Maternal stress has divergent effects on gene expression patterns in the brains of male and female threespine stickleback

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Maternal stress can have long-term effects on neurodevelopment that can influence offspring performance and population evolutionary trajectories. To examine the mechanistic basis for these neurodevelopmental effects of maternal stress, we used RNA-seq to assess differential gene expression across the brain transcriptome of adult male and female threespine stickleback (*Gasterosteus aculeatus*) from stressed and unstressed mothers. We identified sexually divergent effects of maternal stress on the brain transcriptome. In males, genes that were upregulated by maternal stress were enriched for processes involved in synaptic function and organization and steroid hormone-mediated signalling pathways, whereas in females genes that were upregulated by maternal stress were enriched for processes involved in protein translation and metabolic functions. The expression of several genes involved in the hypothalamic–pituitary–interrenal response to stress and epigenetic processes such as the regulation of DNA methylation patterns and miRNAs increased in males and not in females. These data suggest that maternal stress has markedly different effects on cellular pathways in the brains of male and female offspring of mothers that are exposed to stress, which could have important implications when assessing the long-term ecological and evolutionary impacts of stress across generations.

1. Introduction

Maternal exposure to stress has been shown to have persistent effects on the neurodevelopmental processes of offspring across a wide range of taxa, resulting in profound changes to the health and behaviour of subsequent generations [1–3]. For example, in humans, epidemiological studies suggest that prenatal exposure to maternal stress increases the prevalence of neurodevelopmental diseases such as schizophrenia and autism spectrum disorder in adult offspring [4]. By contrast, in a variety of ecological contexts the effects of maternal stress on offspring phenotypes have been suggested to be positive, conferring increased fitness in stressful environments [5]. Whether maladaptive or adaptive, these effects are probably the result of a complex interaction between genes and the environment, mediated through a variety of epigenetic processes [6,7].

In mammals, the effects of maternal stress on adult offspring have been repeatedly demonstrated to differ between males and females [8–10]. These sex-specific effects are thought to be regulated by complex maternal–fetal interactions via the placenta [11]. Thus, it is unclear whether conclusions derived from studies in mammals can be broadly applied across non-placental taxa. Studies in birds have detected sexually dimorphic effects of maternal stress on offspring behaviour [12,13], and the regulation of the hypothalamic–pituitary axis (HPA) [14], but the generality of these findings across oviparous vertebrates remains unknown. This is particularly important in the context of ongoing debates regarding the evolutionary implications of maternal effects in natural populations [5,15,16].
In this study, we use threespine stickleback (*Gasterosteus aculeatus*) as a model to study the effects of maternal stress on the brain transcriptome of male and female offspring. There is compelling evidence from studies in this species that maternally derived stressors have profound and persistent effects on developmental, morphological and behavioural phenotypes of offspring [3] that can persist across generations [17–20] making them an important model in which to study the adaptive potential of transgenerational plasticity, and an ideal candidate in which to explore the sexually dimorphic effects of maternal stress on the offspring of an oviparous vertebrate.

2. Methods

(a) Stress treatment and animal rearing

Adult threespine stickleback (*G. aculeatus*) of the fully plated ‘marine’ ecotype were collected in May 2013 in Oyster Lagoon (British Columbia, Canada, GPS: 49.6121, −124.0314) and acclimated to laboratory conditions for three weeks (see the electronic supplementary material for details). Following the three-week acclimation period, fish were divided into two treatment groups (unstressed and stressed) with three replicate tanks per treatment. Fish in the stressed treatment were chased once daily with a fish net for 30 s before being captured and held out of water for a further 30 s and then returned to their tank. This was intended to mimic similar predatory and handling stressors that have been used to investigate the effects of maternal stress on offspring behaviour in fish [21,22]. Timing of the stress treatment was randomized during each day to avoid conditioning. The stress treatment was applied for two weeks, and significantly increased plasma cortisol levels in mothers (ANOVA $p = 0.0014$; see electronic supplementary material, figure S1, for details). Testes were then harvested from unstressed males and used to generate half-sib crosses, with the same male being used to fertilize the eggs of one stressed female and one unstressed female. A total of six pairs of half-sib crosses were produced.

After 1 year post-hatch, and prior to reaching sexual maturity, brain tissue was dissected from the offspring and immediately frozen in liquid nitrogen and stored at −80°C until further use. Sex of the 1-year-old offspring was determined by PCR amplification of *Idh*, *Gasm1* and *Stri190* as previously described [23]. Electronic supplementary material, table S1, summarizes the family information for the fish that were selected for RNA-seq. Because not all families had the required number of male and female offspring at 1 year post-hatch, we were unable to use a completely balanced design with male and female half-sibs represented in both the unstressed and stressed treatments. However, we attempted to maximize the genetic diversity within the sample by limiting the use of full-sibs where possible (see the electronic supplementary material for details).

