1 **Immune genes, *IL1 β* and *Casp9*, show sexual dimorphic methylation patterns in**
2 **the zebrafish gonads**

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14 gonads

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26 **Abstract**

27 There is a crosstalk between the immune and the reproductive systems in which
 28 sexual dimorphism is a common pattern in vertebrates. In the last years, epigenetics
 29 has emerged as a way to study the molecular mechanisms involved during gonadal
 30 development, which are responsible to integrate environmental information that
 31 contributes to assign a specific sexual phenotype (either an ovary or a testis). In the
 32 fish gonads, it is known of the existence of the reproduction-immune system
 33 interactions although the epigenetic mechanisms involved are far to be elucidated.
 34 Here, we used the zebrafish (*Danio rerio*) as a model to study the DNA methylation
 35 patterns of two well-known innate immune genes: *IL1 β* and *Casp9*. DNA
 36 methylation levels were studied by a candidate gene approach at single nucleotide
 37 resolution and further, gene expression analysis were carried out. Results showed that
 38 there was clear sexual dimorphism in the DNA methylation levels of the two immune
 39 studied genes, being significantly higher in the testes when compared to the ovaries.
 40 In summary, and although much research is needed, here we present two potential
 41 candidates as epimarkers with forthcoming applications in the livestock and fish
 42 farming production, for example, in immune fish diseases or sexual control
 43 programs.

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51 **1. Introduction**

52 Epigenetic definition has been subjected to controversy (Deans and Maggert, 2015)
53 but, in general terms, it could be defined as the set of reactions conditioned by the
54 cellular environment that are responsible to change DNA activity and gene
55 expression without altering the nucleotide sequence (Moore et al., 2013). These
56 mechanisms enclose changes in histone conformation (e.g., chromatin packaging),
57 DNA methylation and regulation by non-coding RNAs (Qiu, 2006). DNA
58 methylation is the best characterized epigenetic modification functioning on the pre-
59 transcriptional control in several biological processes, such as cell differentiation or
60 genomic imprinting (Jones and Takai, 2001). Epigenetic changes (or epimutations)
61 can be inherited cell to cell through mitotic divisions or throughout subsequent
62 generations by meiotic divisions (Dupont et al., 2009; Hanson and Skinner, 2016).
63 Inheritable epimutations need to occur in the primordial germ cells (PGCs) during
64 embryo formation, which in humans appeared during 6-18 weeks of gestation (Jirtle
65 and Skinner, 2007) and in zebrafish (*Danio rerio*) approximately around 1,000 cells
66 (Kopranner et al., 2001). In addition, gametogenesis in the gonads, i.e., sperm and
67 oocyte formation, is a sensitive window to promote transgenerational epimutation
68 changes that will be transferred to the following phenotypes (Daxinger and
69 Whitelaw, 2012). Thus, understanding the underlied molecular mechanisms of these
70 epimutations in the gonads needs to be at the forefront of the research.

71

72 In the fish gonads, the first study showing the interaction between epigenetics and
73 sexual development was published eight years ago in the European sea bass,
74 *Dicentrarchus labrax*, showing that methylation of the gonadal aromatase
75 (*cyp19a1a*) promoter was altered in the ovaries of adult females previously exposed

76 to high temperature during early stages of development (Navarro-Martin et al.,
77 2011). Since then, other studies, including in this fish species (Anastasiadi et al.,
78 2017; Anastasiadi et al., 2018b) have been have evidenced the important role of the
79 epigenetic mechanisms in the gonads. This is the case, for example, in half-smooth
80 tongue sole, *Cynoglossus semilaevis* (Shao et al., 2014), Nile tilapia, *Oreochromis*
81 *niloticus*, (Wang et al., 2017) or barramundi, *Lates calcarifer* (Domingos et al., 2018),
82 among others (reviewed in Piferrer et al. (2019), all of them revealing the importance
83 of the methylation processes in reproduction-related genes in the ovaries or in the
84 testes and so pointing out the sexual dimorphic epigenetic patterns.

