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RESEARCH ARTICLE

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Androgen and prolactin manipulation do not induce changes in immunocompetence measures in a fish with male parental care

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Abstract

Prolactin and 11-ketotestosterone (11-KT) are important reproductive hormones in fishes, which may also influence immunocompetence. The immunocompetence handicap hypothesis states that higher androgen concentrations that support secondary sex traits are traded off against a decrease in immune system function. To test the relationships between these hormones and immunocompetence, we experimentally manipulated 11-ketotestosterone and prolactin in the freshwater fish, bluegill (*Lepomis macrochirus*) during parental care using implants that contained either 11-KT, prolactin, or an inert control. We vaccinated individuals to stimulate the acquired immune response, then measured immunocompetence as the number of granulocytes, lymphocytes and monocytes, and the expression of interleukin 8 in each sample. We did not observe any significant differences in the immune measures among the hormone treatments. Our results indicate that in bluegill, there is no trade-off between androgens or prolactin and immunocompetence.

KEYWORDS
11-ketotestosterone, ICHH, immune response, imunocompetence handicap hypothesis, prolactin

1 | INTRODUCTION

Hormone concentrations have been postulated to mediate investment in mating and parental care (Angelier & Chastel, 2009; Ketterson et al., 2015; Stearns, 1989), with a potential trade-off with immunocompetence (Folstad & Karter, 1992). In the freshwater bluegill sunfish (*Lepomis macrochirus*), androgens and other hormones are involved in nest building, courting females that visit the nest, and offering protection to the offspring for up to 10 days, as well as color displays, sperm production and other secondary sexual characteristics. If the same hormones also influence immunocompetence—the capacity of an organism to respond to a pathogen—it could represent an important constraint on the expression of traits related to reproduction. The immunocompetence handicap hypothesis (ICHH) posits that an increase in androgens, and consequent expression of secondary sexual characteristics, is linked to a decrease in immunocompetence, which can constrain the expression of androgen-regulated traits during breeding (Folstad & Karter, 1992). There is a need to better understand the relationships between reproductive hormones and immunocompetence and trade-offs involved.

The immune response generally has two components. The innate immune response is not specific to a particular pathogen and includes, for example, the phagocytosis of foreign cells by neutrophils and monocytes and the destruction of infected or foreign cells by natural killer lymphocytes (Bouman et al., 2005). Interleukin 8 (IL-8) is a protein that can activate neutrophils and the innate immune response (Bouman et al., 2005). Therefore, innate immunocompetence can be measured by expression of interleukins such as IL-8 and by the number of neutrophils and monocytes present in the blood (Kobayashi & DeLeo, 2009; Ros et al., 2006). Acquired

immunity, in contrast, is the capacity of the organism to respond to previously encountered antigens through the production of antibodies by B lymphocytes. When macrophages phagocyte an antigen, they will expose it to B and T lymphocytes, causing a specific immune response against that antigen. Therefore, acquired immunocompetence can be measured by the number of T and B lymphocytes in the blood (Ros et al., 2006).

The ICHH proposes that androgen concentrations show a negative association with immunocompetence, however, the evidence is not overwhelming, and reviews show both scenarios (see Foo et al., 2016; and Roberts et al., 2004). There are also few studies about the effects of androgens other than testosterone on immunocompetence, which is particularly relevant for fishes, a group that has 11-ketotestosterone (11-KT) rather than testosterone as the most potent androgen in stimulating secondary sexual characteristics, spermatogenesis, and reproductive behaviors (Borg, 1994). Three-spined sticklebacks (*Gasterosteus aculeatus*) implanted with 11-ketoandrostenedione, a precursor to 11-KT, had a reduced innate immune response, measured using the respiratory burst reaction, which is a rapid release of oxygen and peroxides when phagocytosis occur (Kurtz et al., 2007). Rainbow trout (*Oncorhynchus mykiss*) treated with an anti-androgen drug (butachlor) had increased neutrophil concentrations in the blood (Ahmadivand et al., 2015), suggesting that androgens have a negative effect on this measure of innate immunity. In mango tilapia (*Sarotherodon galilaeus*), antibody production in response to an injection with nonpathogenic antigens (sheep red blood cells) was negatively correlated with both testosterone and 11-KT concentrations (Ros et al., 2012). Conversely, Loggie et al. (2018) found no evidence that 11-KT concentrations were correlated with innate immunity measures, measured as mRNA levels of interleukins, respiratory burst luminescence (a measure of phagocyte activity), and lymphocyte and granulocyte counts in bluegill (*Lepomis macrochirus*). In a study of common carp (*Cyprinus carpio*), reduced IgM secretion was observed in cell cultures when testosterone concentrations were experimentally increased, while 11-KT concentrations had no effect on IgM secretion (Saha et al., 2004). Therefore, there is limited causal evidence that 11-KT concentrations directly affect immune responses; most studies are correlational or apply androgen inhibitors that could affect the response of other androgens, not only 11-KT.

