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## Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in *Drosophila melanogaster*

Khushboo Sharma, Nalini Mishra and Mallikarjun N. Shakarad

**Review timeline**

| | |
|---|---|
| Original submission: | 19 December 2019 |
| 1st revised submission: | 30 March 2020 |
| 2nd revised submission: | 19 May 2020 |
| Final acceptance: | 21 May 2020 |

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Note: This manuscript was transferred from another Royal Society journal without peer review.

# Review History

# RSOS-191910.R0 (Original submission)

## Review form: Reviewer 1

**Is the manuscript scientifically sound in its present form?**
No

**Are the interpretations and conclusions justified by the results?**
Yes

**Is the language acceptable?**
No

**Do you have any ethical concerns with this paper?**
No

**Have you any concerns about statistical analyses in this paper?**
Yes

**Recommendation?**
Major revision is needed (please make suggestions in comments)

**Comments to the Author(s)**
This is an interesting paper that explores the effect of prolonged selection for accelerated developmental-time on the critical size phenomenon, using Drosophila melanogaster as a model organism. The authors selected for accelerated developmental time and extended longevity across 231 generations, resulting in a reduction in adult body size and egg-to-adult developmental time. This is in turn correlated with a reduction in critical size, and pre- and post-critical size developmental time, as well as second-instar growth rates. The results of the research are generally clear and support the hypothesis that selection for accelerated development reduces critical size.

Nevertheless, there are a number of relatively substantive problems with the MS that need to be addressed before publication.

1) The authors suggest that the terms minimal viable weight (MVW) and critical size (CS) are interchangeable, while several researchers have demonstrated that they are distinct phenomena. The authors correctly define CS as the size above which starvation does not alter the time course to metamorphosis. The CS is a therefore a physiological switch in the effect of nutrition on the hormone cascade that ends on metamorphosis, and the molecular genetic mechanisms underlying the switch have been well elucidated. The MVW for pupariation (MVWpupariation) is the minimum weight at which >50% of starved larvae pupariate, while the MVW for eclosion (MVWeclosion) is the minimum weight at which >50% of starved larvae eclose as adults. Both of these therefore reflect a larva's nutritional status and its ability to mobilize stored resources and survive to different points in development upon starvation. While the three phenomena are correlated, they are not the same. Generally MVWpupariation< MVWeclosion<CS (see Stieper et al 2008 and Hironaka et al 2019 for more on this). Further, only Drosophila researchers confound CS and MVW: in M. sexta for example, CS is the time at which starvation no longer affects the time course to metamorphosis.

While I think that it is fine forr the authors to use MVWeclosion as a proxy for CS (as it has been used in various publications, e.g. Mirth et al 2014, Hironaka et al 2019), it adds confusion if the distinction is not clearly stated in the text. Further, it is conceivable that an observed reduction in MVWeclosion may not be accompanied by a reduction in CS sensu stricto. Problematically, while the CS phenomenon is well understood at a physiological level, the MVW phenomenon is not, although it likely involves the initiation of ecdysteroidgenesis by factors other than nutrition, through a bail-out response or a 'leaky' prothoracic gland (see Nijhout et al 2014 WIRE Dev Bio for more details). Thus, while the observed response to selection may indeed reflect changes in the mechanisms that initiate ecdysteroidgenesis at attainment of CS, it is also possible that they reflect changes in the ability to initiate a bail-out response, although these mechanisms remain unknown. Without distinguishing between CS and MVW, the distinction is lost.

2) L64 "larvae monitor their size by the relative growth of various organs in proportion to each other [4]". The cited paper does not support the statement (indeed I do not known of any paper that does).

3) L67 "Critical size is suggested to evolve in response to environmental conditions. If the environmental conditions are conducive, then slower growth along with larger body size as a consequence of larger critical size is favoured by natural selection; while under non-conducive environment, faster growth with the smaller critical size is selected". This statement has unclear logic. All traits evolve by natural selection in response to 'environmental conditions [20]'. The question is what is the selective pressure and what is it targeting. Does low nutrition select for smaller body size or accelerated growth? And does it target critical size directly, or is critical size a consequence of selection on body size/growth rate? See Hironaka et al 2019 for more details on

how selection may act to change critical size. Further, the citation is for amphibians and not holometabolous insects.

4) L80. Please provide citations for the selection experiments.

5) L90. Here you correctly argue that the change in critical size is a correlated response to selection for accelerated development. However, in the title you state "Higher instantaneous growth during second larval instar leads to lower critical size in Drosophila melanogaster populations", which is not supported by your data, since both growth rate and change in critical size are a correlated response to changes in developmental time. The title should be changed to reflect this.

6) In the methods you describe the selection regime, and provide a helpful figure. However, you do not explicitly state how many generations you have maintained selection apart from in the introduction. You should state this in the M&M section also.

7) L130. This section is a little difficult to follow. Problematically you use the term 'standardized flies' (L138) before you define it (L143).

8) Minor point: The sampling method for calculating MVW/CS is rather complex. A figure would be helpful to show how larvae were sampled at each time point.

9) L249. The authors report the MVW/CS as 1002.66 $\mu$g which is likely much more precise than the microbalance that they are using. It would be useful to adjust the number of significant figures to reflect the precision of the microbalance.

10) L256 "Furthermore, there was no significant effect of the availability of food on the post-critical developmental duration of larvae in both the selected and control populations" This is an interesting result since many studies have shown that starvation at CS accelerates pupariation in Drosophila (e.g. Steiper et al 2009). Does this reflect the relatively imprecise method used to calculate CS/MVW? The challenge with the methodology used is that even though eggs were collected in a 1h cohort, developmental stage can vary considerably by the time larvae get to the 3rd instar (Ashburner discusses this in his book). Thus the average mass of a cohort of larvae at 70h AEL may hide considerable variation in size. Further, the authors only calculated MVW/CS at a resolution of every 2 hours. Other methods to calculate MVW/CS (e.g. using logistic regression, Steiper et al 2009, Hironaka et al 2019) appear to be more precise and may have been able to detect the acceleration in developmental time upon starvation at CS/MVW.

11) L305 "However, the post-critical duration in the populations selected for faster pre-adult development has significantly reduced- suggesting that these populations are likely to have evolved mechanisms so as to have higher fitness compared to their ancestral control populations." I do not follow the logic of this argument. Why would this lead to higher fitness? Does the author mean higher fitness under the specified selection pressure?

12) L312 "A couple of studies in Drosophila [10, 11] have shown the critical size to be static with respect to nutrition, thus supporting the above suggestion. However, many studies over the past three decades have shown a reduction in the adult size of D. melanogaster flies in response to lack of nutrition [25], increased growth temperatures [12] and selection for faster pre-adult development [24, 39]." This is rather misleading since the authors confound phenotypic plasticity with adaptation. The cited studies [1-,11] suggest that CS/MVW does not vary within a genotype in response to nutrition; that is, it is not nutritionally plastic. This does not mean that there is no genetic variation for CS/MVW nor that it cannot evolve.

13) L323 "Here we show that populations under selection for faster pre-adult development have evolved significantly smaller critical size (Figure 2A) supporting the view that critical size in D. melanogaster is polyphenic under different growth conditions [19, 25]". Again, the authors

confound plasticity with adaptation. Polyphenism is the response of a phenotype to environmental conditions (strictly speaking two or more discrete phenotypes, e.g. castes of bees). Their data support the hypothesis that CS/MVW is genetically variable and can respond to selection, not that it is phenotypically plastic.

14) I encourage the authors to revise their figures. The colors are rather difficult to follow (a key in the figure would help). Also, it is increasingly standard to include all the data points in box plots and bar charts, to give the reader an idea of the distribution of data. Finally, the authors must specify what the error bars are and provide sample sizes for all statistical tests.

15) Statistical Analysis. Details of the statistical analyses need to be included. What statistical models were used the analyze the data? The authors used a GLM but did not specify the link function or probability distribution. How were the regression slopes compared? Why fit a linear regression when your state clearly in the introduction that growth prior to CS/MVW is exponential? Did you test for homoscedasticity?

16) L289 "In D. melanogaster body size is tightly correlated with development time, thus one would expect the body size distributions of the selected and control populations to be non-overlapping". Why would you expect it to be non-overlapping? Different, possible, but not necessarily non-overlapping. Also, I am not sure why the authors examined the impact of selection on body size distribution. Why not just apply a standard two-sample t-test to see whether body size is different?

# Review form: Reviewer 2 (Amitabh Joshi)

**Is the manuscript scientifically sound in its present form?**
Yes

**Are the interpretations and conclusions justified by the results?**
No

**Is the language acceptable?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Have you any concerns about statistical analyses in this paper?**
No

**Recommendation?**
Accept with minor revision (please list in comments)

**Comments to the Author(s)**
This is a nice piece of work. The experimental design is good and statistics are appropriate. The issue is one of interest, especially within the fly experimental evolution community. My main concerns are with the way the Introduction and Discussion are framed, which should be improved in a revision. I am attaching an annotated copy of the manuscript (Appendix A) with detailed comments to this end. Since I choose not to be anonymous, I urge the authors to feel free to contact me if they have any doubts when they undertake a revision.

# Decision letter (RSOS-191910.R0)

27-Feb-2020

Dear Dr SHAKARAD,

The editors assigned to your paper ("Higher instantaneous growth during second larval instar leads to lower critical size in Drosophila melanogaster populations") have now received comments from reviewers. We would like you to revise your paper in accordance with the referee and Associate Editor suggestions which can be found below (not including confidential reports to the Editor). Please note this decision does not guarantee eventual acceptance.

