Abstract

Parasites produce some of the strongest selective pressures on hosts. The interaction between a parasite and its host is a well-studied one, both at a genomic and an epigenomic level. Recent studies have looked at this interaction in conjunction with transgenerational immune priming (TGIP). TGIP is the transfer of parental immunological experiences to its offspring, mechanisms of this transfer are yet to be fully understood. To understand the mechanisms behind TGIP research has been done on factors which could impact it for example, sex, infection load and the parental environment. However, not much has been done on looking at the impact the interaction between sex and infection status of both the offspring and parent has on TGIP. We used the three-spined stickleback *(Gasterosteus aculeatus*) and its natural nematode parasite, *Camallanus lacustris* as the host-parasite interaction. A key aspect of the three-spined stickleback and other members of the teleost fish group is that the males contribute heavily to offspring care. It is hypothesised that the higher the level of resources a parent exerts on their young the better immune response it has, known as Bateman's principle. This is why females of many species have better immune systems. We plan to explore the association of sex and treatment on TGIP as well as see how sticklebacks sexual behaviour and investments fits in with Bateman's principle. By using reduced representation bisulfite sequencing we analysed the inheritance of DNA methylation based on sex and infection status. We found that sex (ANOVA, $F=18.88$, $p<0.01$) and paternal infection status (ANOVA, $F=5.44$, $p<0.05$) had a significantly higher impact on offspring methylation level than offspring's own infection status. We show that 12,559 sites are differentially methylated (DMS') by the sex and treatment interaction. We identified immune genes linked to sex-associated DMS' within the different treatments, with males having more methylated immune genes. We show that the interaction between sex and treatment does impact TGIP. Hypermethylation of the male stickleback immune genes compared to females suggest a better female immune response. Which follows Bateman's principle that the sex which is under more sexual selection must invest in mating rather than young. This allows females, the sex under less sexual selection, to invest in egg production and improve their immunity.

Word count: 5,990

Contents

List of figures

List of tables

Introduction

The adaptive potential is the ability of species or populations to respond to a selective pressure either by means of phenotypic or molecular changes (Eizaguirre and Baltazar-Soares, 2014). Traditionally, populations with high genetic diversity are considered more capable in dealing with selection events and have higher viability probability (Markert *et al.*, 2010). This is because there is a direct link between genetic and phenotypic diversity – the traits on which natural selection acts. As individuals with specific allelic diversity will be able to reproduce, selection will operate to remove the non-adapted part of the population. Genetic variance thus provides a way of determining adaptive potential in the population. An example of an adaption which arose through standing genetic variation is the evolution of the bony lateral plates of the three-spined stickleback. The marine populations have 32-36 sets of bony lateral plates whereas the freshwater populations have 0-9, less armour, the gene responsible for the reduction in defensive armour is more than 2 million years old (Colosimo *et al.*, 2005), but the movement from the oceans to freshwater lakes of the stickleback appeared roughly 10,000 years ago. This means that appearance of the phenotype through evolution was caused by selection of an allele present within the marine stickleback gene pool (BARRETT and SCHLUTER, 2008). This explains how populations can overcome selection pressures over time but not over an individual's lifespan. This is the role of phenotypic plasticity.

Phenotypic plasticity is the ability of genotypes to produce different phenotypes when exposed to different environmental conditions (Pigliucci, Murren and Schlichting, 2006). For example, genetically identical water flea (*Daphina*) clones can produce different morphologies depending on if they are in predator infested environments or not. An integral part of phenotypic plasticity is the epigenome and its mechanisms potentially linking the altering of gene expression in response to environmental conditions (Gibney and Nolan, 2010).

DNA methylation is an example of a well-studied epigenetic mechanism which includes the addition of a methyl (CH_3) group to the 5^{th} carbon on a cytosine (Moore, Le and Fan, 2013), it has been linked with phenotypic plasticity (Ibañez, Masuelli and Marfil, 2021). DNA methylation has a regulatory effect on gene expression especially in promoters where its presence tends to block or silence transcription factors limiting expression of genes (Anastasiadi, Esteve-Codina and Piferrer, 2018). DNA methylation sites undergo

reprogramming during embryogenesis so as to not limit developmental potential (Seisenberger *et al.*, 2013). However, some DNA methylation patterns may be inherited and become stable over generations through incomplete resetting of DNA methylation (Li *et al.*, 2014; Uller, English and Pen, 2015; Kelley *et al.*, 2021). Inheritance may be selected for if it facilitates responses to environmental conditions which are shared between generations (Beemelmanns and Roth, 2017). This phenomenon where parental immunological experience is transferred to offspring is coined transgenerational immune imprinting (TGIP) (Tetreau *et al.*, 2019). This suggests that as well as the genome, the epigenome can grant protection to individuals from environmental conditions, via inheritance of certain DNA methylation patterns. Also, both maternal and paternal DNA methylation has been shown to prepare offspring for stressful events (Venney *et al.*, 2020; Xu *et al.*, 2021).

Parasites exert some of the strongest selection pressures on individual hosts (Hamilton, Axelrod and Tanese, 1990; Brunner *et al.*, 2017), they also effect population dynamics by decreasing population growth (Watson, 2013). The fitness of parasites increases the more they encounter a host susceptible to them and the fitness of a host increases if they can avoid the parasite or deal with it swiftly once infected (Combes, 2000). Parasites and hosts both apply selective pressures onto each other and this is known as an evolutionary arms race (Dawkins and Krebs, 1979). There is continuous adaption in response to each other and one of the best ways we can characterize this coevolution is through the genome, where we are able to identify regions/genes which confer parasite resistance in hosts. For example, the plant parasitic nematodes are parasites which obtain their nutrition from the cytoplasm of their hosts cells (Williamson, 1999). A multitude of these nematode parasites cause significant decrease in crop yield and therefore a loss of an enormous amount of money (Williamson, 1999). However, through the study of the host-parasite interaction parasite-resistance genes have been identified such as the $Hs1^{pro-1}$ and Mi-1, these can then be used to provide other crops with parasite resistance (Williamson, 1999)

Another way in which host resistance is inherited is through the epigenome where beneficial changes within the parental epigenome are inherited, and no change is made to DNA sequences within the genome (Vilcinskas, 2016). Parasites are known to negatively impact individuals' phenotypes. For example, *Camallanus lacustris*, a nematode parasite is known to decrease head kidney and liver size as well as decrease the body conditions and weight of the three-spined stickleback (Sagonas *et al.*, 2020).

A crucial factor, yet rarely investigated, when looking at DNA methylation inheritance is sex (Davegårdh *et al.*, 2019). DNA patterns between sexes usually differ in regions/genes which are sex-determining (Grant *et al.*, 2022). However, immune responses differ between males and females across a multitude of species with females tending to cope better with infection than their male counter parts (Klein and Flanagan, 2016). This can be due to multiple reasons including where females and males use their available resources and any trade-offs associated with where they use them (Zuk, 2009). There are two main causes that influence the differences in immune responses between males and females: gene regulation differences and hormone differences (Klein and Flanagan, 2016). It is postulated that males will maximise their fitness by increasing the rate at which they mate whereas females will maximise their lifespan to increase their fitness, also known as Bateman's principle, (BATEMAN, 1948). To increase lifespan would mean to have a better immune system that would allow for it. Females can transfer immunity via their maternal antibodies, acquired immunity, but also components of the innate immune defence (Roth *et al.*, 2012).

In most animals, parental investment is mostly attributed to the female sex and thus immunity often is better in these cases, however, to truly see this principle in action studies have been performed where the sex-roles, in pipefish which is a teleost, is reversed and males invest in looking after the young, a common feature within teleost fish (Goldberg *et al.*, 2020). It was discovered, in this instance, males had a more active and specific immune response than females (ROTH *et al.*, 2011).

It has been well studied how females pass their immunity to their offspring but to a lesser extent how paternal imprinting occurs and how much information is inherited and how it benefits the offspring (Kaufmann *et al.*, 2014). Studies have investigated how sex and immunity link at the parental level taking into account where each sex invests its resources (Roth *et al.*, 2012). However, they have not really looked at sex on the offspring level and how transgenerational immune imprinting may differ between the sexes at the level of the offspring. Exploring these issues will allow us to identify whether there are differences in inheritance depending on the sex of the offspring and, if there is, where these differences occur.

