

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

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16

17 **Abstract:**

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

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

32 **1. Introduction:**

33 Reproduction is essential for species proliferation and a variety of sexual and asexual reproductive
34 strategies have evolved in multicellular organisms. Vertebrates generally reproduce sexually and, for
35 gonochoristic species, undifferentiated gonads differentiate into either ovaries or testis during
36 development. Compared to mammals, where sex determination is controlled by a cascade of
37 molecular events associated with the presence or absence of the sex determining region in the Y
38 chromosome (SRY) (Eggers et al., 2014), many fish species, including the zebrafish, display a greater
39 degree of plasticity with regards to sexual determination and differentiation (Martínez et al., 2014;
40 Ribas et al., 2017). Studies in medaka have shown that *DMY* (the DM-domain gene on the Y
41 chromosome), an SRY homologue, is the key initiator of male masculinisation (Chakraborty et al.,
42 2016; Matsuda et al., 2007). For zebrafish, evidence for polygenic sex determination mechanisms
43 have been proposed (Liew et al., 2012; Wilson et al., 2014). Despite the variability in sex
44 determination mechanisms, the downstream pathways regulating sex differentiation and
45 reproductive function are well conserved across vertebrates (Aron, 1979; Hewitt and Korach, 2003;
46 Piferrer, 2001; Simerly, 2002). At the level of the gonads, large numbers of transcripts were reported
47 to be differentially expressed between ovaries and testis in a range of vertebrate species including
48 the zebrafish (Santos et al., 2007; Wen et al., 2005), rainbow trout (*Oncorhynchus mykiss*) (Daniel
49 Baron et al., 2005) and mouse (Menke and Page, 2002). Several of these genes have been linked to
50 gonad differentiation including anti-mullerian hormone (*amh*) and sry (sex determining region Y)-box
51 9a (*sox9a*), both predominantly expressed in the testis, and aromatase (*cyp19a1a*), an enzyme
52 responsible for catalyzing the irreversible conversion of androgen to estrogens, predominantly
53 expressed in the ovaries (Rodríguez-Marí et al., 2005; Santos et al., 2007).

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

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

63 Besides being regulated by molecular and endocrine factors, sexual differentiation in fish is sensitive
64 to environmental cues, including temperature and oxygen saturation, and can be disturbed when
65 fish are exposed to altered environmental conditions resulting in altered sex ratios in fish
66 populations (Baroiller et al., 2009; Shang et al., 2006). The role of epigenetic processes on both the
67 endogenous and environmental regulation of reproduction is still poorly understood (Piferrer, 2013).
68 Several epigenetic mechanisms of gene expression regulation have been described to date, including
69 changes in DNA methylation, histone modifications and ncRNAs (Goldberg et al., 2007;
70 Vandegehuchte and Janssen, 2011) and they all have the potential to contribute to the regulation of
71 expression of reproductive genes. Recent reports have focused principally on DNA methylation and
72 evidence from a range of vertebrates have demonstrated its importance for the regulation of
73 reproductive processes in some circumstances including in animals exposed to environmental
74 chemicals (Laing et al., 2016) and increased temperature (Navarro-Martín et al., 2011; Ribas et al.,
75 2017).

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

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

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

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

106 2008; Wilson et al., 2014). Evidence thus far suggests that zebrafish sex determination is polygenic
107 and may require female-dominant genetic factors, and genes influencing sex determination may
108 vary depending on the strain or environmental condition (Anderson et al., 2012; Tong et al., 2010).

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

114

115 **2. Materials and Methods:**

116 *2.1 Fish husbandry*

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

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

140 2.2 DNA and RNA isolation

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

147

148 2.3 Transcript profiling

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

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

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

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

184

185 2.4 Bisulfite-PCR-Pyrosequencing

186 The sequences of promoter regions of *esr1*, *amh* and *dnmt1* were obtained from Ensembl (release
187 83; Cunningham et al 2015) (Cunningham et al., 2015) using the Biomart portal (Kinsella et al., 2011).
188 Zebrafish *esr1* (ENSDARG00000004111) has three known transcripts (*esr1*-001 (3449 bp), *esr1*-201
189 (3502 bp) and *esr1*-202 (212 bp)) and two TSSs. The *dnmt1* gene (ENSDARG000000030756) also has
190 two TSSs and three transcripts (*dnmt1*-001 (4896 bp), *dnmt1*-201 (4893 bp) and *dnmt1*-202 (5031
191 bp)). *amh* (ENSDARG000000014357) has one transcript (*amh*-001, 3243 bp) and one TSS (Figure 1).
192 The promoter regions were screened for the presence of putative binding sites for transcription
193 factors known to be involved in reproduction, including estrogen-responsive elements (EREs),
194 Dmrt3 and Sox9 using JASPAR (Sandelin et al., 2004), and the matrix models ESR1 (MA0112), ESR2
195 (MA0258), DMRT3 (MA0610) and SOX9 (MA0077). PCR and Pyrosequencing assays were designed
196 using the PyroMark Assay design software (Qiagen, Hilden, Germany). Pyrosequencing primers and
197 their corresponding target sequences were previously described in Laing et al., 2016 (Laing et al.,
198 2016) and are shown in Supplementary Information Table S2.