Please submit a copy of your revised paper before 21-Mar-2020. Please note that the revision deadline will expire at 00.00am on this date. If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available, we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.

In addition to addressing all of the reviewers' and editor's comments please also ensure that your revised manuscript contains the following sections as appropriate before the reference list:

• Ethics statement (if applicable)
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data have been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that have been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:

http://datadryad.org/submit?journalID=RSOS&manu=RSOS-191910

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Anita Kristiansen
Editorial Coordinator

Royal Society Open Science
openscience@royalsociety.org

on behalf of Professor Laura Johnston (Associate Editor) and Kevin Padian (Subject Editor)
openscience@royalsociety.org


Associate Editor's comments (Professor Laura Johnston):
Associate Editor: 1
Comments to the Author:
Two expert referees have now reviewed your work, "Higher instantaneous growth during second larval instar leads to lower critical size in Drosophila melanogaster populations". Both reviewers felt the work is of high quality and of significant interest, however, several changes are recommended prior to publication. Review 1, in particular, notes several important points that need to be addressed.  Reviewer 2 suggests making revisions to the text and has included suggestions in the text itself, including more details  regarding statistical methods.

Reviewers' Comments to Author:
Reviewer: 1

Comments to the Author(s)
This is an interesting paper that explores the effect of prolonged selection for accelerated developmental-time on the critical size phenomenon, using Drosophila melanogaster as a model organism. The authors selected for accelerated developmental time and extended longevity across 231 generations, resulting in a reduction in adult body size and egg-to-adult developmental time. This is in turn correlated with a reduction in critical size, and pre- and post-critical size developmental time, as well as second-instar growth rates. The results of the research are generally clear and support the hypothesis that selection for accelerated development reduces critical size.

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13) L323 "Here we show that populations under selection for faster pre-adult development have evolved significantly smaller critical size (Figure 2A) supporting the view that critical size in D. melanogaster is polyphenic under different growth conditions [19, 25]". Again, the authors

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14) I encourage the authors to revise their figures. The colors are rather difficult to follow (a key in the figure would help). Also, it is increasingly standard to include all the data points in box plots and bar charts, to give the reader an idea of the distribution of data. Finally, the authors must specify what the error bars are and provide sample sizes for all statistical tests.

15) Statistical Analysis. Details of the statistical analyses need to be included. What statistical models were used the analyze the data? The authors used a GLM but did not specify the link function or probability distribution. How were the regression slopes compared? Why fit a linear regression when your state clearly in the introduction that growth prior to CS/MVW is exponential? Did you test for homoscedasticity?

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Reviewer: 2

Comments to the Author(s)
This is a nice piece of work. The experimental design is good and statistics are appropriate. The issue is one of interest, especially within the fly experimental evolution community. My main concerns are with the way the Introduction and Discussion are framed, which should be improved in a revision. I am attaching an annotated copy of the manuscript with detailed comments to this end. Since I choose not to be anonymous, I urge the authors to feel free to contact me if they have any doubts when they undertake a revision.

# Author's Response to Decision Letter for (RSOS-191910.R0)

See Appendix B.

# RSOS-191910.R1 (Revision)

## Review form: Reviewer 1

**Is the manuscript scientifically sound in its present form?**
Yes

**Are the interpretations and conclusions justified by the results?**
Yes

**Is the language acceptable?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Have you any concerns about statistical analyses in this paper?**
No

**Recommendation?**
Accept with minor revision (please list in comments)

**Comments to the Author(s)**
1) The authors state: "In holometabolous and some hemimetabolous insects, the process of initiation of metamorphosis is dependent on attaining a certain minimum threshold size called critical size [7-10] beyond which starvation does not alter the time course to metamorphosis [10, 11-17]. Critical size, also called minimum critical size, is similar to minimal viable weight for eclosion in Drosophila melanogaster [16, 18]. Hence, throughout this study we have used the term 'critical size/weight'. "

While I appreciate the authors clarifying the difference between MVW[ecolision] and critical size, this change rather misses the point. The authors define the critical size as the point at which starvation does not alter the time course to metamorphosis, but do not measure this. Rather they measure the size at which 50% of starved larvae survive to eclosion, which is the MVW[eclosion]. It is imprecise to say that MVW[eclosion] is 'similar' to critical size/weight. Rather Drosophilists use MVW[eclosion] as a proxy for critical size weight, which is what the authors do in their study. Thus the penultimate sentence of this paragraph should read something like: "In Drosophila, the size at which 50% of starved larvae successfully eclose as adults (the minimal viable weight for eclosion) is used as a proxy for critical size [16, 18]. We use this proxy for critical size/weight in this study."

2) The authors state: '....in Manduca sexta - a Lepidopteran holometabolous insect, larvae between 4th instar and 5th instar stage, whose head capsule size was greater than 5.4 mm were able to successfully pupate else undergo one more molt to 6th instar thus monitor their size by the growth of head capsule in proportion to body size.'

This is a slight overstatement of the conclusions of the cited paper. Nijhout observed a correlation between head capsule size and probability of pupating, and hypothesized that the larvae are monitoring their size by growth of the head capsule (which only grows between instars). However, this was a hypothesis. I am aware of no subsequent study that has explored this hypothesis further. I would therefore modify this sentence to say that the data 'suggest' that larvae monitor their size by the growth of head capsule.

# Decision letter (RSOS-191910.R1)

12-May-2020

Dear Dr SHAKARAD:

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-191910.R1 entitled "Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in Drosophila melanogaster." has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and Subject Editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:
http://datadryad.org/submit?journalID=RSOS&manu=RSOS-191910.R1

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Please note that we cannot publish your manuscript without these end statements included. We have included a screenshot example of the end statements for reference. If you feel that a given

heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript before 21-May-2020. Please note that the revision deadline will expire at 00.00am on this date. If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create a Revision." You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees.

When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document".
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://figshare.com). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Andrew Dunn
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Professor Laura Johnston (Associate Editor) and Kevin Padian (Subject Editor)
openscience@royalsociety.org

Reviewer comments to Author:
Reviewer: 1

Comments to the Author(s)
1) The authors state: "In holometabolous and some hemimetabolous insects, the process of initiation of metamorphosis is dependent on attaining a certain minimum threshold size called critical size [7-10] beyond which starvation does not alter the time course to metamorphosis [10, 11-17]. Critical size, also called minimum critical size, is similar to minimal viable weight for eclosion in Drosophila melanogaster [16, 18]. Hence, throughout this study we have used the term 'critical size/weight'. "

While I appreciate the authors clarifying the difference between MVW[ecolision] and critical size, this change rather misses the point. The authors define the critical size as the point at which starvation does not alter the time course to metamorphosis, but do not measure this. Rather they measure the size at which 50% of starved larvae survive to eclosion, which is the MVW[eclosion]. It is imprecise to say that MVW[eclosion] is 'similar' to critical size/weight. Rather Drosophilists use MVW[eclosion] as a proxy for critical size weight, which is what the authors do in their study. Thus the penultimate sentence of this paragraph should read something like: "In Drosophila, the size at which 50% of starved larvae successfully eclose as adults (the minimal viable weight for eclosion) is used as a proxy for critical size [16, 18]. We use this proxy for critical size/weight in this study."

2) The authors state: '....in Manduca sexta - a Lepidopteran holometabolous insect, larvae between 4th instar and 5th instar stage, whose head capsule size was greater than 5.4 mm were able to successfully pupate else undergo one more molt to 6th instar thus monitor their size by the growth of head capsule in proportion to body size.'

This is a slight overstatement of the conclusions of the cited paper. Nijhout observed a correlation between head capsule size and probability of pupating, and hypothesized that the larvae are monitoring their size by growth of the head capsule (which only grows between instars). However, this was a hypothesis. I am aware of no subsequent study that has explored this hypothesis further. I would therefore modify this sentence to say that the data 'suggest' that larvae monitor their size by the growth of head capsule.

# Author's Response to Decision Letter for (RSOS-191910.R1)

See Appendix C.

# Decision letter (RSOS-191910.R2)

21-May-2020

Dear Dr SHAKARAD,

It is a pleasure to accept your manuscript entitled "Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in Drosophila melanogaster." in its current form for publication in Royal Society Open Science.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org) and the production office (openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact -- if you are going to be away, please nominate a co-author (if available) to manage the proofing process, and ensure they are copied into your email to the journal.

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**Appendix A**

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## Higher instantaneous growth during second larval instar leads to lower critical size in Drosophila melanogaster populations

| | |
|---|---|
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| Keywords: | critical size, larval growth, adult body size, adaptive-bailout, accelerated pre-adult development |
| Subject Category: | Biology (whole organism) |
| | |

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31
32 *Conflict of interest*
33
34
35 I/We declare we have no competing interests
36
37 *Statement (if applicable):*
38 CUST_STATE_CONFLICT :No data available.
39
40 *Authors' contributions*
41
42
43 This paper has multiple authors and our individual contributions were as below
44
45 *Statement (if applicable):*
46 MS and KS conceived and designed the study.  KS and NM collected data and performed the
47 experiments. KS and MS analysed the data and wrote the manuscript. All authors gave final approval
48 for manuscript submission.
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60

# Title

**Higher instantaneous growth during second larval instar leads to lower critical size in *Drosophila melanogaster* populations**

Khushboo Sharma, Nalini Mishra and Mallikarjun N Shakarad[1]

Evolutionary Biology Laboratory, Department of Zoology, University of Delhi, Delhi-110007.