To investigate the differences in methylation patterns between sexes and individuals of varying degrees of infection, we performed a controlled laboratory split-clutch experiment (Kaufmann *et al.*, 2014). Such a design controls for genetic variation that may influence the

methylation patterns of individuals (Sagonas *et al.*, 2020). The host-parasite system we chose to study involves the three-spined stickleback (*Gasterosteus aculeatus*) fish and its natural nematode parasite *Camallanus lacustris* (Kalbe, Wegner and Reusch, 2002). *Camallanus lacustris* triggers a response both on a phenotypic and DNA methylation level from the three spined stickleback. For example, male infected sticklebacks showed lower sperms concentration hence a smaller number of eggs fertilised. Also DNA methylated sites were more prevalent in infected fish than control ones (Sagonas *et al.*, 2020). Using this interaction system, we will explore which factors including sex, paternal and offspring infection status, have the greatest effect on offspring methylation levels as well as compare our findings to see how it aligns with Bateman's principle of how resource expenditure may impact immunity. We aimed to explore the interaction between sex and infection status and identify locations within the three-spined stickleback genome where DNA methylation level significantly differs between the different infection types and sexes. We will also identify genes that may be associated with these differing DNA methylation sites and get their annotations as well as understand their gene ontology to be able to identify what processes these DNA methylation sites may be impacting.

Methods and Materials

Sampling and infection

We used a split-clutch design to compare individuals who were closely related but differed in treatment. Wild three-spined sticklebacks (*Gasterosteus aceleatus*) were caught in Grosser Plöner See (North Germany, 54°9′21.16″N, 10°25′50.14″E). Male and female sticklebacks from Lake Plön were randomly paired, mating occurred, and the first generation (G1) were obtained, Within G1 multiple brother pairs from different families were selected to control for any genetic variation. In each pair, one brother was exposed (T) with *C. lacustris*, an abundant and natural parasite of *Gasterosteus aceleatus* within North Germany (Kalbe, Wegner and Reusch, 2002). The other brother was kept as control (C), unexposed to the parasite. Within the same generation as the brother pairs a female was assigned to each brother pair and her eggs clutch was split in two, each half fertilised by one brother, providing us with generation 2 (G2). We analysed 8 brother pairs in total with each brother

pair having between 11 and 16 offspring. Further details of the experiment can be found in Kaufmann *et al.* (2014).

Data collection

DNA was extracted from the liver tissue of the sticklebacks of both generations (G1 and G2) using the Qiagen DNeasy Blood and Tissue Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). DNA quality and quantity was assessed using the Qubit fluorometric assay. DNA methylated sites were identified using reduced representation bisulfite sequencing (RRBS) (Meissner *et al.*, 2005). Library preparation was performed at the Institute for Clinical Molecular Biology (Germany) on each of the samples obtaining a 100 bp library for every sample resulting in an average of 11.5 million reads. These libraries were then sequenced on an Illumina HiSeq 2500 platform.

Data processing, and cleaning

The raw reads from 144 individual fish were analysed using FASTQC v0.11.9 and samples were examined for poor sequencing quality. Reads were trimmed using CUTADAPT v2.10 also removing the adapter sequences (NNAGATCGGAAGAGCACAC, AGATCGGAAGAGCACAC, ATCGGAAGAGCACAC). We then used the bisulphite alignment tool BISMARK v0.22.1 which used the aligner BOWTIE2 v2.4.1 to align the raw reads to the genome of a European stickleback (Thorburn et al. in prep). We used a local genome rather than the gold standard American "gasAcu1" reference genome (Peichel *et al.*, 2017) to increase mapping efficiency (Thorburn et al. in prep). Females had an average mapping percentage to the complete European genome of 74.56% and males had an average of 73.03%, these values being significantly different (t-test, p-value<0.05). SAMTOOLS v1.9 (Li *et al.*, 2009) was then used to filter out the reads which mapped to chromosome 19 (sex chromosome) and uncharacterised scaffold. This resulted in a mapping efficiency of 65.91% for females and 65.37% for males, values which did not show significant differences (t-test, p-value>0.05).

Methylation preprocessing

Analysis of the data was performed on v 4.2.0 using the R package "methylKit" v3.15 (Akalin et al., 2012). The coverage files from the methylation calling were read into R and filtered for bases that had a coverage lower than 10X and which presented a percentile of read coverage higher than the 99.9th within each sample, this latter filtration step reducing

potential PCR bias. The reads coverage between the samples were normalized. Eventually, we removed 14 samples which presented either a small number of reads being sequenced (less than 6 million) or sequencing errors visible by PCA (Principal Component Analysis) using the prcomp function in methylKit. Ultimately, we kept for further analyses 111 G2 fish from 8 brother pairs (see table 1).

Table 1, table showing the number of G2 kept by treatment by sex

Treatment	Male	Female
Control-Control	13	15
Control-Exposed	11	16
Exposed-Control	16	12
Exposed-Exposed	16	12

Global methylation

We used linear mixed effects models to explore DNA methylation in context of sex, paternal and offspring infection status. We wanted to test whether sex had any significant interactions with the infection statuses of the father or offspring on offspring methylation and to observe the impact the father's infection status would have on offspring methylation. We did these analyses using brother pairs as a random effect to take into account any genetic variation between families. We considered for this analysis only the CpGs which were present in all samples. A CpG was considered "methylated" if it had a methylation percentage (number of methylated reads at a given position divided by the total number of reads sequenced at this position) of 70 % or more. To correct for the positive correlation between the number of CpGs found and the number reads sequenced, we considered as global methylation value per individual the residuals of the linear model of CpGs given the number of reads sequenced. A Chi-squared test confirmed the absence of detectable correlation between this global methylation value and the number of reads at the 5% significance threshold.

DMS

To test for the genome-wide distribution of DMS associated with treatment by sex , we first calculated the differential methylation of bases using methylkit function calculateDiffMeth comparing between the 8 groups of treatment (N=4) by sex (N=2). We used G1 brother pair

as a covariate to exclude the variation due to underlying genetics. For this analysis, we considered bases which were found in at least 50% of the samples per group, returning a methylbase object. We used the methylKit function getMethylDiff on the methylbase object to get the bases that showed a methylation difference of 25% or above at a 1% significance threshold.

We then tested the variation of difference in methylation between sex, within each treatment, by calculating pairwise differential methylation four times, between males and females within each of the four treatment groups. To do this we took the methylbase object containing bases found in at least 50% of the samples per group and subset it using the positions/chromosomes of the bases which showed a methylation difference of 25% or above at a 1% significance threshold. Using the new filtered methylbase object we separated it into its four treatment groups, each containing the two sex groups. For each treatment group DMS' were identified by using the calculateDiffMeth function, again using the brother pair id as a covariate, and then getMetylhDiff to identify the bases which showed significant sex-differences within each treatment.

Gene Annotation and Ontology

We tested for the presence of differentially methylated genes which were linked with sex-associated DMS' of the four different treatment groups. As well as identifying the over- and under-representation of gene ontology terms of the differentially methylated genes identified previously. The location within the genome of the European stickleback of the differentially methylated sites discovered in our analysis where identified using the "genomation" R package v1.28.0 (Akalin *et al.*, 2015). We associated these differentially methylated sites with genes if they were located within the gene or at most 10kb away from a genes transcription start site (TSS) (Heckwolf *et al.*, 2020). Using the "rentrez" R package v1.2.3 (Winter, David, 2017) we converted the uniprot gene names obtained from the gene annotations into ENTREZ IDs and obtained gene summaries.

Differentially methylated genes were then used for gene ontology enrichment analysis. Where we used the "GOstats" package v2.62.0 (Falcon and Gentleman, 2007) to find significantly over- or under-representation of GO terms, which were characterised as either biological processes (BP), molecular functions (MF), and cellular components (CC). We created a gene universe by sub selecting covered CpGs from the annotation file, and then kept the genes with gene ontology terms. Before we run any statistics to identify significant GO

terms we create sub universes, this is to select for a subset you are interested in. For example, when we wanted to find GO terms for sex-associated DMS genes within the control-control group we would subset our universe using these differentially methylated genes within this group. We did this for all treatment groups and then used GOstats to search for the significantly over- and under-represented GO terms. P values were corrected for using FDR.

Results

Sex and Paternal infection status contribute significantly to offspring methylation

CpGs which were present in all samples were filtered for and using the residuals as the dependent variable and sex, offspring infection status, as well as paternal infection status as the independent variables. We made linear mixed effects models using brother pair IDs as the random effect to consider any genetic variation that may be present between individuals and families. The linear mixed effects model (LMM) had the three independent factors with no interaction between them. Our results from this LMM, shown in table 2, indicate that sex is a strong factor in determining global offspring methylation levels, and that paternal infection status (PAT) also has an effect on global offspring methylation. The direct infection status of offspring was not found to significantly affect global offspring methylation (ANOVA, $F = 0.4732$, $p>0.05$) (Table 2). However, the infection status of the father did alter offspring methylation significantly (ANOVA, $F = 5.4397$, $p<0.05$). Meaning their father's infection status had more of an impact on their methylation levels than their own infection status.

