Epigenetic differences in the innate response after immune stimulation during zebrafish sex differentiation

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Abstract (150 words)

Infections are able to trigger epigenetic modifications; however, epigenetic-mediating infections in the immune system in fish is currently unavailable. Within this purpose, zebrafish were immune-stimulated with three lipopolysaccharides (LPS) during sex differentiation. Methylation patterns of three immune genes were studied by a candidate gene approach together with gene expression analysis, and in adulthood, sex ratios were determined. It was shown that the entrance of LPS was through the gills and accumulated in the pronephros. Significant hypomethylation levels of *CASP9* and a significant CpG site for *IL1β* after *Pseudomonas aeruginosa* LPS exposure were found. No methylation difference was observed for *TNFα.* Gene expression and correlation data differed among studied genes. Sex ratios showed a feminization in dose and LPS strain-dependent manner. Here, it is provided epigenetic regulatory mechanisms derived by innate response and the first evidence of possible epigenetic interactions between the immune and reproductive systems.

Introduction

Epigenetic modifications occur in the host genome after infections altering the transcriptome and the corresponding signaling pathways (Marr et al., 2014). The understanding of epigenetic host-pathogen interactions is a flourishing research field to untangle the sophisticated cellular strategies (Gomez-Diaz et al., 2012; Zhang and Cao, 2019). In humans, plenty of studies give evidence of rapid change in DNA methylation of the innate immune cells to face infections (Sinclair et al., 2015; Wiencke et al., 2016; Pacis et al., 2019) and the importance of the epigenetic mechanisms in the immunity reprograming (Netea et al., 2016; Binnie et al., 2020). In contrast, less data is found in fish. Findings in the guppy (*Poecilia reticulata*) showed changes in DNA methylation dynamics after host-parasite interactions (Hu et al., 2018). In Atlantic salmon (*Salmon salar*) it is shown that stress response together with immune challenges altered transcriptome and methylome of the gills through immune responses (Uren Webster et al., 2018). In zebrafish (*Danio rerio*), viral infections showed that histone modification were able to increase methylation levels of the gene promoters associated with an innate immune response in head kidney, liver, spleen and heart (Medina-Gali et al., 2018). Surprisingly, to our knowledge, there is a lack of data regarding the epigenetic-mediating infections in the immune system in fish, and more in particular, in the fish gonads.

The interaction between reproduction and the immune system is present in fish although it is far to be understood. To prevent germ cells against pathogens, gonads are considered as immune-privileged organs (Maddocks and Setchell, 1990). The presence of immune cells has been described along the gonadal development in gilthead seabream (*Sparus aurata* L.) (Chaves-Pozo et al., 2008; Chaves-Pozo et al., 2009), in turbot (*Scophthalmus maxima*) (Ribas et al., 2016) and in European sea bass (*Dicentrarchus labrax*) (Ribas et al., 2019). Further, the pro-inflammatory cytokines, tumor necrosis factor alpha (*TNFα*) and interleukin 1 beta (*IL1β*) are important to regulate goldfish (*Carassius auratus*) testicular steroid biosynthesis (Lister and van der Kraak, 2002). In zebrafish, it is known that the undifferentiated gonad, first developed as an ovarian-like organ to latter, half of the population develop into testicular phenotype throughout the activation of apoptotic pathways (Liew and Orban, 2014). The nuclear factor kappa beta (NF-κB) has a crucial role by blocking apoptotic pathways promoting cell survival and not allowing to activate the testicular pathway

and, consequently, the ovarian-like organ became ovaries (Orban et al., 2009). By treating zebrafish with heat-killed *Escherichia coli* during the sex differentiation (~15–40 days post fertilization, dpf), a skewed sex ratio towards females was observed, thus demonstrating the existence that the activation of the immune system has consequences in the final sexual phenotype (Pradhan et al., 2012). Strikingly, this is the only available study showing that the activation of the immune system during gonadal development is able to skew sex ratios in fish.

In contrast to mammals in which one master gene leads the sex of an individual (the sex determination region of the Y chromosome, *Sry*), in fish, the genetic but also environmental factors are responsible to determine the final phenotypic sex (Penman and Piferrer, 2008). In the last decade, it has been explored the role of epigenetics in the fish gonads, most of them focus on the temperature effects, demonstrating that the final sexual phenotype depends on the epigenetic-environment interactions (Piferrer, 2019).

In fish, the innate immune response is the most important mechanism to cope with immune challenges (Press and Evensen, 1999), although the adaptive immunity cannot be underestimated by gaining shreds of evidence during the last years of research (Secombes and Belmonte, 2016). The innate immune response is the first response when an infection occurs and requires the pathogen recognition through germline encoded by the pathogen recognition receptors (PRR) (Rauta et al., 2014). This recognition is fast and characterized by activating inflammatory pathways (Bayne and Gerwick, 2001) in which several cytokines, such as *IL1β* together with interleukin 6 (*IL6*) and TNF*α*, are ones of the main players (Smith et al., 2000; Brocker et al., 2010). Caspases, including CASP9, are also important actors in the innate responses in fish by activating apoptotic pathways and the signaling pathways controlled by the inflammasome (McIlwain et al., 2013; Shalini et al., 2015).

Bacterial lipopolysaccharide (LPS) is the major outer surface membrane component present in almost all the Gram-negative bacteria and LPS is considered as a good model for studying acute inflammation in fish as most of the regulatory genes are conserved between zebrafish and mammals (Forn-Cuni et al., 2017). Nevertheless, the receptors triggering cellular activation are not the same. Once it is recognized by the cells, the response to LPS in fish is immediate as gene expression is altered in already 30 minutes

(min) (Novoa et al., 2009) and can last for several hours (h) (MacKenzie et al., 2008) or even for days (Abdel-Mageid et al., 2020).

The main objective of the present study was to evaluate epigenetic modifications undertaken/initiated by the activation of the innate immune system during the gonadal development. Consequently, influences of early immune activation during sex differentiation on the final sexual phenotypes were evaluated. To address it, zebrafish, a well-known experimental animal, was used to develop an *in vivo* model by using different LPS strains from three of the most common bacteria (i.e., *E. coli*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*). First, we ensured LPS entrance in the zebrafish larva by using LPS conjugated with fluorescence; secondly, we performed dose-response experiments to determine the sensitivity to LPS treatments and the final consequences on sex ratios. Following, the DNA methylation patterns of three major players of the fish innate immune system (i.e., *IL1β*, *TNFα* and *CASP9*) were studied in larvae after LPS immune stimulation. For this purpose, a candidate gene approach method (Multiplex Bisulfite Sequencing, MBS) that allows determining epigenetic changes at the level of single CpG sites (Anastasiadi et al., 2018b), were performed. In parallel, gene expression of the three immune genes together with their correlations with methylation levels were determined.

Materials and methods

1. Ethics statement

The experimental protocol was approved by the Spanish National Research Council (CSIC) Ethics Committee within the project AGL2015-73864-JIN and licensed by the Bioethical Committee of the Generalitat de Catalunya under reference code 9977. European regulations of animal welfare (ETS N8 123, 01/01/91) were respected regarding fish maintenance. Likewise, ICM facilities were validated for animal experimentation by the Ministry of Agriculture and Fisheries (certificate number 08039–46–A) in accordance with the Spanish law (R.D. 223 of March 1988).

2. Animal rearing conditions

AB (ZFIN ID: ZDB-GENO-960809-7) laboratory strain zebrafish were housed in the animal facilities of the experimental aquariums at the Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). Zebrafish were reared in an *ad hoc* closed recirculating

system built for this purpose with a water pump of 3000 l h^{-1} and a UV light system to eliminate any possible bacteria in the water. This system was placed in a chamber with a photoperiod of 12 h of light and 12 h of darkness, an air temperature of 26 ± 1°C and a humidity of 50 ± 3%. Physicochemical parameters were monitored daily, staying at appropriate conditions (Ribas and Piferrer, 2014), which included temperature (28 ± 0.2°C), pH (7.2 ± 0.5), conductivity (750–900 µS), and dissolved oxygen (6.5–7.0 mg l^{-1}). Sulfite, sulfate, nitrate and ammonia quality parameters were checked weekly using commercial kits and periodically by the water analysis service of the ICM-CSIC.