Previous studies have indicated a positive relationship between prolactin concentrations and immunocompetence. In mammals, prolactin promotes proliferation and inhibits apoptosis of lymphocytes, thereby promoting immunocompetence (Freeman et al., 2000; and Buckley, 2001). In mammals, increased doses of prolactin stimulate the production of interleukin-12 (Brand et al., 2004). Further effects of prolactin on immunocompetence are indicated by the presence of prolactin receptors on T and B lymphocytes (Buckley, 2001). Prolactin likely has a similar effect on immunocompetence in fishes, although there have been few studies of this relationship. In rainbow trout (*Oncorhynchus mykiss*), prolactin is positively associated with the secretion of antibodies (Yada et al., 1999) and leukocytes (Yada et al., 2004), and the proliferation of lymphocytes (Narnaware et al., 1998; Sakai et al., 1996). In Atlantic salmon (*Salmo salar*), prolactin enhances macrophage activity (Paredes et al., 2013) and in gilthead seabream (*Sparus aurata*), high prolactin concentrations increase NADPH activity in leukocytes (Olavarría et al., 2012). However, the relationship between immunocompetence and prolactin concentrations has rarely been investigated in fishes other than the ones mentioned above.

In bluegill sunfish both 11-KT and prolactin are important reproductive hormones that influence the provisioning of parental care by parental males (Cunha et al., 2019). Bluegill implanted with 11-KT have reduced expression of immune-related genes (e.g., immuno-globulin heavy chain and MHC II alpha) compared to fish implanted with the androgen receptor antagonist flutamide, suggesting that high 11-KT concentrations may reduce immunocompetence in these fish (Partridge et al., 2015). Bluegill that received bromocriptine, a prolactin antagonist, have reduced nurturing behaviors toward their offspring, suggesting a role of prolactin during parental care (Kindler et al., 1991). Here, we measure the effects of experimentally manipulated concentrations of 11-KT and prolactin on immunocompetence in bluegill. We manipulated these hormones with implants that contained either 11-KT, prolactin, or an inert control. We then measured immunocompetence as the number granulocytes, lymphocytes, and monocytes (Németh & Mócsai, 2012) and the expression of interleukin 8 (de Oliveira et al., 2013). We vaccinated individuals to stimulate the acquired immune response, which we then measured as the number of lymphocytes. We hypothesize that 11-KT and prolactin will affect the immune response of parental males. We predict that the immune response will be the highest in the prolactin treatment and lowest in the 11-KT treatment for both innate and acquired immune measures.

2 | METHODS

2.1 | Experimental design

This experiment was conducted in June 2017 in Lake Opinicon, Ontario, Canada (44°34′N, 76°19′W). The fish used here are a subset of those used for a parallel study (Cunha et al., 2019). Parental male bluegill (n = 117) were collected on the day after spawning using dip nets and brought to a nearby boat, during which time their nests were covered to prevent nest predation. Bluegill nest in colonies that can contain dozens of nests, abiotic conditions were not measured due to the similarity of proximity and depth of nests. Fish were then anesthetized using clove oil. Three to four scales were removed from the abdomen and a small incision was made so two experimental implants could be inserted into the peritoneal cavity (for additional detail about the experimental design and treatment of these fish see Cunha et al., 2019). Each fish was assigned randomly to one of three hormone manipulation treatments: (1) control, implants contained castor oil; (2) 11-KT, each implant contained castor oil and 80 μg of 11-KT (Steraloids); and (3) Prolactin, each implant contained castor oil and 1.25 IU of prolactin (Sigma Aldrich #L6520). A saturated solution

of 1 ml of antibiotic oxytetracycline was injected into the incision, which was then closed with liquid bandage (New Skin; Prestige Brand Holdings). Fish were then randomly assigned to a vaccination treatment, receiving an intraperitoneal injection with 0.1 ml of either saline or 0.1 ml of a vibrio vaccine containing inactivated cultures of *Vibrio anguillarum* serotypes I and II and *Vibrio ordalii* (Vibrinogen 2 vaccine, Novartis Animal Health). Fish were assumed to be naïve to the vaccination since Vibrio is not common to freshwater environments (Aykanat et al., 2012). Fish were placed in a water-filled bucket and allowed to recover for at least 5 min, after which they were returned to their nest. Two days later, fish were captured and euthanized with an overdose of clove oil at which time the spleen was collected and preserved in RNAlater (Sigma Aldrich) before being stored at −80°C. Because some fish abandoned their nests following the manipulation our final sample size was $n = 59$.