(b) RNA isolation and sequencing

Total RNA was isolated from stickleback whole brain tissue (three fish per sex per stress treatment; 12 fish total) using TRIzol Reagent (Invitrogen Life Technologies) as previously described [24] followed by DNase treatment (Qiagen RNaseasy). RNA integrity numbers (RIN) were between 7.9 and 9.1 (mean = 8.6 ± 0.4 s.d.). Preparation of Illumina TruSeq cDNA libraries and 100 base-pair paired end sequencing was performed at the UBC Nucleic Acid Protein Service Unit (NAPS) and UBC Biodiversity Research Center’s next generation sequencing facility, and at the McGill University and Genome Quebec Innovation Center. Sequencing depth and sample multiplexing were devised to optimize statistical power given the total sequence generated [25,26]. Mean library size was 19.8 million reads (see electronic supplementary material, table S2, for details of library sizes).

(c) Sequence alignment and expression analysis

Reads were aligned to the stickleback genome (http://www.ensembl.org) using CLC genomics workbench v. 8.5 (Qiagen). Average mapping efficiency of paired and broken reads was 88.5 ± 0.9%. Analysis of total read counts was performed in R v. 3.2.2 with edgeR v. 3.12.0. Recommended RNA-seq analysis guidelines were followed [27] with the addition of RUVr from the RUVSeq protocol v. 1.4.0 [28] to account for batch effects. Genes with no reads were removed from the dataset. Counts were then normalized using the relative log expression (RLE) method [29]. Following normalization, genes with low expression were filtered from the dataset. The minimum criterion for retaining a gene was at least 1 count per million (approx. 10 counts in the smallest library) in each of the three samples of at least one sex by stress treatment group. A total of 16477 genes were retained after normalization and filtering for subsequent expression analysis. Robust dispersions were calculated using the robust method in edgeR [30]. The data were then fit to a negative binomial generalized linear model using glmFit(). Likelihood ratio tests were run to assess effects of sex, maternal stress and the interaction of these main effects with factors calculated from RUVSeq included in the model. The resulting $p$-values were adjusted based on the false discovery rate (FDR) correction [31], and the threshold for significance of these adjusted $p$-values was set at 0.05. Principal component analysis (PCA) was performed using the precomp() function from the base package in R on log2 counts per million expression values. GO and KEGG pathway enrichment analyses were conducted using the goseq (v. 1.22.0) R package [32], with FDR correction (see the electronic supplementary material for details). Cluster analysis of differentially expressed gene ontologies was performed using REVIGO (http://revigo.irb.hr), and the resulting source code was used to generate figures using the ggplot package in R.

3. Results

Analysis of RNA-seq data from the brains of 1-year-old male and female threespine stickleback offspring from stressed and unstressed mothers revealed over 2900 genes with either a significant main effect of sex (1255 genes) or a significant interaction between maternal stress and sex (1650 genes), but very few genes with a main effect of maternal stress (figure 1a). PCA of all expressed genes distinguished four groups (figure 1b). PC1 explained approximately 30% of the variation in the data and separated males and females that came from stressed mothers indicating that maternal stress has different effects on the brain transcriptome in male and female stickleback. PC2 explained approximately 11.5% of the variation in the data and separated males and females that came from unstressed mothers. Combined, PC1 and PC2 accounted for approximately 41% of the variation in the RNA-seq data.

(a) Sexual dimorphism in gene expression

Independent of the effects of maternal stress, there were differences in brain gene expression patterns between male and female threespine stickleback (electronic supplementary material, figure S2 and table S1). In total, 614 genes were
expressed at higher levels in females whereas 641 genes were expressed at higher levels in males (electronic supplementary material, figure S2 and table S3). Enrichment analysis (Gene Ontology (GO)-enrichment, KEGG pathway enrichment) did not reveal any terms or pathways that were significantly enriched among the genes with a significant main effect of sex. However, GO enrichment analyses do not provide comprehensive categorization of the GO terms associated with a differentially expressed gene list [33]. Electronic supplementary material, figure S3, provides a summary of the most commonly represented GO biological process categories for genes that exhibited a bias towards higher expression in either males or females. Genes associated with metabolic processes were expressed at higher levels in females whereas genes associated with cytoskeletal organization, cell adhesion and developmental processes including nervous system development were expressed at higher levels in males.

