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Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinizes female gene expression

Jack G. Rayner, Sonia Pascoal and Nathan W. Bailey

Article citation details
Proc. R. Soc. B **286**: 20190497.
http://dx.doi.org/10.1098/rspb.2019.0497

Review timeline

Original submission:	15 January 2019
1st revised submission:	28 February 2019
2nd revised submission:	29 March 2019
Final acceptance:	1 April 2019

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

Review History

RSPB-2019-0110.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions 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?
Yes

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript tests how a flatwing genotype that eliminates male wings in the Hawaiian field crickets, and subsequently their song production, affects gene expression in both sexes in multiple tissues. This is a really cool study system and I read about the gene expression patterns discovered here with great interest.

The authors find more pronounced differences between the females. There was a pattern of decreased expression of male-biased and/or increased expression of female-biased genes in the flatwing females relative to the genotype that underlies normal wing development, which the authors refer to as demasculinisation of the transcriptome. In the males there were hardly any genes with a significant expression difference between the genotypes. The authors interpret these patterns to result from a release from intra-locus sexual conflict (IaSC), under the premise that development of a secondary sexual signal in males – a male limited trait – is connected to a more masculinized gene expression profile in the females as a pleiotropic effect, which the authors take as an indication that it also reduces female fitness. The authors also report a difference in male testes mass and female body condition, whereby flatwing mutants had a lower and higher values, respectively, relative to the winged genotype.

1.
While I think that testing for gene expression differences, reproductive organ sizes and body condition in these crickets is really interesting, I think the interpretation leaning on IaSC is not as straightforward as authors now suggest. What is the evidence that there is sexually antagonistic selection on the flatwing locus? The authors themselves state that there are no differences in the reproductive output (line 320) in the females, based on the fact that they did not differ in investment into the ovary tissues (although no actual reproductive fitness was measured). I think toning down on this is needed (e.g. drop "Release from intralocus sexual conflict" from the title).

The evidence connecting sex-biased expression, phenotypic sexual dimorphism and IaSC is mostly theoretical and correlational, but the text now suggests as if there is solid evidence to back up this interpretation (e.g. lines 45-47), which is then used to make the case that this paper

directly tests the consequences of song loss on IaSC (line 85). Also lines 43-44 show careless interpretation of the literature: no way there is evidence of a majority of sexually dimorphic phenotypes being associated with autosomal sex-biased expression. Rather, many studies show substantial autosomal sex-biased expression, which can be taken as an indication of this.

The notion that IaSC persists even when sex-limited expression has been achieved goes against the idea that sex-limitation evolves in response to antagonistic selection in order to resolve IaSC. To make an opposite prediction, the authors should make a better case for it to be understood correctly, especially by a general reader. If I got this right, I think the authors want to get across that genetic correlations between traits - due to pleiotropy - can impose IaSC on loci other than those directly underlying the focal trait, even when a trait is under selection only in one sex because of its sex-limited expression (which should have resolved IaSC already for the expression of the genes directly underlying the focal trait). This is certainly interesting, but rather than assuming that the results of this paper directly speak of it, more caution is needed as this is just an interpretation and not in any way proven by the results presented here, in my opinion.

Also connected to this (Line 49-50): Berger et al. 2014 does not come across as a good example of sex-limited traits causing IaSC. All the focal traits in this paper are homologous but dimorphic in the sexes, whilst the selection applied was sex-limited. But I guess the reason to use it here is that it reveals how genetic correlations among traits can impose a conflict?

2.
Related to the point of connecting the degree of sex-bias and its fitness consequences. How confident the authors are in that the time point when they chose to look at the expression differences show patterns that actually affect fitness? The degree of sex-bias changes over ontogeny as well as in response to the physiological status of individuals (e.g. in response to nutritional conditions or reproductive status). Also, given that the authors find the flatwing females to be of higher body condition, the expression patterns can entirely reflect this, without any direct involvement of IaSC. Higher conditioned D. melanogaster males for example have more masculinized transcriptomes (Wyman et al. 2010 Evolution).

