Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the gonads and livers of breeding zebrafish.

L. V. Laing[1,2]*, J. Viana[3], E. Dempster[3], T. M. Uren Webster[1, 4], R. van Aerle[5], J. Mill[3], E. M. Santos[1]*

[1] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, United Kingdom

[2] Present address: Environmental Epigenetics Group, Institute of Experimental Genetics, Helmholtz Zentrum München and German Center for Diabetes Research (DZD),85764 Neuherberg, Germany

[3] University of Exeter Medical School, RILD building, University of Exeter, Exeter, EX2 5DW, United Kingdom

[4] Present address: College of Science, Swansea University, Singleton Park, Swansea, UK, SA2 8PP.

[5] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom

* Corresponding authors

E-mail: ll292@exeter.ac.uk , e.santos@exeter.ac.uk

Abstract:

Reproduction is an essential process for life and is regulated by complex hormone networks and environmental factors. To date, little is known about the contribution of epigenetic mechanisms to the regulation of reproduction, particularly in lower vertebrates. We used the zebrafish (*Danio rerio*) model to investigate the sex-specific transcription and DNA methylation profiles for genes involved in the regulation of reproduction and in epigenetic signalling in the livers and gonads. We found evidence for associations between DNA promotor methylation and transcription for *esr1* (gonads and female livers), *amh* (gonads) and *dnmt1* (livers). In the liver, *esr1* was shown to be significantly over-expressed in females compared to males, and its promoter was significantly hypo-methylated in females compared to males. In the gonads, genes involved in epigenetic processes including *dnmt1, dnmt3 and hdac1* were over-expressed in the ovary compared to the testis. In addition, *dnmt1* and *dnmt3* transcription in the testis was found to be strongly correlated with global DNA methylation. These data provide evidence of the sex-specific epigenetic regulation and transcription of genes involved in reproduction and epigenetic signalling in a commonly used vertebrate model.

Keywords: LUMA assay, pyrosequencing, teleost, gamete, hepatic, germ cells.

1. Introduction:

Reproduction is essential for species proliferation and a variety of sexual and asexual reproductive strategies have evolved in multicellular organisms. Vertebrates generally reproduce sexually and, for gonochoristic species, undifferentiated gonads differentiate into either ovaries or testis during development. Compared to mammals, where sex determination is controlled by a cascade of molecular events associated with the presence or absence of the sex determining region in the Y chromosome (SRY) (Eggers et al., 2014), many fish species, including the zebrafish, display a greater degree of plasticity with regards to sexual determination and differentiation (Martínez et al., 2014; Ribas et al., 2017). Studies in medaka have shown that *DMY* (the DM-domain gene on the Y chromosome), an SRY homologue, is the key initiator of male masculinisation (Chakraborty et al., 2016; Matsuda et al., 2007). For zebrafish, evidence for polygenic sex determination mechanisms have been proposed (Liew et al., 2012; Wilson et al., 2014). Despite the variability in sex determination mechanisms, the downstream pathways regulating sex differentiation and reproductive function are well conserved across vertebrates (Aron, 1979; Hewitt and Korach, 2003; Piferrer, 2001; Simerly, 2002). At the level of the gonads, large numbers of transcripts were reported to be differentially expressed between ovaries and testis in a range of vertebrate species including the zebrafish (Santos et al., 2007; Wen et al., 2005), rainbow trout (*Oncorhynchus mykiss*) (Daniel Baron et al., 2005) and mouse (Menke and Page, 2002). Several of these genes have been linked to gonad differentiation including anti-mullerian hormone (*amh*) and sry (sex determining region Y)-box 9a (*sox9a*), both predominantly expressed in the testis, and aromatase (*cyp19a1a*), an enzyme responsible for catalyzing the irreversible conversion of androgen to estrogens, predominantly expressed in the ovaries (Rodríguez-Marí et al., 2005; Santos et al., 2007).

Although less pronounced, sexual dimorphism in gene transcription has also been reported in other tissues for both mammals and fish, including in the brain, liver, kidney, muscle and adipose tissue (Ribas et al., 2017; Rinn and Snyder, 2005; Santos et al., 2008; Yang et al., 2006; Zheng et al., 2013).

In the liver of fish (and other oviparous vertebrates), vitellogenin (*vtg*) and zona radiata proteins (*zrp*) are strongly over-expressed in females and their expression are regulated by estrogens (Arukwe and Goksøyr, 2003; Tyler et al., 1999). In addition, estrogen receptor 1 (*esr1*) transcription was found to be greater in the livers of females compared to males in the fathead minnow (*Pimephales promelas*), but in contrast estrogen receptor 2b (*esr2b*) was significantly higher in males (Filby and Tyler, 2005).

Besides being regulated by molecular and endocrine factors, sexual differentiation in fish is sensitive to environmental cues, including temperature and oxygen saturation, and can be disturbed when fish are exposed to altered environmental conditions resulting in altered sex ratios in fish populations (Baroiller et al., 2009; Shang et al., 2006). The role of epigenetic processes on both the endogenous and environmental regulation of reproduction is still poorly understood (Piferrer, 2013). Several epigenetic mechanisms of gene expression regulation have been described to date, including changes in DNA methylation, histone modifications and ncRNAs (Goldberg et al., 2007; Vandegehuchte and Janssen, 2011) and they all have the potential to contribute to the regulation of expression of reproductive genes. Recent reports have focused principally on DNA methylation and evidence from a range of vertebrates have demonstrated its importance for the regulation of reproductive processes in some circumstances including in animals exposed to environmental chemicals (Laing et al., 2016) and increased temperature (Navarro-Martín et al., 2011; Ribas et al., 2017).

In mammalian species the SRY gene is normally epigenetically silenced and activated during specific developmental windows (Nishino et al., 2004), and DNA methylation has been associated with sex-linked differential expression of sex steroid hormone related genes in conjunction with histone modifications in human cells (Martinowich et al., 2003; Yamane et al., 2006). In addition, a sex difference in DNA methylation of the estrogen receptor – alpha (*esr1*) promotor region has been

reported in rats, with males exhibiting a greater proportion of DNA methylation in the preoptic area of the brain (Kurian et al., 2010).

Examples of epigenetic regulation of genes involved in reproduction have also been reported in fish species. In the Japanese Flounder (*Paralichthys olivaceus*), the expression of doublesex and Mab-3-related transcription factor 1 (*dmrt1*), a transcription factor involved in sex determination and differentiation in fish, was reported to be 70 times higher in the testis compared to the ovary in mature fish (Wen et al., 2014). The *dmrt1* promotor was found to be predominantly unmethylated in testis cells, while in the ovary the 13 CpG sites measured were found to be relatively hyper-methylated (57.69%). In the same study the expression of *cyp19a1a* was found to be 40 times higher in the ovary than in the testis, with the *cyp19a1a* promoter being notably hyper-methylated on average across 12 CpG sites in the testis compared to the ovary (Wen et al., 2014). This sex related pattern of *cyp19a1a* transcription and promoter DNA methylation has also been described in the European sea bass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2011). In this study, elevated temperature resulted in the masculinization of females and was associated with an increase in *cyp19a1a* promotor DNA methylation and a decrease in gene expression, suggesting that the temperature-dependent masculinization process involves DNA methylation-mediated control of the *cyp19a1a* gene.

The zebrafish is an important model organism frequently used in studies of vertebrate development, disease, behaviour, physiology and as a model for human health research (Wilson et al., 2014). Given its extensive use as a model organism, information about the sex-specific transcription patterns and promoter DNA methylation of reproductive and epigenetic related genes is highly relevant to inform on the design and interpretation of studies investigating how reproduction is regulated and how this process is disrupted by environmental stressors. To date, little is known regarding the primary mode of sex determination, differentiation and maintenance in the zebrafish, and no true conserved sex chromosomes have been determined for lab strains (Kallivretaki et al., 2007; Sreenivasan et al.,

2008; Wilson et al., 2014). Evidence thus far suggests that zebrafish sex determination is polygenic and may require female-dominant genetic factors, and genes influencing sex determination may vary depending on the strain or environmental condition (Anderson et al., 2012; Tong et al., 2010).

The present study aimed to investigate the sex-specific transcription and DNA methylation profiles for reproductive and epigenetic genes in the livers and gonads of breeding zebrafish. To achieve this, we quantified the transcription of key genes involved in epigenetic signalling and reproductive function, together with global and locus-specific DNA methylation in the gonads and livers of mature males and females.

2. Materials and Methods:

2.1 Fish husbandry

Adult wild-type WIK strain zebrafish (originating from a stock population at the University of Exeter) were maintained according to conditions reported by Paull and colleagues (Paull et al., 2008; Westerfield, 1995). Mains tap water was filtered by reverse osmosis and reconstituted with Analar-grade mineral salts. Water was then heated to 28°C in a reservoir and aerated before it was supplied to each aquarium via a flow-through system. Preceding the start of the experiment, fish were allocated randomly into breeding groups (4 males and 4 females). These groups were then kept in individual 15 L tanks and allowed to breed naturally. Tanks were supplied with a flow rate of 48 L/day with constant aeration and maintained at 28 ± 0.5 °C and pH 7-7.5. Fish were kept under a 12h light:dark cycle, including dawn and dusk transition periods of 30 minutes and were fed live *Artemia nauplii* once daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) twice daily, to satiation (Laing et al., 2016). All experiments were approved by the University of Exeter Ethics committee and conducted under approved protocols according to the UK Home Office regulations for use of animals in scientific procedures.

Initially, reproduction was monitored in several colonies and after a 10-day acclimation period, breeding groups that failed to spawn consistently were removed from the experiment. Reproduction (number of eggs produced per female, % of fertilization) was monitored for a total of 25 days on the two breeding groups selected for this study. At the end of this period fish were sacrificed humanely according to UK Home Office regulations, the fork length and weight were measured; the gonads and livers were dissected, weighted, immediately frozen in liquid nitrogen and stored at -80°C for molecular analysis. The gonadosomatic index (GSI) = gonad weight (mg)/[total weight (mg)- gonad weight (mg)] x 100, hepatosomatic index (HSI) = liver weight (mg)/[total weight (mg)- liver weight (mg)] x 100 and the condition factor (k) = [weight (g) x 100]/[fork length (cm)]^3 were calculated for each fish.