At the time of mating for natural egg fertilization, one female per male coming from different families were placed in crossing tanks in order to maintain the interfamily variation observed in this fish species (Ribas et al., 2017a). The total number of fertilized eggs was counted to guarantee fertility according to the reference values for this species and fish post-hatch survival agreed with OECD's guidelines for the Fish Sexual Development Test (OECD, 2011). About 50 eggs were arranged per Petri dish with E3 embryonic medium (pH 7.2 ± 0.5) supplemented with 0.1% methylene blue (Sigma-Aldrich, Madrid, Spain) at 26 ± 1°C until 6 dpf. Then, 25–35 larvae were transferred into 2.8 liter plastic tanks (Aquaneering, mod. ZT280) to avoid masculinization due to rearing density effects (Ribas et al., 2017b).

Fishes were fed three times a day according to their stages of development: 6–15 dpf received specific larvae dried food (17.FO.CE.0611, Sparos I&D Nutrition in Aquaculture) and from 15 dpf onwards, they were fed with pellets of increasing size (AquaSchwarz, Göttingen, Germany). In all stages up to 10 dpf, diets were complemented with *Artemia nauplii* (AF48, INVE Aquaculture, Dendermonde, Belgium).

3. Purification of LPS

Commercial LPS from *E. coli* and *P. aeruginosa* (L2630 and L9143, respectively, Sigma-Aldrich, Madrid, Spain) were dissolved in Milli-Q water at 1 µg ml^{-1} and, subsequently, kept at −20ºC. In order to obtain *A. hydrophila* LPS, bacteria were grown on LB agar plates containing 50 µg ml^{-1} ampicillin. A single isolated colony was inoculated first in 50 ml LB overnight (O/N) culture to later grown O/N in 2.5 l of LB for the large-scale production. The *A. hydrophila* culture was centrifuged at 6,000 rpm,

4°C, 30 min in a Beckman JLA 8.1000 rotor. Then resuspended sequencially in PBS, ethanol, acetone and diethyl ether to finally prepare the dried biomass. Then a phenol-water extraction method was used to separate the LPS from the biomass into two phases after performing four centrifugations at 4ºC. The LPS was further dialyzed at 4ºC for seven days and its concentration was checked using NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The solution was diluted to obtain a final working 1 $\mu g\ \mu l^{-1}$ concentration.

4. LPS entrance to larvae body

The LPS conjugated with fluorescein isothiocyanate (*E. coli O111: B4* LPS-FITC, F3665, Sigma-Aldrich, Madrid, Spain) was dissolved in Milli-Q water at a concentration of 1 µg ml^{-1} and, subsequently, kept in a refrigerator at 4ºC for further uses. All procedures were done in the dark to avoid FITC degradation. To visualize the entrance of LPS-FITC, 20 dpf larvae were bath exposed to *E. coli* LPS-FITC in 1.5 ml glass vials for 3 h (1 larva per tube). The four groups were: control group (CT) (without LPS) (N = 4), 150 µg $ml^{-1}$of *E. coli* LPS (N = 4), and 150 (N = 4) and 750 µg ml^{-1} (N = 4) of *E. coli* LPS-FITC (Fig. S1). After exposures, larvae were fixed with 500 µl of 4% paraformaldehyde in 0.2 M phosphate buffer saline (PBS) in glass vials. Then, the samples were washed with 0.2 M PBS three times for ten min each. Once the fixation was completed, the 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescence staining (D9542, Sigma-Aldrich, Madrid, Spain) was added. For this purpose, 1 µl of DAPI was diluted in 3 ml of 0.2 M PBS remaining in darkness. Then, 500 µl of this solution was placed in borosilicate vials for 5 min. Finally, larvae were washed twice for one minute each with 0.2 M PBS and placed on a glass slide for microscopical analysis under the Zeiss LSM880 microscope using the software ZEN 2 pro.

5. Immune stimulation experiments

5.1. Dose-response experiments

These dose-response experiments were performed to obtain a non-lethal dose capable of trigger an immune response. LPS from three different bacteria species, *E. coli, P. aeruginosa* and *A. hydrophila*, were used for dose-response experiments. A total of 16 larvae at 15 dpf per each of the two-three technical replicates were bath-immersed in different LPS concentrations of the different strains (Fig. S1). Two, four, and three

biological family pairs were used for *E. coli, P. aeruginosa* and *A. hydrophila*, respectively. The total number of larvae used for dose-response experiments were 192, 768, 1008 for *E. coli, P. aeruginosa* and *A. hydrophila*, respectively. For testing LPS from *E. coli*, larvae were bathed in 0, 150 and 300 µg ml^{-1} LPS dilutions. For LPS from *P. aeruginosa*, larvae were subjected to concentrations of 0, 150, 175 and 200 µg ml^{-1}. Lastly, the tested concentrations of *A. hydrophila* LPS were 0, 25, 50, 75, 150, 200 and 300 µg ml^{-1}.

Larvae were placed in 3 ml glass containers for 24 h with the corresponding LPS concentration for the three LPS strains, except for the control group. Glass vessels containing larvae were incubated at 28 ± 1ºC in an oven chamber allowing circadian rhythms (12 h light:12 h dark). After 3 h of *P. aeruginosa* LPS treatment, larvae were washed with PBS and flash-frozen in liquid nitrogen and kept at -80ºC for further DNA methylation (N = 10) and gene expression (N = 10) analyses. The same procedure was followed for *A. hydrophila* LPS treated larvae after 3 h for gene expression analyses (N = 10). During the following hours, survival for the threes LPS strains was recorded.

5.2. Immune stimulations during sex differentiation

Based on the observed survival results from dose-response experiments, to study sex ratio resulting from immune stimulations during sex differentiation we repeated the *E. coli* and *P. aeruginosa* treatments as previously reported with the 150 µg ml^{-1} concentration (Fig. S1). A set of larvae of 15 dpf were treated with *E. coli* LPS and other two batches of larvae of 15 and 25 dpf were treated with *P. aeruginosa* LPS for 24 h. Between two to three technical replicates in one and two different biological family pairs for *E. coli* and *P. aeruginosa* LPS, respectively, were used. The total initial larvae used for this experiment was 96 and 320 for *E. coli* and *P. aeruginosa* LPS, respectively. Once LPS treatments finished, larvae were reared in tanks and placed in the rack system until adulthood to study sex ratios (120 dpf).

Due to the lack of sex ratio differences in adulthood observed from larvae exposed for 24 h of immune stimulation from both *E. coli* and *P. aeruginosa* strains, we extended the treatment along gonadal development based on the methodology reported in Ribas et al. (2017d) to obtain sex ratio differences. Thus, 18 dpf fish were immersed 7 times during sex differentiation period (18–32 dpf) for 3 h using *A. hydrophila* LPS using

concentrations of 25, 75 and 150 µg ml^{-1} (Fig. S1). A total of seven family pairs (N = 2, N = 2 and N = 3) for concentrations of 25, 75 and 150 µg ml^{-1} of *A. hydrophila* LPS, respectively) were used. For each family pair, three technical replicates were performed and a total of 816 initial number of fish was used. After 3h of immune stimulation baths, larvae were washed using the same procedure as described above. At the end of the treatments, fish of 32 dpf were reared in tanks and placed in the rack system until 120 dpf for sex ratio analyses. To evaluate possible stress originated from the experimental procedures, a negative control group (mock) was added to the experiment consisting of offspring of the same batch that was never removed from the tanks.

6. Methylation analysis

Methylation levels of larvae at 15 dpf treated 3 h with *P. aeruginosa* LPS at 150 µg ml^{-1} were studied for three immune genes i.e., *IL1β*, *CASP9* and *TNFα* by MBS technique following the procedures described elsewhere in (Anastasiadi et al., 2018b; Caballero-Huertas et al., 2020). Briefly, genomic DNA from five larvae for each group was extracted by 1 µg of proteinase K (Sigma-Aldrich, St. Louis, Missouri) overnight at 65 °C and the following day, a standard phenol-chloroform-isoamyl alcohol protocol with ribonuclease A (PureLink RNase A, Life Technologies) was performed and bisulphite converted. The targeted portion of the promoter was amplified with two PCR rounds by using costumed primers for the three immune genes (Table S1). Primer specificities were previously validated by Sanger sequencing of amplicons from a pool of samples. Amplicon regions included the promoter, the first exon and the first intron were included to the extent possible. Adaptor sequences for 16S metagenomic library preparation (Illumina) were added to the 5′ ends of the primers designed: Forward-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and Reverse-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. The target regions included a total of nine for *IL1β*, 19 for *CASP9* and seven for *TNFα* CpGs, respectively.

