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Research paper

A test of the effects of androgens on immunity: No relationship between 11-ketotestosterone and immune performance in bluegill (*Lepomis macrochirus*)

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ABSTRACT

The immunosuppressive effects of androgens are a key component of the immunocompetence handicap hypothesis (ICHH). Here, we use bluegill sunfish (*Lepomis macrochirus*) to test two predictions arising from this hypothesis: (1) natural circulating concentrations of the androgen 11-ketotestosterone (11-KT) will be negatively related with measures of immunity, and (2) immune stimulation will lower circulating 11-KT concentration. We found no evidence for a relationship between natural circulating 11-KT concentration and measures of immunity (lymphocyte and granulocyte counts, respiratory burst, cytokine mRNA levels), and an immune stimulation with *Vibrio* vaccine did not affect circulating 11-KT concentration. We also performed a meta-analysis of immune stimulation studies to help interpret our results, and report evidence suggesting that immune stimulation has weaker effects on androgen levels in fishes compared to other vertebrates. These results suggest that the ICHH may not apply to all vertebrates, although it remains premature to state what factors account for the weaker evidence in fishes that androgens are immunosuppressive.

1. Introduction

The immunocompetence handicap hypothesis (ICHH) proposes that androgens linked to the development and maintenance of traits that increase male reproductive success may also act to suppress immunity (Folstad and Karter, 1992). Since the introduction of the ICHH, many studies have been conducted with the aim of testing the hypothesis that androgens are immunosuppressive. Three predictions resulting from this hypothesis have been tested: (1) natural androgen levels will be negatively related with measures of immune function, (2) experimentally elevated androgen levels will suppress immune function, and (3) immune stimulation will lower androgen levels. To date, most of these studies have been conducted in taxa whose primary androgen is testosterone. Studies testing each of the three predictions have produced mixed results, with some finding support for testosterone having immunosuppressive effects (e.g. Alonso-Alvarez et al., 2009; Garamszegi et al., 2004; Halliday et al., 2014), others finding no support (e.g. Kasilima et al., 2004; Ros and Oliveira, 2009), and others finding evidence that contradicted the hypothesis of testosterone having immunosuppressive effects (e.g. Bilbo and Nelson, 2001; Peters, 2000).

Meta-analyses are a useful tool for evaluating the relationship between testosterone and immunity across taxa. The first meta-analysis of ICHH studies focused on studies that had experimentally manipulated circulating testosterone concentrations and measured the effects on immunity, thus testing prediction 2 (Roberts et al., 2004). This analysis found no immunosuppressive effect of testosterone manipulation in birds or mammals, although an immunosuppressive effect of testosterone manipulation was found in reptiles. A second meta-analysis examined studies in birds and mammals in which the effect of immune stimulation on circulating testosterone concentration was tested (prediction 3), and found a significant decrease in testosterone concentration when the immune system is stimulated, regardless of whether it was stimulated with a live pathogen or non-pathogenic antigen (Boonekamp et al., 2008). A third meta-analysis included both testosterone manipulation experiments (prediction 2) and testosterone-immunity correlation studies (prediction 1) and

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found support for prediction 2 but not prediction 1 (Foo et al., 2017). The findings of this recent meta-analysis differ from those of Roberts et al. (2004), who did not find an overall effect of testosterone manipulation on immunity. The large number of studies published since 2004, along with improvements in meta-analytical techniques were cited by Foo et al. (2017) as the most likely causes for the differences in findings between the two analyses. Overall, these analyses show significant support for predictions 2 and 3, which suggests that testosterone is indeed immunosuppressive.

There has been interest in determining if the ICHH can be generalized to all androgens, but relatively few studies have tested the relationship between immunity and androgens other than testosterone. Within the theoretical framework of the ICHH, any androgen that provides benefits to reproduction, i.e. by regulating sexual signaling, should be associated with an immunosuppressive cost. It is therefore important to examine the relationships between androgens other than testosterone and immunity, to determine whether the ICHH applies to all reproductively relevant androgens. In fishes, 11-ketotestosterone (11-KT) is the primary androgen, both in terms of circulating concentrations during breeding and in terms of effects on reproductive traits and behaviours (Borg, 1994). To date, equivocal results have been obtained across studies testing each of the three ICHH predictions in fishes, with both supportive (e.g. Kortet et al., 2003; Kurtz et al., 2007; Partridge et al., 2015) and non-supportive (e.g. Law et al., 2001; Ros et al., 2006a, b; Ros et al., 2012) results obtained. More studies are needed to determine whether 11-KT in fishes demonstrates the same relationship with immunity as testosterone in other vertebrates.

Here, we examine the relationship between 11-KT and immunity in bluegill (*Lepomis macrochirus*). Many aspects of the role of 11-KT in the mating system of bluegill are well-understood. At the onset of breeding activity, circulating 11-KT concentrations are elevated in parental males (Magee et al., 2006). These elevated 11-KT concentrations coincide with high levels of aggressive behaviour amongst parental males, who construct and defend nests in close proximity to one another prior to spawning. A positive relationship between circulating 11-KT concentration and paternity (measured as the proportion of offspring sired by the nest-tending male) has been found (Neff and Knapp, 2009). No correlation between androgen levels (11-KT, testosterone) and cortisol levels has been observed in male bluegill (Magee et al., 2006; Neff and Knapp, 2009), so immunosuppressive effects of cortisol are unlikely to confound the interpretation of the relationship between androgens and immune performance in these fish. Bluegill thus offer a suitable system in which to study the immunosuppressive effects of 11-KT.

Here, we tested predictions 1 and 3 arising from the hypothesis that androgens are immunosuppressive. We examined the relationship between natural circulating 11-KT concentration and components of both resting immunity and the immune response, and used an experimental immune stimulation to determine whether circulating 11-KT concentration decreases during the early innate immune response. We also performed a meta-analysis of immune stimulation studies testing prediction 3, in which we included and differentiated between studies in species that use testosterone or 11-KT as their primary androgen. If androgens are immunosuppressive, we predicted that there would be a negative relationship between natural circulating 11-KT concentrations and measures of both resting immunity and the innate immune response. We also predicted that if androgens are immunosuppressive, circulating 11-KT concentrations would drop following an experimental immune stimulation. Finally, if androgens are immunosuppressive, we predicted that we would find an overall negative effect of immune stimulation on circulating androgen concentrations across taxa.