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

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

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

243 **3. Results:**

244 Zebrafish breeding groups of 4 males and 4 females were allowed to breed naturally and
245 reproduction (egg output and proportion of fertilization) was quantified for 25 days. During this
246 period, fish reproduced normally as demonstrated by consistent egg production and fertilization
247 rates (Supplementary Information Figure S1). The gonadosomatic index (GSI; the ratio of gonad
248 weight to body weight) was also significantly lower in male fish compared to female fish (0.92 and
249 6.75 respectively; $P \leq 0.001$; Supplementary Information Figure S2A). The mean hepatosomatic index
250 (HSI; the ratio of liver weight to body weight) in males was significantly lower than for females (1.04
251 and 3.87 respectively; $P \leq 0.001$; Supplementary Information Figure S2B). There were no significant
252 differences in the condition factors of male and female fish in this study (0.97 and 1.08 respectively;
253 $P = 0.093$).

254

255 *3.1 Sex-Specific Transcription and DNA Methylation Levels in the Gonads*

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

260 In the gonads, sex-specific patterns of transcription were identified for estrogen receptor 1 (*esr1*;
261 4.66 fold; $P = 0.031$), *esr2b* (14.75 fold; $P = < 0.001$), *amh* (21.71 fold; $P = < 0.001$) and *cyp19a1a*
262 (46.47 fold; $P = 0.032$) but not for estrogen receptor 2a (*esr2a*) or the androgen receptor (*ar*; Figure
263 2A). *esr1* and *cyp19a1a* were predominantly expressed in the ovary, while *esr2b* and *amh* were
264 found to be predominantly expressed in the testis. For transcripts involved in epigenetic regulation,
265 sex-specific patterns of transcription were observed for *dnmt1* (45.46 fold; $P = 0.031$), *hdac1* (2.96
266 fold; $P = 0.035$), *dnmt3* (30.13 fold; $P = 0.022$), *mecp2* (7.22 fold; $P = 0.006$), *mbd2* (4.90 fold; $P =$

267 0.028) and *mbd3a* (24.10 fold; $P = 0.033$), where the relative mRNA transcription level for each of
268 these gene was found to be greater in the ovary compared to the testis (Figure 2A).

269 Mean global DNA methylation in the gonads was significantly higher in males (87.76%) compared to
270 females (82.78%; $P \leq 0.001$, Figure 3). Furthermore, in the testis *dnmt1* and *dnmt3* transcription
271 were strongly inversely correlated with global DNA methylation (correlation coefficient = -0.919, $P =$
272 0.010 and correlation coefficient = - 0.927, $P = 0.003$ respectively; Table 1).

273 In the gonads, sex-specific DNA methylation levels were identified in the promoter region of *esr1*
274 (male = 93.13%, female = 18.95%, $P \leq 0.001$, Figure 2B) and *amh* (male = 75.14%, female = 50.88%, P
275 = < 0.001, Figure 2B; DNA methylation levels are reported as an average percentage methylation
276 across all CpG sites measured). However, there was no significant difference in the sex-specific DNA
277 methylation levels for *dnmt1* (Figure 2B) despite the differences in *dnmt1* transcription observed
278 between males and females.

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

284

285 3.2 Sex-Specific Transcription and DNA Methylation Levels in the Liver

286 In the liver, at the transcriptional level, sex-specific differences were identified for a number of
287 transcripts involved in reproductive function including the transcripts encoding vitellogenin (*vtg1*;
288 1322.96 fold; $P \leq 0.001$; Figure 4A), *esr1* (8 fold; $P \leq 0.001$; Figure 4B), and *esr2a* (2.8 fold; $P = 0.012$;
289 Figure 4B), but not for estrogen receptor 2b (*esr2b*; Figure 4A). *vtg1* (Figure 4A) and *esr1* (Figure 4B)
290 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 promoter 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).

302 **4. Discussion:**

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

309

310 *4.1 Sex-Specific Transcription and DNA Methylation in the Gonads*

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

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

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

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

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

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

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

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

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

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

375 maternal transcripts that support embryo development prior to the zygotic genome activation
376 (ZGA), with many maternal transcripts playing important roles well beyond this point (Aanes et al.,
377 2011; Andersen et al., 2013). In zebrafish, ZGA occurs at approximately the 1,000 cell blastula stage,
378 much later in development than in mammalian species including mice (~2 cell) or humans (~4–8 cell)
379 (Andersen et al., 2012; Braude et al., 1988; Flach et al., 1982; Potok et al., 2013). Therefore, in
380 comparison to mammalian models, zebrafish are likely to be significantly more dependent on
381 maternal transcripts to support the critical early stages of embryo development. We hypothesize
382 that, in addition to their role within somatic and germ cells in the gonads, many of these transcripts
383 involved in epigenetic regulation which we have shown to be overexpressed in ovaries, may be
384 related to their role as maternal transcripts during embryo development, and are potentially
385 involved in the dynamics of demethylation and re-methylation occurring prior to ZGA, as well as
386 histone remodelling, occurring during this period (Jiang et al., 2013; Potok et al., 2013). This
387 hypothesis is supported by the fact that several of these transcripts were reported to be strongly
388 expressed during early embryo development prior to ZGA which begins during the maternal-to-
389 zygotic transition as the embryo enters the mid blastula transition (reviewed in (Andersen et al.,
390 2013)).

