High-resolution characterization of male ornamentation and re-evaluation of sex linkage in guppies

Jake Morris, Iulia Darolti, Wouter van der Bijl and Judith E. Mank

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

Review History

RSPB-2020-1677.R0 (Original submission)

Review form: Reviewer 1 (Jessica Stephenson)

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
In this paper the authors present data from high-resolution, automated image analysis of the orange and black colour patterns of male guppies. They apply these methods to 1) a large number of male guppies from a freely breeding population and 2) offspring from a pedigree breeding design. They use the images from the population males to group colour patches (at the pixel level) that were observed in the same place on the body in at least 89% of males into discrete ornaments. Once they had identified these ornaments (4 orange and 5 black), they investigated correlations in the size and saturation between them to identify potential ornament 'modules'. The authors used the pedigree fish to investigate the heritability and inheritance of both the presence/absence, and size of these ornaments. While they found that individual ornaments were heritable, the values they report are surprisingly low, given the previous reports from this system. They additionally report that the presence/absence of the individual ornaments was not Y-linked in their population, but confirm that the overall aggregate area of orange and area of black on the males did appear to be Y-linked.

This is a careful and elegant dissection of the heritability, inheritance, and relationship between male ornaments in this well-studied model, and to my knowledge the first to eliminate the potential for human bias in quantification of colour pattern. The manuscript is overall well-written, particularly the introduction, and my comments should be easily addressed by tweaking the organisation and presentation of the methods and results. I have a few general suggestions and then some more minor specific points.

First – I recommend the authors highlight earlier that their results using the 'aggregate' measures of total area of orange and total area of black do largely support previous results suggesting these are Y-linked. The initial part of the discussion attempts to set the authors' results up as in contradiction to previous work, but I think this actually does the novelty of their results a disservice. The nuances to the story are cool – because of previous methodologies being limited, researchers were forced to use aggregate methods and as a result they picked up on the Y-linkage of potential modifiers, rather than the autosomal/X-linked control of the actual underlying ornaments themselves. This is more clearly the message of the abstract and introduction, and I think the discussion could usefully build on this framing.

Second – I would really like to see an analysis of the heritability/inheritance of saturation of orange and black. I think the authors have the data to do this, and I would be really interested to know what the heritability of saturation is. There is clearly an important environmental component to saturation, particularly in orange, but it would be very useful for researchers in the

field to be able to directly compare metrics of heritability between the area and saturation of these important ornaments. We and others have hypothesised, and I think it is feasible, that variation in the biochemical pathways through which pigments become saturated/unsaturated is heritable, and it would be very exciting to see if there's any evidence that this is correlated with heritable variation in the area of these pigments. These values could usefully be added to table 2.

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Table 1 and associated methods: it is not clear if false discovery rate corrections were applied here – this is a lot of statistical tests. It would be good to know which of these correlations remain significant under more stringent test conditions.

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268-269 – why are these figures not in table 2?
270 – table 2?
356 - typo

Table 2: title is a bit misleading – it's the heritability of both pres/abs and area for each ornament

I hope the authors find my comments helpful as they revise their interesting manuscript.
Jessica Stephenson

Review form: Reviewer 2

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

Is the length of the paper justified?
Yes

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Is it adequate?
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Do you have any ethical concerns with this paper?
No

Comments to the Author
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I think the manuscript would benefit from more detail in the Introduction. For example, please provide an example or two of the ornaments only found in captive bred guppies (Line 80). Are these ornaments also black and orange spots, or are they altogether different to the wild-type ornaments focused upon in this study? If they bear a similarity to the wild-type ornaments, that should also be considered in the Discussion.
In the Methods, software and R package versions are not always provided (e.g. Lines 136, 141,

167), and when provided and only included in the reference (e.g. Line 118). I suggest citing versions directly after program names in the Methods.

It would be nice for the grandfather effects discussed in the Results Lines 272 – 282 to be presented in a simple table, either in the main text or supplement.