(b) Sex-specific effects of maternal stress

The effects of maternal stress on brain gene expression were strikingly different in male and female offspring (figure 2;
Figure 3. Cluster analysis of the significantly enriched gene ontology (GO) terms for biological process over-represented among genes that increased in males (blue circles) or in females (red circles) from stressed mothers compared to unstressed mothers. Size of the circle is representative of the total number of DE genes in that GO category. Note that some names have been omitted from the figure due to semantic similarity. A complete list of all significantly enriched GO categories and the total number of genes with each annotation are provided in supplemental data file 1.

electronic supplementary material, table S4) and these effects were generally in opposing directions between the sexes such that genes that increased in expression in response to maternal stress in female offspring decreased in expression or did not change in response to maternal stress in male offspring and vice versa. This is in direct contrast to the small number of genes that showed a conserved effect of maternal stress in both males and females, with only three genes demonstrating a maternal stress effect without an effect of offspring sex or an interaction (figure 1a; electronic supplementary material, table S5).

GO-enrichment analysis of genes with a significant interaction between maternal stress and offspring sex revealed several enriched GO categories that suggest differential expression of genes involved in protein translation, metabolism and synapse organization and assembly (electronic supplementary material, table S6). Enrichment analysis of KEGG pathways identified those involved in protein synthesis, and several neurodegenerative diseases such as Huntington’s, Parkinson’s and Alzheimer’s disease, as well as pathways associated with synaptic function such as nicotinic addiction and glutamatergic synapse (electronic supplementary material, table S7).

To further explore functional enrichment in the genes exhibiting significant interactions, we also performed separate GO enrichment analysis on genes that were upregulated by maternal stress in female offspring and genes that were upregulated by maternal stress in male offspring. This analysis divided the previously identified enriched GO categories into two distinct groups (figure 3; electronic supplementary material, table S6). The majority of enriched GO categories for genes that were upregulated by maternal stress in female offspring were involved in protein translation and regulation of metabolic processes, and this was reflected in both the biological process (figure 3) and cellular component (electronic supplementary material, figure S4) ontologies. Enriched KEGG pathways (electronic supplementary material, table S7) upregulated by maternal stress in female offspring were those associated with protein translation and diseases such as Parkinson’s, Huntington’s and Alzheimer’s disease, all of which have a metabolic component [34, 35]. A different set of processes was identified as enriched for genes that were upregulated by maternal stress in male offspring. The majority of enriched GO categories for genes upregulated by maternal stress in males were involved in nervous system development and synapse formation, and again this was reflected in both the biological process (figure 3) and cellular component (electronic supplementary material, figure S4) ontologies (electronic supplementary material, table S6). Enriched KEGG pathways upregulated by maternal stress in males were those associated with synaptic function and organization including glutamatergic synapse, GABAergic synapse, long-term depression and nicotine addiction (electronic supplementary material, table S7).

(c) Chromosomal location of differentially expressed genes

Of the 1255 genes that exhibited a main effect of sex, 1170 could be localized to chromosomes, and of these, 585 were expressed at higher levels in females, and 585 were expressed at higher levels in males. A disproportionate number of these genes were located on the sex chromosome (chromosome 19; 441 genes, 38%), and 87% of these sex-chromosome localized genes (383 genes) were expressed at higher levels in females than in males (figure 4a). By contrast, genes that were expressed at higher levels in males than in females were distributed fairly uniformly across the chromosomes. Because similar numbers of genes were expressed at higher levels in females and in males, genes with autosomal locations tended to be more highly expressed in males.

A strikingly different pattern of chromosomal location was observed for genes that differed in their response to maternal stress between male and female offspring (figure 4b). In this case, 1477 genes of the 1650 genes with a significant interaction between offspring sex and maternal stress could be localized to chromosomes. Of these, 504 genes were expressed at higher levels in female offspring from stressed mothers, whereas 973 genes were expressed at higher levels in male offspring from stressed mothers. These genes were uniformly distributed across the 21 stickleback chromosomes in both sexes (figure 4b), with each chromosome harbouring 4.8 ± 1% of the genes that demonstrated a significant interaction between maternal stress and offspring sex.