Table 2, table showing the ANOVA results for the mixed linear effect model using the residual methylation as the dependent factor, sex, paternal and offspring infection status as independent factors and brother pair ID as the random effect.

	Sum Sq	Mean	NumDF	DenDF	F-value	Pr(>F)	
Sex	25011197	25011197	1	102.50	18.8812	$3.282e^{-0.5}$	***
PAT	7205754	7205754	1	100.15	5.4397	0.02169	*

Out-come	626784	626784	1	100.29	0.4732	0.49312	

To look at the strength of effects of sex and paternal infection status on global offspring methylation we visualised the linear mixed effects model with only the significant independent factors, sex and paternal infection status. (fig.1).

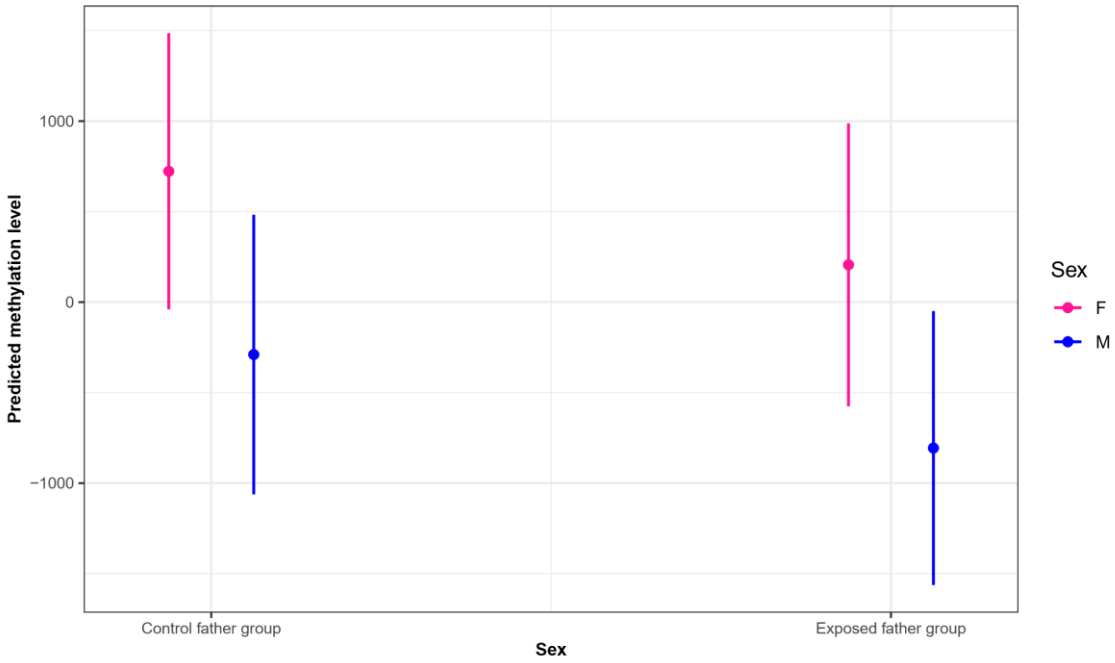

Figure 1, Prediction of offspring methylation levels in relation to paternal infection status and sex, with the methylation values coming from DMS' which were present and covered in all samples.

As shown in figure 1 females tended to have higher levels of methylation when compared to their male counterparts and offspring with infected fathers tended to have overall lower methylation levels. We then used the same dependent and independent factors as well as the random effect of brother pair ID from the previous LMM but with all the terms as interaction terms. This was to test for whether any of the main effects interacted together to impact offspring methylation levels. There was no interaction between sex and paternal infection status (ANOVA, $F = 0.700$, $p>0.05$) in the model it is shown by the gradient between the sexes remaining the same in both paternal infection statuses.

Methylation patterns between individuals within Sex–Treatment interaction

We wanted to observe where differentially methylated CpGs were in context to genomic regions. To do this we filtered for significantly differentially methylated CpGs between the sex and treatment interactions, interaction groups shown in table 2. We observed 12,559 differentially methylated CpGs from 1,092,678 CpGs (1.1%). With the same parameters used for the DMS filtering of the sex-treatment DMS' we observed 712 DMS' for sex-specific methylation differences out of 2,049,290 CpGs (0.03%) and 744 DMS' for treatment-specific methylation differences out of 1,058,914 CpGs (0.07%).

Table 3, Table showing the treatment by sex groups which were used to identify DMS' for distribution across the genome. The total number of differentially methylated sites between these groups was 12,559.

Control-Control Female	Control-Control Male	Control-Exposed Female	Control-Exposed Male	Exposed-Control Female	Exposed-Control Male	Exposed-Exposed Female	Exposed-Exposed Female

For the sex-treatment DMS' we wanted the most significantly different bases, so we selected for DMS' which showed a standard deviation of above 2 in their methylation difference. We then looked at where these most significantly different DMS' were located within the genome. Between the eight groups the percentage of DMS' ranged from 6-8% within promoters, 16-20% within exons, 30-39% within introns, and 36-44% within intergenic regions of the European stickleback genome. We performed a chi-squared test to see whether DMS' where disproportionally distributed across promoters, exons, introns, and intergenic regions. However, there were no significant differences between the expected distribution of DMS' ($\chi^2 = 21.965$, df =21, $p>0.05$). To compare the distribution of the DMS' as well as looking at the DMS' with the biggest differences between sex-treatment groups we compared each group to a chosen standard group, Control G1-Control G2 Female (figure 2).

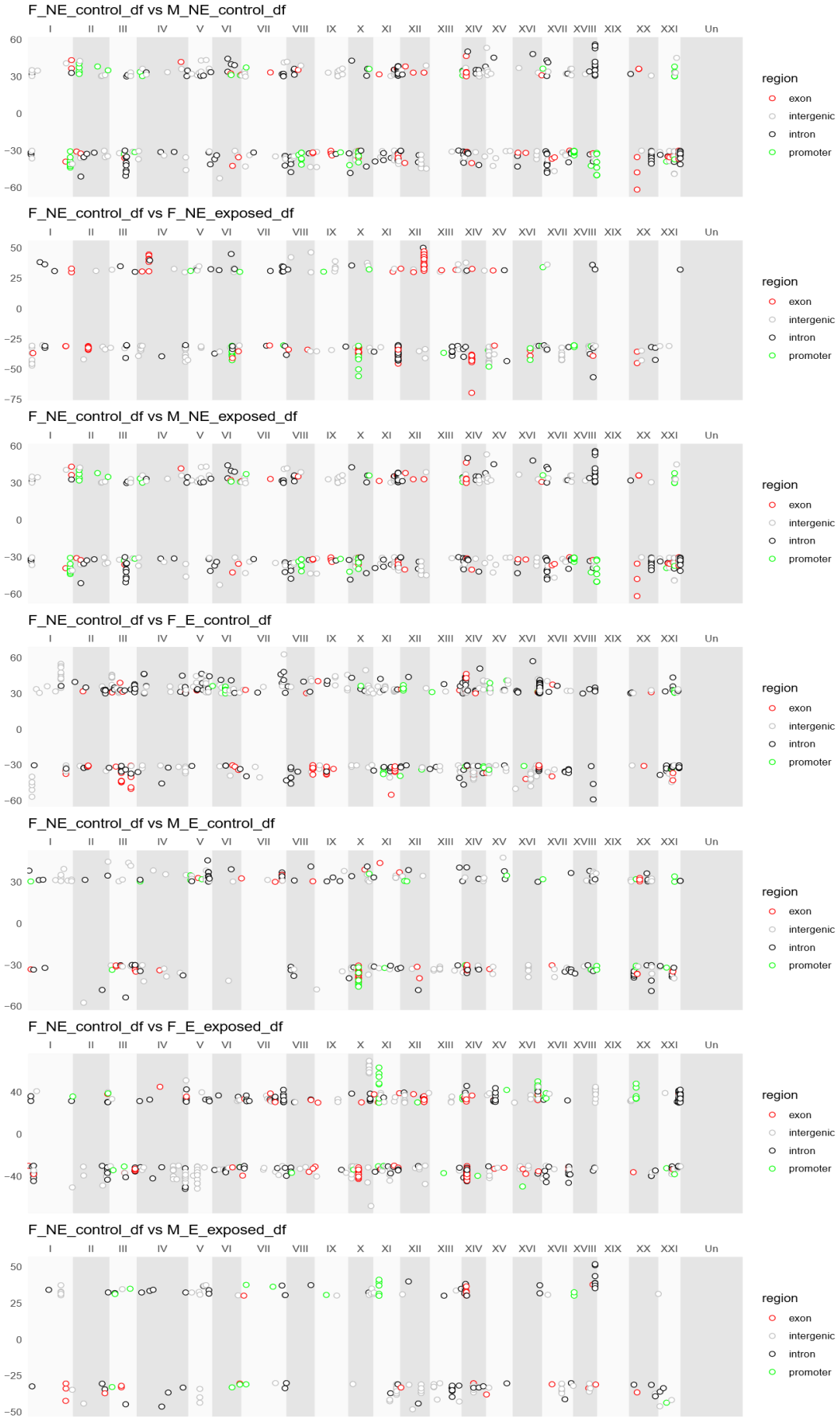
F_NE_control_df vs M_NE_control_df
F_NE_control_df vs F_NE_exposed_df
F_NE_control_df vs M_NE_exposed_df
F_NE_control_df vs F_E_control_df
F_NE_control_df vs M_E_control_df
F_NE_control_df vs F_E_exposed_df
F_NE_control_df vs M_E_exposed_df
I II III IV V VI VII VIII IX X XI XII XIII XIV XV XVI XVII XVIII XIX XX XXI Un
region
exon
intergenic
intron
promoter

