HORMONES AND IMMUNITY: WHAT IS THE ROLE OF ESTROGEN IN IMMUNE FUNCTION?

by

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INTRODUCTION

Studies have repeatedly demonstrated that the immune system is sexually dimorphic, with males and females displaying differences in a variety of immune responses (Marriott & Huet-Hudson 2006; Yet et al. 2018; Dong et al. 2017). In general, studies performed in both teleost and mammalian systems have supported the idea of an enhanced immune response in females as compared to males. For example, studies have found decreased mortality in response to a pathogen challenge in females as well as higher incidences of autoimmune disease (Thornton et al. 2018; Khan & Ansar 2015). While the mechanisms underlying these differences are not well described, a role for sex steroids is suspected.

Initially, androgens were targeted as potential immunosuppressive compounds, as detailed by the immunocompetence handicap hypothesis (Folstad & Karter 1992). This theory states that the fitness cost associated with decreased immune function due to high androgen levels in males is offset by gains associated with the development of androgen-dependent secondary sexual characteristics that attract potential mates, resulting in enhanced reproductive success. However, this hypothesis has been studied with inconclusive results. While Kurtz et al. (2007) found that implantation of 11-ketoandrostenedione correlated negatively with measures of innate immunity in male three-spined sticklebacks (*Gasterosteus aculeatus*), Loggie et al. (2018) found no correlation between levels of naturally circulating 11-ketotestosterone and immune function in male bluegill sunfish (*Lepomis macrochirus*). In addition, a meta-analysis conducted by Roberts et al. (2004) found a significant immunosuppressive effect of testosterone but did not find a significant effect of testosterone on any direct measures of

immune function, suggesting that testosterone may be only indirectly associated with immune function.

More recently, it has also been proposed that differences in estrogen levels may be the causal factor underlying differences in male and female immune function. Generally, estrogens are thought to function in an immunoprotective manner. Thornton et al. (2018) found that female fathead minnows (*Pimephales promelas*) demonstrated significantly lower mortality after a pathogen challenge with *Yersinia ruckeri* than their male counterparts. In a study conducted on Japanese sea bass (*Lateolabrax japonicus*), Thilagam et al. (2009) found that exposure to 17β-estradiol (E2) led to enhanced immunoglobulin production. While it is believed that estrogens have the capacity to alter immunity in teleosts, the way in which this occurs and the precise effects of estrogen on various aspects of immunity are not well understood.

Previously, researchers have attempted to explore the relationship between estrogens and immune function by exposing fish to estrogenic compounds (Wenger et al. 2012; Cabas et al. 2012). These studies have uncovered immune function processes which may be sensitive to estrogens, such as inflammation and complement pathways. While this approach has provided some information regarding the role of estrogens in immunity, other approaches are likely necessary to fully elucidate the role of estrogens in immune processes. Whereas estrogenic exposures test the response of immune processes when faced with higher physiological levels of estrogens than normal, antiestrogenic exposures can test the functioning of these processes in states of reduced estrogen levels. These exposures can lend additional support to the

immunomodulatory ability of estrogens by demonstrating that immune function alterations can occur not only with excess estrogen levels, but also via estrogen suppression.

Thus, the primary objective of the present study was to assess the impacts of decreased estrogen levels on immune function. To accomplish this, immune function was evaluated in female fathead minnows exposed to fadrozole, an antiestrogen which decreases estrogen levels via aromatase inhibition. Immune function was assessed at three biological levels of organization using three separate experiments, which allowed for a more holistic view of immune function impacts. Specifically, mortality in response to a bacterial pathogen, phagocytic cell activity, respiratory burst activity, and the expression of genes which encode inflammatory cytokines and complement proteins were analyzed. Given the notion that estrogens are immunoprotective, it was hypothesized that the immune function of fadrozole-exposed females would be detrimented relative to unexposed females as demonstrated by higher rates of mortality following pathogen infection, decreased phagocytic cell activity, increased respiratory burst activity, and altered expression of target immune function genes. The present study serves to enhance the current understanding of the role of estrogen levels in immune function, which may play a role in the observed differences between male and female fish. Additionally, estrogenic and antiestrogenic contaminants are found in the aquatic environment. If estrogen levels have the capacity to modulate immune function, proper risk assessment necessitates a clearer understanding of this relationship.

MATERIALS AND METHODS

General Experimental Design

Three separate experiments were performed in the present study. All experiments utilized adult female fathead minnows exposed to 0, 10, or 50 µg/L of fadrozole (FAD). The three experiments assessed the ability of FAD to alter: 1) mortality after a pathogen challenge, 2) immune responses 8 hours after pathogen challenge, and 3) function of immune cells. In experiment 1, fish were exposed to FAD for a total of 21 d and their ability to survive pathogen infection was evaluated by exposing them to a pathogen on day 7 of the exposure period and monitoring survival for the remaining 14 d. In experiment 2, fish were similarly challenged by a pathogen after 7 d of FAD exposure but were then sampled 8 hours after pathogen administration so that the expression of immune genes could be evaluated. In experiment 3, fish were exposed to FAD for 7 d, at which point kidney cells were harvested for use in cellular assays to assess phagocytic cell activity and respiratory burst activity.

Animal Husbandry

All experiments were performed utilizing procedures approved by the Texas Christian University (TCU) Institutional Animal Care and Use Committee (protocol 19/04). Female fathead minnows utilized were obtained from Aquatic Research Organisms (ARO) and were ~14 months old at the start date of the exposures. Prior to the exposures, fish were held in 90-L aerated glass aquaria with dechlorinated municipal water at a temperature of 25.5 °C. Densities remained below 0.42 fish/L and a photoperiod of 16:8-h light: dark was maintained. One-third water exchanges were completed each day and commercially available flake food (TetraMin) was provided *ad libitum* twice daily. Fish were acclimated to 75-L aerated glass

aquaria for at least 1 week before the start of each exposure. During the exposures, two-thirds water exchanges were conducted daily.

Chemicals & Water Quality

Stock solutions (2, 2.5, or 3.5 mg/L FAD) were prepared daily for each experiment by dissolving fadrozole hydrochloride (Sigma-Aldrich, ≥ 98% purity) in dechlorinated municipal tap water to achieve desired FAD concentrations. Appropriate volumes of the prepared stock solution were then immediately added to aquaria based on the exposure group (10 or 50 ug/L) and tank size (75 or 27 L). No solvent was used due to the high solubility of FAD in water.

During the exposures, basic water quality parameters were measured weekly. Temperature, pH, conductivity, and salinity were all assessed using a YSI Professional Plus water quality meter, while dissolved oxygen was measured using a Vernier Go Direct Optical Dissolved Oxygen meter. In addition, hardness and alkalinity were assessed using LaMotte water quality test kits. Through the duration of the exposures, mean (standard deviation) temperature, pH, conductivity, and dissolved oxygen in the aquaria were 25.2 (0.4) °C, 7.97 (0.04), 413 (15) uS/cm, and 7.74 (0.515) mg/L, respectively. The mean (standard deviation) temperature, pH, conductivity, hardness and alkalinity of renewal water was 25.1 (0.24) °C, 7.99 (0.02), 393 (11) uS/cm, 152 (8) mg/L and 150 (5) mg/L, respectively.

Bacterial Culture & Injection Procedure

A pathogen resistance assay featuring *Yersinia ruckeri* (a Gram-negative bacterium that is the causal agent of enteric red mouth disease in fish) has been previously described for use in fathead minnows by Thornton et al. (2017), thus this pathogen was utilized in both experiments

1 and 2. A bacterial suspension was produced from bacteria cultured overnight in nutrient broth and kept on an orbital shaker held at 26 °C. In the morning of the day of bacterial injections, the culture was washed and resuspended in Hank's Balanced Salt Solution (HBSS). The absorbance (at 620 nm) of this solution was measured via a spectrophotometer and the solution was then gradually diluted with HBSS to achieve an absorbance of ~0.8. Subsequent nutrient agar plating of the bacterial injection solution demonstrated that the solution contained 1.24 x 10^9 CFU/mL.

