9-20-2016 12:00 AM

A test of the effects of androgens on immunity: no relationship between 11-keto testosterone and immune performance in bluegill sunfish (Lepomis macrochirus)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Abstract

The posited immunosuppressive effects of androgens are a key component of the immunocompetence handicap hypothesis (ICHH). My thesis uses bluegill sunfish (*Lepomis macrochirus*) to test two predictions arising from this hypothesis: (1) natural concentrations of the androgen 11-keto testosterone (11-KT) will be negatively related with immunity, and (2) an immunochallenge will lower 11-KT concentration. I found no evidence for a relationship between natural 11-KT concentration and measures of immunity (leukocyte counts, respiratory burst, cytokine gene expression), and an immunochallenge with *Vibrio* vaccine did not affect 11-KT concentration. I performed a meta-analysis of immunochallenge studies to help interpret my results, and report evidence suggesting that immunochallenges have weaker effects on androgens in fishes compared to other vertebrates. These results suggest that the ICHH may not apply to all vertebrates, though it remains premature to state which factors specific to fish may account for the weaker evidence in favour of immunosuppressive androgens.

Keywords

Immunocompetence handicap hypothesis, sexual selection, androgens, immunity, testosterone, 11-ketotestosterone, bluegill sunfish

Acknowledgments

I'd like to thank my supervisor, Dr. Bryan Neff, for his encouragement and support throughout my degree. I'd like to thank Dr. Shawn Garner for the countless hours of editing, discussing, and planning that he put into this project. I'd like to thank Dr. Charlyn Partridge for her help and guidance in the field and lab, and Dr. Scott Colborne for teaching me how to work with wild fish. I would like to thank Dr. Rosemary Knapp and Dr. Brian Dixon for their intellectual contributions throughout the project. In addition, I share the success of this project's completion with Gurpreet Dhami, Karen Nygard, Sarah Lee, Mat Stephan, Shiny Yang and Sara Chenier. Thanks to all of the fine folks at QUBS who made the field seasons such a blast, and most of all thank you to my family and Kayla for supporting me and keeping me sane (for the most part) throughout this project.

Co-Authorship Statement

A manuscript version of this monograph is planned for submission to *Fish & Shellfish Immunology* with Shawn Garner, Charlyn Partridge, Brian Dixon, Rosemary Knapp, and Bryan Neff as co-authors. **John Loggie** contributed to study design, data collection, data analysis, and drafted the manuscript. **Shawn Garner** contributed to data analysis, and provided input on the manuscript. **Charlyn Partridge** contributed to study design and data collection. **Brian Dixon** contributed to study design and data analysis. **Rosemary Knapp** contributed to data collection and data analysis. **Bryan Neff** contributed to study design and provided input on the manuscript.

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1.1 Introduction

Sexual selection acts on differential success among members of the same sex during competition for reproductive opportunities. As a major contributor to lifetime fitness, sexual selection is an important evolutionary force that has played an extensive role in shaping biological diversity (Kirkpatrick 1982). There are two commonly recognized mechanisms of sexual selection: mate choice and intra-sexual competition. Mate choice involves members of one sex selecting mates of the other sex on the basis of specific traits and behaviours. For example, in scaled quail (*Callipepla squamata*), females evaluate the quality of males based on elaborate courtship displays, preferring males that signal more frequently (Hagelin & Ligon 1999). Alternatively, intra-sexual competition among individuals of the same sex for access to reproductive opportunities can drive sexual selection. For example, in greater spear-nosed bats (*Phyllostomus hastatus*), males compete amongst one another for the control of harems of females, with dominant males securing harems for greater lengths of time (McCracken & Bradbury 1981). In many cases, including the examples described above, male success in competition for reproductive opportunities is linked to circulating concentrations of androgens, which are hormones that regulate male sexual development in vertebrates (Greiner et al. 2011; Preston et al. 2012). Identifying the factors that constrain circulating androgen concentrations is needed in order to understand why individuals differ in their expression of reproductive traits and behaviours. Establishing how differential reproductive success arises is critical to understanding sexual selection.

Given the generally positive relationship between androgen concentrations and reproductive success, there is interest in understanding the physiological constraints on androgen levels. The hypothalamic-pituitary-gonadal axis controls the development and maintenance of reproductive traits and behaviours, as well as androgen production in the testes (Rivier & Rivest 1991). One hypothesis for how androgen concentrations could be constrained is that organisms may be limited in their ability to produce androgens from precursor molecules. However, like other steroid hormones, androgens are synthesized by enzymes that modify

cholesterol, which is endogenously produced and found in high quantities throughout cells (Rudney & Sexton 1986). Furthermore, it is unlikely that circulating androgen concentrations reach high enough levels to impose limits to their production, because they are found in low concentrations compared to cholesterol (Rudney & Sexton 1986). Alternatively, androgens may instead be constrained if high androgen concentrations have a negative effect on other biological processes. Positive relationships between incidences of chronic blood infections and the expression of traits and behaviours used to indicate male quality, such as plumage brightness and song complexity, have been observed across species of passerine birds (Hamilton & Zuk 1982). Additionally, females have been found to have stronger immunity than males across vertebrate taxa (Grossman 1985). Together, these observations led to the development of the immunocompetence handicap hypothesis (ICHH), which posits that androgens linked to the development and maintenance of reproductively-beneficial traits and behaviours may also act to suppress immunity (Folstad & Karter 1992). This hypothesis therefore implicates androgen-mediated immunosuppression as a key constraint on male reproductive success, and an important factor in generating the differential reproductive success that drives sexual selection. Because androgens have conserved roles in reproduction across vertebrates (Norris & Lopez 2011), an immunosuppressive effect of androgens may serve as an important selective force across vertebrates.

Androgens may potentially affect different components of the vertebrate immune system. Vertebrate immunity can be broadly divided into the adaptive and innate branches (Uribe et al. 2011). Adaptive immunity functions in mounting a pathogen-specific immune response. This response is initiated when lymphocyte cells recognize a pathogen via antigen molecules, which bind to receptors located on the plasma membranes of lymphocytes (Magnadóttir et al. 2006). Following antigen binding, there is a multiple-day latent period, followed by a rise in circulating concentrations of antibodies specific to the recognized antigen, along with a proliferation of lymphocytes (Frank 2002). The strength of the activated adaptive immune response can be quantified by measuring the rise in circulating antibody concentration, and an individual's resting capacity to mount an adaptive immune

response is commonly measured by counting the concentration of lymphocytes in blood (e.g. Kurtz et al. 2007; Ros et al. 2006a).

Innate immunity, by contrast, functions in maintaining a constant level of nonspecific protection against potential pathogens, and is active regardless of an individual's infection status (Frank 2002). The most abundant group of innate immunity cells are granulocytes, which destroy pathogens primarily through phagocytosis, using the production of reactive oxygen species to degrade ingested pathogens in a process known as the respiratory burst response (Uribe et al. 2011). Resting immunity can thus be measured as the number of granulocytes, or the intensity of the respiratory burst response (e.g. Ros et al. 2006a; Castillo-Briceño et al. 2013). The initial phase of the activation of the innate immune response involves the production of pro-inflammatory cytokine proteins, which facilitate intercellular communication and coordination of both the adaptive and innate immune responses (Magnadóttir et al. 2006). The strength of the activated innate immune response can thus be measured by exposing individuals to an immunochallenge, and measuring the expression of cytokine genes such as interleukin 1β (IL1β) and interleukin 8 (IL8). These cytokines play important roles in the early inflammatory response and are conserved cross taxa, including fish (Uribe et al. 2011). Owing to the complexity of the vertebrate immune system, reliably assessing an individual's overall immunocompetence requires multiple independent measures.

