Title: Sex-biased gene expression across tissues reveals unexpected differentiation in the gills of the threespine stickleback

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Abstract:

Sexual dimorphism can evolve through sex-specific regulation of the same gene set. However, sex chromosomes can also facilitate this by directly linking gene expression to sex. Moreover, heteromorphic sex chromosomes often exhibit different gene content, which contributes to sexual dimorphism. Understanding patterns of sex-biased gene expression across organisms is important for gaining insight about the evolution of sexual dimorphism and sex chromosomes. Moreover, studying gene expression in species with recently established sex chromosomes can help understand the evolutionary dynamics of gene loss and dosage compensation. The threespine stickleback is known for its strong sexual dimorphism, especially during the reproductive period. Sex is determined by a young XY sex chromosome pair with three non-recombining regions that have started to degenerate. Using the high multiplexing capability of 3′ QuantSeq to sequence the sex-biased transcriptome of liver, gills and brain, we provide the first characterization of sex-specific transcriptomes from ~80 stickleback (40 males and 40 females) collected from a natural population during the reproductive period. We find that the liver is extremely differentiated (36% of autosomal genes) and reflects ongoing reproduction, while the brain shows very low levels of differentiation (0.78%) with no particular functional enrichment. Finally, the gills exhibit high levels of differentiation (5%), suggesting that sex should be considered in physiological and ecotoxicological studies of gill responses in fishes. We also find that sex-biased gene expression in X-linked genes is mainly driven by a lack of dosage compensation. However, sex-biased expression of genes that have conserved copies on both sex chromosomes is likely driven by the degeneration of Y allele expression and a down-regulation of male-beneficial mutations on the X chromosome.

Keywords*:* sex, Liver, Gills, Brain, Stickleback, gene expression, dosage compensation

Introduction

Species with sexual reproduction often exhibit sexual dimorphism (Lande, 1980). Because sexes share most of their genetic material except for potential sex chromosomes, sexual dimorphism can arise from the different regulation of the same set of genes (Ellegren & Parsch, 2007; Tosto et al., 2023). Sex chromosomes thus represent a key step in understanding the evolution of sexes. Sex-biased gene expression has been described in many systems as dependent on both life stages (Djordjevic et al., 2022) and tissues (Rodríguez-Montes et al., 2023). Compiled data for five tissues in five mammals and a bird species show that sex-biased gene expression varies in intensity across tissues, species and sexual maturity, with the contribution of sex chromosomes being also variable (Rodríguez-Montes et al., 2023).

The variety of sex determinism systems and reproductive behaviours present in teleost fish make them an interesting vertebrate group to study sex-biased gene expression (Thresher, 1984; Devlin & Nagahama, 2002; Kobayashi et al., 2013). Patterns of sex-biased gene expression have been described in fish somatic tissues, such as the brain or liver, which are known to play a role in reproduction. Sex-biased gene differentiation in the brain seems highly species dependent, with about a thousand genes identified in salmonids (Hale et al., 2018) but only a handful in the Gulp pipefish *syngnathus scovelli* (Beal et al., 2018) or the zebrafish *Danio renio* (Yuan et al., 2019). In cichlids, gene expression levels in the brain are associated with social status and gonadic sex (Renn et al., 2008; Schumer et al., 2011). Liver is sexually dimorphic, specifically in oviparous species (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). On the contrary, other tissues such as the gills have received strong attention in the context of adaptation to salinity, pollution or hypoxia (Scott et al., 2004; Van Der Meer et al., 2005; Gonzalez et al., 2006) but studies rarely account for potential sex dimorphism in the response of this tissue (but see (Lichilín et al., 2021). Given the dynamic nature of sex-biased gene expression, we need a better characterization of sex-biased gene expression in natural populations if we want to understand the processes underlying the evolution of sexual dimorphism.

Sex chromosomes play an important role in sex dimorphism (Rice, 1984). However, understanding patterns of sex-biased gene expression on sex chromosomes is particularly complex in species with non-recombining sex chromosomes, as they tend to degenerate and may involve dosage compensation (Bachtrog, 2013). The lowered effective size of the 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). Genes that have lost their Y or W copy (hemizygous genes) are expected to exhibit a reduced expression level that does not come from sex-specific gene regulation. However, lowered gene activity on sex-chromosome can have widespread effects on autosomal genes (Wijchers et al., 2010), and mutation reestablishing the ancestral level of expression in the heterogametic sex is expected to be advantageous, leading to the evolution of dosage compensation. Global dosage compensation, where the X (or Z) chromosome is overexpressed to compensate for the loss of Y (or W) genes was long thought to be necessary in systems with degenerated sex chromosomes, but accumulating literature outside

Drosophila and mammals suggest that it is not necessarily the case (Mank, 2013), with many groups exhibiting no or partial dosage compensation. Moreover, many genes are dose insensitive (i.e., their expression level does not affect the phenotypic outcome), therefore they do not need to be compensated. In fish, global dosage compensation has rarely been found (Darolti et al., 2019) and we still lack knowledge about the extent of the evolution of dosage compensation in this highly diverse group.

The three-spined stickleback is a model fish species with a young XY sex-chromosome system, at most 13–26 million years-old (Peichel et al., 2020), and strong sexual dimorphism. The sex chromosome consists of a pseudoautosomal region which is still recombining, and three evolutionary strata which have evolved through successive inversion (Peichel et al., 2020). Studies have found sex-specific splicing and protein expression (Viitaniemi & Leder, 2011; Naftaly et al., 2021), but few have described sex-specific patterns of gene expression in that species. The brain is the most studied tissue (Primmer et al., 2013; Kaitetzidou et al., 2022) and shows limited sex-biased gene expression outside the sex chromosomes (but see (Kitano et al., 2020). Similar results have been observed in the liver (Leder et al., 2010). Accumulation of sex-biased genes on sex chromosomes seems to be associated with a lack of global dosage compensation (Leder et al., 2010; Schultheiß et al., 2015; White et al., 2015) in that species, coupled with a potential partial dosage compensation in one of the evolutionary strata of the Y chromosome. However, these studies used laboratory-raised individuals outside the reproductive period when sticklebacks exhibit the strongest sexual dimorphism, and thus more studies are needed to understand sex-biased gene expression in natural populations during the reproductive period.