Immediately prior to injections, fish were anesthetized in buffered tricaine methanesulfonate (MS-222) at a concentration of 0.1 g/L. Intraperitoneal injections were performed using a syringe pump (NE-500, New Era Pump Systems, Inc., Farmingdale, NY) with a 27-gauge needle, and 10 µL of *Y. ruckeri* solution/g body mass was administered to achieve a dose of 1.24 x 10^7 CFU/g body mass. Sham-injected fish in experiment 2 received the equivalent volume (10 µL/g body mass) of HBSS through the same injection procedure.

Confirmation of Endocrine Disruption

Plasma and tissue samples were taken from subsets of fish in experiments 2 and 3 to provide confirmation of endocrine disruption. No samples were taken from experiment 1, as fish used in experiments 1 and 2 were housed together prior to injections and thus received identical FAD dosing. Expression of the genes vitellogenin (*vtg*), an egg yolk protein, and estrogen receptor alpha (*ERα*) was measured in liver samples, as both genes are known to be estrogen regulated (Sumpter & Jobling 1995; MacKay et al. 1996).

Experiment 1: Pathogen Resistance

Fish were exposed to 0, 10, or 50 µg/L FAD for a total of 21 d (n= 26-27/group). On day 7 of the exposure period, fish were subjected to an intraperitoneal injection of *Y. ruckeri* as described above. Post-injection, mortality was recorded for 14 d, with checks occurring at least twice daily. At the conclusion of the exposure period, surviving fish were euthanized via immersion in buffered MS-222 at a concentration of 0.3 g/L.

Experiment 2: Stimulated Immune Response

To analyze the innate immune responses to *Y. ruckeri*, a similar procedure was used as described in experiment 1. In total, 21 fish (n=7/group) were injected with HBSS to control for the possible effect of the injection itself, while 33 fish (n=11/group) were injected with a solution of *Y. ruckeri.* Instead of monitoring mortality after the *Y. ruckeri* injection, all fish were sacrificed 8 hours post-injection via immersion in buffered MS-222 (0.3 g/L). Fish were blotted dry and weighed. Blood was collected by severing the caudal artery and a 2-3 µL aliquot was utilized to create blood smears. Blood slides were later stained using Camco Quik Stain II (Wright - Giemsa) and photographed using a Leica DMi1 inverted scope. For each fish, 200 cells were counted in total and the percentage of white blood cells was recorded. Spleen tissue and liver tissues were extracted and weighed; spleen and hepatosomatic index (SI and HSI, respectively) were calculated as tissue mass (mg)/body mass (mg) x 100. Extracted livers were frozen on dry ice, stored at -80 °C, and subsequently utilized to quantify the expression of immune-related genes.

Experiment 3: Cellular Assays

To assess changes in immune cell function as a result of FAD exposure, 75 fish (n=25/group) were exposed to 0, 10, or 50 ug/L of FAD for 7 d. At the conclusion of the exposure, all fish were sacrificed via immersion in buffered 0.3 g MS-222/L. Fish were first bled by severing the caudal artery. Liver tissue was extracted, frozen on dry ice, and stored at -80 °C for confirmation of endocrine disruption via gene expression analysis. Kidney tissue was collected from each fish and kidneys from 5 fish from the same exposure group were randomly pooled to generate a cell suspension to ensure a sufficient number of cells for use in the cellular assays. A total of 5 cell suspensions/group were generated, per Thornton Hampton et al. (in prep), by lightly homogenizing the pooled kidney tissue with a plastic pestle in supplemented L-15 media that had been filtered through a 27-gauge syringe and washed three times prior to use. Cells were subsequently resuspended in L-15 media and counted using a hemocytometer. The concentration of cells in each sample was diluted to 6 x 10^6 cells/mL through addition of L-15 supplemented media. Cells suspensions were then plated in triplicate in 96-well plates and stored overnight at 30 °C in a humidified incubator. The following morning, the health of the cells was inspected before performing the phagocytic cell and respiratory burst assays as described below.

Assessment of Phagocytic Cell Activity

The ability of immune cells to phagocytose a pathogen, FITC-labeled *Escherichia coli*, was measured via fluorescence levels at four separate timepoints: 0, 1, 2, and 4 hours after *E. coli* addition. Plates were centrifuged after the addition of *E. coli* to facilitate particle:cell interactions and trypan blue was used to quench extracellular fluorescence immediately before measurement on a plate reader. In between measurements, plates were returned to the 30 °C

humidified incubator. To account for background fluorescence, the mean fluorescence intensity at 0 hours for each sample was subtracted from the recorded fluorescence at all following timepoints.

Assessment of Respiratory Burst Activity

The ability of cells to produce superoxide, a reactive oxygen species associated with immune function, was measured by a modified colorimetric nitroblue tetrazolium (NBT) assay. This NBT assay measures the amount of formazan crystals produced as a result of NBT reduction by superoxide. Samples were plated in triplicate under four different reaction mixture conditions based on presence or absence of superoxide dismutase (SOD) and phorbol 12-myristate 13-acetate (PMA): (1) -SOD/-PMA, (2) +SOD/-PMA, (3) -SOD/+PMA, and (4) +SOD/+PMA. Bovine SOD was included to account for possible intracellular superoxide production. Immune cells were stimulated using PMA to promote higher levels of superoxide production. Absorbance was measured at 620 nm.

Gene Expression Analysis

Gene expression analysis was performed to validate FAD-induced endocrine disruption and to assess the impacts of FAD on *Y. ruckeri*-stimulated molecular immune responses. A list of the genes tested, their function, and the associated primer sequences and annealing temperatures is provided in Table 1. Tissue samples were homogenized via sonication with a Q125 sonicator (Qsonica) and total RNA was extracted using a Maxwell 16 LEV simplyRNA Purification Kit and Maxwell Research System (Promega) per the manufacturer's instructions. A NanoDrop 1000 (ThermoFisher Scientific) was used to quantify total RNA and to assess RNA purity. All samples were confirmed to have 260/280 absorbance ratios > 1.8 indicating suitable purity for

downstream analysis. First-strand cDNA synthesis reactions, containing 2 µL of qScript cDNA Supermix (Quantabio) and 8 µL of RNA, were carried out using a TC100 thermal cycler (BioRad) with a program of 5 min at 25°C followed by 30 min at 42°C and 5 min at 85°C. Each 10 µL quantitative polymerase chain reaction (qPCR) reaction, containing 0.4 µL of cDNA, 4.3 µL of nuclease-free water, 0.3 µL of 10 mM primer mix, and 5 µL of PerfeCta SYBR Green FastMix (Quantabio), was performed in triplicate using a BioRad CFX Connect real-time PCR detection system with a cycling program consisting of an activation step (95 °C, 30 s) and 40 cycles of denaturing (95 °C, 10 s) and annealing (primer-specific temperature, 15 s).

Table 1. Target genes, gene functions, and primer sequences utilized for testing gene expression levels in hepatic tissue. AT = annealing temperature.