2.2 *DNA and RNA isolation*

RNA and DNA were extracted from the livers and gonads of 8 male and 8 female fish using the AllPrep DNA/RNA Micro Qiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extraction of both RNA and DNA was performed from the same tissue sample to allow for comparisons of the DNA methylation and transcription for the same gene within the same individual. In order to assess RNA and DNA purity and concentration, samples were analysed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.3 Transcript profiling

Transcript profiling of genes encoding epigenetic regulatory proteins was performed. Epigenetic marker genes were selected based on their role in DNA methylation maintenance and *de novo* DNA methylation, including DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) and DNA (cytosine-5)-methyltransferase 3 (*dnmt3*) respectively. Methyl binding domains and binding proteins such as methyl-CpG-binding protein 2 (*mecp2*), methyl-CpG-binding domain protein 2 (*mbd2*) and methyl-CpG-binding domain protein 3a (*mbd3a*) were also measured, representing further key targets associated with indirect regulation of transcription as a result of promoter methylation. In addition, we explored the differential transcription of histone modifiers, including histone deacetylase 1 (*hdac1*) and histone deacetylase 3 (*hdac3*), which are known to play a key role in the regulation of eukaryotic gene expression, and therefore hypothesized to play a role in differential transcriptional regulation between male and female gonads and livers.

We also measured the transcription of a number of target genes involved in reproductive processes including, aromatase (*cyp19a1a*), estrogen receptor 1 (*esr1*), estrogen receptor 2a (*esr2a*), estrogen receptor 2b (*esr2b*), androgen receptor (*ar*), anti-Mullerian hormone (*amh*), and vitellogenin (*vtg*)).

Transcription profiling was conducted using real-time quantitative PCR (RT-QPCR) as previously described (Laing et al., 2016). Primers for each target gene were designed using Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) and using zebrafish NCBI RefSeq sequences. Primers were purchased from MWG-Biotech (Ebersburg, Germany). Assays were optimized for each transcript and standard curves were generated as previously described (Uren-Webster et al., 2014). Primer specificity was confirmed by the observation of a single amplification product of the expected melting temperature throughout the range of detection of the assays. The linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.86 - 2.24. The primer sequences, annealing temperatures, PCR product sizes and PCR efficiencies for each primer pair were previously described in Laing et al., 2016 and are shown in Supplementary Information Table S1.

RNA was treated with DNase I (Qiagen) to remove any potential DNA contamination. 2 µg of total RNA was then converted to cDNA using random hexamers (MWG-Biotech, Ebersberg, Germany) and M-MLV reverse transcriptase (Promega, Madison, USA), according to manufacturer's instructions. cDNA was then diluted 1:2 and RT-QPCR was performed in duplicate using an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Green chemistry as previously described (Laing et al., 2016). A template-minus negative control was run in duplicate to verify the absence of contamination on each plate. Efficiency-corrected relative expression levels were determined using the ΔΔ Ct method (Livak and Schmittgen, 2001). Data were normalized to the control gene, ribosomal protein l8 (*rpl8*), shown to have stable expression in the livers and gonads in another cyprinid fish species (Filby and Tyler, 2007, 2005).

2.4 Bisulfite-PCR-Pyrosequencing

The sequences of promoter regions of *esr1*, *amh* and *dnmt1* were obtained from Ensembl (release 83; Cunningham et al 2015) (Cunningham et al., 2015) using the Biomart portal (Kinsella et al., 2011). Zebrafish *esr1* (ENSDARG00000004111) has three known transcripts (*esr1*-001 (3449 bp), *esr1*-201 (3502 bp) and *esr1*-202 (212 bp)) and two TSSs. The *dnmt1* gene (ENSDARG00000030756) also has two TSSs and three transcripts (*dnmt1*-001 (4896 bp), *dnmt1*-201 (4893 bp) and *dnmt1*-202 (5031 bp)). *amh* (ENSDARG00000014357) has one transcript (*amh*-001, 3243 bp) and one TSS (Figure 1). The promoter regions were screened for the presence of putative binding sites for transcription factors known to be involved in reproduction, including estrogen-responsive elements (EREs), Dmrt3 and Sox9 using JASPAR (Sandelin et al., 2004), and the matrix models ESR1 (MA0112), ESR2 (MA0258), DMRT3 (MA0610) and SOX9 (MA0077). PCR and Pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). Pyrosequencing primers and their corresponding target sequences were previously described in Laing et al., 2016 (Laing et al., 2016) and are shown in Supplementary Information Table S2.

Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturers' standard protocol. Template preparation and pyrosequencing in a Qiagen Pyromark Q24 pyrosequencer was carried out as described by Tost and Gut (2007) (Tost and Gut, 2007) on bisulfite-treated DNA from the gonads and liver of 8 individual fish per treatment group. In this technique, the degree of methylation at each CpG position in a sequence is determined from the ratio of T and C and the analytical sensitivity is approximately 5%–10% for individual CpG dinucleotides (Mikeska and Craig, 2014). To verify the absence of DNA contamination, negative controls were run in duplicate. Bisulfite-PCR amplification was performed in duplicate using the primers and assay conditions provided in Supplementary Information Table S2. In order to confirm primer specificity for bisulfite-modified DNA, unmodified DNA samples were included during primer optimization. For figures 2-4, data are presented as an average of CpG sites within each amplicon.

211

212 *2.5 Luminometric-Based Assay (LUMA) for Global DNA Methylation*

213 The LUMA assay was performed using DNA extracted from gonad samples from 8 individual fish per
214 sex, as described by Karimi and colleagues (Karimi et al., 2006). Analyses of global DNA methylation
215 were conducted only for gonad samples since sufficient quantities of DNA were not available to
216 perform the LUMA assay in liver samples. 250ng of each DNA sample were digested in duplicate with
217 both HpaII and MspI, and data were normalized to the EcoRI peak to account for any technical
218 differences between samples (Head et al., 2014). HpaII and MspI are restriction endonucleases
219 which are sensitive and insensitive to CpG methylation in the sequence CCGG, respectively, while
220 EcoRI is included in all reactions as a normalization reference. Global DNA methylation values were
221 calculated according to the formula (HpaII(G)/EcoRI(T))/(MspI(G)/EcoRI(T)), where G and T refer to
222 the peak heights for HpaII or MspI (DNA methylation) and EcoRI (input DNA), respectively.

223

224 *2.6 Statistical analysis*

225 Statistical analyses were carried out using R (version 3.0.2) (R Core Team, 2012). Data were tested
226 for equal variance and for normality using the Shapiro–Wilk test prior to analysis. Comparisons
227 between male and female groups were performed using the Student's t-Test. P values of ≤0.05 were
228 considered to be significant. All data are presented as mean ± SEM.

229 For transcript profiles, data points classified as outliers (using Chauvenet's criterion) and data points
230 for which the expression was below the assay detection limit were excluded from analysis. Where
231 for both of the sexes amplification was detected in more than 70% of individuals, data were
232 represented as relative expression. Where amplification was detected in less than 70% of the
233 individuals from one or more of the sexes, data were represented as the proportion of individuals
234 for which the target genes were detected.

In order to determine if there were associations between the DNA methylation levels for specific loci in the promoter regions of genes of interest and their transcription, correlation analysis was conducted. Pearson correlation was used when data was normally distributed, and where data did not meet the assumptions of parametric testing, Spearman correlation analysis was performed. Correlation analyses were also conducted to determine the relationship between global DNA methylation and transcription for *dnmt1* and *dnmt3*, as above.

All graphs were created using the R packages ggplot2 (Wickham, 2009), gplots (Warnes et al., 2015), beeswarm (Eklund, 2015) and ggbiplot (Vincent Q. Vu, 2011).

3. Results:

Zebrafish breeding groups of 4 males and 4 females were allowed to breed naturally and reproduction (egg output and proportion of fertilization) was quantified for 25 days. During this period, fish reproduced normally as demonstrated by consistent egg production and fertilization rates (Supplementary Information Figure S1). The gonadosomatic index (GSI; the ratio of gonad weight to body weight) was also significantly lower in male fish compared to female fish (0.92 and 6.75 respectively; $P \leq 0.001$; Supplementary Information Figure S2A). The mean hepatosomatic index (HSI; the ratio of liver weight to body weight) in males was significantly lower than for females (1.04 and 3.87 respectively; $P \leq 0.001$; Supplementary Information Figure S2B). There were no significant differences in the condition factors of male and female fish in this study (0.97 and 1.08 respectively; $P = 0.093$).

3.1 Sex-Specific Transcription and DNA Methylation Levels in the Gonads

We measured the transcription of target genes of interest using quantitative PCR, locus specific methylation using bisulfite pyrosequencing in selected loci (chosen based on their proximity to the transcription start site (TSS) and binding sites for transcription factors of recognised importance for reproductive function; Figure 1) and global methylation using the LUMA assay.

In the gonads, sex-specific patterns of transcription were identified for estrogen receptor 1 (*esr1*; 4.66 fold; $P = 0.031$), *esr2b* (14.75 fold; $P = < 0.001$), *amh* (21.71 fold; $P = < 0.001$) and *cyp19a1a* (46.47 fold; $P = 0.032$) but not for estrogen receptor 2a (*esr2a*) or the androgen receptor (*ar*; Figure 2A). *esr1* and *cyp19a1a* were predominantly expressed in the ovary, while *esr2b* and *amh* were found to be predominantly expressed in the testis. For transcripts involved in epigenetic regulation, sex-specific patterns of transcription were observed for *dnmt1* (45.46 fold; $P = 0.031$), *hdac1* (2.96 fold; $P = 0.035$), *dnmt3* (30.13 fold; $P = 0.022$), *mecp2* (7.22 fold; $P = 0.006$), *mbd2* (4.90 fold; P =

0.028) and *mbd3a* (24.10 fold; P = 0.033), where the relative mRNA transcription level for each of these gene was found to be greater in the ovary compared to the testis (Figure 2A).

Mean global DNA methylation in the gonads was significantly higher in males (87.76%) compared to females (82.78%; P ≤ 0.001, Figure 3). Furthermore, in the testis *dnmt1* and *dnmt3* transcription were strongly inversely correlated with global DNA methylation (correlation coefficient = -0.919, P = 0.010 and correlation coefficient = - 0.927, P = 0.003 respectively; Table 1).

In the gonads, sex-specific DNA methylation levels were identified in the promoter region of *esr1* (male = 93.13%, female = 18.95%, P ≤ 0.001, Figure 2B) and *amh* (male = 75.14%, female = 50.88%, P = < 0.001, Figure 2B; DNA methylation levels are reported as an average percentage methylation across all CpG sites measured). However, there was no significant difference in the sex-specific DNA methylation levels for *dnmt1* (Figure 2B) despite the differences in *dnmt1* transcription observed between males and females.

The *esr1* gene transcription was significantly inversely correlated with promoter DNA methylation in the ovary (positions 1 and 2; Table 1) and in the testis (CpG position 1; Table 1). For the *amh* gene, transcription levels were strongly inversely correlated with promotor DNA methylation in the ovary (CpG position 1; Table 1) and the testis (CpG positions 2 and 3; Table 1). The *dnmt1* transcription did not correlate with promotor DNA methylation in the ovary or testis.