391 In the gonads, there were no significant differences in the DNA methylation patterns for *dnmt1*,
392 despite the fact that this gene was significantly overexpressed in ovaries at the transcript level.
393 However, *the* expression of *dnmt1* and *dnmt3* was significantly inversely correlated with global DNA
394 methylation in the testis, supporting the idea that these enzymes may be important in regulating
395 global DNA methylation levels (Bestor, 2000). For example; the expression of *dnmt1* has been
396 associated with changes in global DNA methylation, and inactivation of *dnmt1* has been shown to
397 cause global demethylation of the genome (Bestor, 2000). In addition, it has been demonstrated
398 that *dnmt3* is important in the maintenance of DNA methylation patterns during *de novo*
399 methylation processes (Okano et al., 1999; Potok et al., 2013). The mean promoter DNA
400 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 *mbd3a* 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* promoter 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

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

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

467

468 **5. Conclusions:**

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

478

479 **6. Acknowledgements:**

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

486

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692 sexual dimorphism of the Zebrafish Liver and the effect of sex hormones. *PLoS One* 8, 1–12.

693

694 **8. Figure captions and Tables:**

695

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

Anti-Müllerian Hormone (*amh*) - GRCz10 22:20,736,779-20,737,279

```

...ACTTAAACTTCCACTTATGTGTTTCAATCCCAAAAACACGACTGTTGTAAACAACAGGCAAAATGTATAAAACATTACCTGTTTGGCTGAAAACATTTTTGTGAATGACCCG3TTA
                                     TF SOX9                               TF DMRT3                               TF SOX9
GTAATGCCAAATGCG2TTCCCTGAACATAAAACATCCCCATTACG1TATGGTCCAATGTACTTCAAGCAGGTAATAGTTCTAGGAACTGTAAAAAGTTCTTAATGTGAACATCTTACAGTTT
GGTGGGTTAACAAAAATTCGCCGGACAGAAAAAGAGCCTAACTGCCATCCCATGGGAATGAAGGCCTAAACTAAGAAATAGACTTTTGTGCAATTGCCACAAGGAGTCTTAAACCGTTATCCGCT
                                     TF ESR2                               TF SOX9
ACTACAGATGTGTCTGAGGTCTATACAAATATTCATTTGTCGTATGAGTTCCTCCTCACCTTATCAAACCTCAAGGCATGTGATGCCATTCCACTATCCCTCAAATTCACACGTCCG...>>>
                                     TF SOX9   TF SOX9                               TF ESR2
                                                                                                     TSS amh-001
  