In this study, we took advantage of the high sample multiplexing capability offered by QuantSeq 3′-UTR sequencing (Moll et al., 2014) to profile the transcriptome of ~40 samples per sex in three tissues (liver, brain, and gills) in adults from a natural population of threespine stickleback from eastern Canada. We aimed at 1) describing the sex-specific transcriptome of this species in each tissue and 2) making use of the recently sequenced Y chromosome data to understand the dynamics of dosage compensation. According to work in other species, we expected the liver to be the most differentiated somatic tissue between sexes, as it plays an important role during reproduction in teleost fish (Meng et al., 2016). The brain is known to be sexually dimorphic during the reproduction of the threespine stickleback (Kotrschal et al., 2012) and we expected substantial sex-biased gene expression for that tissue. Finally, sex is not a factor usually accounted for when studying gills, an extremely important tissue for physiological regulation, and when it is, there is almost no sex-biased expression (Lichilín et al., 2021). We therefore predicted that this would be a neutral tissue with little to no sex-biased expression. We also expected to find that most genes on sex-chromosome exhibit sex-biased gene expression caused by the lack of global dosage compensation in that species.

Material and methods

Ethics statement

This study was approved by the Comité de Protection des Animaux de l'Université Laval (CPAUL, approval number SIRUL 109096). A fish permit was issue by the Ministère des Forêts, de la Faune et des Parcs du Québec (permit number 2018 04 11 005 01 S P) for sampling.

Sampling and Sequencing:

We collected adult anadromous three-spined sticklebacks from tide pools of the St Lawrence River at Baie de l'Isle verte (48.009961, −69.407070). Brain, liver, and gills were dissected (under five, seven and ten minutes respectively) and preserved in RNAlater at -20 C. In 2022, we performed RNA extractions using the RNeasy mini Kit (Qiagen). We disrupted samples is 700 µL (brain) or 1400 µL (Gills, Liver) of trizol using a mixermix 400 at 30 Hz for 3 minutes or until complete tissue disruption. After three minutes, we added 140 µL (or 280 µL) of chloroform, homogenized the solution and waited five minutes before centrifuging for 15 min at 12,000g. We collected the upper phase and added 550 µL (or 1100 µL) of ethanol before transferring 700 µL into a RNeasy Mini column (Qiagen). We then centrifuged for 15s at 11,000g, discarded the flow-through and repeated the operation until all the collected phase has been used. We then proceeded with the extraction protocol following manufacturer's instruction, including a DNase step, but replacing buffer RW1 by buffer RWT from miRNeasy Mini Kit (Qiagen), as it yielded better results for the brain. We then generated QuantSeq libraries using 3′ mRNA-Seq Library Prep Kit (Lexogen) with dual indexing to identify each individual and 18 PCR cycles for library amplification. After quality check on a 2100 Bioanalyzer (Agilent Technologies) and concentration estimation using Quant-iT PicoGreen (Invitrogen), 227 libraries were pooled to equimolarity and sent to the Centre d'Expertise et de Services Génome Québec (Montréal, QC, Canada) for 50 bp single-end sequencing on 2 lanes of an Illumina Novaseq X. Tissue disruption and quality check were performed at the Plateforme d'Analyse Genomique of Laval university (Québec, QC, Canada).

Alignment and Expression Counts

After quality check using FASTQC v0.11.8, we used fastp v 0.15.0 (Chen et al., 2018) to trim poly-A tails, Illumina adapters, and the first 12bp of each read, as they showed biased composition. We discarded reads shorter than 20bp long and proceeded with the alignment. We used STAR v2.7.2b (Dobin et al., 2013) two-pass mode to align reads to the stickleback reference genome V5 (Peichel et al., 2020; Nath et al., 2021), accessible on NCBI as RefSeq assembly GCF_016920845.1), excluding the Y reference sequence for the females and its pseudo-autosomal region (PAR) for males. 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) in union mode after filtering out multi-mapping reads. Counts from each sample were merged using custom R scripts, leading to four datasets: the total datasets comprising all samples, and one dataset for each tissue (Liver, Gills and Brain).

Filtering, normalization, and quality check

Unless stated otherwise, analyses were performed in R v 4.3.2 (R Core Team, 2021) and python 3.9.12 (Rossum & Drake, 2010). For each dataset, we applied the same filtering procedure. First, samples with fewer than 5,000,000 raw reads counts were excluded, as preliminary principal component analysis (PCA) showed that they clustered together (result not shown). Then, we kept only genes with one count per million (cpm) in at least 10 samples. To identify potential errors in the dataset (in particular, samples where tissue was misidentified), we first performed a PCA on autosomal genes across all samples, using blind Vst normalization as implemented in DESeq2 v1.40.2 (Love et al., 2014). For all other analyses, read counts normalization was carried out independently for each tissue using the average of ratio normalization factor implemented in DESeq2. Given the specificity of genes on sex chromosomes, only autosomal genes were used for the calculation of the scaling factors in each tissue, which were then used to normalize sex-chromosome genes independently. Note that gene length was not accounted for in the normalization process, as it could generate a bias in our dataset as QuantSeq only sequences the poly-A tail of each transcript, not the full transcript.

Differential Expression Analysis of Autosomal Genes

Within each tissue, we used Wilcoxon rank-sum tests to identify differentially expressed genes (DEG), using the Benjamini-Hochberg procedure to control the false discovery rate (Benjamini & Hochberg, 1995). This approach was selected as a recent study suggested 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 also calculated the log-fold change (LFC) of gene expression between sexes as, for each gene:

$$LFC_g = \frac{\sum_{m=1}^{N_m} log2(g_m + 0.5)}{N_m} - \frac{\sum_{f=1}^{N_f} log2(g_f + 0.5)}{N_f}$$

with gm and gf being the normalized read count for each male and female, and Nm and Nf the number of individuals from each sex.

Functional Characterization of DEG

To explore common functions among sex-biased genes, we performed Gene Ontology (GO) enrichment analysis. To do so, we used blastX (Camacho et al., 2009) to gather the Swiss-Prot (Schneider et al., 2009) annotation for each sequence of the three-spined stickleback transcriptome available on NCBI (RefSeq assembly GCF_016920845.1) and gathered gene ontology information from the associated UniProt entry (The UniProt Consortium, 2023). We then summarized transcript-level GO at the gene level using information from the NCBI annotation of our reference genome with a custom python script (lien?). 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. We also combined Zfin (zfin.atlassian.net), genecards (www.genecards.org) and Uniprot (www.uniprot.org) databases to provide a more precise

functional characterization of 1) the 10 genes with the lowest q-value and 2) the 10 genes with the highest LFC for each tissue, as well as all DEG shared by all tissues. Functional annotations described in the results section are based on information from these databases unless otherwise noted.