The Discussion can be somewhat repetitive, and I suggest that some content is moved from the Discussion to the Introduction and expanded upon there. For example, Lines 296 – 301 could be moved to the Introduction, and Lines 325 – 328 mostly repeat Lines 74 – 83. In addition, Lines 74 – 86 would benefit from a one or two sentence summary of current knowledge of the guppy genome, which will help provide context for later discussions of the Y-chromosome region (as in Lines 337 – 343).

Alternatively, for some sections of the Discussion, I suggest that the authors restructure many paragraphs in the Discussion to begin with the findings of this study, and then refer back to previous research. If you read the manuscript in one go, the Discussion often seems to tread back through the previous literature, which is frustrating when you want to hear the discussion of this study's own interesting findings.

The Discussion could be more explicit about the potential of applying the developed pattern digitisation method to the mentioned captive bred ornament phenotypes, and to compare multiple wild-type populations (e.g. 381 – 382). In addition, what other systems could this digitisation method be applied to? This would provide an opportunity to expand the scope of interest in this paper beyond guppy research.

More general thoughts for discussion:
• 99 semilandmarks were used to digitise the fish shape. Did the authors try a landmark reduction method, as this could have increased statistical power for these analyses and reduced computational time?
o Likewise, could fixed landmarks have been used instead, as again these provide greater statistical power?
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Line-by-line comments:
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presence/absence for black ornaments, and not the correlation of orange and black ornaments for size.

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- Line 349: is there literature that can be cited to support this statement about genetic diversity and bottlenecks?
- Line 384: I recommend removing "Surprisingly" in this instance.
- In Supplementary Tables 1 – 3, p-values listed as 0.000 should be changed to <0.001.

Decision letter (RSPB-2020-1677.R0)

25-Aug-2020

Dear Dr Morris,

Thank you for submitting your manuscript 'HIGH-RESOLUTION CHARACTERIZATION REEVALUATES SEX LINKAGE OF ORNAMENTATION IN GUPPIES' to Proceedings B. The 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 and Associate Editor are all agreed that this is a very interesting set of results as well as presenting useful methods, and I totally agree with this assessment. However they 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.

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 at all, please do not hesitate to get in touch.

Finally, I hope you and your co-authors are well in these strange times.

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

Associate Editor
Board Member: 1
Comments to Author:

This manuscript examines the genetic basis of orange and black ornaments in guppies using a pedigree approach. These ornaments have previously been determined to be Y-linked, but the manuscript finds that this is not the case. Rather, the results were more consistent with an autosomal and/or X-linked genetic basis, since males frequently had a different phenotype than their fathers, which is inconsistent with direct Y-linked effects. However there did seem to be evidence of mediation of the overall amount of ornamentation by one or more Y-linked loci, since total orange and black area were both more highly correlated between males and their paternal grandfather, than between males and their maternal grandfather. The discrepancy between the results presented here and previous results may therefore be a consequence of the fact that previous results have generally examined composite traits such as total area.

I think that this manuscript should be of interest to quite a wide range of readers for two reasons. Firstly, guppies are a popular system for the study of sexual selection and, more recently, sex chromosome evolution. Secondly, the manuscript presents a new approach for measuring colour ornaments at higher resolution. The manuscript is therefore likely to be of interest both to researchers working in sexual selection and those who work on colour pattern analysis.

A couple of minor points:
In the methods it seems like there are a few places where the words black and orange have been switched. This needs to be corrected since it causes unnecessary confusion.
It's not clear whether the phenotypic correlation matrices are corrected for multiple testing or not. If not, then such correction should be implemented.