3.2 Sex-Specific Transcription and DNA Methylation Levels in the Liver

In the liver, at the transcriptional level, sex-specific differences were identified for a number of transcripts involved in reproductive function including the transcripts encoding vitellogenin (*vtg1*; 1322.96 fold; P ≤ 0.001; Figure 4A), *esr1* (8 fold; P ≤ 0.001; Figure 4B), and *esr2a* (2.8 fold; P = 0.012; Figure 4B), but not for estrogen receptor 2b (*esr2b*; Figure 4A). *vtg1* (Figure 4A) and *esr1* (Figure 4B) were found to be predominantly expressed in female compared to male livers, while *esr2a* was

291 found to be predominantly expressed in males (Figure 4B). For transcripts involved in epigenetic
292 regulation, differences in transcription between males and females were identified only for *hdac1* (P
293 ≤ 0.001; over-expressed in males; Figure 4A).

294 Analysis of DNA methylation in the promoter region for *esr1* revealed consistently higher average
295 DNA methylation at 2 CpG sites in males compared to females (male = 13.17%, female = 9.03%, P =
296 0.013, Figure 4C), but no significant differences in methylation were detected in the promoter region
297 of *dnmt1*.

298 The transcription of *esr1* was significantly inversely correlated with its promoter DNA methylation in
299 the liver of female fish (CpG positions 1 and 2; Table 1). In addition, for the *dnmt1* gene,
300 transcription was significantly inversely correlated with promotor DNA methylation both in the livers
301 of females (CpG positions 5, 7 and 10; Table 1) and males (CpG positions 2, 3, 9 and 11; Table 1).

4. Discussion:

A wealth of information exists about zebrafish reproductive biology at the physiological and molecular levels, but little is known about the role of epigenetic regulation on reproductive function in adult tissues. Here we report for the first time sexually dimorphic DNA methylation profiles, and associations between transcription and DNA methylation in the promoters of *esr1*, *amh* and *dnmt1*, providing a valuable contribution to our understanding of the epigenetic regulation of reproduction in this important model organism.

4.1 Sex-Specific Transcription and DNA Methylation in the Gonads

Comparisons of global DNA methylation between males and females revealed that the DNA in the testes was significantly hyper-methylated compared to that in the ovaries. The relative proportion of germ cells to somatic cells in oviparous animals differs in the male and female gonads, with testis containing a far greater proportion of maturing germ cells, including sperm, compared to ovaries, which contain large oocytes surrounded by many somatic cells (Leal et al., 2009; Schulz et al., 2010; Selman et al., 1993). The differences in global DNA methylation between testes and ovaries could be due to the differences in cell type composition between these two organs and in particular to the high proportion of maturing gametes, including mature sperm which are known to be hyper-methylated across vertebrates (Jiang et al., 2013; Potok et al., 2013).

In the gonads*, amh* was over-expressed in the testis compared to the ovary, as previously reported in fish (Ribas et al., 2017; Schulz et al., 2007) and other vertebrates (Houmard et al., 2009) where *amh* is known to play an important role in testis development. Studies in zebrafish have associated the upregulation of *amh* with the gonadal transcriptional profile in heat-induced masculinization of female fish (Ribas et al., 2017). In the ovary, *amh* is expressed in granulosa cells and is thought to have multiple functions including the regulation of germ cell proliferation and follicular development

(Morinaga et al., 2007), while in males *amh* is expressed by Sertoli cells, inhibits the development of the Müllerian ducts during development and maintains the differentiation of the gonads in adult fish (Schulz et al., 2007). Comparisons between the mean gonadal DNA methylation levels in the 5' flanking region of *amh* revealed that the transcription of this gene and the level of DNA methylation measured in its promotor region were significantly inversely correlated both in testicular and ovarian tissues. DNA methylation of CpG sites located in gene promotors has previously been associated with the regulation of transcript expression in a number of organisms, suggesting that for *amh,* transcription may be influenced by DNA methylation in this region of the promotor (Jones, 1999).

In addition, the *amh* promotor region analysed was significantly hyper-methylated by 24.26% in testes compared to ovaries, despite the fact that transcription of *amh* is higher in males. We hypothesise that this may be due to the low proportion of cells expressing *amh* (Sertoli cells), compared to germ cells in the testis, which would have masked the DNA methylation profiles present in Sertoli cells. This result highlights the issues of conducting epigenetic studies in tissues with multiple cell types, each with unique DNA methylation patterns, where changes in DNA methylation on a specific cell type may not reflect the dynamics of the methylome in other cell types.

Comparisons between the relative expression levels for the three estrogen receptor subtypes show that the predominantly expressed receptor in both males and females was *esr2a*, but no differences were observed between the sexes. In contrast, for *esr1*, there was a sex specific pattern of transcription and DNA methylation; we found hyper-methylation of the 5' flanking region of the *esr1* gene in the testis (93.13%) compared to the ovary (18.95%). We also observed that *esr1 was* predominantly expressed in the ovaries, similar to previous reports in adult *Oryzias latipes* (Chakraborty et al., 2011) and *Pimephales promelas* (Filby and Tyler, 2005), associated with the important role of *esr1* in estrogen signalling in females. In addition, for both ovarian and testicular tissues, *esr1* expression was found to be significantly inversely correlated with promotor DNA

methylation, suggesting relative hypo-methylation of CpG sites in the promoter may be associated with elevated transcription for this gene.

It is important to note that the very prominent hyper-methylation of the targeted *esr1* promotor region reported in testicular tissue is potentially associated with the proportion of germ cells, including mature sperm, within the testis in addition to specific regulation within cell types where *esr1* is expressed (Jiang et al., 2013; Potok et al., 2013).

In contrast to that observed for *esr1*, *esr2b* was overexpressed in the testis, similarly to previous reports in the Korean rockfish (*Sebastes schlegeli)* (Mu et al., 2013). Our findings support the hypothesis that *esr2b* may play a role in mediating the effects of estrogen on testicular function and spermatogenesis in fish, as previously proposed for another cyprinid species (Filby and Tyler, 2005).

In ovaries, we found significant overexpression of *cyp19a1a*, as previously reported in a number of species including in *Perca flavescens* and *Pimephales promelas* (Halm et al., 2002; Lynn et al., 2008). Aromatase is responsible for the irreversible conversion of androgens into estrogens and it is fundamental for female sex differentiation and development, with its inhibition resulting in masculinization of the population (Fenske and Segner, 2004; Navarro-Martín et al., 2011), accounting for its overexpression in ovaries (granulosa cells) compared to testes. For example, a study in zebrafish associated the downregulation of *cyp19a1a* with the heat-induced masculinization of female fish (Ribas et al., 2017).

For the transcripts involved in epigenetic pathways, in the gonads, sex-specific levels of transcription were observed for six of the seven transcripts measured, and all were significantly overexpressed in ovaries compared to testes. These findings suggest an important role for these transcripts during oogenesis and/or during embryonic development. It is important to note that sperm cells contain very little cytoplasm, and within this, few transcripts are stored and delivered to the embryo during fertilization (Krawetz, 2005; Sauvan et al., 2004). In contrast, oocytes contain large reserves of

maternal transcripts that support embryo development prior to the zygotic genome activation (ZGA), with many maternal transcripts playing important roles well beyond this point (Aanes et al., 2011; Andersen et al., 2013). In zebrafish, ZGA occurs at approximately the 1,000 cell blastula stage, much later in development than in mammalian species including mice (~2 cell) or humans (~4–8 cell) (Andersen et al., 2012; Braude et al., 1988; Flach et al., 1982; Potok et al., 2013). Therefore, in comparison to mammalian models, zebrafish are likely to be significantly more dependent on maternal transcripts to support the critical early stages of embryo development. We hypothesize that, in addition to their role within somatic and germ cells in the gonads, many of these transcripts involved in epigenetic regulation which we have shown to be overexpressed in ovaries, may be related to their role as maternal transcripts during embryo development, and are potentially involved in the dynamics of demethylation and re-methylation occurring prior to ZGA, as well as histone remodelling, occurring during this period (Jiang et al., 2013; Potok et al., 2013). This hypothesis is supported by the fact that several of these transcripts were reported to be strongly expressed during early embryo development prior to ZGA which begins during the maternal-to-zygotic transition as the embryo enters the mid blastula transition (reviewed in (Andersen et al., 2013)).

In the gonads, there were no significant differences in the DNA methylation patterns for *dnmt1*, despite the fact that this gene was significantly overexpressed in ovaries at the transcript level. However, *the* expression of *dnmt1* and *dnmt3 was* significantly inversely correlated with global DNA methylation in the testis, supporting the idea that these enzymes may be important in regulating global DNA methylation levels (Bestor, 2000). For example; the expression of *dnmt1* has been associated with changes in global DNA methylation, and inactivation of *dnmt1* has been shown to cause global demethylation of the genome (Bestor, 2000). In addition, it has been demonstrated that *dnmt3* is important in the maintenance of DNA methylation patterns during *de novo* methylation processes (Okano et al., 1999; Potok et al., 2013). The mean promoter DNA methylation of the 11 CpG sites measured was very low, 1.52% and 2.62% for males and females,

401 respectively. It is important to note, that while pyrosequencing is considered to be one of the most
402 accurate methods for measuring DNA methylation available, at this level of methylation the
403 detection accuracy of the Pyrosequencer is compromised. In addition, previous studies have
404 reported little correlation between transcription and DNA hypomethylation, suggesting that hypo-
405 methylated promoters create a transcriptionally permissive state (Andersen et al., 2013), with no
406 predictive value on gene activation (Emerman et al., 2010). The fact that the promoter of *dnmt1* was
407 found to be hypo-methylated indicates that it was likely available to be regulated by other
408 mechanisms including transcription factors, histone recruitment and modifications (reviewed in
409 (Andersen et al., 2013)). In addition to repression of transcription through DNA hyper-methylation,
410 genes may be blocked indirectly through the recruitment of methyl-binding proteins or methyl-CpG-
411 binding domain proteins, and these in turn may recruit co-repressors such as histone deacetylases
412 (Klose and Bird, 2006). Interestingly, in this study *hdac1* was the most highly expressed gene of those
413 we studied in the gonads and was significantly over-expressed in ovarian tissue. *Mecp2, mbd2* and
414 *mdb3a* were also significantly over-expressed in ovarian tissue compared to the testis; therefore, it
415 is possible that the transcription of *dnmt1* in the ovaries may be regulated indirectly through histone
416 modifications or the recruitment of methyl-CpG-binding domain proteins.

417

418 *4.2 Sex-Specific Transcriptional and DNA Methylation in the Liver*

419 We found significant over-expression of the transcripts encoding *esr1* and *vtg1* in female livers, likely
420 associated with the role of these genes in vitellogenesis (Filby and Tyler, 2005). In parallel, the 5'
421 flanking region of the *esr1* gene was hyper-methylated in male compared to female livers. The
422 transcription for *esr1* was found to be significantly inversely correlated with *esr1* promotor DNA
423 methylation in the livers of female fish, suggesting that the level of promoter DNA methylation
424 contributes to regulation of transcription for this gene. In contrast, the transcript encoding *esr2a*
425 was over-expressed in male livers compared to females. This estrogen receptor subtype is thought

426 to be responsible for maintaining basal *esr1* levels and to act as an adjustment mechanism for
427 estrogen function (Griffin et al., 2013; Nelson and Habibi, 2010). The *esr2a* transcript profile levels
428 are therefore variable and have been reported to be higher either in females (Lynn et al., 2008) or in
429 males (Filby and Tyler, 2005; Halm et al., 2004).