Gene	Gene Function	Primer sequence (5' → 3')	AT (°C)
Ribosomal protein *(l8)*	Reference gene	F: gcccatgtcaagcacagaaaa R: acggaaaaccaccttagccag	60
Acidic ribosomal protein *(arp)*	Reference gene	F: ctgaacatctcgcccttctc R: gacacacactggcgatgttc	57
Vitellogenin (*vtg)*	Egg yolk protein; expression regulated by estrogens	F: tatgcacgagaaaatcgccac R: agcatgacgacttcacgcag	60
Estrogen receptor α *(ERα)*	Estrogen receptor; expression regulated by estrogens	F: cggtgtgcagtgactatgct R: ctcttcctgcggtttctgtc	57
16S ribosomal RNA *(16S rRNA)*	Bacterial ribosomal protein	F: gcgaggaggaagggttaagtg R: gttagccggtgcttcttctg	60
Interleukin 1β *(il-1β)*	Pro-inflammatory cytokine involved in cell proliferation, differentiation, and apoptosis	F: agaccaatctctacctcgcttgtac R: ttaatggtgtttaatgtttcactgatctc	60
Interleukin 11 *(il-11)*	Pro-inflammatory cytokine which stimulates T-cell-dependent development of B cells	F: tgttagcatctgccttccct R: tcgttctgttcagccactca	60
Interleukin 10 *(il-10)*	Anti-inflammatory cytokine which downregulates Th1 expression and enhances B cell survival	F: ctcatttgtggagggctttc R: tacagctgttggcagaatgg	61
Tumor necrosis factor α *(tnfα)*	Pro-inflammatory cytokine involved in cell proliferation, apoptosis, lipid metabolism, and coagulation	F: caagcaattggcgagtgtgt R: cagttccactttcctgattactctga	54
Complement component 3 *(c3)*	Required for activation of both the classical and alternative complement pathways	F: gtgccagtgtgcagaagaaa R: ttcccctcaacatcctcatc	60
Complement component 9 *(c9)*	Component of complement membrane attack complex (MAC), inducing pores on cell membranes and causing lysis	F: cagtgcagcctcagcagtag R: ggcagaatggtgatggtctt	60

The expression of each gene was quantified via the standard curve method using serially diluted cDNA samples. The expression of *vtg* and *ERα* in experiments 1 and 2 was normalized using the geometric mean of the reference genes ribosomal protein L8 (*l8*), acidic ribosomal protein (*arp*), and elongation factor 1α (*ef1*) due to issues in finding a single suitable reference gene. This issue did not occur for the samples in experiment 3, and as such the expression of *vtg* and *ERα* was normalized to the reference gene *l8*. Expression of each target immune function gene was normalized using *l8*. No statistically significant differences in the expression of *l8* was observed across treatment groups indicating its suitability as a reference gene. Samples with mean cycle quantification values (Cq) ≥ 34 were considered non-detects and assigned a starting quantity corresponding to a Cq of 34.

Statistical Analysis

Morality data were analyzed using PRISM (GraphPad), while all other data were analyzed using the statistical software package JMP 11.0 (SAS Institute). For analysis of pathogen-induced mortality (experiment 1), a Kaplan-Meier curve was generated. Remaining fish at the end of the experiment were censored and assigned a value of 336 hours. Differences between FAD exposure groups were tested via a log-rank test. Gene expression data collected to confirm FAD-induced endocrine disruption was analyzed via one-way analysis of variance (ANOVA). A Levene's test was utilized to check for unequal variances, and if variance was found to be unequal a nonparametric Steel-Dwass test was used. If variances were equal, a Wilcoxon test was used. All immune-related data obtained in experiment 2 (*i.e.*, SI, HIS, cell counts, gene expression) was assessed via two-way ANOVA with exposure group and pathogen exposure as factors. For the phagocytic cell activity assay, a two-way ANOVA was performed with exposure group and time as factors followed by a post-hoc Tukey HSD test.

Respiratory burst activity in the absence (-PMA) and presence (+PMA) was analyzed using one-way ANOVA. An α value of 0.05 was used to determine statistical significance throughout.

RESULTS

Confirmation of Endocrine Disruption

When hepatic *vtg* and *ERα* expression were assessed for confirmation of endocrine disruption in experiments 1 and 2, significant downregulation was observed for *ERα*, but not *vtg* (ANOVA, p = 0.04 and 0.20, respectively, Fig. 1). However, fold changes between groups in *vtg* expression demonstrated a dose-dependent decline. The 10 µg/L FAD group had a 40.1-fold decrease in *vtg* expression relative to the control group, while the 50 µg/L FAD group had a 272.6-fold decrease.

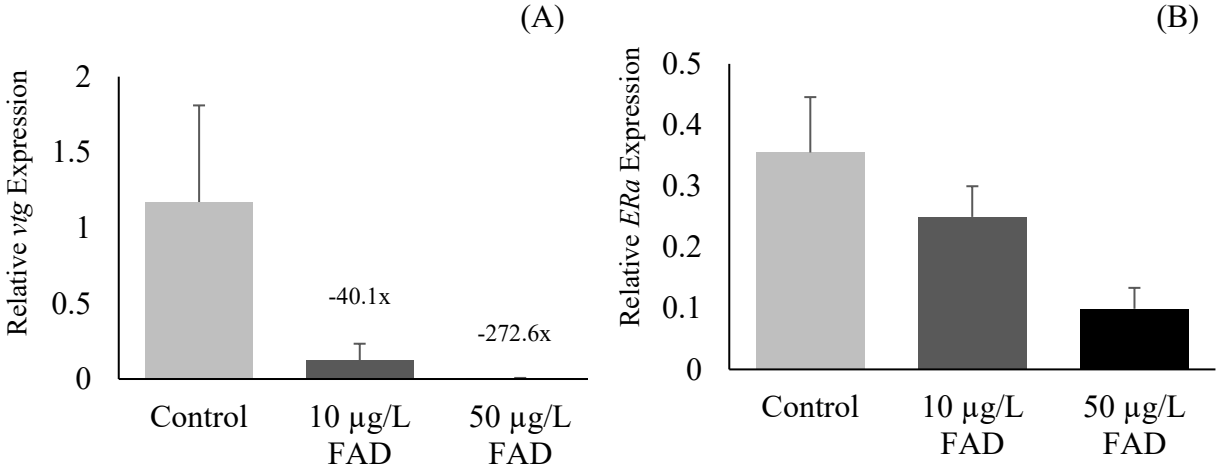

Figure 1. Relative expression of (A) vitellogenin (*vtg,* p = 0.20*)* and (B) estrogen receptor alpha (*ERα;* p = 0.04) for control, 10 µg/L FAD, and 50 µg/L FAD-exposed fish (n = 18/group). Numbers above columns indicate fold change relative to control group. Error bars represent standard error. ANOVA.

Assessment of endocrine disruption fish from experiment 3 revealed significant differences in the expression of both hepatic *vtg* and *ERα* between groups (ANOVA, $p < 0.01$ for each gene, Fig. 2). Specifically, *vtg* and *ERα* were significantly downregulated in both the 10 µg/L and 50 µg/L FAD-exposed fish relative to the controls but did not differ from one another.