4. Discussion

The results presented here clearly demonstrate two distinct classes of sexual dimorphism in brain gene expression in threespine stickleback: (i) differences between males and females that are present under all conditions, and (ii) differences in the response of the male and female brain to maternal stress. Sexual dimorphism in brain gene expression is known for a wide variety of taxa [9], and has been previously observed in several fish species including threespine stickleback [36–41]. By contrast, the critical importance of the interaction between maternal stress and offspring sex has been pointed out in mammals [10], but has been poorly studied in other taxa and is often neglected in ecological and evolutionary studies. Our finding that maternal stress affects the brains of male and female stickleback offspring differently emphasizes the importance of taking sex into account when studying the
effects of maternal stress on gene expression, neurodevelopment and behaviour and has important implications for the assessment of the ecological and evolutionary impacts of stress across generations.

(a) Sex-specific effects of maternal stress
In this study, we detected 1650 genes that demonstrated a significant interaction between maternal stress and offspring sex, while only six genes demonstrated a significant effect of maternal stress with no interaction. This pattern is surprising because the power to detect differential expression is greater for the main effect of maternal stress ($n = 6$ individuals per group) than it is for the interactive effects of maternal stress and offspring sex ($n = 3$ individuals per group). Simulation studies suggest that six individuals per group should be sufficient to detect differential expression given the sequencing depth per sample used here [25], and thus we should be able to detect a general effect of maternal stress, if present. The lack of significant main effects of maternal stress is probably driven by the observation that genes upregulated by maternal stress in male offspring were generally downregulated in female offspring, and vice versa.

We had relatively low power to detect differences between males and females in the effects of maternal stress (i.e. the interaction between offspring sex and maternal stress), suggesting that the 1650 genes for which we detected significant interactions may represent an underestimate of the sex-specific effects of maternal stress. Another key question is the extent of false positives within this dataset, as rates of false positives increase at low samples sizes [25]. Two possible approaches to reduce this ‘false discovery cost’ are to apply a very stringent FDR threshold, or to apply an effect-size filter and retain only genes above a certain fold-change cut-off as differentially expressed, which greatly reduces the probability of detecting false positives. We applied each of these approaches (see electronic supplementary material, figure S5) and these analyses support the conclusion of sex-specific effects of maternal stress on brain gene expression patterns.

We also detected clear divergence in the pathways identified as enriched for genes upregulated by maternal stress in male versus female offspring of stressed mothers. This pattern suggests that maternal stress has physiologically distinct effects on the brains of male and female offspring of stressed mothers.

Enrichment analysis indicated that maternal stress upregulates genes involved in mitochondrial respiration in females, while upregulating genes involved in synaptic function in males, and these patterns were supported when very stringent effect size and FDR filters were applied to the data (see electronic supplementary material). Although differences in gene expression do not necessarily result in changes in protein activity, the striking differences in expression patterns between the sexes and the sexual dimorphism in the biological processes identified and the enrichment of different biological processes between the sexes in response to maternal stress strongly suggests important differences in the physiological impacts of maternal stress between male and female offspring in threespine stickleback. In combination with similar patterns that have been observed in mammals [8], these data suggest that sexually dimorphic effects of maternal stress may be common across a wide range of taxa.

(b) Sources of sexual dimorphism in brain gene expression
We also detected significant sexual dimorphism in brain gene expression independent of maternal stress. Although the specific functional consequences of the sexually dimorphic patterns of brain gene expression that we and others [36,42] observe in threespine stickleback remain unknown, they may be either causes or consequences of the many morphological and behavioural differences between male and female threespine stickleback [43–45]. For example, male stickleback perform several complex behaviours that females do not, such as nest building, courtship behaviours and paternal care of offspring [43]. Associated with this sexual dimorphism in behaviour, there is striking sexual dimorphism in brain size, with males having larger brains compared with females [45–47], and similar patterns have been observed in other fish species [48,49]. It has been proposed that the larger brain size of male stickleback may be due to the increased cognitive demand on males compared with females [45,46,50]. In addition to differences in brain size and behaviour between males and females, another possible cause of differences in brain gene expression between the sexes could be differences in the relative sizes of different brain regions. Such a pattern has been observed in a variety of species [51], including stickleback [52].
In addition to differing in size and morphology between the sexes, the vertebrate brain also has substantial capacity for plasticity in many species [53,54], including stickleback [55]. Interestingly, it has been shown that male stickleback have greater brain plasticity compared with female stickleback [46]. The effects of maternal stress on brain gene expression represent a form of transgenerational plasticity. Here, we observed increased expression of genes associated with neurodevelopmental processes in males from stressed mothers but not in females from stressed mothers. This observation raises the intriguing possibility that the differential effects of maternal stress on the male and female brain could be due to sex differences in the plasticity of different brain regions [46,55–57].