3.
The fact that there were no concomitant changes in males, argues against the interpretation that the female gene expression patterns in the normal winged genotypes would reflect more the male optimum. IaSC leans on there being a genetic constraint for sexual dimorphism, and here clearly male and female responses are not that tightly correlated as only a handful (N=19) of genes showed a concordant DE in the sexes. If the correlation was tight and the sexes constrained, relaxed selection in the flatwing males coupled with selection for demasculinisation in females should result in a similar response in both sexes, if a release from IaSC plays a major role. If, on the other hand, there are sex differences in the genetic architecture in place, as it now seems to be the case, why should then NW genotype females be constrained by selection on males in the first place? Alternative explanation to the results here is that NW females are actually selected to show a more masculinized expression pattern.

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I was missing information about the expression in the flatwing locus itself. What is in this locus? (sorry if I missed this info somewhere). Could you do some kind of pathway analysis or expression correlation to try to connect the genes that show differential expression with the gene(s) in the flatwing locus, to understand more the pleiotropic effect at the molecular level?

5.
I was missing a figure and supplementary table that would show what kind of effect sizes we are talking about (logFC) for the sex-biased genes.

6.
How about differential expression of un-biased genes? Anything interesting there? It seems odd to leave them out. Was there more differentially expressed sex-biased than un-biased genes? Such enrichment would strengthen the interpretation of the consequences on sex-specific fitness.

Review form: Reviewer 2

Recommendation
Accept as is

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

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

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

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
This manuscript presents a study of transcriptome-wide differences in gene expression between two wing morphs of a field cricket, compared between the sexes and across 3 tissues. The authors investigate hypotheses about the feminization (in males) and masculinization (in females) of gene

expression in relation to the flatwing morph, and relate the data to ideas about intralocus sexual conflict.

This study presents a compelling example of changes in sex-specific gene expression with respect to relatively masculinized and feminized males, compared with females. It nicely complements some related examples from lab-based Drosophila populations, and other animals (turkeys, mites) where males have alternative reproductive tactics. Here male differences relate to a recent traceable mutation in a natural population, an important model system for rapid evolution and sexual selection. The additional experiment testing differences in reproductive tissues and measures of condition strengthens the paper. Notably, the authors have dissects out neural tissue; this is a strength of the study and will allow more precise resolution compared with whole heads. This study is an important addition to this model system and I expect it will be of broad interest.

The paper is very nicely written and presented. Conceptual figure 1 is clear and useful.

I reviewed this manuscript in a previous version and my suggestions have been addressed with care in the present version. I have no further suggestions.

Decision letter (RSPB-2019-0110.R0)

06-Feb-2019

Dear Mr Rayner:

I am writing to inform you that your manuscript RSPB-2019-0110 entitled "Release from intralocus sexual conflict: Evolutionary loss of a male sexual trait demasculinises female gene expression" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees and the Associate Editor, who have recommended that revisions are necessary, although the overall opinion is one of optimism if the revisions are satisfactory. With this in mind we would be happy to consider a resubmission, provided the comments of the referees and the Associate Editor are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

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Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Reviewer 1 raises some very insightful points about the sexual conflict framework employed in this paper. I agree that although the changes in expression may represent the release of conflict, they could also be thought of in different ways. It will be important to address the points raised by the reviewer with regards to conflict, which will also strengthen the interpretation of the paper.

Having read the paper myself, I agree with Reviewer 1's points, and had several questions about the methodology.

1 – Were all the reads from all samples used in a single transcriptome assembly?

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is unclear. Also, were the results validated to make sure that the inclusion of multiple isoforms is minimized?

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Referee: 1

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Referee: 2

Comments to the Author(s)
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The paper is very nicely written and presented. Conceptual figure 1 is clear and useful.

I reviewed this manuscript in a previous version and my suggestions have been addressed with care in the present version. I have no further suggestions.

Author's Response to Decision Letter for (RSPB-2019-0110.R0)

See Appendix A.

RSPB-2019-0497.R0

Review form: Reviewer 1

Recommendation
Accept as is

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

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

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
This was the second time I read this manuscript and think that the authors have done a great job addressing both my and AE's comments. I have no further comments to make, and think that this very interesting manuscript is a great addition to the field.

Decision letter (RSPB-2019-0497.R0)

22-Mar-2019

Dear Mr Rayner

I am pleased to inform you that your Review manuscript RSPB-2019-0497 entitled "Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinises female gene expression" has been accepted for publication in Proceedings B.