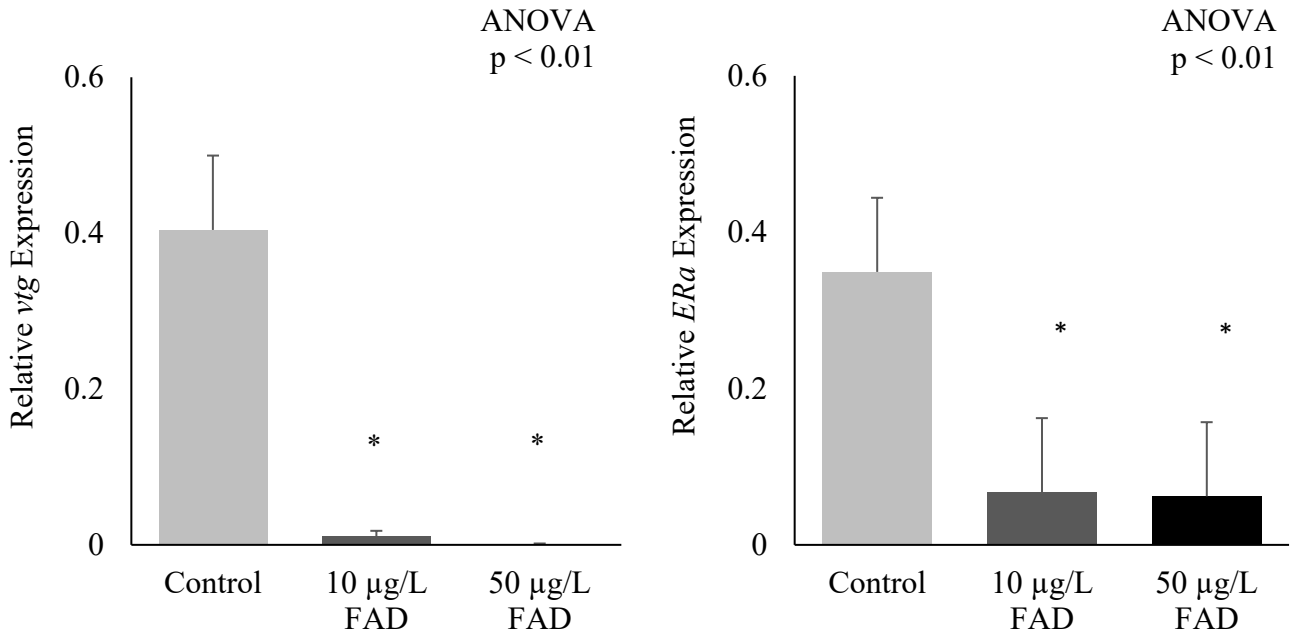

Figure 2. Relative expression of (A) *vitellogenin* (*vtg)* and (B) *estrogen receptor alpha* (*ERα*) for control, 10 µg/L FAD, and 50 µg/L FAD-exposed fish (n = 8/group). Asterisks indicate significant difference from the control group. Error bars represent standard error.

Experiment 1: Pathogen Resistance

Mortality did not differ between groups over the 14 d monitoring period ($p = 0.87$, Fig. 3A). Mean overall survival (standard error) was 36.82% (10.86) for control fish, 40.74% (9.80) for fish exposed to 10 µg/L FAD, and 35.65% (13.42) for fish exposed to 50 µg/L FAD. Overall survival was not found to differ significantly between groups ($p = 0.95$, Fig. 3B).

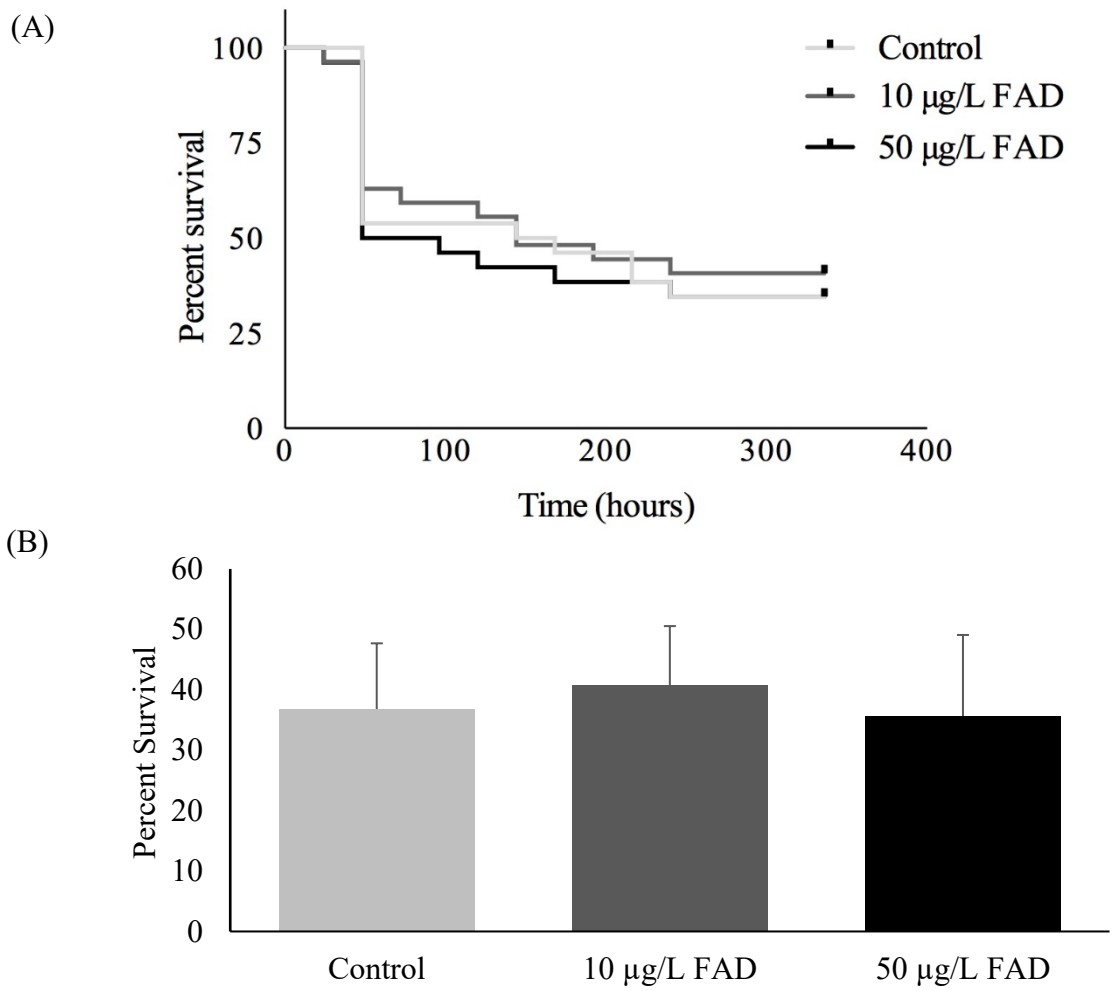

Figure 3. (A) Kaplan-Meier survival curve for fish exposed to 0, 10, and 50 µg/L FAD over 14-day monitoring following injection with *Yersinia ruckeri* (n = 26-27/group, log-rank test, p = 0.87). (B) Percent survival 14 days after pathogen injection for 0, 10, and 50 µg/L FAD exposure (n = 26-27/group, ANOVA, p = 0.95). Error bars represent standard error.

Experiment 2: Stimulated Immune Response

No significant differences were found in fish mass, SI, HSI, or percentage of leukocytes as a result of FAD exposure ($F_{2, 48}$ = 0.05, 0.79, 0.21, and 0.01, respectively, all p values > 0.46, Table 2). Similarly, there was not a significant effect of pathogen injection on fish mass, SI, or HSI (F = 0.20, 1.44, and 1.72, respectively, all p values > 0.20). Pathogen-injected fish did demonstrate a significant increase in percentage of leukocytes as compared to sham-injected

fish ($p < 0.01$). None of the tested morphometric endpoints demonstrated an effect of the interaction between FAD exposure group and pathogen injection (Table 2).

Analysis of hepatic *16S rRNA* gene expression revealed a significant main effect of pathogen exposure ($F_{2, 47} = 29.0$, $p < 0.01$, Fig. 4), but not of exposure group ($F = 0.51$, $p = 0.60$) nor the interaction between pathogen exposure and exposure group ($F = 0.51$, $p = 0.60$). While pathogen-injected fish experienced a significant decrease in hematocrit level as compared to sham-injected fish (2-way ANOVA, $F_{2, 45} = 46.3$, $p < 0.01$, Fig. 5), no significant differences in hematocrit were seen between FAD exposure groups ($F = 0.70$, $p = 0.50$, Fig. 5) or due to the interaction between FAD exposure group and pathogen injection ($F = 1.79$, $p = 0.18$).

Table 2. Means, standard error, and statistical output for fish mass, spleen index, hepatosomatic index, and leukocyte percentage (n = 7-11/group). All tests performed via two-way analysis of variance. FAD = fadrozole, FAD*SP = interaction between FAD and Sham/Pathogen factors.