85
86 Sexual dimorphism refers to phenotypic differences between the two sexes within a
87 species. In fish, sexual dimorphism is present in a large number of species being
88 females much larger than males, for example in turbot, *Scophthalmus maximus*
89 (Imsland et al., 1997) or E. sea bass (Saillant et al., 2001) or conversely, males much
90 larger than females, for example in Nile tilapia (Toguyeni et al., 1997) or rainbow
91 trout, *Oncorhynchus mykiss* (Bonnet et al., 1999). Sexual dimorphism not only
92 manifests by morphological traits such as growth, but also by behavior (Magurran
93 and Garcia, 2000) or by molecular mechanisms (e.g., gene expression). Sex-biased
94 gene expression has implications beyond evolutionary biology due to the existence of
95 a predominant expression in one sex (Ellegren and Parsch, 2007). Thus, in human,
96 for example, transcriptomic sex-related differences are more remarkable in the
97 gonads, although other tissues such as brain, muscle or liver also exist (Mele et al.,
98 2015). In fish, transcriptomic studies identifying both the number of expressed genes
99 between ovaries and testes and the interactions of sex-specific pathways have been
100 carried out in several fish species such as zebrafish (Small et al., 2009), turbot (Ribas

101 et al., 2016), guppy, *Poecilia reticulata* (Sharma et al., 2014) or E. sea bass (Ribas et
102 al., 2019).

103

104 With clinical purposes, in human, many epigenetic studies address sexual
105 dimorphism, for example, in brain, showing differences in the methylation levels
106 (Matsuda et al., 2012) but the vast majority are focusing on the sex-related
107 differences in diseases responses. It is well documented that woman are more
108 resilient against infectious and some non-infectious diseases such as cancer but at the
109 same time, they are more susceptible to autoimmune diseases (Kirsch-Volders et al.,
110 2010; Ghosh and Klein, 2017). Such research is an emerging field in which the
111 underlying mechanisms of these sexual dimorphic responses are far to be elucidated,
112 though it is known, that the epigenetics mechanisms play important roles (reviewed
113 in Klein and Flanagan (2016).

114

115 Gonads are immune-privileged sites as they need to prevent immune responses
116 against meiotic germ cells (Maddocks and Setchell, 1990). Immune cells present
117 sexual steroid receptors not only in mammals (Kovats et al., 2010) but also in fish
118 (Chaves-Pozo et al., 2018). Thus, sexual steroids regulate the immune system in
119 addition to classical reproduction-related functions in vertebrates. In fish,
120 immunotoxicology field has developed more of the research concerning the
121 interactions between the reproduction and the immune systems because large number
122 of endocrine disruptors can mimic sex steroids and in turn, activate the immune
123 system (Milla et al., 2011). Further, expression of immune-related genes during
124 gonadal development have been described in some fish species like gilthead
125 seabream, *Sparus aurata* L. (Chaves-Pozo et al., 2008; Chaves-Pozo et al., 2009) or

126 turbot (Ribas et al., 2016). In sea bream, a protandrous hermaphroditic teleost, a
127 massive infiltration of an immune cell type (acidophilic granulocyte) is required to
128 degenerate the testis for phenotype sex-change (Liarte et al., 2007). In zebrafish,
129 gonads are first developed as an ovarian-like organ to latter half of the population
130 activates apoptotic and tp53 signaling pathways that drive to the testis phenotype
131 (Rodríguez-Marí et al., 2010; Liew and Orban, 2014).