The referee and the Associate Editor do not recommend any further changes. Therefore, please proof-read your manuscript carefully and upload your final files for publication. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let me know immediately.

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2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and the file name should contain the author's name and journal name, e.g authorname_procb_ESM_figures.pdf
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 see: https://royalsociety.org/journals/authors/author-guidelines/

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It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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http://datadryad.org/submit?journalID=RSPB&manu=RSPB-2019-0497 which will take you to your unique entry in the Dryad repository.

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Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your final version. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

Proceedings B
mailto:proceedingsb@royalsociety.org

Comments to Author:
One of the original reviewers and I have both read through the revision. We both agree - the authors have done an excellent job revising their paper, and there are no further suggestions for revision.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
This was the second time I read this manuscript and think that the authors have done a great job addressing both my and AE's comments. I have no further comments to make, and think that this very interesting manuscript is a great addition to the field.

Decision letter (RSPB-2019-0497.R1)

01-Apr-2019

Dear Mr Rayner

I am pleased to inform you that your manuscript entitled "Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinises female gene expression" 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.

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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,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Dear Editor,

Thank you for your comments on the previous version of our manuscript, ID RSPB-2019-0110, and for the opportunity to resubmit. We are pleased to present our updated manuscript, which we have substantially revised in accordance with your comments and those of reviewers.

While both reviewers agreed that our study addressed an interesting question in a compelling study system, Reviewer 1 requested clarifications regarding the rationale underpinning our interpretation of IASC and suggested alternative explanations for our results, which we address in our revision and responses.

We have also performed and include analyses suggested by the editor to evaluate sex- and tissue-specificity of genotype-associated changes in gene expression. The results support our interpretation of release from sex-associated constraints on gene expression, by illustrating most of the transcripts affected are those which do not show sex- or tissue-specific expression. These results help address some of the concerns of Reviewer 1. We also added a new paragraph in our discussion, which more thoroughly considers interpretations of our results suggested by Reviewer 1.

These revisions and additional analyses in accordance with the comments on our previous version strengthen and clarify our results and their interpretation. We hope you will agree our manuscript is

suitable for publication in Proc B, and we thank you and each of the reviewers for your time reading, and your helpful feedback on, our earlier version.

Yours sincerely,
Jack Rayner
Nathan Bailey

Associate Editor

Reviewer 1 raises some very insightful points about the sexual conflict framework employed in this paper. I agree that although the changes in expression may represent the release of conflict, they could also be thought of in different ways. It will be important to address the points raised by the reviewer with regards to conflict, which will also strengthen the interpretation of the paper.

Having read the paper myself, I agree with Reviewer 1's points, and had several questions about the methodology.

Thank you for your comments on our manuscript, and for the opportunity to submit a revised version. We have responded to comments below, as well as those of the reviewers, and made corresponding changes in our revised MS.

1 – Were all the reads from all samples used in a single transcriptome assembly?

Yes – all samples were used to construct a single transcriptome assembly. This is now mentioned on **line 150** of our revised MS.

2 – How were multiple isoforms dealt with? I assume this was done, at least in part, with CD-hit-est, but how is unclear. Also, were the results validated to make sure that the inclusion of multiple isoforms is minimized?

We were concerned with differences in expression at the level of genes, so performed analyses at the level of Trinity genes. As noted, we clustered any genes showing 95% sequence similarity using CD-hit-est to remove any duplicated genes. To ensure that the transcriptome was not highly duplicated (potentially indicating multiple isoforms of the same gene identified as different genes), we used BUSCO statistics which indicate low levels of duplication (1.8% of complete conserved genes). These points are made in our revised transcript, at **lines 160-165** of the methods and **line 237** of the results.

3 – What threshold was used for filtering out lowly expressed transcripts? How was this applied across tissues, between sexes and among lines?

We filtered the entire transcriptome once, prior to constructing models, by removing any transcripts not expressed at >1 count per million in at least 3 samples. We have now moved this information from the ESM to the methods section, at **line 153**.

It was necessary for our study to obtain a single set of transcripts to compare between sexes and across tissues, and this is why we filtered prior to constructing separate models. Our goal in filtering lowly expressed transcripts was to remove those with little empirical support, rather than define a set of transcripts expressed at high levels in each of the sex*tissue combinations. We note that lowly expressed transcripts are unlikely to be identified as DE (EdgeR manual; Robinson et al. 2010).

