Dear Pr. Schwander,

Please find enclosed a revision of our manuscript (PCI Evol Biol #484) entitled "Masculinization of the X-chromosome in aphid soma and gonads".

As recommended, we have taken at heart to consider the comments, and made appropriate modifications. Our point-by-point responses appear below.

We thank you and the reviewers for the useful comments and for considering this revised manuscript.

Sincerely,

J. Jaquiéry and co-authors

Invitation to revise

Dear authors,

Thank you for submitting your preprint "Masculinization of the X-chromosome in aphid soma and gonads" to PCI Evol Biol. Your manuscript has been read by two reviewers, whose comments are enclosed. As you will see, the reviews are largely positive, and, based on these reviews as well as my own evaluation, I would recommend your manuscript to be eventually included in PCI Evol Biol. However, before reaching a final decision, I would ask you to revise your manuscript according to the recommendations by the reviewers. Please address the main highlighted points, including:

- Addressing the issue of using a cut-off of 70% for deciding if a gene is morph or tissue specific (or specific to tissues within morphs). Reviewer#2 suggests using different cut-offs. An alternative would be to use a quantification of specificity for each gene (eg. across the 9 sample types) and then testing for enrichment of highly specific genes in different conditions. A good quantification for specificity is for example Tau (see Yanai et al. 2004. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue) specification. Bioinformatics 21:650-659)

The identification of gene specific of a given class of samples was indeed not trivial in our 3 morphs x 3 tissues design. The use of Tau was explored as an alternative strategy. Tau is highly efficient to identify genes expressed in a single condition (e.g. gene specific of male gonads, e.g. see fictive genes 1 and 2 in table A below). However, it is less appropriate to identify genes specific of a morph (and expressed at similar rate within the different tissues of that morph) or genes expressed in a tissue in all morphs. Indeed, while genes 3 to 6 were highly specific of males (from 75% to 100% of the reads were found in males), the tau value was quite low (especially for genes 5 and 6). Identifying genes of interest (sex-biased genes across tissues or specific to a tissue) using tau would require measuring tau on different subsets of samples and then combining them, which would have complicated the analyses. Furthermore, tau identifies genes with highly biased expression, but not in which condition.

Nevertheless, we present below analyses based on tau to demonstrate that the evidence for a masculinization of the X still hold when using this approach (Figure A).

Table A: Tau measured for different types of fictive gene expression.

	MG	MH	ML	FG	FH	FL	PG	PH	PL	Tau (on log+1 transformed expression)
Gene1	100	0	0	0	0	0	0	0	0	1,00
Gene2	92	1	1	1	1	1	1	1	1	0,85
Gene3	33	33	33	0	0	0	0	0	0	0,75
Gene4	31	31	32	1	1	1	1	1	1	0,60
Gene5	29	29	29	3	2	1	3	2	1	0,52
Gene6	25	25	25	6	6	7	3	2	1	0,40

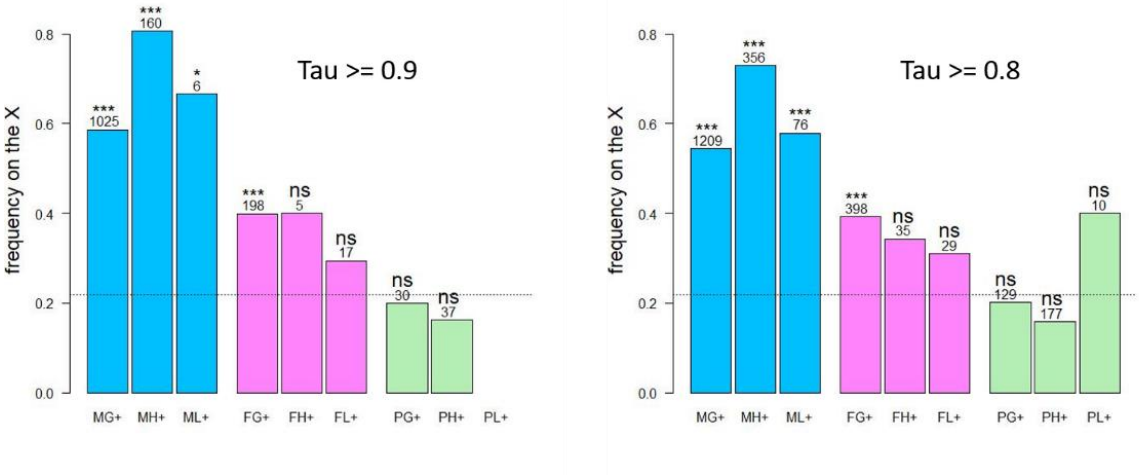

Figure A: Frequency of X-linkage for genes with tau values above 0.9 and 0.8. These genes were then assigned as specific of a condition based on the condition in which expression was the highest.