2. Methods

2.1. Resting immunity study

Field collections took place at Queen's University Biological Station, located on Lake Opinicon (44°34′N, 76°19′W). Our first objective was to measure circulating 11-KT concentrations as well as levels of resting immunity in a wild population of bluegill (*Lepomis macrochirus*) during the breeding season. From June 4 to 19 2015, parental male bluegill (n = 24) were collected either by angling in pelagic areas or with dip nets in littoral areas while males were guarding nests that contained few or no eggs (≤10% of the nest surface covered by eggs, such that males were assumed to be in the early stages of spawning). For all fish, 400 μL of whole blood was drawn from the caudal vessel using a heparinized syringe. The fish were then measured for total body length and released. The time from collection to the end of blood sampling was recorded for each fish (always <2 min from capture). For the respiratory burst assay, 200 μL of whole blood was then transferred into tubes containing 20 μL of 600 units/mL ammonium heparin. The remaining 200 μL of blood was transferred into non-heparinized tubes for blood smear cell counts (lymphocyte and granulocyte) and hormone analyses. Blood samples were kept on ice after collection and then stored at −20 °C prior to 11-KT analysis.

2.2. Immune stimulation study

To test whether circulating 11-KT concentrations decline following immune stimulation, on June 15, 2015 we captured a group of parental males (n = 11) using dip nets while they were guarding nests that contained few or no eggs. We measured total body length and clipped a unique combination of dorsal spines on each individual to allow subsequent identification. We then transferred each individual to a separate 60 × 60 × 60 cm floating net pen situated in the littoral zone of the lake. Previous trials have shown that floating net pens support confinement of bluegill for up to 14 days with low mortality and no elevation of glucose levels that would indicate an energetic response to a stressor (Gutowsky et al., 2015).

Individuals were given 24 h to become accustomed to the net pens, and were then randomly assigned to one of two treatments: one group received an intraperitoneal injection of 0.1 mL of a sterile 0.85% NaCl solution (n = 6), and the other group received an intraperitoneal injection of 0.1 mL of Vibrogen 2 vaccine (n = 5; Novartis Animal Health, Charlottetown, PEI, Canada). The vaccine contained formalin-inactivated cultures of *Vibrio anguillarum* serotypes I & II and *Vibrio ordalii*, and was administered to stimulate both innate and adaptive immune responses (e.g. Aykanat et al., 2012). Following the injections, all fish were placed back into their net pens. After 24 h we removed fish from their net pens and drew 200 μL of whole blood from the caudal vessel using a heparinized syringe for 11-KT analysis. Fish were then euthanized via immersion in a concentrated solution of clove oil. Immediately after euthanization, the head kidneys were collected to examine cytokine mRNA levels via qPCR. Head kidneys were placed into RNAlater (Life Technologies, Carlsbad, CA) and stored at 4 °C overnight to ensure the RNAlater penetrated the tissue. Samples were then transferred into liquid nitrogen, and were later transported on dry ice to Western University and stored at −80 °C.

To address our third objective, measuring the relationship between circulating 11-KT concentrations at the time of capture and the cytokine response following vaccination, we caught another group of parental males (n = 11) on June 24, 2015. The males were caught while guarding nests (<10% of nest surface covered by eggs) using a dip net. For each fish, 200 μL of whole blood

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was immediately drawn from the caudal vessel using a heparinized syringe for 11-KT analysis. These fish were then transferred to individual floating net pens and all individuals were vaccinated the following day, as described above. These fish were similarly euthanized 24 h after vaccination, with head kidney samples collected from each individual for analysis of cytokine mRNA levels. It is unlikely that males that had blood sampled in the resting immunity study were also included in the immune stimulation study, as the population of bluegill in Lake Opinicon is large and males were collected at different sites.

2.3. Measuring granulocyte oxidative activity

As part of our assessment of resting immunity, we used a respiratory burst assay to measure the oxidative activity of granulocytes undergoing phagocytosis (as in Marnila et al., 1995). Assays were carried out on blood samples within 60 min of their collection. Each well of a white 96-well plate was pre-filled with 110 μL of Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO). For each individual, 10 μL of heparinized whole blood was added to three test wells and three control wells. All wells then received 60 μL of 1 mM luminol (Sigma-Aldrich, St. Louis, MO) diluted in HBSS. Test wells then received 20 μL of 20 mg/mL zymozan A (Sigma-Aldrich, St. Louis, MO) and control wells received 20 μL HBSS. Wells were mixed via aspiration, and luminescence was then measured using a Glomax 96 luminometer (Promega, Madison, WI), with a program that took 99 readings with 150 s between readings, and an integration time of 0.5 s. Net luminescence at each time point was calculated by subtracting the mean luminescence of the control wells from the mean luminescence of the test wells. The peak luminescence of an individual's respiratory burst (maximum net luminescence observed during a 3-h trial) was used as a measure of granulocyte activity (see Pérez-Casanova et al., 2008).

2.4. Blood cell counts

Blood cell counts were used as a second measure of resting immunity. Specifically, we were interested in the abundance of granulocytes, which contribute to phagocytosis, and the abundance of lymphocytes, which are involved in antibody production (Ros et al., 2006a). Blood smears were prepared within 60 min of blood sampling by spreading a drop of blood from each individual over a glass slide and allowing it to air dry overnight. The following day, blood smears were stained for 20 min in Giemsa's azur eosin methylene blue solution (Sigma-Aldrich, St. Louis, MO) at a 1:10 dilution in distilled water. One blood smear was damaged in storage, so that fish was excluded from the cell count analyses. Blood smears were examined at 100X magnification under an Axioimager Z1 confocal light microscope (Zeiss, Toronto, ON, Canada). Image-Pro Premier software (Media Cybernetics, Rockville, MD) was used to automatically tally the total number of cells in each image, after which an observer manually identified cell types. A minimum of 9500 total blood cells were automatically counted and manually verified for each fish. Three cell types were counted: (i) erythrocytes: elliptical cells with a centrally located nucleus; (ii) granulocytes: large spherical cells with granules present in the cytoplasm; and (iii) lymphocytes: small spherical cells with little cytoplasm (Ros et al., 2006a). Granulocyte and lymphocyte counts were expressed as percentages of the total blood cells counted.

2.5. Cytokine mRNA levels

For the immune stimulation study, the levels of cytokine mRNA were used as a measure of activated innate immunity. First, RNA was extracted from head kidney tissue samples using TRIzol (Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions. Genomic DNA was removed from the extracted RNA using an Ambion DNA-free kit (Life Technologies, Burlington, ON, Canada). The RNA was then converted to cDNA using a qScript kit (Quanta Biosciences, Gaithersburg, Maryland).

