Dear reviewers / recommenders,

We are pleased to submit a revised and improved version of our manuscript entitled “Sex-biased gene expression across tissues reveals unexpected differentiation in the gills of the threespine stickleback”

We are thankful for the positive reception of our manuscript and the constructive comment and suggestions on this first version. As suggested by the reviewers and recommender, we aimed for this new version of the manuscript to improve the presentation of known sexual dimorphism in three-spined stickleback and better link our discussion to its biology whenever possible. The introduction now provides a detailed view of intersex differences in morphology and reproductive behavior of the three-spined stickleback during the reproductive period, as well as a more comprehensive presentation of known sex-biased gene expression from previous study. In each section of the discussion, we have improved the link with existing literature, in particular for the brain, and provide a more accurate picture of how our results stand within pre-existing knowledge. Along those changes, we took into account all reviewers suggestions to improve clarity and readability, as well as corrected remaining mistake from the manuscript. We believe that this version of the manuscript allows for a better understanding of sex-biased gene expression in three-spined stickleback during the reproductive period.

Please find below our detailed answers to reviewers’ comments,

Sincerely,

Florent Sylvestre on behalf of all the authors.

Dear authors,

Thanks for submitting this preprint to the PCI Evol Biol. I found the manuscript attractive due to the general interest of the topic (sex-biased gene expression, sex chromosome evolution) and the quality of the data you gathered. Three colleagues have now reviewed your work. They all agree about the strengths of the study, while also consistently asking for a more elaborate discussion of its implications, in the light of the existing literature. I find the reviews quite positive and useful. I suggest addressing all the comments in a revised version which, I anticipate, should make a valuable contribution to the literature.

Please find below a couple of additional line-by-line comments by myself. Some of these are redundant with the reviewers' as they were made independent.

Looking forward to reading the revision, best regards,

Nicolas Galtier, CNRS - University Montpellier

Nicolas Galtier's comments

- l41: "Sex chromosome thus": the logical link between this sentence and the previous one does not appear obvious

This sentence was removed based on another reviewer's comment

- l51-57: how meaningful is the comparison of numbers of detected sex-biased genes across distinct studies? do they share the same approaches, power, thresholds, etc...?

The raw comparison is indeed not meaningful for the reason you mention. We have made that clear at l66-68 by specifying that limitations of between-studies comparison is precisely why studies like ours are important: "Of course, caution should be taken when comparing level of sex-bias across studies with different life stages, protocol, power and methods, which is why we need to characterize variation in sex-biased gene expression across tissue in a single framework if we want to better understand it."

- l67: I would suggest saying: "The lowered effective size of the Y (or W) chromosome, present in only one sex and therefore non-recombining, ..." as this is the main point of the cited Charlesworth & Charlesworth article.

We clarified this point, as it is indeed central to the prediction. L72 know says: "The lowered effective population size of non-recombining Y (or W) chromosome, present in only one sex, leads to lowered efficiency of natural selection and the degeneration of Y chromosomes (Charlesworth & Charlesworth, 2000)"

- l182: reference to "(lien?)" to be removed/modified

This place holder was removed.

- l83-97: I feel like a better job could be made connecting this bibliographic survey to your study. What questions are left open following the reviewed studies, which you would like to address? Are there

limitations/heterogeneity across studies (sample size, methodology, data analysis) that need to be overcome? You mention the use of lab-raised individuals, which is fine; could you perhaps elaborate on why your study is warranted and what novel interesting knowledge is expected to be produced, given the existing literature?

We improved the connection between the bibliography and study by 1) emphasizing the importance of sexual dimorphism during the reproductive period in contrast to the fact that most studies only cover the non-reproductive period, which represent an important gap in our knowledge of the three-spined stickleback biology. We also described previous results in stickleback in greater details, to emphasize the variability of observation and scarcity of knowledge (l88-119)

- A related comment would be that the discussion hardly compares your results to the existing ones, or addresses the reasons for the discrepancies, when there are some.