Another potential source of sexual dimorphism in gene expression is the biased genomic localization of these genes on divergent sex chromosomes [58–62]. Consistent with previous studies in threespine stickleback [36], we observed that genes that were more highly expressed in females, independent of maternal stress, were heavily biased towards localization on the sex chromosome (figure 4). By contrast, the genes that exhibited divergent expression patterns in the response to maternal stress between male and female offspring (i.e. those that showed a significant interaction between maternal stress and offspring sex) did not exhibit biases in genomic localization. This pattern strongly suggests that trans-acting mechanisms, such as regulation by sexually dimorphic genes localized on the sex chromosome, or via sexually dimorphic hormones, are likely to be responsible for the different responses to maternal stress observed in male and female brains.

(c) Effects of maternal stress on stress-related genes
One of the clear conclusions emerging from previous studies in mammals is that stress experienced during early development, such as during prenatal development or during childhood, can induce changes in the hypothalamic–pituitary–adrenal stress response [63,64], which can lead to the sensitization of adults to stress-related disorders [65]. Similar effects have been observed in a variety of fish species, including threespine stickleback [66]. To explore the effects of maternal stress on the expression of genes associated with the glucocorticoid stress response, we examined the expression patterns of a suite of candidate genes known to be involved in this pathway.

Both of the isoforms of nr3c1, which encode glucocorticoid receptors, were upregulated by maternal stress in male offspring and were downregulated by maternal stress in female offspring. In addition, there was evidence for sexual dimorphism and sex differences in the response to maternal stress in the expression of several of the key regulators of the glucocorticoid receptor (see the electronic supplementary material). Brain glucocorticoid receptors are important for the negative feedback regulation of the stress hormone axis [63]. If the changes in mRNA levels that we detect are predictive of protein levels, this implies that female stickleback from stressed mothers may show reduced ability to downregulate the stress hormone axis compared with male offspring, although current evidence suggests that these effects are likely to be subtle, if present [66]. However, taken together, these data suggest that maternal stress re-shapes the stress hormone axis, and that these effects differ between male and female stickleback.

(d) Effects of maternal stress on epigenetic processes
Epigenetic mechanisms such as DNA methylation are known to be involved in modulating the effects of maternal stress on offspring phenotypes [1] including changes in the expression of the glucocorticoid receptor [67]. We identified multiple sex-specific effects of maternal stress on the expression of DNA-demethylases suggesting a potential role for epigenetic mechanisms influencing the dimorphic expression of genes in males and females from stressed mothers (see the electronic supplementary material). Similarly, GO enrichment analysis identified post-translational gene silencing by RNA (GO: 0035194, electronic supplementary material, table S4) as a process that is over-represented in the set of genes that are upregulated by maternal stress in male, but not female, offspring. The differential regulation of genes involved in a variety of epigenetic processes suggests a potential mechanism via which maternal stress is differentially transduced to male and female offspring.

5. Conclusion
Our data demonstrate that the effects of maternal stress on brain gene expression are sex-specific in threespine stickleback. Maternal stress has the potential to result in adaptive alterations in offspring physiology and behaviour, enabling mothers to prepare their offspring for the environment they will encounter as adults. Alternatively, this phenomenon can also be maladaptive when temporary stressors induce permanent developmental changes that are suboptimal for the environment or when they lead to the development of disease [5,68,69]. Determining whether a specific offspring phenotype is the result of adaptive or maladaptive plasticity is a significant challenge in ecology and evolutionary biology. Our observation of sex-specific effects of maternal stress in stickleback thus has important implications for the analysis of the evolutionary significance of maternal effects on transgenerational plasticity.

Ethics. All animal use was conducted under approved UBC animal care protocol A11-0372.

Data accessibility. Figures and tables supporting this article can be found in the electronic supplementary material. RNA-seq data will be deposited in the NCBI Gene Expression Omnibus database (link to be provided prior to publication). A list of genes identified as differentially expressed, log2 counts per million expression values, and associated GO annotations are provided in the electronic supplementary material. Total read counts for all transcripts from sequence alignments are available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.30ns8) [70]. Short read sequence files can be downloaded from the NCBI sequence read archive (SRA study accession # SRP084036) [71].

Authors’ contributions. D.C.H.M. conceived of the study, executed all experiments, carried out bioinformatics analysis of the RNA-seq data and drafted the manuscript. P.M.S. conceived of and coordinated the study and helped draft the manuscript. All authors give final approval for publication.

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