Primers were developed to measure the levels of the cytokines interleukin 1β (IL1β) and interleukin 8 (IL8), with ribosomal protein subunit 18 (RPS18) used as a reference gene. Transcript sequences compiled from five closely related species were used to design primers (Table 1). PCR product from each primer pair was sequenced at the London Regional Genomics Centre (London, ON, Canada) to confirm that the intended genes were amplified. A nucleotide-BLAST search for each sequence identified matches with ≥85% similarity to transcript sequences of the expected genes.

The mRNA levels were then measured using PerfeCTa SYBR Green FastMix for IL1β and RPS18 (Quanta BioSciences, Gaithersburg, MD) and SensiFAST SYBR No-ROX for IL8 and RPS18 (Bioline, London, UK), according to the manufacturers' instructions. Serial dilutions (1:10) of cDNA template were used to generate a standard curve for each primer pair's qPCR amplification efficiency. All individuals and controls were analyzed in triplicate. Fluorescence measurements were performed in a CFX thermocycler (Bio-rad, Hercules, CA, USA) set to the following program: initial 3 min denaturation at 95.0 °C, 40 cycles of 10 s at 95.0 °C and 30 s at a primer-specific annealing temperature (Table 1). Following Livak and Schmittgen (2001), cytokine mRNA levels were normalized using RPS18.

2.6. 11-KT enzyme immunoassays

Within 60 min of blood collection, blood was centrifuged to collect the hormone-containing plasma. Circulating 11-KT concentrations were subsequently determined using a commercial enzyme-linked immunoassay (ELISA) kit according to the manufacturer's guidelines, including the optional purification step (Cayman Chemical, Ann Arbor, Michigan). Specifically, for each individual, a 1:1 solution of hexane (Sigma-Aldrich, St. Louis, MO) and ethyl acetate (Sigma-Aldrich, St. Louis, MO) was used to extract 11-KT from a 10 μL aliquot of plasma diluted in 490 μL of water. Four mL of the hexane:ethyl acetate solution was added to each diluted plasma sample and vortexed. The organic phase was decanted into a new test tube, and this procedure was repeated three more times (a total of four extractions). The hexane:ethyl acetate solution was evaporated overnight, and the extracted 11-KT was resuspended in ELISA buffer the following day. Samples were analyzed in triplicate and read at 412 nm on a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA). Precision of the 11-KT assay was determined using standards of known concentration, which were purified using the same protocol as the plasma samples, then quantified using separate ELISA kits. Across samples, the coefficient of variation for the 11-KT assay was 22%.

2.7. Statistical analyses

Unpaired two-tailed *t*-tests were used to compare body length and circulating 11-KT concentration between groups of parental males in each of our studies, as well as for comparisons between fish used in the two studies.

For the resting immunity study, respiratory burst peak and circulating 11-KT concentration data were log-transformed to achieve normal distributions. Body length and cell count data were normally distributed without transformation (Kolmogorov-Smirnov tests, $P > .10$). Pearson correlations were used to examine pairwise relationships between body length and: 11-KT concentration, peak respiratory burst luminescence, granulocyte counts, and lymphocyte counts. No significant pairwise relationships were

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Table 1
Details of primers used in qPCR reactions for bluegill (*Lepomis macrochirus*).

Gene	Direction	Sequence (5′-3′)	Annealing temperature (°C)	qPCR efficiency (%)
RPS18	Forward	TCAAGGACGGCAAATACAGCC	62.0	90.0
	Reverse	TTGGACACACCGACGGTGC		
IL1β	Forward	GAGAAGAGGAGCTTAGTTMKGG	62.0	118.0
	Reverse	AGAAAYCGYACCATGTCGC		
IL8	Forward	CAGAGAGCAAACCCATAGG	57.0	81.7
	Reverse	ATCACTTTCTTCACCCAGG		

found (all $p > .05$), so body length was excluded from subsequent analyses.

To examine the relationship between 11-KT concentration and immune parameters, peak respiratory burst luminescence, granulocyte counts, and lymphocyte counts were each analyzed using general linear models that included circulating 11-KT concentration as a covariate, with method of capture (dip net, angling) as a random factor. The time between blood collection and the start of the respiratory burst assay was included as an additional covariate for the general linear model relating maximum luminescence with 11-KT. Method of capture and the time between blood collection and the start of the respiratory burst assays had non-significant effects in all models and were removed from the final models (all $P > .05$). Pearson correlations were used to examine the pairwise relationships between our three immunity measures: respiratory burst peak, granulocyte counts, and lymphocyte counts.

For the immune stimulation study, Pearson correlations were used to examine pairwise relationships between body length and 11-KT concentration, IL1β levels, and IL8 levels. Body length was not significantly associated with any of these traits, so was excluded from the subsequent analyses. Circulating 11-KT concentrations and cytokine levels were compared amongst the three experimental groups (bled at capture and vaccinated, vaccinated, or saline-treated) using pairwise *t*-tests. Pearson correlations were used to examine the relationships between circulating 11-KT concentration and the levels of each of the two cytokine genes.

2.8. Immune stimulation meta-analysis

We selected studies that introduced an immune stimulation in male adults and then measured the response of plasma or serum androgen concentrations. Specifically, we included studies that met the following criteria: (1) the immune system was stimulated with a live pathogen or non-pathogenic antigen (2) androgen concentrations (testosterone or 11-KT) were measured following the immune stimulation, (3) the effect of the immune stimulation on androgen concentrations could be compared with a separate control group, and (4) the data were presented in such a way that the effect size could be extracted. We searched for studies using combinations of the following keywords in ISI Web of Knowledge: testosterone, 11-ketotestosterone, androgen, immunity, immunocompetence, immunosuppression, parasites, vaccination, antigen, immunochallenge, immune stimulation, immunocompetence handicap hypothesis, ICHH. We also included all studies analyzed by Boonekamp et al. (2008) in an earlier meta-analysis of the effects of immune stimulation on testosterone, and evaluated each subsequent study that had cited Boonekamp et al. (2008).

The effect size (r) corresponding to the effect of immune stimulation on androgen concentrations was calculated as in Boonekamp et al. (2008). When data were available for both absolute androgen concentrations and relative change in androgen concentrations associated with an immune stimulation, effect sizes were calculated based on absolute androgen concentrations.

To determine whether the mean effect size across all studies was significantly different from zero, we used a one-sample Student's *t*-test. The variance in effect size across taxa was analyzed using a general linear model that included taxon (mammal, bird or fish) as a fixed factor, with effect size as the dependent variable.