To explore the genomic distribution of DEGs, we performed a Fisher test for enrichment analysis for each chromosome to test whether 1) some chromosomes are enriched in DEG considering their number of genes and 2) DEG within a chromosome are enriched toward male-biased or female-biased genes considering the global distribution of sex bias toward each sex. We used a Benjamini-Hochberg procedure to control the false discovery rate independently for the two hypotheses tested.

Identification of Shared Genes Between X and Y

The study of sex-biased gene expression on sex chromosomes is complexified by independent gene gain or loss on their non-recombining region. To identify gene loss or gain, we first extracted transcript sequences from the reference genome using gffread (Pertea and Pertea 2020) and the NCBI genome annotation for our genome version, using -C option to remove transcripts with no CDS. We did the same using the available ninespine stickleback reference genome (Varadharajan et al., 2019) and annotation (GenBank accession GCA_949316345.1), then used Orthofinder v2.5.5 (Emms & Kelly, 2019) to identify orthogroups with default parameters. We used a custon python script to identify transcripts likely to be orthologs between X and Y chromosomes and summarize the information at the gene level, as each gene can have several transcripts. We accepted an orthologous relationship if 1) an orthogroup was composed of one transcript from X, one from the Y and other members originated from the nine spined stickleback. To account for the fact that one gene can have several transcripts, we accepted an orthogroup with many X or Y transcripts if they belonged to the same X or Y gene, but filtered out genes for which transcripts did not all belong to the same orthogroup (except if the transcript was not associated with any other orthogroup). Transcripts that fell in an orthogroup with only Y or X transcripts were categorized as hemiploid Y (no gene copy on X) or hemiploid X (no gene copy on Y). Orthogroups that contained autosomal genes, likely reflecting gene gain on the sex chromosome, were removed from analysis as well as genes with multiple copies on either sex chromosome. Finally, genes for which transcripts couldn't be assigned to any orthogroup were considered as hemiploid X or Y. Gene counts for genes still shared between X and Y chromosomes were pooled in males. We assigned each gene to one of the three known evolutionary strata of sex chromosomes using its central position, and breakpoints defined in Peichel et al. (2020). Given that the pseudoautosomal region of the Y chromosome was excluded from the read mapping step, read counts for that region were already correct and genes were considered as still sharinga copy ignoring Orthofinder results.

Sex-Biased Gene Expression on the Sex Chromosomes

We used the merged counts to test for sex-biased gene expression on sex chromosome using the same method as for the autosome. To test for potential dosage compensation, we estimated the autosome to sex chromosome median expression ratio for all genes, and independently for

hemiploid X and Y genes for males and females. The autosomal median was calculated as the median across all genes of the mean log2 normalized read counts across all samples. Median expression for genes on the sex chromosome was calculated similarly but separating individuals by sex. We estimated one median for all genes, genes that still have a copy on both chromosomes and genes specific to each sex chromosome. This calculation was done for the whole chromosome and for each stratum independently (PAR, strata I, strata II and strata III). Confidence intervals (CI) were calculated using bias correction bootstraping for both autosomal and sex chromosome genes and significance assessed using overlap of the CI with 0. Finally, to understand the processes underlying the evolution of sex-biased gene expression for genes with a copy conserved on each chromosome, we estimated X and Y allele expression in males. We then compared the expression level of female, male and X and Y alleles in sex-biased genes to their expression levels in unbiased genes using Wilcoxon rank-sum test.

Results and discussion

Sequencing, Mapping and Filtering

The two lines combined rendered 4,247,758,804 reads with an average of 20,319,408 [CI $_{95\%}$: 18,047,379 – 22,591,436] for the gills, 18,847,630 [17,937,603 – 19,757,656] for the brain and 17,073,329 [14,606,317 – 19,540,341] for the liver. The average read length after quality trimming was 38 bp. After removing samples with fewer than 5,000,000 reads (ten for the liver, one for the brain), we kept 67 samples in the liver (35 Females; 32 Males), 77 for the brain (38; 39) and 72 in gills (36; 36). Dataset include 59 samples sequenced in all tissue, but 10 were specific to gills and brain, five to brain and liver and three to gills and liver. Three samples were sequenced only in brain. We had a percent mapping of uniquely mapped reads to the reference genome ranging from 44.3% to 88.61% (median 76.76%). After filtering, we identified five overexpressed genes in the liver but none in the gills or the brain. Liver was the tissue with the lowest number of expressed autosomal genes (14,402) compared to gills (17624) and brain (17930), with 13957 autosomal genes expressed in all three tissues. Using PCA to screen our dataset for potential cross tissue contamination or mislabelling revealed no evident issue (Fig1 A) as each tissue formed a distinct group, confirming the quality of the dataset.

Tissues Differ in the Magnitude of the Sex-Biased Expression

Patterns of sex-biased gene expression varied greatly between liver, gills and brain, both in terms of the number of genes differentially expressed and their function (figure 1B). Liver transcriptomes showed strong sex specificity, with 5303 sex-biased genes (hereafter SBG, “sex-biased gene”) with a q-value ≤ 5% after a Benjamini-Hocheberg correction. In comparison, using the same threshold, we found 973 SBG in gills and only 141 in the brain (Figure 1B). Genes also exhibited a wider range of differential expression in the liver compared to gills and brain (Figure 1 C-D-E). Across all tissues, no chromosome showed enrichment for SBG (Fig. 2, Fisher’s test q-value> 0.4).

Sex-biased genes found in all tissues are implicated in cell physiology, cell-cell signalling and gene expression modulation

Most SBG are unique to a tissue, with only 42 genes significant in all three tissues (figure 1B, table S1). Eight of them are related to basic cell physiology such as growth, cytoskeleton, and differentiation. Five genes are involved in cell-cell signalling or adhesion and are mainly known to play a role in neuron communication and development. Seven genes are involved in gene expression modulation and affect either DNA methylation, transcription, or alternative splicing. If fish, sex-specific methylation is known for its role in modulating the expression level of reproduction-related genes (Laing et al., 2018; Li, Chen, et al., 2022) and in sex determinism