[1]Corresponding Author- email id: beelab.ms@gmail.com

36

## **Background**

38

39 Holometabolous insect species are characterized by two distinct phases in their life cycle *viz.,* the
40 pre-adult phase which consist of (i) larval and (ii) pupal stages; and adult phase. During the larval
41 life, the energy required for metamorphosis from larval to adult tissue and for the early adult life
42 is accumulated [1, 2, 3]. Further, the duration of the larval stages determines the final adult body
43 size. Contrary to common belief, unrestricted growth occurs even at the time of moulting due to
44 the presence of unsclerotized body surface [4]. However, the timing of metamorphosis imposes
45 restriction on larval duration which directly affects the final adult size and associated life-history
46 traits [5, 6]. Different mechanisms of final body size assessment exist in insects that are
47 prerequisite for metamorphosis initiation. In holometabolous and some hemimetabolous insects,
48 the process of initiation of metamorphosis is dependent on attaining a certain minimum threshold
49 size called critical size [7-10] beyond which starvation does not alter the time course to
50 metamorphosis [10, 11-17]. Critical size is similar to minimal viable weight in *Drosophila*
51 *melanogaster* [10, 11, 14, 18]. Hence, throughout this study we have used the term 'critical
52 size/weight'.

53 Critical size being an essential checkpoint during the larval life, acts as a developmental switch
54 for the irreversible process of metamorphosis [13, 14, 17]. The early phase of larval life consists
55 of the exponential growth phase that ends in the attainment of critical size while later-post critical
56 phase is marked by linear growth period on the arithmetic scale [19]. In *Drosophila* sp*.* the final
57 adult body size is determined during this post-critical phase. Thus the larval duration is split into
58 (i) pre-critical duration which is defined as the development time spent in attaining the minimum
59 size necessary to complete metamorphosis and emerge as an adult [10], and (ii) post-critical
60 duration, during which additional energy required for maximizing Darwinian fitness is acquired
61 [1,15]. Once critical size is attained variable size controlling mechanisms operate in different
62 species before they undergo metamorphosis indicating that these species have unique modes of
63 determining the body size with critical size at its core [4, 9, 19]. For example, in *Manduca sexta* -
64 a Lepidopteran holometabolous insect, larvae monitor their size by the relative growth of various
65 organs in proportion to each other [4]; while in *Oncopeltus fasciatus* -a Hemipteran
66 hemimetabolous insect, larval growth and its size is estimated by abdominal stretch receptors [9].
67 Critical size is suggested to evolve in response to environmental conditions. If the environmental
68 conditions are conducive, then slower growth along with larger body size as a consequence of

69   larger critical size is favoured by natural selection; while under non-conducive environment, faster

70   growth with the smaller critical size is selected [20]. *Drosophila melanogaster* is known to occupy

71   ephemeral habitat with limited food and high density and this holometabolous species is under

72   strong selection for faster pre-adult development [21]. Previously, it has been reported that

73   *Drosophila* populations under conscious selection for shorter pre-adult duration have reduced

74   body size [22- 25]. It has been speculated that critical size might reduce if exposed to conscious

75   selection for accelerated pre-adult development [24]. However, every extant species should have

76   evolved a species-specific critical size that has been optimised over the course of evolution, as

77   the critical size is crucial to survival itself. Previous studies have demonstrated evolution of critical

78   size in *Drosophila melanogaster* populations under conditions of malnutrition [25] and selection

79   for body size [26], thus exhibiting genetic variablity for the trait [11]. For example under direct

80   artificial selection for change in body size there is reduction of critical size while in another study

81   populations under nutritional stress leads to smaller critical size. In this study, we test the

82   hypothesis that selection for faster pre-adult development reduces the critical size in *Drosophila*

83   *melanogaster* [24].

84   We used six populations of *D. melanogaster*, of which three were ancestral controls maintained

85   on a 21-day discrete generation cycle and three were simultaneously selected for faster pre-adult

86   development and extended reproductive longevity. The control *Drosophila melanogaster*

87   populations had been through 232 generations of maintenance on 21 days, egg-to-egg discrete

88   generation cycles while the selected populations had been through 126 generations of

89   simultaneous selection for faster pre-adult development and indirect selection for extended adult

90   longevity at the time of initiation of these experiments. We first assessed the pre- and post-critical

91   duration and critical size in the control and selected populations. Then we evaluated the impact

92   of non-availability of food on the post-critical larval duration, pupal duration and adult body size in

93   control and selected populations. Further, we assessed the impact of selection on larval growth

94   rate. We found that the selected populations have evolved a significantly reduced pre- and post-

95   critical duration and smaller critical size as a correlated response to selection for faster pre-adult

96   development. Interestingly, the selected populations have higher growth rate during the second

97   larval instar suggesting that they might have preponed their growth owing to a very short post-

98   critical duration.

99

100   **Methods**

101   **a) Fly husbandry**

102  A total of six *Drosophila melanogaster* populations were used in this study. Of the six populations,
103  three were ancestral control, maintained on a 21-day egg-to-egg discrete generation cycle. The
104  other three were simultaneously selected for faster pre-adult development and extended adult
105  life-span. All six populations were maintained as outbred populations in Power Scientific Inc.
106  USA environmental chamber/incubators under standard laboratory conditions (SLC) of 25 ± 1 °C
107  temperature, 70 ± 5% RH (Relative Humidity) and 24:0 L:D (Light: Dark) cycle. The pre-adult
108  stages were reared in glass vials (9.5 cm × 2.3 cm) with 6 mL standard media-SM, (Table 1) and
109  the adults were reared in plexiglass cages (25 cm × 20 cm × 15 cm). The pre-adults were on a
110  single meal of SM in glass vials till emergence as adults, while the adults (in plexiglass cages)
111  were provided fresh SM every alternate day. All population cages were provided with yeast-acetic
112  acid supplement along with fresh SM 3 days prior to collection of embryos for starting the next
113  cycle. Each of the control populations was generated in 40 vials, with 50-60 eggs in 6 mL SM per
114  vial and incubated at SLC for 12 days in vials. All the emerging adults of a given population were
115  transferred to a clean, sterile pre-labelled population cage with a fresh plate of SM (Figure 1).
116  Selected populations were derived from corresponding ancestral controls by transferring 60-80
117  eggs into 6 mL SM vials under SLC. Egg density was kept low so as to avoid larval crowding [27],
118  and the difference in the egg densities of control and selected populations is marginal and is
119  unlikely to differentially affect any traits in the two population types. A total of 160 vials per
120  replicate population were set up. Only the first 15-20 flies emerging from each of 160 vials were
121  transferred to pre-labelled clean breeding cages through the process of 2 hourly vigil checks. The
122  initial population size of each of the selected populations was 2400-3200 individuals. In order to
123  avoid crowding during the adult stage, the emerging adult flies were maintained in two sister
124  cages, with each cage housing adults from 80 vials. Eggs for initiating the subsequent generation
125  were collected after 50% adult mortality was noticed in either of the cages, thus ensuring a
126  breeding population size of ~1600 flies (like control populations) at the time of egg collection for
127  initiation of next generation. The eggs from the two sister cages were mixed and redistributed into
128  160 vials to avoid independent evolutionary trajectories in the two sister cages (Figure 1).

129

130  **b) Generation of flies for experiments**
131  In order to remove the non-genetic parental effects, eggs were collected from both, selected and
132  control populations, and reared under similar conditions wherein the selection criteria were
133  relaxed in the selected populations prior to experimentation [22, 28]. Eggs were collected on a
134  sterile media plate and exact counts of 50 eggs were dispensed into vials with 6 mL of SM. Forty
135  such vials were maintained per population. Though selected populations were maintained at 60-

136    80 eggs per 6 mL SM in running stock, they are unlikely to experience scramble competition;

137    especially due to their reduced feeding rates [29]. Further, the marginal difference in the egg

138    density used for generation of standardised flies and that of running stocks is unlikely to influence

139    our results. The egg collection from the selected and control populations was staggered by the

140    developmental time difference to obtain adult flies of the same age. All emerging flies were

141    transferred to pre-labelled clean and dry population cages with SM plate either on day 10

142    (selected) or day 12 (control) from the day of egg collection. These flies are referred to as

143    standardized flies. In the experiments that required large number of ~~embryos~~, two sister cages of

144    standardized flies were generated by incubating 80 vials of 50 eggs each, per population.

145

**c) Larvae collection and fly media**

147    Prior to the collection of synchronized eggs, standardized fly populations were supplemented with

148    a generous amount of live-yeast and acetic acid paste for 3 days to boost their egg-laying. After

149    3 days, they were provided with fresh sterile SM plate for one hour (h) and at the end of 1 h, SM

150    plate was replaced by uncontaminated non-nutritive agar plate at every one-hour interval for 3

151    successive intervals. SM and first two agar plates were discarded and eggs laid on 3rd agar plate

152    (4th plate in the series) hereafter referred to as "synchronised eggs" were used in all experiments

153    unless otherwise mentioned.

154    The composition (Table 1) and preparation of the standard media are as specified in

155    Chandrashekara and Shakarad [30]. In the present experiments, in addition to SM for

156    maintenance of populations, liquid media (without agar-agar) was prepared to facilitate the

157    sampling of larvae with ease and hence called as Liquid Standard Media –LSM (Table 1).