3. Results

Parental male bluegill ($N = 24$) used in the resting immunity study were captured in equal numbers either by angling in pelagic areas or by dip-netting in littoral areas (Table 2). Body length did not differ between angled and dip-netted males ($t = 0.79$, $df = 22$, $P = .44$), nor did concentrations of circulating 11-KT ($t = 0.97$, $df = 22$, $P = .34$). Males captured for the immune stimulation study were larger (189 ± 2 mm) than males captured for the resting immunity study (182 ± 2 mm; $t = 2.62$, $df = 44$, $P = .01$). Body length did not differ between saline- and vaccine-treated fish used in the immune stimulation study ($t = 0.03$, $df = 9$, $P = .97$). Mean circulating 11-KT concentration was significantly higher in males from the resting immunity study (29.1 ± 5.7 ng/ml) than males from the immune stimulation study (5.4 ± 0.7 ng/ml; $t = 3.98$, $df = 44$, $P < .01$).

3.1. Resting immunity study

We examined the relationship between circulating 11-KT concentration and three resting immunity measures. Contrary to prediction 1 of the ICHH—that androgen concentrations will be negatively related to immunity—we found no relationship between circulating 11-KT concentration and respiratory burst peak luminescence ($F = 0.03$, $df = 1, 19$, $P = .86$; Fig. 1a), granulocyte counts ($F = 0.03$, $df = 1,19$, $P = .87$; Fig. 1b), or lymphocyte counts ($F = 0.39$, $df = 1,19$, $P = .54$; Fig. 1c).

Next, we compared the relationships among measures of resting immunity. There was a strong positive relationship between granulocyte counts and respiratory burst peak luminescence (Pearson $R = 0.75$, $n = 23$, $P < .001$; Fig. 2). In contrast, lymphocyte percentage was unrelated to respiratory burst peak luminescence (Pearson $R = -0.21$, $n = 23$, $P = .33$). Granulocyte counts and lymphocyte counts were also unrelated to each other (Pearson $R = -0.33$, $n = 23$, $P = .29$).

3.2. Immune stimulation study

We examined the effect of saline or vaccine treatment on circulating 11-KT concentrations. Compared to individuals that were captured and bled immediately, neither saline-treated ($t = 1.51$, $df = 15$, $P = .15$) nor vaccine-treated ($t = 0.38$, $df = 14$, $P = .71$) fish had significantly different 11-KT concentrations 24 h following treatment. 11-KT concentration of saline- and vaccine-treated groups was not significantly different from one another ($t = 1.16$, $df = 9$, $P = .28$). These results do not support prediction 3 of the ICHH, i.e. immune stimulation would lower androgen concentration.

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Table 2
Description of parental male bluegill (*Lepomis macrochirus*) examined in this study. The vaccine- and saline-treated groups in the immune stimulation study had 11-ketotestosterone (11-KT) concentrations measured 24 h following treatment, whereas all other groups had 11-KT concentrations measured at the time of capture. Body length and 11-KT concentrations are presented as mean ± SE.

Study	Group	n	Dates collected	Body length (mm)	[11-KT] (ng mL^{-1})
Resting immunity study	Angled	12	June 4–19	181 ± 2	23.6 ± 6.3
	Dip-netted	12	June 4–19	183 ± 2	34.6 ± 9.5
Immune stimulation study	Saline-treated	6	June 15	191 ± 5	7.3 ± 2.2
	Vaccine-treated	5	June 15	191 ± 3	4.4 ± 1.0
	Vaccine-treated and bled at capture	11	June 24	187 ± 4	4.8 ± 0.5

Next, we compared levels of cytokine mRNA between saline- and vaccine-treated groups. Vaccinated fish had higher mRNA levels for both IL1β ($t = 2.30$, $df = 11$, $P = .021$) and IL8 ($t = 2.30$, $df = 10$, $P = .022$; Fig. 3). IL1β and IL8 levels were unrelated to each other (Pearson $R = 0.12$, $n = 10$, $P = .75$). No relationship was found between circulating 11-KT concentration and either IL1β (Pearson $R = -0.43$, $n = 10$, $P = .22$, Fig. 4), or IL8 (Pearson $R = 0.16$, $n = 11$, $P = .64$). Thus, although the vaccination induced an immune response, the strength of the response was unrelated to 11-KT concentration prior to vaccination. These results do not support the hypothesis of androgens having immunosuppressive effects.

3.3. Immune stimulation meta-analysis

Overall, immune stimulation was found to lower circulating androgen concentration across taxa (mean effect size $r = -0.43$; $t = 8.48$, $df = 22$, $P < .0001$; Table 3). When we analyzed the variation in effect size across taxonomic groups, there were a significant difference in effect size among taxa ($F = 5.60$, $df = 2, 20$, $P = .012$). The effect of immune stimulation on circulating androgen concentrations was smaller in fishes (mean $r = -0.12$) than birds (mean $r = -0.48$) or mammals (mean $r = -0.50$).

4. Discussion

If 11-KT is immunosuppressive, natural variation in the circulating concentration of this androgen is predicted to be negatively associated with immunity. We measured components of resting immunity (lymphocyte and granulocyte counts, and respiratory burst activity of circulating granulocytes) as well as the innate immune response (cytokine mRNA levels following vaccination), and found no relationship between circulating 11-KT concentrations and any of these measures. Correlative studies of androgens and immunity show mixed results across vertebrates, with some studies finding the predicted negative relationships (e.g. Duffy and Ball, 2002; Halliday et al., 2014) and other studies finding no relationship (e.g. Hasselquist et al., 1999; Prall et al., 2015; Ros and Oliveira, 2009) or positive relationships (e.g. Li et al., 2015; Peters, 2000; Rantala et al., 2012). One reason there may be inconsistent relationships between natural androgen concentrations and immunity, even if androgens are immunosuppressive, is that high-quality males may be able to better cope with the immunosuppressive effects of androgens, allowing them to maintain immunity while having higher concentrations of androgens than low-quality males (Getty, 2006). High-quality males could even have the highest levels of both androgens and immunity, so positive relationships between androgens and immunity might emerge even if androgens have immunosuppressive effects among the general population of males. Such may be the case in bluegill where high-quality parental males, as measured by body condition and likelihood of nesting during multiple spawning bouts, also have high androgen levels (Magee et al., 2006). Thus, correlative studies of natural androgen concentrations and immunity may not provide the best test of the ICHH.