Figure 2, Manhattan plot showing average methylation percentage differences above 30 between treatment groups to the Female control-control group, using DMS data obtained by subsetting all CpGs with the positions of DMS' associated with sex-treatment interaction

The group which had the highest number of CpGs with an average methylation difference of 30% was the female exposed-control group. The group with the fewest was the male exposed-exposed, shown in figure 2 with the fewest points in the plot. In context to sex and treatment interaction in figure 2 we can see that when comparing males of the different infection statuses with the control-control females that males tended to show higher methylation of CpGs on promoters in chromosomes 10, 18, and 21. However, within the exposed-exposed group, the males showed a lower difference in methylation of these CpGs, shown by the lack of promoters within the plot at these chromosomes. Also, within chromosomes 1 and 2 there were CpGs, within promoters, which showed methylation differences of above 30. These CpGs were methylated higher within the males of controlled fathers. These sex differences again seem to decrease in difference within males of the exposed fathers.

Sex-associated DMS' within treatments

Using the DMS' which were calculated between sexes within each treatment group (table 4), we wanted to test how much these comparisons had in common. It also allowed us to identify whether paternal treatment affected the number of sex-associated DMS'.

Table 4, table showing how sex-associated DMS' within each treatment group were calculated. These DMS' were used for gene annotations and gene ontology.

Control-Control	Control-Exposed	Exposed-Control	Exposed-Exposed
Female vs. Male	Female vs. Male	Female vs. Male	Female vs. Male

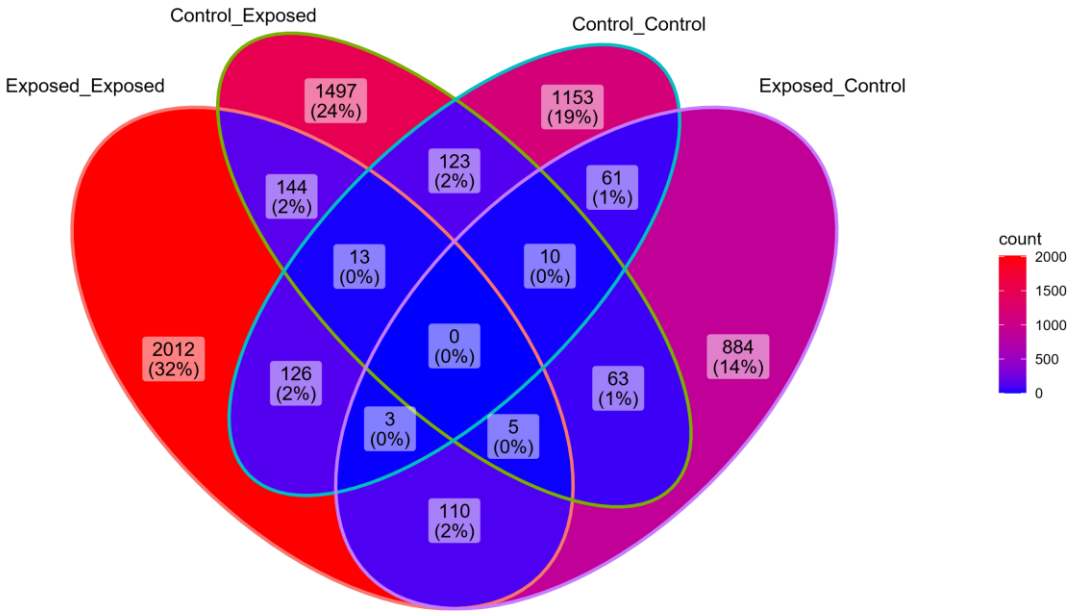

Figure 3, Venn diagram showing the overlap of sex-associated DMS' from each treatment, DMS' obtained from the pool of sex-treatment DMS' and split up into the four treatment groups where DMS' between the sexes of each treatment were calculated.

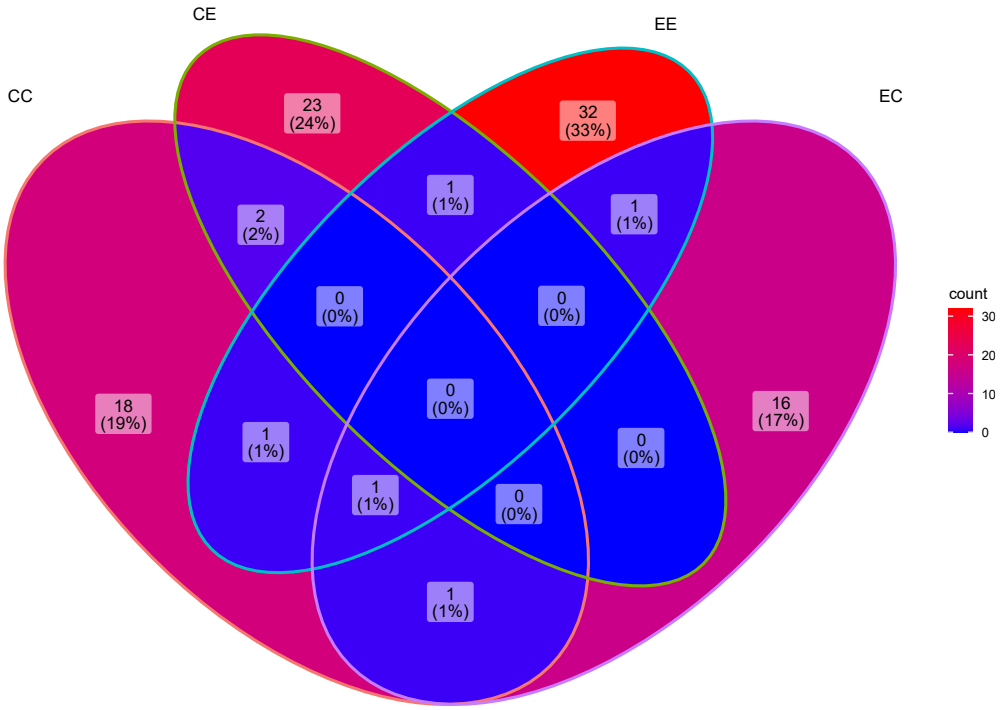

Figure 4, Venn Diagram showing the overlap of sex-associated genes from each treatment, DMS' obtained from the pool of sex-treatment DMS' and split up into the four treatment groups where DMS' between the sexes of each treatment were calculated.

We found far more DMS' found in only one comparison than those that are shared, with the exposed-exposed treatment having the most sex-associated DMS' at 2012. We also built a Venn Diagram for genes because although the DMS' may not be the same, multiple different DMS' can be associated to one gene. However, this Venn diagram also showed very low overlap. There was no sex-associated DMS' found across all of the four treatments-sex comparisons and the same pattern was observed for genes

Gene annotations and Gene Ontology

Using the DMS' calculated using the comparisons in table 4 we were able to test for genes that were associated with these DMS' thus differentially methylated. We could then test for over- under-represented GO terms. The control-control infection status had 1153 sex-associated DMS', control-exposed had 1497, exposed-control had 884, and the exposed-exposed infection status had 2012 sex associated DMS'. These DMS' where then associated with the genes from the European stickleback genome. The control-control treatment group had 30 genes which were associated with sex DMS', some of which were linked to cell-cell interaction (e.g., *vstm2l, dchs2, ccn1*). Some genes were linked to DNA translation and repair (e.g., *strap, zbtb26, rad51b*), and some even linked with immunity (e.g., *ccr2*, and *fkbp3*). The control-exposed group showed genes associated with DNA repair (e.g., triap1-a, zbtb26) and showed some immune related genes associated with sex DMS' (e.g., casp1, cr1) and these genes showed a higher methylation in males than in females (casp1, cr1). Within the exposed-control infection group immune related genes associated with sex DMS' were identified. For example, *fkbp3* and *ptges,* with females having higher methylation levels in *ptges* and males having higher methylation levels in *fkbp3*. More genes associated with immunity were found in the exposed-exposed group (e.g., *anxa2-b, cd22, fkbp3, hvcn1, mreg, usp2*). With *cd22, hvcn1, usp2,* and *mreg* having higher methylation in males than females, and females having higher methylation levels in *anxa2-b,* and *fkbp3*. Based on the gene sets formed before we computed hypergeometric p-values for over and under-represented gene ontology terms using a p-value cutoff of 0.05. First, we looked at the sex-associated DMS' within each infection group and their genes shown in figure 4, here we obtained 70 biological processes, 12 cellular components and 56 molecular functions.