430 For transcripts involved in epigenetic regulation, one gene (*hdac1*) was differentially expressed
431 between males and females in the liver and was over-expressed in males. The significance of this
432 finding is unknown, and to our knowledge this is the first time that a sex specific transcription
433 pattern for this gene is reported for hepatic tissue. For *dnmt1*, significant inverse correlations were
434 found between transcription and promotor DNA methylation in both male and female livers,
435 suggesting that the DNA methylation present on the promotor of this gene may play a role in the
436 regulation of its transcription in this tissue, even though the promoter region analysed was strongly
437 hypo-methylated, and therefore likely regulated by other mechanisms of transcriptional regulation.
438 This demonstrates the importance of DNA methylation of the promoter region analysed for the
439 functional regulation of this gene.

440 Given the functional role of the hepatic tissue in sexually mature females, including the production
441 of vitellogenins and chorion proteins that are incorporated into developing oocytes, the physiology
442 of the liver in females differs from that of males. This is reflected in the higher hepatosomatic index
443 of females compared to males and it is possible that the proportion and volume of hepatocytes
444 compared to other cell types within the liver vary between males and females. If this is the case, this
445 could also contribute to the differences in transcription and promoter DNA methylation observed
446 between the livers of males and females.

447 There are some limitations to the methodologies used in this study: the locus- specific DNA
448 methylation measurements were conducted only on a small number of CpG sites (2-11), within the
449 regulatory regions of select target genes, hypothesized to be play important roles in the regulation
450 of reproduction. CpG sites were chosen based on their proximity to the TSSs and putative binding

sites for transcription factors known to be key regulators of reproduction, including estrogen-responsive elements (EREs). This analysis therefore does not provide a comprehensive view of the changes of methylation potentially occurring at other CpG sites, including those within gene bodies and potentially important for the regulation of splicing (Laurent et al., 2010).

In the future, we advocate that studies should perform measurements of transcription and methylation at the genome wide level at the various stages of sex development, and ideally on isolated populations of cells to avoid the cellular heterogeneity of complex tissues, to better understand the potential for DNA methylation to play a role in transcriptional regulation and contribute to the establishment and maintenance of sexual dimorphism in zebrafish. In addition, it would have been interesting to expand our studies to other important tissues involved in the regulation of reproduction (brain and pituitary) to build a more complete picture of the epigenetic regulation of reproduction in this species. Further to this, while this study focused on methylation, epigenetic regulation of transcription is not limited to DNA methylation. Our finding that *hdac1* was differentially expressed between males and females in both gonads and livers advocates for the importance of measuring histone acetylation, as well as other histone and DNA modifications, in further studies.

5. Conclusions:

We provide evidence for sexual dimorphism in transcription and DNA methylation profiles in the livers and gonads of an important model organism. We report novel evidence for associations between DNA promotor methylation and transcription for *esr1, amh* and *dnmt1,* and between *dnmt1* and *dnmt3* transcription and global DNA methylation in the gonads. In addition, we document for the first time a significant overexpression of a group of genes involved in DNA and histone modifications in ovaries compared to testis, likely associated with their role as maternal transcripts to support embryo development. In addition, our data highlight critical considerations for investigating epigenetics in multicellular tissues, where each cell population is characterized by its own unique transcriptional and epigenetic signature.

6. Acknowledgements:

We thank the Aquatic Resources Centre technical team for support with zebrafish husbandry. This work was funded by a PhD studentship from the Fisheries Society of the British Isles (http://www.fsbi.org.uk/) and the University of Exeter (http://www.exeter.ac.uk/) to LVL and EMS. TMUW was funded by a Natural Environment Research Council CASE PhD studentship (grant no. NE/I528326/1) and the Salmon & Trout Association (http://www.salmon-trout.org/). RVA was supported by Cefas Seedcorn funding (DP385 & DP371).

487 **7. References:**

488 Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G.P., Lim, A.Y.M., Hajan, H.S.,
489 Collas, P., Bourque, G., Gong, Z., Korzh, V., Aleström, P., Mathavan, S., 2011. Zebrafish mRNA
490 sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition.
491 Genome Res. 21, 1328–1338.

492 Andersen, I.S., Lindeman, L.C., Reiner, A.H., Østrup, O., Aanes, H., Aleström, P., Collas, P., 2013.
493 Epigenetic marking of the zebrafish developmental program. Curr. Top. Dev. Biol. 104, 85–112.

494 Andersen, I.S., Reiner, A.H., Aanes, H., Aleström, P., Collas, P., 2012. Developmental features of DNA
495 methylation during activation of the embryonic zebrafish genome. Genome Biol. 13, R65.

496 Anderson, J.L., Mari, A., Braasch, I., Amores, A., Hohenlohe, P., Batzel, P., Postlethwait, J.H., 2012.
497 Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD
498 mapping and population genomics. PLoS One 7.

499 Aron, C., 1979. Mechanisms of Control of the Reproductive Function by Olfactory Stimuli in Female
500 Mammals. Physiol. Rev. 52, 229–284.

501 Arukwe, A., Goksøyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next
502 generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp.
503 Hepatol. 2, 4.

504 Baroiller, J.F., D'Cotta, H., Saillant, E., 2009. Environmental effects on fish sex determination and
505 differentiation. Sex. Dev. 3, 118–135.

506 Baron, D., Houlgatte, R., Alexis, F., Guiuen, Y., 2005. Large-scale temporal gene expression profiling
507 during gonadal differentiation and early gametogenesis in Rainbow trout. Biol. Reprod. 73, 959–966.

508 Bestor, T.H., 2000. The DNA methyltransferases of mammals. Hum. Mol. Genet. 9, 2395–402.

509 Braude, P., Bolton, V., Moore, S., 1988. Human gene expression first occurs between the four- and
510 eight-cell stages of preimplantation development. Nature 332, 459–461.

511 Chakraborty, T., Shibata, Y., Zhou, L.Y., Katsu, Y., Iguchi, T., Nagahama, Y., 2011. Differential
512 expression of three estrogen receptor subtype mRNAs in gonads and liver from embryos to adults of
513 the medaka, *Oryzias latipes*. Mol. Cell. Endocrinol. 333, 47–54.

514 Chakraborty, T., Zhou, L.Y., Chaudhari, A., Iguchi, T., 2016. *Dmy* initiates masculinity by expression in
515 medaka (*Oryzias latipes*). Nat. Publ. Gr. 1–11.

516 Cunningham, F., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P.,
517 Coates, G., Fitzgerald, S., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S.E., Flicek., S., Zerbino,
518 D.R., Paul, 2015. Ensembl 2015. Nucleic Acids Res. 43.

519 Eggers, S., Ohnesorg, T., Sinclair, A., 2014. Genetic regulation of mammalian gonad development.
520 Nat. Rev. Endocrinol. 10, 673–683.

521 Eklund, A., 2015. Beeswarm: The Bee Swarm Plot, an Alternative to Stripchart. R Packag. version
522 0.2.0.

523 Emerman, A.B., Zhang, Z.-R., Chakrabarti, O., Hegde, R.S., 2010. Compartment-restricted
524 biotinylation reveals novel features of prion protein metabolism *in vivo*. Mol. Biol. Cell 21, 4325–
525 4337.

526 Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing
527 zebrafish (*Danio rerio*). Aquat. Toxicol. 67, 105–126.

528 Filby, A.L., Tyler, C.R., 2007. Appropriate “housekeeping” genes for use in expression profiling the
529 effects of environmental estrogens in fish. BMC Mol. Biol. 8, 10.

530 Filby, A L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a, and 2b and their
531 tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). Biol. Reprod. 73,
532 648–62.

533 Flach, G., Johnson, M.H., Braude, P.R., Taylor, R. a, Bolton, V.N., 1982. The transition from maternal
534 to embryonic control in the 2-cell mouse embryo. EMBO J. 1, 681–686.

535 Goldberg, A.D., Allis, C.D., Bernstein, E., 2007. Epigenetics: A landscape takes shape. Cell 128, 635–
536 638.

537 Griffin, L.B., January, K.E., Ho, K.W., Cotter, K. a, Callard, G. V, 2013. Morpholino-mediated
538 knockdown of ER alpha, ER betaa, and E Rbetab mRNAs in Zebrafish (*Danio rerio*) embryos reveals
539 differential regulation of estrogen-inducible genes. Endocrinology 154, 4158–4169.

540 Halm, S., Martínez-Rodríguez, G., Rodríguez, L., Prat, F., Mylonas, C.C., Carrillo, M., Zanuy, S., 2004.
541 Cloning, characterisation, and expression of three oestrogen receptors (ERα, ERβ1 and ERβ2) in the
542 European sea bass, *Dicentrarchus labrax*. Mol. Cell. Endocrinol. 223, 63–75.

543 Halm, S., Pounds, N., Maddix, S., Rand-Weaver, M., Sumpter, J.P., Hutchinson, T.H., Tyler, C.R., 2002.
544 Exposure to exogenous 17 beta-oestradiol disrupts P450aromB mRNA expression in the brain and
545 gonad of adult fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 60, 285–299.

546 Head, J. a., Mittal, K., Basu, N., 2014. Application of the LUminometric Methylation Assay to
547 ecological species: Tissue quality requirements and a survey of DNA methylation levels in animals.
548 Mol. Ecol. Resour.

549 Hewitt, S.C., Korach, K.S., 2003. Oestrogen receptor knockout mice: roles for oestrogen receptors
550 alpha and beta in reproductive tissues. Reproduction 125, 143–149.

551 Houmard, B., Small, C., Yang, L., Naluai-Cecchini, T., Cheng, E., Hassold, T., Griswold, M., 2009. Global
552 gene expression in the human fetal testis and ovary. Biol Reprod 443, biolreprod.108.075747.

553 Jiang, L., Zhang, J., Wang, J.-J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., Zhang, J.,
554 Huang, X., Yu, M., Wang, X., Liu, F., Wu, C.-I., He, C., Zhang, B., Ci, W., Liu, J., 2013. Sperm, but not
555 oocyte, DNA methylome is inherited by zebrafish early embryos. Cell 153, 773–84.

556 Jones, P.A., 1999. The DNA methylation paradox. Trends Genet. 15, 34–37.

557 Kallivretaki, E., Eggen, R.I.L., Neuhauss, S.C.F., Kah, O., Segner, H., 2007. The zebrafish, brain-specific,
558 aromatase *cyp19a2* is neither expressed nor distributed in a sexually dimorphic manner during
559 sexual differentiation. Dev. Dyn. 236, 3155–3166.