Since the introduction of the ICHH, many studies have been conducted with the aim of testing the hypothesis that androgens are immunosuppressive. Namely, three predictions resulting from this hypothesis have been tested: (1) natural androgen levels will be negatively related with immunity, (2) experimentally elevated androgen levels will suppress immunity, and (3) immunochallenges will lower androgen levels. To date, most of these studies have been conducted in taxa whose primary androgen is testosterone. Studies testing prediction 1 have showed mixed results regarding the relationship between natural testosterone levels and immunity. For example, in Yarrow's spiny lizards (*Sceloporus jarrovii*), circulating testosterone concentration was positively related to ectoparasite load, which supports the hypothesis of androgens having immunosuppressive effects (Halliday et al. 2014). In peacock blennys (*Salaria pavo*), circulating concentration of testosterone was

unrelated to leukocyte counts, which does not support the hypothesis that androgens have immunosuppressive effects (Ros & Oliviera 2009). In superb fairy-wrens (*Malurus cyane*), circulating testosterone concentration was positively related to the strength of the primary antibody response (Peters 2000), which contradicts the hypothesis of androgens having immunosuppressive effects.

Studies testing prediction 2 have also produced mixed results regarding the relationship between experimental testosterone elevation and immunosuppression. For example, in red-legged partridges (*Alectoris rufa*), experimental testosterone elevation was found to decrease the proliferation of lymphocytes in response to an antigen in old individuals, but had no effect in younger individuals (Alonso-Alvarez et al. 2009). In dark-eyed juncos (*Junco hyemalis*), experimental testosterone elevation was found to decrease the proliferation of lymphocytes in response to an antigen in wild individuals. However, this effect was not observed in captive individuals (Casto et al. 2001). In Siberian hamsters (*Phodopus sungorus*), castration led to decreased proliferation of lymphocytes, and was restored by experimentally elevating circulating testosterone concentration in castrated individuals, in contradiction of the hypothesis that androgens have immunosuppressive effects (Bilbo & Nelson 2001).

Studies testing prediction 3 have also showed mixed results regarding the effect of immunochallenges on testosterone levels. For example, in male collared flycatchers (*Ficedula albicollis*), an inoculation with sheep red blood cells led to decreased circulating testosterone concentration (Garamszegi et al. 2004). In short-tailed fruit bats (*Carollia perspicillata*), an inoculation with 1,4-dinitrofluorobenzene was found to decrease circulating testosterone concentration (Greiner et al. 2010). In New Zealand rabbits (*Oryctolagus cuniculus*), an experimental infection with a live pathogen was not found to affect circulating testosterone concentration (Kasilima et al. 2004). Overall, individual studies have shown mixed support for each of the three predictions, leading to some uncertainty about the immunosuppressive effects of androgens.

Given the large number of studies that have tested each of the three predictions arising from the hypothesis that testosterone is immunosuppressive, meta-analyses are an important tool

for evaluating the relationship between testosterone and immunity. The first meta-analysis of ICHH studies included those that had experimentally elevated circulating testosterone concentrations and measured the effects on immunity, thus testing prediction 2 (Roberts et al. 2004). This first analysis found no immunosuppressive effect of testosterone manipulation within birds and mammals, although an immunosuppressive effect of testosterone manipulation was found within studies of reptiles. The authors speculated that this difference among taxa may occur because androgen concentrations are generally higher in reptiles than in other vertebrates (Roberts et al. 2004). A second meta-analysis examined studies in which the effect of an immunochallenge on circulating testosterone concentration was tested (prediction 3), and found a significant negative effect of immunochallenges on circulating testosterone concentration across studies (Boonekamp et al. 2008). This meta-analysis only included studies conducted in birds and mammals. A third meta-analysis examined both testosterone manipulation experiments (prediction 2) and testosterone-immunity correlation studies (prediction 1) and found support for prediction 2 but not prediction 1 (Foo et al. 2016). The findings of this recent meta-analysis differ from those of Roberts et al. (2004), whose meta-analysis 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 and colleagues (2016) 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 whether or not 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 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. Owen-Ashley et al. (2004) began this line of study by experimentally elevating circulating concentrations of either testosterone or dihydrotestosterone in song sparrows (*Melospiza melodia*). Elevated circulating

concentrations of testosterone but not dihydrotestosterone were found to lead to immunosuppression, despite the fact that dihydrotestosterone binds to androgen receptors with a greater affinity than testosterone (Owen-Ashley et al. 2004). The authors speculated that dihydrotestosterone may not be immunosuppressive because it cannot be converted to estradiol via the enzyme aromatase, unlike testosterone. Another study in chickens (*Gallus gallus domesticus*) similarly found support for the immunosuppressive effects of estradiol and testosterone but not dihydrotestosterone (Al-Afaleq & Homeida, 1998). It is possible that the aromatizablity of androgens affects their relationship with immunity. 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). For example, 11-KT but not testosterone induces the production of spiggin, a gluelike protein necessary for nest construction in male three-spine sticklebacks (*Gasterosteus aculeatus*; Jakobson et al. 1999). Like dihydrotestosterone, 11-KT is non-aromatizable. It is thus unclear whether 11-KT will be related to immunity as predicted by the ICHH.

To date, equivocal results have been obtained across studies of the ICHH in fishes. Across studies in fishes that have tested prediction 1 — namely, that natural androgen levels with be negatively related with immunity — mixed results have been obtained. For example, in roach (*Rutilus rutilus*) circulating 11-KT concentration was positively related to papillomatosis infection severity, in support of prediction 1 (Kortet et al. 2003). In St. Petersfish (*Sarotherodon galilaeus*) circulating 11-KT concentration was negatively related to the strength of the primary antibody response, also in support of prediction 1 (Ros et al. 2012). In rockpool blennys (*Parablennius parvicornis*) and in peacock blennys, circulating 11-KT concentration did not relate to leukocyte counts, which does not support prediction 1 (Ros et al. 2006a; Ros & Oliviera 2009). Across 11-KT studies that have addressed prediction 2 — namely, that experimentally elevated androgen levels will suppress immunity— mixed results have been obtained. For example, in common carp (*Cyprinus carpio*) and in three-spined sticklebacks, 11-KT elevation suppressed the phagocytic activity of leukocytes isolated from the head kidney, in support of prediction 2 (Watanuki et al. 2002; Kurtz et al. 2007). In bluegill sunfish (*Lepomis macrochirus*), 11-KT elevation

lowered the expression of several immunity-related genes, in support of prediction 2 (Partridge et al. 2015). In tilapia (*Oreochromis niloticus*), common carp, and rockpool plennys, 11-KT elevation did not affect the phagocytic activity of circulating leukocytes, which does not support prediction 2 (Law et al. 2001; Ros et al. 2006b). Finally, only one study to date has examined the effect of an immunochallenge on circulating 11-KT concentration (prediction 3), and it found no effect of an immunochallenge in the form of sheep red blood cells on circulating 11-KT concentration (Ros et al. 2012). Overall, the relationship between 11-KT and immunity remains unclear, and more studies testing each of the three predictions are needed.

My thesis examines the relationship between 11-KT and immunity in bluegill sunfish (*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 highly elevated in males (Rodgers et al. 2012). These elevated 11-KT concentrations coincide with high levels of aggressive behaviour amongst males, who construct and defend nests in close proximity to one another prior to spawning. Within 24 hours following spawning, circulating 11-KT concentrations drop. This decrease in 11-KT is accompanied by a behavioural shift away from aggression towards nurturing parental care behaviours (Kindler et al. 1989; Magee et al. 2006). Experimental 11-KT elevations have been found to increase aggressive behaviour and decrease parental care behaviour in bluegill (Rodgers et al. 2013). The reproductive benefits of 11-KT have been directly demonstrated in this species, with a positive relationship between circulating 11-KT concentration and paternity (measured as the proportion of offspring sired by the nest-tending male) having been reported (Neff & Knapp 2009). With the role of 11-KT well described in this species, bluegill offer an suitable system in which to study the posited immunosuppressive effects of this androgen.