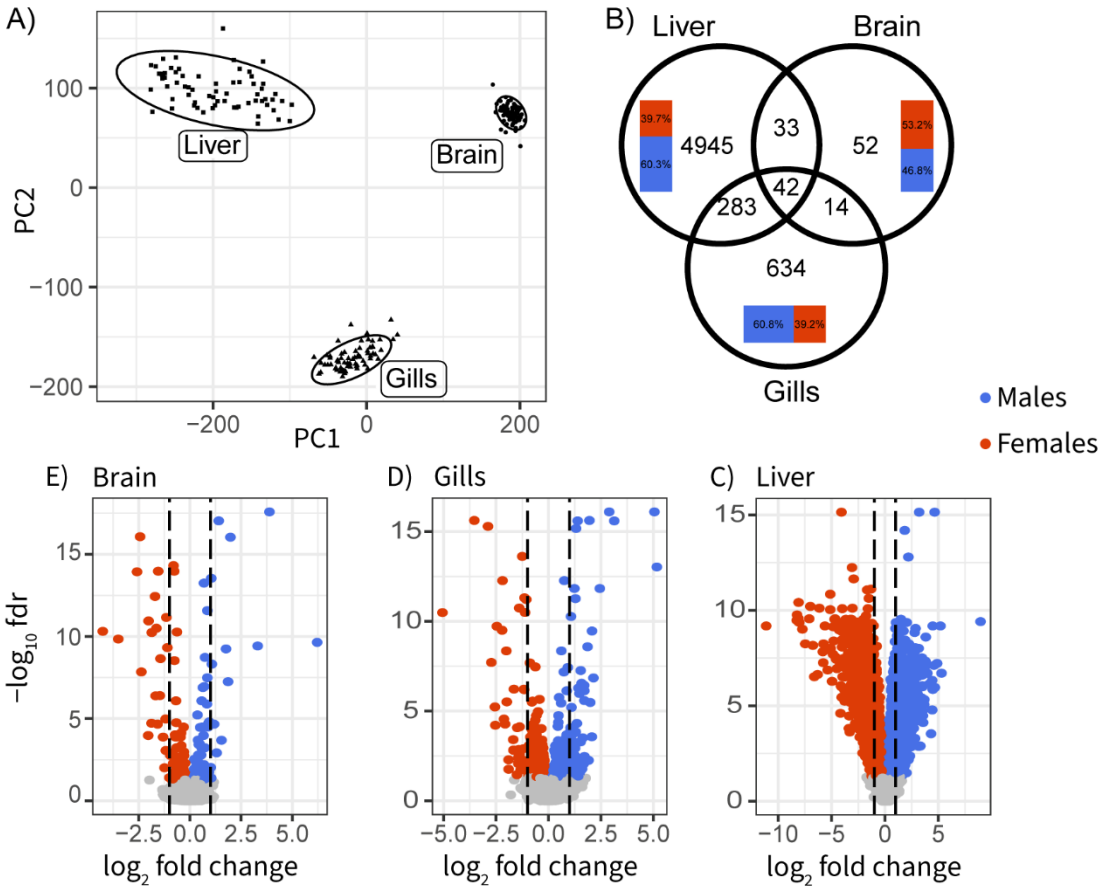

Figure 1: Patterns of autosomal sex-biased gene expression in threespine stickleback. A) PCA of gene expression on all tissues. B) Overlap of sex-biased genes between tissues. Inset barplot represents tissue specific repartition of male (blue) and female-biased genes. Volcano plots of sex-biased gene expression in C) liver, D) gills and E) brain. Coloured dots correspond to genes significant at a 5% false discovery rate based on a Wilcoxon-rank-sum test. Dotted lines represent a log-fold change of 1 (doubled expression).

(Gemmell et al. 2019). Similarly, sex-specific transcript usage can provide an alternative route from gene regulation to generate sexual dimorphism (Telonis-Scott et al., 2009; Naftaly et al., 2021). Hence, those genes could play an important role in regulating sex-biased gene expression and

dimorphism across tissues. Other functions found in shared SBG among tissues involve the immune system, testosterone response (one gene) and two nuclear genes with mitochondrial function.

In most cases, genes showed the same directionality in all tissues (Fig. 3), except for two genes: slc16a13, a monocarboxylic acid transporter, and esr2b, an estrogen receptor. Both are female-biased in liver but male-biased in the brain and gills. While the function of slc16a13 is hard to interpret in our context, as the substrate of this member of a large family of solute transporter is unknown (Halestrap, 2012), the gene esr2b code for an estrogen receptor. In teleosts, estrogen receptors are involved in several biological processes, including reproductive processes (Nelson & Habibi, 2013). In the brain, esr2b is thought to play a role in reproduction through the

