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PROCEEDINGS B

Doublesex mediates species-, sex-, environment- and trait-specific exaggeration of size and shape

Patrick T. Rohner, David M. Linz and Armin P. Moczek

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Review timeline
Original submission: 29 January 2021
Revised submission: 6 April 2021
Final acceptance: 1 June 2021

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

Review History

RSPB-2021-0241.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Comments to the Author (Rohner et al. Proceedings of the Royal Society B)

Main comments

The authors tried to find "master mediator gene" for coordinating sex- nutrition- and segment-specific growth in sexually-dimorphic beetles by using RNAseq based comparison. As results, they identified doublesex gene as candidate for "master mediator" (context-dependent master regulator). By knocking down dsx via RNAi, nutritional specificity and segment specificity in trait growth were weakened, which suggests both of nutrition specificity and segment specificity are governed by dsx expression.

I think aim of the study is clear and experimental approach is suitable for their question. Well designed experiments and analyses support their conclusion. I think the manuscript can be published in the journal after minor revision.

Specific comments
Abstract:
Line 20
On term of "context-dependent master regulator", I think this term does not correctly describe doublesex function. Considering that context-dependent dsx expression likely to be regulated by multiple upstream factors (nutrition dependent endocrinal signals and Hox gene dependent segment identity), "master regulator" sounds somewhat overstatement. How about "master mediator"?

Materials and Methods:
Line 104
Authors wrote that pronotum width were used as estimate of size.
Is there any sexual difference of pronotum size and/or shape? Considering that males have longer foreleg, pronotum muscle volume likely to be different between sexes. If pronotum size shows sexual dimorphism, authors should use alternative character as size estimate.

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Line 205 "Nutritional plasticity caused the weakest change in tibia length"
Is this true? According to Fig. 1C, low nutrition male tibia (ii) is even shorter than female tibia (iii). This result can be interpreted as nutrition difference can affect tibia length more than sexual

difference.

Conclusion:
I think it is better to add conclusion diagram to describe position of dsx as regulator (mediator) for coordinating context-dependent trait growth.

Other minor suggestions:
Line 1, Line 252, Line 294, Line 310
"Doublesex" and "Dsx" should be "doublesex" and "dsx"

Line 307
"regulation of dsx" should be "regulation of dsx expression" for making it clearer.

I could not find any supplementary figures in PDF for review. So, I could not judge a part of results.

Review form: Reviewer 2

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

Scientific importance: Is the manuscript an original and important contribution to its field?
Acceptable

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Acceptable

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
Yes

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
No

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Rohner et al examine the developmental, genetic and evolutionary processes underlying a secondary sexual trait, also known as exaggerated trait. To do so, they focus their experiments on the fore tibia of the beetle Digitontophagus gazella, where males have a much longer tibia of the forelegs compared to females. The foreleg tibia of the females, however, is thicker and presents a particular shape. The foretibia of the male is highly condition dependent, whereby males reared on rich food have a significantly longer tibia than those reared on poor food. The scaling relationship between fore tibia and body is hyper-allometric.
The authors generated populations of males that were fed on rich or poor diet. They dissected the fore and hind limb tibia from those, but also from females that were said to have been raised on 'high resource availability.' The authors sequenced these samples (six replicates each) and conducted a comparative transcriptomics analysis (using an assembled transcriptome as a reference) to extract transcripts that are differentially expressed between treatments (rich vs poor; male vs female; fore vs hind limb). From this analysis, the authors come to conclude that the treatment that leads to the smallest divergence in fore tibia size (nutrition) shows the highest number of differentially expressed genes. They also interrogated these transcriptomics datasets in search for genes that are common (in terms of expression profiles) between various contexts where the trait becomes exaggerated (nutrition, serial homology, sex). They found little overlap between these contexts, which led them to conclude that there is a disparity in the developmental pathways underlying trait exaggeration in each of these contexts.