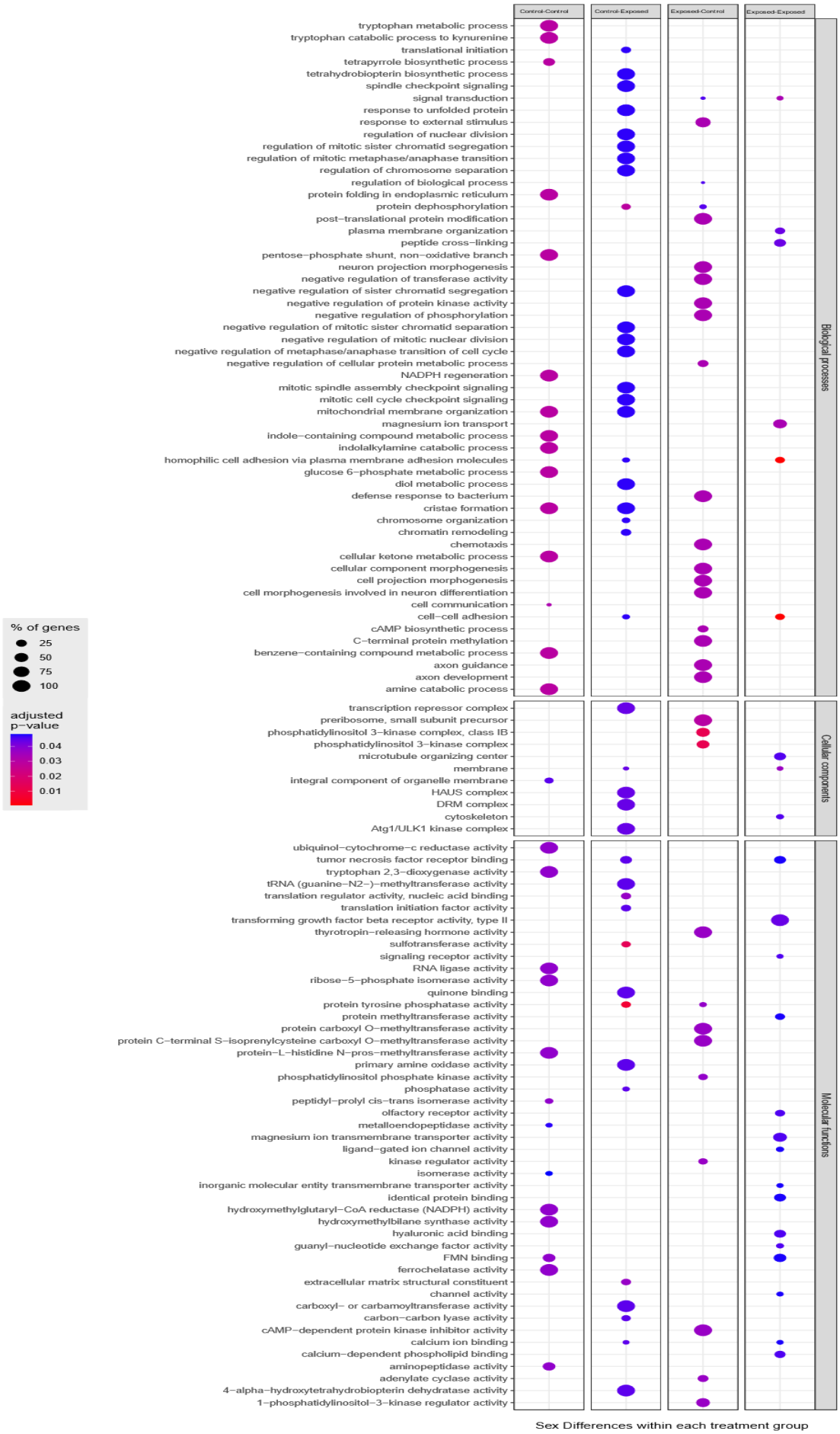
Control-Control
Control-Exposed
Exposed-Control
Exposed-Exposed
tryptophan metabolic process
tryptophan catabolic process to kynurenine
translational initiation
tetrapyrrole biosynthetic process
tetrahydrobiopterin biosynthetic process
spindle checkpoint signaling
signal transduction
response to unfolded protein
response to external stimulus
regulation of nuclear division
regulation of mitotic sister chromatid segregation
regulation of mitotic metaphase/anaphase transition
regulation of chromosome separation
regulation of biological process
protein folding in endoplasmic reticulum
protein dephosphorylation
post−translational protein modification
plasma membrane organization
peptide cross−linking
pentose−phosphate shunt, non−oxidative branch
neuron projection morphogenesis
negative regulation of transferase activity
negative regulation of sister chromatid segregation
negative regulation of protein kinase activity
negative regulation of phosphorylation
negative regulation of mitotic sister chromatid separation
negative regulation of mitotic nuclear division
negative regulation of metaphase/anaphase transition of cell cycle
negative regulation of cellular protein metabolic process
NADPH regeneration
mitotic spindle assembly checkpoint signaling
mitotic cell cycle checkpoint signaling
mitochondrial membrane organization
magnesium ion transport
indole−containing compound metabolic process
indolalkylamine catabolic process
homophilic cell adhesion via plasma membrane adhesion molecules
glucose 6−phosphate metabolic process
diol metabolic process
defense response to bacterium
cristae formation
chromosome organization
chromatin remodeling
chemotaxis
cellular ketone metabolic process
cellular component morphogenesis
cell projection morphogenesis
cell morphogenesis involved in neuron differentiation
cell communication
cell−cell adhesion
cAMP biosynthetic process
C−terminal protein methylation
benzene−containing compound metabolic process
axon guidance
axon development
amine catabolic process
Biological processes
transcription repressor complex
preribosome, small subunit precursor
phosphatidylinositol 3−kinase complex, class IB
phosphatidylinositol 3−kinase complex
microtubule organizing center
membrane
integral component of organelle membrane
HAUS complex
DRM complex
cytoskeleton
Atg1/ULK1 kinase complex
Cellular components
ubiquinol−cytochrome−c reductase activity
tumor necrosis factor receptor binding
tryptophan 2,3−dioxygenase activity
tRNA (guanine−N2−)−methyltransferase activity
translation regulator activity, nucleic acid binding
translation initiation factor activity
transforming growth factor beta receptor activity, type II
thyrotropin−releasing hormone activity
sulfotransferase activity
signaling receptor activity
RNA ligase activity
ribose−5−phosphate isomerase activity
quinone binding
protein tyrosine phosphatase activity
protein methyltransferase activity
protein carboxyl O−methyltransferase activity
protein C−terminal S−isoprenylcysteine carboxyl O−methyltransferase activity
protein−L−histidine N−pros−methyltransferase activity
primary amine oxidase activity
phosphatidylinositol phosphate kinase activity
phosphatase activity
peptidyl−prolyl cis−trans isomerase activity
olfactory receptor activity
metalloendopeptidase activity
magnesium ion transmembrane transporter activity
ligand−gated ion channel activity
kinase regulator activity
isomerase activity
inorganic molecular entity transmembrane transporter activity
identical protein binding
hydroxymethylglutaryl−CoA reductase (NADPH) activity
hydroxymethylbilane synthase activity
hyaluronic acid binding
guanyl−nucleotide exchange factor activity
FMN binding
ferrochelatase activity
extracellular matrix structural constituent
channel activity
carboxyl− or carbamoyltransferase activity
carbon−carbon lyase activity
cAMP−dependent protein kinase inhibitor activity
calcium ion binding
calcium−dependent phospholipid binding
aminopeptidase activity
adenylate cyclase activity
4−alpha−hydroxytetrahydrobiopterin dehydratase activity
1−phosphatidylinositol−3−kinase regulator activity
Molecular functions
% of genes
25
50
75
100
adjusted p−value
0.04
0.03
0.02
0.01
Sex Differences within each treatment group

Figure 5, Gene ontology plot showing over/underrepresented biological processes using the genes that were associated with sex DMS' within each treatment.