560 Karimi, M., Johansson, S., Ekström, T.J., 2006. Using LUMA: A luminometric-based assay for global
561 DNA-methylation. Epigenetics 1, 45–48.

562 Kinsella, *et al*., 2011. Ensembl BioMarts: a hub for data retrieval across taxonomic space. Database
563 (Oxford) 2011.

564 Klose, R.J., Bird, A.P., 2006. Genomic DNA methylation: the mark and its mediators. Trends Biochem.
565 Sci. 31, 89–97.

566 Krawetz, S. a, 2005. Paternal contribution: new insights and future challenges. Nat. Rev. Genet. 6,
567 633–642.

568 Kurian, J.R., Olesen, K.M., Auger, A.P., 2010. Sex differences in epigenetic regulation of the estrogen
569 receptor-alpha promoter within the developing preoptic area. Endocrinology 151, 2297–2305.

570 Laing, L. V., Viana, J., Dempster, E.L., Trznadel, M., Trunkfield, L. a., Uren Webster, T.M., van Aerle,
571 R., Paull, G.C., Wilson, R.J., Mill, J., Santos, E.M., 2016. Bisphenol A causes reproductive toxicity,
572 decreases *dnmt1* transcription, and reduces global DNA methylation in breeding zebrafish (*Danio*
573 *rerio*). Epigenetics 11, 526–538.

574 Laurent, L., Wong, E., Li, G., Huynh, T., Tsirigos, A., Ong, C.T., Low, H.M., Kin Sung, K.W., Rigoutsos, I.,
575 Loring, J., Wei, C.-L., 2010. Dynamic changes in the human methylome during differentiation.
576 Genome Res. 20, 320–331.

577 Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009.
578 Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an
579 emphasis on spermatogonial generations. Biol. Reprod. 81, 177–87.

580 Liew, W.C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K.R., Orban, L., 2012. Polygenic sex
581 determination system in zebrafish. PLoS One 7.

582 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
583 quantitative PCR. Methods 25, 402–408.

584 Lynn, S.G., Birge, W.J., Shepherd, B.S., 2008. Molecular characterization and sex-specific tissue
585 expression of estrogen receptor α (*esr1*), estrogen receptor βa (*esr2a*) and ovarian aromatase
586 (*cyp19a1a*) in yellow perch (*Perca flavescens*). Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 149,
587 126–147.

588 Martínez, P., Viñas, A.M., Sánchez, L., Díaz, N., Ribas, L., Piferrer, F., 2014. Genetic architecture of sex
589 determination in fish: Applications to sex ratio control in aquaculture. Front. Genet. 5, 1–13.

590 Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G., Sun, Y.E., 2003. DNA
591 methylation – related chromatin remodeling in activity- dependent *Bdnf.* Gene Regulation 302, 890–
592 894.

593 Matsuda, M., Shinomiya, A., Kinoshita, M., Suzuki, A., Kobayashi, T., Paul-Prasanth, B., Lau, E.,
594 Hamaguchi, S., Sakaizumi, M., Nagahama, Y., 2007. DMY gene induces male development in
595 genetically female (XX) medaka fish. Proc. Natl. Acad. Sci. U. S. A. 104, 3865–3870.

596 Menke, D.B., Page, D.C., 2002. Sexually dimorphic gene expression in the developing mouse gonad.
597 Gene Expr. Patterns 2, 359–367.

598 Mikeska, T., Craig, J.M., 2014. DNA methylation biomarkers: Cancer and beyond. Genes (Basel). 5,
599 821–864.

600 Morinaga, C., Saito, D., Nakamura, S., Sasaki, T., Asakawa, S., Shimizu, N., Mitani, H., Furutani-Seiki,
601 M., Tanaka, M., Kondoh, H., 2007. The hotei mutation of medaka in the anti-Mullerian hormone
602 receptor causes the dysregulation of germ cell and sexual development. Proc. Natl. Acad. Sci. U. S. A.
603 104, 9691–9696.

604 Mu, W.J., Wen, H.S., Shi, D., Yang, Y.P., 2013. Molecular cloning and expression analysis of estrogen
605 receptor betas (ERβ1 and ERβ2) during gonad development in the Korean rockfish, *Sebastes*
606 *schlegeli*. Gene 523, 39–49.

607 Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA
608 methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex
609 ratio shifts in the European sea bass. PLoS Genet. 7.

610 Nelson, E.R., Habibi, H.R., 2010. Functional significance of nuclear estrogen receptor subtypes in the
611 liver of goldfish. Endocrinology 151, 1668–1676.

612 Nishino, K., Hattori, N., Tanaka, S., Shiota, K., 2004. DNA methylation-mediated control of *Sry* gene
613 expression in mouse gonadal development. J. Biol. Chem. 279, 22306–22313.

614 Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases *Dnmt3a* and *Dnmt3b* are
615 essential for *de novo* methylation and mammalian development. Cell 99, 247–257.

616 Paull, G.C., Van Look, K.J.W., Santos, E.M., Filby, A.L., Gray, D.M., Nash, J.P., Tyler, C.R., 2008.
617 Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and

implications for studies addressing population-level effects of environmental chemicals. Aquat. Toxicol. 87, 115–26.

Piferrer, F., 2013. Epigenetics of sex determination and gonadogenesis. Dev. Dyn. 242, 360–370.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229–281.

Potok, M.E., Nix, D. a, Parnell, T.J., Cairns, B.R., 2013. Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. Cell 153, 759–72 (2013).

R Core Team, 2012. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna, Austria.

Ribas, L., Liew, W.C., Díaz, N., Sreenivasan, R., Orbán, L., Piferrer, F., 2017. Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. Proc. Natl. Acad. Sci. U. S. A. 201609411.

Rinn, J.L., Snyder, M., 2005. Sexual dimorphism in mammalian gene expression. Trends Genet. 21, 298–305.

Rodríguez-Marí, A., Yan, Y.L., BreMiller, R.A., Wilson, C., Cañestro, C., Postlethwait, J.H., 2005. Characterization and expression pattern of zebrafish anti-Müllerian hormone (*amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. Gene Expr. Patterns 5, 655–667.

Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W., Lenhard, B., 2004. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res. 32.

Santos, E.M., Kille, P., Workman, V.L., Paull, G.C., Tyler, C.R., 2008. Sexually dimorphic gene expression in the brains of mature zebrafish. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 149, 314–324.

640 Santos, E.M., Workman, V.L., Paull, G.C., Filby, a L., Van Look, K.J.W., Kille, P., Tyler, C.R., 2007.
641 Molecular basis of sex and reproductive status in breeding zebrafish. Physiol. Genomics 30, 111–22.

642 Sauvan, C., Lalanne, P., Hugonin, J.-P Delivering Spermatozoan RNA to the oocyte. Nature 429,
643 (2004).

644 Schulz, R.W., Bogerd, J., Male, R., Ball, J., Fenske, M., Olsen, L.C., Tyler, C.R., 2007. Estrogen-Induced
645 Alterations in *amh* and *dmrt1* Expression Signal for Disruption in Male Sexual Development in the
646 Zebrafish Estrogen-Induced Alterations in *amh* and *dmrt1* Expression Signal for Disruption in Male
647 Sexual Development in the Zebrafish. Environ. Sci. Technol. 41, 6305–6310.

648 Schulz, R.W., de França, L.R., Lareyre, J.-J., Le Gac, F., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H.,
649 Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

650 Selman, K., Wallace, R., Sarka, A., Qi, X., 1993. Stages of oocyte development in the zebrafish,
651 Brachydanio rerio. J. Morphol. 218, 203–224.

652 Shang, E.V.A.H.H., Yu, R.M.K., Wu, R.S.S., 2006. Hypoxia affects sex differentiation and development,
653 leading to a male-dominated population in Zebrafish (*Danio rerio*) 40, 3118–3122.

654 Simerly, R.B., 2002. Wired for reproduction: Organization and Development of Sexually Dimorphic
655 Circuits in the Mammalian Forebrain. Annu. Rev. Neurosci. 25, 507–536.

656 Sreenivasan, R., Cai, M., Bartfai, R., Wang, X., Christoffels, A., Orban, L., 2008. Transcriptomic
657 analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain.
658 PLoS One 3.

659 Tong, S.K., Hsu, H.J., Chung, B.C., 2010. Zebrafish monosex population reveals female dominance in
660 sex determination and earliest events of gonad differentiation. Dev. Biol. 344, 849–856.

661 Tost, J., Gut, I.G., 2007. DNA methylation analysis by pyrosequencing. Nat. Protoc. 2, 2265–75.

662 Tyler, C.R., van Aerle, R., Hutchinson, T.H., Maddix, S., Trip, H., 1999. An *in vivo* testing system for
663 endocrine disruptors in fish early life stages using induction of vitellogenin. Environ. Toxicol. Chem.
664 18, 337–347.

665 Uren-Webster, T.M., Laing, L., Florence, H., Santos, E., 2014. Effects of glyphosate and its
666 formulation, Roundup®, on reproduction in zebrafish (*Danio rerio*). Environ. Sci. Technol. 48, 1271–
667 1279.

668 Vandegehuchte, M.B., Janssen, C.R., 2011. Epigenetics and its implications for ecotoxicology.
669 Ecotoxicology 20, 607–24.

670 Vincent Q. Vu, 2011. ggbiplot: A ggplot2 based biplot. R Packag. version 0.55. 2011.

671 Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T., Maechler, M.,
672 Magnusson, A., Moeller, S., Schwartz, M., Venables, B., 2015. gplots: Various R programming tools
673 for plotting data. R packag. version 2.17.0. 2015.

674 Wen, A.Y., You, F., Sun, P., Li, J., Xu, D.D., Wu, Z.H., Ma, D.Y., Zhang, P.J., 2014. CpG methylation of
675 *dmrt1* and *cyp19a* promoters in relation to their sexual dimorphic expression in the Japanese
676 flounder *Paralichthys olivaceus*. J. Fish Biol. 84, 193–205.

677 Wen, C., Zhang, Z., Ma, W., Xu, M., Wen, Z., Peng, J., 2005. Genome-wide identification of female-
678 enriched genes in zebrafish. Dev. Dyn. 232, 171–179.

679 Westerfield, M., 1995. The Zebrafish Book. A guide for the laboratory use of Zebrafish (*Danio rerio*),
680 3rd Edition.

681 Wickham, H., 2009. ggplot2: elegant graphics for data analysis. Springer New York.

682 Wilson, C.A., High, S.K., McCluskey, B.M., Amores, A., Yan, Y. -l., Titus, T.A., Anderson, J.L., Batzel, P.,
683 Carvan, M.J., Schartl, M., Postlethwait, J.H., 2014. Wild sex in zebrafish: loss of the natural sex
684 determinant in domesticated strains. Genetics 198, 1291–1308.