Next, the authors focused their attention on the gene doublesex (dsx), a major developmental regulator of sex determination whose role is quite conserved across insects. Dsx was enriched in the fore tibia of males both when compared to the hind tibia and when well-fed males were compared to poorly fed ones. The authors were also able to distinguish between male and female dsx isoforms. Following a series of RNAi experiments, the authors examined the role of dsx in the growth of the fore leg tibia. They found that dsx RNAi reduces fore limb tibia in males and increases it in females reared in standard diet. They also did this experiment following diet treatment and found that dsx RNAi had a more pronounced effect on well fed individuals. Finally, the authors asked if the observed results of dsx were common to another beetle that does not show exaggerated fore limbs; Onthophagus taurus. Based on quantitative difference of dsx RNAi phenotypes between the two beetles, the authors conclude that the role of dsx in trait exaggeration is labile and that there was a divergence in the function of dsx that explained the differences in the degree of sexual dimorphism between species.

This paper addresses a fundamentally important question dealing with how diversification of sexually dimorphic characters is mediated through the intricate interaction between genetics and the environment (here nutrition). I think it would be of interest to the audience of Proc. B. I have however a number of issues that the authors might want to address and hopefully increase the quality of this manuscript.

Methods:
1- Sampling: The authors state (lines 116-119) that the males were reared in poor or rich food condition. In line 119, that the females were reared on 'high resource availability.' Why is this stated this way? Were these samples treated differently at different times or was this one designed experiment? Please clarify. And if it is the latter, please make it clear and discuss the potential limitation of having treated differently samples that are meant to be compared.

2- Dissections: the tibiae were dissected from the imaginal discs. This is a hard task that will inevitably introduce biases between samples. Can the authors acknowledge this?

3- Reference transcriptome: There is a lot of critical information missing. The authors state in line 127 that a full transcriptome was assembled. From what raw data? Was it the combined

samples of the tibias? Was it from specific libraries meant for full transcriptome assembly? What coverage? What read length? What are the metrics of this full transcriptome (N50; BUSCOs; missing; duplicated etc…). If the reference transcriptome was assembled from the 24 tibia samples, this would be a heavily truncated dataset. The authors would want a reference transcriptome that has the highest representation possible of the gene set of this species. It is hard to make reliable claims with this information missing. Please provide the methods and the metrics of your reference transcriptome and make sure it is actually sufficiently representative (BUSCO over 90%). The fasta file supplemented with in the SOM along with the raw data should be submitted to a public database and accession numbers supplemented with the paper.

4- Comparative transcriptomics: I couldn't find any data describing the sequencing raw results (coverage, read length etc…). More importantly, the authors need to look closer at their replicates and see if they all make sense or whether there are outliers. Finally there is no explanation about possible batch effects and corrections for those, if necessary.

5- Doublesex RNAi: This experiment typically makes the dimorphism (whatever it is) reduced or entirely removed. One main difficulty is knowing the sex of the individuals at the moment of injection so they are not confused when they hatch. How did the authors deal with this? Were they able to sex the larvae before injection? If not, are they able to discriminate males and females that hatch with dsx RNAi phenotypes? If so, could the authors please describe how and preferably provide images in the supplement showing this?

6- In figure 2, I wonder why the authors chose to do box plots rather than the more informative scaling relationship as in Figure 1. Is this because of low number of individuals?

7- Dsx will reduce dimorphism in any trait and this effect is going to be stronger with higher dimorphism. Just as male fore tibia goes closer to female's, the opposite is also true (Fig. 2). So the role of dsx is not necessarily restricted to the exaggerated trait, but affects any trait that is dimorphic. Fig 2D also shows that the dimorphism in O. taurus in weak, so is dsx RNAi. These realities of dsx should be acknowledged throughout the manuscript.

8- The lability of dsx function: I am not at all convinced by the argument that, because dsx RNAi effect is stronger in the horns than in the tibia, its function must have diverged. Again, the horns are much more dimorphic than the tibia and the result is not surprising. In addition, RNAi by nature being a partial depletion of transcripts, it is hard to conclude about divergence in function from a quantitative effect. This part is one of the weakest in this paper and should be stricken. The authors are pushing the interpretation of RNAi too far. Specific experiments are needed to test this, including expression data and genetic interactions across species and tissues.