We then separated the sexes into their own group, being left with 8 groups of treatment by sex. This was so we could attribute different processes and ontology terms to the specific sex where it was present. This is shown in figure 5 where there are 126 biological processes, 24 cellular components and 84 molecular functions. Within the control-exposed treatment there are a few processes involved in the cell cycle and its regulation which are over-represented. Whereas the exposed-control treatment group shows a response to an external stimulus and seems to have a negative regulatory effect on phosphorylation and protein kinase activity. In contrast to the other treatment groups and following a trend seen in the Manhattan plots of figure 2, the exposed-exposed treatment group had very few processes that were over or under-represented within our gene sets.


CC-F
CC-M
CE-F
CE-M
EC-F
EC-M
EE-F
EE-M
tubulin complex assembly
tryptophan metabolic process
tryptophan catabolic process to kynurenine
translational termination
tetrapyrrole biosynthetic process
tetrahydrobiopterin biosynthetic process
spindle checkpoint signaling
spindle assembly
signaling
response to unfolded protein
response to other organism
response to hormone
response to external stimulus
response to biotic stimulus
regulation of nuclear division
regulation of mitotic sister chromatid segregation
regulation of mitotic metaphase/anaphase transition
regulation of mitotic cell cycle
regulation of DNA-templated transcription, elongation
regulation of chromosome separation
regulation of chromosome organization
regulation of cellular process
pyrimidine nucleotide biosynthetic process
pyrimidine-containing compound metabolic process
pteridine-containing compound metabolic process
protein peptidyl-prolyl isomerization
protein folding in endoplasmic reticulum
protein dephosphorylation
protein alkylation
post-translational protein modification
post-chaperonin tubulin folding pathway
positive regulation of transcription elongation from RNA polymerase II promoter
plasma membrane raft organization
pentose metabolic process
pentose-phosphate shunt, non-oxidative branch
Notch signaling pathway
neuronal signal transduction
neuron projection morphogenesis
negative regulation of translational initiation
negative regulation of transferase activity
negative regulation of sister chromatid segregation
negative regulation of protein modification process
negative regulation of protein kinase activity
negative regulation of phosphorylation
negative regulation of phosphorus metabolic process
negative regulation of molecular function
negative regulation of mitotic sister chromatid separation
negative regulation of mitotic nuclear division
negative regulation of mitotic cell cycle phase transition
negative regulation of metaphase/anaphase transition of cell cycle
negative regulation of cell cycle
NADPH regeneration
mitotic spindle assembly checkpoint signaling
mitotic cell cycle checkpoint signaling
mitochondrial respiratory chain complex I assembly
mitochondrial membrane organization
metal ion transport
membrane raft assembly
membrane lipid catabolic process
membrane biogenesis
melanosome transport
meiotic DNA double-strand break formation
lipopolysaccharide metabolic process
L-arabinose metabolic process
interstrand cross-link repair
indole-containing compound metabolic process
indolalkylamine catabolic process
hormone-mediated signaling pathway
homophilic cell adhesion via plasma membrane adhesion molecules
heme biosynthetic process
glycosylceramide metabolic process
glycosphingolipid catabolic process
glucose 6-phosphate metabolic process
galactosylceramide catabolic process
galactolipid metabolic process
G protein-coupled receptor signaling pathway
establishment of pigment granule localization
diol metabolic process
dephosphorylation
defense response to bacterium
defense response
cyclic nucleotide biosynthetic process
cristae formation
chromatin remodeling
chemotaxis
ceramide catabolic process
centrosome cycle
cellular response to stimulus
cellular response to endogenous stimulus
cellular pigmentation
cellular ketone metabolic process
cellular component morphogenesis
cellular biogenic amine metabolic process
cellular amino acid catabolic process
cell projection morphogenesis
cell morphogenesis involved in neuron differentiation
cell differentiation
cell communication
cell adhesion
cell-cell adhesion
caveola assembly
cAMP biosynthetic process
calcium-mediated signaling
C-terminal protein methylation
biological regulation
benzene-containing compound metabolic process
axon guidance
axon development
aromatic amino acid family catabolic process
amine catabolic process
alpha-amino acid catabolic process
Biological processes
% of genes
25
50
75
100
adjusted p-value
0.04
0.03
0.02
0.01

Figure 6, Gene ontology plot showing over/underrepresented biological processes for each sex within each treatment, using the genes that were associated with sex DMS' within each treatment.

When looking at the 8-group gene ontology plot we can see that males from the exposed-control group tend display over-representation of the response to external factor processes and even more important is that within the exposed-control group the males make up nearly all of the effect seen in figure 3 exposed-control treatment group. This pattern where the males have more significantly over/under-represented ontology terms is followed by all but the control-exposed group, where females tended to have more terms associated to them then the males.

Discussion

The epigenome has been shown to have great importance in the transfer of information through to offspring (Lacal and Ventura, 2018) from both maternal and paternal sides (Beemelmanns and Roth, 2017). Essentially, epigenetic information involved in the immunity of the parents has been shown to be inherited through a process called transgenerational immune priming (TGIP). Where diseases/infections experienced from either of the parents can be passed down and allow for offspring to fight of the disease/infection, especially if they are born and raised within the same environment (Tetreau *et al.*, 2019). Previous studies have suggested that the sex of the parent experiencing the environmental cue plays a role in how much and what type of epigenetic information is inherited (Hellmann *et al.*, 2020). The mechanisms behind the partial inheritance of immunity from parents are not fully understood, and looking at a multitude of factors including sex at all levels may help understand TGIP more.

We show that sex and paternal infection status impact offspring global DNA methylation levels significantly. However, offspring infection status itself is not significant in determining their own global DNA methylation levels. We identify no sites which are differentially methylated between sexes in all 4 treatment groups. We highlight that differential methylation of the exposed G1-exposed G2 infection group show little sex associated DMS'. Suggesting a instances of methylation patterns homogenizing between the sexes. This is also shown with the decreased number of significantly over/underrepresented sex-associated processes between the sexes of the exposed-exposed treatment group. We also find that male immune genes are hypermethylated in comparison to females. This shows that males are disproportionately impacted by parasite infection. As the sex which is under the most sexual

selection and male sticklebacks provide less of their resources to offspring and more to mating. Females are under less sexual selection and thus invest more into egg development and therefore better immune responses. These results align with Bateman's principles.

The results of the present study showed that females tended to have higher DNA methylation levels within the autosomes compared to males. The model took into account the sex, paternal and offspring infection statuses of the samples as non-interaction terms. This suggests that sex contributes significantly towards offspring methylation levels. The hypermethylation of female stickleback genomes is in line with other studies examining sex-associated differences within sticklebacks (Metzger and Schulte, 2018). Due to DNA methylation having a repressive nature on gene expression (Moore, Le and Fan, 2013) this increase in DNA methylation could be associated with sex differences between male and female sticklebacks.

We also tested for the significance of offspring and paternal infection status on offspring methylation along with sex in another model, all the factors were interaction terms in this model. We found that the infection status of the offspring was not a significant factor in determining its own methylation levels. However, the infection status of the father was significant in determining the offspring methylation levels. This phenomenon where parental experiences have a greater impact on offspring methylation then offspring's own experiences has been shown to occur (Venney *et al.*, 2022). Our results point out that paternal treatment/the experience of the fathers are more indicative of offspring methylation. The offspring of the control father group had higher methylation than those of the exposed father groups. This could then suggest that exposed fathers passed down lower methylated genomes to their offspring in response to their environments. Which seems to highlight that there is a form of transgenerational epigenetic inheritance based on infection status occurring. Exploring where these site-specific differences occur will allow us to determine what time of information is being inherited.

Our DMS results looked at sites differentially methylated by the interaction of sex and treatment. We found that the distribution of these DMS' in terms of genomic features they were associated to did not significantly change between promoters, intergenic regions, exons, and introns. We also identified that sex differences decrease when the offspring's father was infected. There does not seem to be a significant difference in where the DMS' are in context to the genome depending on the interaction between sex and infection status. DMS results

showed an average of about 85% of DMS' within genes and the rest within promoters. It has been shown that methylation of promoters can silence genes (Yang and Park, 2012) but genes can be altered via DNA methylation within the gene. It has also been shown that DNA methylation can alternatively splice genes (Lev Maor, Yearim and Ast, 2015) producing different forms of genes, which can have different functions. Suggesting that the sex by treatment DMS' identified may play a role in the differential expression/transcription of their associated genes. An interesting point within the Manhattan plot is that at the exposed G1-exposed G2 infection group does not show some of the DMS's which seemed to be associated with sex. This could suggest that epigenetic inheritance from the father can decrease sex associated differential methylation once the offspring is also infected. One limitation of the analysis is that we were unable to normalise coverage before the removal of the sex chromosome. It has been shown that normalisation before the removal of the sex chromosome may introduce bias into data. This could falsely label CpGs as associated to sex when they are not (Grant *et al.*, 2022). We explored how DMS' between the sex-infection interaction where distributed. We then wanted to explore sex-associated DMS' within the treatments and how infection status impacted these DMS'.