In my thesis, I test predictions 1 and 3 arising from the hypothesis that androgens are immunosuppressive. I examine the relationship between natural circulating 11-KT concentration and components of both resting immunity and the immune response, along with an experimental immunochallenge to determine whether circulating 11-KT concentration drops during the early innate immune response. I also perform a meta-

analysis of immunochallenge studies testing prediction 3, in which I include and differentiate between studies in species that use testosterone or 11-KT as their primary androgen. I predict that if androgens are immunosuppressive, I will find a negative relationship between natural circulating 11-KT concentration and measures of both resting immunity and the innate immune response. I also predict that if androgens are immunosuppressive, circulating 11-KT concentrations will drop following an experimental immunochallenge. Finally, I predict that if androgens are immunosuppressive, an overall negative effect of immunochallenges on circulating androgen concentrations will be found across taxa in my meta-analysis.

1.2 Methods

1.2.1 Field collections

1.2.1.1 Study site and species

Field collections took place at Queen's University Biological Field Station, which lies along the shore of Lake Opinicon (44°34'N, 76°19'W; Ontario Ministry of Natural Resources Licence No. 1079753; Animal Use Protocol # 2010-214). Bluegill sunfish (*Lepomis macrochirus*) are common throughout the 900-hectare lake, and this population has been sampled annually as part of a long-term series of experiments focusing on the behavioural ecology of this species (e.g. Colgan et al. 1979; Neff & Knapp 2009). Bluegill sunfish are characterized by alternative male reproductive tactics. The parental male reproductive tactic provides sole parental care to its young, whereas the cuckolder tactic steals fertilizations from parental males but provides no parental care (Gross and Charnov 1980). In Lake Opinicon, bluegill spawning typically begins in late May or early June. The initiation of spawning activity involves the movement of parental male bluegill into the littoral areas of the lake, where they construct nests in the substrate. Nests are typically aggregated in colonies that form in areas with favourable substrate. Parental males behave aggressively toward cuckolders, brood predators, and other parental males during spawning, which occurs when females arrive at a colony and deposit eggs into nests (Gross 1982). Following spawning, parental males protect and care for the developing eggs and larvae for 7-10 days before the larvae and parental males leave the nest, moving into pelagic areas of the lake to forage. Multiple spawning "bouts" occur each summer, with parental males foraging in shoals and behaving non-aggressively towards conspecifics in the periods between bouts.

1.2.1.2 Resting immunity study

My first objective was to measure circulating 11-KT concentrations as well as levels of resting immunity in a wild population during the breeding season. From June 4 - 19 2015, parental male bluegill (n = 24) were collected in Lake Opinicon. I captured equal numbers of males either with dip nets while they were guarding nests that contained few to no eggs (≤ 10% of the nest surface covered by eggs, such that males were assumed to be in the early stages of spawning), or by angling. For all fish, 400 μL of whole blood was drawn from the

caudal vessel using a heparinized syringe that was prepared on the same day as collections by drawing and expelling a solution of 600 units/mL ammonium heparin (Sigma, #H6279 Oakville, Ontario). The fish were then measured for total body length and released. The time it took to collect the blood samples was recorded for each fish (it was always less than 2 minutes from capture). 200 µL of whole blood was then transferred into microcentrifuge tubes containing 20 µL of 600 units/mL ammonium heparin for use in a respiratory burst assay. The remaining 200 µL of blood was transferred into non-heparinized microcentrifuge tubes for blood smear cell counts and hormone analyses, as detailed below. All blood samples were kept on ice prior to analysis.

1.2.1.3 Immunochallenge study

My second objective was to test whether circulating 11-KT concentrations drop following an immunochallenge. To address this objective, on June 15, 2015 I captured a group of males (n = 11) using dip nets while they were guarding nests that contained few (<10% of nest surface covered) to no eggs. I measured total body length and clipped a unique combination of dorsal spines on each individual to allow subsequent identification. I transferred captured fish to floating net pens situated in the littoral area of the lake. Floating net pens have previously been shown to provide the least-stressful confinement conditions for bluegill, compared to fixed net pens in shallow water or indoor aquaria (Gutowsky et al. 2015). Each individual was placed into a separate 60 × 60 × 60 cm net pen and given 24 h to become accustomed to the new conditions. I removed fish from their net pens after the 24-hour rest interval and randomly assigned individuals to one of two treatments: one group received an intraperitoneal injection of 0.1 mL 0.85% NaCl (Sigma, #S9888 Oakville, Ontario) sterilized saline solution (n = 6), and the other group received an intraperitoneal injection of 0.1 mL Vibrogen 2 vaccine (n = 5; Novartis Animal Health, #870BA Charlottetown Prince Edward Island). 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. 24 h later, I removed fish from their net pens and drew 200 µL of whole blood from the caudal vessel using a heparinized syringe. Collected blood was transferred into non-heparinized microcentrifuge tubes and kept on ice. Fish were euthanized

via immersion in a concentrated solution of clove oil. Immediately after euthanization, the head kidneys were collected and placed into cryovials containing 1 mL of RNAlater solution (Sigma, #R0901 Oakville, Ontario). Head kidney samples were kept 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 the laboratory at Western University and stored in a -80°C freezer.

To address my third objective, namely, to examine the relationship between circulating 11-KT concentrations at the time of capture and the cytokine response following vaccination, I 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 was immediately drawn from the caudal sinus using a heparinized syringe. 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 hours after vaccination, with head kidney samples collected from each individual.

1.2.2 Laboratory analyses

1.2.2.1 Measuring granulocyte oxidative activity.

As part of my assessment of resting immunity, I used a respiratory burst assay to measure the oxidative activity of neutrophils undergoing phagocytosis (as in Marnila et al. 1995). Assays were carried out on blood samples within 60 minutes of their collection. Each well of a white 96 well plate (Fisher Scientific, #3917 Ottawa, Ontario) was pre-filled with 110 μL of Hank's balanced salt solution (HBSS; Sigma #H9394 Oakville, Ontario). 10 μL of heparinized whole blood from each individual was added to three test wells and three control wells. 60 μL of 1 mM luminol (Sigma, #A8511 Oakville Ontario) diluted in HBSS was then added to all wells. 20 μL of 20 mg/ mL zymozan A (Sigma, #Z4250 Oakville, Ontario) was then added to test wells, with control wells receiving 20 μL HBSS. The control wells were used as a measure of background respiratory burst activity not caused by the addition of zymozan A. Immediately prior to the first reading of the plate, all wells were mixed via aspiration. Luminescence was measured using a Glomax 96 luminometer (Promega, Madison, Wisconsin), with a program that took 99 repeated readings with 150 seconds

between readings, and an integration time of 0.5 seconds. Luminescence was recorded in relative light units (RLU). A respiratory burst curve was generated for each individual by subtracting the average luminescence values of the control wells from the average of the test wells, and plotting the net luminescence as a function of time. The peak luminescence of an individual's respiratory burst curve (maximum net luminescence observed during a 3 hour trial) was used as a measure of neutrophil activity (see Pérez-Casanova et al. 2008). The highest rate of increase in luminescence measured over a 10-minute interval (calculated as the slope of the respiratory burst curve) was used as a second measure of neutrophil activity. This latter measurement captures the rate of increase in granulocyte oxidative activity rather than the maximum activity. Peak luminescence and slope were highly correlated (Pearson $R = 0.97$, $n = 24$, $P < 0.001$), so I elected to present only the peak luminescence data.