9- Lines 323-326: There seems to be a confusion between sexual conflict and trade-offs. Sometimes the authors write 'intralocus sexual conflict' and other times just 'intralocus conflict.' Could the authors please be more accurate? If we are talking about conflict between the sexes, that is intralocus sexual conflict. If the authors refer to within sex (benefit of horns for mating success and their cost for survivorship or manoeuvrability), then we are talking about trade-offs.

10- Line 331: I couldn't follow the argument about epistasy, and I certainly didn't see any data showing this. Can the authors please elaborate. Do they mean that dsx isoforms would have different interactions based on environment?

Decision letter (RSPB-2021-0241.R0)

24-Mar-2021

Dear Dr Rohner,

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

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 Associate Editor, 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. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage. I would also ask you to make sure that the Abstract and Title are understandable to the broad general-biology readership of Proceedings B (see my detailed comments below).

To submit your revision please log into http://mc.manuscriptcentral.com/prsb 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.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Please see our Data Sharing Policies (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession

number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. 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. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions, please do not hesitate to get in touch.

Best wishes,
Professor Loeske Kruuk
Editor
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Thank you for the submission of your manuscript. The two reviewers and I see much to like about this work. It is a fascinating study. However, the reviewers have voiced a number of suggestions and queries. I agree with many of their comments and also have wondered about your choice of measurement for body size. Based on the reviews, much work is needed, including a careful response to each point they bring up.

Editor (LK)
Comments to Author(s):
I note that the two versions of the abstract (in the pdf versus in the Abstract box) are quite different; the latter works better for a general audience, but I do still have some comments. It

reads clearly for the first three sentences, but these are rather disconnected from the results sentence 'We show...', which is too dense to be generally accessible. I would reduce the first three sentences and provide more explanation of what the results are. Also, you mention antagonistic selection twice in this version, but I'm not clear if you have an evidence of antagonistic selection in this system?

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
Comments to the Author (Rohner et al. Proceedings of the Royal Society B)

Main comments

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Referee: 2

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4- Comparative transcriptomics: I couldn't find any data describing the sequencing raw results (coverage, read length etc…). More importantly, the authors need to look closer at their replicates and see if they all make sense or whether there are outliers. Finally there is no explanation about possible batch effects and corrections for those, if necessary.

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Author's Response to Decision Letter for (RSPB-2021-0241.R0)

See Appendix A.

RSPB-2021-0241.R1 (Revision)

Review form: Reviewer 1 (Abderrahman Khila)

Recommendation
Accept as is

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I am quite satisfied with the new version of the paper. I have no further comments.

Decision letter (RSPB-2021-0241.R1)

01-Jun-2021

Dear Dr Rohner

I am pleased to inform you that your manuscript entitled "*Doublesex* mediates species-, sex-, environment-, and trait-specific exaggeration of size and shape" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Data Accessibility section
Please remember to make any data sets live prior to publication, and update any links as needed when you receive a proof to check. It is good practice to also add data sets to your reference list.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. 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.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Professor Loeske Kruuk
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member: 1
Comments to Author:
(There are no comments.)

Board Member: 2
Comments to Author:
(There are no comments.)

Appendix A

Dear Editors,

Thank you very much for the constructive feedback and for giving us the opportunity to submit a revised version of our manuscript entitled *Doublesex mediates species-, sex-, environment-, and trait-specific exaggeration of size and shape*. We have thoroughly revised our manuscript, added information, and ran additional bioinformatic analyses. All changes made to the text are tracked in the submitted version (highlighted in blue) except for changes in the numbering of the references.

We would like to apologize that the previously submitted version did not include all the supplementary files. To make sure that this information is now visible, the supplementary tables S1-S10 and figures S1-S9 are now attached to the main document.

Below we respond to each comment in detail (highlighted in bold).

Associate Editor

Board Member: 1

Comments to Author:

Thank you for the submission of your manuscript. The two reviewers and I see much to like about this work. It is a fascinating study. However, the reviewers have voiced a number of suggestions and queries. I agree with many of their comments and also have wondered about your choice of measurement for body size. Based on the reviews, much work is needed, including a careful response to each point they bring up.

Thank you very much for your generally positive response to our manuscript.