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s)
In this paper the authors present data from high-resolution, automated image analysis of the orange and black colour patterns of male guppies. They apply these methods to 1) a large number of male guppies from a freely breeding population and 2) offspring from a pedigree breeding design. They use the images from the population males to group colour patches (at the pixel level) that were observed in the same place on the body in at least 89% of males into discrete ornaments. Once they had identified these ornaments (4 orange and 5 black), they investigated correlations in the size and saturation between them to identify potential ornament 'modules'. The authors used the pedigree fish to investigate the heritability and inheritance of both the presence/absence, and size of these ornaments. While they found that individual ornaments were heritable, the values they report are surprisingly low, given the previous reports from this system. They additionally report that the presence/absence of the individual ornaments was not Y-linked in their population, but confirm that the overall aggregate area of orange and area of black on the males did appear to be Y-linked.

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Referee: 2
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• Line 384: I recommend removing "Surprisingly" in this instance.
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Author's Response to Decision Letter for (RSPB-2020-1677.R0)

See Appendix A.

RSPB-2020-1677.R1 (Revision)

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?
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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

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   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
The authors have addressed my comments and the manuscript is much clearer. A few questions remain that the authors should be able to address:

Line 199: why use mean saturation weighted by the size of the ornament?
Table 2: could include the h2 estimates of saturation, and therefore save space by removing the need for paragraph ln 280-283?
Line 289: I think the correlations among ornaments were done separately for size and saturation, but it would be interesting to see if e.g. larger ornaments were on average less saturated than smaller ones? Is there a trade-off between more orange area and more orange saturation?

Discussion: this is missing a discussion of the heritability of saturation – to my knowledge this is a very novel result - particularly black: I was surprised to see that the heritability of saturation of these ornaments is higher than the heritability of either their presence/absence or size.

Overall I enjoyed the paper and look forward to seeing it in print!

Jessica Stephenson

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?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
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Do you have any ethical concerns with this paper?
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Comments to the Author
I am satisfied that the authors have responded adequately to the comments and recommendations made by myself and the other referees. I particularly appreciate the new clarity over the pedigree breeding regime, landmark digitisation method, and the restructuring and new text in the Discussion improves the manuscript overall. The analysis of saturation heritability suggested by Referee 1 has been implemented, and it improves the manuscript further. The corrections for multiple testing are also appreciated.

I think this manuscript will be an interesting contribution to Proceedings B.

Decision letter (RSPB-2020-1677.R1)

21-Sep-2020

Dear Dr Morris

I am pleased to inform you that your Review manuscript RSPB-2020-1677.R1 entitled "HIGH-RESOLUTION CHARACTERIZATION OF MALE ORNAMENTATION AND REEVALUATION OF SEX LINKAGE IN GUPPIES" has been accepted for publication in Proceedings B.

The referee(s) 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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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,
Professor Loeske Kruuk
Editor, Proceedings B
mailto:proceedingsb@royalsociety.org

Associate Editor Board Member: 1
Comments to Author:
Both reviewers were satisfied on the whole with the revisions, although reviewer 1 had a few additional minor comments that should be addressed.

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s)
The authors have addressed my comments and the manuscript is much clearer. A few questions remain that the authors should be able to address:

Line 199: why use mean saturation weighted by the size of the ornament?
Table 2: could include the h2 estimates of saturation, and therefore save space by removing the need for paragraph ln 280-283?
Line 289: I think the correlations among ornaments were done separately for size and saturation, but it would be interesting to see if e.g. larger ornaments were on average less saturated than smaller ones? Is there a trade-off between more orange area and more orange saturation?

Discussion: this is missing a discussion of the heritability of saturation – to my knowledge this is a very novel result - particularly black: I was surprised to see that the heritability of saturation of these ornaments is higher than the heritability of either their presence/absence or size.

Overall I enjoyed the paper and look forward to seeing it in print!

Jessica Stephenson

Referee: 2

Comments to the Author(s)
I am satisfied that the authors have responded adequately to the comments and recommendations made by myself and the other referees. I particularly appreciate the new clarity over the pedigree breeding regime, landmark digitisation method, and the restructuring and new text in the Discussion improves the manuscript overall. The analysis of saturation heritability suggested by Referee 1 has been implemented, and it improves the manuscript further. The corrections for multiple testing are also appreciated.

I think this manuscript will be an interesting contribution to Proceedings B.