```

DNA (cytosine-5-)-methyltransferase 1 (*dnmt1*) - GRCz10 3:54,352,519-54,352,819

```

...TGAGCTTAATATTTGTTATTCCTTTACCCTTAACGTATACATTAAATAAACCGAATTAGACCCAGATACACTCACAACACTCTCACTGATTTATTTTAAACGAAACACAACGCACAACTTCTTT
GACACCGTCCATCCCCCTGCTTTATGAATCGTAGCG11TCG10ACTCACCG9CG8TTTCCG7CG6CG5AAAAGGCG4GCCCG3CG2CG1TATTCTCAGCTGTAGCGACAATTCATAAA
TSS dnmt1-202   TF SOX9
GCCCGTTGAGCCTCTCCAGCGCGCGCTGCAGTTGGCACTGCACAATTAATACCCTTTTAT...>>>
TSS dnmt1-001   TF ESR2
TSS dnmt1-201
  
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Estrogen receptor 1 (*esr1*) - GRCz10 20:26,483,369-26,484,513

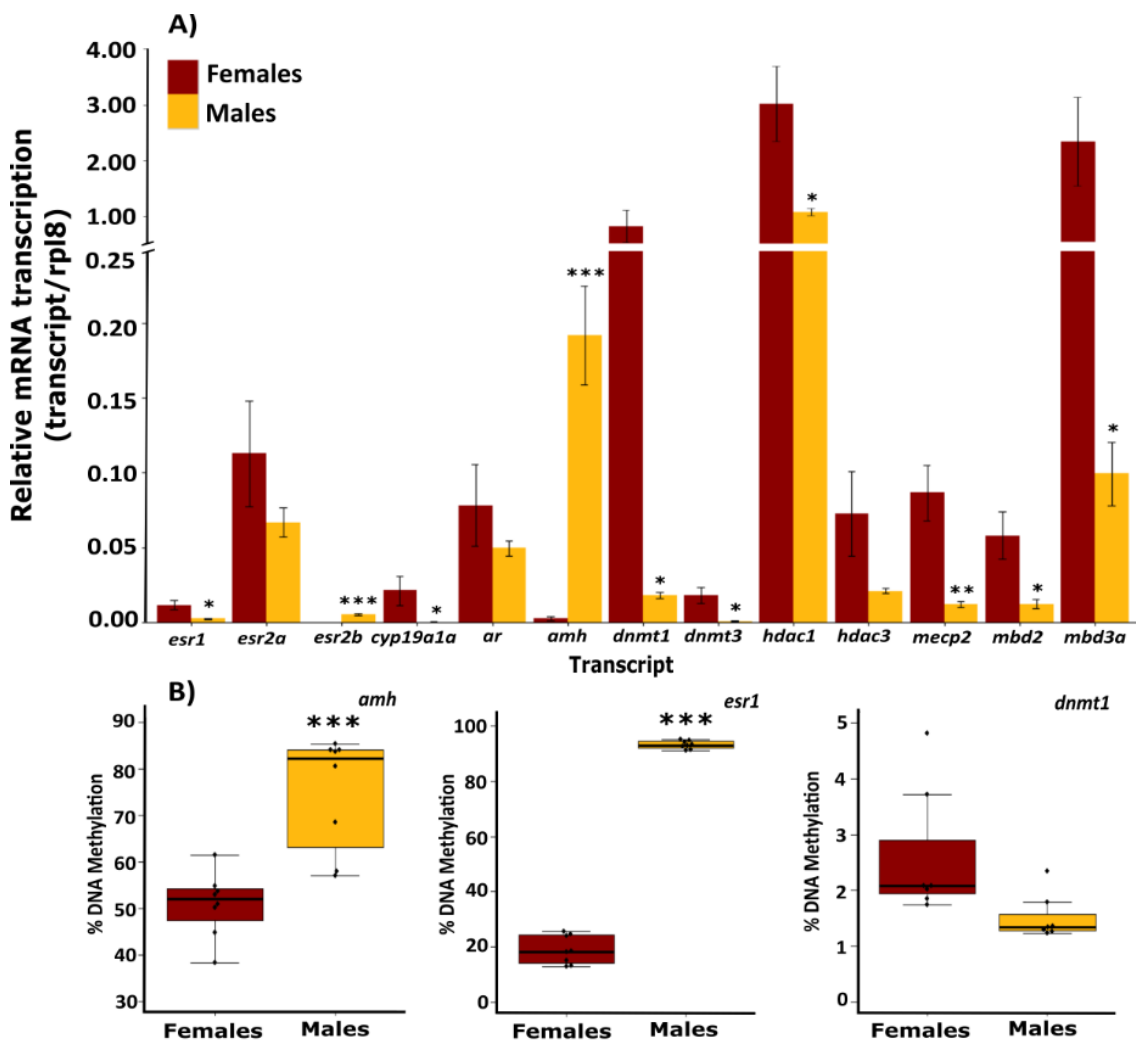
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...TAATTTCCCATGGCAGCAGCATGTAAGTGGTTTCGCAGCGCATCACCTGTAAACTCAAAGGTTTGGGCAAGTGAATCAAGTGGTGACCTCCATCTCTTGTTTACTGGTGCATGACCGTCT
                                     TF DMRT3                               TF SOX9   TF ESR1/ESR2
CTGAGAAGTGACCG2TCAGTATCG1AGCTGGCTGTCTTTAATTTGTTTACTCTCTTTTTTTAAAGCCAGAGACAGACAGAGAAAGTAAGAAGGACACAGGAAGTCAGAGATCATCAGTGAGAGA
                                     TF DMRT3                               TSS esr1-001   TF DMRT3
CAGAAAGCATCCAGCCTGTAATGGGACTCAAGTAAACATCAGGAGAGTGAACATTTTGGTGAGAGTCTGGAAGAATTCAGCAACCAATTTTTCAGCTTTTGTTTCTAGTAATGATATCCATGT
                                     TF SOX9
GTCTTTTACTCTAGCGTTTGTAAATCGAGATGCTTCTATGATGTGCTTTCAGTTGAGTTTCTGTCTGTTGGTTTCTGTTTATAGAAAAGCACACCTTTCAGTTGGGTTTATCCTTTCTTAC
                                     TF SOX9   TF SOX9
ACTGCAAAATAAATAGCTTTTGTGCTTTGTCTAGTCCAAATACCTAACAATTTCTAAAAAATGATGTAAAAAATCTGTTTGTGTTGTTTGTCCCTTAAAAAATAATCTGCCAATGAAGTGGGTA
                                     TF SOX9
AATAAATCTTAATTTCCGGGTGAAATAAGATCATTTTAAATCATTTTGTGTTGACATAAAATCTCCCTCAATTTTGACATATTTCTGTTTCAAGTAAAACAAACAATTTTTACTTGTCT
                                     TF SOX9   TF SOX9
AGAAAATGCTTCCGTAATGCTCTCTCCAAAAAATAAAGGCAAAAGAAATCAAAATGAAATGTGTTTAAAGGGAAAGACAGGAAAGAGAAAGAAAGCAAGCCTCACGTATCTTAGAAAAACATCATA
GAACAATGAGGGGAAAAGATGGGATAAAAGAAAGGCAAAAGAAATCAAAATGAAATGTGTTTAAAGGGAAAGACAGGAAAGAGAAAGAAAGCAAGCCTCACGTATCTTAGAAAAACATCATA
TF SOX9
CTTTGTCTCTCAGCCCAAGGAGGACTGTAGGAGAGGAAGGTAGTTTTTTTTTAGGAGGAGTAGGCAGAGCAGCGAGAGAGTGAAGCGGAAAGCGAAGCAGAACAAGCGGGAGAGAGTTT...>>>
                                     TF SOX9                               TSS esr1-201
                                                                                                     TSS esr1-201
  