158

**d) Critical size, post-critical duration and body size (in terms of weight)**

160    Twenty-two hours post-egg-laying, 30 newly hatched (first instar) larvae were transferred to small

161    Petri-dishes (5.5 mm in diameter, Tarson) containing 2000 µL of LSM. Ten such plates were set

162    up per population per time point and incubated at SLC. The same is followed for other experiments

163    unless stated otherwise. Through pilot experiments, the average duration to attain critical size

164    (*aka* minimum viable size) was estimated to be 62 h and 74 h for the selected and control

165    populations respectively. Hence, the sampling of larvae for this experiment was initiated at 60 h

166    and 70 h for the selected and control populations respectively. The sampling consisted of

167    harvesting 300 larvae each from the selected and control populations. The larvae were washed

168    with RO (Reverse Osmosis) water (in order to remove food particles sticking to the body surface)

169    and rolled on a tissue towel to remove excess water. Thereafter the larvae were sorted into 10

170 groups of 30 individuals each. Five groups were transferred to 5 pre-labelled vials containing 5

171 mL of non-nutritive agar and incubated at SLC while the other 5 groups were weighed on Citizen

172 (CM11) micro-balance. The entire process of harvesting larvae, sorting them into batches,

173 weighing and incubating was repeated at every 2 h interval till they started wandering. We adopted

174 the criterion described in previous studies [10, 11] with some modifications, for calculation of

175 critical size time point. The average critical size was the weight at which at least 50% of larvae

176 undergo metamorphosis into adults even under non- availability of food. The total developmental

177 time by which this weight is attained is referred to as 'critical duration'.

178 Average post-critical duration of both population types was estimated as the difference in the time

179 lag between the attainment of critical size and the average duration to pupation under availability

180 of *ad libitum* food. Synchronised first instar larvae from non-nutritive agar plates were harvested,

181 washed and transferred to 5 mL SM vials. The vials were incubated at SLC. At the pre-determined

182 critical duration (estimated from previous experiment) the larvae were re-harvested and either

183 transferred to non-nutritive agar vials or SM vials and incubated again under SLC. Five vials each

184 with 30 larvae per treatment per population were set up. Time duration from critical duration till

185 pupation was calculated by observing larvae at two-hour interval till no further fresh pupations

186 were observed. The number of pupae was scored and recorded. Emerging adults from both

187 treatment vials were sorted based on gender and weighed on microbalance in order to estimate

188 body size differences (in terms of weight).

189

### e) Larval growth rate, feeding rate and development time

191 In this experiment, synchronised eggs were collected, transferred to Petri-plates containing a thin

192 film of LSM on 10% agar base and incubated under SLC. Triplicate sample of each of the six

193 populations with twenty larvae per replicate per time point was washed, rolled on tissue towel and

194 weighed on microbalance at every 4 h interval till the pupation time point. The first reading was

195 taken at 24 h from the mid-point of synchronized egg collection window- marked as zero-hour

196 reading. Weight of an average single larva was deduced by dividing the group larval weight by

197 the number of larvae.

198 Post-development of red eyespots in pupae, vials were checked at every four-hour interval for the

199 emergence of adult flies. The emerging flies were collected into pre-labelled empty dry vials,

200 sorted according to gender under mild $CO_2$ anaesthesia and recorded in data books. The mid-

201 point between two successive 4 h checkpoints was taken as the time of emergence. The average

202 development time was estimated from this primary data. The flies of a given treatment and gender

203 were pooled and held in neatly labelled clean dry vials, freeze killed at -80 °C and five replicate

204 groups of flies per gender per population were weighed to obtain the size of the fly measured as

205 fresh weight.

206 A colourimetric assay was performed [31, 32] with some modifications, to assess feeding rate

207 during different larval time points. For feeding rate assay, the newly hatched larvae from the

208 synchronized batch of eggs were transferred to LSM in batches of 100 larvae per petri-dish. The

209 Petri-dishes were incubated at SLC in Power Scientific, USA, Incubators. In all, there were 9 such

210 Petri dishes per population. Three Petri-dishes per population were taken out after 12 h

211 incubation, for L1 (mid-L1). Fifty larvae per population were harvested from the plates, washed

212 with RO water, rolled over tissue towel and transferred to fresh agar plates overlaid with 2 mL

213 double-distilled water. The larvae were allowed to be in double-distilled water for 10 minutes. After

214 this starvation period of 10 minutes, the larvae were rolled on Kimwipe towel and transferred to

215 fresh agar plate overlaid with 5 mL of 4% (w/v) blue dye (Erioglaucine disodium salt –Sigma

216 Aldrich) mixed with 2 g yeast. The larvae were allowed an acclimatization period of 2 min,

217 subsequent to which they were allowed to feed on the dye-yeast mix for 2 h. Immediately after 2

218 h interval, chilled (4 °C) water was poured on to the larvae to arrest further feeding. Larvae were

219 washed twice with distilled water to remove debris and rolled on Kimwipe tissue towel. A batch of

220 50 larvae was homogenized in 500µL of PBS (1X). Samples were centrifuged at 13,5000 rpm

221 (Eppendorf, 5430R) for 10 min. Then 100 µL of supernatant was used for OD (Optical density)

222 reading. Absorbance was taken at 625 nm on ELISA plate reader (ECIL micro scan MS5605A).

223 The entire process from harvesting of larvae till measuring of OD was repeated at 36 (mid-L2)

224 and 48 h for selected, and 36 and 52 h for control populations post transfer of freshly hatched L1

225 to LSM. The time durations chosen were believed to have caught the larvae in mid-L1, mid-L2

226 and L3 (prior to attainment of critical size) stages respectively. The differential time point was

227 chosen for the L3 so as to assess larvae of similar physiological age [24].

228

229 **f) Statistical Analysis**

230 Univariate analysis of Variance, under General linear model (GLM) using SPSS version 22 [33]

231 was carried out on critical, post-critical and pupal duration; feeding rate and adult weight with

232 treatment and selection as fixed factors and replications as random factors. Since, in all cases,

233 the population means were used as the units of analysis only fixed-factor effects and interactions

234 could be tested for significance [24].

235 To understand the impact of selection on the growth rate, linear regression analysis was

236 performed on the larval stage-specific weight gain with L1- 24 h: initial 6 data points, L2- 24 h:

237  data points 7-12 and L3: 13$^{th}$data point and beyond [34]. The regression slope 'b' of the three

238  larval stages were compared between selected and control populations [35, 36].

239  We also ascertained the impact of selection on body size distribution by fitting a normal probability

240  density function:

$$f(x) = \frac{1}{\sigma 2\pi} e^{-}(x_i - \mu)/2\sigma^2$$

241

242  {Where s represented standard deviation (σ), s$^2$ represented variance (σ²), x represented mean

243  (μ), x$_i$ = (μ+ σ) or (μ+ 2σ) or (μ+ 3σ).  π =3.14, $e$ = 2.71}

244

## **Results**

246  **a)  Selection for accelerated development leads to the evolution of smaller critical size**

247  There was significant effect of selection on the critical size ($F_{1, 2}$ = 24.45, p = 0.0385; Figure 2A)

248  and critical duration ($F_{1, 2}$ = 192.66, p = 0.0034; Figure 2B). The selected populations attained

249  their critical size at an average wet weight of 1002.66 μg in an average duration of 62.5 h

250  compared to their ancestral control whose average wet weight was 1308.71 μg attained in 74 h.

251  A reduction of 23.38% in critical weight was attained with a reduction of 15.31% in critical

252  developmental duration.

253  Further, there was significant impact of selection on post-critical developmental duration ($F_{1, 2}$ =

254  344.32, p = 0.003; Figure 2C). The developmental duration, post- attainment of critical size was

255  reduced by 56.8% in the selected populations as compared to their ancestral control populations.

256  Furthermore, there was no significant effect of the availability of food on the post-critical

257  developmental duration of larvae in both the selected and control populations ($F_{1, 2}$ = 0.763, p =

258  0.473; Figure 2C). In addition, the reduction in the pupal duration was also non-significant ($F_{1, 2}$ =

259  5.960, p = 0.135; Figure 2D) between the selected (86.36 h) and control (89.96 h) populations.

260  Overall, the egg to adult development time significantly ($F_{1, 2}$ = 363.701, p = 0.003; Figure 2D)

261  reduced by 17.5% in selected populations compared to their ancestral controls. An average adult

262  from populations under selection for faster pre-adult development took 188.34 h to eclose from

263  the egg, while control populations took 228.3 h to eclose.

264

**b) Selection for accelerated pre-adult development affects larval growth rate at second instar**

The reduction in the critical size associated with a reduction in larval developmental duration of the selected compared to the control populations could be a correlated response without any change in the larval growth rate. To address this, the larval growth (measured as wet weight) trajectories of the two population types were ascertained at every 4 h intervals from the time of hatching till pupation (Figure 3A). Linear regression analysis of the three larval stages showed no significant difference in the slope during the L1 (First 24 h, t = 0.98; Figure 3A) and L3 stages (Post 48 h till wandering stage mid-point, t = 0.16; Figure 3A; Table 2). However, the slope of the selected populations was significantly higher than that of their ancestral control during the L2 stage (t = 3.54, p < 0.01; Figure 3A; Table 2). The increased growth rate was not due to increase in the feeding rate that was not significantly different between the selected and control populations ($F_{1,2}$ = 16.14, p = 0.057; Figure 3B).

**c) Impact of selection for accelerated pre-adult development on adult body size and its distribution**

We found a significant reduction ($F_{1,2}$ = 35.682, p = 0.027; Figure 4A) in the fresh/wet weight of adults as a function of selection. There was a reduction of 19.13% in the wet weight of an average fly from the selected populations (689.57µg) in comparison to an average fly from control population (852.73µg) when they had access to *ad libitum* food. Further, there was a significant effect of feeding regimen on the wet weight of the flies ($F_{1,2}$ = 498.54, p = 0.002; Figure 4A). The overall reduction in the weight of the flies that emerged after feeding only up to critical duration in comparison to those that fed till they naturally wondered off to pupate was 51.33%. There was no selection × feeding duration interaction effect ($F_{1,2}$ = 0.031, p = 0.877).