We also have evaluated the robustness of our conclusions to different cut-offs in term of the % of reads found in a condition or a set of conditions to identify specific genes. We tested cutoffs of 50%, 60%, 80% and 90% in addition to the 70% cutoff initially presented in the ms. While the number of genes satisfying the more stringent threshold (90%) is low, these analyses showed that our conclusions are robust to the threshold used (see supplementary figure S2 which presents these new analyses). Indeed genes that were preferentially expressed in males or male tissues were always more likely to locate on the X than expected, and those preferentially expressed in parthenogenetic females were less likely in most cases (significantly or not depending on sample sizes).

Finally, we also investigated whether the genes we identified as specific of a condition, of a sex or of a tissue (with the 70% cutoff) were also identified as statistically significant (after FDR correction, at FDR <0.05) by EdgeR. For genes identified as specific of a sex (i.e. M+, F+ and G+ genes), we expect them to be significantly DE in the contrasts involving the three

morphs. The vast majority of them (96.5%, 87.9% and 93.1%, respectively) were indeed significant. 97.7% of the G+, 99.7% of the H+ and 99.8% of the L+ genes were also significantly DE in the contrast between tissues. For genes identified as specific of a condition (e.g. MG+), we expect them to be significantly DE between the three different tissues within the morph (here male) AND between the focal tissue (here gonads) of the three morphs. For each of the different classes of genes (i.e. MG+, MH+, ML+, FG+, FH+, FL+, PG+, PH+ and PL+), 100% of the genes were found significantly DE in the two respective conditions at the same time. This demonstrates that the approach we used is conservative and largely supported by alternative approaches.

From these new analyses, we believe that our approach based on the % of reads found within different sets of samples is the most straightforward and simple. We provide as supplementary Figure S2 the results obtained with different cut-offs (50%, 60%, 80% and 90%) to show its robustness to the arbitrary chosen threshold. We also have modified the methods and results accordingly (lines 184-186 and 549-561).

-Contrary to reviewer#1, I do not see the lack of single-cell resolution of gonads as a fundamental problem for this study. It might be if the X was enriched for female-biased genes (a pattern which can be mimicked by X-inactivation in male gonads), but is difficult to use as an explanation here since the X is enriched for male-biased genes and there is no evidence for reduced expression of the X in females. Nevertheless, the authors may want to extend their current discussion of how tissue allometries or different cell types within composite tissues can affect interpretations of dosage compensation in their study.

We mention that single-cell RNAseq on different cell types of the aphid testis would be important (lines 459-462) and point out that the single-cell approach is the most accurate method for studying dosage compensation (lines 465-467).

In addition to the points raised by the reviewers, I suggest investigating sex-biased expression and dosage compensation along the X chromosome. Are there any clusters of strongly male-biased genes?

We have now included a supplementary figure showing the distribution of the different types of sex-biased genes along chromosomes (Supplementary figure S3). Male-biased genes appears to be distributed throughout the X chromosome, even if some regions of the genome show high density of some types of biased genes (notably the end of the X chromosome).

Dosage compensation along the X chromosome is now illustrated in supplementary file S6. When removing sex-biased genes with FC >2 (see Ann Kathrin Huylmans's comment), the pattern of dosage compensation appears rather homogeneous.

We mention these new analyses and results in the ms (lines 213-215, 282-285, supplementary figure S3 and S6).

I also find the lack of (detected) expression of a large portion of X-linked genes overall quite interesting and suggest the authors include a discussion of this finding in their ms. Is this because X-linked genes are generally lowly expressed (so they do not pass the filtering) or

highly tissue-specific (for tissues not present in the sequenced body fragments)? Are they detected in the whole-body samples in the previous studies?

It is difficult to disentangle among the two hypotheses (low expression and/or high tissue specificity).

Among the genes considered as "not expressed" based on our 18 samples and our filters, 24 % (of X-linked) and 12% (for those on autosomes) show 0 reads when summing the 18 libraries. 50% of the X-linked genes show less the 5 reads (against 26% for those on autosomes). It thus appears that the lack of expression for a larger proportion of X-linked genes compared to those on autosomes is independent of our filtering criteria.