Endpoint	Means (Standard Error)	Factor: F statistic, p value
Fish mass (g)		
Sham-injected		FAD exposure group: 0.04, 0.95
Control	2.09 (0.59)	
10 µg/L FAD	1.70 (0.34)	Sham/Pathogen: 0.20, 0.66
50 µg/L FAD	1.64 (0.69)	
Pathogen-injected		FAD*SP: 3.07, 0.06
Control	1.63 (0.26)	
10 µg/L FAD	1.92 (0.76)	
50 µg/L FAD	2.09 (0.54)	
Spleen index		
Sham-injected		FAD exposure group: 0.79, 0.46
Control	0.09 (0.03)	
10 µg/L FAD	0.14 (0.08)	Sham/Pathogen: 1.44, 0.24
50 µg/L FAD	0.15 (0.11)	
		FAD*SP: 1.54, 0.22
Pathogen-injected		
Control	0.12 (0.05)	
10 µg/L FAD	0.09 (0.05)	
50 µg/L FAD	0.11 (0.05)	
Hepatosomatic index		
Sham-injected		
Control	1.57 (0.66)	
10 µg/L FAD	1.87 (1.08)	FAD exposure group: 0.21, 0.81
50 µg/L FAD	1.38 (0.68)	
		Sham/Pathogen: 1.72, 0.20
Pathogen-injected		
Control	1.93 (0.63)	FAD*SP: 1.10, 0.34
10 µg/L FAD	1.73 (0.62)	
50 µg/L FAD	1.92 (0.56)	
Leukocyte percentage		FAD exposure group: 0.01, 0.99
Sham-injected		Sham/Pathogen: 10.0, < 0.01
Control	4.57 (2.79)	
10 µg/L FAD	5.29 (4.41)	FAD*SP: 0.48, 0.62
50 µg/L FAD	4.57 (2.27)	
Pathogen-injected		
Control	2.64 (2.92)	
10 µg/L FAD	1.82 (1.52)	
50 µg/L FAD	2.73 (2.38)	

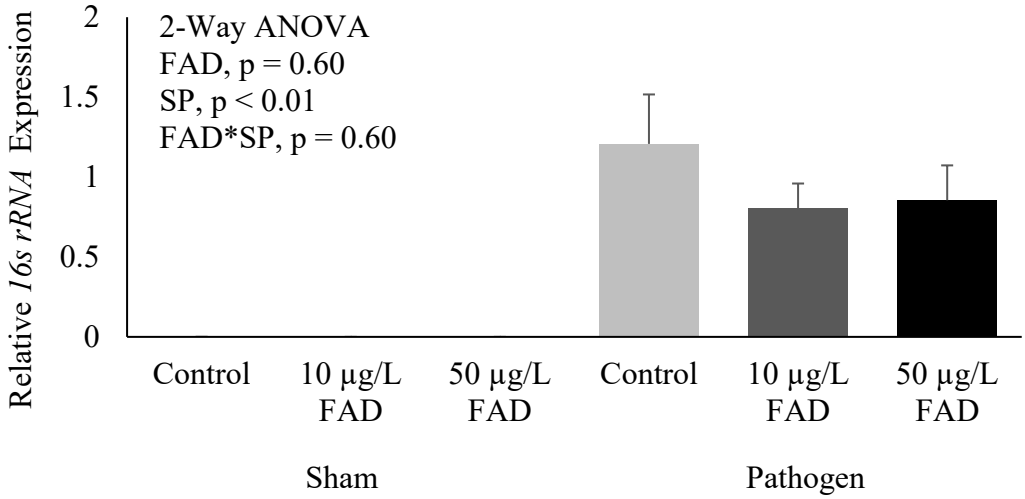

Figure 4. Relative hepatic *16S rRNA* expression in sham- and-pathogen injected fish from the control and fadrozole (FAD)-exposed groups (n=7-11/group). Error bars represent standard error.

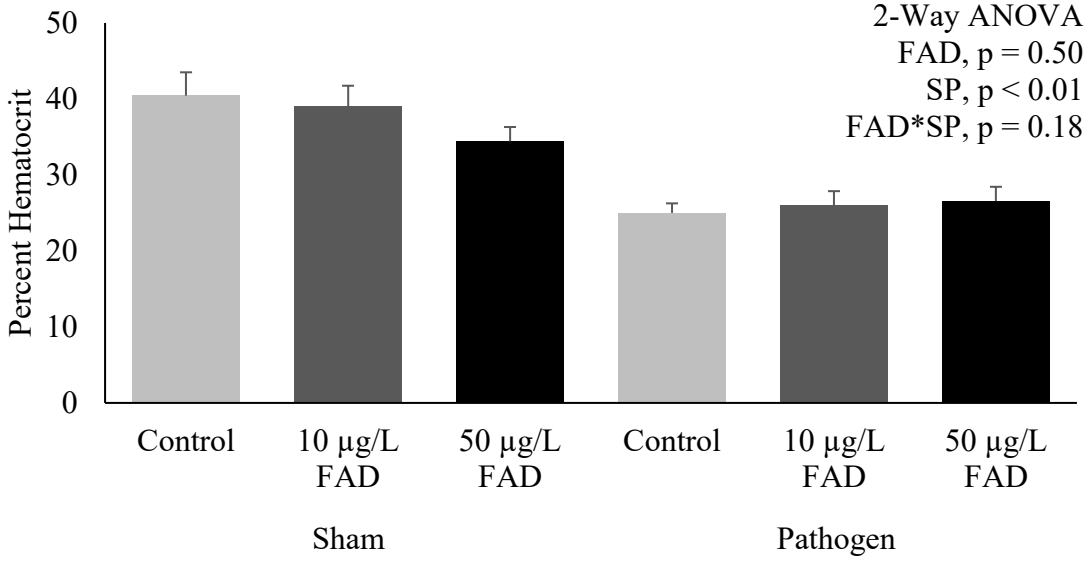

Figure 5. Percent hematocrit in sham- and pathogen-injected fish in the control and fadrozole (FAD)-exposed groups (n = 7-11/group). Error bars represent standard error.

Analysis of genes involved in inflammation (*il-1β, il-11, il-10,* and *tnfα)* showed no significant differences as a result of FAD exposure (2-way ANOVA, $F_{2, 47} = 0.27$, 0.68, 0.41, and 0.38, respectively, all p-values ≥ 0.50, Fig. 6) nor the interaction between FAD exposure and

pathogen injection ($F_{2, 47}$ = 0.29, 0.74, 0.38, and 0.31, respectively, all p-values ≥ 0.48). However, pathogen-injected fish demonstrated significantly upregulated expression of *il-1β, il-11, il-10, and tnfα* as compared to sham-injected fish ($F_{2, 47}$ = 52,8, 45.2, 43.1, and 59.6, respectively, all p-values ≤ 0.01).