In *D. melanogaster* body size is tightly correlated with development time, thus one would expect the body size distributions of the selected and control populations to be non-overlapping. In order to test this hypothesis, we constructed a normal distribution using a normal probability density function. The normal probability distribution of the adult size (measured as wet weight at emergence) of the flies from the selected populations was shifted to the left of the normal probability distribution of control population flies (Figure 4B).

**Discussion**

In *D. melanogaster,* although intricate regulatory hierarchy is suggested to respond to variations

299    in nutrient availability and ensure uniformity of species-specific final body size [37], selection for

300    faster pre-adult development have reported a significant reduction in body size [22, 24, 38]. The

301    reduced adult size concomitantly resulted in reduced lifespan and fecundity [3,8]. However, in an

302    earlier study, we reported higher fecundity in flies that were significantly smaller [39]. In *D.*

303    *melanogaster*, it has also been reported that much of the resources that are utilized for survival

304    during metamorphosis and early adult activities are acquired in the late L3 stage, post attainment

305    of critical size and are stored in larval fat bodies [1, 2]. However, the post-critical duration in the

306    populations selected for faster pre-adult development has significantly reduced- suggesting that

307    these populations are likely to have evolved mechanisms so as to have higher fitness compared

308    to their ancestral control populations.

309    Adult body size in insects is an important fitness governing trait that is determined by three factors:

310    (i) the number of larval instars, (ii) the size increment at each larval moult, and (iii) the size at

311    which the last larval instar stops feeding and initiates metamorphosis [40]. Many earlier studies

312    have suggested the critical size to be species-specific [12, 25]. A couple of studies in *Drosophila*

313    [10, 11] have shown the critical size to be static with respect to nutrition, thus supporting the above

314    suggestion. However, many studies over the past three decades have shown a reduction in the

315    adult size of *D. melanogaster* flies in response to lack of nutrition [25], increased growth

316    temperatures [12] and selection for faster pre-adult development  [24, 39]. The decreased adult

317    size could be due to reduction in the number of larval instars, decrease in the size increment at

318    each larval moult, and or the size at which the last larval instar stops feeding and initiates

319    metamorphosis. An additional factor that can alter the final adult size is the duration of each of

320    the larval stages. A reduction in the duration of 1st and 3rd larval instar contributing to reduction in

321    final adult size has been reported by Prasad *et al.* [24]. Contrary to many studies [11, 12], Prasad

322    *et al*. [24] suggested that the critical size in *D. melanogaster* can evolve under selection for faster

323    pre-adult development. Here we show that populations under selection for faster pre-adult

324    development have evolved significantly smaller critical size (Figure 2A) supporting the view that

325    critical size in *D. melanogaster* is polyphenic under different growth conditions [19, 25]. *Drosophila*

326    *melanogaster* inhabits ephemeral environment where nutritional conditions are deteriorating

327    continuously thus under the constant pressure of faster development. Larvae exposed to poor

328    dietary condition since the start of larval life exhibit higher metabolic efficacy and accelerated

329    development rate through evolution of smaller critical size [25].   *Scathophaga stercoraria* like

330    *Drosophila melanogaster* adopt adaptive bail out under the condition of continuously deteriorating

environment and accelerates its development along with lower critical size attainment indicating critical weight to be the target of selection in the yellow dung fly *Scathophaga stercoraria* [41]. The body size is a highly plastic trait, influenced by both genotype as well as the environment. The plasticity of body size helps the organisms to survive fluctuating food availability, both quantitatively and qualitatively [42]. A general phyletic trend in the evolution of larger body size among insects has been through decrease in the number of larval instars accompanied by size increment at each larval instar [40]. However, adaptive evolutionary processes operate on the variation present within a population rather than in different populations let alone species, unless they occupy similar/identical niches. In *M. sexta*, evolution of large body size is accompanied by an increase in size increment and not an increase in the number of larval instars [43]. Contrary to the expectation based on phyletic trend and data on *M. sexta*, the reduced adult size in our study was not accompanied by reduction in size increment at each larval moult (Figure 3). As opposed to previous study where lines selected for small body size grew slowly [26], populations under selection for faster pre-adult development demonstrated higher growth rate than control populations. There was no significant difference in the larval growth rate of the selected populations and their ancestral control populations during the first as well as the third larval instars (Figure 3B). Vijendravarma et al. [25] also reported no difference in the growth trajectories of their selected and control populations till attainment of critical size. However, the growth rate of the faster developing populations in our study was significantly higher during the second larval instar, unlike that of Partridge et al. [26] study where the growth rate were lower for the smaller adult size selections and higher for the larger adult size selections. The increased growth rate during second larval instar accompanied by reduced critical size could be responsible for the significant reduction in the post-critical duration of the larvae in our selected populations. The observed differences in the growth rates in the three studies might be due to the difference in the genotypes of the populations under study and/or genuienly indicate the dynamic nature of responses due to different components being the target of selection. The reduction in adult body size could be due to reduction in the critical size and/or reduction in the post-critical duration. This could be another form of adaptive-bailout [44] as in the case of *S. stercoraria* in response to food limitation [41, 44]. The populations under selection for faster development might be exhibiting adaptive-bail out [44] not due to external food limitation but due to internal trigger.

**Conclusion**

365   Overall, our study provides the experimental evidence for increased larval growth rate specifically

366   in a developmentally important stage of second instar ==leading to a reduced critical size== and

367   eventually reduced adult size in *Drosophila melanogaster* populations under selection for faster

368   development.

369

370   **Data availability**

371   Data is available on Dryad Digital Repository with the following link

372   https://doi.org/10.5061/dryad.k6djh9w32 [45]

373

374   {Reviewer URL: https://datadryad.org/stash/share/4dXJwU3WSx-

375   xFAZVFm15zTrFMNvVj5u9jBMFWiP17h0}

376

377   **Authors' contributions**

378   MS and KS conceived and designed the study. KS and NM collected data and performed the

379   experiments. KS and MS analysed the data and wrote the manuscript.

380

381   **Competing interests**

382   Author declares no competing interests.

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394   **References**

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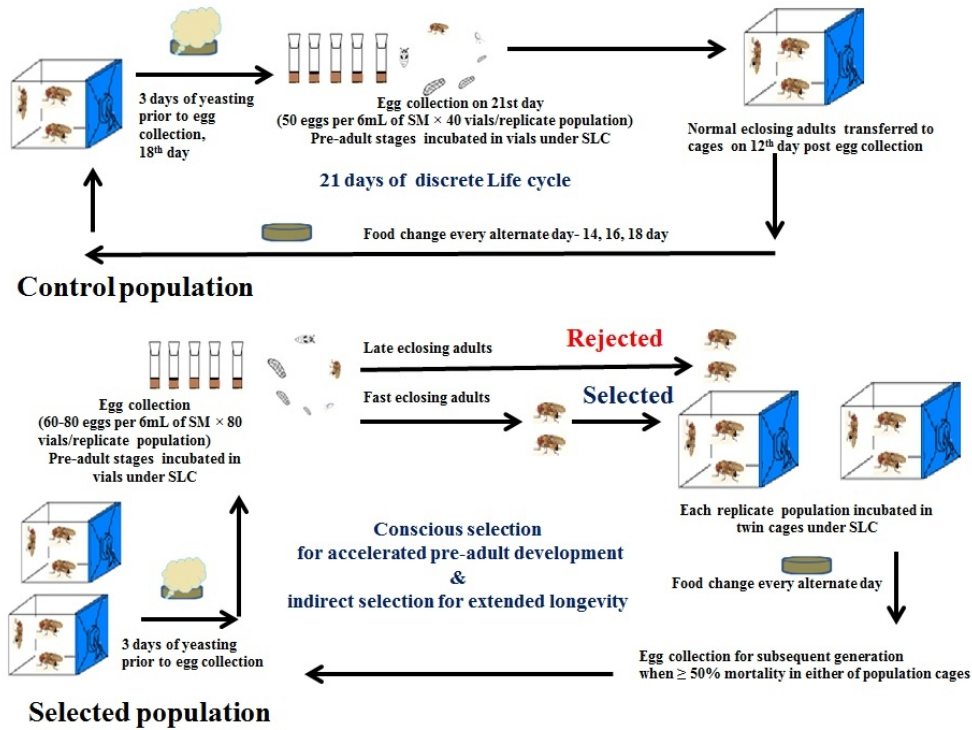
Figure 1 Schematics of control and selected population life cycle. The control population was maintained on 21 days of egg-to-egg discrete life cycle, one cage per population for adult stages. The selected population is under conscious selection for accelerated development and indirect selection for extended longevity. The selected population is maintained in twin cages per replicate to avoid over-crowding during adult stage. Both control and selected populations are maintained under SLC in Power Scientific USA incubators.