1.2.2.2 Leukocyte counts

Leukocyte counts were used as a second measure of resting immunity. Blood smears were prepared within 60 minutes 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 minutes in Giemsa's azur eosin methylene blue solution (Sigma, #48900 Oakville, Ontario) at a 1:10 dilution in distilled water. One blood smear was damaged in storage, and a reliable leucocyte count could not be obtained, so this fish was excluded from the cell count analyses. Blood smears were examined at 100× magnification under an Axioimager Z1 confocal light microscope (Zeiss, Toronto, Ontario). ImagePro Premier software (MediaCybernetics, Rockville, Maryland) was used to automatically tally the total number of cells in each image, after which an observer manually tagged granulocytes and lymphocytes. A minimum of 9,500 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; (iii) lymphocytes: small spherical cells with little cytoplasm (Ros et al. 2006). Granulocyte and lymphocyte counts were each expressed as a percentage of all cells counted.

1.2.2.3 Gene expression

For the immunochallenge study, the expression of cytokine genes was used as a measure of activated innate immunity. First, RNA was extracted from head kidney tissue samples using 1 mL TRIzol (Thermo Fisher, #15596 Mississauga Ontario) and 0.2 mL chloroform (Sigma, #288306 Oakville Ontario), following the TRIzol manufacturer's instructions. Samples were centrifuged for 15 minutes at 12,000 rcf, and the aqueous phase was transferred to a fresh microcentrifuge tube and the organic phase was discarded. RNA was precipitated using 0.5 mL isopropyl alcohol (Sigma, #W292907 Oakville Ontario) and spun at 12,000 rcf for 10 minutes. The nucleotide-containing pellet was washed twice with 75% ethanol and then air dried until no moisture was visible (approximately 20 minutes). The dried pellet was resuspended in 25 μL of DEPC-treated water (Sigma, #95284 Oakville Ontario) and RNA concentration was determined with a Nanodrop 2000 (Thermo Fisher, #ND-2000, Mississauga, Ontario), and aliquoted into a 50 uL aqueous solution with a final RNA concentration of 200 μg/μL, such that each microcentrifuge tube contained 10 μg of RNA. Genomic DNA was removed from the solution using a commercial DNA-free kit (Thermo Fisher, #AM1906 Mississauga, Ontario). Following DNA removal, the 50 uL RNA-containing solution underwent a salt-removal protocol in which 5 μL of 3 M sodium acetate solution (Thermo Fisher, #AM9740 Mississauga, Ontario) and 150 μL of 100% ethanol were added to each sample and frozen at -80°C for 60 minutes. Samples were then centrifuged at 12,000 rcf for 10 minutes, and the RNA pellet was washed twice with a 75% ethanol solution. The pellet was left to air dry until no moisture was visible before being resuspended in 50 μL of DEPC-treated water. RNA concentration was determined, and samples were aliquoted into a 15 μL aqueous solution with a final RNA concentration of 66 μg/μL, such that each tube contained 1 μg of RNA. RNA was converted to cDNA using a commercial kit (VWR, #95047 Mississauga Ontario) and stored at -20°C.

Primers were developed to measure the expression 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 available on GenBank (NCBI) were used to design primers (Table 1). PCR product from each primer pair was sequenced at the London Regional Genomics Centre (London, Ontario) to confirm that the

intended genes were amplified. Nucleotide-BLAST searches for each sequence identified matches that had at least 85% similarity to transcript sequences of the expected genes.

Gene expression was then measured using PerfeCTa SYBR Green FastMix for IL1β and RPS18 (VWR, #95054 Mississauga, Ontario) and SensiFAST SYBR for IL8 and RPS18 (FroggaBio, #BIO-98005 North York, Ontario), according to the manufacturers' instructions. Negative controls containing water instead of cDNA template were included for each primer pair. Serial dilutions 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. Florescence measurements were performed in a Biorad CFX thermocycler (Biorad, #1855195 Mississauga, Ontario) set to the following program: initial 3 minute denaturation at 95.0°C, followed by 40 cycles of 95.0°C for 10 seconds with a 30 second annealing step at a primer-specific temperature followed by a florescence measurement.

Immediately after cycling, a melting curve protocol was run consisting of one cycle at 95°C for 5 seconds, a drop to 55°C, followed by a gradual increase in temperature to 95°C with fluorescence readings taken at every 0.5°C increase. Relative normalized expression for each individual was determined as follows: ΔCT (cycle threshold; the cycle number at which florescence crosses a pre-determined threshold) for each individual was calculated as the difference in CT between RPS18 and cytokine wells. The efficiency of each cytokine primer pair was raised to the power of ΔCT and divided by the efficiency of the RPS18 primer pair raised to the power of ΔCT (Livak & Schmittgen 2001). For each gene, the fish with the highest normalized expression served as the reference sample for the expression levels of all other fish.

1.2.2.4 11-KT enzyme immunoassays

Within 60 minutes of blood collection, a blood sample from each fish was centrifuged to separate the hormone-containing plasma from cellular blood components. Plasma from each individual was decanted into a new microcentrifuge tube and stored at -20°C. Circulating 11-KT concentrations were subsequently determined using a commercial enzyme immunoassay (EIA) kit according to the manufacturer's guidelines, including the optional purification step (Cayman Chemical, Ann Arbor, Michigan). For each individual, a 1:1 solution of hexane

(Sigma, #296090 Oakville, Ontario) and ethyl acetate (Sigma, #270989 Oakville, Ontario) was used to extract 11-KT from a 10 µL aliquot of plasma diluted in 490 µL of water. 4 mL of the hexane: ethyl acetate solution was added to each diluted plasma sample and vortexed. The organic phase was decanted into a new vial, and this procedure was repeated four times. The hexane:ethyl acetate solution was evaporated overnight, and the extracted 11-KT was reconstituted in 1,000 µL of EIA buffer the following day. Hormone samples underwent a final 1:10 dilution in EIA buffer, for a final dilution factor of 1:1,000. Extracted plasma samples were then analyzed in triplicate.

1.2.3 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 my studies, as well as for comparisons between the fish used in the two studies.

1.2.3.1 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, $P > 0.10$). 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 found, so body length was excluded from subsequent analyses.

To examine the relationship between immune parameters and 11-KT concentration, peak respiratory burst luminescence, granulocyte counts, and lymphocyte counts were each analyzed using a general linear model 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. Pearson

correlations were used to examine the pairwise relationships between my immunity measures: respiratory burst peak, granulocyte counts, and lymphocyte counts.

1.2.3.2 Immunochallenge study

Pearson correlations were used to examine pairwise relationships between body length and: 11-KT concentration, IL1β expression, and IL8 expression. No significant relationships were found amongst the pairwise relationships tested, so body length was excluded from subsequent analyses. Pairwise t-tests were used to compare circulating 11-KT concentration and cytokine gene expression level amongst the three experimental groups (bled at capture and vaccinated, vaccinated, or saline-treated). Pearson correlations were used to examine the relationships between circulating 11-KT concentration and the expression of each of the two cytokine genes. All statistical tests were performed using SPSS v.23 (IBM, Armonk, New York).

1.2.4 Immunochallenge meta-analysis

We selected studies that introduced an immune challenge in male adults and then measured the response of androgen concentrations. I searched for studies using the ISI Web of Knowledge, using combinations of the following keywords: testosterone, 11-ketotestosterone, androgen, immunity, immunocompetence, immunosuppression, parasites, vaccination, antigen, immunochallenge, immunocompetence handicap hypothesis, ICHH. I also included all studies analyzed by Boonekamp et al. (2008), and evaluated each study that had cited Boonekamp et al. 2008. I broadened Boonekamp et al. (2008)'s selection criteria by including studies in which an immunochallenge took place *in vitro* (e.g. Lister et al. 2002), and by including studies that were conducted in fishes. Specifically, I included studies that met the following criteria: (1) the immune system was challenged or experimental strains with genetic differences in immunity were produced, such that two or more groups with different levels of immune activity were directly compared, (2) androgen concentrations (testosterone or 11-KT) were measured following the immunochallenge, (3) the effect of the immune challenge could be compared with a control group, and (4) the data

were presented in such a way that the test statistics required to calculate effect size could be extracted.