Resulting PCR products were indexed by Nextera XT index Kit Set A (Illumina; FC-131–2001) according to Illumina's protocol for 16 S metagenomic library preparation and were pooled in an equimolar manner to obtain a single multiplexed library which was sequenced in a MiSeq (Illumina, San Diego, California) using the paired-end (PE) reads 250 bp protocol at the National Center of Genomic Analysis (CNAG, Barcelona).

Raw sequencing data were submitted in Gene Expression Omnibus (GEO) from NCBI (https://www.ncbi.nlm.nih.gov) with the accession number: GSE134400.

7. Gene expression analysis

RNA was extracted from larvae treated for 3 h with *P. aeruginosa* LPS at 150, 175 and 200 µg ml^{-1} concentrations and with *A. hydrophila* LPS at 25, 75 and 150 µg ml^{-1}. RNA was individually extracted from 5 larvae each group with TRIzol (T9424, Sigma-Aldrich, St. Louis, Missouri) according to manufactured procedures. RNA pellets were suspended in 25 µl DEPC–water and kept at −80°C. RNA concentration was determined by ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality was checked on a 1% agarose/formaldehyde gel. By following supplier protocols, 200 ng of RNA were treated with DNAse I, Amplification Grade (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and retrotranscribed to cDNA with SuperScript III RNase Transcriptase (Invitrogen, Spain) with Random hexamer (Invitrogen, Spain). Quantitative PCR (qPCR) was carried out in technical triplicates for each sample with the SYBR Green chemistry (Power SYBR Green PCR Master Mix; Applied Biosystems). The conditions in the thermocycler: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min in a 384-well plate (CFX-386, Touch BioRad). The qPCRs were run in optically clear 384-well plates. Finally, a temperature-determining dissociation step was performed at 95°C for 15 seconds (sec), 60°C for 15 sec and 95°C for 15 sec at the end of the amplification phase. Dissociation step, primers efficiency curves and PCR product sequencing confirmed the specificity for each primer pair. The qPCR primers used in this analysis are shown in Table S2.

8. Bioinformatics analysis

Raw data from the sequencer were demultiplexed based on the index codes by the Illumina software and adapters were removed using the Trim Galore! software (v. 0.4.5) (Babraham Bioinformatics). Quality controls of the samples were carried out during pre- and post-trimming using the FastQC software (v. 0.11.8) (Andrews, 2010) to ensure adapters were cut off correctly (Ewels et al., 2016). Low-quality bases were filtered (Phred score <20) and only PE reads were used for this analysis.

We used *in silico* bisulfite-converted zebrafish genome (danRer11, GCA_000002035.4) as a reference to align the PE using both steps procedures Bismark software (v.20.0) (Krueger and Andrews, 2011). Bisulfite conversion efficiency was calculated for each sample with a minimum threshold of 99.0%. All samples passed the minimum threshold. The "*BSgenome.Drerio.UCSC.danRer11*" package was used to obtain the coordinate positions of all CpG sites (TBD, 2019). Data were cleaned up, labelled and tabulated using the Python 3 web-based environment Jupyter Notebook (v. 5.7.4). Every single CpG site coordinate was checked after deleting those whose coverage was below 10 reads. Then, we calculated the methylation levels by averaging the CpG site values in each gene for each sample and then were averaged by treatment. For individual CpGs sites, the methylation values were averaged by the coordinate CpG position by the treatment.

9. Statistical analysis

All statistical analyses were performed using RStudio (v. 1.1.456). Data were expressed as mean ± S.E.M and the differences were considered significant when $P < 0.05$. Graphs were generated either by Sigma Plot software (v. S13.0) and by using the "*ggplot2*" package (v. 3.1.0) (Wickham, 2009).

DNA methylation data

To work with a methylation database, the "*dyplir*" package were used (Wickham et al., 2020). DNA methylation differences between control and LPS groups were determined by Student t-tests. Previously, homoscedasticity was checked by Levene's test for every single group, as well as normality was tested by the Shapiro-Wilk test for each group. When normality was not followed, a Kruskal-Wallis test was performed.

Gene expression and correlation analyses

Data obtained from qPCR were collected by SDS 2.3 and RQ Manager 1.2 software. For each sample, the relative quantity (RQ) values of the genes of interest were used to normalize against the geometric mean value of two references genes (*EF*α and *RPL3A*) validated for zebrafish (Tang et al., 2007) and the fold change was calculated using the $2^{\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). One-way ANOVA was used to detect differences in gene expression between treatments. Previously, homoscedasticity was checked by Levene's test for every single group, as well as normality was tested by the

Shapiro-Wilk test for each group. When normality was not followed, a Kruskal-Wallis test was performed. Tukey's test was used to perform *post hoc* multiple comparisons. Correlation analyses between methylation and gene expression were performed by a Spearman's rank correlation coefficient (r_s) test using the *corrplot* package (v. 0.84).

Survival data from dose-response experiments

For dose-response experimental data, the Kolmogorov–Smirnov's and Levene's tests were used to check data normality and the homoscedasticity of variances, respectively. Then, a one-way analysis of variance (ANOVA) was used to detect possible differences among groups. Tukey's test was used to perform *post hoc* multiple comparisons.

Sex ratio statistics from immune stimulation experiments

Statistical significance of the resulting sex ratio was calculated by using the Chi-square test (χ^2) with the application of the Yates correction (Yates, 1934).

Results

1. LPS penetrates through the gills and recruited in the pronephros

Positive LPS-FITC signal was observed inside the larvae, in particular in the pronephros area, after 3 h of bath incubation at 150 µg ml^{-1} (Fig. 1C). No signal was observed in the negative control group neither in none-LPS conjugated (Fig. 1B and D). Likewise, after bathing with a higher concentration (750 µg ml^{-1}), fluorescence was observed in the gill arches (Fig. 1E). To facilitate the observation of LPS localization inside the larvae, figure 1A is a diagram of the dorsal view of illustrating the location of pronephros, branchial arches and developing gonad.

2. Fish survival depends on LPS strain

E. coli LPS treatments did not affect larva survival as both concentrations used showed similar results as the control group after 24, 48 and 72 h post-treatment (Fig. S2A). No differences in larva survival were observed during the 24 h LPS treatment (data not shown).

In *P. aeruginosa* LPS immune stimulation experiments, the larva survival rate behaved in a dose-dependent manner (Fig. S2B). After 3 h onwards at 200 µg ml^{-1} a significant decrease in survival was observed when compared to control group. At 175 µg ml^{-1} a

significant decrease in larva survival was observed after 24 h whereas no differences were found at 150 µg ml^{-1}.

Survival observed from *A. hydrophila* LPS experiments showed a clear dose-dependent pattern although not enough biological replicates were obtained for all the concentrations (Fig. S2C). Nevertheless, with the dose of 150 µg ml^{-1}, a significant decrease ($P < 0.05$) in fish survival was observed after 24 h.

3. LPS altered the DNA methylation of immune genes

After treating 15 dpf larvae for 3 h with *P. aeruginosa* LPS at 150 µg ml^{-1}, no significant differences in *IL1β* mean methylation were found between control and LPS groups (Fig. 2A). However, at individual CpG sites, a significant ($P = 0.00902$) methylation difference were found in the CpG9 site (position +189 after first ATG) with hypomethylation in the LPS group (Fig. 2B).

Significant differences ($P = 0.001305$) in mean DNA methylation between control and LPS groups were detected in *CASP9* (Fig. 2C), being methylation levels in LPS group lower than the control group. The same pattern was found in all CpG sites were individually studied and concretely, CpG11 site (position -95 to first ATG) was significantly hypomethylated in LPS group when compared to control ($P = 0.01337$) (Fig. 2D). *TNFα* did not show significant differences in DNA methylation levels between control and treatment groups both by the mean or by CpG sites analysis as DNA methylation patterns were not altered (Fig. 2E and F).