Decision letter (RSPB-2020-1677.R2)

22-Sep-2020

Dear Dr Morris

I am pleased to inform you that your manuscript entitled "HIGH-RESOLUTION CHARACTERIZATION OF MALE ORNAMENTATION AND REEVALUATION OF SEX LINKAGE IN GUPPIES" 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.

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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,
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Dear Editor,

Many thanks for your positive remarks and helpful suggestions on our manuscript, "High resolution characterization of male ornamentation and reevaluation of sex-linkage in guppies". We also thank the reviewers for their extremely thoughtful and constructive suggestions, addressed below in bold. The revised manuscript is greatly improved, and we hope that it is now suitable for publication in Proceedings B.

Many thanks,

Jake Morris and Judith Mank, on behalf of all co-authors.

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).

On acceptance all data will be made available via Figshare at DOI: 10.6084/m9.figshare.12640766. Prior to this data can be accessed via this private link: https://figshare.com/s/c81614b4b2eb29e0f7c9

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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.

Associate Editor
Board Member: 1
Comments to Author:

This manuscript examines the genetic basis of orange and black ornaments in guppies using a pedigree approach. These ornaments have previously been determined to be Y-linked, but the manuscript finds that this is not the case. Rather, the results were more consistent with an autosomal and/or X-linked genetic basis, since males frequently had a different phenotype than their fathers, which is inconsistent with direct Y-linked effects. However there did seem to be

evidence of mediation of the overall amount of ornamentation by one or more Y-linked loci, since total orange and black area were both more highly correlated between males and their paternal grandfather, than between males and their maternal grandfather. The discrepancy between the results presented here and previous results may therefore be a consequence of the fact that previous results have generally examined composite traits such as total area.

I think that this manuscript should be of interest to quite a wide range of readers for two reasons. Firstly, guppies are a popular system for the study of sexual selection and, more recently, sex chromosome evolution. Secondly, the manuscript presents a new approach for measuring colour ornaments at higher resolution. The manuscript is therefore likely to be of interest both to researchers working in sexual selection and those who work on colour pattern analysis.

Many thanks for the positive comments!

A couple of minor points:
In the methods it seems like there are a few places where the words black and orange have been switched. This needs to be corrected since it causes unnecessary confusion.

Many thanks for noticing this – corrected!

It's not clear whether the phenotypic correlation matrices are corrected for multiple testing or not. If not, then such correction should be implemented.

We have now used Holm's method to calculate corrected P-values accounting for multiple testing, as you can see many of these correlations for saturation remain significant. Figure 2 has also been updated, as have supplementary tables 1-3.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
In this paper the authors present data from high-resolution, automated image analysis of the orange and black colour patterns of male guppies. They apply these methods to 1) a large number of male guppies from a freely breeding population and 2) offspring from a pedigree breeding design. They use the images from the population males to group colour patches (at the pixel level) that were observed in the same place on the body in at least 89% of males into discrete ornaments. Once they had identified these ornaments (4 orange and 5 black), they investigated correlations in the size and saturation between them to identify potential ornament 'modules'. The authors used the pedigree fish to investigate the heritability and inheritance of both the presence/absence, and size of these ornaments. While they found that individual ornaments were heritable, the values they report are surprisingly low, given the previous reports from this system. They additionally report that the presence/absence of the

individual ornaments was not Y-linked in their population, but confirm that the overall aggregate area of orange and area of black on the males did appear to be Y-linked.

This is a careful and elegant dissection of the heritability, inheritance, and relationship between male ornaments in this well-studied model, and to my knowledge the first to eliminate the potential for human bias in quantification of colour pattern. The manuscript is overall well-written, particularly the introduction, and my comments should be easily addressed by tweaking the organisation and presentation of the methods and results. I have a few general suggestions and then some more minor specific points.