We agree that the discussion lacked a comparison with existing literature. We corrected that in each section. L377-381, we discuss whether the low overlap in sex-biased genes between tissues is concordant with results found in other tissue. L404-437, we improved our discussion on sex-biased gene expression in the brain, linking our results to known variations in hormonal levels across reproduction in other systems. L 459-470, we are now more explicit about the potential importance of sex-biased gene expression in the gills given the lack of prior information in the literature. We have also improved our discussion of highly expressed genes in the liver to better link it to the reproductive biology of the three-spined stickleback, L490-493, L509-513, 538.

l106: shouldn't the reference to Kotrschal et al. 2012 be part of the previous paragraph? (l83-91). This sounds like an important piece of the state of the art, but it is disconnected from the rest of the bibliographic survey.

While this was not made clear, Kotrschal et al. 2012 did not study gene expression, but reported strong sexual dimorphism in brain size in stickleback. We clarified that point and linked it to previous information. We also more clearly stated why we expected the brain to be differentiated, and how that is linked to previous studies (l142)

- l166: you mention using a method that differs from the classical negative binomial model, but no description of the method is made and no reference is given, besides the generic Benjamini-Hochberg paper. Is the method described in the DESEQ2 manual? If yes please state it, maybe refer to the manual section, and maybe add a couple of words describing it.

The methods are described at L213, as it corresponds to using the non-parametric Wilcoxon rank-sum test to compare expression levels between males and females instead of the traditional DESEQ2 models. This test is described in Li, Ge, et al., 2022 (https://doi.org/10.1186/s13059-022-02648-4), which was cited in the previous text. We reformulate the sentence to make the link between the test and the new method clearer. It now reads:

“Within each tissue, we used Wilcoxon rank-sum tests to compare gene expression between sexes and identify differentially expressed genes (DEG), as suggested by as a recent study which showed

that the classically used negative binomial models implemented in Deseq2 are subject to increased false positive rates in large sample size datasets (Li, Ge, et al., 2022). We used the Benjamini-Hochberg procedure to control the false discovery rate (Benjamini & Hochberg, 1995), using a 5% q-value for significance. »

- l332: "Synaptic signaling..." -> this sentence seems to say the same thing twice?

Indeed, we removed the second part of the sentence

- l337: "This suggests that gene ontology analysis in gills suffer from the lack of gill-specific information." I'm not sure what exactly this sentence is intended to mean, but it relates to a comment I'm having on this and the next (liver) section. GO enrichment analysis was here performed by comparing the annotations associated to DEGs in gills to the whole set of annotations associated to the three-spine stickleback genome. This is fine but we're left with the following question: are gill sex-biased genes enriched in ion-related and immunity genes because they are sex-biased, or because they are gill-expressed? This could be examined by contrasting annotations between gill DEGs and all gill-expressed genes, or between all gill-expressed genes and whole genome (and same for liver).

In our case, it is enriched compared to gill-expressed genes, as we compared the enrichment of significant genes to the distribution of gene ontology in gill-expressed genes only. We added this information to the methods. L230-233 now reads “We used goatools (Klopfenstein et al. 2018) to perform Fisher’s exact test for enrichment at a q-value threshold of 0.05 using the Benjamini-Hochberg procedure, using the set of expressed genes in each tissue as the reference set as to not confound the enrichment of sex-biased genes with enrichment driven by the specific role of each tissue. “.

- Not being a fish expert, I was not aware that extensive sex-biased gene expression in the liver is documented in this group. Your results strongly corroborate this previous finding. Maybe would you like to recall what is known or thought about the reasons for this pattern? Why the liver and not the brain, for instance? Is this specific to fishes? Feel free to comment on others' suggestions if any, and to share your own hypotheses.

We have improved our presentation of why the liver is sexually dimorphic in teleost, by stating its function in egg production. L59-61 ; “Liver is sexually dimorphic, specifically in oviparous species, where it produces many proteins or proteins precursors which will be stored in the eggs (Qiao et al., 2016; Darolti & Mank, 2023), and many genes have been identified as sexually biased in salmonids (Sutherland et al., 2019) and across cichlid taxa (Lichilín et al., 2021).”