```

703

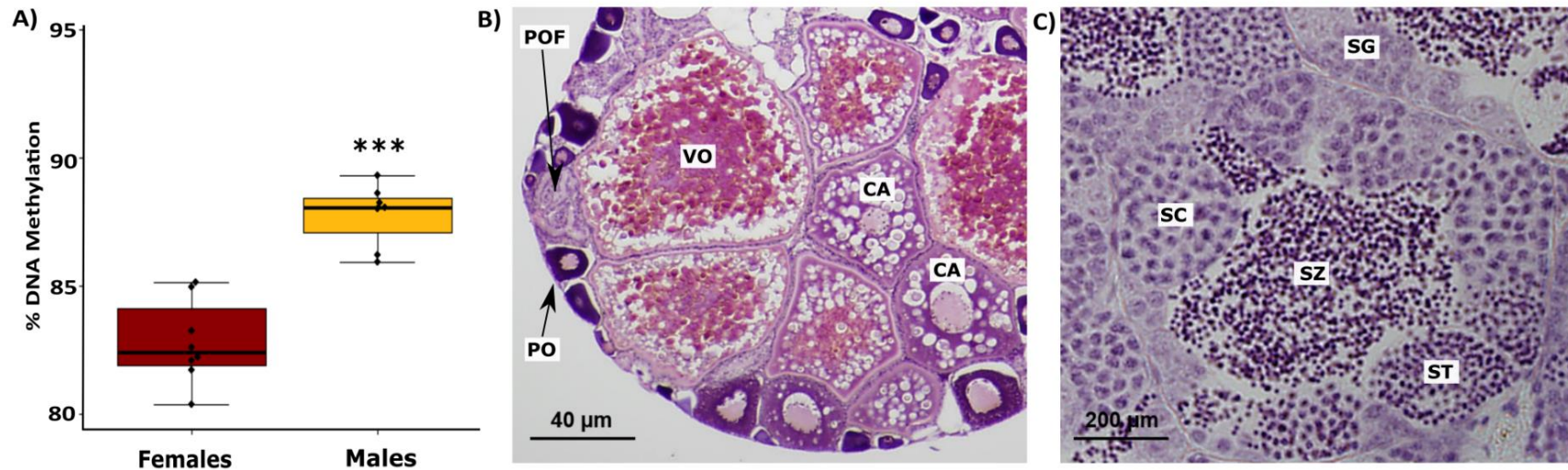
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705 **Figure 2. A)** Comparison of the relative transcript profiles (target gene transcription/*rpl8*
 706 transcription) between females and males in adult zebrafish gonads (n= 6-8 for each group). **B)**
 707 Comparison of gene specific average promoter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