132

133 Taken together, there is evidence of immune-reproduction interactions in the fish
134 gonads that are able to maintain physiological and molecular changes. Nevertheless,
135 the role of epigenetic mechanisms in regulating these changes by a sex-dependent
136 manner is unknown. We address this gap by studying the methylation levels in the
137 zebrafish ovaries and testes of two well-known innate immune genes: interleukin 1
138 beta, *IL1β*, a proinflammatory multifunctional cytokine involved in a variety of
139 cellular activities (Secombes et al., 2011) and caspase 9, *Casp9*, a cysteine proteases
140 that plays an essential role in the mitochondria-mediated cell death pathway
141 (Sakamaki and Satou, 2009). We performed a candidate gene approach method
142 (Methylation Bisulfite Sequencing, MBS) which allowed to analyze at single
143 nucleotide resolution, high coverage and single CpG sites in the desired regions
144 (Anastasiadi et al., 2018b). In parallel, gene expression of these two immune genes were
145 studied together with methylation levels by correlation analysis.

146

147 **2. Material and methods**

148 **2.1. Fish husbandry**

149 AB zebrafish were housed in the fish facilities at the Institute of Marine Sciences
150 (ICM- CSIC, Barcelona) following standard conditions (Ribas and Piferrer, 2014)

151 with an appropriate density levels (8 fish/L) to avoid induced masculinization (Ribas
152 et al., 2017). Fish were reared in a close-circuit system (Aquaneering, San Diego,
153 CA, USA) in a monitored chamber: photoperiod of 12:12 hours, environmental
154 temperature of $26 \pm 1^\circ\text{C}$ and humidity of $50 \pm 3\%$. Physicochemical parameters of
155 the water were monitored daily: temperature ($28 \pm 0.2^\circ\text{C}$), pH (7.2 ± 0.5),
156 conductivity (750-900 μS) and dissolved oxygen (6.5-7.0 mg l^{-1}). Ammonia, nitrate
157 and nitrite water levels were checked weekly by a commercial colorimetric test (Test
158 kit Individual Aquarium Water Test Kit, API, United Kingdom).

159

160 **2.2 Ethics statement**

161 The animal procedure for the present study was licensed by Bioethical Committee of
162 the Generalitat de Catalunya with the reference code 9977 and approved by the
163 Spanish National Research Council (CSIC) Ethics Committee within the project
164 AGL2013-41047-R. European regulations of animal welfare (ETS N8 123, 01/01/91
165 and 2010/63/EU) were respected regarding fish rearing and maintenance. Fish
166 facilities in the ICM were validated for animal experimentation by the Ministry of
167 Agriculture and Fisheries (certificate number 08039-46-A) in accordance with the
168 Spanish law (R.D. 223 of March 1988).

169

170 **2.3 Fish sampling**

171 A total of ten females and ten males from adult zebrafish at 90 dpf were euthanized
172 on iced water followed by decapitation, and total weight (precision ± 0.05 g),
173 standard length (SL, precision ± 0.01 cm) were recorded. Under magnifying glass,
174 gonads were sexed, dissected and flash frozen in liquid nitrogen and kept at -80°C for
175 further analysis.

176

177 **2.4 Methylation analysis**

178 Methylation levels of the ovaries and testes were analyzed following the protocol
179 described in (Anastasiadi et al., 2018b). Briefly, genomic DNA was isolated from 20
180 gonads and a unique library was prepared by pooling the *IL1 β* and *Casp9* amplicons
181 for all the samples. For each fish, DNA was extracted from half of the gonad which
182 was lysed by by one microgram (1 μ g) of proteinase K (Sigma-Aldrich, St. Louis,
183 Missouri) overnight at 65°C and the following day, a standard phenol-chloroform-
184 isoamyl alcohol protocol with ribonuclease A (PureLink RNase A, Life
185 Technologies) was performed. Five hundred nanograms of DNA per sample were
186 bisulfite-converted using the EZ DNA Methylation-Direct™ Kit (ZymoResearch;
187 D5023).