We then looked at the expression in whole body samples obtained in a previous study (Jaquiéry et al. 2013). Most X-linked gene considered as "not expressed" based on our tissue samples also showed little evidence of expression in the whole body samples. Indeed, 40% of the "not expressed" X-linked genes showed 0 reads (when summing 6 whole body samples of males, sexual females and parthenogenetic females), while it was only 23% for the autosomal counterparts. Again, this suggests that a large part of the "not expressed" X-linked genes tend to be truly unexpressed (or at least lowly expressed). Expression levels in whole body samples for genes considered as not expressed and expressed in the tissue samples differed strikingly (see the figure included in supplementary text S1).

However, this does not rule out the hypothesis that X-linked genes could have a more tissue- or time specific expression, and thus that some of the genes identified as "not expressed" in the 18 tissue samples are expressed in unsampled tissues or time points. This hypothesis is further supported by the higher tau for X-linked genes ($tau_X = 0.71$) than for autosomal genes ($tau_A = 0.33$).

We now mention in the ms that both effects (lower expression and narrower expression) probably contribute to the large fraction of unexpressed X-linked genes, but that we cannot quantify their relative contribution (lines 152-155, 439-455). We also have added the τ analyzes in the ms (lines 216-220, 569-570) and the analyses presented above in the supplementary (supplementary text S1).

Furthermore, generating strongly male-biased expression of X-linked genes in an XX/X0 species will require extreme up-regulation in males. Is there evidence for increased gene duplication on the X relative to autosomes (with both/all copies located on the X)? Such a pattern could facilitate strong expression from a single X.

Gene duplication in the pea aphid genome has been studied in Li et al. 2019 MBE. They found that the X chromosome is the one that contains the highest number of within chromosome paralogues (X: 449, A1: 413, A2: 261, A3: 61) though its large size also partly accounts for this high number (X: 132 Mb, A1: 170, A2: 119, A3: 42).

Based on our analyses, we also found that the X chromosome contains slightly more genes that belong to multicopies families. However, we doubt that the 10 points increase we observe in the % of duplicated or multicopy gene families on the X can account for the highly male-biased or male-specific genes expression of genes from this chromosome. Indeed, we

also observed that for certain gene families, each of the X-linked copy on its own already showed increased expression in males compared to the autosomal copies. Of course, we cannot rule out that recent X-linked duplicates have not been detected (hence that the reads from many duplicates map to an artifactually merged copy), but we lack power to detect this with our current dataset. On the other hand, there is evidence that that the structure of the chromatin and methylation landscapes differ between X and autosomes among sexes (Mathers et al 2019, Richard et al 2017), a characteristic that might contribute to expression patterns. We now discuss these different hypotheses (lines 426-438).

I also encourage the authors to revise their manuscript according to the more minor suggestions from the reviewers, which will certainly improve it. To additional minor comments:

I suggest reformulating "By contrast, segregation distortion cannot affect the aphid X chromosome during spermatogenesis, as all sperm cells that do not carry an X degenerate.". This can be considered the most extreme form of segregation distortion (100% transmission instead of the expected 50%).

Right. We have modified the sentence (lines 479-481).

Please tone down "Here, we predicted that if masculinization of the aphid X chromosome evolved solely in response to intra-locus sexual conflicts, masculinization would occur in all tissues." to "a strong driver of" or something like this ("soley" would require excluding other factors, which I do not think is the authors intention. Furthermore there is no direct evidence that genes with male-biased expression are actually generally beneficial for males, even though this is certainly an implicit assumption in many studies)

Thank you, it was indeed not our intention, we have tone down this sentence (lines 129-130): *"Here, we predicted that if intra-locus sexual conflict is a strong driver of the masculinization of the aphid X chromosome, masculinization would occur in all tissues."*

Best regards,

Tanja Schwander

Reviews

Reviewed by anonymous reviewer, 04 Oct 2021 11:47

In this study, the authors predicted that if masculinization of the pea aphid X chromosome evolved solely in response to intra-locus sexual conflicts, masculinization would occur in all tissues. To verify this prediction they used chromosome-scale assembly and bulk RNA-seq of different sexes/tissues/morphs of the pea aphid to measure gene expression levels of the X and autosomes, across samples. They found masculinization of the X in each type of tissue. Furthermore, authors stated that the X-linked copy of a duplicated gene is more likely to show a male-biased expression than its autosomal copy or an X-linked single copy gene. The findings suggest that duplications facilitate sub or neofunctionalization toward the sex-specific optimum. Overall, the ms is an extension of prevoius theoretical and empirical works from some of the authors supporting the hypothesis of that the large excess of male-biased

genes observed on the pea aphid X chromosome compared to autosomes has evolved in response to sexual conflicts, by restricting the product of a sexually antagonistic allele to the sex it benefits. These observations provide new information about the atypical genome-wide pattern of gene expression in pea aphid, with a high degree of masculinization of the X chromosome in both somatic and gonadic tissues. The methodology and the details of the experiments are clearly described and appropriate. I have only some minor concerns that I think the authors should address before the ms progress it further.