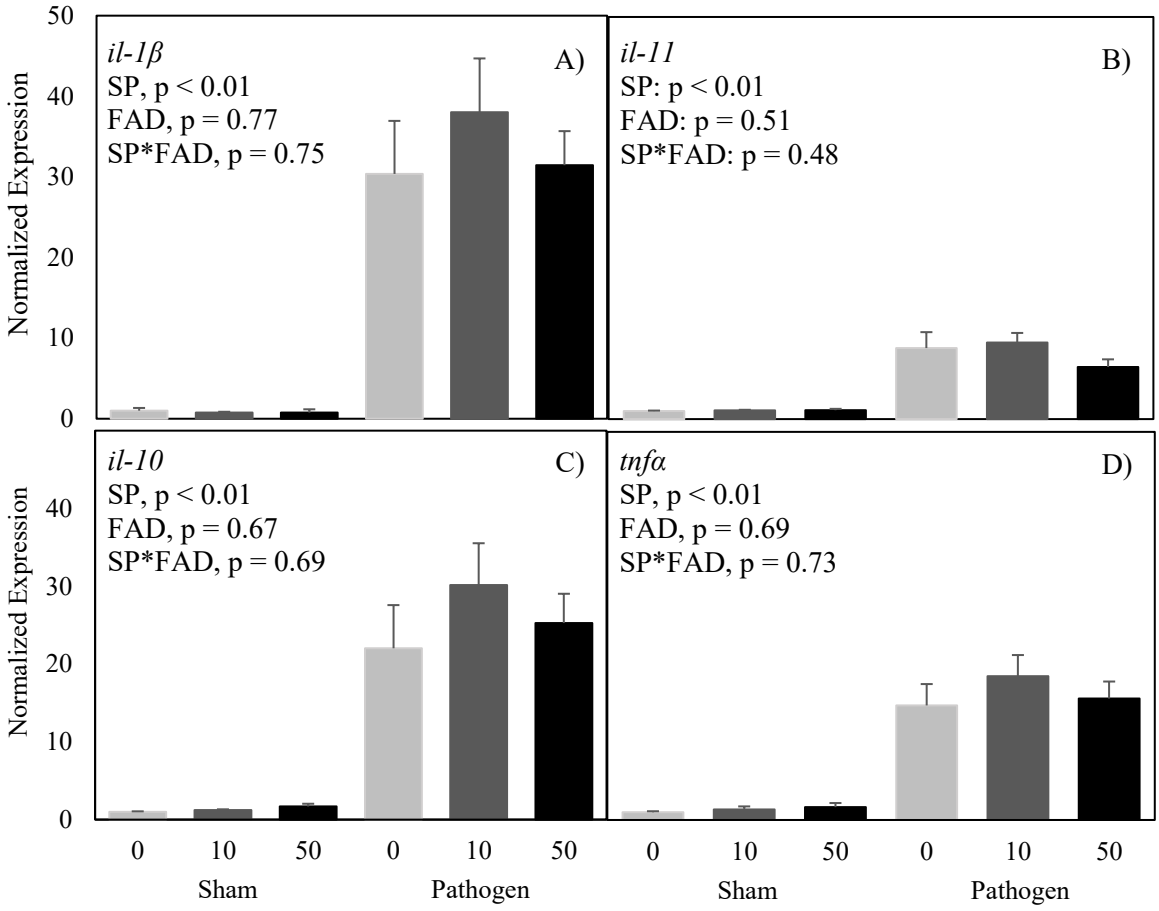

Figure 6. Relative expression of the target immune function genes (A) *interleukin 1β* (*il-1β),* (B) *interleukin 11* (*il-11),* (C) *interleukin 10 (il-10),* and (D) *tumor necrosis factor alpha (tnfα)* in sham- and pathogen-injected fish from the control (0), 10 μg/L fadrozole (FAD) (10) and 50 μg/L FAD (50) groups (n=16-18/group). All values are normalized to control sham values. Error bars represent standard error. All analyses were completed via two-way analysis of variance.

Analysis of the complement genes *c3* and *c9* similarly showed no significant differences resulting from the FAD exposure (2-way ANOVA, $F_{2, 47} = 0.62$ and 0.41, respectively, all p values ≥ 0.54, Fig. 7) or the interaction between FAD exposure and pathogen injection (2-way ANOVA, F = 1.23 and 0.01, respectively, all p values ≥ 0.30). While a significant upregulation of *c9* was noted in the pathogen-injected fish as compared to the sham-injected fish ($F = 49.5$, $p < 0.01$), this same relationship was not observed for *c3* ($F = 0.51$, $p = 0.48$).

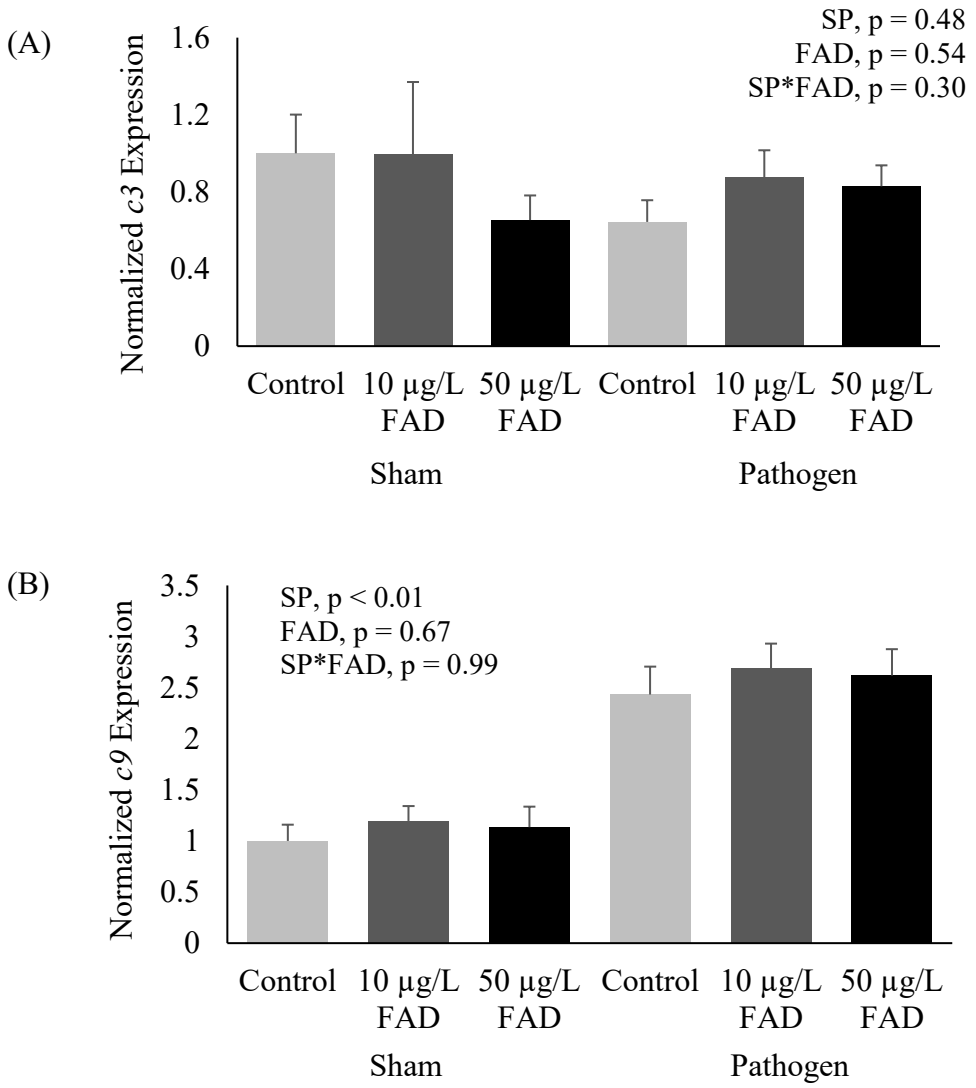

Figure 7. Relative expression of the target immune function genes (A) *complement component c3* (*c3) and* (B) *complement component c9* (*c9)* in sham- and pathogen-injected fish from the control, 10 µg/L fadrozole, and 50 µg/L fadrozole groups (n=16-18/group). All values are normalized to control sham values. Error bars represent standard error. 2-way ANOVAs.