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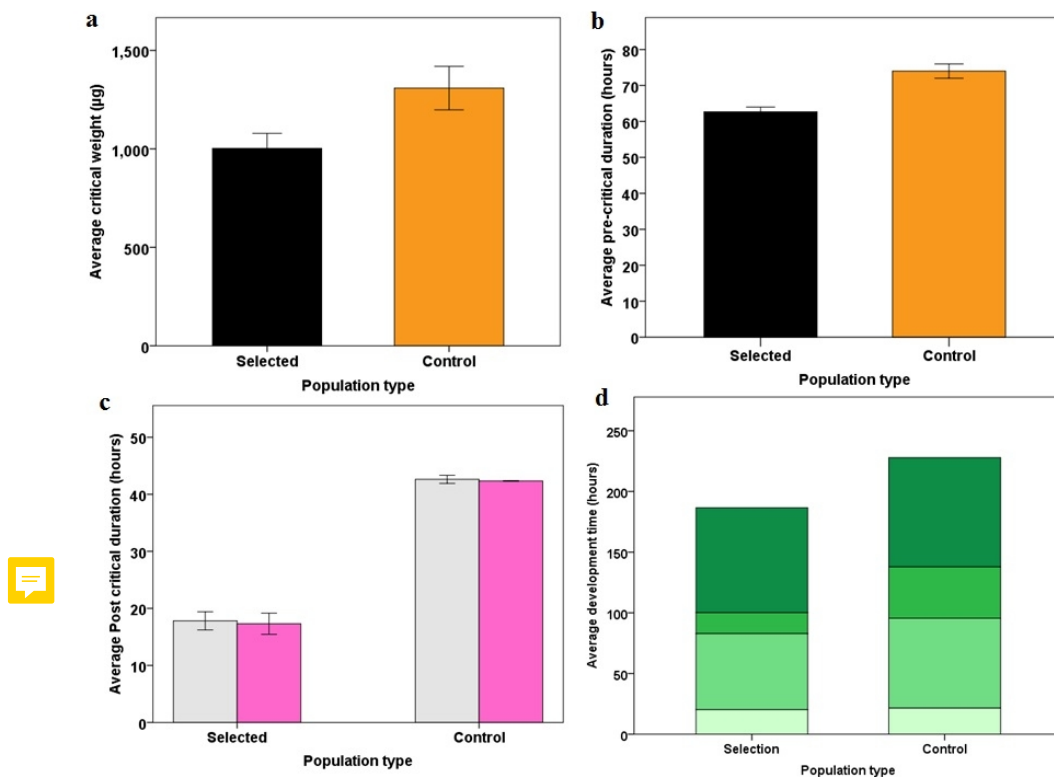
Figure 2 (a) Average critical size, (b) Average pre-critical duration, (c) Average post-critical duration, and (d) Average development duration from egg to adult eclosion. The black bar represents selected population and orange stands for control population. Grey bar, pink bar represents food availability until critical size time point only and ad libitum food during larval duration in both populations respectively. Different shades of green bars from light to dark stands for egg, pre-critical, post-critical and pupal duration respectively.

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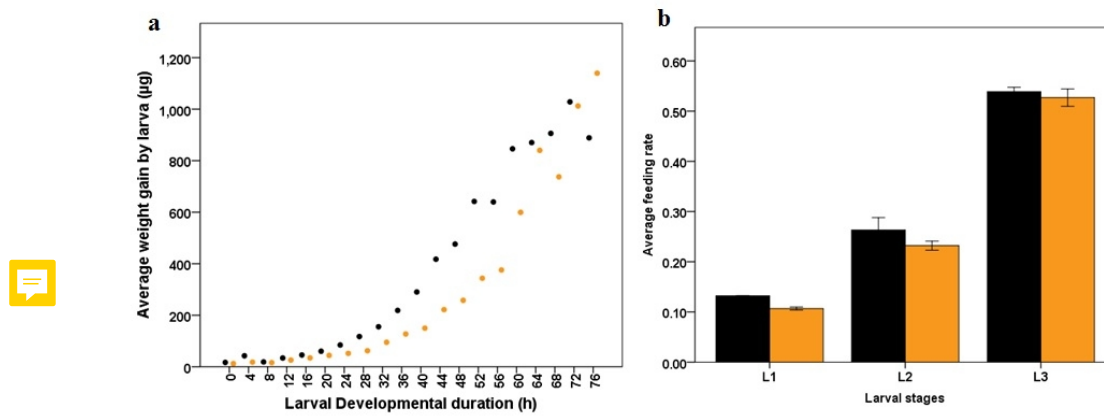


Figure 3 (a) Larval growth rate in terms of weight gain in control population (orange) is up to 108 h prior to pupation and in the selected population (black) is maximal up to 76 h and then it undergoes metamorphosis. (b) Larval feeding rates at L1, L2 and L3 stages.

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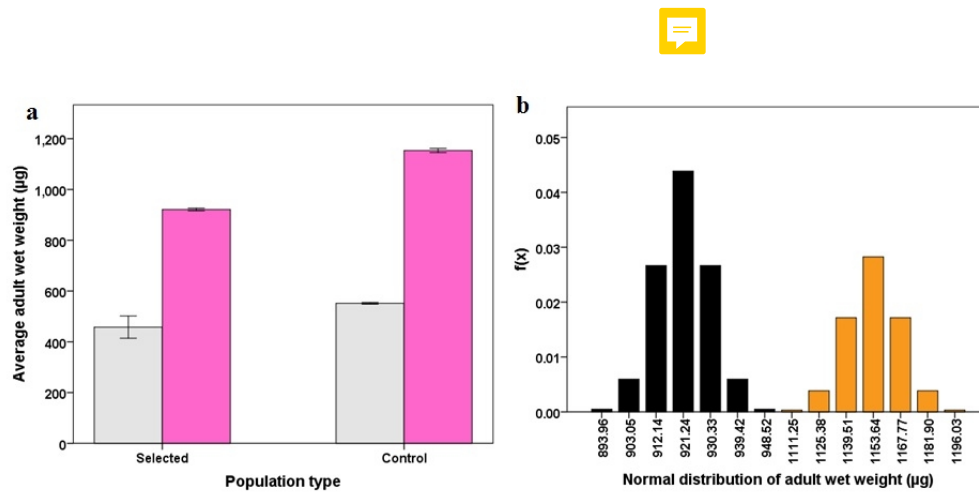
Figure 4 (a) Adult body weight (μg), Grey- when larvae were on non-nutritive agar post attaining critical weight, and Pink- when larvae fed till they naturally stopped feeding and wandered to pupate. (b) Adult weight probability density functions of selected (black) and control (orange) populations.

246x115mm (96 x 96 DPI)

| Diet composition (1 L) | Standard Media (SM) | Liquid Standard Media (LSM) |
|---|---|---|
| Water | 1180 mL | 1180 mL |
| Banana | 205 g | 205 g |
| Jaggery | 35 g | 35 g |
| Barley flour | 25 g | 25 g |
| Yeast | 36 g | 36 g |
| Methyl paraben | 2.4 g | 2.4 g |
| Ethanol | 45 mL | 45 mL |
| Agar-agar | 12.5 g | Zero |

Table 1 **Diet composition:** Standard media and Liquid standard media differ only with respect to agar-agar composition.

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| Larval duration- β values | Control populations | Selected populations | t- values |
|---|---|---|---|
| L1- Zero to 24h post hatching | 1.66 | 2.17 | 0.97 |
| L2- 24h to 48h post hatching | 8.62 | 17.21 | 3.54 |
| L3- 48h to wandering stage mid- point* | 49.77 | 29.49 | 0.16 |

*For selected populations, as L3 is of small duration thus midpoint value is considered for the analysis. L1, L2 and L3 stand for first, second and third larval stages.

Table 2 **Regression table:** β value of control and selected populations and respective t values at different larval stages.

# Appendix B

1) The authors suggest that the terms minimal viable weight (MVW) and critical size (CS) are interchangeable, while several researchers have demonstrated that they are distinct phenomena. The authors correctly define CS as the size above which starvation does not alter the time course to metamorphosis. The CS is a therefore a physiological switch in the effect of nutrition on the hormone cascade that ends on metamorphosis, and the molecular genetic mechanisms underlying the switch have been well elucidated. The MVW for pupariation (MVWpupariation) is the minimum weight at which >50% of starved larvae pupariate, while the MVW for eclosion (MVWeclosion) is the minimum weight at which >50% of starved larvae eclose as adults. Both of these therefore reflect a larva's nutritional status and its ability to mobilize stored resources and survive to different points in development upon starvation. While the three phenomena are correlated, they are not the same. Generally MVWpupariation< MVWeclosion<CS (see Stieper et al 2008 and Hironaka et al 2019 for more on this). Further, only Drosophila researchers confound CS and MVW: in M. sexta for example, CS is the time at which starvation no longer affects the time course to metamorphosis.

While I think that it is fine for the authors to use MVWeclosion as a proxy for CS (as it has been used in various publications, e.g. Mirth et al 2014, Hironaka et al 2019), it adds confusion if the distinction is not clearly stated in the text. Further, it is conceivable that an observed reduction in MVWeclosion may not be accompanied by a reduction in CS sensu stricto. Problematically, while the CS phenomenon is well understood at a physiological level, the MVW phenomenon is not, although it likely involves the initiation of ecdysteroidgenesis by factors other than nutrition, through a bail-out response or a 'leaky' prothoracic gland (see Nijhout et al 2014 WIRE Dev Bio for more details). Thus, while the observed response to selection may indeed reflect changes in the mechanisms that initiate ecdysteroidgenesis at attainment of CS, it is also possible that they reflect changes in the ability to initiate a bail-out response, although these mechanisms remain unknown. Without distinguishing between CS and MVW, the distinction is lost.