We looked at differentially methylated sites between the sexes within the 4 different treatment groups. Here we identified no DMS' found in all comparison, suggesting that the inheritance of the sex-associated DMS' was based very strongly on infection status. For paternal infection status not to have an impact on sex-associated DMS' we would expect all the DMS' between the control-control treatment and the exposed-control treatment to be the same. This pattern would also have to be seen within the control-exposed and exposed-exposed treatments. However, they were not, with both these comparisons having very little similarity. Showing that there was clear infection-based inheritance of DNA methylation. To identify the type of inheritance obtained by the offspring we then looked at which genes were associated with the sex by treatment DMS'.

Our gene annotation results show a large number of immune genes which are hypermethylated in males when compared to females. This shows that one of the types of epigenetic inheritance is transgenerational immune priming. This also suggest that females could have a better immune response then their male counterparts, due DNA methylations repressive nature. As the sex which is under less sexual selection Bateman's principle suggests that females should have better immune responses. Within the control-exposed group we found differentially methylated genes associated with the olfactory response such

as *OR11A1* and *OR52D1*. Parasites within the stickleback host have been known to impact host olfactory predatory cues that expose the host to greater predation risk (Eghbal, 2020). These genes where hypermethylated in females which could have an impact in their mate choice, female sticklebacks use male MHC-associated olfactory cues for mate choice (Gahr, Boehm and Milinski, 2018). Within the group there are also immune related genes which show differential methylation in one sex such as *casp1*, the gene is hypermethylated in males within this group. Casp1 is an essential mediator of the inflammatory process (Denes, Lopez-Castejon and Brough, 2012). Another immune gene is CR1, which is important in the regulation of the complement cascade (Oliveira *et al.*, 2019), and is also hypermethylated in males. The hypermethylation of *casp1* and *cr1* could be an evolutionary response from the parasite to decrease the hosts defence mechanisms. A similar number of sex-associated differentially methylated genes were identified in the exposed-control infection group but when offspring of exposed fathers were themselves exposed 5 sex-associated differentially methylated genes relating to immunity were identified. Following the same trend seen in the control-exposed infection group most of the immune genes within the exposed-exposed infection group were also hypermethylated in males compared to females. Regarding Bateman's principle although fathers protect the nest which contains the offspring, they do not carry them. This points to a partial sex-reversal system. The investment of males making sperm is not higher than that of females making eggs. It has been shown that in sticklebacks even though they care for offspring, sexual selection still acts more strongly on them (Rios-Cardenas, 2005). This means that males must invest mating in order to maximise mating success. This allows for females to invest in egg production and increase lifespan. Our data shows hypermethylation of immune genes within the male sticklebacks. This suggest that females have stronger immune responses than males. This matches what is described within Bateman's principle.

Gene ontology results indicate males having a larger share of the over- /underrepresented biological processes in most treatment groups. The sex of the parent being infected could be a factor influencing this, in our case this was the father. It has been shown within sticklebacks that predator exposure to fathers produces male offspring that are more risk prone compared to females. Exposed mothers produce more anxious offspring (Hellmann *et al.*, 2020). Showing that there could be sex-biased inheritance of the epigenome in the male sticklebacks due to the father being infected.

General Avenues for improvement

We used the Bismark alignment tool because it is easy to use as well as very flexible, being able to handle single end mapping as well as paired. Interpretation of results is also easy to understand (Krueger and Andrews, 2011). We could have also used other aligners to compare mapping efficiency with Bismark. One such software being BS-bolt (Farrell *et al.*, 2021). We used RRBS in conjunction with illumina sequencing to obtain reads which we aligned to a genome. RRBS captures interesting genomic regions such as promoters of the genome saving the customer a lot of money in sequencing costs (Beck, Ben Maamar and Skinner, 2022). We could have also used Whole genome bisulfite sequencing (WGBS) which captures all of the genome and allows for full representation of the methylome but at the expense of cost.

Conclusion

Overall, our results suggest that on global methylation level sex and treatment significantly impact the offspring but do not have a significant interaction. However, at a more site-specific level there are plenty of instances where sex-differences are impacted by infection status. Also, numerous genes are associated with these differentially methylated sites especially genes related to immune function. We show that the sex-infection interaction does have a link to transgenerational immune priming and that further research will only help understand the mechanisms behind TGIP. Bateman's principle of the sex which invests in the young having a better immune response is shown in our data. With females having lower methylation levels in immune related genes than males who are under higher sexual selection pressure.

References

Akalin, A. *et al.* (2015) 'genomation: a toolkit to summarize, annotate and visualize genomic intervals', *Bioinformatics*, 31(7), pp. 1127–1129. doi: 10.1093/bioinformatics/btu775.

Anastasiadi, D., Esteve-Codina, A. and Piferrer, F. (2018) 'Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species', *Epigenetics & Chromatin*, 11(1), p. 37. doi: 10.1186/s13072-018-0205-1.

BARRETT, R. and SCHLUTER, D. (2008) 'Adaptation from standing genetic variation', *Trends in Ecology & Evolution*, 23(1), pp. 38–44. doi: 10.1016/j.tree.2007.09.008.

BATEMAN, A. J. (1948) 'Intra-sexual selection in Drosophila.', *Heredity*, 2(Pt. 3), pp. 349–68. doi: 10.1038/hdy.1948.21.

Beck, D., Ben Maamar, M. and Skinner, M. K. (2022) 'Genome-wide CpG density and DNA methylation analysis method (MeDIP, RRBS, and WGBS) comparisons', *Epigenetics*, 17(5), pp. 518–530. doi: 10.1080/15592294.2021.1924970.

Beemelmanns, A. and Roth, O. (2017) 'Grandparental immune priming in the pipefish Syngnathus typhle.', *BMC evolutionary biology*, 17(1), p. 44. doi: 10.1186/s12862-017-0885-3.

Brunner, F. S. *et al.* (2017) 'Experimental evidence that parasites drive eco-evolutionary feedbacks', *Proceedings of the National Academy of Sciences*, 114(14), pp. 3678–3683. doi: 10.1073/pnas.1619147114.

Colosimo, P. F. *et al.* (2005) 'Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles.', *Science (New York, N.Y.)*, 307(5717), pp. 1928–33. doi: 10.1126/science.1107239.

Combes, C. (2000) '[Selective pressure in host-parasite systems].', *Journal de la Societe de biologie*, 194(1), pp. 19–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11107545.

Davegårdh, C. *et al.* (2019) 'Sex influences DNA methylation and gene expression in human skeletal muscle myoblasts and myotubes', *Stem Cell Research & Therapy*, 10(1), p. 26. doi: 10.1186/s13287-018-1118-4.

Dawkins, R. and Krebs, J. R. (1979) 'Arms races between and within species.', *Proceedings of the Royal Society of London. Series B, Biological sciences*, 205(1161), pp. 489–511. doi: 10.1098/rspb.1979.0081.

Denes, A., Lopez-Castejon, G. and Brough, D. (2012) 'Caspase-1: is IL-1 just the tip of the ICEberg?', *Cell Death & Disease*, 3(7), pp. e338–e338. doi: 10.1038/cddis.2012.86.

Eizaguirre, C. and Baltazar-Soares, M. (2014) 'Evolutionary conservation-evaluating the adaptive potential of species', *Evolutionary Applications*, 7(9), pp. 963–967. doi: 10.1111/eva.12227.

Falcon, S. and Gentleman, R. (2007) 'Using GOstats to test gene lists for GO term association', *Bioinformatics*, 23(2), pp. 257–258. doi: 10.1093/bioinformatics/btl567.

Farrell, C. *et al.* (2021) 'BiSulfite Bolt: A bisulfite sequencing analysis platform', *GigaScience*, 10(5). doi: 10.1093/gigascience/giab033.

Gahr, C. L., Boehm, T. and Milinski, M. (2018) 'Female assortative mate choice functionally validates synthesized male odours of evolving stickleback river–lake ecotypes', *Biology Letters*, 14(12), p. 20180730. doi: 10.1098/rsbl.2018.0730.

Gibney, E. R. and Nolan, C. M. (2010) 'Epigenetics and gene expression', *Heredity*, 105(1), pp. 4–13. doi: 10.1038/hdy.2010.54.

Goldberg, R. L. *et al.* (2020) 'The costs and benefits of paternal care in fish: a meta-analysis', *Proceedings of the Royal Society B: Biological Sciences*, 287(1935), p. 20201759. doi: 10.1098/rspb.2020.1759.