#1. A major potential caveat of this study is the expression level of the X chromosome during spermatogenesis. Germ cells enact a very specialized program of differentiation and bulk RNA-seq alone is not enough to accurately determine the transcriptional profile of the X chromosome along the spermatogenesis. Two recent Drosophila papers that used single-cell RNA-seq experiments (see below) have showed that the X undergone dosage compensation earlier in spermatogenesis and inactivation in secondary spermatocytes. Folowing this idea, the masculinization of the X chromosomes (or the male-biased gene expression) in pead aphid may be because dosage compensation in spermatogonia; however the picture could be different during the transition to meiosis. That being said, how authors exclude this posiblity uisng bulk RNA expression alone? There may be other ways to do this too, but the current approach does not seem sufficient. Furthermnore, previous indiret evidences in other X0 systems (e.g. crickets and grasshoppers) have suggested that the X chromosome is inactived during male meioses similar to the XY body in mammals because of the differential chromathin condensation patthern of the X (called heteropicnosis) regarding autosomes.

We agree that single-cell RNAseq on different cell types of the aphid testis would be interesting (we mention this point in the ms lines 459-462). Note however that - before the present study - only two other studies generated transcriptomes in male aphids, and in both cases it was whole body transcriptomes). Our logical approach was thus to first have an overview of what happens in different types of male tissues, before going to more precise analyses such as single-cell approaches. Furthermore, as noted by the editor, the fact that males have only one X copy but nevertheless show increased expression compared to females (which have to Xs) makes our results quite robust despite our rather crude entire organ approach.

#2 Page 18 (Discussion), middle of the first paragraph:
- It said that "Indeed, sex chromosomes of other dosage-compensated species are generally not compensated in the gonads of the heterogametic sex in diptera and lepidoptera (Vicoso and Bachtrog 2015; Gu et al. 2017; Gu and Walters 2017)". I would suggest not generalizing the idea. Using single-cell RNA-sequencing, two recent Drosophila paper (https://doi.org/10.1038/s41467-021-20897-y; https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1009728) have showed that X chromosome is expressed at a higher rate in spermatogonia than expected based on DNA copy number alone, supporting the idea of X chromosome dosage compensation in the premeiotic male germline. However, authors also showed that the X chromosome is specifically inactivated in primary spermatocytes (MSCI) based on measuring all genes or a set of broadly expressed genes in testis they identified (https://doi.org/10.1038/s41467-021-20897-y). Please correct this sentence accordengly.

Thank you for this recent reference. We have corrected our sentence to account for these new results and we now cite these articles (lines 459-471).

#3 Page 18 (Discussion), at the end of the first paragraph:
- It is said that "The silencing of sex-linked genes during gametogenesis could have evolved to protect from the invasion of segregation distorters that would bias sex-ratio (Meiklejohn and Tao 2010)". Other transcriptional silencing mechanisms can evolve to protect the largely dissimilar XY or X0 chromosomes by defending un-synapsed regions from stealthy invasive transposons that cannot be detected as foreign in the absence of a homolog. Silencing may also protect against unwanted recombination between the X and Y, or to repair damage created by lack of recombination in XY or X0. I would suggest that the authors add these potential mechanisms in the text, and stress that these models are unlikely in the case of aphid males X0, which completely lack meiotic recombination.

Thank for these information. We now mention these additional mechanisms (lines 471-484).

Reviewed by Ann Kathrin Huylmans, 10 Oct 2021 19:31

In this paper, the authors test in the pea aphid whether masculinisation of the X chromosome is present in all tissues or restricted to only a few. In the latter case, sexual antagonism cannot explain the distribution of sex-biased genes as this presumably affects all tissues equally. They find that the enrichment of male-biased genes concerns all tissues and the authors take this as further support that sexual antagonism together with the interesting mode of reproduction (XX/X0 and cyclic parthenogenesis) drive the masculinisation of the X in this system.

The study is nicely designed and carried out. All the ground work on sex-biased genes in pea aphid has already been laid by previous work by Jaquiéry et al. and it is a natural next step to look at individual tissues (or compound structure as it is mostly the case here) to if this is maybe driven by individual tissues such as the gonads or the brain as has been found in other systems.