First – I recommend the authors highlight earlier that their results using the 'aggregate' measures of total area of orange and total area of black do largely support previous results suggesting these are Y-linked. The initial part of the discussion attempts to set the authors' results up as in contradiction to previous work, but I think this actually does the novelty of their results a disservice. The nuances to the story are cool – because of previous methodologies being limited, researchers were forced to use aggregate methods and as a result they picked up on the Y-linkage of potential modifiers, rather than the autosomal/X-linked control of the actual underlying ornaments themselves. This is more clearly the message of the abstract and introduction, and I think the discussion could usefully build on this framing.

This is a great point, and we apologize if we did not emphasize this enough. We have revised the discussion, which now reads:

However, our results are consistent with studies which have characterized aggregate ornamental area, which often identify significant Y contributions. For example, Postma *et al* [34] and Brooks and Endler [21] both identified significant levels of Y-linked genetic variation when assessing total ornamental areas. Our analysis suggests that although the presence or absence of individual ornaments are not often Y-linked in natural populations, the Y chromosome does carry one or more modifier loci that affect many ornaments in aggregate. Therefore when studies use the total orange or black area as a trait they are in large part measuring the mode of inheritance of these loci rather than any loci related to the presence or absence of any individual ornament [21,33].

Second – I would really like to see an analysis of the heritability/inheritance of saturation of orange and black. I think the authors have the data to do this, and I would be really interested to know what the heritability of saturation is. There is clearly an important environmental component to saturation, particularly in orange, but it would be very useful for researchers in the field to be able to directly compare metrics of heritability between the area and saturation of these important ornaments. We and others have hypothesised, and I think it is feasible, that variation in the biochemical pathways through which pigments become saturated/unsaturated is heritable, and it would be very exciting to see if there's any evidence that this is correlated with heritable variation in the area of these pigments. These values could usefully be added to table 2.

This is a very good idea! We have now carried out the analysis and do indeed find heritable genetic variation for average orange (h2 = 0.232) and black saturation (h2 = 0.207). We have included these results in the text. In addition, we also looked for Y effects. We found that the coefficient for average offspring orange saturation vs paternal grandfather orange saturation was 0.313, while the coefficient for average offspring orange saturation vs maternal grandfather orange saturation was -0.186. This suggests there may be Y-linked modifier loci that increase orange saturation. For black saturation we found that the coefficient for both paternal and maternal grandfathers were negative, and so there is no evidence for Y-linked modifier loci.

Finally – I found the structure of the methods a bit challenging to follow. I think it would be helpful to have a summary paragraph explaining the overall rationale and steps their analysis followed, and then dive into the details of the process. Bits of the paragraph at the start of my review may help to give an example of what I mean (assuming I understood the methods!). Alternatively, or additionally, more explicit explanations of the rationale behind each of the steps as they are described would be useful. I think more judicious use of the word 'ornament' at the start of the methods process would also help. It's not until the pixels have been clustered and found in at least 89% of fish that the authors consider them to be 'ornaments', yet they use the word beforehand to describe orange and black pigment more generally. This made it quite challenging to keep track of what they meant, and what stage of the process they were referring to. Moving the flow chart from the supplement to the main text, and adding labels and explanations to it, would help the reader follow these methods and why they were employed.

Unfortunately, we are not able to move the chart into the main text due to space constraints. However, we have extensively revised the methods as suggested.

Table 1 and associated methods: it is not clear if false discovery rate corrections were applied here – this is a lot of statistical tests. It would be good to know which of these correlations remain significant under more stringent test conditions.

This is a good point. We have now used Holm's method to calculate corrected P-values accounting for multiple testing, as you can see many (although not all) of these correlations for saturation remain significant. It does however mean that some of the correlations in size between orange ornaments which were previously weakly significant are no longer significant. We have still discussed these in the text, but made clear that they are not significant when multiple testing is taken into account.

Minor comments with line numbers:

93 – should be 'area'?

Corrected.

103 – I really needed this clear summary of sample size here, but unfortunately this sentence is very unclear – three generations and 19 crosses I understand, but '15 generations two and three'? What does this mean? For this section I recommend the authors clearly set up that they used both the freely breeding general population, and the pedigree fish, in this study, and explain how they used each and why.