However, as our work does not aim at comparing pattern between species, and our introduction quickly center our discussion around teleost and sticklebacks, we do not think that adding more information about other group such as mammals or bird will help the understanding of our work – specifically as the introduction is already heavy in information. This pattern is not restricted to teleost, and the liver is dimorphic in other systems like mice, although it is not the case in all mammals studied.

Review by anonymous reviewer 1, 27 Aug 2024 11:51

Sylvestre et al. describe a fantastic dataset for exploring sex-specific patterns of expression in wild-caught sticklebacks, with over 30 individuals of each sex sequenced for RNA of brain, gill and liver. They describe substantial heterogeneity between tissues in the amount and functional nature of sex-biased genes in the three tissues and confirm that dosage compensation has not evolved in this group in response to Y chromosome degeneration.

I have some questions below about data acquisition and analysis, which I think are worth clarifying in the manuscript.

* In response to the specific questions asked by PCI: I found the title a bit disconnected from the article, since gills are a small part of the results, and most of the sex-bias is found in the liver. But that's a matter of personal taste, I also understand why you would chose to emphasize the most surprising result.

We understand the reviewer's comment but chose to keep the original title.

* "We collected adult anadromous three-spined sticklebacks from tide pools of the St Lawrence 120 River at Baie de l'Isle verte (48.009961, −69.407070). "
--> Do you have a sense of how old the individuals were? Would age heterogeneity affect the results?

Samples were roughly balanced based on size (which is correlated to age in stickleback) to try to limit this factor. We did not include this information in the manuscript as it is still imprecise in our opinion.

* "In fish, global dosage compensation has rarely been found (Darolti et al., 2019) and 81 we still lack knowledge about the extent of the evolution of dosage compensation in this highly 82 diverse group."

--> this sounds slightly confusing, as it could mean that global compensation has not been found in fish with differentiated sex chromosomes, or that it has not been studied because there are not so many fish species with differentiated sex chromosomes. Could you clarify?

The former hypothesis is true, and this have been made explicit in the text on L83-87 "Moreover, many genes are dose insensitive (i.e., their copy number does not affect their expression level or protein abundance), therefore they do not need to be compensated. In fish, global dosage compensation has rarely been found when studied (Darolti et al., 2019) and we still lack knowledge about the extent of the evolution of dosage compensation in this highly diverse group."

* "Identification of Shared Genes Between X and Y"
--> I could not understand how this was done. How exactly do the custom scripts work? What information is used to know if you have X and/or Y-derived transcripts? Are X- and Y-linked genes already annotated? Could you make the methods more explicit?

Orthofinder provides a simple table where one row is a group of transcripts, with one column for each species (one for ninespine and one for threespine in our context). The custom script parses this table, and using the annotation of X and Y chromosome from NCBI applies to each row the rules we described in the paper:

1) Orthologues between X and Y correspond to row where all the transcript in the threespine stickleback column belong to exactly one gene on the X and one gene on the Y
2) Genes for which transcripts were scattered across multiple orthogroups (multiple lines) were filtered out, as the relationship is not clear
3) Genes with scattered transcript were rescued only if the scattered transcript were alone in their group, as it simply suggests that orthofinder failed to cluster them together.
4) Orthogroups constituted of transcript from only one gene on the X or one gene on the Y were classified as hemiploid for that chromosome and having lost their copy on the other chromosome.
5) Orthgroups containing multiple genes of either or both sex chromosomes were filtered out
6) Orthogroups which also contains transcript from autosomal genes were filtered out as they rather correspond to gene duplication from the autosomes
7) Genes for which transcript were not assign any orthogroups were considered as hemiploid.