Experiment 3: Cellular Assays

In the respiratory burst assay, mean (standard error) absorbances at 620 nm of unstimulated cells were 0.14 (0.02), 0.15 (0.03), and 0.11 (0.02) for the control, 10 µg/L FAD, and 50 µg/L FAD fish, respectively. No significant differences were found in absorbances due to FAD

exposure (ANOVA, p = 0.47, Fig. 8). In the stimulated cells, absorbances were 0.69 (0.07), 0.65 (0.04), and 0.50 50 (0.05) for control, 10 µg/L FAD, and 50 µg/L FAD fish, respectively. Similarly, no significant differences were found in the stimulated cells due to FAD exposure (ANOVA, p = 0.06)

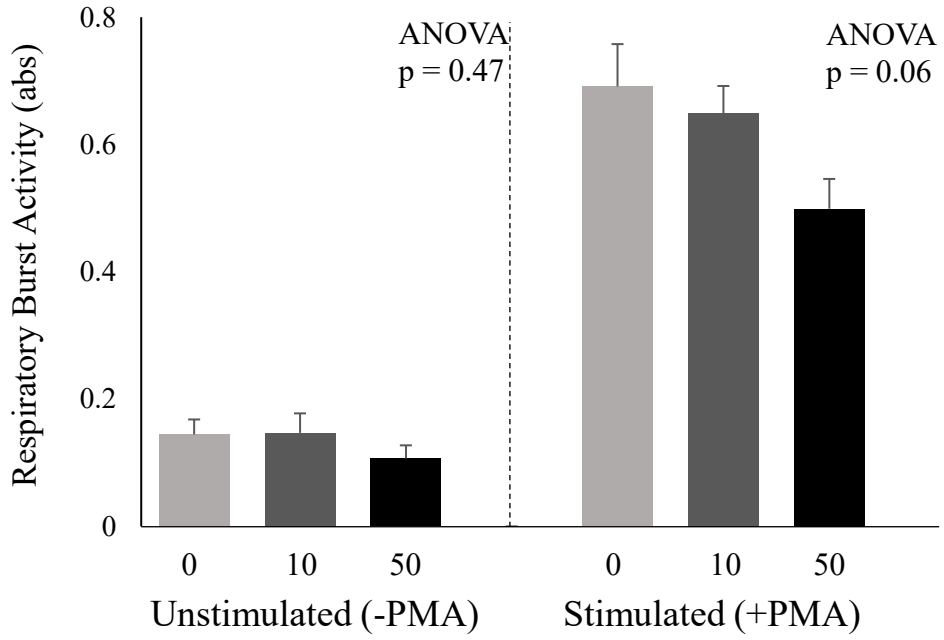

Figure 8. Respiratory burst activity (as measured via absorbance at 620 nm). ANOVAs performed for differences between control (0), 10 µg/L fadrozole (10) and 50 µg/L fadrozole (50) groups for both unstimulated (p=0.47) and stimulated cells (0.06) (n=5/group). Error bars represent standard error.

In the phagocytosis assay, a significant effect was seen due to both FAD exposure (2-way ANOVA, $F_{2, 36} = 4.10$, p = 0.03, Fig. 9) and time (F = 50.1, p < 0.01), but not due to the interaction between group and time (F = 1.40, p = 0.25). With regard to time, all tested time points were significantly different from each other, with fluorescence intensity increasing at each time interval. With regard to FAD exposure history, the 10 µg/L and 50 µg/L FAD

exposure groups were found to differ significantly from one another. The control group was not found to differ significantly from either the 10 µg/L or 50 µg/L FAD exposure groups.

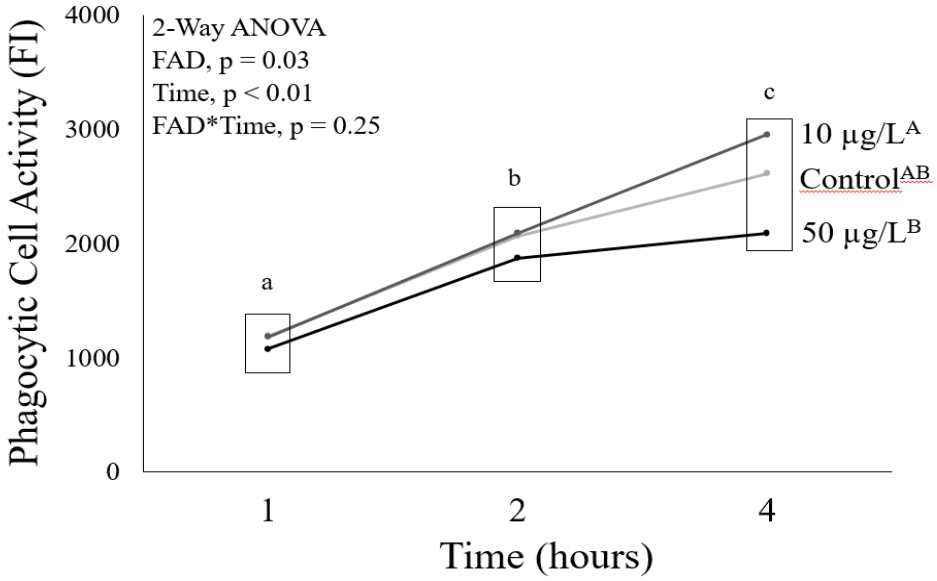

Figure 9. Phagocytic cell activity after 1, 2 and 4 hours of incubation with FITC-labeled *E. coli*, as measured by fluorescence intensity (FI), in control and fadrozole (10 and 50 µg/L) fish (n=5/group). Differences in lowercase letters indicate significant differences between time points. Differences in uppercase letters indicate significant differences between fadrozole exposure groups.

DISCUSSION

The goal of the present study was to assess the impact of estrogen suppression on immune function parameters in female fathead minnows. This approach represents an alternative study design by which the impact of estrogen levels on immune function can be tested, as estrogenic exposures are most commonly employed. A dose-dependent decline in estrogen levels, as assessed by hepatic expression of *vtg* and *ERα*, was demonstrated with statistical significance in experiment 3 and in *ERα* in experiments 1 and 2, signaling the effectiveness of the exposure regimen in suppressing estrogen levels. While a statistically significant decrease in *vtg*

expression was not found for experiments 1 and 2, the fold-change decreases seen in *vtg* expression suggest biologically-significant estrogen suppression.

Pathogen Resistance

No differences in pathogen resistance were observed in either of the FAD-exposed groups relative to those in the control group, providing evidence that reduced estrogen signaling induced by FAD exposure did not alter pathogen resistance. Two potential conclusions could be drawn from these results. The first is that estrogen levels do not influence immune function. However, this seems unlikely given the results of previous studies demonstrating ability of estrogens to modulate immunity. In a study done using rainbow trout (*Oncorhynchus mykiss*), Shelley et al. (2013) found that exposure to E2 without immune stimulation led to alterations in hematocrit levels and lymphocyte proliferation. Similarly, Dong et al. (2017) demonstrated the ability of an EE2 exposure to alter the plasma complement profile in marine medaka. A second conclusion is that while estrogens may impact immune function, the consequence to the overall wellbeing of the organism is not sufficient to alter mortality in the face of a pathogen challenge. For example, enhancement of immune cell processes such as phagocytosis, while a benefit, may not provide a substantial enough alteration to the immune function of an organism such that lesser mortality is seen when faced with a pathogen. While the present study did not find alterations in many of the tested sublethal immune endpoints, alterations in these endpoints have previously been noted in response to both estrogenic and antiestrogenic exposures (Seemann et al. 2013; Law et al. 2001; Watanuki et al. 2002). It is also possible that the effects of estrogen levels on immune function are dependent on factors such as the dose of the estrogenic compound as well as the species, sex, and life stage of the study organism.