Apologies, this is indeed very complicated. We have revised this section to the best of our abilities, and we hope that the supplemental figure detailing the pedigree and inheritance pattern (Supp Fig 1) helps both the reviewer and future readers.

126 – why use k=20 and 40? How were these numbers chosen, were more tested, why use both 20 and 40? Clustering to me suggested the pixels were moved, but I think this confusion would be eliminated with clearer rationale setting up this step (i.e. to highlight patches of same-coloured pixels that could be considered ornaments, I think..).

Thank you. K=20 and k=40 were chosen as together these provided a level of granularity that enabled reasonable extraction of ornaments across all fish in our dataset. We have added this explanation to the manuscript (L130-131). As mentioned, we then '*used the k-image that gave the most distinct and defined pixel patterns*' for a given image. Other values were tested, but k=20 and K=40 proved to be most useful for our dataset, with lower numbers not partitioning images well enough and higher partitioning images to much. You are right that this was used to 'highlight patches of same-coloured pixels that could be considered ornaments' (a rationale we have now included L129-130) and that this step does not involve the movement of any pixels.

132 – would the clustering step have changed RGB values?

The clustering step does not change RGB values. At this step a new image is generated, an image that has pixels clustered into RGB clusters, but this is then used to extract out individual pixel RGB values from the original image, and it is these individual pixels RGB values that are used in downstream analyses. This is stated on L138-139:

We did however extract the original RGB values from the non k-means image.

134 – clarify that this is now morphometrics – what are semi-landmarks? Also the authors could provide rationale here by reorganising the paragraph and putting something about the general mean reference shape of the males in the topic sentence. I appreciate the word count is super tight and I'm asking for a lot of additional explanations.

We have now made clear that this is a morphometric approach and mentioned the reference shape in the first sentence of this section. We have also clarified that semi-landmarks were used as '*equally spaced points along a curve*'.

145 – each pixel in each ornament in each fish?

Correct, we have now adjusted this text (L150):

After aligning the orange and black extracted images of all 171 males from the stock population, we had RGB scores for each pixel in each ornament in each fish.

154 – typo – is this meant to be orange?

Fixed!

181 – add commas to these large numbers

Done!

191 – topic sentence not a sentence

Revised. It now reads:

Unfortunately, we were unable to photograph and phenotype the grandfathers of our generation two offspring with our phenotype analysis pipeline. We therefore have a sample of 73 males across seven families from generation three.

208 – very unclear what is happening here?

Apologies, this has been revised. It now reads:

Although all orange ornaments overlapped with at least one black ornament, the overlap regions were small, and 59% of potential ornament pixels in Fig 1. were orange, 35% black, and only 6% both (grey).

227 – loci controlling both colours?

Apologies, we are unsure of what the reviewer is suggesting. Given the generally low correlations between orange and black ornament size and the fact that orange and black pigmentation arise from separate biochemical pathways, we do not think that it is likely that there are loci that control both colour ornaments simultaneously. If the reviewer intended something else, we would very much appreciate clarification.

244-249 – the text explaining the authors' hypotheses about epistatis is saturation is much clearer than the text explaining their hypothesis about area above.

We have revised our hypotheses about area. We hope that it is clearer. It now reads:

This perhaps indicates epistatic interactions between loci controlling ornaments, increasing the size of one ornament while reducing the size of others that are adjacent. Alternatively, it may reflect that where orange and black ornaments overlap, only the black area is visible.

268-269 – why are these figures not in table 2?

Thank you. They are now!

270 – table 2?

Corrected!

356 – typo

Corrected!

Table 2: title is a bit misleading – it's the heritability of both pres/abs and area for each ornament

Renamed.

I hope the authors find my comments helpful as they revise their interesting manuscript.
Jessica Stephenson

Many thanks for the very helpful comments!