If 11-KT is immunosuppressive, the concentration of this androgen is predicted to drop following immune stimulation. In bluegill, we found no effect of immune stimulation on the circulating concentration of 11-KT. Relative to the control treatment, immune stimulation was associated with a significant increase in cytokine mRNA levels, indicating that immune stimulation had the expected effect on immune activation. Consequently, low immune activation in both treatments (e.g. due to handling or confinement stress) cannot explain the absence of an effect of immune stimulation on 11-KT levels. Our result agrees with the only other study that examined the effect of immune stimulation on circulating concentration of 11-KT, which found that in St. Petersfish (*Sarotherodon galilaeus*) there was no decrease in 11-KT following immune stimulation (Ros et al., 2012). Similarly, studies in fishes that examined the effect of immune stimulation on circulating concentration of testosterone found no effect in tench (*Tinca tinca*) or St. Petersfish (Ros et al., 2012; Vainikka et al., 2005). Together these studies do not support an effect of immune stimulation on circulating androgen concentration in fishes.

In a meta-analysis of studies that examined the effect of immune stimulation in birds and mammals, a significant negative effect of immune stimulation on testosterone concentrations was found (Boonekamp et al., 2008). We performed a similar meta-analysis, but also included testosterone and 11-KT studies in fishes, along with studies in birds and mammals that had been published since the previous meta-analysis. We found an overall significant effect of immune stimulation on androgens, but the mean effect size across all studies was smaller in our meta-analysis than what was previously found ($r = -0.43$ vs. $r = -0.52$; Boonekamp et al., 2008). This reduction in effect size was largely driven by our inclusion of immune stimulation studies in fishes, which had significantly lower effect sizes than studies of birds and mammals. Overall, our meta-analysis found support for the testosterone-suppressing effects of immune stimulation in birds and mammals, but to date there is little evidence in fishes that either testosterone or 11-KT respond to immune stimulation.

There are a number of potential reasons why immune stimulation appears to have a weaker effect on androgen levels in fishes relative to other groups of vertebrates. Fishes use 11-KT as a primary androgen, rather than testosterone as in other vertebrates (Borg, 1994). The fact that 11-KT is non-aromatizable, unlike testosterone, may affect how circulating concentrations of this androgen respond to immune stimulation. For example, testosterone and non-aromatizable dihydrotestosterone have been shown to differ in their relationship with immunity, with testosterone having stronger immunosuppressive effects (Owen-Ashley et al., 2004). Alternatively, the smaller effect sizes of immune stimulation on circulating androgen concentration in fishes could be a result of differences in the immune system between fishes and other vertebrates, such as their lower diversity of immunoglobulins or higher specialization of B-cell lineages; however, the general structure of the fish immune system is remarkably similar to that of other vertebrates (Magadan et al., 2015). Regardless of the specific mechanism, immune stimulation appears to have

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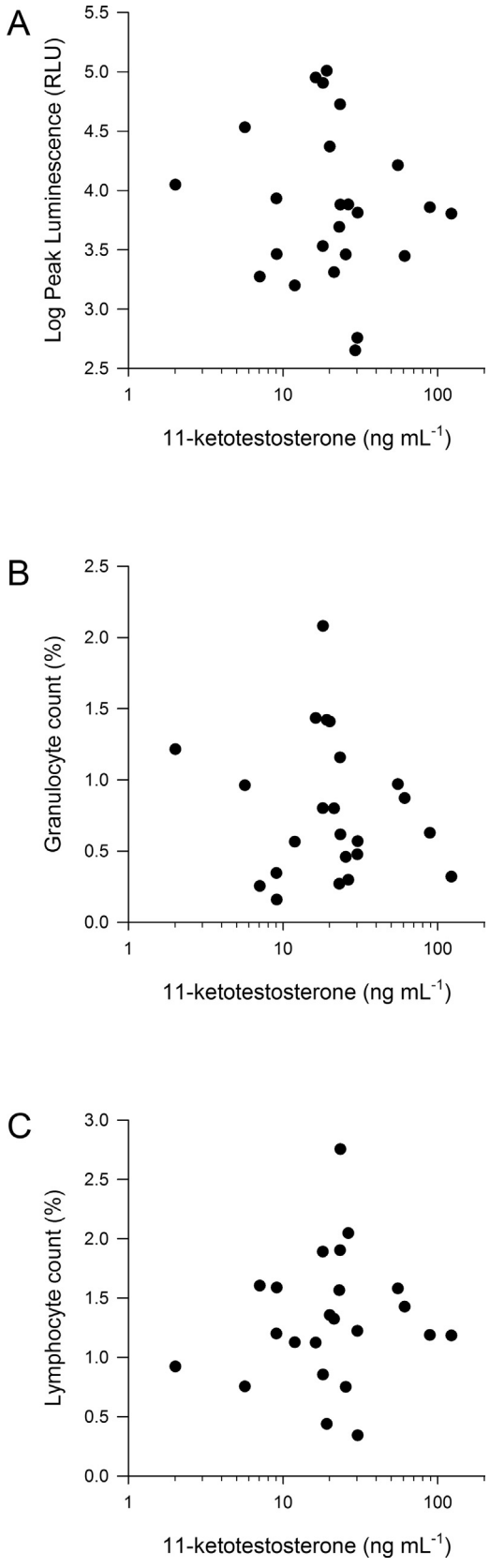

Fig. 1. Relationship between circulating 11-ketotestoserone (11-KT) concentrations and three measures of immunity in bluegill (*Lepomis macrochirus*): respiratory burst peak luminescence (Panel A), granulocyte count (Panel B), and lymphocyte count (Panel C). Granulocyte and lymphocyte counts are each expressed as the percentage of all blood cells counted.