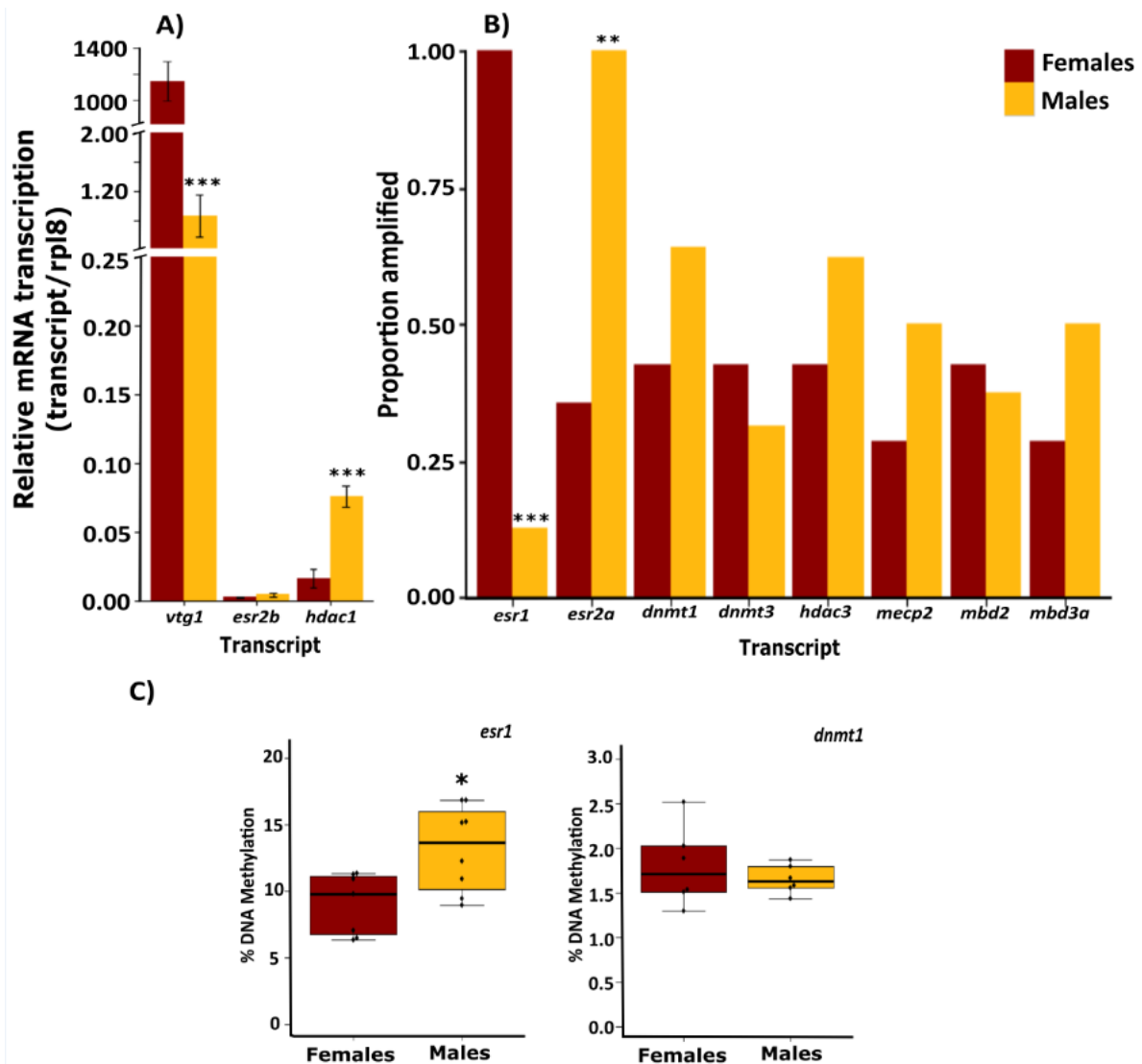
711

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



719

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



729

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 <i>dnmt1</i> and <i>dnmt3</i>.				
Tissue	Gene	-	Correlation coefficient	P value
Testis	<i>dnmt1</i>	-	-0.919	0.010
Ovary	<i>dnmt1</i>	-	-0.225	0.668
Testis	<i>dnmt3</i>	-	-0.927	0.003
Ovary	<i>dnmt3</i>	-	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	<i>esr1</i>	1	-0.765	0.045
		2	-0.470	0.029
	<i>amh</i>	1	-0.271	0.659
		2	-0.957	0.017
		3	-0.547	0.341
	<i>dnmt1</i>	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	<i>esr1</i>	1	-0.875	0.004
		2	-0.697	0.055
	<i>amh</i>	1	-0.072	0.878
		2	-0.818	0.025
		3	-0.920	0.003
	<i>dnmt1</i>	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	<i>esr1</i>	1	-0.971	0.006
		2	-0.906	0.034
	<i>dnmt1</i>	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	<i>esr1</i>	1	-0.065	0.878
		2	-0.268	0.522
	<i>dnmt1</i>	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	