Effect size (r) was calculated using the methods of Boonekamp et al. 2008. The p value corresponding to the test statistic that measured the effect of the immune challenge, along with sample size, were used to calculate r for each study. In some cases, r or r^2 values had been calculated by the authors of the studies, in which case I used that value as the study's effect size. Additional description of how the effect size was calculated for each study can be found in the Appendix.

Mean circulating androgen concentration for each study was estimated by determining the mean androgen concentration of control-treatment animals. For studies in which animals had a hormone measurement taken prior to an immunochallenge, the mean concentration value at this sampling point was used. For studies in which hormone concentrations were only measured following an immunochallenge, I used the mean concentration value of control animals at the earliest point of sampling. For studies in which animals with divergent levels of immunity were selected for, the mean androgen concentration of the study's entire sample prior to the immunochallenge was used. In cases where mean androgen concentration values were not reported in the text, I used Datathief (Tummers 2006) to extract the values from the appropriate figures.

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. I also noted whether studies were conducted on wild or captive animal populations, although the small number of studies using wild animals (4 out of 24 studies) prevented us from including this factor in my model. To determine whether the mean effect size across all studies was significantly different from zero, I used a one-sample t-test.

1.3 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 1). Body length did not differ between angled and dip-netted males ($t = 0.79$, $df = 22$, $P = 0.44$), nor did concentrations of circulating 11-KT ($t = 0.97$, $df = 22$, $P = 0.34$). Males captured for the immunochallenge study (189 ± 2 mm) were larger than males captured for the resting immunity study (182 ± 2 mm; $t = 2.62$, $df = 44$, $P = 0.01$). Body length did not differ between saline- and vaccine-treated fish used in the immunochallenge study ($t = 0.03$, $df = 9$, $P = 0.97$). Mean circulating 11-KT concentration was significantly higher in males from the resting immunity study (29.1 ± 5.7 ng) than males from the immunochallenge study (5.4 ± 0.7 ng; $t = 3.98$, $df = 44$, $P < 0.01$).

1.3.1 Resting immunity study

I examined the relationship between circulating 11-KT concentration and three resting immunity measures. Contrary to prediction 1 of the ICHH — namely, that androgen concentrations will be negatively related to immunity — I found no relationship between circulating 11-KT concentration and respiratory burst peak luminescence ($F = 0.03$, $df = 1, 19$, $P = 0.86$; Figure 1a), granulocyte counts ($F = 0.03$, $df = 1,19$, $P = 0.87$; Figure 1b), or lymphocyte counts ($F = 0.39$, $df = 1,19$, $P = 0.54$; Figure 1c).

Next, I compared the relationships among measures of resting immunity. There was a strong positive relationship between granulocyte counts and respiratory burst peak luminescence (Pearson $R = 0.75$, $n = 23$, $P < 0.001$; Figure 2). In contrast, lymphocyte percentage was unrelated to respiratory burst peak luminescence (Pearson $R = -0.21$, $n = 23$, $P = 0.33$). Granulocyte counts and lymphocyte counts were also unrelated to each other (Pearson $R = -0.33$, $n = 23$, $P = 0.29$).

1.3.2 Immunochallenge study

I examined the effect of treatment (saline or vaccine) on circulating 11-KT concentration following treatment. Compared to individuals that were captured and bled immediately,

neither saline-treated ($t = 1.51$, $df = 15$, $P = 0.15$) nor vaccine-treated ($t = 0.38$, $df = 14$, $P = 0.71$) fish had significantly different 11-KT concentration 24 h following treatment. 11-KT concentration of saline- and vaccine-treated groups was not significantly different from one another ($t = 1.16$, $df = 9$, $P = 0.28$). These results do not support prediction 2 of the ICHH: namely, that immunochallenges will lower androgen concentration.

Next, I compared levels of cytokine gene expression between saline- and vaccine-treated groups. Vaccinated fish had higher levels of expression for both IL1β ($t = 2.30$, $df = 11$, $P = 0.021$) and IL8 ($t = 2.30$, $df = 10$, $P = 0.022$; Figure 4). IL1β and IL8 expression levels were unrelated to each other (Pearson $R = 0.12$, $n = 10$, $P = 0.75$). No relationship was found between IL1β expression and circulating 11-KT concentration at the time of capture (Pearson $R = -0.43$, $n = 10$, $P = 0.22$), or between IL8 expression and 11-KT concentration at the time of capture (Pearson $R = 0.16$, $n = 11$, $P = 0.64$). Thus, although the vaccination induced an immune response, the strength of the response was unrelated to 11-KT concentration. These results do not support the hypothesis of androgens having immunosuppressive effects.

1.3.3 Immunochallenge meta-analysis

Overall, immunochallenges were found to lower circulating androgen concentration across taxa (mean effect size $r = -0.40$; $t = 5.64$, $df = 24$, $P < 0.0001$; Table 3). When I analyzed the variation in effect size across taxonomic groups, there were no significant differences in effect size among taxa; however, there was a trend in which the effect of immunochallenges on circulating androgen concentrations was smaller in fishes (mean $r = -0.1$) than birds (mean $r = -0.48$) or mammals (mean $r = -0.53$; $F = 3.29$ $df = 2, 21$, $P = 0.059$). For all studies in which circulating testosterone concentration was measured, testosterone concentration in control-treatment animals did not relate to the effect size of the immunochallenge ($F = 0.089$, $df = 3, 19$, $P = 0.769$).

Table 1. Description of parental male bluegill (*Lepomis macrochirus*) examined. The vaccine- and saline-treated groups in the immunochallenge study had their 11-KT concentrations measured 24 h following treatment, whereas all other groups had their 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		183 ± 2	34.6 ± 9.5
Immunochallenge study	Vaccine-treated	5	June 15	191 ± 3	4.4 ± 1.0
	Saline-treated	6		191 ± 5	7.3 ± 2.2
	Vaccine-treated and bled at capture	11	June 24	187 ± 4	4.8 ± 0.5

Table 2. Details of primers used in qPCR reactions for bluegill sunfish (*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		