4. Gene expression varies for each immune gene and depends on LPS strain

No significant up or downregulation in gene expression of the three studied genes were found at 15 dpf larvae treated with *P. aeruginosa* LPS. *IL1β* gene was repressed more than four and seven times after 3 h of incubation at 150 µg ml^{-1} and 175 µg ml^{-1} being the former concentration almost significant ($P = 0.0512$) (Fig. 3A).

Gene expression of *IL1β* gene in larvae treated 3h with *A. hydrophila* LPS upregulated significantly ($P < 0.01$) for 75 and 150 µg ml^{-1} concentrations when compared to control group. Gene expression was significantly upregulated in a dose-dependent manner with more than twenty-fold change when compared to the lowest concentration (i.e., 25µg

437 ml^{-1}) (Fig. 3B). No significance in gene expression differences was observed in *CASP9*
438 (Fig. 3D) while TNFα expression was significantly downregulated in the highest dose
439 (Fig. 3F).
440
441 **5. Negative correlation observed are mostly not significant**
442 Correlation analyses were studied between DNA methylation and gene expression
443 levels in *P. aeruginosa* LPS treated larvae during 3h at 150 µg ml^{-1}. Results for five out
444 of the six studied groups showed a negative tendency (Fig. 4). Nevertheless, only one
445 correlation was significant in *TNFα* LPS group (P = 0.01667; r_s = -1). No significant
446 correlations between methylation and gene expression levels were found in *IL1β* (P =
447 0.35; r_s = 0.6 and P = 0.6833; r_s = -0.3, in control and LPS, respectively) or in *CASP9*
448 (P = 0.35; r_s = -0.1 and P = 0.6833; r_s = -0.3, in control and LPS, respectively).
449
450 **6. Feminization depends on the duration of the exposure and the LPS strain**
451 Percent of males of control groups of the eleven families used for immune stimulation
452 ranged among standard levels in AB strain (53–78%). Larvae of zebrafish treated during
453 24 h with *E. coli* or *P. aeruginosa* LPS at 150 µg ml^{-1} was not able to skew sex ratios in
454 populations (Fig. 5A and B). However, in the *P. aeruginosa* LPS treatments a
455 feminization tendency was observed. Likewise, 25 µg ml^{-1} repeated exposures for 3 h
456 during seven times along the gonadal development with *A. hydrophila* LPS did not
457 interfere sex ratio (Fig. 6A). Interestingly, those larvae treated with a concentration of
458 75 µg ml^{-1} of *A. hydrophila* LPS showed a significant (P <0.01) increase in the number
459 of females (Fig. 6B). In contrast, this feminization tendency was not observed after
460 increasing the concentration up to 150 µg ml^{-1} of *A. hydrophila* LPS but without
461 statistical significance (Fig. 6C). Nevertheless, a significant difference was found in the
462 mock group when compared to control and LPS treated (P <0.05).
463
464 **Discussion**
465 In the present work, we used the LPS, a molecule described as a good inflammatory
466 model in zebrafish (Yang et al., 2014), to activate the immune system in the developing
467 gonads to study DNA methylation impact and the possible consequences on the final
468 sexual phenotype. To ensure that the LPS penetrated the larvae body, as well as to
469 visualize which tissues it interacts with, larvae were submerged during 3 h in water with

E. coli LPS conjugated with FITC. It was observed that LPS penetrated through the gills, with clear evidence in the highest LPS concentration. Penetration of labelled antigens by bath immersion through the gills was observed in other fish species such as rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio* L.) (Kiryu et al., 2000; Huising et al., 2003). It is known that gills, together with the nose, the skin, the gut and the urogenital system constitute the first line of fish defense (Rombout et al., 2014). Gills are mucosal tissues with rapid renewing allowing to recover from minor epithelial damages (Lyndon and Houlihan, 1998), and after infections, locally secreted factors allowing the migration of circulating leukocytes such as neutrophils (Huising et al., 2003) or lymphocytes (Castro et al., 2014). In addition, LPS fluorescence was detected near the head of the larvae where pronephros is located (Outtandy et al., 2019). Pronephros consists of glomerulus connected by two bilateral ducts and persist in the same position during the first month after fertilization (Drummond et al., 1998). The role of pronephros is essential in the osmoregulation in fish but also in functions as an immune organ with the presence of B lymphocytes and immunoglobulins two weeks after fertilization (Zapata et al., 2006). In common carp, pronephros increased up to 53% of the platelet-forming cells when infected (Rijkers et al., 1980). After ontogenic development, pronephros becomes the head kidney which is considered the principal immune organ in fish and it is homologous to mammalian bone marrow (Meseguer et al., 1995; Rauta et al., 2012). In adult fish, LPS insults in the head kidney increased cell proliferation to cope with the infection (Ribas et al., 2008). Therefore, our observation in the pronephros belongs to the recruitment of labeled LPS-FITC by the activation of the immune cells responsible to cope with the insult. No fluorescence was detected in the abdominal area where the differentiating gonads are located at any of the concentrations of LPS tested, maybe due to the short exposure time or the experimental procedure. Liposomes containing labeled LPS showed accumulation in zebrafish gills after bath immersion but an intraperitoneal injection was required to be visible in the internal tissues (i.e., spleen) (Ruyra et al., 2013). With the help of flow-cytometry, observation of the liposome positive cells were also possible in the head kidney (Ruyra et al., 2014). Thus, to identify labeled LPS in the developing gonads further experimental procedures should be accomplished.

Once we knew that LPS was inside the larvae, we studied the DNA methylation levels of three important innate immune-related genes at 15 dpf zebrafish when sex

differentiation takes place in this fish species. In all of the genes studied, LPS was able to reduce DNA methylation levels on the promoter regions after only 3 h of treatment, although only a significant alteration of mean DNA methylation was observed for *CASP9* gene. These results showed the rapid activation of the immune system by epigenetic regulatory mechanisms. When looking in *CASP9* at individual CpG sites, only the CpG11 was differentially hypomethylated when compared to the control group although hypomethylation tendency was observed in all the 19 CpGs studied. In Atlantic salmon fry, *in silico* associations between transcriptomic data obtained after *A. aeruginosa* LPS treatment with methylation data obtained after chronic stress treatments showed that some immune genes, including *CASP3a*, suffered hypermethylation and reduced expression, although other were hypomethylated (Uren Webster et al., 2018). Thus, denoting that in fish there is a crosstalk between the environment and DNA methylation of genes related to the immune system.

Here, despite not finding differences in *IL1β* global methylation levels between control and LPS groups, there were significant DNA hypomethylation differences in the CpG9 site after LPS treatment. The rest of the CpG sites showed similar methylation levels between control and LPS groups. In adult zebrafish, dimorphic differences in the methylation patterns of *IL1β* between ovaries and testes were observed (Caballero-Huertas et al., 2020). Strikingly, all CpGs studied in the mature zebrafish gonads showed significant dimorphic differences except for the CpG9 site, in which similar methylation levels were found in both sexes. Recent findings in mice gonads showed dimorphic epigenetic marks in the Wnt/beta-catenin signalling pathway that allows activating the ovary formation and maintenance (Koth et al., 2020). Overall, and although more research is needed specifically in infected adult gonads, CpG9 site might function as an important player of the immune response in fish and acts independently to the sex of an individual.

LPS mimics bacterial infections and consequently, a cascade of inflammatory pathways are triggered, including the expression of interleukins and cytokines (Bayne and Gerwick, 2001; Novoa et al., 2009). Gene expression levels of *IL1β* gene was significantly increased after *A. hydrophila* LPS immersion treatments but not significance was found after *P. aeruginosa* LPS thus showing that *A. hydrophila* likely stimulated the innate response to a greater extent than *P. aeruginosa*. Nevertheless,

IL1β gene expression at 175 µg ml^{-1} in *P. aeruginosa* LPS was almost significant. Thus, in both LPS, *IL1β* expression was upregulated after only 3h of LPS treatments in a dose dependent manner while no difference was found in CASP9. In contrast, *TNFα* was inhibited at the highest dose after 3h of *A. hydrophila* LPS treatment. Another published study in zebrafish larvae treated with *E. coli* LPS observed a decrease of *IL1β* expression in a low dose but an increase in a high dose at 3 h (Novoa et al., 2009). These dose-related differences could be linked with the tolerance response to LPS in which it may protect the host from developing a shock syndrome caused by hyperactivation of the immune system (Henricson et al., 1991; Medvedev et al., 2000). Thus, when the immune system is hyperactivated, there is a suppression of the production of many cytokines which can reprogramme the immune cells setting up an adaptation to future bacterial infections (Shnyra et al., 1998). Thus, low doses of LPS may exert beneficial effects by enhancing the protection against different fish diseases and, in fact, LPS is used as adjuvant in some fish vaccines (LaFrentz et al., 2004; Selvaraj et al., 2009; Vallejos-Vidal et al., 2016).