We improved the manuscript to better reflect the file structure and the fact that we have this annotation (L254-261, 263-265)

*"We had a percent mapping of uniquely mapped reads to the reference genome ranging from 44.3% to 88.61% (median 76.76%)."
--> This seems low. Could you provide numbers for each sample (perhaps this is already in a supplementary table, I could not find these), and perhaps repeat analyses with only samples with good mapping rates as an additional line of evidence that the conclusions are robust? More importantly, are there systematic differences in mapping rates between tissues? These could affect your power to detect sex-bias.

After exploration, variations in mapping rates can be attributed mainly to ribosomal RNA and internal and external transcribed spacer, both of them related to cellular activity and possessing multiple, undifferentiable copies across the genome. It is not associated with reads or sample quality, and variation in total number of reads should be controlled by the normalization process. To test that, we measured Mean gene expression in two datasets: the whole liver and a dataset with only 10 individual between 70-80% of mapping rate for each sex (close to our average). Correlation of both mean expression value are >99%, suggesting that it does not affect our analysis. Information about this was added in the manuscript, L307-312 : “Unmapped reads in low quality samples mainly corresponded to ribosomal RNA and external transcribed spacer, genes known to have multiple copies across the genome. Correlation between mean expression levels estimated on the whole dataset and in a subset of 10 males and 10 females between 70-80% of mapping rates were all above .99%, suggesting that our normalization process was effective in correcting the differences in total coverage in samples with low mapping rates »

* On a similar note, while the PCA is reassuring, could you also provide correlation values between replicates? Or more generally a measure of heterogeneity within the sexes as well as between them? From the PCA it seems that liver is highly heterogeneous independent of sex, brain is highly homogeneous, and gills are somewhere in between, so sex-bias may just mirror general patterns of heterogeneity.

Correlation value between all replicates would result in huge tables (36*36 for the gills in female alone?), and we are not sure which additional information it would actually provide. Yes, the liver is globally less homogenous than the brain and the gills, but we expect this to ultimately reduce our power to detect differences between any two groups, but this is the tissue in which we have the most differentiation between sexes. Thus, we would like to maintain the focus on the PCA analysis.

* Along those lines, would it be possible to color males and females in the PCA?

We added colors for males and females on the PCA

* Do you have any kind of mininum expression filtering? If not, it could be reassuring to see that patterns hold when very low expression genes are removed, or you could justify why this was not deemed useful.

We kept only genes with at least 1cpm in 10 samples, as stated at L195.

* "After filtering, we identified five overexpressed genes in the liver but none in the gills or the brain."
--> overexpressed relative to what? Could you define this more specifically?

Indeed, we omitted to define this term. Overexpressed genes correspond to genes representing at least 10% of total read counts in one sample. This element was forgotten in the methods, but is now defined at 209-211, with a renaming to “highly expressed genes” to better reflect the method: “Additionally, we identified genes representing more than 10% of total read counts in any individual (hereafter named “highly expressed genes”)”

* 418 Sex-biased Gene Expression on Sexual Chromosome Mostly Reflects Gene Loss in Non-Recombining Regions
It would be helpful to know how many genes are being considered in each of the categories (diploid, hemiploid X, hemiploid Y)

We added this information to the text, L551-554

We identified 235 expressed genes still having both their X and Y copy in the brain, 545 having lost their Y copy and 144 their X copy. Numbers are similar in the gills (respectively 242, 528 and126 expressed genes) and liver (204, 405 and 85). Detailled results by strata are presented in Table S9.

* Fig 5: Why do you have all and diploid in the PAR? Should all PAR genes not be diploid?

Thank you for noticing that! This is caused by the merging of two dataset in processing the data for this figure, which lead to the same data being present twice with different label. This has been corrected, as indeed “all” and “diploid” genes contain the same information for the PAR

Review by anonymous reviewer 2, 23 Aug 2024 05:14

Review of the manuscript «Sex-biased gene expression across tissues reveals unexpected differentiation in the gills of the threespine stickleback»