2.2 | Immune gene expression

Total RNA was extracted from spleens using TRIzol (Life Technologies), and genomic DNA was removed using a Turbo DNA-free kit (Thermo Fisher Scientific). A qScript cDNA Synthesis kit (QuantaBio) was then used to create cDNA. Two reference genes (eukaryotic translation elongation factor 1 beta 2 (EF1-beta) and ribosomal protein subunit 18 (RPS18)) and one immune gene (interleukin 8 (IL8)) were examined. Primers for RPS18 and IL8 were from Loggie et al. (2018), while EF1-beta primers were designed based on sequences from related species (5′ and 3′ Forward: CGTGGGTTACGGCATCAAGA, Reverse: GATCTTGTTGAAAGCGGCGA). Gene expression was measured in duplicate using SensiFAST SYBR on a Biorad CFX thermocycler. The cycle was divided as follows: initial 3 min of denaturation (95°C), 40 cycles of 10 s (95°C) and 30 s of annealing (57°C), followed by fluorescence measurements before the start of a new cycle. Serial dilutions of pooled samples were used to measure each primer pair's qPCR amplification efficiency. RNA-free water was used as negative control for every gene. Gene expression of IL8 was calculated relative to the mean of EF1B and RPS18 following the ΔCT method of Livak and Schmittgen (2001). Expression values were normalized so that the fish that exhibited the highest expression for each gene had a value of one. Gene expression was measured in a total of 59 individuals, with a range of 8–12 individuals from each group.

2.3 | Cell counts

Blood smears were prepared within 4 h of blood collection and later stained with Giemsa's azur-eosin-methylene blue solution. Images of the blood smears were then recorded using a light microscope using a ×100 oil immersion lens. Cells were manually counted by an observer blind to the treatments, with an average of 3661 cells counted per individual (Range: 2009–8355). Erythrocytes, monocytes, granulocytes, and lymphocytes were counted. Erythrocytes were identified as elliptical cells with an oval nucleus; monocytes were identified as nongranular cells with an ellipsoidal nucleus; granulocytes were identified as large cells with granules on their cytoplasm (basophils, neutrophils, eosinophils, and mast cells), and lymphocytes were identified as small cells with a large nucleus and little cytoplasm (Ros et al., 2006). Cell counts for monocytes, granulocytes, and lymphocytes were expressed as a percentage of the total number of blood cells counted for each individual. Cell counts were performed in a total of 38 individuals, with a range of five to nine individuals from each combination of treatments.

2.4 | Hormone validation

11-KT concentrations were measured and published in Cunha et al. (2019). Hormone implants increased the plasma concentrations of the hormone. Prolactin and 11-KT implants were effective in changing the behavior of the treatments (Cunha et al., 2019), prolactin concentrations were not measured.

2.5 | Statistical analyses

Statistical analyses used SPSS version 25 (IBM) and JMP version 4.0.2 (SAS Institute Inc.). Expression of IL8 (data were log-transformed to achieve normal distributions) and cell counts (granulocytes, monocytes, or lymphocytes) were examined using ANOVA models that included hormone treatment (placebo, 11-KT, prolactin), vaccination treatment (vaccine, saline), and the interaction between hormone treatment and vaccination treatment as factors. When a model term was nonsignificant, we further calculated the least significant number of sampled individuals at which the observed effect size would be significant for that model term based on the observed data and the same ANOVA model structure, as implemented in JMP.

3 | RESULTS

Forty-eight hours following vaccination, we observed that vaccinated fish had higher expression of IL-8 than saline injected fish (control) ($F_{1,53} = 5.04$, $p = 0.029$; Figure 1a); however we did not observe a significant difference in the expression of IL-8 among hormone treatments ($F_{2,53} = 1.12$, $p = 0.34$) nor was there an interaction between vaccination and hormone treatment ($F_{2,53} = 0.62$, $p = 0.54$).