Stimulated Immune Response

Significant upregulation of the *16s rRNA* gene in the pathogen-injected fish relative to the sham-injected fish provides support that the pathogen injection regimen was successful in infecting the fish. Likewise, the statistically significant upregulation of genes associated with complement and inflammation in pathogen-exposed fish relative to sham-injected fish indicates that the pathogen exposure was successful in initiating immune responses. Despite the differences between sham- and pathogen-exposed fish, none of the target immune genes differed significantly in expression as a result of the FAD exposure. These genes, involved in the inflammation and complement pathways, have previously been shown to have been modulated in hepatic tissue in response to estrogenic exposures both in resting and activated immune states (Wenger et al. 2012; Dong et al. 2017; Seemann et al. 2013). Thus, the lack of significant differences in the expression of inflammation and complement between control and FAD-exposed fish was unexpected given the demonstrated estrogen suppression in the FAD-exposed groups. These results suggest that FAD exposure did not impact either the inflammation or complement pathways at the molecular level. No differences were found between FAD exposure groups in the morphometric endpoints assessed in experiment 2: mass, SI, HSI, leukocyte percentage, or hematocrit (Table 2), providing further evidence that immune function was not impacted by the exposure regimen.

Inflammation has been a primary target in the attempt to understand estrogenic modulation of immune function. In a study involving gilthead seabream (*Sparus aurata*), Cabas et al. (2012) found that exposure to 17α-ethinylestradiol (EE2) led to inhibition of *il-1β* gene expression as well as increased mRNA levels of *il-6* and *tnfα* in non-activated macrophages. Seemann et al.

(2013) likewise found that E2 exposure caused downregulation of *il-1β* and *tnfα* in juvenile sea bass (*Dicentrarchus labrax*). Support has also been demonstrated for the ability of estrogen levels to impact complement pathways. In a gene ontology study conducted in juvenile rainbow trout, Wegner et al. (2011) demonstrated that the expression of 62 genes involved in the immune response was altered in hepatic tissue resulting from E2 exposure. Of these, 16 were involved in complement activation. Dong et al. (2017) found that plasma levels of the complement proteins C3dg and C1Q in marine medaka (*Oryzias melastigma*) were suppressed in response to EE2 exposure. In a similar study, Ye et al. (2018) demonstrated that EE2 exposure led to the downregulation of hepatic *c3* expression in both male and female marine medaka.

Overall, the expression of only six genes was tested in the present study: 4 involved in inflammation and 2 involved in complement pathways. It is possible that assessment of the expression of different genes within these pathways, or of genes within separate immune function pathways, could yield statistically significant differences in expression resulting from the exposure. However, the genes selected for analysis in the present study reflect those most well-studied and well-supported in the current body of literature. Expression of all of the genes targeted in this study was measured in hepatic tissue. The liver is an important immunological organ which has been demonstrated to actively transcribe and translate proteins in the inflammation and complement pathways, making it a logical choice for analysis (Thornton et al. 2016). However, the kidney and spleen are also important tissues which contribute to immune function in fish (Cabas et al. 2012; Casanova-Nakayama et al. 2011). It is possible that changes in expression not seen in hepatic tissue could be occurring in these tissues.

Immune Cell Function

While no statistically significant differences in respiratory burst were observed due to FAD exposure, a slight dose-dependent decline was noted in the stimulated (+PMA) immune cells that may be indicative of biologically significant alterations. The 10 µg/L FAD-exposed group demonstrated a ~6% decrease in respiratory burst activity relative to the control group, whereas the 50 µg/L FAD-exposed group demonstrated a ~28% decrease relative to the control group. Respiratory burst is a key process employed by in the immune system to destroy pathogens, thus if decreases in estrogen levels correlate with decreased production of superoxide, this could indicate a weaker response to invading pathogens. Previous studies have demonstrated the ability of both estrogenic and antiestrogenic compounds to modulate respiratory burst activity. For example, mice (*Mus musculus*) neutrophils treated with E2 by Abid et al. (2017) demonstrated increased respiratory burst activity, yet also exhibited decreased ability to eliminate the pathogen *Pseudomonas aeruginosa.* The antiestrogen Tamoxifen, an estrogen receptor modulator, has correspondingly been found to inhibit respiratory burst activity in equine neutrophils (Olave et al. 2019). Collectively, these results indicate that the process of respiratory burst is likely impacted by estrogen levels.

The phagocytic cell activity assay measures the ability of the immune cells to phagocytose a pathogen, FITC-labeled *E. coli*. Thus, an increase in phagocytic cell activity indicates enhanced immune function and a higher capacity to clear pathogens from the system. Conversely, decreased phagocytic cell activity indicates a lower capacity to clear pathogens, which may contribute to the onset of disease. The results of this experiment did not show a clear relationship between estrogen suppression and phagocytic cell activity. While a

significant effect of the FAD exposure was observed, post-hoc analysis showed that only the 10 ug/L FAD and 50 ug/L FAD groups were significantly different from each other, with phagocytic cell activity being higher in the 10 ug/L FAD group than in the 50 ug/L FAD group and the control group falling in between the two FAD exposure groups. This could be interpreted as a nonmonotonic response, where small decreases in estrogen actually improved phagocytic cell activity and larger decreases in estrogen decreased phagocytic cell activity. This type of response has frequently been associated with estrogenic EDCs (Xu et al. 2017; Vandenberg et al. 2012; Arkoosh et al. 2015). Law et al. (2001) demonstrated that both E2 and EE2 exposure were capable of suppressing phagocytosis in two species of tilapia (*Oreochromis niloticus, O. aureus*). Similarly, Cabas et al. (2012) showed that EE2 exposure suppressed phagocytosis in gilthead seabream macrophages. These studies support the notion that estrogenic compounds may impact the process of phagocytosis.

Conclusions & Future Directions

Overall, the present study did not find significant differences in the immune function of female fathead minnows, as measured by pathogen resistance, hematocrit, leukocyte percentage, respiratory burst activity, and the expression of genes associated with inflammation and complement, in response to estrogen suppression induced by FAD exposures. Limited evidence suggests that cellular immune function may be altered as evidenced by the observed changes in phagocytic cell activity due to FAD exposure. However, these changes were subtle at best and did not occur in a dose-response fashion. This, combined with the lack of response in other endpoints measured, does not provide sufficient evidence to support the hypothesis that estrogen suppression leads to decreased female immune capacity. Furthermore, it is does

provide support for the hypothesized that differences in sex steroid hormone levels between males and females lead to sex-specific differences in immunity. However, this should not be taken as conclusive evidence that estrogen levels do not play a role in immunity, as a relatively small suite of immune pathways within a single model system were tested.

To further study potential impacts of estrogen levels on immune function in teleosts, future studies could use antiestrogens which operate through a different mode of action, employ a wider range of exposure concentrations, or analyze different suites of target genes. Characterizing the relationship between estrogen levels and immune function will help us to better understand the basis for the difference in male and female immunity in fathead minnows, and lead to more informed evaluation of estrogenic or anti-estrogenic contaminants in the environment. This line of research will also continue to describe crosstalk between the endocrine and immune systems, which is incompletely characterized in this study organism.

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ABSTRACT

HORMONES AND IMMUNITY: WHAT IS THE ROLE OF ESTROGEN IN IMMUNE FUNCTION?

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Observed differences in various aspects of immune function between males are females are hypothesized to stem, in part, from differences in estrogen levels. Though estrogens are generally considered to enhance immune function, the extent to which estrogen influences various immune processes is not well characterized. The objective of the present study was to assess the impacts of decreased estrogen levels on immune function. Fadrozole hydrochloride, an aromatase inhibitor, was utilized to reduce estrogen levels within female fathead minnows. Immune function was assessed at three biological levels of organization, allowing for a holistic view of immune function impacts. Specifically, pathogen resistance, phagocytic cell activity, respiratory burst activity, leukocyte percentage, and the expression of genes associated with inflammation and complement were analyzed. It was hypothesized that the immune function of the fadrozole-exposed females would be detrimented. However, only phagocytic cell activity was significantly altered by the exposure. Thus, the present study provides limited evidence supporting the ability of estrogen levels to modulate immunity.