In the manuscript untitled «Sex-biased gene expression across tissues reveals unexpected differentiation in the gills of the threespine stickleback», the authors explore the patterns of sex-biased gene expression in brain, liver and gills during the reproductive period in males and females of the sexually dimorphic threespine stickleback. Confirming previous results, they found that expression in the liver is extremely sex-biased, whereas the brain is not, and that there is a lack of global dosage compensation in this young sex chromosome system. They also found that gills exhibit high levels of differentiation, an interesting result as sex is rarely considered in physiological and ecotoxicological studies of gill responses in fishes. They outline a few genes and gene functions that are particularly sex-biased, and thus likely determinant in sexual dimorphism in this species. Those descriptive results bring some insights into the role of sex biased expression in sexual dimorphism in sticklebacks. It would be even more apparent if the authors added a brief description, for instance in the introduction, of the sexually dimorphic phenotypic traits observed in the focal species for the non-specialist reader. The manuscript is well written, the topic is well introduced, the used methodology is sound (notably the choice of sampling during the reproduction period, and to use the QuanSeq 3' UTR technology for sequencing) and the analyses are clear and well conducted (only a few details of the material and method require some clarification) and the conclusions adequately supported by the results.

Comments:

1. A non-specialist reader would greatly benefit from a brief description of the phenotypic traits that are under sexual dimorphism in the threespine stickleback (for instance in the introduction at around line 84). It would also be interesting to link the gene ontology observations to those phenotypic traits. For instance, you found several GO enrichment terms related to immune system in SBG, are there any indication that male and female show different immune response in some context?

We agree and have added a description of sexual dimorphism in threespine stickleback at l88-130 and added some link between known dimorphism and GO when possible. However, most gene functions are vague and relationships with phenotype are usually not possible.

2. I really appreciated that the a priori expectations were clearly stated at the end of the introduction. It would be even better if they were more deeply discussed later in the manuscript: for instance the expectation that the brain would be highly sex-biased during the reproduction period (line 106-107) is eventually not met in the results: what could be some possible explanations?

We agree that the relationship between prediction and results could be better highlighted in the brain. We improved this part of the discussion by comparing to previous results in our species L 404-437.

3. Maybe briefly explain the principle and advantages of the use of the QuanSeq 3' UTR technology to quantify gene expression level, and how this might impact comparisons with other studies based on classic RNAseq.

We added this information L168-172: “This approach reduces the required coverage for estimating gene expression levels but removes the possibility of study alternative transcript and sequences variation. This tool allows the study of many samples at a reasonable cost.” And L175-177 : “A read length of 50 bp was selected as longer sequencing will only result in longer sequencing of the poly-A tail, which does not contain helpful information about the gene sequence.”

4. In the “Alignment and expression counts” section of the material and method:

-What HTSeq parameters/functions have been used ?

The section has been updated: L185-189 “We used STAR two-pass mode to discover reads junctions and improve mapping accuracy in the second pass, then quantified gene expression using HTSeq v0.11.3. (Anders et al., 2015) using htseq-count in union mode and no strand constrain (-s no) after filtering out multi-mapping reads using samtools”

-Multi-mapping reads have to be filtered out to obtain reliable read counts. However, for X-linked genes still retaining a Y copy outside the PAR, I expect reads could map on both the X and Y copy in males. How was this handled for the quantification of expression of XY gametologs? A related question: how was X and Y allele expression measured for those gametologs?

In males, we have a Y and X reference genome. For gene still sharing a copy on both chromosomes, XY expression was estimated as the sum of the reads mapped on the X and Y copy, which we obtained thanks to mapping to both the X and Y reference genome. Because multiple-mapping reads are removed prior to this step, it could in theory lead to reduced expression in males compared to females. However, Peichel et al. 2020 showed that cross-mapping between the two reference genomes, while existing, was not frequent. Addition, this shouldn’t affect specifically the Y or X copy, and we find that female-biased genes in sex chromosome are driven mainly by Y-specific down regulation. Because of that, we do not think that cross-mapping reads strongly impacted this study.

5. For the non-specialists, briefly explain what the different normalization methods in different analyzes normalize for (within sample/within dataset/between samples comparisons) : cpm, Vst (it should be stated that it stands for Variance stabilizing transformation), and the the (average? Median?) ratio normalization factor.