There were no significant differences in the monocyte counts among hormone treatments ($F_{2,32} = 0.86$, $p = 0.43$; Figure 1b), vaccination treatments ($F_{1,32} = 1.92$, $p = 0.18$), or based on the interaction of vaccination by hormone treatment ($F_{2,32} = 1.15$, $p = 0.33$). There were no significant differences in the granulocyte counts among hormone treatments ($F_{2,32} = 1.27$, $p = 0.12$; Figure 1c), vaccination treatments ($F_{1,32} = 3.26$, $p = 0.080$), or the interaction of vaccination by hormone treatment ($F_{1,32} = 0.70$, $p = 0.51$). There were no significant differences in the lymphocyte counts among hormone

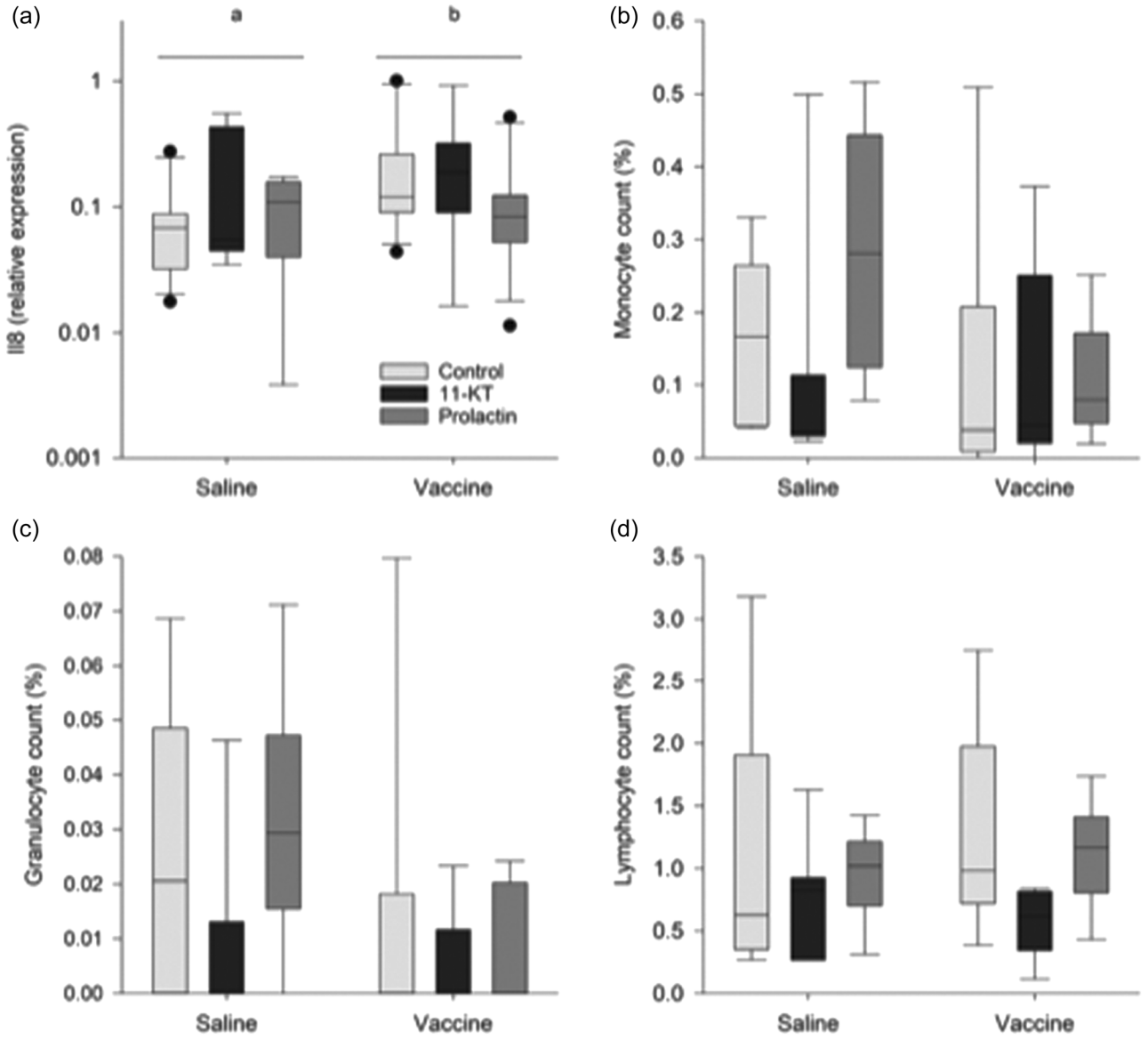

FIGURE 1 Immunocompetence measures for nest-tending bluegill (*Lepomis macrochirus*) exposed to experimental hormone manipulation and vaccination treatments. The immunocompetence measures are relative IL8 expression (a); monocyte count (b); granulocyte count (c) and lymphocyte count (d) Fish received either a control, 11-ketotestosterone or prolactin hormone manipulation treatment and either a saline or vaccine vaccination treatment. The boxplots display median, 25th, and 75th percentiles, with the whiskers representing the 10 and 90th percentiles. Data outside this range are shown. Significant differences among treatments are indicated by different letters above the bars.

treatments ($F_{2,32} = 1.77$, $p = 0.19$; Figure 1d), vaccination treatments ($F_{1,32} = 0.16$, $p = 0.69$), or the interaction of vaccination by hormone treatment ($F_{2,32} = 0.38$, $p = 0.69$).