4 – Given that the authors have gene expression data from multiple

tissues, they could actually test the rule of pleiotropy in sex-bias, or in this case, change in sex-bias. For example, is sex-bias more common for tissue-specific genes? And are tissue-specific genes more likely to be differentially expressed in the FW genotype?

These are very interesting questions, which we have subsequently investigated. We now describe the methodology used on **lines 198-203** and results on **lines 293-313**. The results are discussed in the final paragraph of the discussion (**lines 405-411**).

The results support the view that the *flatwing* genotype mostly affects the expression of genes that are shared in their expression between sexes. They also support our interpretation that we are observing pleiotropic effects of the *flatwing* genotype on non-wing tissues, by illustrating that transcripts DE between the wing genotypes tend not to be tissue-specific (and moreover, that changes are correlated between tissues – see Fig. S1).

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This manuscript tests how a flatwing genotype that eliminates male wings in the Hawaiian field crickets, and subsequently their song production, affects gene expression in both sexes in multiple tissues. This is a really cool study system and I read about the gene expression patterns discovered here with great interest.

The authors find more pronounced differences between the females. There was a pattern of decreased expression of male-biased and/or increased expression of female-biased genes in the flatwing females relative to the genotype that underlies normal wing development, which the authors refer to as demasculinisation of the transcriptome. In the males there were hardly any genes with a significant expression difference between the genotypes. The authors interpret these patterns to result from a release from intra-locus sexual conflict (IaSC), under the premise that development of a secondary sexual signal in males – a male limited trait – is connected to a more masculinized gene expression profile in the females as a pleiotropic effect, which the authors take as an indication that it also reduces female fitness. The authors also report a difference in male testes mass and female body condition, whereby flatwing mutants had a lowesr and higher values, respectively, relative to the winged genotype.

Thank you for your time reviewing our manuscript, and for your helpful comments which we have taken on board to clarify our rationale for the study and interpretation of the results. We have responded to each of your points below.

1.

While I think that testing for gene expression differences, reproductive organ sizes and body condition in these crickets is really interesting, I think the interpretation leaning on IaSC is not as straightforward as authors now suggest. What is the evidence that there is sexually antagonistic selection on the flatwing locus? The authors themselves state that there are no differences in the reproductive output (line 320)

in the females, based on the fact that they did not differ in investment into the ovary tissues (although no actual reproductive fitness was measured). I think toning down on this is needed (e.g. drop “Release from intralocus sexual conflict” from the title).

We have toned down this language as suggested (see title change below), and also added a paragraph discussing caveats to be taken into account when interpreting the results of our study (**lines 381-398**). There is good reason to expect sexual conflict at shared loci under contrasting selection between sexes *a priori*, and that pleiotropic changes associated with a mutant genotype in males will also affect females (this is discussed further below). Male song, which requires the expression of sound-producing structures on wing membranes, benefits males in the context of sexual selection. Females do not sing, and never express sound-producing structures irrespective of genotype, however the current study and others have demonstrated that genotype at the *flatwing* locus, which determines male wing vein morphology, has associated, or pleiotropic, effects upon multiple phenotypes and in both sexes (now mentioned on **line 87**). Males and females share all of the same genes in *T. oceanicus* (now mentioned on **line 82**), so evidence of effects of genotype at the flatwing locus upon phenotypes in non-wing tissues in males and females illustrates the opportunity for sexual conflict at this and associated loci. That is, pleiotropic effects can drive IASC, as we mention on **line 88**, with reference to Figure 1. Few studies have addressed IASC in association with sex-limited traits, likely owing to the view that sex-limited expression of traits should resolve IASC, however in one study where this was explicitly tested it was shown that IASC does indeed

persist despite sex-limited expression of a sexual ornament (Harano et al. 2010).

Another reason for our prediction of release from IASC associated with *flatwing* which we clarify in our revised MS (**lines 85-89** & **95-96**), and which is based on existing empirical data, derives from the feminised (or demasculinised) phenotypes that have been observed in flatwing males (Bailey et al. 2010; Pascoal et al. 2018b; see MS). These effects suggest that females could benefit from a similar feminising/demasculinising effect.