Reply:
L48-50: We agree with the reviewer's reasoning and accordingly made the necessary changes in the text. We had used the term critical size (CS) and minimum viable weight

(MVW) interchangeably as in the manuscript (L48-50) as it is widely used in *Drosophila* community. We have now modified the terminology to Minimum viable weight for eclosion instead of MVW and cited Steiper et al., 2008 and Hironaka et al., 2019.

2) L64 "larvae monitor their size by the relative growth of various organs in proportion to each other [4]". The cited paper does not support the statement (indeed I do not known of any paper that does).

Reply:

There was an error in citing the research article. We have now corrected the error and cited appropriate reference (Nijhout 1975- A Threshold Size for Metamorphosis in the Tobacco Hornworm, Manduca sexta (L.). Accordingly, the correction now reads (L62-65) '….in *Manduca sexta* - a Lepidopteran holometabolous insect, larvae between 4th instar and 5th instar stage, whose head capsule size was greater than 5.4 mm were able to successfully pupate else undergo one more molt to 6th instar thus monitor their size by the growth of head capsule in proportion to body size.'

3) L67" Critical size is suggested to evolve in response to environmental conditions. If the environmental conditions are conducive, then slower growth along with larger body size as a consequence of larger critical size is favoured by natural selection; while under non-conducive environment, faster growth with the smaller critical size is selected". This statement has unclear logic. All traits evolve by natural selection in response to 'environmental conditions [20]'. The question is what is the selective pressure and what is it targeting. Does low nutrition select for smaller body size or accelerated growth? And does it target critical size directly, or is critical size a consequence of selection on body size/growth rate? See Hironaka et al 2019 for more details on how selection may act to change critical size. Further, the citation is for amphibians and not holometabolous insects.

Reply:

We agree with the reviewer in that 'all traits in response to environmental conditions'. We have now modified the section starting with L67 to read as 'Critical size is suggested to evolve in response to environmental conditions. For example in Drosophila genus, large sized species like *D. repleta* have higher critical size- which is larger than the final larval

size of small sized *D. willistoni*. Critical size change, thus, can be one of the drivers of adult body size evolution (Hironaka et al., 2019) [18].'

4) L80. Please provide citations for the selection experiments.
Reply:
L79 In accordance with the text we cited appropriate research papers- Vijendravarma et al., 2011 [25] and Partridge et al., 1999 [26].

5) L90. Here you correctly argue that the change in critical size is a correlated response to selection for accelerated development. However, in the title you state "Higher instantaneous growth during second larval instar leads to lower critical size in Drosophila melanogaster populations", which is not supported by your data, since both growth rate and change in critical size are a correlated response to changes in developmental time. The title should be changed to reflect this.

Reply:
We thank the reviewer for the kind appreciation. As per both the reviewers' suggestion, we have adopted the title suggested by 2$^{nd}$ Reviewer and the modified title is:

"Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in *Drosophila melanogaster.*"

6) In the methods you describe the selection regime, and provide a helpful figure. However, you do not explicitly state how many generations you have maintained selection apart from in the introduction. You should state this in the M&M section also.

Reply:
We have added the generation number in Materials and methods section in L146-149.

7) L130. This section is a little difficult to follow. Problematically you use the term 'standardized flies' (L138) before you define it (L143).

Reply:

As per the reviewer's suggestion we have reorganized the text and explicitly defined the 'standardized flies' from L129 to L136 prior to its use.

8) Minor point: The sampling method for calculating MVW/CS is rather complex. A figure would be helpful to show how larvae were sampled at each time point.

Reply:
We have added a figure (Figure 1*c*) for the larval sampling method as suggested by the reviewer.

9) L249. The authors report the MVW/CS as 1002.66 µg which is likely much more precise than the microbalance that they are using. It would be useful to adjust the number of significant figures to reflect the precision of the microbalance.

Reply:
We agree with the reviewer that we would not be able to accurately measure any biological parameter. However, the precision of microbalance (Citizen- CM 11) used in our experiments is up to 5 decimal places but we had rounded off the data values to two decimals places only.

10) L256 "Furthermore, there was no significant effect of the availability of food on the post-critical developmental duration of larvae in both the selected and control populations" This is an interesting result since many studies have shown that starvation at CS accelerates pupariation in Drosophila (e.g. Steiper et al 2009). Does this reflect the relatively imprecise method used to calculate CS/MVW? The challenge with the methodology used is that even though eggs were collected in a 1h cohort, developmental stage can vary considerably by the time larvae get to the 3rd instar (Ashburner discusses this in his book). Thus the average mass of a cohort of larvae at 70h AEL may hide considerable variation in size. Further, the authors only calculated MVW/CS at a resolution of every 2 hours. Other methods to calculate MVW/CS (e.g. using logistic regression, Steiper et al 2009, Hironaka et al 2019) appear to be more precise and may have been able to detect the acceleration in developmental time upon starvation at CS/MVW.

Reply:

We agree that average mass would mask the variability. However, we do not agree with the reviewer that our method is imprecise. Measurements at every two hour interval is based upon a pilot run done prior to assay initiation (replicated thrice). It is humanly not possible to proceed with the experiment at less than two hour interval. As handling of larvae itself takes enormous time thus prolonging the process. This would have resulted in greater variability due to ever changing larval growth. At every time point, we handled a total of 600 larvae (300 larvae per selection line) for weighing and incubation at SLC as per the protocol used.

Further, we agree with the reviewer that no experimental design can remove inter-individual variability, at best one can attempt to minimize it and that is what we have attempted achieve through our experimental protocol.

11) L305 "However, the post-critical duration in the populations selected for faster pre-adult development has significantly reduced- suggesting that these populations are likely to have evolved mechanisms so as to have higher fitness compared to their ancestral control populations." I do not follow the logic of this argument. Why would this lead to higher fitness? Does the author mean higher fitness under the specified selection pressure?

Reply:
We have modified the statement to read '…suggesting that these populations are likely to have evolved mechanisms such as, energy acquisition, storage and utilization during the adult stage due to long adult life owing to the selection protocol, and thus have fitness comparable to their ancestral control populations (Handa et al. 2014) [40]. Incidentally, there was no fitness cost in terms of viability during the pre-adult stages (Supplementary figure 1) suggesting that the long adult life-span seems to have mitigated the viability cost during the pre-adult stage of our selected (FLJ) populations, unlike that reported by Prasad et al. [24] in their FEJ populations selected for faster pre-adult development and early reproduction (L314-323).

12) L312 "A couple of studies in Drosophila [10, 11] have shown the critical size to be static with respect to nutrition, thus supporting the above suggestion. However, many studies over the past three decades have shown a reduction in the adult size of D. melanogaster flies in response to lack of nutrition [25], increased growth temperatures [12]

and selection for faster pre-adult development [24, 39]." This is rather misleading since the authors confound phenotypic plasticity with adaptation. The cited studies [1-,11] suggest that CS/MVW does not vary within a genotype in response to nutrition; that is, it is not nutritionally plastic. This does not mean that there is no genetic variation for CS/MVW nor that it cannot evolve.

Reply:

We have removed all citations that were dealing with phenotypic plasticity and restricted our citations to selection studies throughout the discussion section.

13) L323 "Here we show that populations under selection for faster pre-adult development have evolved significantly smaller critical size (Figure 2A) supporting the view that critical size in D. melanogaster is polyphenic under different growth conditions [19, 25]". Again, the authors confound plasticity with adaptation. Polyphenism is the response of a phenotype to environmental conditions (strictly speaking two or more discrete phenotypes, e.g. castes of bees). Their data support the hypothesis that CS/MVW is genetically variable and can respond to selection, not that it is phenotypically plastic.

Reply:

We have restricted our discussion section to include studies pertaining to adaptation only and modified the text accordingly.

14) I encourage the authors to revise their figures. The colors are rather difficult to follow (a key in the figure would help). Also, it is increasingly standard to include all the data points in box plots and bar charts, to give the reader an idea of the distribution of data. Finally, the authors must specify what the error bars are and provide sample sizes for all statistical tests.

Reply: We have revised our figures and amended them as per both reviewer's suggestions.

15) Statistical Analysis. Details of the statistical analyses need to be included. What statistical models were used to analyze the data? The authors used a GLM but did not

specify the link function or probability distribution. How were the regression slopes compared? Why fit a linear regression when your state clearly in the introduction that growth prior to CS/MVW is exponential? Did you test for homoscedasticity?

Reply:
We included the statistical analysis details as per both reviewers' comments.
We fitted linear regression and slopes were compared using t test (L243-247).

16) L289 "In D. melanogaster body size is tightly correlated with development time, thus one would expect the body size distributions of the selected and control populations to be non-overlapping". Why would you expect it to be non-overlapping? Different, possible, but not necessarily non-overlapping. Also, I am not sure why the authors examined the impact of selection on body size distribution. Why not just apply a standard two-sample t-test to see whether body size is different?

Reply:
L297: We have provided the actual data (figure 4*a*). Since we did not weigh individual flies, we tried to recapture the population distribution based on theoretical model so as to have a near realistic picture of the distribution of adult body sizes in the two types of populations. The body size distributions of the two populations were non-over lapping (figure 4*b*). These results are similar to those reported based on the actual wing measurements reported by our laboratory (Handa et al., 2014) [40]. This will also provide us a view how selection for faster pre-adult development actually affect the body weight as there is no difference in body size at critical size (figure 2*c* and 4*a*) implying the significance of post-critical duration in determining the final adult body size (Hironaka et al., 2019) [18].

Reviewer: 2

This is a nice piece of work. The experimental design is good and statistics are appropriate. The issue is one of interest, especially within the fly experimental evolution community. My main concerns are with the way the Introduction and Discussion are framed, which should be improved in a revision. I am attaching an annotated copy of the manuscript with detailed comments to this end. Since I choose not to be anonymous, I urge the authors to feel free to contact me if they have any doubts when they undertake a revision.