4 | DISCUSSION

The literature on the ICHH is far from being resolved (Foo et al., 2016; Roberts et al., 2004), and the effect of 11-KT on immunocompetence in fish is even less clear. In the present study, we did not find evidence that experimentally increasing 11-KT concentrations (Cunha et al., 2019) affected measures of either the innate immune response (granulocyte count, monocyte count, and IL-8 expression) or the acquired immune response (lymphocyte count), thus our results do not support the immunocompetence handicap hypothesis. By returning the fish to their nests, where they continued to perform parental care and nest defense, we were able to behaviorally assess the effects of the hormones. The experimental 11-KT manipulation approach used in this study was previously shown to increase plasma 11-KT concentrations and nest defense in bluegill (Cunha et al., 2019), so it is very unlikely that our null results on immunity arises from a lack of increase in 11-KT concentrations. An earlier correlational study in bluegill similarly showed no relationship between 11-KT and immunocompetence (Loggie et al., 2018), while an in vitro study in

carp found no effect of 11-KT on immune measures in blood cells and also did not support the ICHH in fish. One of the few studies to contrast with this pattern involved three-spined sticklebacks, in which 11-ketoandrostenedione injections led to a change in respiratory burst response (Kurtz et al., 2007). This study used 11-ketoandrostenedione, a precursor of 11-KT, so it is possible that this result was specific to 11-ketoandrostenedione rather than 11-KT. Although there is currently limited evidence that 11-KT influences immunocompetence directly, differentiating which androgens and intermediaries specifically affect immunocompetence might shed light on the possible, if any, control mechanisms involved in androgen actions in fish immune systems.

Prolactin has a well-documented effect of promoting immunocompetence in mammals (e.g., Redelman et al., 2008), although studies examining this relationship in fish are less common. Our data does not show an effect of prolactin on the immune response of bluegill sunfish, as prolactin implants had no effect on the abundance of lymphocytes, granulocytes, monocytes, or the expression of IL-8. These findings contrast with a number of previous studies that have shown a positive relationship between prolactin and immune measures, including enhanced IgM expression in rainbow trout (Yada et al., 1999), increased phagocytic expression in Atlantic salmon (Paredes et al., 2013), higher mitosis rate of lymphocytes in chum salmon (Sakai et al., 1996), increased expression of NADPH oxidase subunit p47phox (a signal of phagocyte activity) in gilthead seabream (Olavarría et al., 2012), and increase in phagocytosis and lymphocyte count in silver seabream (Narnaware et al., 1998). Previous studies of prolactin effects on immunocompetence in fishes have predominantly used prolactin injections in the peritoneal cavity as the method of administration (e.g., Cuesta et al., 2006; Narnaware et al., 1998; Paredes et al., 2013), whereas our study used slow-releasing implants. This difference might explain the absence of an effect on immunocompetence if the implants had a limited impact on prolactin concentrations. However, our implants included a higher overall dosage of prolactin than other studies (e.g., 118 μg/g fish compared to 5 μg/g fish in Cuesta et al., 2006). Our implants have previously been shown to increase nurturing behavior at the same dosage in bluegill (Cunha et al., 2019). The rate of hormone release of our implants might have not been a sufficient increase to stimulate the immune response but was enough to cause an increase in parental behaviors (Cunha et al., 2019). Another possible explanation for the null effects on immune responses might be in the immune measurements we chose; a broader selection might show which are influenced by the hormone, such as immunoglobulin concentration, phagocytosis, and determination of other inflammatory cytokines.

In this study using bluegill, neither 11-KT nor prolactin had an effect on immunocompetence. Prolactin, in bluegill, does not have the same positive effect on the immunocompetence as reported in tetrapods. Interestingly, immunocompetence does not limit individual 11-KT concentrations. Given the positive effects of 11-KT on other aspects of mating and parental care, and if the ICHH is to be supported, we would expect a trade-off between the immune response and androgen concentrations, which androgen, if any, will affect the immune response in fish remains to be determined.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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