I also like that the authors also look at duplicated genes that can alleviate sexual antagonism and in a system with sex chromosomes, can also be translocated to either the autosomes or the sex chromosomes (in this case only the X). This study represents an interesting case where it is indeed possible to show that specifically male-biased copies are retained on or translocated to the X.

Thank you!

The one major point in the methodology that I find strange is the definition of biased genes. Why do the authors use 70% of all reads as a cut-off? At least it should be demonstrated that 60% or 80% does deliver the same patterns. This cut-off seems somewhat arbitrary to me and is not explained. Furthermore, what happens if you instead use traditional differential expression analysis? Do the patterns hold and you just loose statistical power because of fewer genes in your analysis or does this change any thing more fundamentally? Because the programmes do have a point of throwing out very lowly expressed genes or those with strong heterogeneity among replicates. As the whole paper builds on this analysis, I think it is crucial to clarify this point, i.e. explain why use 70% and show that it does not change the results qualitatively if another method to define differential expression is used.

Thank you for this comment. We have responded to it in detail in our response to the editor, as this was also a point of concern for her. We have now included additional analyses in the ms that show the robustness of our approach.

In addition, I have only a few minor comments that could be rather easily addressed:

1) Abstract: “We observed a masculinization of the X at the tissue-level, with male-biased genes being 2.5 to 3.5 more frequent on the X than expected.”

This is not quite clear. Upon first reading, I thought this meant that masculinisation is only observed in some tissues.

Thanks, we have now changed the text (lines 31-33).

2) I think there is a typo/mistake here in the parenthesis, at least it is not clear to me what the “whether sexual or parthenogenetic” means:

“The mode of Log2 ratio of male-to-female log2(RPKM+1) (whether sexual or parthenogenetic, figure 4CFI and Supplementary figure S2) lies close to 0 for both autosomal and X-linked genes”

We have now clarified this point (lines 272-273).

3) “This indicates an overexpression of some of the genes located on the single X chromosome of male cells, which exceeds dosage compensation. This pattern was expected, given that male-biased genes are significantly more frequent on the X than on autosomes (figure 2).”

So when sex-biased genes (more than 2-fold different) are excluded, is there full dosage compensation? This point was not entirely clear to me and should be tested as the enrichment of highly male-biased genes could mask the underlying patterns.

Right. We have redone the analyses by removing genes with a larger than 2-fold change in expression between males and sexual females in at least one of the tissues (i.e. sex-biased genes). We still find evidence for dosage compensation in all three tissues. The new figure is now included as supplementary file S5, and we have also modified the text accordingly (lines 279-282, 595-599). We have also added a supplementary figure investigating dosage compensation along the X (supplementary figure S6).

4) While in the figures, I find the abbreviation for the sample types helpful, they reduce readability in the text a bit. Maybe it makes sense to write “parthenogenetic female legs” rather than “PL” for example here: “Conversely, parthenogenetic female-biased genes were significantly less frequent on the X, except for PL+ genes due to lack of power[...]”

Changed as suggested (lines 350).

We also checked that the definition is always nearby when the abbreviation is used so as not to lose the reader.

5) Concerning dosage compensation of the testes in the discussion: Is it known whether the X chromosome has fewer TEs than other arthropods where X-inaktivation or X-downregulation occurs?

TEs seem to be frequent in two genomic regions on the pea aphid (see figure 4 from Li et al. 2019 MBE): One region is located at the end of the X chromosome, and the second one on autosome 2. Transposons also seem more frequent on the X (though no statistical test was done in Li et al. 2019). Based on our analyses, it also appears that the end of the X (which is rich in TE) is characterized by high frequency of male-biased genes (see supplementary figures S3 and S6). However, we prefer not to speculate on that point as we think we have no good data or clear ideas to make a significant contribution to this topic.

6) I do not understand this part of the methods: "Given the low number of genes satisfying this criterion (n=210), gene classes with male-biased expression (i.e., M+, MG+, ML+ and MH+ genes) were aggregated. " What do you mean by aggregated? Did you use additional genes, i.e. ones other than those with 1 copy on the X and 1 copy on an autosome?

No, we exclusively worked with the 210 pairs of genes. We wanted to test if - among these 210 pairs of genes - the frequency of the different classes of expression differed between the X and autosomal copies. Testing this for each of the formerly described class of genes (MG+, MH+, ML+, M+, FG+ etc, 16 classes in total) would obviously suffer from lack of statistical power. We therefore decided to group the 16 different classes of genes into 4 classes only (male-biased, sexual female-biased, parthenogenetic-female biased, not morph-biased) to have sufficient power. We have now clarified this point in the ms (lines 580-588). We also show in supplementary table S3 the raw data for each of the 16 classes of expression patterns.