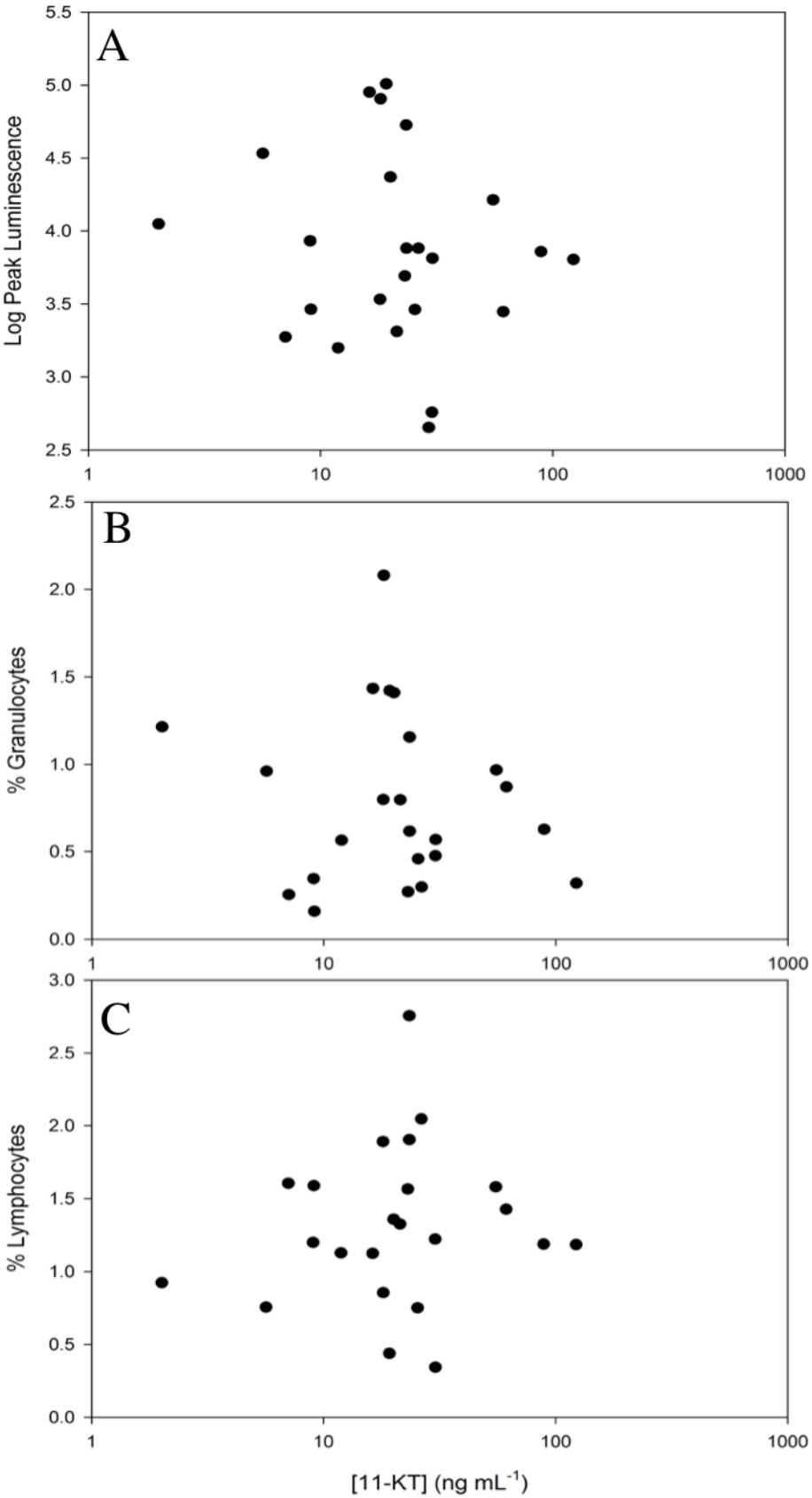
Table 3. Meta-analysis of the effect of immunochallenges on androgens. The data comprise the taxa, species, and whether the study animals were from wild or captive populations, in addition to effect size, total sample size and mean circulating androgen concentrations in control-treatment animals.

Reference	Taxa	Species	Wild or captive animals	Effect size (r)	N	Mean circulating testosterone (ng mL^{-1})	Mean circulating 11-KT (ng mL^{-1})
Barthelemy et al. 2004	Mammals	mouse	captive	-0.711	16	0.1	
Greiner et al. 2010		short-tailed fruit bat	wild	-0.645	25	6.6	
Hales et al. 2000		mouse	captive	-0.442	55	4.0	
He et al. 2000		mouse	captive	-0.708	18	NA	
Isseroff et al. 1986		mouse	captive	-0.827	42	2.2	
Kasilima et al. 2005		rabbit	captive	-0.261	16	0.7	
Mutayoba et al. 1997		sheep	captive	-0.516	9	1.9	
O'Bryan et al. 2000		rat	captive	-0.513	92	6.4	

O'Bryan et al. 2000		rat	captive	-0.386	136	3.2
Rogovin et al. 2015		hamster	captive	-0.196	24	0.7
Weil et al. 2006		mouse	captive	-0.575	62	11.2
Boltz et al. 2004	Birds	chicken	captive	-0.497	16	7.0
Boltz et al. 2007		chicken	captive	-0.511	19	3.0
Casagrande & Groothuis 2011		diamond dove	captive	-0.391	94	3.7
DeVaney et al. 1977		chicken	captive	-0.388	26	1.5
Garamszegi et al. 2004		collared flycatcher	wild	-0.584	13	NA
Muller et al. 2013		canaries	captive	-0.645	16	7.8
Verhulst et al. 1999		chicken	captive	-0.328	80	2.7
Lister et al. 2002	Fishes	goldfish	captive	-0.937	8	NA

Lister et al. 2002	goldfish	captive	0.966	8	NA	
Loggie et al. 2016	bluegill sunfish	wild	-0.358	11		7.3
Ros et al. 2012	St. Petersfish	captive	-0.008	47	16.7	
Ros et al. 2012	St. Petersfish	captive	-0.147	18		8.8
Vainikka et al. 2006	tench	wild	-0.093	40	1.1	

See appendix for details on how effect sizes were calculated for each study.


A
B
C
Log Peak Luminescence
% Granulocytes
% Lymphocytes
[11-KT] (ng mL-1)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
10
100
1000

Figurel 1. Relationships between circulating 11-KT concentrations and three measures of immunity in bluegill (*Lepomis macrochirus*). The relationships are plotted for log circulating 11-KT concentration and log respiratory burst peak luminescence (Panel A), log circulating 11-KTconcentration and granulocyte counts (Panel B), and log circulating 11-KT concentration and lymphocyte counts (Panel C).

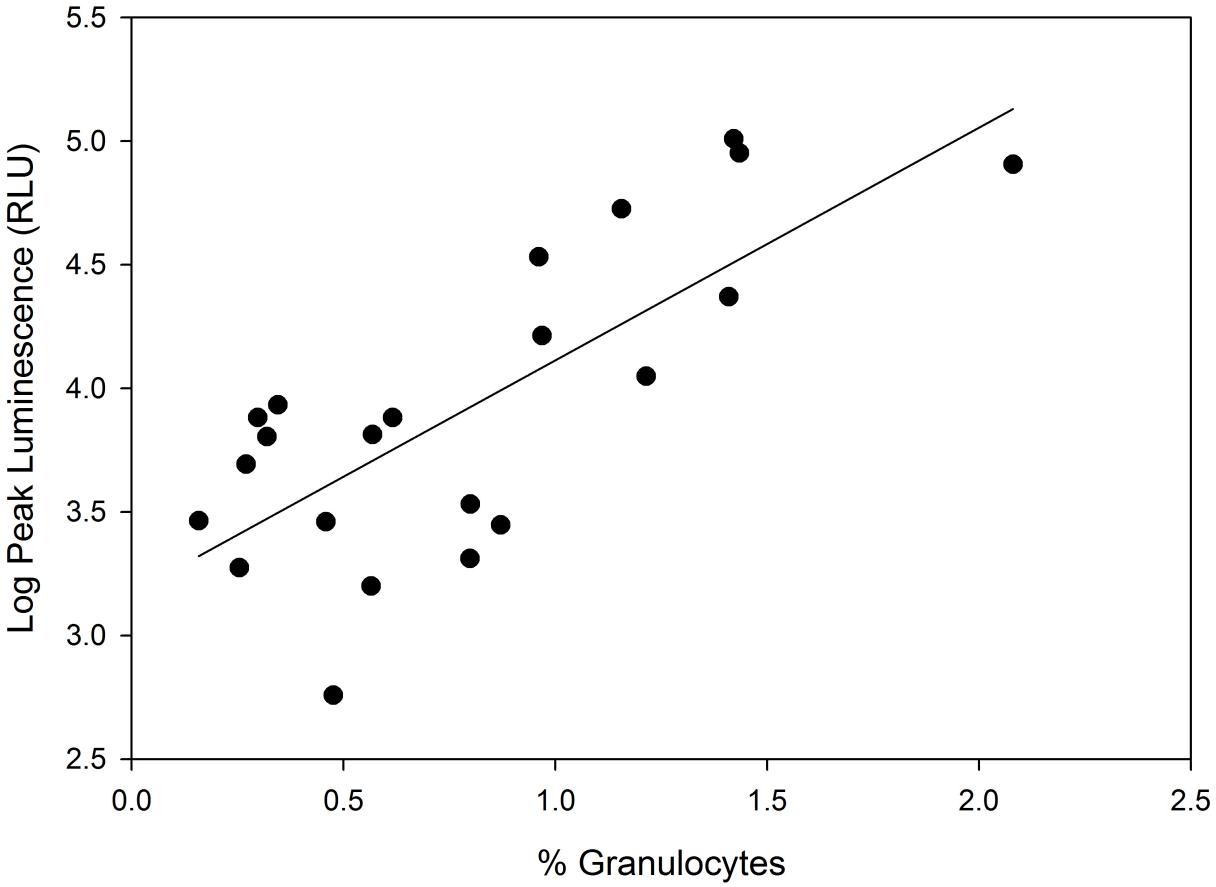


Figure 2. Relationship between granulocyte counts in blood samples and peak respiratory burst activity in bluegill (*Lepomis macrochirus*).