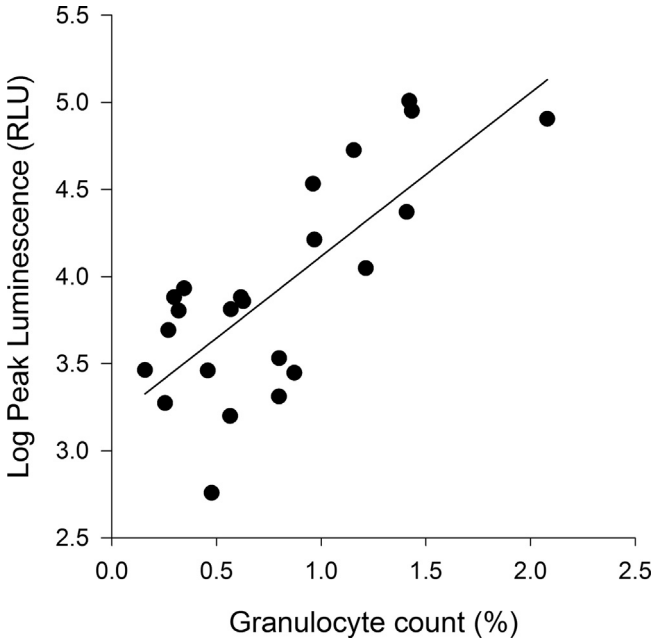

Fig. 2. Relationship between granulocyte count and peak respiratory burst activity in bluegill (*Lepomis macrochirus*). Granulocyte count is expressed as the percentage of all blood cells counted.

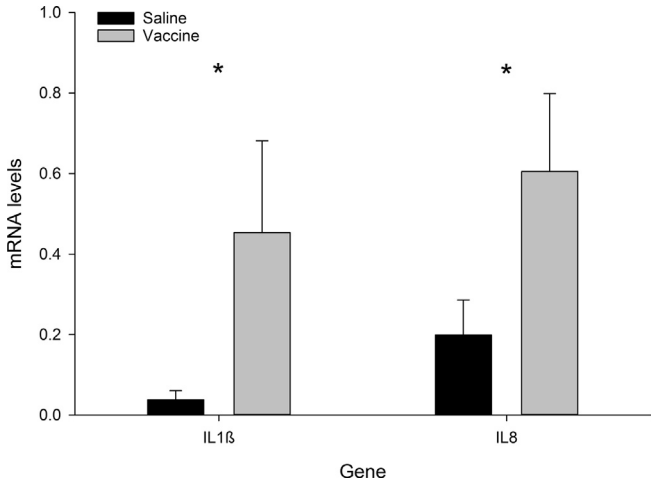

Fig. 3. Mean ± SE mRNA levels of cytokines IL1β and IL8 in bluegill (*Lepomis macrochirus*) 24 h following a saline or vaccine treatment. Levels were normalized using RPS18 as a reference gene. A significant difference in mRNA levels between the saline and vaccine treatments is indicated by *.

weaker effects on androgen concentrations in fishes than in other groups of vertebrates.

In conclusion, the immunosuppressive effect of testosterone has generally been well-supported experimentally, whereas the effects of 11-KT on immunity are largely unresolved, and at present it is unclear whether these two androgens share the same relationship with immunity. The ICHH depends on the immunosuppressive effect of androgens as a means of explaining how the honest signaling of androgen-mediated traits is maintained across a wide range of taxa. If testosterone is immunosuppressive but other androgens, such as 11-KT, that facilitate reproductive success are not found to be immunosuppressive, this finding will necessitate a re-evaluation of the ICHH. It is therefore critical that androgens other than testosterone continue to receive attention in the context of the ICHH, as it remains to be determined whether Folstad and

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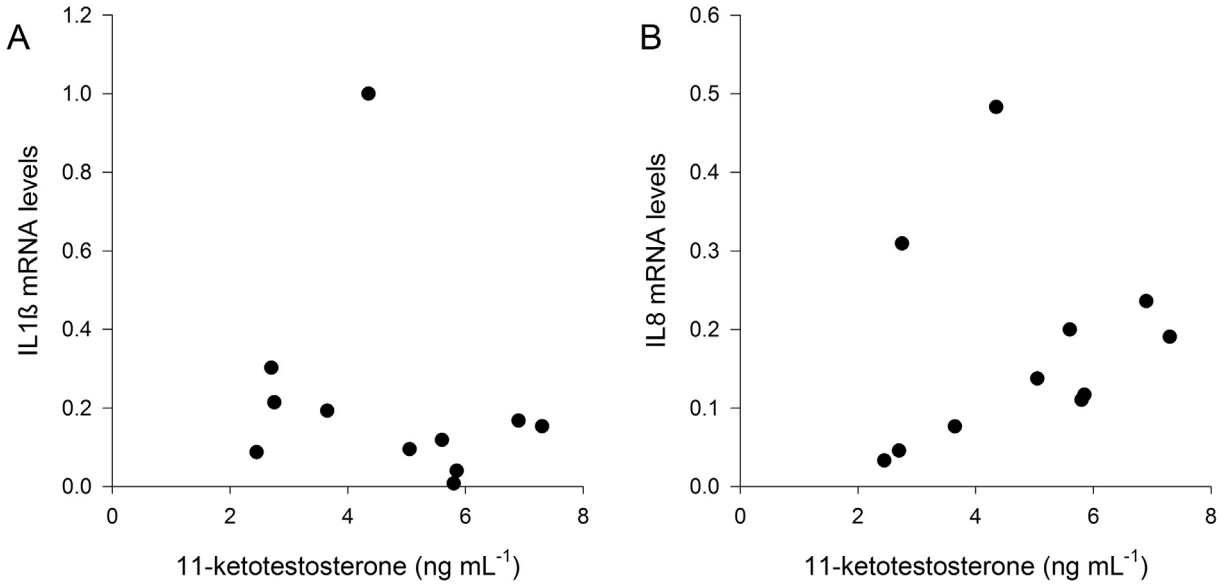

Fig. 4. Relationships in bluegill (*Lepomis macrochirus*) between circulating 11-ketotestoreone concentrations at the time of capture and the mRNA levels of cytokines (A) IL1β and (B) IL8 24 h following vaccination. Levels were normalized using RPS18 as a reference gene.

Table 3
Individual studies examined in the meta-analysis of the effect of immune stimulation on androgens. The data comprise the taxon, species, effect size, sample size, androgen, and reference. Negative effect sizes indicate lower androgen concentrations in individuals that received an immune stimulation.