685 Yamane, K., Toumazou, C., Tsukada, Y. ichi, Erdjument-Bromage, H., Tempst, P., Wong, J., Zhang, Y.,
686 2006. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen
687 receptor. Cell 125, 483–495.

688 Yang, X., Yang, X., Schadt, E.E., Schadt, E.E., Wang, S., Wang, S., Wang, H., Wang, H., Arnold, A.P.,
689 Arnold, A.P., Ingram-Drake, L., Ingram-Drake, L., Drake, T. a, Drake, T. a, Lusis, A.J., Lusis, A.J., 2006.
690 Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res.

691 Zheng, W., Xu, H., Lam, S.H., Luo, H., Karuturi, R.K.M., Gong, Z., 2013. Transcriptomic analyses of
692 sexual dimorphism of the Zebrafish Liver and the effect of sex hormones. PLoS One 8, 1–12.

693

8. Figure captions and Tables:

Figure 1. Promoter regions of *amh*, *dnmt1* and *esr1*, showing the location of the CpG sites (indicated in bold), the target sequences used for pyrosequencing (underlined) and putative transcription factor binding sites (EREs, DMRT3, and SOX9; highlighted in blue, orange and green, respectively) in relation to the transcription start sites (TSSs; highlighted in yellow). The sequences shown were derived from Ensembl Zv9 (release 83; assembly GRCz10) and correspond to the following genomic positions: chr22:20,736,779-20,737,279 (*amh*), chr3:54,352,519-54,352,819 (*dnmt1*) and chr20:26,483,369-26,484,513 (*esr1*).

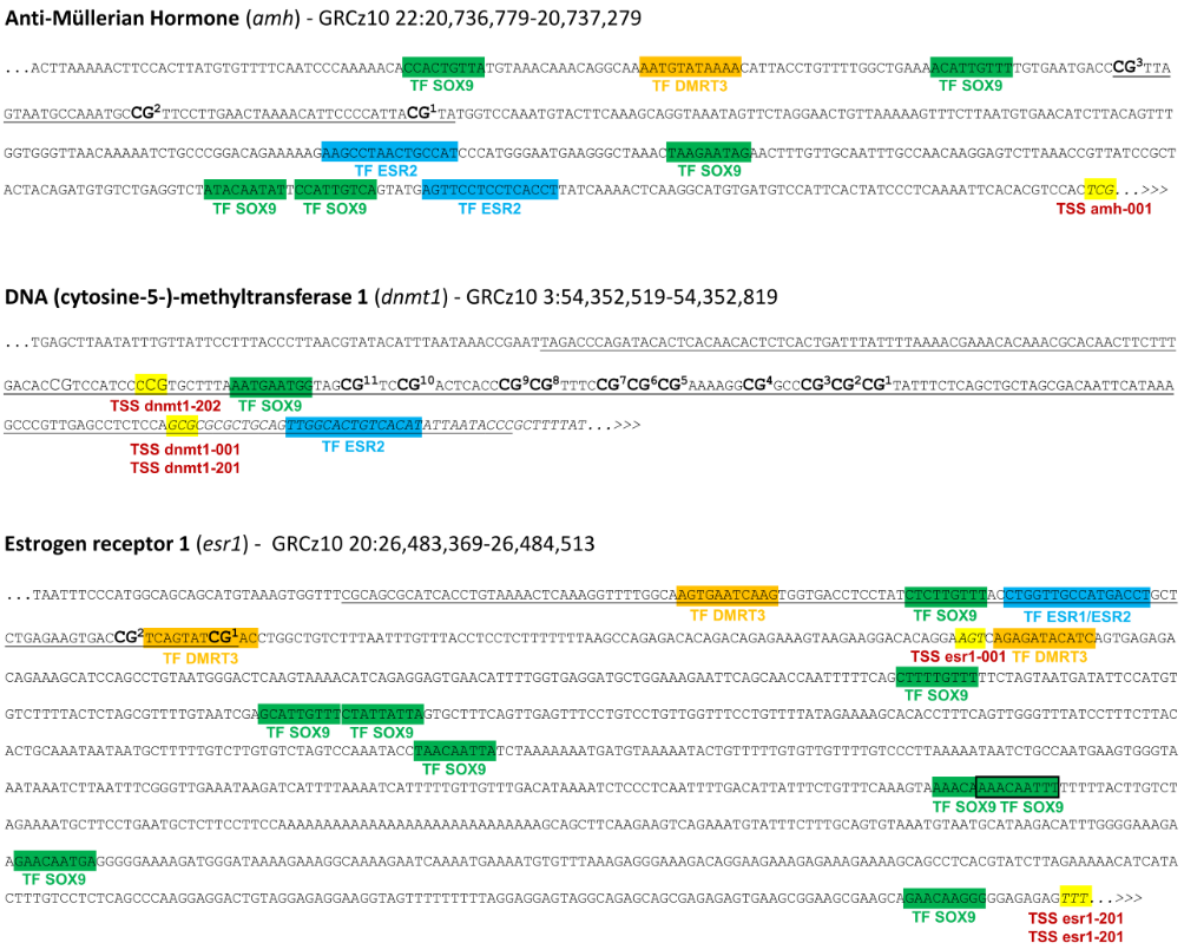


Figure 2. A) Comparison of the relative transcript profiles (target gene transcription/*rpl8* transcription) between females and males in adult zebrafish gonads (n= 6-8 for each group). **B**) Comparison of gene specific average promoter methylation between females and males in adult zebrafish gonads. Data for individual CpG sites are presented in supplementary figure 3. Asterisks indicate significant differences between males and females (Student's t-Test; *P<0.05 **P<0.01 ***P<0.001)

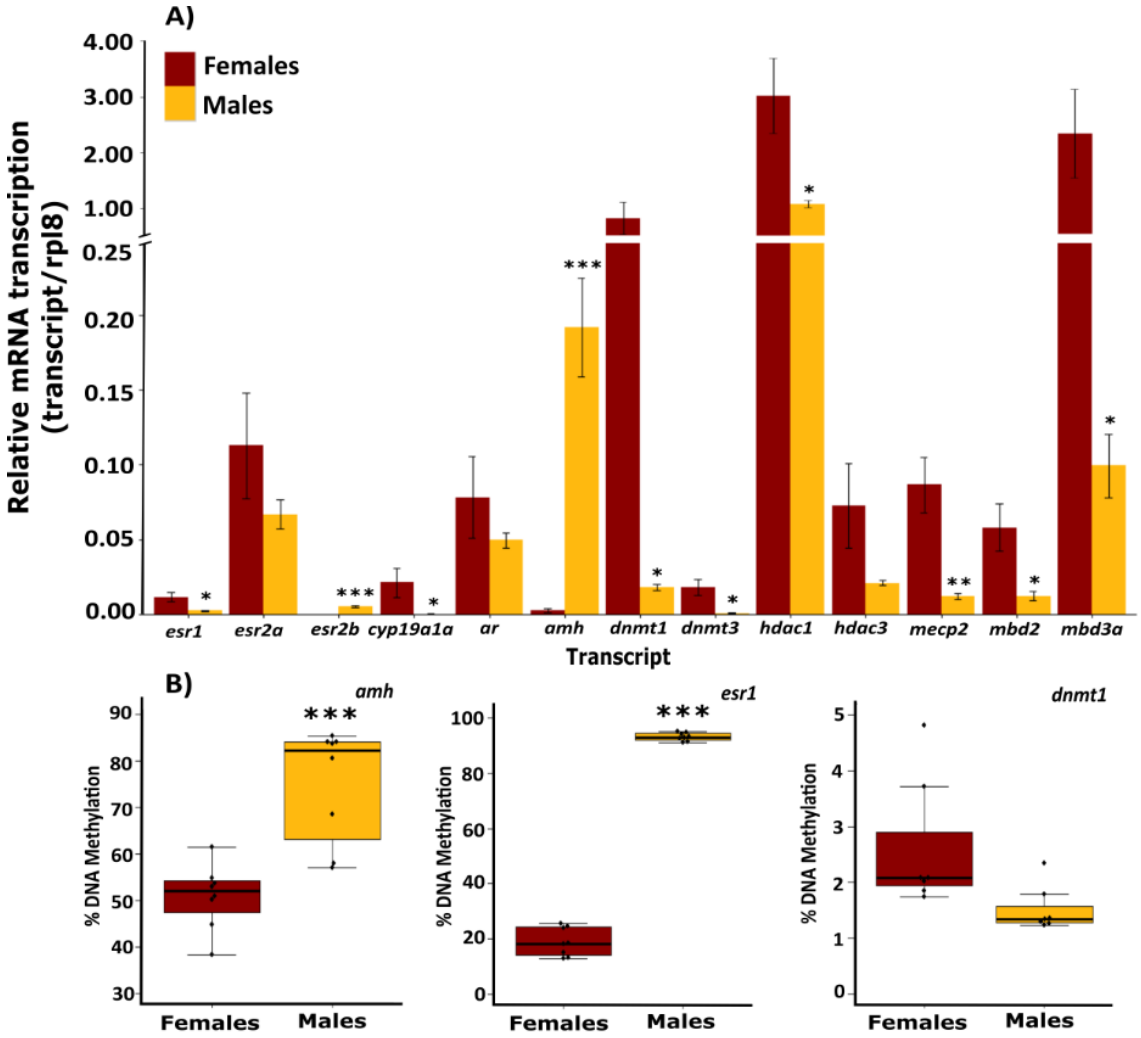


Figure 3. A) Comparison of the proportion of global DNA methylation (measured using the LUMA assay) between ovaries and testis in control adult zebrafish (n = 6-8 for each group). Asterisks indicate significant differences between males and females (Student's t-Test; ***P<0.001). Example of histological sections of zebrafish testicular (**B**) and ovarian tissue (**C**) of mature fish to illustrate the histological structure and proportion of the various cell types in ovaries and testes. The testes section is a normal testes showing different stages of spermatogenesis; SG spermatogonia, SC Spermatocytes, ST Spermatids and SZ spermatozoa. The ovarian section shows a normal ovary with oocytes at different stages of development; POF post ovulatory follicle, CA, Cortical alveoli, VO vitellogenic oocyte and PO primary oocytes. Histological images were obtained from samples analyzed as part of a different experiment but kept under similar husbandry conditions.