Regarding the body size estimate: There are two primary reasons why we use (logarithmized) pronotum width as a measure of overall size. Firstly, this is a standard measure of size in this group and helps comparing studies among each other. Secondly, and more importantly, this measure scales isometrically with overall size in both sexes when applying a multivariate morphometric framework (see Cheverud, 1982). In brief, we previously measured eight different linear traits that are not strongly exaggerated in either sex and performed a principal component analysis on the covariance matrix of all log-transformed raw values. Multiplying the loadings of each variable on the dominant eigenvector by the square root of the total number of traits renders each variable's relationship to an overall multivariate assessment of size (for more details see: (Cheverud 1982; Klingenberg 1996)). To put another way, this approach generates a classic allometric slope of trait size against a multivariate composite of overall body size.

	loading on PC1	Multivariate allometric slope (loading * sqrt(8))
pronotum width	**0.35**	**1.00**
pronotum length	**0.41**	**1.15**
elytra length	**0.25**	**0.71**
elytra width	**0.36**	**1.03**
metatibia length	**0.29**	**0.82**
profemur length	**0.45**	**1.26**
profemur width	**0.37**	**1.05**
head width	**0.31**	**0.87**

This effort suggests that pronotum width (β = 1.00), elytra width (β = 1.03), and profemur width (β = 1.05) scale isometrically (or nearly so) with body size and are thus a reasonable body size estimates in this species. Because the loading of pronotum width is identical to the value expected under isometry, we are confident that this measurement is a good index of body size.

We explicitly mention this now on lines 106-108 but do not go into much detail as this is a rather technical aspect.

Editor (LK)

Comments to Author(s):

I note that the two versions of the abstract (in the pdf versus in the Abstract box) are quite different; the latter works better for a general audience, but I do still have some comments. It reads clearly for the first three sentences, but these are rather disconnected from the results sentence 'We show...', which is too dense to be generally accessible. I would reduce the first three sentences and provide more explanation of what the results are. Also, you mention antagonistic selection twice in this version, but I'm not clear if you have an evidence of antagonistic selection in this system?

We revised the abstract and hope it is now better suited for a general audience.

We currently lack direct evidence for antagonistic selection in this specific species but there are a number of studies suggesting the action of sexually antagonistic selection in the close relative *O. taurus*. We explicitly mention this in section 3e) (in the context of intralocus conflict), but also tuned this aspect down in the abstract.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Comments to the Author (Rohner et al. Proceedings of the Royal Society B)

Main comments

The authors tried to find “master mediator gene” for coordinating sex- nutrition- and segment-specific growth in sexually-dimorphic beetles by using RNAseq based comparison. As results, they identified doublesex gene as candidate for “master mediator” (context-dependent master regulator). By knocking down dsx via RNAi, nutritional specificity and segment specificity in trait growth were weakened, which suggests both of nutrition specificity and segment specificity are governed by dsx expression. I think aim of the study is clear and experimental approach is suitable for their question. Well designed experiments and analyses support their conclusion. I think the manuscript can be published in the journal after minor revision.

Thank you very much for your positive comments.

Specific comments

Abstract:
Line 20

On term of “context-dependent master regulator”, I think this term does not correctly describe doublesex function. Considering that context-dependent dsx expression likely to be regulated by multiple upstream factors (nutrition dependent endocrinal signals and Hox gene dependent segment identity), “master regulator” sounds somewhat overstatement. How about “master mediator”?

We now changes master regulator to master mediator. We agree that this is a more fitting term.

Materials and Methods:

Line 104

Authors wrote that pronotum width were used as estimate of size.

Is there any sexual difference of pronotum size and/or shape? Considering that males have longer foreleg, pronotum muscle volume likely to be different between sexes. If pronotum size shows sexual dimorphism, authors should use alternative character as size estimate.

This is a great comment. Please see our response to the Editor above.

Results and Discussion:

Line 205 “Nutritional plasticity caused the weakest change in tibia length”

Is this true? According to Fig. 1C, low nutrition male tibia (ii) is even shorter than female tibia (iii). This result can be interpreted as nutrition difference can affect tibia length more than sexual difference.