Referee: 2

Comments to the Author(s)
Overall I enjoyed reading the paper and I think that study uses an elegant experimental design to investigate an interesting question. I only have some minor criticisms and suggestions.

Many thanks for the positive remarks!

I think the manuscript would benefit from more detail in the Introduction. For example, please provide an example or two of the ornaments only found in captive bred guppies (Line 80). Are these ornaments also black and orange spots, or are they altogether different to the wild-type ornaments focused upon in this study? If they bear a similarity to the wild-type ornaments, that should also be considered in the Discussion.

This is a very interesting point. We are actually quoting Lindholm and Breden (2002), who suggest that many of the phenotypes assayed in these previous studies are not found in the wild. Looking at the hand drawn plates from Winge (1927), it is very difficult to know how to

compare the colouration patterns he identifies with patterns from natural populations. This is why we are so excited about our automated method, which will make it far easier to compare ornamentation across populations.

In the Methods, software and R package versions are not always provided (e.g. Lines 136, 141, 167), and when provided and only included in the reference (e.g. Line 118). I suggest citing versions directly after program names in the Methods.

Thank you, we have now added all versions of programs we used.

It would be nice for the grandfather effects discussed in the Results Lines 272 – 282 to be presented in a simple table, either in the main text or supplement.

We have added a small table to the text with the results of our four analyses.

The Discussion can be somewhat repetitive, and I suggest that some content is moved from the Discussion to the Introduction and expanded upon there. For example, Lines 296 – 301 could be moved to the Introduction, and Lines 325 – 328 mostly repeat Lines 74 – 83. In addition, Lines 74 – 86 would benefit from a one or two sentence summary of current knowledge of the guppy genome, which will help provide context for later discussions of the Y-chromosome region (as in Lines 337 – 343).

We have added a brief explanation (to L78-82) of the proposed role of colour and its proposed role in recombination suppression on the sex chromosomes, mentioning both 'the small region of the sex chromosomes that never recombines, as well as a larger region of the sex chromosomes where recombination occurs infrequently' (as in Lines 337-343 *of original manuscript)

Alternatively, for some sections of the Discussion, I suggest that the authors restructure many paragraphs in the Discussion to begin with the findings of this study, and then refer back to previous research. If you read the manuscript in one go, the Discussion often seems to tread back through the previous literature, which is frustrating when you want to hear the discussion of this study's own interesting findings.

The Discussion could be more explicit about the potential of applying the developed pattern digitisation method to the mentioned captive bred ornament phenotypes, and to compare multiple wild-type populations (e.g. 381 – 382). In addition, what other systems could this digitisation method be applied to? This would provide an opportunity to expand the scope of interest in this paper beyond guppy research.

We have extensively revised the discussion as suggested.

More general thoughts for discussion:
• 99 semilandmarks were used to digitise the fish shape. Did the authors try a landmark

reduction method, as this could have increased statistical power for these analyses and reduced computational time?
o Likewise, could fixed landmarks have been used instead, as again these provide greater statistical power?

We did not try a landmark reduction method. It is perhaps useful to note that the goal of the morphometrics methodology was limited to transforming the images to a reference shape to facilitate direct comparison, rather than to perform analyses on the shape itself.

Unfortunately, due to the nature of dataset (photos of live guppies, rather than say a skeleton) we found only a few reproducible landmarks that could be used. For example, we found even the size of fins to be variable in our population and so even body/fin intersections could not be used as reliable landmarks. He sliding landmark method we use is therefore more accurate than using the few available reliable landmarks.

• Has the digitsation method been applied to females? Are there patterns that human observers have missed but the image clustering method can detect? Are females as bland traditionally assumed?

This is an interesting point and we do think that an analysis of females would be worthwhile. However, given that our question was focused on Y inheritance, an analysis of females is somewhat beyond the scope of this paper, particularly given the tight page limit at this journal.

Line-by-line comments:
• I like the title, but it does not seem to make grammatical sense. The process of characterization cannot itself reevaluate anything. I suggest changing "characterization reevaluates sex linkage" to "characterization prompts reevaluation of sex linkage" or "characterization suggests reevaluation of sex linkage".