We added this information on l196-197: Cpm normalization allows comparison of gene expression level by normalizing read counts by the total number of reads per sample; l199-200 variance stabilization transformation (Vst) as implemented in DESeq2 v1.40.2 (Love et al., 2014), which normalize for the increases in variance with mean gene expression and is recommended for clustering-based analysis; and l202-204 For all other analyses, read counts normalization was carried out independently for each tissue using the average of ratio normalization factor implemented in DESeq2, which account for differences in number of reads and gene composition of each samples to allow precise comparison of gene expression across samples.

Average was also corrected by median, as you pointed out.

6. Foie/ Cerveau / Branchie are kept in french in supplementary table 1 and 2.

This was corrected from supplementary tables 1,5 and 7

7. At line 121: under five, seven and ten minutes respectively → after death?

Yes, we added this information on L157

8. At line 119: What year were the samples collected?

In July 2018, we added this information

9. At line 122: We disrupted samples in

This was corrected

10. At line 182: (lien ?) was kept in the text → https://zenodo.org/records/11477976

It was removed, as the information is in the data availability statement

11. At line 312: (0.78%) of expressed genes) → (0.78% of expressed genes)

This was corrected

12. At line 361: Is it chromosome V, as stated in the text, or chromosome VII, as stated in Figure 2 legend, which is enriched in male-biased genes?

In fact, chr IV and VII are enriched in male-biased genes and chrXVIII toward female-biased genes. This has been corrected in the text (L474-477) and the figure

13. At line 366: total gene expression

This was corrected

14. At line 429-431: Precise the statistics that are stated there.

This information was clarified

15. At line 450-453: "In sticklebacks, hemizigous genes tend to be dosage insensitive, meaning that protein quantities are independent from its expression level (Peichel et al., 2020)." → I would alleviate this sentence, as the Peichel et al., 2020' result only indicate that genes with a retained X and Y copy were more likely to exhibit haploinsufficiency based on orthology with human genes.

"This suggests that there is no selective pressure to evolve dosage compensation and is corroborated by the fact that conserved genes are dosage-sensitive and evolving under purifying selection (White et al., 2015)." → I am not sure I understand the second part of this sentence: is it that as Y-linked genes show signs of degeneration, they are likely to be under relaxed selective pressure and thus to be dosage-insensitive?

To answer these two questions, we reformulated the sentences to better identify what is known from what is speculation: l573-578 In sticklebacks, conserved genes between the X and Y chromosomes are enriched in haplo-insufficient function (Peichel et al., 2020) and evolving under purifying selection (White et al., 2015) , meaning that losing one copy would affect their expression

level, potentially affecting their stoichiometry with other cellular component. which might suggest that lost genes were not as impacted by deleterious mutations affecting expression levels. This, coupled with the apparent lack of dosage compensation, suggests that there is no selective pressure to evolve dosage compensation.

16. At line 477-484: (p-value=2.10-2)

We homogenized the notation of p-value across the text. The format is now: $2x10^{-2}$

Review by Qi Zhou, 21 Jul 2024 23:16

Sex biased genes are widely found throughout the animal kingdom as potentially the result of resolved sexual conflict. This work inspected the pattern of sex biased genes in gill, brain and liver from the wild population of threespine sticklebacks, a model species for teleost sex chromosome evolution. The species has been previously reported to evolved three recombination suppression events (evolutionary strata) between the XY chromosomes. This work found liver harbors many more sex biased genes than the other tissues, with also many sex biased genes unexpectedly in the gill.

Overall I found the work interesting and the conclusions are generally consistent with what one would expect or what the previous results reported.

Since there are several works (Kitano et al. 2010, Leder et al. 2010, Kaitetzidou et al. 2022) did a similar study but with different techiniques, or sample sources, I suggest deeper comparison with them, particulary in the regard of lab vs. wild collected samples, as stated in the introduction by the authors.