Supporting Information

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

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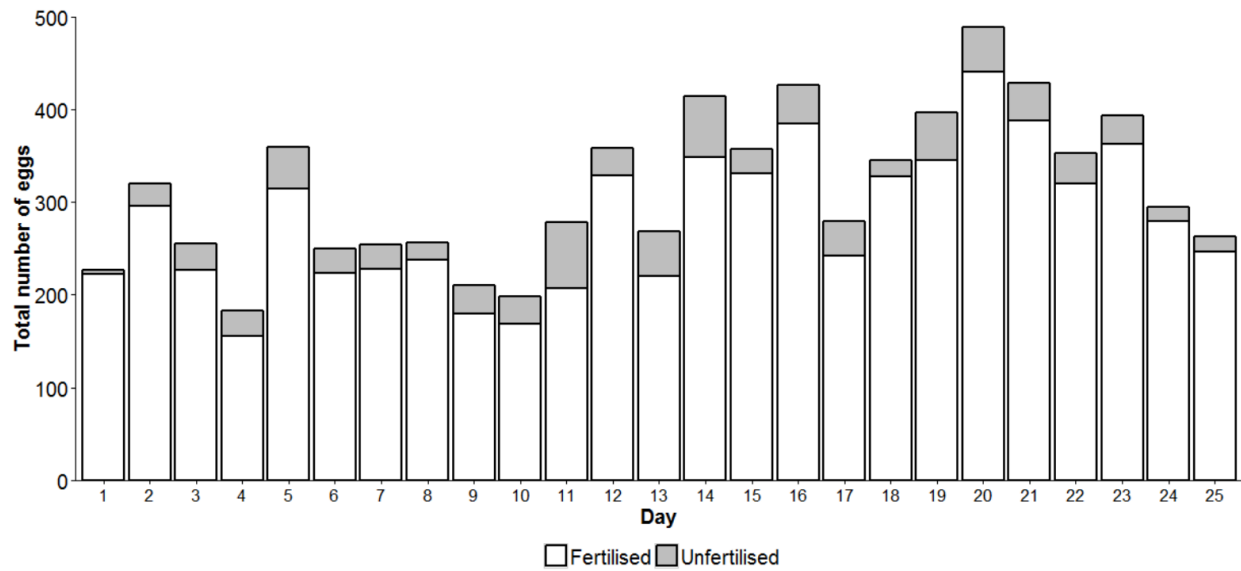
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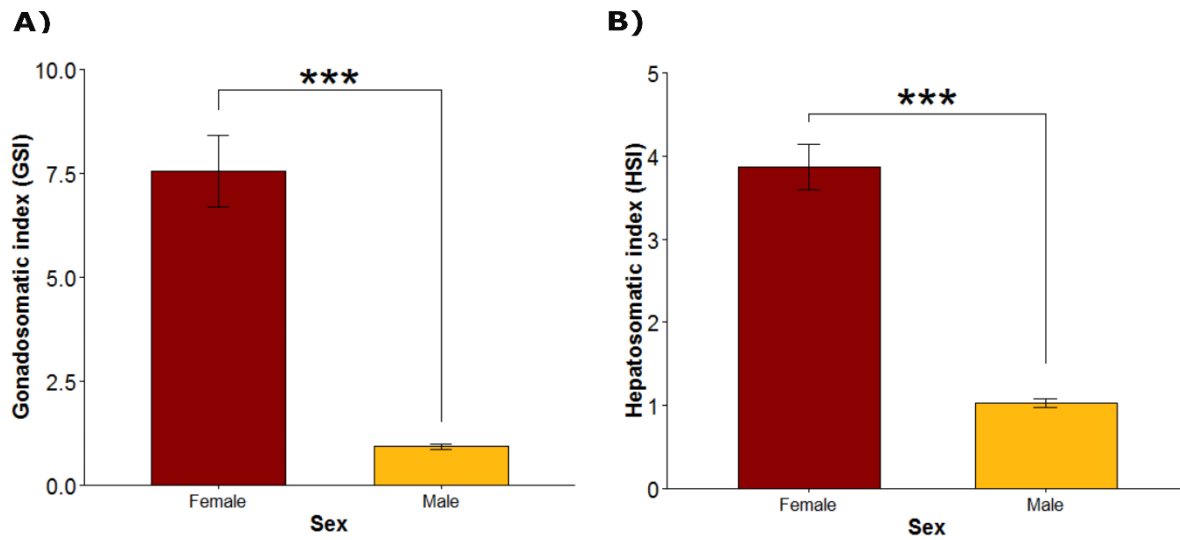
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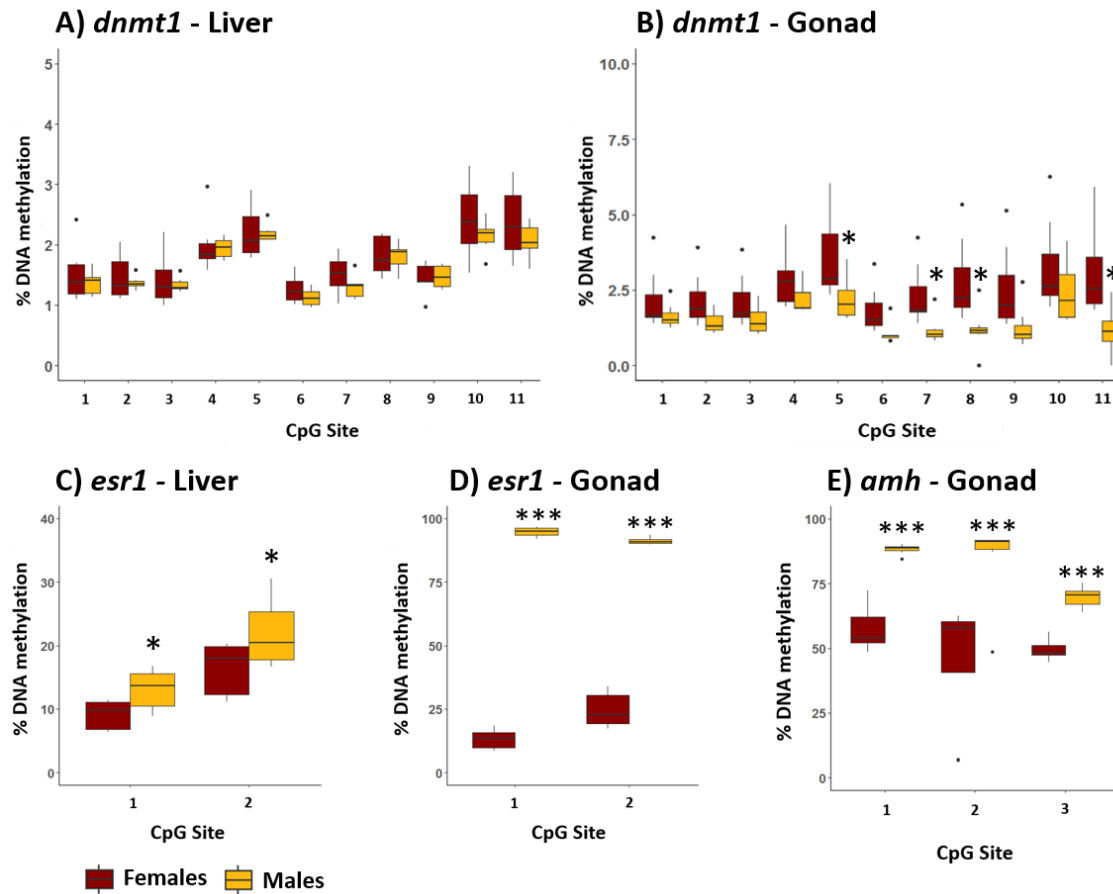
Supplementary Information Figure S1. Total number of eggs spawned for all females (n=8) during the 25 day period prior to sampling. Proportion of eggs unfertilized and fertilized eggs are shown in grey and white, respectively.