188

189 **2.5 Primer design**

190 Primers were designed for bisulfite converted DNA using MethPrimer tool (Li and
191 Dahiya, 2002) with an amplicon size of 459 and 441 for *IL1 β* and *Casp9*,
192 respectively. Amplicon regions included the promoter, the first intron and the first
193 exon as much as possible (Figure 1, Table S1). The adaptor sequences for 16S
194 metagenomic library preparation (Illumina) were added to the 5' ends of the primers
195 designed: Forward-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and
196 Reverse-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. The target
197 regions included a total of nine for *IL1 β* and 19 for *Casp9* CpGs, respectively. Primer
198 specificities were previously validated by Sanger sequencing of amplicons from a pool
199 of samples.

200

201 **2.6 Amplification of target regions and library preparation**

202 Targeted-regions were obtained from bisulfited DNA (25 ng per sample) by 30
 203 cycles of PCR using specific primers: 7 min at 95°C, followed by 40 cycles of 95°C
 204 for 1 min, annealing temperature (55°C and 54°C for *IL1β* and *Casp9*, respectively)
 205 for 2 min and 65°C for 2 min, with a final step at 65°C for 10 min. Obtained targeted
 206 amplicons were size-selected and normalized by magnetic beads was (Fisher
 207 09981123) following the protocol described in (Anastasiadi et al., 2018b). PCR
 208 products were indexed by Nextera XT index Kit Set A and Set D (Illumina; FC-131–
 209 2001 and FC-131–2004) according to Illumina’s protocol for 16S metagenomic
 210 library preparation. PCR products were pooled in an equimolar manner to obtain a
 211 single multiplexed library which was sequenced on a MiSeq (Illumina, San Diego,
 212 California) using the paired-end 250 bp protocol at the National Center of Genomic
 213 Analysis (CNAG, Barcelona).

214

215 **2.7 Biocomputational analysis**

216 Raw data from the sequencer were demultiplexed and Nextera adapters were
 217 removed using Trim Galore! software (v. 0.4.5) (Babraham Bioinformatics) and low
 218 quality bases were filtered (Phred score < 20). Pre- and post-trim quality control was
 219 carried out with MultiQC to ensure that the adapters were cut off correctly (Ewels et
 220 al., 2016). Bismark (v.20.0) was used to generate an *in silico* bisulfite converted
 221 zebrafish genome (danRer11) as a reference template for alignments (Krueger and
 222 Andrews, 2011). Bisulfite conversion efficiency was calculated for each sample with
 223 a minimum threshold of 99.0%. One ovarian sample did not passed the minimum
 224 thresholds and therefore was deleted. The coordinate positions of all CpG sites were
 225 obtained by using “BSgenome.Drerio.UCSC.danRer10” package from BSgenome

(Pagès, 2018). The coordinates of the CpG sites of the targeted genes was assessed by BED tools methodology (Quinlan and Hall, 2010) through bedr package (v. 1.0.4) (Haider et al., 2017). From the targeted CpG sites, only those that showed coverage >10 times were retained for statistical analysis. Raw sequencing data was submitted in the NCBI with the accession number: GSE134291.

231

232 **2.8 Gene expression analysis**

233 Gene expression analyses were carried out with half of the gonadal tissue from the
234 same individuals that were used for methylation analysis except for two fish per sex
235 in which no more samples were available. RNA was extracted with chloroform by
236 TRIzol (T9424, Sigma-Aldrich, St. Louis, Missouri) according to manufacturer's
237 procedures. Quality of the RNA was measured by ND-1000 spectrophotometer
238 (NanoDrop Technologies) and quality was checked on a 1% agarose gel. By
239 following supplier indications, 150 ng of RNA were treated with DNase I,
240 Amplification Grade (Thermo Fisher Scientific Inc., USA) and retrotranscribed to
241 cDNA with SuperScript III RNase Transcriptase (Invitrogen, Spain) and Random
242 hexamer (Invitrogen, Spain). Quantitative PCR (qPCR) was carried out in triplicate
243 for each sample and two housekeeping genes were used. cDNA samples were mixed
244 with Eva Green chemistry (87H24-001; CultiGen), being the cycling parameters: 50°C
245 for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1
246 min. qPCRs were run in triplicate in optically clear 384-well plates (CFX-386, Touch
247 BioRad). Dissociation step, primers efficiency curves and PCR product sequencing
248 by Sanger, confirmed the specificity for each primer pair. Primer sequences used for
249 gene expression study are shown in Table S2.