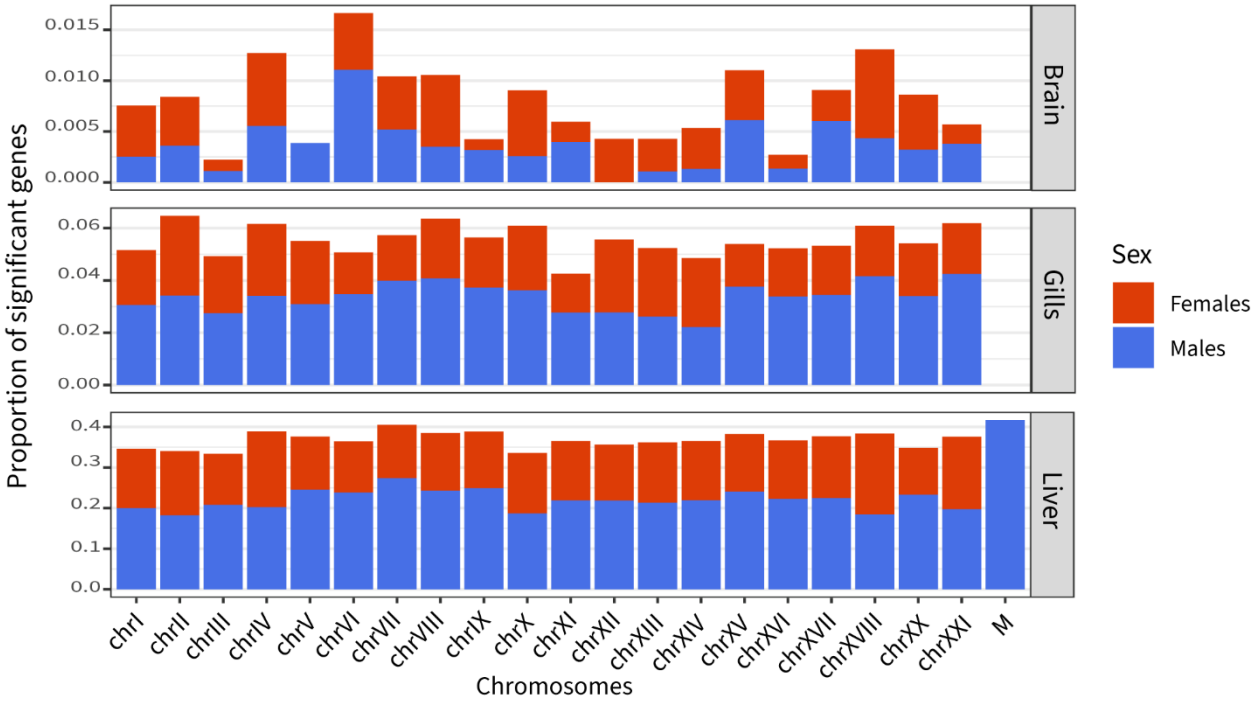

Figure 2: Proportion of sex biased genes across chromosomes and sex in each tissue. Chromosome VII and XVIII show enrichment toward male-biased function in liver (q-value ≤0.05), as well as the mitochondria with lower confidence (q-value = 0.12. No chromosomes are significantly enriched in sex-biased genes.

regulation of gonadotropin production, which is involved in gametogenesis (Muriach et al., 2008) in both sexes and have been found to have a higher expression in males in the pituitary gland of the fathead minnow (Pimephales promelas) (Filby & Tyler, 2005). 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). We still lack knowledge on the effect of sex hormones in the gills.

In the case of a pattern of directionality in gene expression shared by only two tissues, we only found discordance when the comparison included the liver (Fig. S1). While part of this is explained

by the liver having both more SBG and more shared SBG with other tissues in general, it suggests that this tissue might have a different usage of the same gene set.

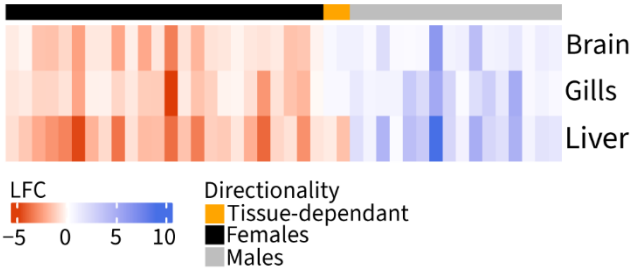

Figure 3: Heatmap of log-fold change in gene expression between sexes for 42 genes significantly differentially expressed in all tissues. Bar on top shows concordance in the direction of expression bias.

Sex-biased genes in the brain are few and not enriched for particular functions

The brain showed the lowest number of SBG with 141 genes differentially expressed between sexes on the autosomes (0.78%) of expressed genes), equally distributed between males and females and across chromosomes (Figure 2). We found no enrichment for any gene ontology term using the 5% threshold (supplementary tables). These results are in accordance with a similar study in the same species in which they found a higher number of expressed genes but included sex chromosomes in their analyses (Kaitetzidou et al., 2022). Looking into the most significant genes (10 with highest p-value and 10 with highest LFC), we found that only 12 of them are specific to the brain (Table S2). We were unable to annotate three of them (LOC120812970, LOC120817963 and LOC120833148). The remaining 9 genes were associated with various biological functions. The growth hormone-releasing hormone (ghrh), more highly expressed in males, is the first hormone secreted in the growth hormone axis, which in fish is not only involved in growth but also reproduction, metabolism and immune function (Chang & Wong, 2009). TTC29 is involved in cilium movement and is mainly described in sperm flagellum (Bereketoğlu et al., 2022). Other genes included ihhb (LOC120819658), which is involved in neural and chondrocye development (Wu et al., 2001; Chung et al., 2013), hcn3 (si:dkey-197j19.6), an ion channel which is essential for neuronal function, MMP13 or 18 (LOC120822795), which modulates angiogenesis in the brain (Ma et al., 2016), and ecm1a (LOC120810788), which codes for an extracellular matrix protein involved in signal transduction.

Sex-biased genes in gills are associated with ion-related functions and immune defense

We identified 973 SBG in the gills (5.5% of expressed genes), of which 60.8% are male-biased. Significant genes are distributed homogeneously in the genome, with no chromosome significantly enriched in SBG of biased toward a sex (Fig. 2). Synaptic signaling and organisation represent 48% of significantly enriched GO terms (Table S3), most of them (48% of total significant terms) related to synaptic signalling and organization. However, looking at descriptions of genes within those GO categories on Zfin or Genbank revealed that many genes code for ion channels or pumps, which

are a core function of the gill tissue (Perry et al., 2003), yet the associated GO functions have only been described in the brain (Table S4). This suggests that gene ontology analysis in gills suffer from the lack of gill-specific information. Other GOs include various biosynthetic and metabolic processes, as well as cell adhesion, communication, and development. Most strongly differentiated genes (Table S5) in gills involve two genes with potential role in pathogen resistance, hhipl1 and CLEC4M (LOC120817010) and genes with basic cellular functions (LOC120828377, si:dkeyp-92c9.4 and LOC120810538). Asic2 codes for an ion channel, and this gene family is involved in Na+ intake in rainbow trout gills (Dymowska et al., 2014). Three genes are involved in neuronal function, including one ion channel, rem2, and a galanin receptor, galr2b. Dpysl3, is also male biased in the liver is supposed to have an effect of peripheric axon growth. As for the gene ontology, the function of these genes in the gills is unclear. Finally, three genes (LOC120816929, LOC120817829 and LOC120821053) could not be annotated.

Most studies on gills transcriptomes focused on their role in osmoregulation and respiratory processes in responses to anoxia, salinity, or various contaminants (Scott et al., 2004; Van Der Meer et al., 2005; Gonzalez et al., 2006). Works have also been interested in gills' function in defence against pathogens, as they represent a direct entry for infection and parasites (Mitchell & Rodger, 2011). However, a survey of the physiology literature illustrates that these studies do not usually account for sex. When they do, few SBG are identified, for example, in African cichlids (Lichilín et al., 2021). However, our results identify numerous SBG in animals all found in the same environmental conditions. This unexpected result highlights the importance of accounting for sex when studying gills, as the extent to which this tissue might respond differently to various challenges between sexes is also poorly understood.