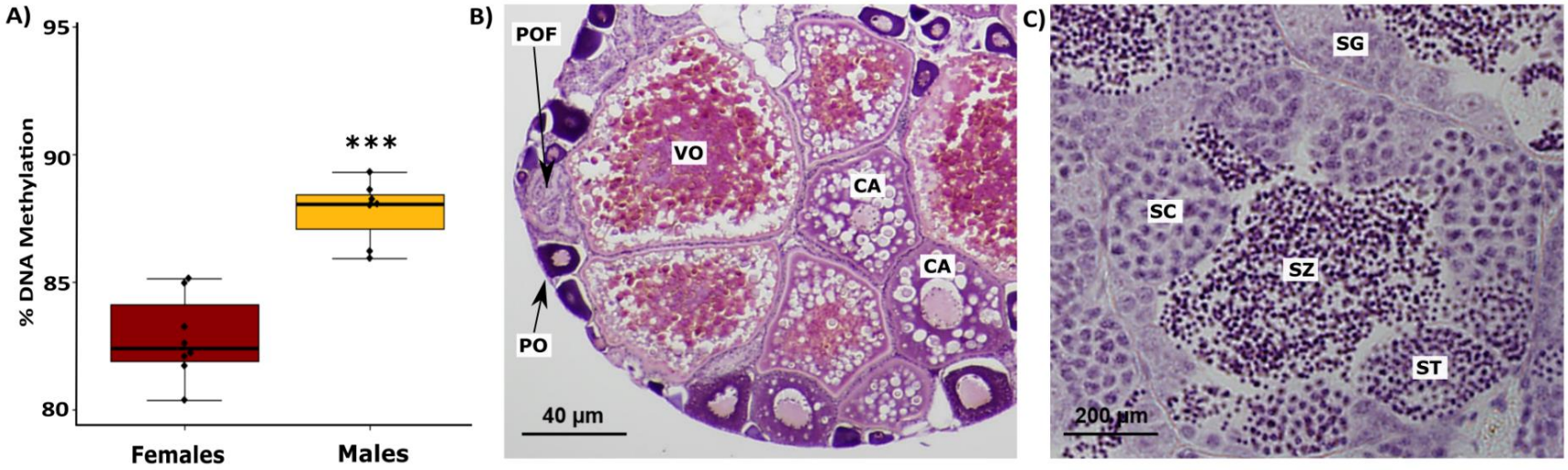
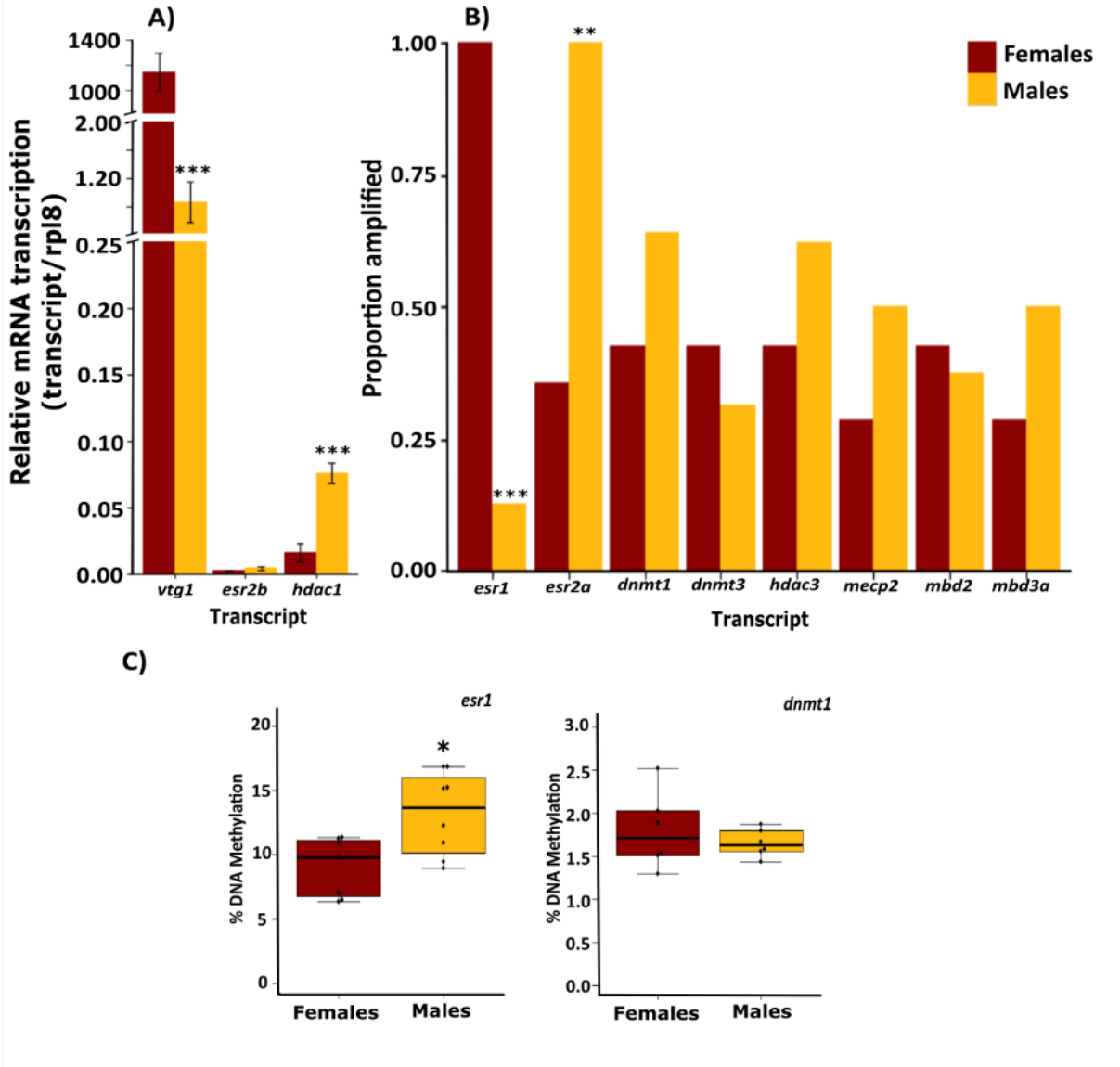


Figure 4. A and **B**) Comparison of the relative transcript profiles (target gene transcription/*rpl8* transcription) between females and males in adult zebrafish livers (n= 6-8 for each group). For each gene of interest, where amplification was detected in more than 70% individuals in both sexes, data are represented as relative expression compared to *rpl8*. Where amplification was detected in less than 70% of the individuals of one or both sexes, data are presented as the proportion of individuals for which the target genes of interest were detected**. C**) Comparison of gene specific average promoter methylation between females and males in adult zebrafish livers. Data for individual CpG sites are presented in supplementary figure 3. Asterisks indicate significant differences between males and females (Student's t-Test; *P<0.05 **P<0.01 ***P<0.001)



730 **Table 1.** Relationships between transcription and global methylation for *dnmt1* and *dnmt3* (**A**) and
731 gene transcription and promoter CpG loci methylation for specific target genes (**B**).Red shading
732 indicates significant correlations (P < 0.05).

Table 1A. Correlation analysis between transcript expression and global methylation for *dnmt1* and *dnmt3*.				
Tissue	**Gene**	**-**	**Correlation coefficient**	P value
Testis	*dnmt1*	-	-0.919	**0.010**
Ovary	*dnmt1*	-	-0.225	0.668
Testis	*dnmt3*	-	-0.927	**0.003**
Ovary	*dnmt3*	-	0.052	0.934
Table 1B. Correlation analysis between transcript expression and specific CpG loci methylation.				
Tissue	**Gene**	**CpG Position**	**Correlation coefficient**	P value
Ovary	*esr1*	1	-0.765	**0.045**
		2	-0.470	**0.029**
	amh	1	-0.271	0.659
		2	-0.957	**0.017**
		3	-0.547	0.341
	dnmt1	1	-0.584	0.224
		2	-0.573	0.234
		3	-0.580	0.227
		4	-0.540	0.269
		5	-0.582	0.225
		6	-0.552	0.256
		7	-0.603	0.206
		8	-0.625	0.160
		9	-0.701	0.121
		10	-0.625	0.185
		11	-0.461	0.358
		Mean	-0.148	0.779
Testis	*esr1*	1	-0.875	**0.004**
		2	-0.697	0.055
	amh	1	-0.072	0.878
		2	-0.818	**0.025**
		3	-0.920	**0.003**
	dnmt1	1	0.526	0.284
		2	0.522	0.288
		3	0.525	0.285
		4	0.509	0.302

		5	0.503	0.309
		6	0.447	0.374
		7	0.461	0.358
		8	0.449	0.371
		9	0.532	0.277
		10	0.599	0.401
		11	0.484	0.516
		Mean	0.071	0.893
Liver Females	*esr1*	1	-0.971	**0.006**
		2	-0.906	**0.034**
	dnmt1	1	-0.761	0.079
		2	-0.686	0.132
		3	-0.751	0.085
		4	-0.268	0.663
		5	-0.912	**0.011**
		6	-0.736	0.993
		7	-0.813	**0.049**
		8	-0.689	0.199
		9	-0.717	0.173
		10	-0.836	**0.038**
		11	-0.758	0.080
		Mean	-0.843	**0.035**
Liver Males	*esr1*	1	-0.065	0.878
		2	-0.268	0.522
	dnmt1	1	-0.736	0.095
		2	-0.818	**0.047**
		3	-0.901	**0.014**
		4	-0.534	0.275
		5	0.431	0.394
		6	-0.843	0.352
		7	-0.683	0.135
		8	-0.669	0.146
		9	-0.826	**0.043**
		10	-0.683	0.135
		11	-0.847	**0.034**
		Mean	-0.872	**0.024**

733

Supporting Information

Sex-specific transcription and DNA methylation profiles of reproductive and epigenetic associated genes in the gonads and livers of breeding zebrafish.