250

251 **2.9 Statistical analysis**

252 All statistical analyses were carried out using R software (v. 3.4.4). Data were
253 expressed as mean $\pm$ SEM and the differences were considered significant
254 when $P < 0.05$. Graphs were generated either by using the ggplot2 package (v. 3.1.0)
255 (Wickham, 2016) or Sigma Plot (v. S13.0).

256
257 Methylation levels were calculated by averaging the values of each CpG per gene
258 and per sample, whereas for the CpG-specific approach the means per individual
259 CpGs per sample were considered. DNA methylation were evaluated by one-way
260 ANOVA followed by a Tukey's HSD test. Normality was tested by the Shapiro-Wilk
261 test and, when normality was not followed, methylation proportions logit
262 transformed and a robust non parametric two-way ANOVA was applied by using the
263 WRS2 package (v. 0.9.2) (Mair and Wilcox, 2016).

264
265 Statistics on gene expression data was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and
266 Schmittgen, 2001; Schmittgen and Livak, 2008). Data from qPCR were analyzed for
267 normality and homogeneity of variances by using the Kolmogorov–Smirnov and
268 Levene's test, respectively and significance between two groups was analyzed by t-
269 Student test. For DNA methylation and gene expression correlation analyses, a
270 Spearman's rank correlation (ρ) test was applied using the corplot package (v. 0.84)
271 (Wei et al., 2017).

272

273 **3. Results**

274 **3.1 Sequencing results**

275 The total number of read-pairs that passed the Illumina filter for all 20 samples
276 ranged between 30,000–243,000 read-pairs with an average of 97894.7 ± 47209.8
277 (mean $\pm$ SD) and the bisulfite conversion rate was $>99.5\%$ for all samples producing
278 0.936 Gb of data. MultiQC summary confirmed that sequence quality post trimming
279 passed the quality controls and were within the accepted thresholds.

280

281 **3.2 Methylation patterns show sexual dimorphism**

282 Global methylation were significantly higher in testes than ovaries of adult zebrafish
283 for *IL1 β* ($P < 0.001$) and *Casp9* ($P < 0.05$) genes (Fig. 2A and Fig.3A). The percent
284 levels for *IL1 β* were 89.7 ± 3.8 and 61.4 ± 5.5 in testes and ovaries, respectively and
285 for *Casp9* were 0.62 ± 0.04 and 0.56 ± 0.12 in testes and ovaries, respectively.

286

287 In *IL1 β* , eight out of nine analyzed CpGs showed significant differences of the
288 methylation levels between ovaries and testes ($P < 0.001$) (Fig.2B). Only CpG9 was
289 not significant. In *Casp9*, four out of nineteen CpGs showed significant difference in
290 the methylation levels between ovaries and testes which significant levels varied
291 among CpG positions: CpG2 and CpG18 $P < 0.001$; CpG3 $P < 0.01$ and CpG 6 $P <$
292 0.05 (Fig.3B).

293

294 **3.3 Gene expression**

295 The fold change of *IL1 β* expression in the testis was seven times higher than that of
296 ovary ($P < 0.01$) in adult zebrafish (Fig.4A). However, no significant differences in
297 gene expression were found for *Casp9* gene when testes and ovaries were compared
298 (Fig.4B).

299

300 **3.4 Correlations methylation vs. gene expression**

301 Negative correlation patterns between methylation and gene expression levels were
 302 observed for both *IL1 β* and *Casp9* genes in testes and ovaries of adult zebrafish,
 303 though no significance differences were obtained (Fig. 5). The strongest correlation
 304 was found in the testes for *IL1 β* gene ($\rho = -0.5$).