The Liver is a Hotspot of Sex-Biased Gene Expression

We identified 5303 SBG in the liver (36.8% of total expressed genes), 60.3% of which are male-biased. SBG are uniformly distributed among chromosomes (Fisher's exact test qvalue ≥ 0.45, Figure 2), except for chromosomes V and XVIII, which show enrichment in male-biased genes, and the mitochondria which shows marginal enrichment for male-biased function (FDR of 0.12). While comparing the number of genes between studies is complex as life stages, condition and filtering have a deep impact on detected SBG, widespread sex-biased gene expression has been found in the liver of other fish species, such as *Salvelinus fontinalis*, in which SBG represents 16.1% of the total gene expression using more stringent filtering (LFC>= 1.5) than we applied (Sutherland et al., 2019). Similarly, very low levels of SBG are identified in cichlids (Lichilín et al., 2021) using a LFC of 2 as a cutoff but these observations vary across species. In our study, only 396 (2.6%) in the liver passed this cutoff (644 for the 1.5 cutoff), suggesting that sex-specific regulation of gene expression in the liver mostly occurs through subtle regulatory changes, with a median absolute LFC for significant genes at 0.63. Enriched gene ontology terms in the liver are mainly related to metabolic and biosynthetic processes. The immune system also seems differentiated between the sexes, with enriched processes such as humoral immune response and response to external stimulus. We also identified an enrichment in hemostasis and coagulation regulation (Table S6). Among the most significant genes in terms of p-value and fold change (Table S7), numerous genes were involved in

response to estrogen and estradiol (fam20cl, vtg3, LOC100190882, LOC100190880, LOC120823934 blasting to vtg1, and two vtg2). Other genes showed functions related to gene expression regulation (e.g. lbx2, st18), response to pathogens (LOC120810467, LOC120820940, blasting to the fucolectin-1 and CHIT1), and cellular differentiation (LOC120824638, blasting to srda3a).

We identify five genes representing more than 15% of reads in at least one sample: apoa2, lect2.1, LOC120808851, LOC120810467 and LOC120823934, hereafter referred to as overexpressed genes (Figure 4). All five overexpressed genes showed sex-biased gene expression, according to Wilcoxon rank-sum tests (p.value <10-6). Apoa2 (apolipoprotein A-II) and lect2.1 (leukocyte cell derived chemotaxin 2.1), which were more highly expressed in males, have functions related to lipid transport and immune system. Apolipoproteins are involved in lipid transports in vertebrates but have also been found to have antimicrobial activity in teleost fish (Concha et al., 2003), among other functions. Leukocyte cell derived chemotaxin2 have been known to have chemotactic activity in humans, but also have antibacterial activity in other vertebrates and in teleost fish. BlastX results for females-biased genes (LOC120808851, LOC120810467 and LOC120823934, figure 4) are indicative of ongoing preparation for reproduction. LOC120808851 is located on the sex chromosome and shows similarity to the ZP3 (Zona pellucida sperm-binding protein 3) Uniprot entry, a protein that mediates sperm-binding during fertilization. According to Orthofinder results, it is part of a cluster of duplicated genes on the chrXIX (LOC120808849, LOC120809240, LOC120808850, LOC120808851) with a single copy conserved on the Y chromosome, which supports the importance of this function for females. LOC120810467 shows similarity to the Fucolectin-1 entry, which belongs to a family of genes involved in innate immunity (Honda et al., 2000) that has been found to be accumulating in European seabass' (Dicentrachus labras) eggs (Parisi et al., 2010). Finally, LOC120823934 shows similarity to Vitellogenin-2, which is a precursor to several egg-yolk proteins (Tata, 1976). This result is concordant with the observation of high levels of estrogen receptors in liver as described before, and further confirmed by the presence of vtg3, another vitellogenin coding gene, and vtg-2 among the list of most significant genes in liver (LFC = -5.69). Moreover, as described before, other genes related to response to estrogen also are among the most significant genes. Moreover, both ZP3 and vitellogenin are genes known to be expressed in liver (Sano et al., 2017), at least in recent teleosts, further confirming the quality of the dataset

The liver is the only tissue in which we observed sex-biased expression of mitochondrial genes. We identified 13 genes with sex-biased gene expression (~56% of expressed genes in the liver's mitochondria), all male-biased. These genes include ATP6 and 8, COX2, ND1,2,3,5 and two transfer RNA. In sticklebacks, parental care from the male during the reproductive period, coupled with gonadal development, is associated with a strong depletion of energy reserves (Chellappa et al., 1989; Huntingford et al., 2001). While the development of eggs is also costly for females, the strong involvement in nest building, defence, and parental care by males could generate a high energetic need in males associated with the metabolic processing of energy reserves in the liver.

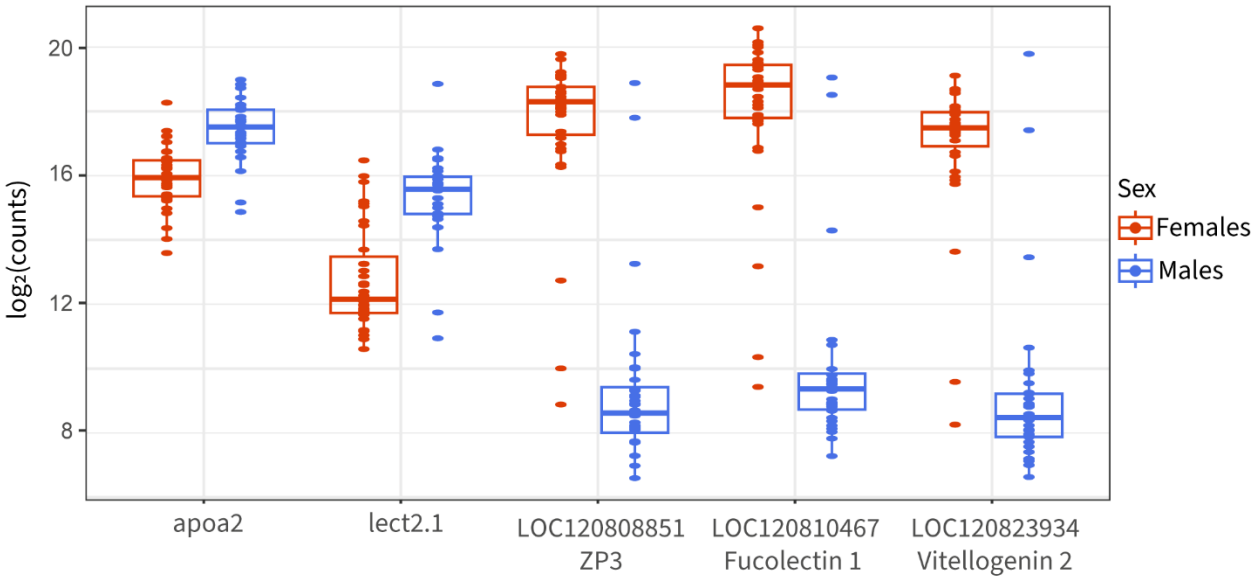

Figure 4: Normalized read counts distribution between sexes for five genes representing more than 15% of total read counts in at least one sample. All between-sex comparisons are significant using an FDR threshold of 5%.