The MBS methodology here performed for DNA methylation analyses was based on a high resolution at locus-specific but amplifies limited regions of DNA (~500 bp), and although, targeted regions intended to include promotors, first intron and first exon, the entire gene body and possible enhancer were not fully covered. Consequently, our correlation analyses were not significant in any of the genes tested except for *TNFα* in the LPS group. Nevertheless, data showed slight inverse correlations between DNA methylation levels and gene expression for *CASP9* and *TNFα* genes following the pattern dictated by the classical dogma; low DNA methylation of CpG-rich promoters is associated with the activation of the gene transcription machinery (Jones and Takai, 2001; Deaton and Bird, 2011). Nevertheless, current research shows that DNA methylation patterns are more dynamic and complex as hypomethylation of the promoters is associated with gene silencing through the blockage of the transcription initiation machinery (Ambrosi et al., 2017; Smith et al., 2020). Thus, other genomic elements rather than the promoters contribute to transcriptional regulation like the exons (Brenet et al., 2011), gene body (Blattler et al., 2014), the introns (Anastasiadi et al., 2018a), as well as post transcription modifications (Shilatifard, 2006). That could be the case of the pattern observed in the *IL1β* correlation analysis.

In fish, abiotic factors such as temperature (Ribas et al., 2017a) or hypoxia (Shang et al., 2006) and biotic factors such as density (Ribas et al., 2017b, c) are able to skew sex ratios towards males in the final population. In contrast, zebrafish populations treated with chemical compounds i.e. demethylation agent 5-aza-2′-deoxycytidine (5-aza-dC) (Ribas et al., 2017e), or with biotic factors such as *E. coli* heat-killed bacteria (Pradhan et al., 2012) during sex differentiation, showed sex ratio bias towards females. In this study, we have tested different bacterial strains at different concentrations to determine whether activation of the immune system was able to alter the final phenotypic sex in the developing zebrafish larvae. Larvae treated with *E. coli* and *P. aeruginosa* LPS at 150 µg ml^{-1} for a short period (i.e., 24 h) during sex differentiation did not have any effect in the final sex ratios. However, repeated exposures along the sex differentiation with *A. hydrophila* LPS at 75 µg ml^{-1} demonstrated a significant feminization of the populations (P <0.01) in a similar manner previously described by Pradhan et al. (2012). Although we did not find significant feminization at lower (25 µg ml^{-1}) and higher doses (150 µg ml^{-1}), a tendency towards females was observed in the former. Thus, higher number of biological family replications should be further tested. Nevertheless, overall sex ratio results showed the existence of a crosstalk between the immune system and the reproduction system in zebrafish.

Needs to be discussed that despite an observed trend towards the increase in the number of females under 150 µg ml^{-1} *A. hydrophila* LPS, significant results were only found when compared LPS treated and control groups to mock control group. Results indicated that experimental procedure performed to treat larvae of zebrafish with LPS along sex differentiation caused stress. It is known that cortisol released by stress response is able to masculinize fish populations (Fernandino et al., 2012) and in the case of zebrafish, synthetic cortisol was able to fully masculinized populations (Ribas et al., 2017b). Thus, based on the present data, LPS treatment might attenuate the masculinization by counteracting the stress, thus reinforcing that the activation of the immune system during sex differentiation is able to feminize zebrafish populations. Not finding sex ratio differences in other LPS strains tested or other doses might be explained by the fact that sensitivity to the LPS response depends on the LPS bacterial origin species, serotype, doses, experimental concentrations and the time of duration of the exposure (Novoa et al., 2009). It is known that fish, together with amphibians, are

resistant to the toxic effect of LPS as high doses are required to trigger an immune response when compared to mammals (Berczi et al., 1966; Sepulcre et al., 2009) and these differences were originated by diverse molecular strategies to recognize LPS (Chu and Mazmanian, 2013; Yang et al., 2018).

Conclusions

Epigenetics integrates environmental cues and genomic information to determine the final phenotype. Here, we have shown that the immune stimulation by LPS in the developing zebrafish rapidly hypomethylates *CASP9* and *IL1β* genes identifying, in the former, a CpG which might play an important role in the immune system in zebrafish. Gene expression data is altered only after 3h of immune challenges with higher sensitivity of *A. hydrophila* when compared to *P. aeruginosa*. LPS entrance by immersion is through the gills and recruited in the pronephros. Further, LPS is able to skew sex ratios towards females but in a dose and strain-dependent manner. The present work emphasizes the importance of epigenetic control in the response of zebrafish immune system during gonadal development and reinforces the existence of the crosstalk between reproductive and immune systems.

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Conflicts of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. Immune stimulation with *E. coli* LPS-FITC labelled. Images obtained from fluorescence microscopy of 20 days post fertilization zebrafish larvae (blue by DAPI

labelling). **(A)** Dorsal view of a larva illustrating the location of pronephros, branchial arches and developing gonad. **(B)** Control (without the addition of LPS). **(C)** Immune stimulated larva with 150 µg ml^{-1} of LPS-FITC (green). **(D)** Immune stimulated larva with 150 µg ml^{-1} of LPS. **(E)** Immune stimulated larva with 750 µg ml^{-1} of LPS-FITC (green).

Figure 2. Percent of DNA methylation levels of immune genes of 15 dpf larvae exposed to *Pseudomonas aeruginosa* LPS for 3 h. **(A)** *IL1β* percentage of mean DNA methylation levels under *P. aeruginosa* immersion treatment. **(B)** DNA methylation percentage for each of the nine CpG analyzed in *IL1β*. **(C)** *CASP9* percentage of mean DNA methylation under *P. aeruginosa* immersion treatment. **(D)** DNA methylation percent for each of the 19 CpG analyzed in *CASP9*. **(E)** *TNFα* percentage of mean DNA methylation levels under *P. aeruginosa* immersion treatment. **(F)** DNA methylation percentage for each of the seven CpG analyzed in *TNFα.* Significant differences among groups (P <0.05) were analyzed by Student t-tests and when normality was not followed, a Kruskal-Wallis test was performed.

Figure 3. (A) *IL1β,* (**C)** *CASP9* and **(E)** *TNF*α expression profiles after 3 h of treatment with *Pseudomonas aeruginosa* LPS at different concentrations in 15 dpf larvae. **(B)** *IL1β,* **(D)** *CASP9* and **(F)** *TNFα* expression profiles after 3 h of treatment with *Aeromonas hydrophila* LPS at different concentrations in 15 dpf larvae. Data are shown as mean ± SEM of fold change using control values set at 1. Sample size N = 5 larvae per treatment. Significant differences (P <0.05) are symbolized by letters between treated groups and by asterisks between treated and control groups: * (P <0.05), ** (P <0.01). Statistics were analyzed by one-way ANOVA.

Figure 4. Correlations among DNA methylation and gene expression of **(A)** *IL1β*, **(B)** *CASP9* and **(C)** *TNFα* genes. Correlations were analyzed by Spearman's rank correlation coefficient (r_s).

Figure 5. Zebrafish sex ratio at 120 days post fertilization (dpf) after 24 h of 150 µg ml^{-1} LPS challenges. **(A)** Sex ratio of fish treated with *Escherichia coli* LPS at 15 dpf. The total number of fish are 35 and 64 in control and LPS groups, respectively. Each data

point is the mean ± SEM, corresponding to three technical replicates for one breeding pair. **(B)** Sex ratio of fish treated *Pseudomonas aeruginosa* LPS at 15 and 25 dpf. Total numbers of fish are 98 and 150 in 15 and 25 dpf, respectively. Each data point is the mean ± SEM, corresponding to two to three technical replicates for each of the two breeding pairs. Significant differences among groups (P <0.05) were analyzed by the Chi-squared test with Yate's correction.