The specific male tibia shown in panel ii) is indeed shorter than the female tibia in panel iii). However, according to the mean length measures presented in figure 1C the average nutritional effect (Δ1.67) was still smaller than sexual dimorphism (Δ 1.85). The differences therefore hold up, but the figure depicted an atypically small individual (which we picked at random). We now revised the figure and included a picture of a more representative individual. Thank you for spotting this!

Conclusion:
I think it is better to add conclusion diagram to describe position of dsx as regulator (mediator) for coordinating context-dependent trait growth.

We liked this suggestion very much and attempted to develop such a figure. However, while the mechanism underpinning sex-specific DSX action is well understood, the mechanisms that allow DXS to act in a nutrition and region specific manner mostly remain to be documented. Presumably interactions with growth regulators such as insulin signaling and regional identifiers such as HOX proteins are likely candidates, but there is not a ton of evidence yet for these conjectures. In the end we decided that that would leave any conceptual figure rather non-specific and thus likely not add much to enhance the quality of the manuscript. Other minor suggestions:

Line 1, Line 252, Line 294, Line 310

“Doublesex” and “Dsx” should be “doublesex” and “dsx”

Thank you, we corrected this accordingly.

Line 307

“regulation of dsx” should be “regulation of dsx expression” for making it clearer.

Done.

I could not find any supplementary figures in PDF for review. So, I could not judge a part of results.

We are sorry that we did not upload the data correctly. It should now all be attached to the main document.

Referee: 2

Comments to the Author(s)

Rohner et al examine the developmental, genetic and evolutionary processes underlying a secondary sexual trait, also known as exaggerated trait. To do so, they focus their experiments on the fore tibia of the beetle Digitontophagus gazella, where males have a much longer tibia of the forelegs compared to females. The foreleg tibia of the females, however, is thicker and presents a particular shape. The foretibia of the male is highly condition dependent, whereby males reared on rich food have a

significantly longer tibia than those reared on poor food. The scaling relationship between fore tibia and body is hyper-allometric.

The authors generated populations of males that were fed on rich or poor diet. They dissected the fore and hind limb tibia from those, but also from females that were said to have been raised on 'high resource availability.' The authors sequenced these samples (six replicates each) and conducted a comparative transcriptomics analysis (using an assembled transcriptome as a reference) to extract transcripts that are differentially expressed between treatments (rich vs poor; male vs female; fore vs hind limb). From this analysis, the authors come to conclude that the treatment that leads to the smallest divergence in fore tibia size (nutrition) shows the highest number of differentially expressed genes. They also interrogated these transcriptomics datasets in search for genes that are common (in terms of expression profiles) between various contexts where the trait becomes exaggerated (nutrition, serial homology, sex). They found little overlap between these contexts, which led them to conclude that there is a disparity in the developmental pathways underlying trait exaggeration in each of these contexts.

Next, the authors focused their attention on the gene doublesex (dsx), a major developmental regulator of sex determination whose role is quite conserved across insects. Dsx was enriched in the fore tibia of males both when compared to the hind tibia and when well-fed males were compared to poorly fed ones. The authors were also able to distinguish between male and female dsx isoforms. Following a series of RNAi experiments, the authors examined the role of dsx in the growth of the fore leg tibia. They found that dsx RNAi reduces fore limb tibia in males and increases it in females reared in standard diet. They also did this experiment following diet treatment and found that dsx RNAi had a more pronounced effect on well fed individuals.

Finally, the authors asked if the observed results of dsx were common to another beetle that does not show exaggerated fore limbs; Onthophagus taurus. Based on quantitative difference of dsx RNAi phenotypes between the two beetles, the authors conclude that the role of dsx in trait exaggeration is labile and that there was a divergence in the function of dsx that explained the differences in the degree of sexual dimorphism between species.

This paper addresses a fundamentally important question dealing with how diversification of sexually dimorphic characters is mediated through the intricate interaction between genetics and the environment (here nutrition). I think it would be of interest to the audience of Proc. B. I have however a number of issues that the authors might want to address and hopefully increase the quality of this manuscript.

Thank you very much for your very constructive comments. We apologize again for the missing supplementary material that would have resolved several issues and hope that this became clearer now.

