out. There were 34 flies, not 32 (we forgot how to add!). This has been updated in line 579. Only female choice was studied in this assay. In each group mate choice vial, 10 individuals of one female type were exposed to 12 of both types of males.	579