Figure 6. Sex ratio of fish treated with *Aeromonas hydrophila* LPS during sex differentiation (18-32 dpf). **(A)** Sex ratio results obtained at 25 µg ml^{-1} of *A. hydrophila* LPS. Total numbers of fish are 121 and 116 in control and treated groups, respectively. **(B)** Sex ratio results obtained at 75 µg ml^{-1} of *A. hydrophila* LPS. Total numbers of fish are 84 and 87 in control and treated groups, respectively. **(C)** Sex ratio results obtained at 150 µg ml^{-1} of *A. hydrophila* LPS. Total numbers of fish are 143, 146 and 72 in control, treated and mock control group, respectively. Each data point is the mean ± SEM, corresponding to breeding families (N = 2 families, N = 2 families, N = 3 families for 25, 75 and 150 µg ml^{-1}, respectively) for each of the two or three technical replicates used for each concentration. Significant differences among groups (P <0.05) were analyzed by the Chi-squared test with Yate's correction.

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1039

Figure 1

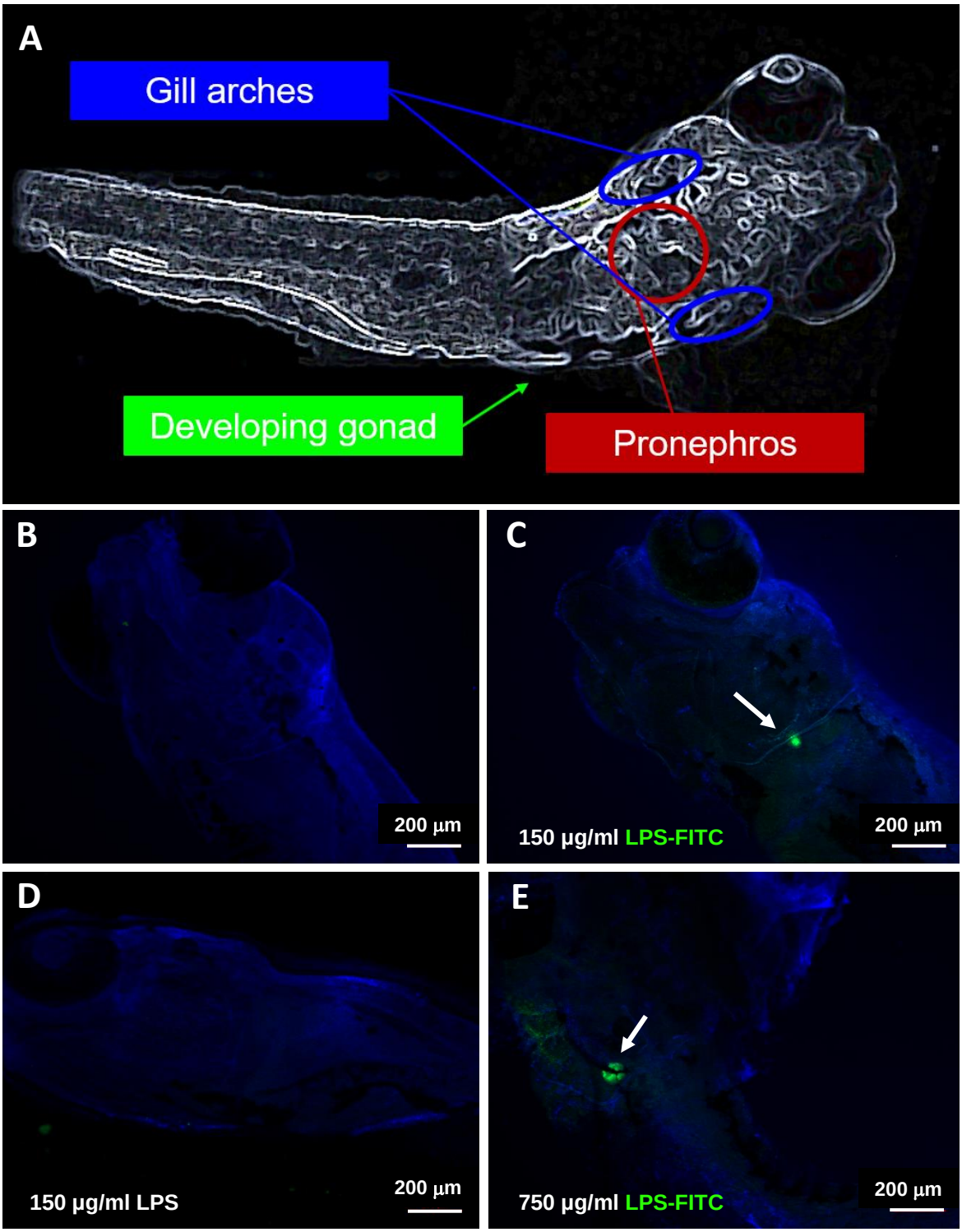

A
Gill arches
Developing gonad
Pronephros
B
200 µm
C
150 µg/ml LPS-FITC
200 µm
D
150 µg/ml LPS
200 µm
E
750 µg/ml LPS-FITC
200 µm