Methods:
1- Sampling: The authors state (lines 116-119) that the males were reared in poor or rich food condition. In line 119, that the females were reared on 'high resource availability.' Why is this stated this way? Were these samples treated differently at different times or was this one designed experiment? Please clarify. And if it is the latter, please make it clear and discuss the potential limitation of having treated differently samples that are meant to be compared.

All individuals were treated in the exact same way. We now rephrased this section to make this clearer. Thank you for pointing this out.

2- Dissections: the tibiae were dissected from the imaginal discs. This is a hard task that will inevitably introduce biases between samples. Can the authors acknowledge this?

At the time of dissection, the tissue that gives rise to the adult leg is relatively large and quite easy to dissect. Specifically, adult legs do not derive from imaginal discs (at least not as they are understood from the *Drosophila* literature) but instead derive from the larval leg precursors. By the time of the dissection the larval leg epidermis has undergone apolysis from the cuticle and completed most if not all of the cell proliferation needed to produce the future pupal leg. By this time in development the parts of the prepupal leg that give rise to the adult femur, tibia, and tarsi are quiet easy to separate (see fig S6). We cannot exclude that some biases exist in our data, but

given the small transcriptomic variation within groups relative to variation among groups (see fig S9) we believe this to be of minor concern.

3- Reference transcriptome: There is a lot of critical information missing. The authors state in line 127 that a full transcriptome was assembled. From what raw data? Was it the combined samples of the tibias? Was it from specific libraries meant for full transcriptome assembly? What coverage? What read length? What are the metrics of this full transcriptome (N50; BUSCOs; missing; duplicated etc…). If the reference transcriptome was assembled from the 24 tibia samples, this would be a heavily truncated dataset. The authors would want a reference transcriptome that has the highest representation possible of the gene set of this species. It is hard to make reliable claims with this information missing. Please provide the methods and the metrics of your reference transcriptome and make sure it is actually sufficiently representative (BUSCO over 90%). The fasta file supplemented with in the SOM along with the raw data should be submitted to a public database and accession numbers supplemented with the paper.

We added a lot of information to the manuscript that was previously missing (or was only part of the inaccessible supplementary files - once again, our apologies). Among others, we included a supplementary table including read length and number per sample before and after trimming (table S1). Information on contig length, N50, and BUSCO statistics are listed in table S2. Note that, according to BUSCO, our de novo assembled transcriptome includes a little more than 90% of the expected genes even though we only used information from two tissue types and one developmental stage (C:91.7% [S:91.1%, D:0.6%], F:4.8%, M:3.5%, n:2124). We thus believe that the transcriptome is suitable for our analysis.

In the materials section, we now also explicitly state that the de novo transcriptome was generated from the 24 tibia samples. Although this transcriptome is not expected to include all possible genes, it is expected to include genes expressed in the relevant biological context. We therefore see no a priori reason why a transcriptome should be based on a more inclusive (larger) set of tissue samples. We previously also mapped the reads gathered in this study to a transcriptome generated in the same species de novo assembled from completely different source tissues (head tissues) and used for another experiment. As the results were qualitatively similar, this gave us confidence in our approach used here.

We now also indicate that all raw reads will be made available at https://www.ncbi.nlm.nih.gov/sra/PRJNA718544 (see data availability section).

4- Comparative transcriptomics: I couldn't find any data describing the sequencing raw results (coverage, read length etc…). More importantly, the authors need to look closer at their replicates and see if they all make sense or whether there are outliers. Finally there is no explanation about possible batch effects and corrections for those, if necessary.

This information is now included in supplementary table S1. We also provide correlation matrices and principal components to show consistency among replicates in figure S9. Samples were all pooled in each lane of sequencing to control for batch effects. This is now mentioned in the Methods section (line 130).

5- Doublesex RNAi: This experiment typically makes the dimorphism (whatever it is) reduced or entirely removed. One main difficulty is knowing the sex of the individuals at the moment of injection so they are not confused when they hatch. How did the authors deal with this? Were they able to sex the larvae before injection? If not, are they able to discriminate males and females that hatch with dsx RNAi phenotypes? If so, could the authors please describe how and preferably provide images in the supplement showing this?