305

306 **4. Discussion**

307 The main immune signaling pathways are conserved in fish (Stein et al., 2007) in
 308 which the innate response is the essential immunity to cope immune challenges
 309 (Press and Evensen, 1999) although evidence of the required role of the adaptive
 310 immunity have been emerged in the last years (Secombes and Belmonte, 2016). The
 311 innate response is the earliest immune mechanism characterized by being nonspecific
 312 and therefore not depending upon previous recognition of the surface structures of
 313 the invader (Tort et al., 2003). It has also the advantage of being inducible by
 314 external molecules but at the same time is constitutive and reacts within a very short
 315 time scale thorough inflammatory pathways (Bayne and Gerwick, 2001).
 316 Interleukins constitute a large group of cytokines involved in the specific immune
 317 defense by recruiting leukocytes to a site of infection to initiate inflammation
 318 (Brocker et al., 2010). *IL1 β* , together with IL6 and tumor necrosis factor alpha
 319 (TNF α), are one of the main actors involved in the first inflammatory response
 320 (Smith et al., 2000). *IL1 β* , once it is cleaved by caspase 1 into a mature form, it is
 321 able to bind to its receptor leading the activation of the nuclear factor (NF)-
 322 κ B pathway (Martin and Falk, 1997). Caspases, including *Casp9*, are a family of
 323 protease enzymes that play essential roles in the apoptotic pathways and in the
 324 inflammasome of the cells (McIlwain et al., 2013; Shalini et al., 2015).

325

326 In vertebrates, and in some invertebrates such as parasites, reproductive and immune
 327 systems are interconnected playing sexual-dependent molecular functions (Folstad
 328 and Karter, 1992; Nunn et al., 2009). In human, testes contain immune cells such
 329 leukocytes and macrophages in the interstitial compartment that are involved in
 330 gametogenesis and Leyding cell development (Li et al., 2012). *IL1 α* is mostly
 331 produced by spermatocytes but also by Sertoli cells and its production is stage-
 332 specific (Haugen et al., 1994; Lie et al., 2012). In human ovaries, *IL1 α* mediates
 333 inflammatory processes during ovulation, fertilization and oocyte implantation
 334 (Polan et al., 1991; Garcia-Velasco and Arici, 1999) and it is highly expressed in
 335 ovarian cancers (Aggarwal and Gehlot, 2009). In sea bream gonads, *IL1 β* was found
 336 in the cytoplasm of spermatogonia, spermatocytes and oogonia and its expression
 337 was higher during reproductive cycles suggesting a role in promoting proliferation in
 338 gametogenesis and spermatocyte differentiation (Chaves-Pozo et al., 2008). The two
 339 cytokines, *IL1 β* and *TNF α* modulated the *in vitro* testosterone production in goldfish
 340 testis indicating their role in regulating sex steroids (Lister and van der Kraak, 2002).
 341 In the present work, we report a higher expression of *IL1 β* in testes when compared
 342 to ovaries in adult zebrafish according to what previously found by Lee et al. (2017),
 343 thus indicating that *IL1 β* is expressed in a sex-dependent manner in fish. We did not
 344 find significant difference in *Casp9* expression between testes and ovaries although
 345 higher expression in testes was also observed. In contrast, in protogynous black sea
 346 bass (*Centropristis striata*) ovaries expressed *Casp9* twice as much as testes,
 347 although gene expression patterns related to atresia differs among gonochoristic and
 348 hermaphroditic species (Breton et al., 2019). It is described that in zebrafish, during
 349 gonad development, activation of apoptotic pathways are required for testes

350 development (Liew and Orban, 2014), including specifically the expression of *Casp9*
 351 (Pradhan and Olsson, 2014), thus we might have observed the remaining activation
 352 of the pro-male machinery during testicular development.