Our study largely addresses sexual conflict, and we believe it is important to communicate this in the title. To address the comment above, we have edited the title as follows: 'Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinises female gene expression'. This is a small but important change – the title does not now presuppose a particular outcome about IASC. We have also made changes to wording throughout the text to clarify that release from IASC is an interpretation of our results that is consistent with our hypothesis of female benefit from the loss of a male sexual trait.

The evidence connecting sex-biased expression, phenotypic sexual dimorphism and IaSC is mostly theoretical and correlational, but the text now suggests as if there is solid evidence to back up this interpretation (e.g. lines 45-47), which is then used to make the case that this paper directly tests the consequences of song loss on IaSC (line 85). Also lines 43-44 show careless interpretation of the literature: no way there is evidence of a majority of sexually dimorphic

phenotypes being associated with autosomal sex-biased expression. Rather, many studies show substantial autosomal sex-biased expression, which can be taken as an indication of this.

In response to the first point, we have made changes to our introduction to clarify the background to our study, many of which are also described in the response above (examples include **lines 42-45; 48-57; 87-90; 97-98**). There is a strong theoretical framework underpinning the argument that shared genes under contrasting selection pressures will result in sexual dimorphism, at least partially through sex differences in gene expression, which we have strengthened and clarified at **lines 42-51**. In response to the second point, we have removed the sentence about autosomal sex-biased expression to better focus our opening paragraph (**line 42**).

The notion that IaSC persists even when sex-limited expression has been achieved goes against the idea that sex-limitation evolves in response to antagonistic selection in order to resolve IaSC. To make an opposite prediction, the authors should make a better case for it to be understood correctly, especially by a general reader. If I got this right, I think the authors want to get across that genetic correlations between traits - due to pleiotropy - can impose IaSC on loci other than those directly underlying the focal trait, even when a trait is under selection only in one sex because of its sex-limited expression (which should have resolved IaSC already for the expression of the genes directly underlying the focal trait). This is certainly interesting, but rather than assuming that the results of this paper directly speak of it, more caution is needed as this is just an interpretation and not in any way proven by the results presented here, in my opinion.

We respectfully disagree with the initial statement above; interpretation of incomplete resolution of IASC still views sex-limited expression of traits (and sex-biased gene expression) as evolving in response to contrasting selection pressures, but this sex-limited expression does not necessarily resolve underlying conflict. This hypothesis of incomplete resolution is not a novel prediction of ours; it receives attention in prominent discussions of IASC in the literature (e.g. Bonduriansky & Chenoweth 2009; Mank 2017), and has empirical support (Cox & Calsbeek 2009; Harano et al. 2010). For example, spillover effects of male sexual trait loss in females are observed in horned beetles, where knockdown of *doublesex* reduces horn size in males but actually causes horn development in ordinarily horn-less females (Kijimoto et al. 2012, PNAS). Our results are therefore consistent with, but we do not suggest they 'prove', our hypothesis of release from sexual conflict associated with the loss of the male sexual trait, and we have made changes to wording throughout the abstract (e.g. **line 34**), introduction (e.g. **lines 108, 115**) and discussion (e.g. **lines 346, 418**) to emphasise this.

As summarized in the comment above, in *T. oceanicus*, incomplete resolution of IASC could be due to pleiotropic effects of the *flatwing/normal-wing* locus. The large differences in gene expression between female genotypes, as well as correlations in changes across tissues within each sex and disproportionate involvement of non-tissue-specific genes in somatic tissues, support this interpretation. There are potential other interpretations: for example, male-beneficial genes could be genetically linked with the *NW* genotype, and loss of the male sexual trait could have knock-on effects for IASC by affecting

these genes' functions, as well. In either scenario our interpretation of release from IASC would apply.

We clarify in our revised introductory paragraph that sex-biased and sex-limited expression are expected to have evolved in response to sexual conflict (**lines 42** & **49**), even if this resolution is incomplete due to constraints associated with opposing selection in the opposite sex, and why incomplete resolution may be due to pleiotropic effects (**lines 51-57**).

Also connected to this (Line 49-50): Berger et al. 2014 does not come across as a good example of sex-limited traits causing IaSC. All the focal traits in this paper are homologous but dimorphic in the sexes, whilst the selection applied was sex-limited. But I guess the reason to use it here is that it reveals how genetic correlations among traits can impose a conflict?