Sex-biased Gene Expression on Sexual Chromosome Mostly Reflects Gene Loss in Non-Recombining Regions

Most genes located on sex chromosomes exhibited sex-biased gene expression (499 in liver, 755 in gills and 813 in brain). Contrasting with the autosomal pattern of sex-biased gene expression, gills and brain exhibited the strongest pattern of SBG, and the higher number of genes expressed in those tissues is not sufficient to explain it (respectively 71%, 83% and 87% of expressed genes in liver, gills and brain). Most SBG are caused by genes having lost their Y copy (83.9%, 71.6% and 70.2% of significant genes in liver, gills and brain), suggesting a lack of global dosage compensation in all studied tissues. Genes orthology relationships for sex chromosomes are available in Table S8. When looking at the ratio of gene expression between sex chromosomes and autosomes, we found that gene expression was greatly lowered uniquely in males for genes having lost their Y copy (95% confidence interval: [-1.77; -1.29] in brain, [-1.5;-1.00] in gills and [-1.50;-1.01] in liver for males; [-0.71; -0.28], [-0.41; -0.03] and [-0.70; -0.07] in females), but not for genes still having a Y orthologue ([-0.48; 0.24], [-0.59,0.22] and [-0.26, 0.18] in males; [-0.23; 0.39], [-0.27;0.5] and [-0.34, 0.71] in females). This pattern holds across all evolutionary strata and tissue (Fig. 5, Table S9). Estimated ratios for the pseudo-autosomal regions or genes with coding sequences on both chromosomes in non-recombining strata did not statistically differ from 0 (Table S9), except for female-biased expression in stratum II in the brain ([0.10, 1.35], and stratum I in the gills ([0.39, 1.61]). Genes having lost their X copy exhibhit a similar pattern but with overall higher median sex-chromosome to autosome expression ratio. In stratum I, it is not statistically different from 0 ([-1.31, 0.02], [-1.44, 0.10] and [-0.92, 0.69]).

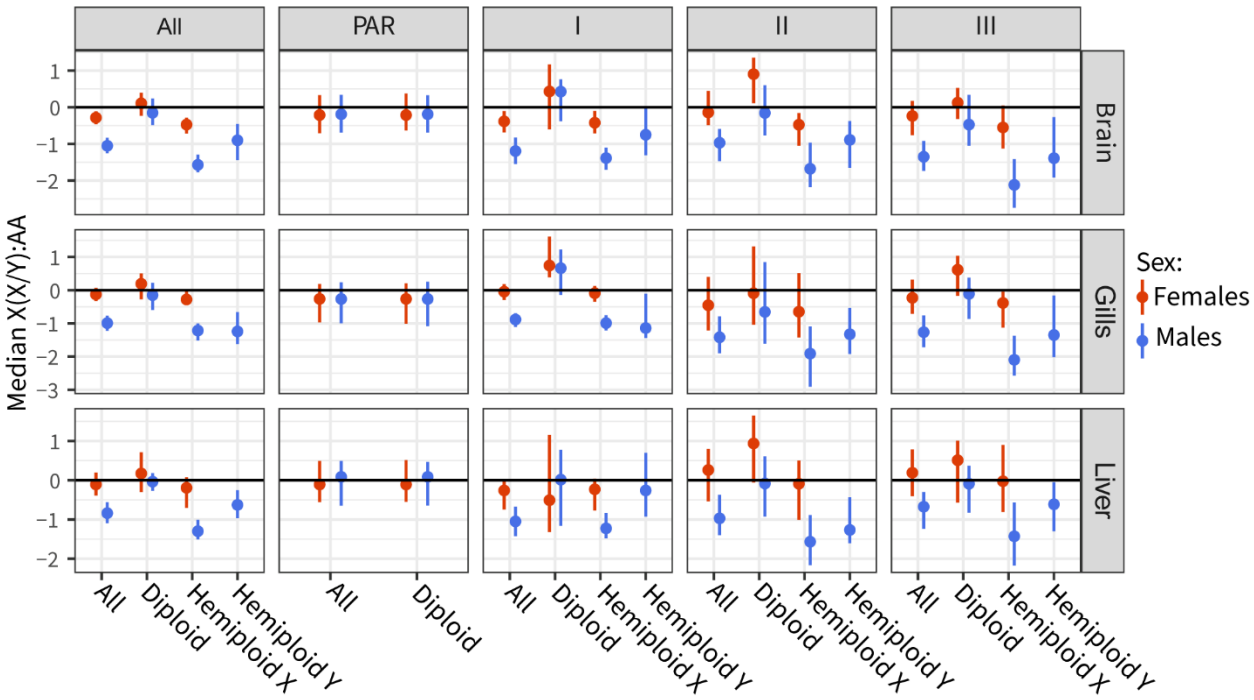

Figure 5: Sex chromosomes to autosome expression ratio across sex-chromosome evolutionary strata. Hemizygous X and hemizygous Y genes respectively lost their Y- and X-coding sequence, while diploid genes are still have a copy on both chromosome. For diploid genes, strata was defined using the Y copy position Confidence intervals from 1000 bootstraps are shown. Sample sizes for each median are indicated in Table S8.

Lack of dosage compensation has already been described in the brain (Schultheiß et al., 2015; White et al., 2015). and our results extend this conclusion to liver in and gills, and to genes with a lost X copy which had not been included in previous studies. Dosage compensation is expected to evolve when reduced expression in the heterogametic sex affects phenotype, i.e., affects the protein level and its interaction network within the organism. In sticklebacks, hemizigous genes tend to be dosage insensitive, meaning that protein quantities are independent from its expression level (Peichel et al., 2020). 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).

Apart from chromosome degeneration, gene expression on sex chromosomes is expected to evolve through several processes. First, as the X chromosome is more often transmitted to females, it is expected to accumulate dominant female-beneficial mutations that could lead to an increase in expression of the X copy (Bachtrog et al., 2011). Sex chromosomes are also expected to be enriched in both active and resolved sexual conflicts, in which case gene expression should increase depending on the sex in which they are beneficial (Vicoso & Charlesworth, 2006; Bachtrog et al., 2011). Finally, the lack of recombination and lowered sample size of the Y chromosome can lead to the accumulation of loss-of-function mutations, leading to the progressive loss of Y-copy expression (Charlesworth & Charlesworth, 2000; Bachtrog, 2013). We observed a feminization of

stratum I in gills and II in brain, which had previously been described for stratum II (Leder et al., 2010; White et al., 2015), suggesting a role for female-beneficial mutations in the evolution of gene expression on the X chromosome. We did not observe feminization of the pseudo-autosomal region as previously reported by White et al (2015). This could be caused by the use of autosomal expression level instead of a closely related species to estimate ancestral expression rate.