353

354 Epigenetic modifications occurred in the host genome by pathogens after infections
 355 altering the transcription and the corresponding signaling pathways of the host (Marr
 356 et al., 2014). The understanding of epigenetic host-pathogen interactions is a
 357 flourishing research field to untangle the sophisticated cellular strategies (Gomez-
 358 Diaz et al., 2012). In humans, plenty of studies give evidence of rapid change in
 359 DNA methylation of the innate immune cells to face infections (Sinclair et al., 2015;
 360 Wiencke et al., 2016; Pacis et al., 2019) and the importance of the epigenetic
 361 mechanisms in reprogramming the immunity (Netea et al., 2016). In contrast, less data
 362 is found in fish. Recent findings in guppy (*Poecilia reticulata*) showed changes in
 363 DNA methylation dynamics after host–parasite interactions (Hu et al., 2018) and in
 364 Atlantic salmon (*Salmon salar*) showed that stress response together with immune
 365 challenges, altered transcriptome and methylome of the gills through immune
 366 responses (Uren Webster et al., 2018). In zebrafish, viral infections showed that
 367 histone modification was able to increase methylation levels of the gene promoters
 368 associated with innate immune response in head kidney, liver, spleen and heart
 369 (Medina-Gali et al., 2018). Surprisingly, to our knowledge, no data on epigenetic
 370 mediating infections in the immune system in the fish gonads are currently found.

371

372 It is estimated that in zebrafish, global methylation in sperm is nearly 95% of CG
 373 dinucleotides are methylated while in oocytes are ~75% methylated (Jiang et al.,
 374 2013; Potok et al., 2013). Therefore, it may be said that the product of mature germ

375 cells already possess methylation sexual dimorphism. Here, by a candidate gene
 376 approach method, we found higher methylation levels in the two studied immune
 377 genes (*IL1 β* and *Casp9*) in the testes when compared to ovaries thus evidencing sex-
 378 specificity of the methylation programs in the fish gonads. Likewise, significant
 379 differences were found in specific CpG sites for each of the targeted regions, in
 380 which eight out of nine and four out of nineteen of the studied CpGs for *IL1 β* and
 381 *Casp9*, respectively, showed significant differences in the methylation levels
 382 between the two sexes. Global DNA methylation levels between these two immune
 383 cells differ in magnitude being ~73% and ~0.62% (percent methylation mean in
 384 ovaries and testes) in *IL1 β* and *Casp9*, respectively. Although low levels were found
 385 in *Casp9* gene, percent difference between ovaries and testes of the four CpG
 386 significantly methylated was ~2%. In a similar manner, differences in the
 387 methylation levels (5.2%) were enough to observed epigenetic transgenerational
 388 effects in the insulin growth factor (IGF) 2 promoter in the siblings of those mothers
 389 famished in the Second World War (methylation levels for IGF2 ranged from 0.517-
 390 0.488 in all studied groups) (Heijmans et al., 2008). Thus, low but significant
 391 changes in DNA methylation levels might induce epigenetic changes.

392

393 Analysis of gene expression and methylation levels showed negative, although not
 394 significant, correlations among the four studied comparisons. Conventionally, higher
 395 methylation levels in the gene promoters have been associated with transcriptional
 396 silencing (Illingworth and Bird, 2009; Strausman et al., 2009). However, gene
 397 expression inhibition have been related to other genomic features like the first exon
 398 and the first intron of the gene body not only in human (Blattler et al., 2014) but also
 399 in fish (Anastasiadi et al., 2018a). Further, the approach here performed for studying

400 the methylation levels is limited to amplicons about ~500 bp, that even with the
401 intention of include promotor, first intron and first exon in the targeted regions, the
402 entire gene body and possible enhancers involved in the gene regulation were not
403 fully studied. Thus, to tackle this physical barrier, wide genomic approaches need to
404 be performed.