Figure 2

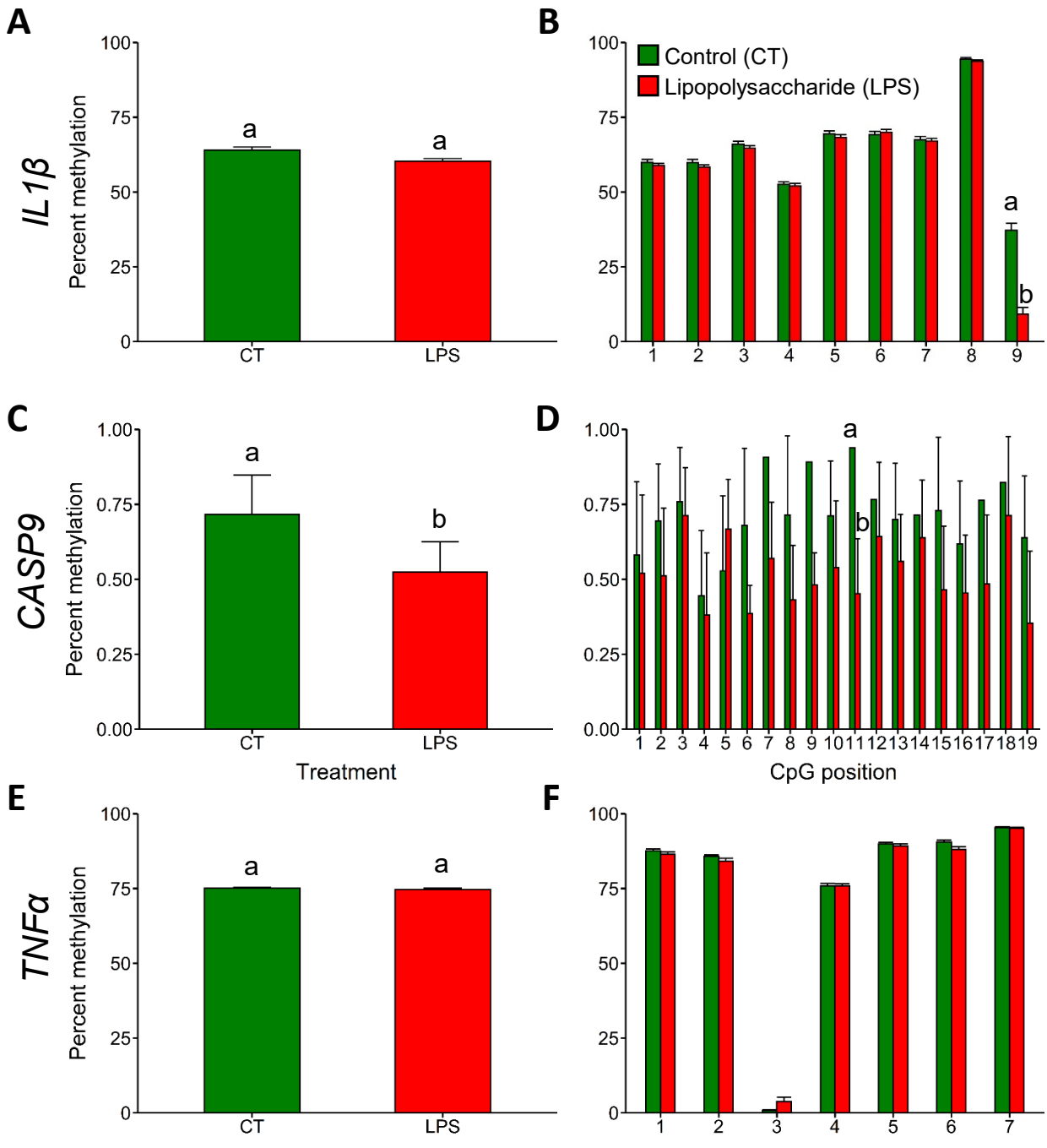

A
IL1β
Percent methylation
100
75
50
25
0
a
a
CT
LPS
B
Control (CT)
Lipopolysaccharide (LPS)
a
b
1 2 3 4 5 6 7 8 9
C
CASP9
Percent methylation
1.00
0.75
0.50
0.25
0.00
a
b
CT
LPS
Treatment
D
a
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
CpG position
E
TNFα
Percent methylation
a
a
CT
LPS
F
1 2 3 4 5 6 7

Figure 3

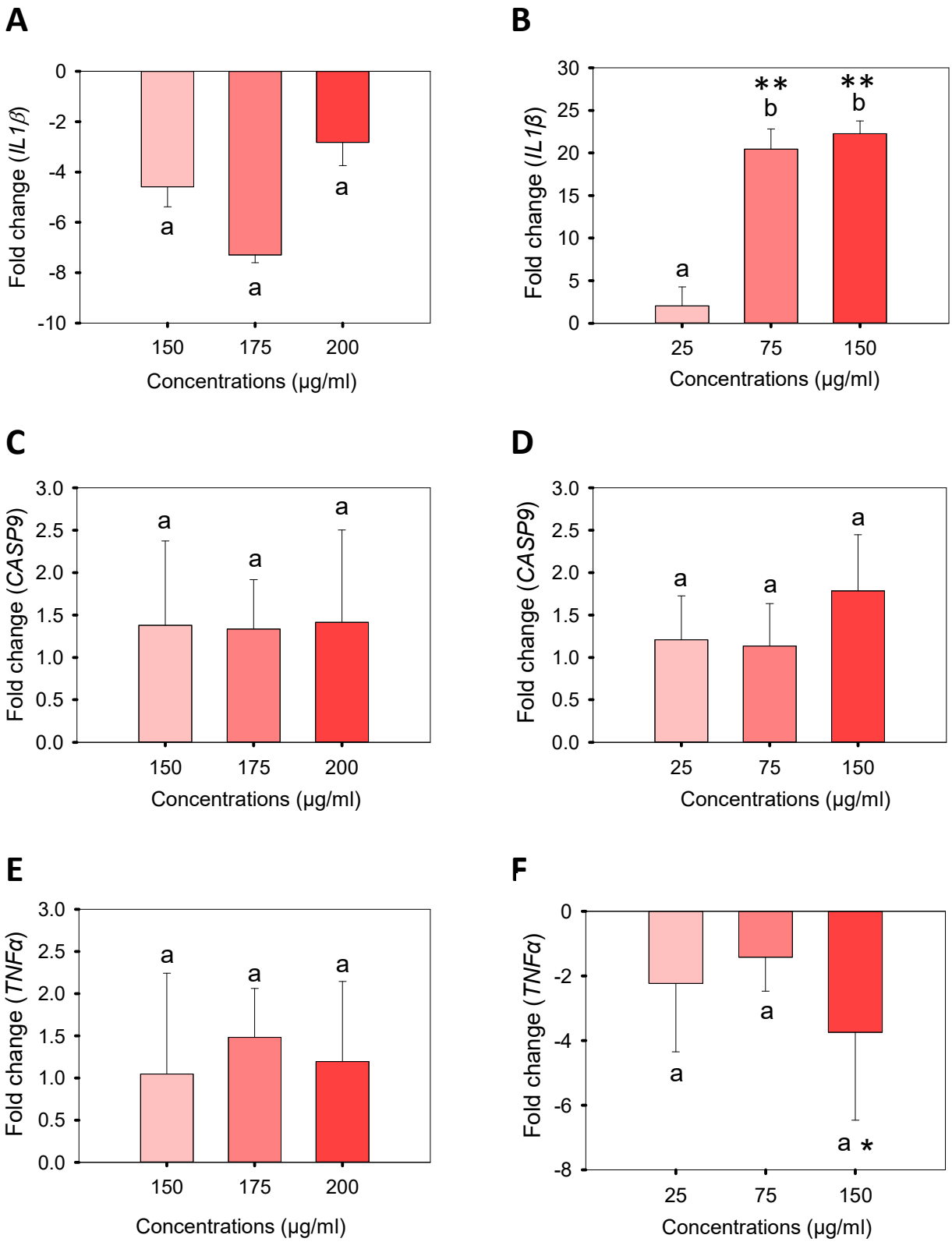

A
Fold change (IL1β)
0
-2
-4
-6
-8
-10
a
a
a
150
175
200
Concentrations (µg/ml)
B
Fold change (IL1β)
30
25
20
15
10
5
0
a
** b
** b
25
75
150
Concentrations (µg/ml)
C
Fold change (CASP9)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
150
175
200
Concentrations (µg/ml)
D
Fold change (CASP9)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
25
75
150
Concentrations (µg/ml)
E
Fold change (TNFα)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
150
175
200
Concentrations (µg/ml)
F
Fold change (TNFα)
0
-2
-4
-6
-8
a
a
a *
25
75
150
Concentrations (µg/ml)