As suggested by you and other reviewers, we improved our comparison to previous study, in particular in the brain where many results are available. L404-437 now compares our results for the brain to previous results and discusses potential causes for the differences. L 98-116 now does a better job a describing expected results based on existing literature for sticklebacks

Other aspects that need to be addressed is the techinical aspect of the study, including the definition of sex biased genes, that could lead to very different conclusions. For example, the authors first stated over 5000 sex biased genes in the liver, but then it was reduced to around 300 if the authors applied the same cutoff with one of the previous study, if I understand it correctly.

We are not sure where this comment wants to go. This aspect is addressed and discussed in our results, as cited by the reviewer. We made it clearer from the introduction that studies spanning multiple tissues in a single framework are essential because many biological and technical aspects of gene expression analysis are not strictly reproducible across studies, making comparison difficult. This aspect is also addressed in the discussion.

Another contradiction is the feminization pattern in the PAR, that the author found a different result compared to a previous study, due to the usage of autosome or outgroup orthologs, but which one is more appropriate?

The latter is more appropriate, as it allows us to directly compare the ancestral expression level of each gene rather than an estimation from autosomal expression level. This has been made more explicit on L582-583: “This could be caused by the use of autosomal average expression level as the ancestral level of expression instead of the use of a closely related species to estimate each gene ancestral expression rate, which is more accurate.”

Finally, a general issue is that the paper is organized in a way that the authors reported the number or general patterns of sex biased genes, then decribe invidiual genes in these tissues. Somehow the reader cannot have a coherent conclusion from these pattern throughout the paper. For example, why gill, a tissue that is not expect to have many sex biased genes show so many in this study? This has not been discussed before and newly found in this study. The author might look deeper into this, just to draw a difference from all previous studies. I have many detailed commments that I marked in the pdf uploaded together with these comments.

This comment matches other reviewers and editor’s opinion that we should improve the organization of the paper to better deliver our main findings and conclusions. We believe that we have addressed it through our previous responses, and that this manuscript is indeed clearer and more useful in its new state, and we thank you for all your suggestions.

Answers to individual comments extracted from the PDF:

L17 : do you mean between species or between sexchr vs. autosomes?

Neither. We meant between sex chromosome pairs. This has been made explicit on L17 “Moreover, differences in gene content between heteromorphic sex chromosomes contributes to sexual dimorphism”

L23: do you mean one non-recombing region with three strata?

Yes. This has been corrected: 23: “Sex is determined by a young XY sex chromosome pair with a non-recombining region divided in three strata, which have started to degenerate”

L32: you mean hemizygous genes?

Yes, it has been made explicit on L32: “in hemizygous genes”

L41: this sounds ambiguous, do you mean sex determination, sexual reproduction or sex dimorphism here. As you start with one of these.

This line was removed from the text as it was too ambiguous and an unnecessary precision

L84: do you mean in phenotype? Please provide more information about this

We now provide more information concerning sexual dimorphism in stickleback at different biological levels. From line 88 to 119

L88: this is incorrect, the introduction should describe briefly what Kaitetizdou et al. 2022, Kitano et al. 2020 found, and compare this paper's results to theirs.

This section was completely rewritten to better reflect pre-existing literature (L88-119), as suggested by you and other reviewers

L91: How are genes identified by Leder et al. 2010 compared to your results, both of which used livers.

We addressed the two precedent comments by largely rewriting this section, which more accurately describe previous knowledge in sex-biased gene expression in stickleback. (L88-119), as suggested by you and other reviewers.

L91: sex chromosomes

This has been corrected, as well as other occurrence of “sexual chromosomes” in the text

L94: which strata

We added this information (stratum I) at L124

L104: But why not tissues that exhibit strong sexual dimorphism during reproduction. Hence I am interested to know what tissues or what phenotypes do threespine sticklebacks exhibit SD during reproduction period

As specified earlier, we improved our introduction concerning the stickleback biology, which should have answered this comment

L247: what do you mean by overexpressed?