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	<i>rpl8</i>	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Aromatase	<i>cyp19a1a</i>	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Estrogen receptor 1	<i>esr1</i>	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	<i>esr2a</i>	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAAGCTAATG	173	59.0	1.86
Estrogen receptor 2b	<i>esr2b</i>	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	<i>ar</i>	ACGAGGGTGTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
Anti-Mullerian hormone	<i>amh</i>	TGTCTCAACCATCGTCTTCAAG	CAGTCAATCCATCCATCCAAAC	124	61.0	2.24
Vitellogenin	<i>vtg1</i>	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	1.84
DNA (cytosine-5)-methyltransferase 1	<i>dnmt1</i>	CGCTGTCGTGTTGAGTATGC	TCCCTTGCCCTTCTCTTCC	180	58.5	2.06
DNA (cytosine-5)-methyltransferase 3	<i>dnmt3</i>	TGATGCCGTGAAAGTGAGTC	TTGCCGTGTAGTGATAGTGC	172	58.5	2.19
Histone deacetylase 1	<i>hdac1</i>	TGACAAACGCATCTCCATTCG	CTCTTCTCCATCCTTCTTCTTC	157	58.0	2.04
Histone deacetylase 3	<i>hdac3</i>	GAATGTGTGGAGTTTGTGAAGG	CTGGATGAAGTGTGAAGTCTGG	190	57.0	1.98
Methyl CpG binding protein 2	<i>mecp2</i>	GAGGCAGAAACAGGACAG	TGGTGGTGATGATGATGG	176	58.0	2.13
Methyl-CpG-binding domain protein 2	<i>mbd2</i>	AACAGCCTCCATCTTCAAG	CGTCTCAGCACTTCTTC	166	59.0	2.19
Methyl-CpG-binding domain protein 3a	<i>mbd3a</i>	ACTCTTCTTTCGGCTCTG	TCTTCTGCTTCTGATG	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	<i>esr1</i>	AGAGGAGGTAAATAAATTAAGATAGTTAG	Biotin-TACTCCTTAAACATATAATTTCCCATAACA	GGTAAATAAATTAAGATAGTTAGG	TYGATATTGAYGGTTATTTTTAGAGTAGGTTATGGTAATAG	58.0
Anti-Mullerian hormone	<i>amh</i>	GTTTTTATTTTTATGGGATGGTAGTTAGG	Biotin-AAACACAACCTAAAACTTCCACTTATAT	TTGTTTTGAAGTATATTTGGAT	TATAYGTAATGGGAATGTTTTAGTTAAGGAAYGGTATTTGGTATTATAAYGGGTTATTTATAAAATAATGTTTTTA	58.0
DNA (cytosine-5)-methyltransferase 1	<i>dnmt1</i>	GGGTATTAATATGTGATAGTGTTAATTGTAG	Biotin - TAAACCCAAATACACTCACAACA C	TTATGAATTGTAGTTAGTAGTTGA	GAAATAYGYGGGTYGTTTTTYGYGGAAAYGYGGGTGAGTYGGAYGTTATT	58.0