405

406 In tilapia, genome-wide methylation analysis identified sexually dimorphic
407 methylated regions along specific genomic regions (Wan et al., 2016) as well as in
408 zebrafish brain, global methylation differences were found between males and
409 females (Chatterjee et al., 2016). Thus, both studies, pointed out the importance of
410 the sexual dimorphic methylation patterns in fish. Our results supports that sexual
411 dimorphism defined the methylation patterns in two immune genes in the gonads and
412 so the sex-biased differences need to take into account when analyzing the immune
413 responses in fish. In fact, human research on sex-based differences in immune
414 responses are bringing to light the importance of considering sex when analyzing the
415 data in such a way that funding agencies and journals have launched policies to
416 analyze and publish research based on sex and gender in biomedical sciences (Klein
417 and Flanagan, 2016). Thus, sexual dimorphic differences need to be included for
418 good data interpretation.

419

420 Over the last few years, epigenetic marks have been implemented to predict, among
421 others, phenotypic features. This is the case, for example, in the identification of
422 clinical biomarkers to predict cancer predisposition or other diseases (Jubb and
423 Harris, 2010; Taberero et al., 2015). In animal production, epigenetic biomarkers to
424 predict the sex of the offspring in E. sea bass has been stated as an important step

425 towards improving sex control programs in aquaculture farms (Anastasiadi et al.,
426 2018b). The present study shows, for the first time, the presence of sexual
427 dimorphism in the DNA methylation profiles of two immune genes in the fish
428 gonads that might be useful, for example, to develop sexual epimarkers but also to
429 other applications such as fish diseases research. Further studies need to cover the
430 methylation patterns of these two immune genes when fish are subjected to immune
431 challenges and to see whether dimorphic sex-specific patterns are present when
432 facing immunological diseases.

433

434 **6. Declaration of interest**

435 The authors declare no competing interests.

436

437 **7. Funding**

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439 JIN “Ambisex” to LR. LR and JM were supported by Ambisex contracts.

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450 **Figure legends**

451 **Figure 1. Schematic view of the amplicon region studied by Methylation**

452 **Bisulfite Sequencing (MBS) method for *IL1 β* (A) and *Casp9* (B) genes.** The
453 amplicon length for the *IL1 β* was 459 bp and for *Casp9* 441 bp. A total of 9 CpG
454 were studied for *IL1 β* and 19 CpG for *Casp9*. Accession numbers were NM212844.2
455 and NM001007404.2 for *IL1 β* and *Casp9*, respectively.

456

457 **Figure 2. Methylation levels of *IL1 β* gene in adult zebrafish gonads.** (a) Global
458 DNA methylation percent found in ovaries and testes. (b) DNA methylation percent
459 for each of the nine CpG analyzed. Number of individuals studied; N = 9 ovaries and
460 N = 10 testes. Data shown as mean $\pm$ sem. Significant differences *** (P < 0.001).

461

462 **Figure 3. Methylation levels of *Casp9* gene in adult zebrafish gonads.** (a) Global
463 DNA methylation percent found in ovaries and testes. (b) DNA methylation percent
464 for each of the 19 CpG analyzed. Number of individuals studied; N = 9 ovaries and
465 N = 10 testes. Data shown as mean $\pm$ SEM. Significant differences * P<0.05, ** P <
466 0.01, ***P < 0.001.

467

468 **Figure 4. *IL1 β* and *Casp9* expression profiles in adult zebrafish gonads.** Data shown
469 as mean $\pm$ SEM of fold change using control values set at 1. Sample size $n = 10$
470 gonad per sex. Significant differences between sexes were analysed by Student's t
471 test, *** $P < 0.001$.

472

473 **Figure 5. Correlations DNA methylation and gene expression of *IL1 β* and *Casp9***
474 **genes.** Correlations are shown by Spearman's rank correlation coefficient (ρ). The

475 direction of the long axis of the ellipses and the color indicate the type of correlation:
 476 negative is shown in red and positive is in blue shade. The short axis of the ellipse
 477 and the intensity of the color are proportional to the correlation coefficients.
 478 Significant differences are considered when $* = P < 0.05$; $** = P < 0.01$; $*** = P <$
 479 0.001 .

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Figure 1.

Figure 2.

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Figure 3.

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Figure 4.

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Figure 5.