Thanks for pointing this out. We have changed the title to "High resolution characterization of male ornamentation and reevaluation of sex linkage in guppies.

• The Abstract doesn't explicitly mention the breeding programme or the pedigrees to estimate inheritance. As such Lines 32 – 37 seem a bit misleading, as a digitisation method is introduced and then Y-linkage is discussed. A reader just checking the Abstract might think that this study used genetic sequencing or that no breeding occurred at all.

Thanks for pointing this out. We have revised the abstract, and the key sentence now reads:

Here we developed a pipeline for automated ornament identification and high-resolution image analysis of male guppy colour patterns and applied it to a multigenerational pedigree.

• Lines 54 – 55: I suggest adding a brief clause that Y-linkage is the mechanism in male heterogametic XY systems.

Revised. Now reads:

For species with male heterogametic sex chromosomes, one important way to establish separate male and female genetic architecture, and thereby resolve sexual conflict, is Y-linkage.

• Line 97: when (what year) was the pedigree established? Furthermore, how long did it take to breed the three generations used in this study?

Revised. Now reads:

We established pedigrees from this population in 2016 by pairing four males and virgin females randomly in the first generation. We then paired offspring in subsequent generations from different families to maximise the number of grandparents in each brood, to minimize inbreeding which can make it difficult to differentiate different modes of inheritance, and to help increase the diversity of different phenotypes segregating in different families. This led to a minimum of six great-grandparents per family in generation three (see Supplementary Fig. 1). In all, our pedigree stretched across three generations and 19 crosses, and produced 214 male offspring across generations two and three (average per family = 14). We allowed between 4 and 8 months for each generation to allow for multiple clutches in most pairs.

• Lines 103 – 105: this sentence is confusing and seems like it needs to be reworded. Is '15' a typo here, because it seems from Supplementary Figure 1 and the rest of the methods that there are only three generations?

We have revised this sentence, which now reads:

In all, our pedigree stretched across three generations and 19 crosses, and produced 214 male offspring across generations two and three (average per family = 14).

• Line 154: I think "black" should be "orange" here, and it is a typo.

Fixed!

• Line 177: there is an extra space between "We" and "used".

Fixed!

• Line 188: "affecting" instead of "effecting".

Fixed!

• Line 208: typo "blackblack".

Fixed!

• Line 225: I think the reference to Figure 2B is a typo here, as Figure 2B shows presence/absence for black ornaments, and not the correlation of orange and black ornaments for size.

Agreed and removed.

• Line 287: recommend changing to "without human or experimenter bias" as clustering methods still contain their own statistical biases.

We agree that our methods are not without any bias, and that is why we do use the term "human bias" throughout. We have removed the phrase "without bias" from the succeeding sentence as this was perhaps ambiguous.

• Line 317 – 318: it is unclear which evidence (a table or the discussion of grandfather effects?) that this sentence is referring to. I suggest adding a citation.

Apologies, this was based on our observations. We have revised this section, which now reads:

This high correlation among ornaments of the same pigmentation type, and higher similarity between grandsons and their paternal grandfathers compared to maternal grandfathers in in ornament size suggests a Y-linked modifier locus that globally affect total amount of orange and black pigmentation.

• Line 349: is there literature that can be cited to support this statement about genetic diversity and bottlenecks?

Not to our knowledge. Our reasoning is based on standard population genetic theory, rather than a specific case of diversity modeling. If the reviewer knows of any relevant citation, we would very much appreciate it.

• Line 384: I recommend removing "Surprisingly" in this instance.

We actually did find this quite surprising, given previous work on this topic. We have therefore left this sentence as is, but would be happy to hear the reviewer's reasons for suggesting the change.

• In Supplementary Tables 1 – 3, p-values listed as 0.000 should be changed to <0.001.

Done! (with our new adjusted for multiple testing p-values).