Figure 4

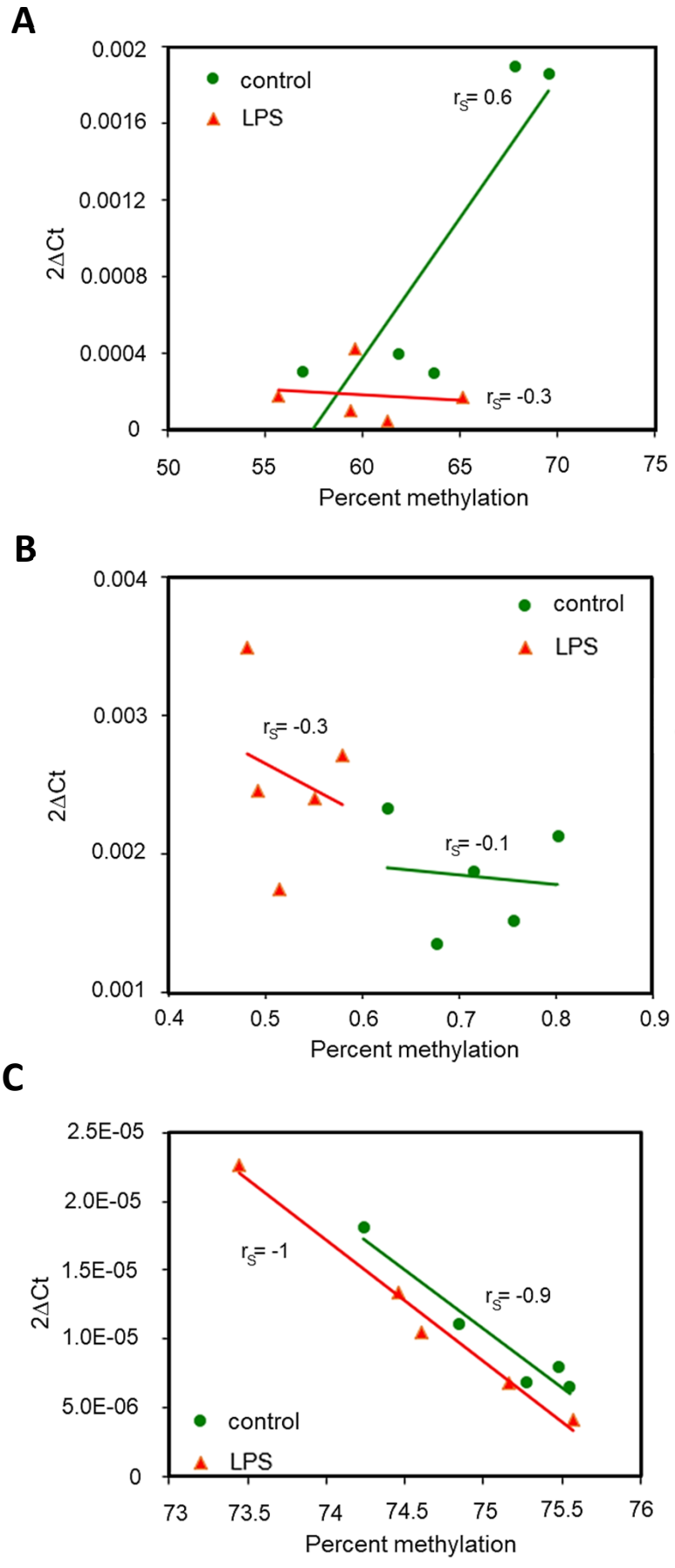

A
0.002
0.0016
0.0012
0.0008
0.0004
0
2ΔCt
control
LPS
r_S= 0.6
r_S= -0.3
50
55
60
65
70
75
Percent methylation
B
0.004
0.003
0.002
0.001
2ΔCt
control
LPS
r_S= -0.3
r_S= -0.1
0.4
0.5
0.6
0.7
0.8
0.9
Percent methylation
C
2.5E-05
2.0E-05
1.5E-05
1.0E-05
5.0E-06
0
2ΔCt
r_S= -1
r_S= -0.9
control
LPS
73
73.5
74
74.5
75
75.5
76
Percent methylation

Figure 5

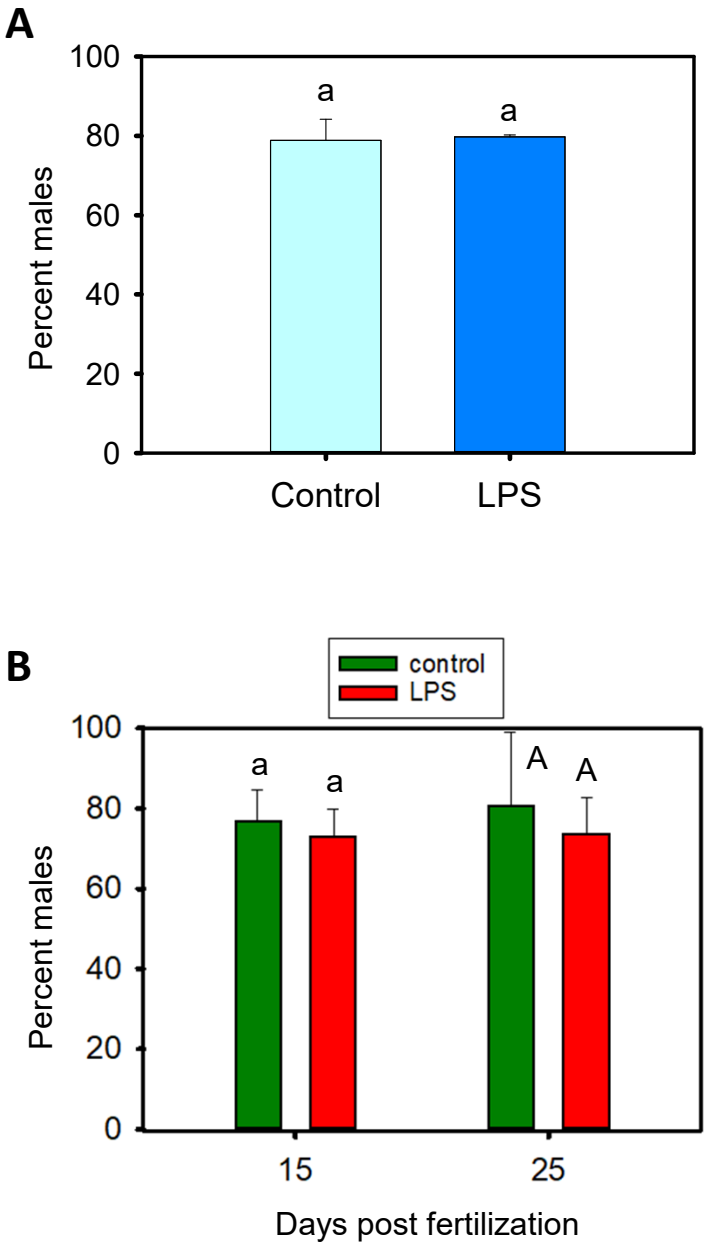
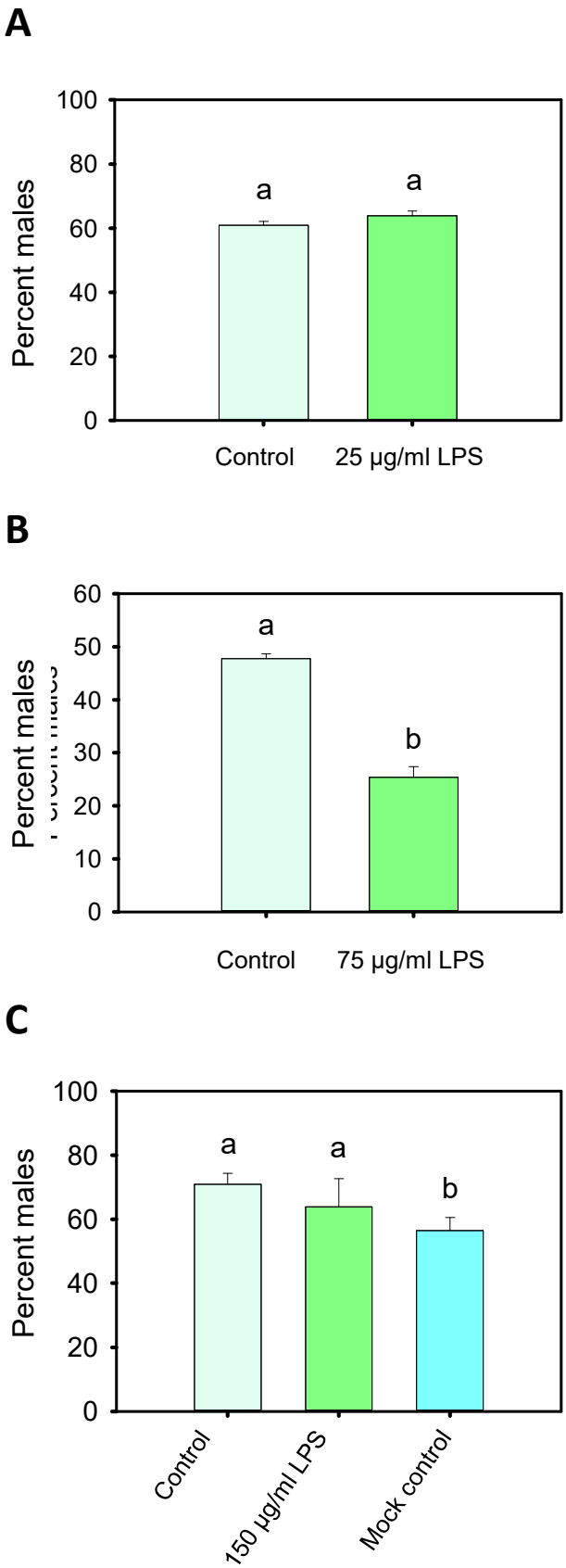

A
100
80
60
40
20
0
Percent males
a
a
Control
LPS
B
control
LPS
100
80
60
40
20
0
Percent males
a
a
A
A
15
25
Days post fertilization

Figure 6


A
100
80
60
40
20
0
Percent males
a
a
Control
25 µg/ml LPS
B
60
50
40
30
20
10
0
Percent males
a
b
Control
75 µg/ml LPS
C
100
80
60
40
20
0
Percent males
a
a
b
Control
150 µg/ml LPS
Mock control