Grant, O. A. *et al.* (2022) 'Characterising sex differences of autosomal DNA methylation in whole blood using the Illumina EPIC array', *Clinical Epigenetics*, 14(1), p. 62. doi: 10.1186/s13148-022-01279-7.

Hamilton, W. D., Axelrod, R. and Tanese, R. (1990) 'Sexual reproduction as an adaptation to resist parasites (a review).', *Proceedings of the National Academy of Sciences*, 87(9), pp. 3566–3573. doi: 10.1073/pnas.87.9.3566.

Heckwolf, M. J. *et al.* (2020) 'Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation', *Science Advances*, 6(12). doi: 10.1126/sciadv.aaz1138.

Hellmann, J. K. *et al.* (2020) 'Sex-specific plasticity across generations I: Maternal and paternal effects on sons and daughters', *Journal of Animal Ecology*. Edited by S. Plaistow, 89(12), pp. 2788–2799. doi: 10.1111/1365-2656.13364.

Ibañez, V. N., Masuelli, R. W. and Marfil, C. F. (2021) 'Environmentally induced phenotypic plasticity and DNA methylation changes in a wild potato growing in two contrasting Andean experimental gardens', *Heredity*, 126(1), pp. 50–62. doi: 10.1038/s41437-020-00355-z.

Kalbe, M., Wegner, K. M. and Reusch, T. B. H. (2002) 'Dispersion patterns of parasites in 0+ year three-spined sticklebacks: a cross population comparison', *Journal of Fish Biology*, 60(6), pp. 1529–1542. doi: 10.1111/j.1095-8649.2002.tb02445.x.

Kaufmann, J. *et al.* (2014) 'Experimental parasite infection reveals costs and benefits of paternal effects', *Ecology Letters*. Edited by D. Marshall, 17(11), pp. 1409–1417. doi: 10.1111/ele.12344.

Kelley, J. L. *et al.* (2021) 'Epigenetic inheritance of DNA methylation changes in fish living in hydrogen sulfide–rich springs', *Proceedings of the National Academy of Sciences*, 118(26). doi: 10.1073/pnas.2014929118.

Klein, S. L. and Flanagan, K. L. (2016) 'Sex differences in immune responses', *Nature Reviews Immunology*, 16(10), pp. 626–638. doi: 10.1038/nri.2016.90.

Krueger, F. and Andrews, S. R. (2011) 'Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications', *Bioinformatics*, 27(11), pp. 1571–1572. doi: 10.1093/bioinformatics/btr167.

Lacal, I. and Ventura, R. (2018) 'Epigenetic Inheritance: Concepts, Mechanisms and Perspectives', *Frontiers in Molecular Neuroscience*, 11. doi: 10.3389/fnmol.2018.00292.

Lev Maor, G., Yearim, A. and Ast, G. (2015) 'The alternative role of DNA methylation in splicing regulation', *Trends in Genetics*, 31(5), pp. 274–280. doi: 10.1016/j.tig.2015.03.002.

Li, H. *et al.* (2009) 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, 25(16), pp. 2078–2079. doi: 10.1093/bioinformatics/btp352.

Li, Q. *et al.* (2014) 'Inheritance Patterns and Stability of DNA Methylation Variation in Maize Near-Isogenic Lines', *Genetics*, 196(3), pp. 667–676. doi: 10.1534/genetics.113.158980.

Markert, J. A. *et al.* (2010) 'Population genetic diversity and fitness in multiple environments.', *BMC evolutionary biology*, 10, p. 205. doi: 10.1186/1471-2148-10-205.

Meissner, A. *et al.* (2005) 'Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis.', *Nucleic acids research*, 33(18), pp. 5868–77. doi: 10.1093/nar/gki901.

Metzger, D. C. H. and Schulte, P. M. (2018) 'The DNA Methylation Landscape of Stickleback Reveals Patterns of Sex Chromosome Evolution and Effects of Environmental Salinity', *Genome Biology and Evolution*, 10(3), pp. 775–785. doi: 10.1093/gbe/evy034.

Moore, L. D., Le, T. and Fan, G. (2013) 'DNA Methylation and Its Basic Function', *Neuropsychopharmacology*, 38(1), pp. 23–38. doi: 10.1038/npp.2012.112.

Oliveira, L. C. *et al.* (2019) 'Complement Receptor 1 (CR1, CD35) Polymorphisms and Soluble CR1: A Proposed Anti-inflammatory Role to Quench the Fire of "Fogo Selvagem" Pemphigus Foliaceus', *Frontiers in Immunology*, 10. doi: 10.3389/fimmu.2019.02585.

Peichel, C. L. *et al.* (2017) 'Improvement of the Threespine Stickleback Genome Using a Hi-C-Based Proximity-Guided Assembly', *Journal of Heredity*, 108(6), pp. 693–700. doi: 10.1093/jhered/esx058.

Pigliucci, M., Murren, C. J. and Schlichting, C. D. (2006) 'Phenotypic plasticity and evolution by genetic assimilation', *Journal of Experimental Biology*, 209(12), pp. 2362–2367. doi: 10.1242/jeb.02070.

Rios-Cardenas, O. (2005) 'Patterns of Parental Investment and Sexual Selection in Teleost Fishes: Do They Support Bateman's Principles?', *Integrative and comparative biology*, 45(5), pp. 885–94. doi: 10.1093/icb/45.5.885.

Roth, O. *et al.* (2012) 'Male pregnancy and biparental immune priming.', *The American naturalist*, 180(6), pp. 802–14. doi: 10.1086/668081.

ROTH, O. *et al.* (2011) 'Bateman's principle and immunity in a sex-role reversed pipefish', *Journal of Evolutionary Biology*, 24(7), pp. 1410–1420. doi: 10.1111/j.1420-9101.2011.02273.x.

Sagonas, K. *et al.* (2020) 'Experimental Parasite Infection Causes Genome-Wide Changes in DNA Methylation', *Molecular Biology and Evolution*. Edited by B. Gaut, 37(8), pp. 2287–2299. doi: 10.1093/molbev/msaa084.

Seisenberger, S. *et al.* (2013) 'Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1609), p. 20110330. doi: 10.1098/rstb.2011.0330.

Tetreau, G. *et al.* (2019) 'Trans-generational Immune Priming in Invertebrates: Current Knowledge and Future Prospects', *Frontiers in Immunology*, 10. doi: 10.3389/fimmu.2019.01938.

Uller, T., English, S. and Pen, I. (2015) ‘When is incomplete epigenetic resetting in germ cells favoured by natural selection?’, *Proceedings of the Royal Society B: Biological Sciences*, 282(1811), p. 20150682. doi: 10.1098/rspb.2015.0682.

Venney, C. J. *et al.* (2020) ‘DNA Methylation Profiles Suggest Intergenerational Transfer of Maternal Effects’, *Molecular Biology and Evolution*. Edited by C. Mulligan, 37(2), pp. 540–548. doi: 10.1093/molbev/msz244.

Venney, C. J. *et al.* (2022) ‘Thermal regime during parental sexual maturation, but not during offspring rearing, modulates DNA methylation in brook charr (Salvelinus fontinalis)’, *Proceedings of the Royal Society B: Biological Sciences*, 289(1974). doi: 10.1098/rspb.2022.0670.

Vilcinskas, A. (2016) ‘The role of epigenetics in host–parasite coevolution: lessons from the model host insects Galleria mellonella and Tribolium castaneum’, *Zoology*, 119(4), pp. 273–280. doi: 10.1016/j.zool.2016.05.004.

Watson, M. J. (2013) ‘What drives population-level effects of parasites? Meta-analysis meets life-history’, *International Journal for Parasitology: Parasites and Wildlife*, 2, pp. 190–196. doi: 10.1016/j.ijppaw.2013.05.001.

Williamson, V. M. (1999) ‘Plant nematode resistance genes’, *Current Opinion in Plant Biology*, 2(4), pp. 327–331. doi: 10.1016/S1369-5266(99)80057-0.

Xu, X. *et al.* (2021) ‘Epigenetic Mechanisms of Paternal Stress in Offspring Development and Diseases’, *International Journal of Genomics*. Edited by C. Meng, 2021, pp. 1–10. doi: 10.1155/2021/6632719.

Yang, M. and Park, J. Y. (2012) ‘DNA Methylation in Promoter Region as Biomarkers in Prostate Cancer’, in, pp. 67–109. doi: 10.1007/978-1-61779-612-8_5.

Zuk, M. (2009) ‘The Sicker Sex’, *PLoS Pathogens*. Edited by G. F. Rall, 5(1), p. e1000267. doi: 10.1371/journal.ppat.1000267.