Taxon	Species	Effect size (r)	N	Androgen	Reference
Mammal	Mouse (*Mus musculus*)	−0.711	16	Testosterone	Barthelemy et al. (2004)
		−0.442	55	Testosterone	Hales et al. (2000)
		−0.708	18	Testosterone	He et al. (2000)
		−0.827	42	Testosterone	Isseroff et al. (1986)
		−0.303	42	Testosterone	Lopes and König (2016)
		−0.575	62	Testosterone	Weil et al. (2006)
		−0.03	20	Testosterone	Zhang and He (2014)
	Rabbit (*Oryctolagus cuniculus*)	−0.261	16	Testosterone	Kasilima et al. (2004)
	Rat (*Rattus norvegicus*)	−0.794	16	Testosterone	Hublart et al. (1990)
		−0.513	92	Testosterone	O'Bryan et al. (2000)
		−0.386	136	Testosterone	O'Bryan et al. (2000)
	Sheep (*Ovis aries*)	−0.516	9	Testosterone	Mutayoba et al. (1997)
Bird	Canary (*Serinus canaria*)	−0.37	16	Testosterone	Müller et al. (2013)
	Chicken (*Gallus gallus*)	−0.497	16	Testosterone	Boltz et al. (2004)
		−0.511	19	Testosterone	Boltz et al. (2007)
		−0.388	26	Testosterone	DeVaney et al. (1977)
		−0.328	80	Testosterone	Verhulst et al. (1999)
	Collared flycatcher (*Ficedula albicollis*)	−0.584	13	Testosterone	Garamszegi et al. (2004)
	Diamond dove (*Geopelia cuneata*)	−0.686	24	Testosterone	Casagrande and Groothuis (2011)
Fish	St. Petersfish (*Sarotherodon galilaeus*)	0.03	47	Testosterone	Ros et al. (2012)
	Tench (*Tinca tinca*)	−0.211	9	Testosterone	Vainikka et al. (2005)
	Bluegill (*Lepomis macrochirus*)	−0.358	11	11-KT	Present study
	St. Petersfish (*Sarotherodon galilaeus*)	0.056	19	11-KT	Ros et al. (2012)

Karter's (1992) hypothesis applies to all species that use androgens during reproduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.01.016.

References

Alonso-Alvarez, C., Pérez-Rodríguez, L., Garcia, J.T., Viñuela, J., 2009. Testosterone-mediated trade-offs in the old age: a new approach to the immunocompetence handicap and carotenoid-based sexual signalling. Proc. R. Soc. B 276, 2093–2101.

Aykanat, T., Heath, J.W., Dixon, B., Heath, D.D., 2012. Additive, non-additive and maternal effects of cytokine transcription in response to immunostimulation with *Vibrio* vaccine in Chinook salmon (*Oncorhynchus tshawytscha*). Immunogenetics 64, 691–703.