This is a great question. In this, as well as in previous studies, we were able to use larval phenotypes (the development of male gonads) as a reliable trait to tell males from females. We now mention it on line 151. Additional detail can be found in Moczek AP, Nijhout HF 2002b. A method for sexing final instar larvae of the genus Onthophagus LATREILLE (Coleoptera: Scarabaeidae). Coleopterist Bulletin 56: 279-284., which we also cite.

6- In figure 2, I wonder why the authors chose to do box plots rather than the more informative scaling relationship as in Figure 1. Is this because of low number of individuals?

Compared to many other similar studies, these analyses are based on a relatively large number of individuals. The reason we show mean values and corresponding 95% confidence limits is that we wanted to highlight residual (i.e., relative) trait size.

7- Dsx will reduce dimorphism in any trait and this effect is going to be stronger with higher dimorphism. Just as male fore tibia goes closer to female's, the opposite is also true (Fig. 2). So the role of dsx is not necessarily restricted to the exaggerated trait, but affects any trait that is dimorphic. Fig 2D also shows that the dimorphism in O. taurus in weak, so is dsx RNAi. These realities of dsx should be acknowledged throughout the manuscript.

As far as we understand this comment we 100% agree! DSX acts on both male and female fore tibiae via sex specific isoforms, promoting tibial enlargement in males and inhibiting or lessening it in females, thereby creating a relatively strong sexual dimorphism in this structure, but much less so in the hind leg. We went over the manuscript to ensure our writing consistently reflects these observations.

8- The lability of dsx function: I am not at all convinced by the argument that, because dsx RNAi effect is stronger in the horns than in the tibia, its function must have diverged. Again, the horns are much more dimorphic than the tibia and the result is not surprising. In addition, RNAi by nature being a partial depletion of transcripts, it is hard to conclude about divergence in function from a quantitative effect. This part is one of the weakest in this paper and should be stricken. The authors are pushing the interpretation of RNAi too far. Specific experiments are needed to test this, including expression data and genetic interactions across species and tissues.

Thank you for bringing this up. The key argument here is that *dsx*RNAi has, in comparison to its effects on horns, a weak effect on tibiae in *O. taurus* while the reverse is true in *D. gazella*. Differences in RNAi penetrance between species alone can thus not explain the patterns observed. At the same time, *dsx* knockdown affected hind tibia length in *D. gazella* but not in *O. taurus*. While we cannot assess whether this is due to sequence evolution of *dsx*, or changes in its downstream effects, these findings indicate species differentiation and hence evolutionary divergence related to *dsx* function. However, we are also aware of the limitations of our data and methodological approach and explicitly mention these drawbacks in the Results & Discussion (at the end of the third section: (c) doublesex-mediated context-dependent trait exaggeration is evolutionarily labile)

9- Lines 323-326: There seems to be a confusion between sexual conflict and trade-offs. Sometimes the authors write 'intralocus sexual conflict' and other times just 'intralocus conflict.' Could the authors please be more accurate? If we are talking about conflict between the sexes, that is intralocus sexual conflict. If the authors refer to within sex (benefit of horns for mating success and their cost for survivorship or maneuverability), then we are talking about trade-offs.

This section has now been revised and we hope that the distinction between trade-offs and conflict have become clearer. As intralocus conflict can also arise between genotypes or genetic elements within a genome we think that the use of intralocus conflict and sexual conflict is acceptable in the few instances where it is noted.

10- Line 331: I couldn't follow the argument about epistasy, and I certainly didn't see any data showing this. Can the authors please elaborate. Do they mean that dsx isoforms would have different interactions based on environment?

The link to epistasis is rooted in a model that was proposed by Russell Bonduriansky (Bonduriansky 2007), however, we agree that it does not add much to this study a whole. We thus removed the references to it throughout.

References

Bonduriansky, R. (2007) The evolution of condition-dependent sexual dimorphism. American Naturalist 169:9-19.

Cheverud, J.M. (1982) Phenotypic, genetic, and environmental morphological integration in the cranium. Evolution 36:499-516.

Klingenberg, C.P. (1996) Multivariate allometry. Advances in Morphometrics 284:23-49.