This was a misusage of the word “overexpressed”. We replaced it by “highly expressed genes” and explain that this term corresponds to gene representing more than 10% of total reads in any individual at L209-210: “Additionally, we identified genes representing more than 10% of total read counts in any individual (hereafter named “highly expressed genes”).

L264: overlapped

This has been corrected

L278 : do you mean sex-specific alternative splicing? did you find any case of this?

We did mean sex-specific alternative splicing, but were not referring to signal from our dataset, but to possibilities according to the literature. Our Quant-seq approach does not allow us to examine alternative transcript, as we only sequence a short part of the mRNA. This is now specified in the methods (L166-170), and the sentences have been clarified in the discussion. L 348-349: “Similarly, sex-specific alternative splicing can provide an alternative route from gene regulation to generate sexual dimorphism”

L298: is there any report of this gene in threespine stickleback? If the sex biased pattern varies across species, one could not draw conclusions from the result in threespine sticklebacks

We did not find reports of esr2b for the three-spined stickleback, and updated our discussion to reflect the fact that we cannot draw conclusion for this gene. L362-365: “In the liver, esr2b showed strong expression levels and was female biased (LFC of -0.35), and could be associated with

vitellogenesis process, although it seems to vary across species (Dominguez et al., 2014; Chen et al., 2019) , which prevent us to interpret it in the context of the three-spined stickleback."

L315: How are the numbers compared to each other if sex chromosomes are excluded?

Kaitetzidou et al. also provided the number for the autosomes alone. The wording of this section was convoluted for no reason and was improved to directly compare the results. L406-408 "These results are in the same order of magnitude than the ones from Kaitetzidou et al. (2022), which identified 104 sex-biased autosomal genes in the brain."

L330: what are the explanation for the male biased genes in gills, while the tissue is assumed to not show many sex biased genes?

The tissue is assumed to not be biased based on the only previous study, and the fact that study in the gills usually overlook sex in their study design, which suggest they don't expect this factor to play a significant role in gene expression. Therefore, we do not discuss our results as in contradiction with the literature, but rather as a new avenue to explore in studies interested in gills

L355: I think the difference with previous results is mainly because of the stage or the animal source (lab vs. wild). Discussions should be focused on these aspects.

We agree that sex vs wild condition and stage is a key factor driving potential differences with the literature. To address this, when literature was available, we specified whether it was from experimental or wild condition. However, this aspect in particular is difficult to discuss at length, as 1) comparison between study is done to give a referential but should be taken cautiously as condition / analytical framework have a strong impact and 2) Sex-biased gene expression during reproduction have not been reported previously in stickleback, so it is complex to draw conclusions relative to the origin of the differences.

L365: is this also a wild sample?

It is lab raised, which is now specified in the text (L481)

L368 then the large number of SBG is because a different cutoff used in this vs. previous studies?

We answered this in your general comment above

L420: how much % of the entire sex chromosome genes

The information was specified later in the text but has been regrouped in one sentence to make the information easier to find. L545: "Most genes located on sex chromosomes exhibited sex-biased gene expression (499 in liver, 755 in gills and 813 in brain, respectively 71%, 83% and 87% of expressed genes on sex chromosomes)."

L458: what do you mean by active sexual conflicts? This is somewhat contradictory to resolved conflicts.

This was not clear, and not pertinent to the discussion. Distinction between resolved and unresolved sexual conflict have been removed from the text

L463: are there any Y-linked genes left in stratum 1&2? As all previous hypotheses were for hemyzigous X-linked genes

Yes, we found Y linked genes in all strata, as you can see on Figure 5 (which now includes the number of genes for each category as a response to a previous comment). Results for Y-linked genes can be found at line 551-555, and we added a short discussion of these results

L466: which one is more appropriate?

The use of ancestral gene expression should provide more precision for such a question, as it allows to account for each gene ancestral state and genetic background. This precision is now discussed at L598-600: “This could be caused by the use of autosomal average expression level as the ancestral level of expression instead of the use of a closely related species to estimate each gene ancestral expression rate, which is more accurate.”