Thank you for pointing this out. We have moved this citation to the previous sentence (**line 48**), which discusses incomplete resolution of sexual conflict resulting from sex-biased gene expression.

2.
Related to the point of connecting the degree of sex-bias and its fitness consequences. How confident the authors are in that the time point when they chose to look at the expression differences show patterns that actually affect fitness? The degree of sex-bias changes over ontogeny as well as in response to the physiological status of individuals (e.g. in response to nutritional conditions or reproductive status). Also, given that the authors find the flatwing females to be of

higher body condition, the expression patterns can entirely reflect this, without any direct involvement of IaSC. Higher conditioned D. melanogaster males for example have more masculinized transcriptomes (Wyman et al. 2010 Evolution).

While patterns of gene expression and fitness effects may vary over development, gene expression at adult stages has been shown to be associated with fitness (e.g. Wyman et al. 2010; Dean et al. 2018), and we expect that this should also be the case in *T. oceanicus*.

We agree sampling these tissues at an earlier stage of development could be informative, but we do not have these data available. In particular, it is clear that flatwing and normal-wing males differ phenotypically, above and beyond the wing morphology for which they are named, and we anticipate that sampling tissues at an earlier stage could reveal patterns of pleiotropy associated with – for example – reduced testes mass and feminised cuticular hydrocarbons of males (Pascoal et al. 2018). This is now mentioned on **lines 343-347**. We have also clarified that our hypotheses of release from IASC, and feminisation of males, are not mutually exclusive – but that we do not find transcriptomic evidence of the latter, and the expression profiles in adult tissues we observe are consistent with the former. (**line 99**).

It is true that higher body condition of *flatwing*-carrier females could be related to the demasculinised transcriptomes. Given that the genotype at which they differ is associated with the male sexual trait, this would indicate that females benefit from carrying the male trait loss locus, consistent with our initial hypothesis of release from sexual conflict at that locus. We mention this at **line 385**.

3.

The fact that there were no concomitant changes in males, argues against the interpretation that the female gene expression patterns in the normal winged genotypes would reflect more the male optimum. IaSC leans on there being a genetic constraint for sexual dimorphism, and here clearly male and female responses are not that tightly correlated as only a handful (N=19) of genes showed a concordant DE in the sexes. If the correlation was tight and the sexes constrained, relaxed selection in the flatwing males coupled with selection for demasculinisation in females should result in a similar response in both sexes, if a release from IaSC plays a major role. If, on the other hand, there are sex differences in the genetic architecture in place, as it now seems to be the case, why should then NW genotype females be constrained by selection on males in the first place? Alternative explanation to the results here is that NW females are actually selected to show a more masculinized expression pattern.

The reviewer discusses selection pressures associated with the *flatwing* genotype in the sexes – we wish to make clear that in our study we looked at differences between males and females derived from a single wild population, which carry alternative genotypes, rather than any evolved differences between lines or populations (clarified on **line 144**). That is, due to the recent nature (15 years ago) of the mutation's appearance and spread in this population, we do not expect to detect gene expression differences attributable to differential selection on the morphs at non-*flatwing* loci, except under a highly unlikely scenario in which positively selected variants are held in gametic phase disequilibrium with *flatwing* over generations of

breeding in the lab. Evolved differences in IASC between flatwing/normal-wing predominated populations in the wild are nevertheless an interesting area for future study, which we discuss on **lines 394-398**.

Regarding the lack of strongly concordant genotype-associated changes between sexes, results from the AE's suggested analyses are useful in addressing this question. These results show very few of the transcripts we find to be DE are sex-limited in their expression, indicating transcripts affected by genotype are those shared between sexes (**lines 293-300**). We were surprised not to identify more genotype-associated differences between males, but nevertheless note that changes in expression (for transcripts DE in one or both sexes) associated with genotype were positively correlated in neural and gonad tissues (Spearman's rank: $r=0.920$, $N=26$, $P<0.001$; and $r=0.203$, $N=193$, $P=0.005$, respectively), and that, across all transcripts, expression in counts per million is strongly correlated between sexes in all tissues ($r=0.97$, 0.96 and 0.52 in neural, muscle and gonad tissues, respectively; all $P<2.2e^{-16}$). Additionally, in RNA-seq data for developing non-adult wing tissues that we recently collected for a separate study, using the same experimental design, we have found that genes DE between male and/or female Kauai genotypes show strongly correlated changes (Spearman's rank $rho=0.771$, $P<0.001$, $N=41$ genes) (Rayner & Bailey, in prep). There is, therefore, strong evidence that morph genotype has partially overlapping effects between sexes in at least some tissues, and across developmental stages.