L. V. Laing[1,2]*, J. Viana[3], E. Dempster[3], T. M. Uren Webster[1, 4], R. van Aerle[5], J. Mill[3], E. M. Santos[1]*

[1] Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, United Kingdom

[2] Present Address: Environmental Epigenetics Group, Institute of Experimental Genetics, Helmholtz Zentrum München and German Center for Diabetes Research (DZD),85764 Neuherberg, Germany

[3] University of Exeter Medical School, RILD building, University of Exeter, Exeter, EX2 5DW, United Kingdom

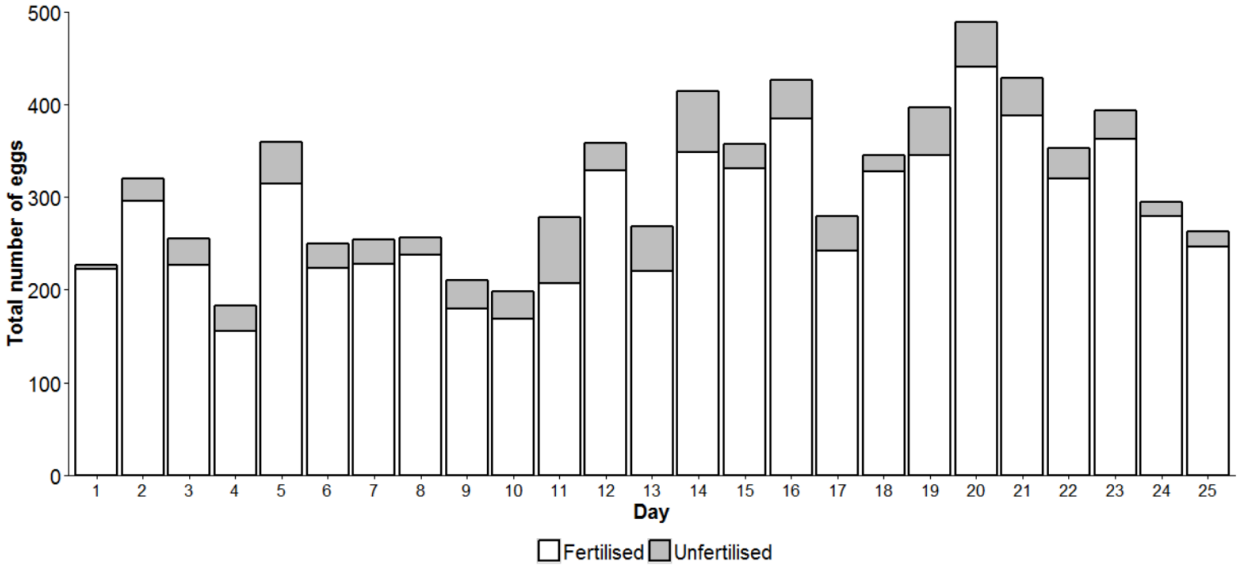
[4] Present Address: College of Science, Swansea University, Singleton Park, Swansea, UK, SA2 8PP.

[5] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom

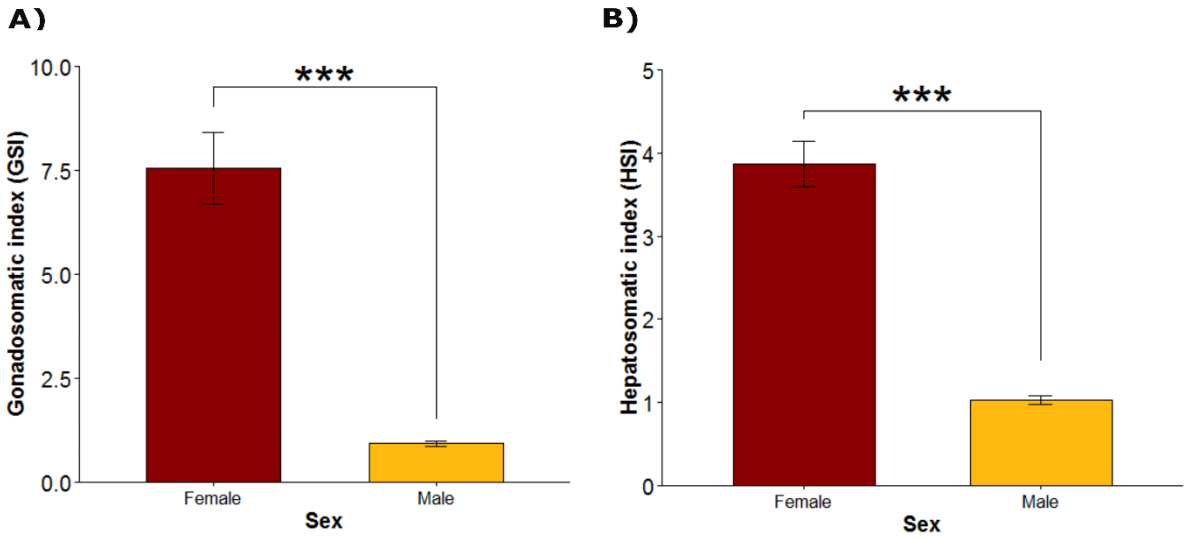
* Corresponding authors

E-mail: ll292@exeter.ac.uk, e.santos@exeter.ac.uk

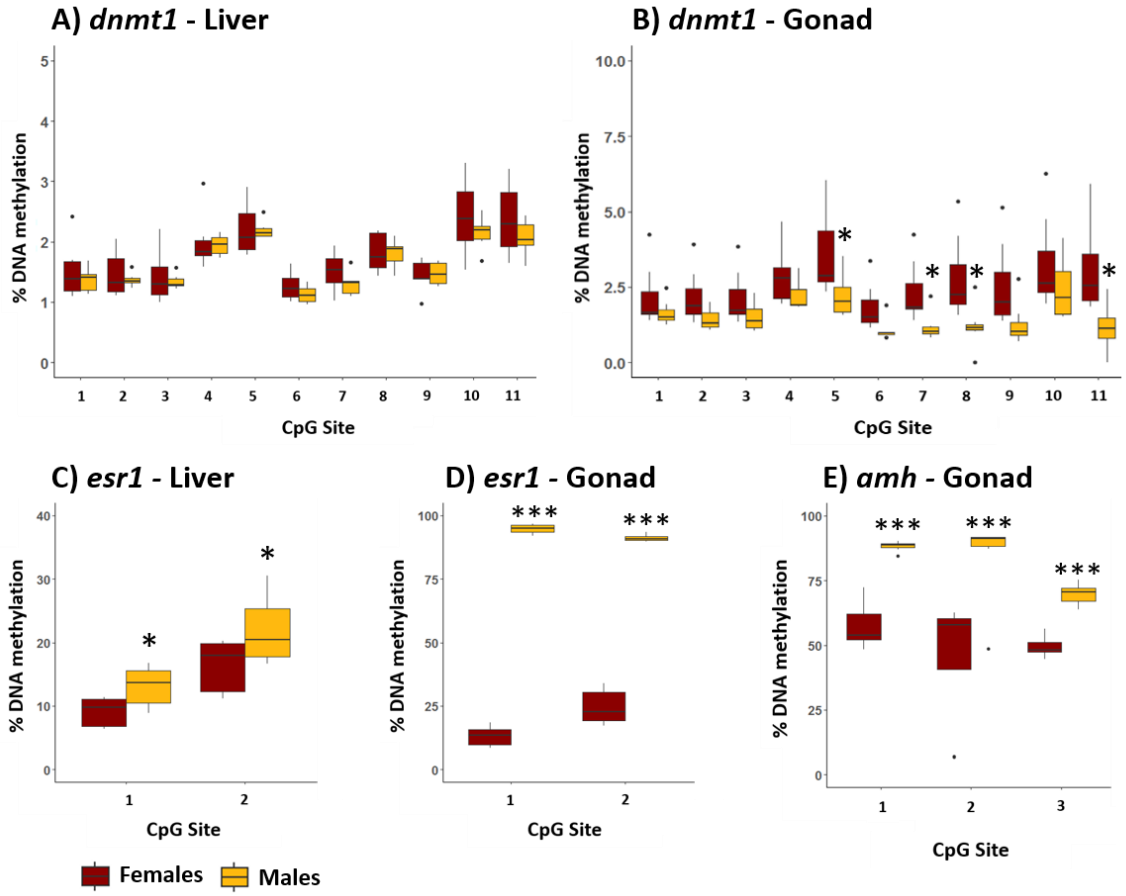
Supplementary Information Figure S1. Total number of eggs spawned for all females (n=8) during the 25 day period prior to sampling. Proportion of eggs unfertilized and fertilized eggs are shown in grey and white, respectively.



Supplementary Information Figure S2. Morphometric parameters for female (red) and male (yellow) fish (n=8 individuals per group). Individual plots represent the gonadosomatic index (**A**) and hepatosomatic index (**B**)**.** Asterisks indicate significant differences between males and females (Student's t-Test, ***p < 0.001).



Supplementary Information Figure S3. Gene specific DNA methylation profiles for the individual CpG sites (shown in Figure 1 of the main manuscript) in the promoter region of *dnmt1* **(A– liver, B - gonad),** *esr1* **(C – liver, D - gonad)** and *amh* **(E - gonad)** in adult zebrafish. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences between males and females (*P<0.05 **P<0.01 ***P<0.001).



Supplementary Information Table 1. Target genes, primer sequences and assay details for the RT-QPCR analysis.

Name	Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR efficiency
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Estrogen receptor 1	*esr1*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
Anti-Mullerian hormone	*amh*	TGTCTCAACCATCGTCTTCAG	CAGTCAATCCATCCATCCAAAC	124	61.0	2.24
Vitellogenin	*vtg1*	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	1.84
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	CGCTGTCGTGTTGAGTATGC	TCCCTTGCCCTTTCCTTTCC	180	58.5	2.06
DNA (cytosine-5)-methyltransferase 3	*dnmt3*	TGATGCCGTGAAAGTGAGTC	TTGCCGTGTAGTGATAGTGC	172	58.5	2.19
Histone deacetylase 1	*hdac1*	TGACAAACGCATCTCCATTCG	CTCTTCTCCATCCTTCTCTTCTTC	157	58.0	2.04
Histone deacetylase 3	*hdac3*	GAATGTGTGGAGTTTGTGAAGG	CTGGATGAAGTGTGAAGTCTGG	190	57.0	1.98
Methyl CpG binding protein 2	*mecp2*	GAGGCAGAAACAGGACAG	TGGTGGTGATGATGATGG	176	58.0	2.13
Methyl-CpG-binding domain protein 2	*mbd2*	AACAGCCTCCATCTTCAAG	CGTCCTCAGCACTTCTTC	166	59.0	2.19
Methyl-CpG-binding domain protein 3a	*mbd3a*	ACTCTTCTTTCGGCTCTG	TCTTCCTGCTTCCTGATG	164	57.0	1.99

Supplementary Information Table 2. Bisulfite-pyrosequencing primers and assay details for the gene promoters analyzed.

Name	Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Sequence Primer (5'-3')	Sequence analysed (5'-3')	Ta (°C)
Oestrogen receptor 1	*esr1*	AGAGGAGGTAAATAAATTAAAGATAGTTAG	Biotin-TACTCCTTTAACATATAATTTCCCATAACA	GGTAAATAAATTAAAGATAGTTAGG	TYGATATTGAYGGTTATTTTTTAGAGTAGGTTATGGTAATTAG	58.0
Anti-Mullerian hormone	*amh*	GTTTTTTATTTTTATGGGATGGTAGTTAGG	Biotin-AAACACAACTTAAAAACTTCCACTTATAT	TTGTTTTGAAGTATATTTGGAT	TATAYGTAATGGGGAATGTTTTAGTTTAAGGAAYGGTATTTGGTATTATTAAYGGGTTATTTATAAAATAATGTTTTTA	58.0
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	GGGTATTAATATGTGATAGTGTTAATTGTAG	Biotin - TAAACCCAAATACACTCACAACAC	TTATGAATTGTAGTTAGTAGTTGA	GAAATAYGYGYGGGTYGTTTTTTYGYGYGGAAAYGYGGGTGAGTYGGAYGTTATT	58.0