To better understand the drivers of sex-biased gene expression of sex chromosomes, we compared expression of X and Y alleles in sex-biased genes with conserved copies on both chromosomes (excluding the still recombining PAR) to the allelic expression of unbiased genes (Fig. 6). In all tissues, we found a lowered expression of the Y allele of female-biased genes compared to unbiased genes (wilcoxon rank-sum test p-value: $1x10^{-4}$ in brain, $8x10^{-4}$ in gills and $2x10^{-2}$ in liver) while X expression remained similar (all p-value > 0.7), which also resulted in lowered expression in males in the brain (p-value$1x10^{-2}$). This suggests that the degeneration of the Y chromosome coupled with the absence of dosage compensation in this species is the main driver of female-biased gene evolution, as suggested by previous work (White et al., 2015). Note that we also found that in the liver overexpression in females also occurred in female-biased genes ($2x10^{-2}$).

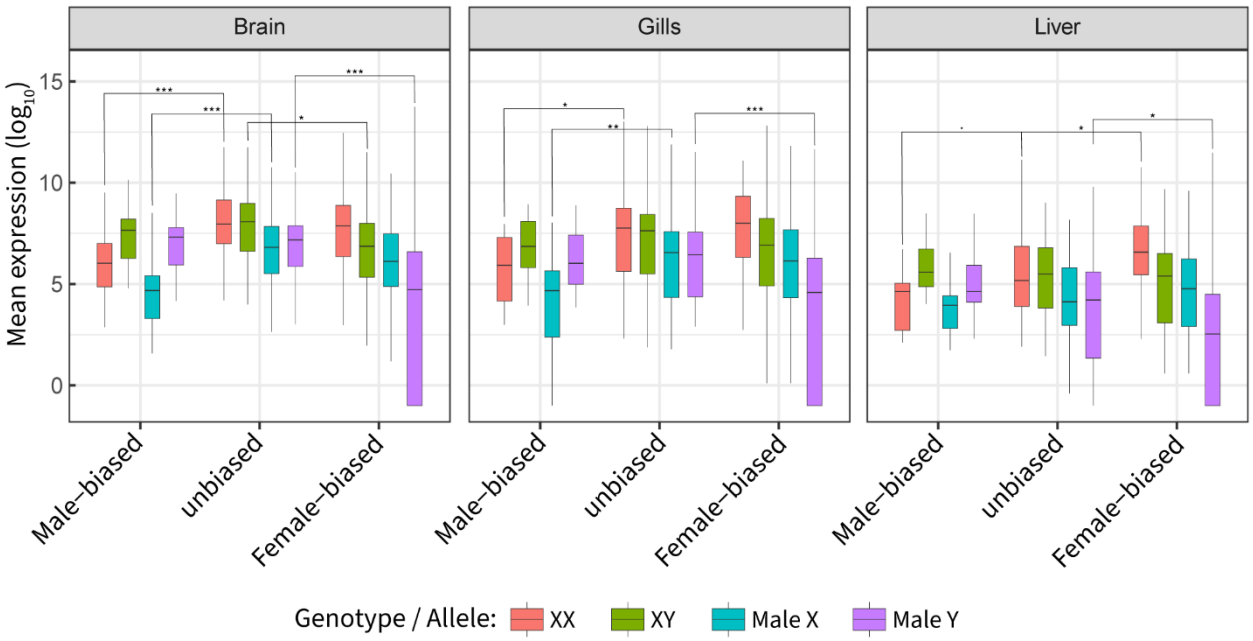

Figure 6: Genotype and allele-specific gene expression across male-biased, unbiased and female-biased genes. Male-biased and female-biased genes were defined as genes with log2 fold change in expression under -0.5 and above 0.5. We assessed significance using Wilcoxon rank-sum test comparing within genotype expression levels of male-biased and female-biased genes to unbiased genes. *: p-value <0.05, ** p-value <0.01, *** p-value <0.001

Similarly, we observed that male-biased gene expression in females ($6x10^{-5}$, $1x10^{-2}$ and $7x10^{-2}$) but not males (all p-value > $2x10^{\wedge-1}$) is reduced compared to unbiased genes. We also observed reduced expression of the X allele in males in the brain and gills ($1x10^{-4}$, $7x10^{-3}$), suggesting that this could be the result of a systematic down-regulation of some genes on the X-chromosome, which

could be a signal of ongoing demasculinization, i.e. the loss of male-advantageous gene on the X chromosome (Gurbich & Bachtrog, 2008) Concordant with that hypothesis, genes identified as specific to the Y chromosome tended to have higher expression than genes specific to the X chromosome in males, suggesting that they are associated with male-beneficial functions.

Conclusion

Our study characterizes the sex-specific transcriptome of brain, gills and liver for the threespine stickleback during its reproductive period. We find low levels of differentiation between sexes in the brain compared to the level of dimorphism shown in behavioural and morphological studies. On the opposite, the gills exhibit pronounced sexual dimorphism that is usually not reported or accounted for in the literature, suggesting that the importance of sex as a cofactor in gill studies has been underestimated. The liver appeared to be strongly differentiated between the sexes, as expected for teleost fish. Sex chromosomes are a hotspot of intersex differentiation in all tissues, with ~70% of genes being differentially expressed. This pattern seems to be caused both by an ongoing degeneration of the non-recombining region of this sex chromosome coupled with the absence of dosage compensation mechanism, and a potential repression of male-advantageous mutations on the X chromosome, although further investigation of gene sequence evolution would be necessary.

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Data accessibility:

Raw sequencing reads and unfiltered read counts are available through NCBI GEO, accession GSE269432. All analysis in the manuscript and related code are available at https://doi.org/10.5281/zenodo.11477976

Authors contributions:

L.B and N.A.H conceptualized and supervised the project. F.S did the fieldwork, generated and filtered the dataset, and performed analyses. N.A.H helped with interpretation. F.S wrote the draft of the manuscript, and N.A.H reviewed and approved it.

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Conflict of interest

The authors have no conflict of interest to declare.

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