4.

I was missing information about the expression in the flatwing locus itself. What is in this locus? (sorry if I missed this info somewhere). Could you do some kind of pathway analysis or expression correlation to try to connect the genes that show differential expression with the gene(s) in the flatwing locus, to understand more the pleiotropic effect at the molecular level?

Currently, relatively little is known about the *flatwing* genotype. A recent study, available on BioRXiv (https://www.biorxiv.org/content/early/2018/12/09/489526), has produced a number of candidate genes, but the exact region and nature of the causative genotype remains unclear. We appreciate this was not previously clear, and we now mention it on **line 83** of our revised MS.

Further study of the *flatwing* genotype is certainly an interesting area for research, and we note results of our functional enrichment analysis (Table S1, called at **line 250**) share similarity with those in the preprint. However, we believe it is important to maintain focus in our current MS on the original hypotheses posed when designing the experiment.

5.

I was missing a figure and supplementary table that would show what kind of effect sizes we are talking about (logFC) for the sex-biased genes.

We now include volcano plots showing logFC and expression for each of the sex comparisons (both with and without a fold-change threshold of >2) in Fig. S3.

6.

How about differential expression of un-biased genes? Anything interesting there? It seems odd to leave them out. Was there more differentially expressed sex-biased than un-biased genes? Such enrichment would strengthen the interpretation of the consequences on sex-specific fitness.

We include numbers of DE genes in Table 1 and Fig. 1A, and performed functional enrichment tests for the total set of DE genes for each sex (Table S1, discussed on **line 244-249**). We also now discuss on **lines 388-393** that while we are unable to make direct inferences about fitness-associated effects of changes to non- sex-biased genes, they are nevertheless an important consideration in interpreting the results of our study.

As above, our focus here was on sex-biased transcripts, which enabled us to compare directions of change between morphs and sexes within a hypothesis-testing framework, but it is important to note many DE transcripts did not show sex-bias. However, it is difficult to make similar inferences about the substance of these changes. Additionally, it is difficult to robustly test whether sex-biased transcripts were disproportionately likely to show DE; a chi-squared test between proportions of sex-biased versus non- sex-biased transcripts showing DE would likely report overrepresentation of sex-biased transcripts, but it is also likely that genes which do not differ between sexes include – for example – essential housekeeping genes, and genes with lower average expression across samples, which are less likely to be DE between genotypes OR sexes, potentially confounding this

comparison. To err on the side of caution in analysing our results, we have refrained from performing such an analysis.

Referee: 2

Comments to the Author(s)
This manuscript presents a study of transcriptome-wide differences in gene expression between two wing morphs of a field cricket, compared between the sexes and across 3 tissues. The authors investigate hypotheses about the feminization (in males) and masculinization (in females) of gene expression in relation to the flatwing morph, and relate the data to ideas about intralocus sexual conflict.

This study presents a compelling example of changes in sex-specific gene expression with respect to relatively masculinized and feminized males, compared with females. It nicely complements some related examples from lab-based Drosophila populations, and other animals (turkeys, mites) where males have alternative reproductive tactics. Here male differences relate to a recent traceable mutation in a natural population, an important model system for rapid evolution and sexual selection. The additional experiment testing differences in reproductive tissues and measures of condition strengthens the paper. Notably, the authors have dissects out neural tissue; this is a strength of the study and will allow more precise resolution compared with whole heads. This study is an important addition to this model system and I expect it will be of broad interest.

The paper is very nicely written and presented. Conceptual figure 1 is

clear and useful.

I reviewed this manuscript in a previous version and my suggestions have been addressed with care in the present version. I have no further suggestions.

Thank you for your positive comments regarding our study. We are pleased that our revisions have addressed your previous comments.