Reply:

We are grateful to the reviewer Prof. Amitabh Joshi for his appreciation of our work.

1) It is not at all clear that the lower critical size is BECAUSE of the higher growth rate during the 2nd instar. Indeed, one could argue that faster growth could permit a faster attainment of the same critical size, thereby not requiring a reduction in critical size while achieving a reduction in critical feeding time, in the context of selection for reduced egg to adult development time. Why not title it something like "Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in Drosophila" and frame the story in terms of "faster dev can be achieved by faster growth, leading to attaining minimum size faster, and/or by reducing the minimum size"

Reply:

We are thankful to the reviewer for suggesting a more appropriate title. Title of the manuscript has been changed as per both the reviewers' suggestion.

The new title is "Evolution of reduced minimum critical size as a response to selection for rapid pre-adult development in *Drosophila melanogaster*".

2) Do you mean replicate populations? If so, were they treated as random blocks (for ancestry/handling) or as random nested replicate populations within selection regimes?

Reply:

Yes, we mean replicate populations and accordingly were treated as random blocks (for ancestry/handling) as in Prasad et al., 2001 [24], Rajamani et al., 2006 [28].

3) Not clear why you did this as opposed to simply showing the actual distribution of measured body sizes.

Reply:

We have provided the actual data (Figure 4*a*). Since we did not weigh individual flies, we tried to recapture the population distribution based on theoretical model so as to have a near realistic picture of the distribution of adult body sizes in the two types of populations. The body size distributions of the two populations were non-over lapping (Figure 4*b*). These results are similar to those reported based on the actual wing measurements reported by our laboratory (Handa et al., 2014) [40].

4) (L260) This actually suggests an overall reduction in average rate of weight gain during the 1st and 2nd instars, since a lot of that critical size weight is lost for a disproportionally smaller reduction in time taken to critical weight. This needs to be addressed.

Reply: We have discussed this elaborately in the discussion (L 350-363). The higher growth rate could possibly be another reason for the lack of pre-adult viability cost in our selected populations. The higher pre-adult mortality in Prasad *et al.* [24] could perhaps be due to lower growth rate in their selected (FEJ) populations. Further, a reduction of 23.38% in critical weight with a reduction of only 15.31% in critical developmental duration suggests that the control (JB) populations might be gaining disproportionately higher weight during the last 12 hours prior to reaching critical size (Supplementary table 1). Unlike the study of Prasad *et al.* where selection for faster development leads to reduction in pupal duration [24], we found no significant difference in the pupal duration of our selected (FLJ) populations (figure 2*d*). The differences in the results of the two studies despite the ancestral populations being the same, could be due to the differences in the selection pressure in the adult phase. The FEJ populations [24] are under pressure to maximize their fitness by day 3 post emergence while the selected (FLJ) populations used in this study have long adult life. The importance of such cross-life-stage effects in life history evolution have been reviewed in Prasad and Joshi [43].

5) Since the selected populations have retained about 45% of their post-critical duration, a time when weight gain rises quite rapidly, it is not clear what is gained by increasing the growth rate in the 2nd instar. Had the selected populations basically evolved to pupate after attaining

critical size, the pressure to increase 1st or 2nd instar growth rate would be more intuitively understandable. This should be taken up in the Discussion.

Reply:

Perhaps the increased growth rate is helping in putting on more weight and buffering them from paying pre-adult viability cost. We have discussed this in detail (L350-363). Besides, the 45% retention in post-critical duration may not necessarily be sufficient enough to build up the required energy reserves.

6) No reduction in pupal duration is very different from the obs in the FEJ populations of Prasad et al 2001, which is referenced, This needs to be addressed in the Discussion.

Reply:

We have discussed the differences in the results of the two studies in detail (L356-363). In now reads 'Unlike the study of Prasad *et al.* where selection for faster development leads to reduction in pupal duration [24], we found no significant difference in the pupal duration of our selected (FLJ) populations (Figure 2*d*). The differences in the results of the two studies despite the ancestral populations being the same, could be due to the differences in the selection pressure in the adult phase. The FEJ populations [24] are under pressure to maximize their fitness by day 3 post emergence while our selected (FLJ) populations have long adult life. The importance of such cross-life-stage effects in life history evolution have been reviewed in Prasad and Joshi [43].'

7) Feeding rate (sclerite retractions per min) in the FEJ and FLJ populations used in earlier studies was reduced, relative to controls. Here, of course, you are measuring something different i.e. food ingestion rate but, nevertheless, it would be good to discuss this discrepancy between previous studies and the present one. One speculation I have is that 2 h is long enough for the gut to get fully loaded with the dyed food, and this allows slower feeding selected population larvae to "catch up". Some discussion of this is needed.

Reply:

L212-233, L282-284 & L370-377- We agree with the observation and interpretation of Prof. Joshi with respect to the feeding rate discrepancies in the two studies and modified the terminology to 'larval food ingestion assay' and discussed the results accordingly.

8) You should explain why this round-about method is used, as opposed to just showing the reader the body size distributions in the actual data.

Reply:

We have provided the actual data (figure 4*a*). Since we did not weigh individual flies, we tried to recapture the population distribution based on theoretical model so as to have a near realistic picture of the distribution of adult body sizes in the two types of populations. The body size distributions of the two populations were non-over lapping. These results are similar to those reported based on the actual wing measurements reported by our laboratory (Handa et al., 2014) [40].

9) Unclear what you are trying to convey here. Is it the idea that these selected populations having a long adult life before egg-collection have time to feed and put on lipids and hence can sacrifice post-critical feeding without fitness consequences? Incidentally, how is the pre-adult mortality across the selected and control populations?

Reply:

Yes, we meant the non-significant fitness difference despite having lower weight at eclosion (Handa et al., 2014) [40]. We have clearly discussed it in the revised version of the manuscript. For pre-adult mortality, we found non-significant difference in the survival of pre-adult stages till adult eclosion ($F_{1,2} = 4.75$, $p= 0.161$ for larva to adult eclosion up to critical sized fed larva then eclosed as adults; $F_{1,2} = 2.31$, $p=0.268$ for larva fed up to natural pupation time and eclosed as normal sized adults). We have added the data as supplementary figure (Supplementary figure 1) and we have discussed the implications of these results too (L319-323).

10) To my mind, this Discussion is a bit dissipated. Changes in larval instar number would seem largely irrelevant to Drosophila. It would be better to organize the discussion of each major Result you have: critical size, critical duration, feeding rate, body size around a comparison of previous work (FEJs) and what we might predict an optimal pre-adult life-history for your selected lines to be.

Reply:

In the revised manuscript we have restricted discussion on interpreting our results and relevant literature and made it more precise.

11) Not clear why you think that critical size reduction is because of increased 2nd instar growth rate. The data do not address any causal link between the two and, on the face of it, they would appear to be two independent results in response to the selection.

Reply:

Again, as per both reviewers' kind suggestions we have modified the conclusion and title of the manuscript.

# Appendix C

**Comments to the Author(s)**

1) The authors state: "In holometabolous and some hemimetabolous insects, the process of initiation of metamorphosis is dependent on attaining a certain minimum threshold size called critical size [7-10] beyond which starvation does not alter the time course to metamorphosis [10, 11-17]. Critical size, also called minimum critical size, is similar to minimal viable weight for eclosion in Drosophila melanogaster [16, 18]. Hence, throughout this study we have used the term 'critical size/weight'. "

While I appreciate the authors clarifying the difference between MVW [eclosion] and critical size, this change rather misses the point. The authors define the critical size as the point at which starvation does not alter the time course to metamorphosis, but do not measure this. Rather they measure the size at which 50% of starved larvae survive to eclosion, which is the MVW [eclosion]. It is imprecise to say that MVW [eclosion] is 'similar' to critical size/weight. Rather Drosophilists use MVW [eclosion] as a proxy for critical size weight, which is what the authors do in their study. Thus the penultimate sentence of this paragraph should read something like: "In Drosophila, the size at which 50% of starved larvae successfully eclose as adults (the minimal viable weight for eclosion) is used as a proxy for critical size [16, 18]. We use this proxy for critical size/weight in this study."

**Reply:**

We agree with the reviewer's reasoning and accepted the text suggested by the Reviewer and have replaced the sentences in L48-50. The penultimate sentence of this paragraph should read something like: "In *Drosophila*, the size at which 50% of starved larvae successfully eclose as adults (the minimal viable weight for eclosion) is used as a proxy for critical size [16, 18]. We use this proxy for critical size/weight in this study."

2) The authors state: '....in Manduca sexta - a Lepidopteran holometabolous insect, larvae between 4th instar and 5th instar stage, whose head capsule size was greater than 5.4 mm were able to successfully pupate else undergo one more molt to 6th instar thus monitor their size by the growth of head capsule in proportion to body size.'

This is a slight overstatement of the conclusions of the cited paper. Nijhout observed a correlation between head capsule size and probability of pupating, and hypothesized that the larvae are monitoring their size by growth of the head capsule (which only grows between instars). However, this was a hypothesis. I am aware of no subsequent study that has explored this hypothesis further. I would therefore modify this sentence to say that the data 'suggest' that larvae monitor their size by the growth of head capsule.

**Reply:**

We have changed the sentence in L64-65 as suggested by the Reviewer. The sentence now reads as 'thus the data suggest that larvae monitor their size by the growth of head capsule [20]; ….'