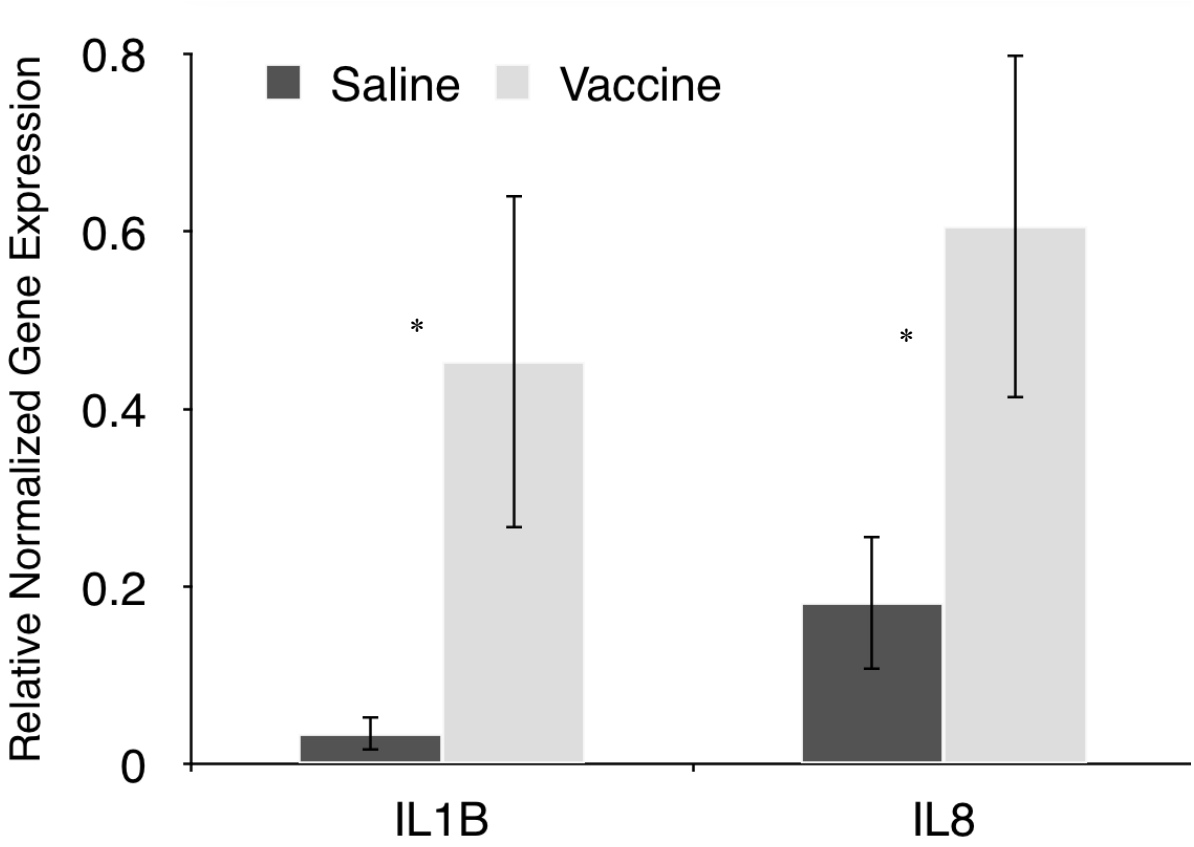


Figure 3. Cytokine gene expression in bluegill (*Lepomis macrochirus*) 24 hours following a saline or vaccine treatment. Means ± SE are plotted for IL1β and IL8. Expression levels were normalized using RPS18 as a reference gene. The individual with the highest normalized expression served as the reference sample for the expression levels of all other fish. * indicates significant differences.

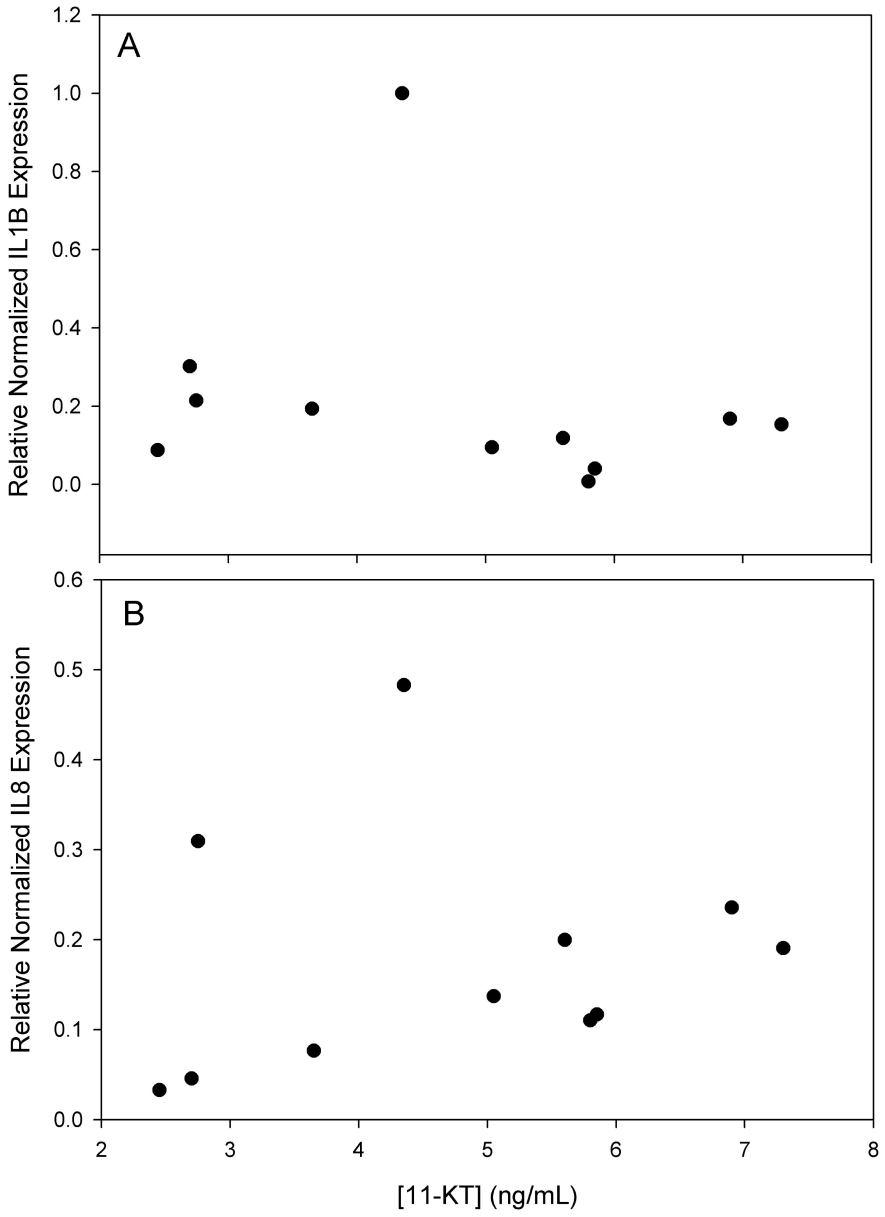


Figure 4. Relationships between circulating 11-KT concentrations at the time of capture and the expression of cytokine genes (A) IL1β and (B) IL8 24 hours following vaccination in bluegill (*Lepomis macrochirus*).

