ZEBRAFISH AND LPS, MODEL TOOLS FOR DECIPHERING EPIGENETIC CHANGES DURING SEX DIFFERENTIATION

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Introduction

Fish farmed for human consumption are reared in artificial environments that are very different from conditions that species experience in the wild. Consequently, the environment influences many aspects of the biology of cultured animals, including sexual phenotype through epigenetic mechanisms (Feeney et al., 2014). Disease outbreaks occur eventually in fish farms, causing economic loses. However, the epigenetic consequences that these reiterating infections can potentially cause in fish during their development and whether these can alter the reproductive system, remain unknown. Here we present a study for which two model research tools have been selected; the zebrafish as a suitable model for aquaculture research (Ribas and Piferrer, 2014) and the lipopolysaccharide (LPS) from Gram negative bacterial wall as a model to stimulate the immune system in fish (Forn-Cuní et al., 2017). The aim of this work is to develop a suitable *in vivo* system to study whether infections occurring in fish during sex differentiation are able to alter the final gonadal phenotype throughout epigenetic changes.

Materials and methods

AB zebrafish were housed following standard conditions (Ribas et al., 2014;) (Bioethical Committee code: 9977). Zebrafish juvenile at 15 days post fertilization (dpf) were stimulated with three different strains of LPS; *Escherichia coli*, *Pseudomonas aeruginosa* or *Aeromonas hydrophila*, during sex differentiation. First, and in order to find the median lethal dose (LD50), animals were exposed to different LPS concentrations and survival were recorded. Samples were taken at 3, 6, 24, 48 and 72 hours after LPS stimulations for studying the expression levels of three immune genes (*IL-1β*, *TNF-α*, *Casp9*) involved in the inflammatory response. Methylation patterns of these immune genes were studied by Methylation Bisulfite Sequencing (MBS), a candidate gene approach (Anastasiadi et al., 2018), by high throughput sequencing (Illumina, paired-end). Surviving individuals were transferred into tanks and kept until adulthood to check final sex ratios

Results

Fish survival was dependent to the dose and the LPS strain, being the *A. hydrophila* strain with the highest toxicity. Gene expression of the studied immune genes were downregulated after *P. aeruginosa* exposition but a significant upregulation was found when zebrafish were exposed to *A. hydrophila*. Methylation levels in the juvenile larvae showed significant changes in the methylation levels of specific CpGs of the three studied genes (Fig.1). Short exposition of LPS was not able to change sex ratios but a feminization tendency was observed after longer exposition to *A. hydrophila* in a dose-response and family-dependent manner.

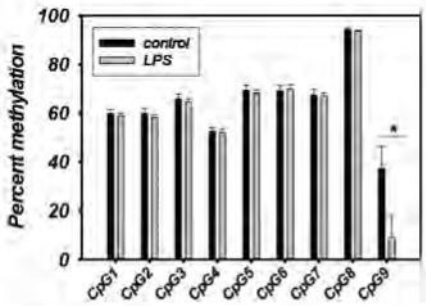

Figure 1. Methylation of immune genes (IL1β) of 15 dpf zebrafish expose during 3h to LPS of *P. aeruginosa*. Data showing as mean ± SEM. Sample size n = 6-7 zebrafish per treatment. Significant differences *($P < 0.05$) were analyzed by Student's *t*-test.

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Discussion and conclusions

Here we have developed an *in vivo* model to study the effects of immune stimulation during gonad differentiation that results in long-term influences in the sexual phenotype and it is able to alter methylation and gene expression patterns. We have identified the LD50 that is able to induce an activation of the immune system by reducing fish survival proving that LPS from *A. hydrophila* has a higher toxicity than the LPS of *P. aeruginosa* but less than *E. coli*, as partly shown in other studies in zebrafish (Novoa *et al.,* 2009). Zebrafish is a gonochoristic species in which all individuals starts developing as female and later, during sex differentiation, male differentiation processes appear throughout apoptotic and p53 signaling pathways that are responsible of the testes development (reviewed in Liew and Orban, 2014). Here, by stimulating zebrafish fish LPS from *A. hydrophila* we have been able to alter sex ratios by decreasing the number of males in a dose and family dependent manner. Intra-family variation of the other environmental factors (i.e., temperature and density) have been observed in zebrafish (Ribas et al., 2017a, 2017b). Previous studies in zebrafish treated with heat-killed bacteria from *E. coli* during sex differentiation showed a female bias due to the activation of NF-κB pathway that induced anti-apoptotic effects in the differentiating gonad (Pradhan *et al*., 2012). We observed that methylation and expression mechanisms were involved in the LPS stimulation in the juvenile larva and so interfered in the final sexual phenotypic fate. However, the epigenetic and transcriptomic mechanisms involved in the gonadal changes need to be further elucidated. In conclusion, our results showed interactions between immune–reproduction systems and we identified methylation differences for particular CpGs of the studied immune genes that can be worth for developing markers that can improve breeding programs (e.g., selection of high quality broodstocks).

Acknowledgments

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