Barthelemy, M., Gabrion, C., Petit, G., 2004. Reduction in testosterone concentration and its effect on the reproductive output of chronic malaria-infected male mice. Parasit. Res. 93, 475–481.
Bilbo, S.D., Nelson, R.J., 2001. Sex steroid hormones enhance immune function in male and female Siberian hamsters. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, R207–R213.
Boltz, C.R., Boltz, D.A., Bunick, D., Scherba, G., Bahr, J.M., 2007. Vaccination against the avian infectious bronchitis virus affects sperm concentration, sperm quality and blood testosterone concentrations in cockerels. Brit. Poultry Sci. 48, 617–624.
Boltz, D.A., Nakai, M., Bahr, J.M., 2004. Avian infectious bronchitis virus: a possible cause of reduced fertility in the rooster. Avian Dis. 48, 909–915.
Boonekamp, J.J., Ros, A.H.F., Verhulst, S., 2008. Immune activation suppresses plasma testosterone level: a meta-analysis. Biol. Lett. 4, 741–744.
Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C 109, 219–245.
Casagrande, S., Groothuis, T.G.G., 2011. The interplay between gonadal steroids and immune defence in affecting a carotenoid-dependent trait. Behav. Ecol. Sociobiol. 65, 2007–2019.
DeVaney, J.A., Elissalde, M.H., Steel, E.G., Hogan, B.F., Petersen, H.D.E.L.V., 1977. Effect of the Northern Fowl Mite, *Ornithonyssus sylviarum* (Canestrini and Fanzago) on White Leghorn Roosters. Poultry Sci. 56, 1585–1590.
Duffy, D.L., Ball, G.F., 2002. Song predicts immunocompetence in male European starlings (*Sturnus vulgaris*). Proc. R. Soc. B 269, 847–852.
Folstad, I., Karter, A.J., 1992. Parasites, bright males, and the immunocompetence handicap. Am. Nat. 139, 603–622.
Foo, Y.Z., Nakagawa, S., Rhodes, G., Simmons, L.W., 2017. The effects of sex hormones on immune function: a meta-analysis. Biol. Rev. 92, 551–571.
Garamszegi, L.Z., Møller, A.P., Török, J., Michl, G., Péczely, P., Richard, M., 2004. Immune challenge mediates vocal communication in a passerine bird: an experiment. Behav. Ecol. 15, 148–157.
Getty, T., 2006. Sexually selected signals are not similar to sports handicaps. Trends Ecol. Evol. 21, 83–88.
Gutowsky, L.F.G., Aslam, W., Banisaeed, R., Bell, L.R., Bove, K.L., Brownscombe, J.W., Burrows, G.J.J., Chu, E., Magel, J.M.T., Rous, A.M., Cooke, S.J., 2015. Considerations for the design and interpretation of fishing release mortality estimates. Fish. Res. 167, 64–70.
Hales, K.H., Diemer, T., Ginde, S., Shankar, B.K., Roberts, M., Bosmann, H.B., Hales, D. B., 2000. Diametric effects of bacterial endotoxin lipopolysaccharide on adrenal and Leydig cell steroidogenic acute regulatory protein. Endocrinology 141, 4000–4012.
Halliday, W.D., Paterson, J.E., Patterson, L.D., Cooke, S.J., Blouin-Demers, G., 2014. Testosterone, body size, and sexual signals predict parasite load in Yarrow's Spiny Lizards (*Sceloporus jarrovii*). Can. J. Zool. 92, 1075–1082.
Hasselquist, D., Marsh, J.A., Sherman, P.W., Wingfield, J.C., 1999. Is avian humoral immunocompetence suppressed by testosterone? Behav. Ecol. Sociobiol. 45, 167–175.
He, H.B., Ohta, N., Kawaguchi, H., 2000. Effect of *Schistosoma japonicum* infection on serum testosterone levels in mice. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 18, 173–175.
Hublart, M., Tetaert, D., Croix, D., Boutignon, F., Degand, P., Boersma, A., 1990. Gonadotropic dysfunction produced by *Trypanosoma brucei brucei* in the rat. Acta Tropica 47, 177–184.
Isseroff, H., Sylvester, P.W., Held, W.A., 1986. Effects of *Schistosoma mansoni* on androgen regulated gene expression in the mouse. Mol. Biochem. Parasit. 18, 401–412.
Kasilima, Y.S., Wango, E.O., Kigondu, C.S., Mutayoba, B.M., Nyindo, M., 2004. Plasma bioactive LH and testosterone profiles in male New Zealand rabbits experimentally infected with *Schistosoma mansoni*. Acta Tropica 92, 165–172.
Kortet, R., Vainikka, A., Rantala, M.J., Jokinen, I., Taskinen, J., 2003. Sexual ornamentation, androgens and papillomatosis in male roach (*Rutilus rutilus*). Evol. Ecol. Res. 5, 411–419.
Kurtz, J., Kalbe, M., Langefors, Å., Mayer, I., Milinski, M., Hasselquist, D., 2007. An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. Am. Nat. 170, 509–519.
Law, W.-Y., Chen, W.-H., Song, Y.-L., Dufour, S., Chang, C.-F., 2001. Differential *in vitro* suppressive effects of steroids on leukocyte phagocytosis in two teleosts, tilapia and common carp. Gen. Comp. Endocrinol. 121, 163–172.
Li, D., Hao, Y., Liu, X., Yao, Y., Du, C., Zhang, X., Cui, S., Wu, L., Wu, Y., 2015. Changes in phytohaemagglutinin skin-swelling responses during the breeding season in a multi-brooded species, the Eurasian Tree Sparrow: do males with higher testosterone levels show stronger immune responses? J. Ornithol. 156, 133–141.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Lopes, P.C., König, B., 2016. Choosing a healthy mate: sexually attractive traits as reliable indicators of current disease status in house mice. Anim. Behav. 111, 119–126.
Magadan, S., Sunyer, O.J., Boudinot, P., 2015. Unique features of fish immune repertoires: particularities of adaptive immunity within the largest group of vertebrates. In: Pathogen-Host Interactions: Antigenic Variation v. Somatic Adaptations. Springer, pp. 235–264.
Magee, S.E., Neff, B.D., Knapp, R., 2006. Plasma levels of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. Horm. Behav. 49, 598–609.
Marnila, P., Tiiska, A., Lagerspetz, K., Lilius, E.M., 1995. Phagocyte activity in the frog *Rana temporaria*: whole blood chemiluminescence method and the effects of temperature and thermal acclimation. Comp. Biochem. Physiol. A 111, 609–614.
Müller, W., Heylen, D., Eens, M., Rivera-Gutierrez, H.F., Groothuis, T.G.G., 2013. An experimental study on the causal relationships between (ecto-)parasites, testosterone and sexual signalling. Behav. Ecol. Sociobiol. 67, 1791–1798.
Mutayoba, B.M., O'Shaughnessy, P.J., Jeffcoate, I.A., Eckersall, P.D., Cestnik, V., Holmes, P.H., 1997. Effect of experimental infection with *Trypanosoma congolense* and scrotal insulation on leydig cell steroidogenesis in the ram. Theriogenology 48, 411–422.
Neff, B.D., Knapp, R., 2009. Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill. Horm. Behav. 56, 239–245.
O'Bryan, M.K., Schlatt, S., Phillips, D.J., de Kretser, D.M., Hedger, M.P., 2000. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels *in vivo*. Endocrinology 141, 238–246.
Owen-Ashley, N.T., Hasselquist, D., Wingfield, J.C., 2004. Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. Am. Nat. 164, 490–505.
Partridge, C., Rodgers, C.M.C., Knapp, R., Neff, B.D., 2015. Androgen effects on immune gene expression during parental care in bluegill sunfish (*Lepomis macrochirus*). Can. J. Zool. 93, 9–13.
Pérez-Casanova, J.C., Rise, M.L., Dixon, B., Afonso, L.O.B., Hall, J.R., Johnson, S.C., Gamperl, A.K., 2008. The immune and stress responses of Atlantic cod to long-term increases in water temperature. Fish Shellfish Immunol. 24, 600–609.
Peters, A., 2000. Testosterone treatment is immunosuppressive in superb fairy-wrens, yet free-living males with high testosterone are more immunocompetent. Proc. R. Soc. B 267, 883–889.
Prall, S.P., Ambu, L., Nathan, S., Alsisto, S., Ramirez, D., Muehlenbein, M.P., 2015. Androgens and innate immunity in rehabilitated semi-captive orangutans (*Pongo pygmaeus morio*) from Malaysian Borneo. Am. J. Primatol. 77, 642–650.
Rantala, M.J., Moore, F.R., Skrinda, I., Krama, T., Kivleniece, I., Kecko, S., Krams, I., 2012. Evidence for the stress-linked immunocompetence handicap hypothesis in humans. Nat. Commun. 3, 694.
Roberts, M.L., Buchanan, K.L., Evans, M.R., 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim. Behav. 68, 227–239.
Ros, A.F.H., Bouton, N., Santos, R.S., Oliveira, R.F., 2006a. Alternative male reproductive tactics and the immunocompetence handicap in the Azorean rock-pool blenny, *Parablennius parvicornis*. Proc. R. Soc. B 273, 901–909.
Ros, A.F.H., Ferreira, C., Santos, R.S., Oliveira, R.F., 2006b. Regulation of immunocompetence by different androgen metabolites in a blenny with alternative reproductive tactics. J. Exp. Zool. A 305A, 986–994.
Ros, A.F.H., Oliveira, R.F., 2009. Androgens and immune function in male alternative reproductive morphotypes of the peacock blenny *Salaria pavo*. Ethology 115, 555–565.
Ros, A.F.H., Oliveira, R.F., Dijkstra, P.D., Groothuis, T.G.G., 2012. Immune activation is inversely related to, but does not cause variation in androgen levels in a cichlid fish species. Fish Shellfish Immunol. 33, 130–133.
Vainikka, A., Jokinen, E.I., Kortet, R., Paukku, S., Pirhonen, J., Rantala, M.J., Taskinen, J., 2005. Effects of testosterone and β-glucan on immune functions in tench. J. Fish Biol. 66, 348–361.
Verhulst, S., Dieleman, S.J., Parmentier, H.K., 1999. A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. Proc. Natl Acad. Sci. USA 96, 4478–4481.
Weil, Z.M., Bowers, S.L., Pyter, L.M., Nelson, R.J., 2006. Social interactions alter proinflammatory cytokine gene expression and behavior following endotoxin administration. Brain Behavior Immun. 20, 72–79.
Zhang, T., He, H., 2014. Test of immunocompetence handicap hypothesis in mice (*Mus musculus*) infected with *Trichinella spiralis*. Can. J. Zool. 92, 817–821.

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