1.4 Discussion

It has been nearly 25 years since Folstad & Karter (1992) introduced the immunocompetence handicap hypothesis (ICHH), which attempts to explain the evolution of androgen-mediated traits and behaviours as honest indicators of male quality. The ICHH proposes that androgens have immunosuppressive effects. To determine if androgens are immunosuppressive, three predictions have been tested in multiple studies: (1) natural androgen concentrations will be negatively related with immunity, (2) experimentally elevated androgen concentrations will suppress immunity, and (3) immunochallenges will lower androgen concentrations. To date, most of these studies have been conducted in taxa whose primary androgen is testosterone, with predictions 2 and 3 having received significant support across studies (Boonekamp et al. 2008; Foo et al. 2016). Here, I tested two of these predictions (1 and 3) in a fish where 11-KT is the primary androgen (Borg 1994). I found no relationship between natural variation in circulating 11-KT and any measure of immunity, nor did 11-KT concentrations drop following an immunochallenge. Below, I discuss these findings within the larger context of the ICHH and its relevance in systems that do not have testosterone as the primary androgen.

If 11-KT is immunosuppressive, natural variation in the circulating concentration of this androgen should be negatively associated with immunity. I measured components of resting immunity (leukocyte counts and respiratory burst activity of circulating granulocytes) as well as the innate immune response (cytokine gene expression following vaccination), and found no relationship between circulating 11-KT concentrations and any of these measures of immunity. Similarly, in other studies of fishes, there was no relationship between 11-KT and leucocyte counts, or between 11-KT and the antibody response in Azorean rock-pool blennys (*Parablennius parvicornis*; Ros et al. 2006), and no relationship between 11-KT and lymphocyte counts in peacock blennys (*Salaria pavo*; Ros and Oliveira 2009). However, other studies of 11-KT in fishes have produced results that are consistent with the prediction that androgen concentrations will be negatively related with immunity: a negative relationship was observed between 11-KT and the antibody response in St. Petersfish (*Sarotherodon galilaeus*; Ros et al. 2012) and a positive relationship was found between 11-KT and papillomatosis infection severity in roach (*Rutilus rutilus*; Kortet et al. 2003).

Overall, two of the five studies (including my study) that have examined the relationship between natural concentrations of 11-KT and immunity have found evidence supporting the immunosuppressive effect of androgens, with no contradictory findings (e.g. positive relationships between 11-KT and immunity) having been reported. Correlative studies of testosterone and immunity show mixed results across vertebrates, with some studies finding the predicted negative relationships (e.g. Duffy & Ball 2002; Halliday et al. 2014) and other studies finding no relationship (e.g. Hasselquist et al. 1999; Ros & Oliviera 2009; Prall et al. 2015) or positive relationships (e.g. Peters 2000; Rentala et al. 2012; Dongming et al. 2015). One reason that 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. Thus, correlative studies of natural androgen concentrations and immunity may not provide a good test of the ICHH.

If 11-KT is immunosuppressive, concentrations of this androgen should drop following an immunochallenge. I examined the effect of an immunochallenge on the circulating concentration of 11-KT and found no significant effect of a vaccination on 11-KT concentrations 24 hours following treatment. This result agrees with the only other study that examined the effect of an immunochallenge on circulating concentration of 11-KT, which found no decrease in 11-KT following an immunochallenge in St. Petersfish (Ros et al. 2012). The effect of immunochallenges on testosterone has also been examined in fishes, with three studies to date. In goldfish (*Carassius auratus*), the effect of an immunochallenge on testosterone was dependent on the relative size of the testes (Lister et al. 2002). In this study the testes were surgically removed and incubated before being administered an immunochallenge in the form of cytokines IL1β and TNFα. For testes isolated from fish with relatively high gonadosomatic indexes, the immunochallenge led to lower testosterone production. For testes isolated from fish with relatively low gonadosomatic indexes, the immunochallenge led to higher testosterone production (Lister et al. 2002). In tench (*Tinca tinca*) and in St. Petersfish, immunochallenges had no effect on circulating testosterone

concentration (Vannika et al. 2005; Ros et al. 2012). Together these five studies do not support an effect of immunochallenges on circulating androgen concentration in fishes.

In a meta-analysis of studies that examined the effect of immunochallenges in taxa other than fishes, a significant negative effect of immunochallenges on testosterone concentrations was found (Boonekamp et al. 2008). I 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. I found an overall significant effect of immunochallenges on androgens, but the mean effect size across all studies was smaller in my meta-analysis than what was previously found ($r = -0.40$ vs. $r = -0.52$; Boonekamp et al. 2008). This reduction in effect size was largely driven by my inclusion of immunochallenge studies in fishes, which on the whole tended to have weak effect sizes regardless of whether testosterone or 11-KT was studied. Overall, my meta-analysis found support for the testosterone-suppressing effects of immunochallenges in birds and mammals, but to date there is little evidence in fishes that either testosterone or 11-KT respond to immunochallenges.

There are a number of potential reasons for why the evidence in favour of immunochallenges lowering androgens appears to be weaker in fishes relative to other groups of vertebrates. This difference may occur because fishes use 11-KT as a primary androgen, rather than testosterone as in other vertebrates (Borg 1994). In fishes, 11-KT plays many of the roles traditionally occupied by testosterone in other taxa (Pall et al. 2002; Ros et al. 2006b; Mayer et al. 2004; Neff & Knapp 2009). Consequently, testosterone in fishes may act primarily as a precursor to 11-KT rather than having direct effects on reproduction and immunity, which may explain why immunochallenges do not appear to lower circulating testosterone concentration in fishes. However, in the context of the ICHH, if 11-KT has taken over the role of regulating male secondary sexual characters and reproductive behaviour, 11-KT concentration would be expected to drop following an immunochallenge, but no evidence for an effect of immunochallenges on 11-KT has been found, including in my study. The fact that 11-KT is non-aromatizable, unlike testosterone, may affect how circulating concentrations of this androgen respond to an immunochallenge. 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). However, this explanation assumes that testosterone is cleared from the body following an immunochallenge via increased aromatization to estradiol. The mechanism by which testosterone concentrations drop following an immunochallenge is currently unknown. Rather than actively removing androgens from circulation through pathways such as aromatization, the most efficient way for individuals to lower their androgens following an immunochallenge may be to cease androgen production, as steroid hormones in fishes have relatively short half-lives ranging from 2-18 hours (Idler et al. 1963; Baroiller et al. 1987; Querat et al. 1985).

Alternatively, the smaller effect sizes of immunochallenges on circulating androgen concentration in fishes could be a result of differences in the immune system between fishes and other vertebrates, rather than differences in their primary androgens. It has been suggested that fever production may play a causal role in the decrease in androgens following an immunochallenge in endotherms (Ros et al. 2012). However, within immunochallenge studies in endotherms, fever induction is not typically observed, so it seems unlikely that an elevated body temperature is required in order to elicit a drop in circulating androgen concentration (e.g. O'Bryan et al. 2000; Greiner et al. 2010). Moreover, fishes have been shown to be capable of undergoing behavioural fever (by swimming into warmer water) which serves as a means to elevate their body temperature in response to an immunochallenge, thereby eliciting a similar physiological change as an endothermic fever (Reynolds et al. 1976). Other differences in the immune systems of fishes, such as their lower diversity of immunoglobulins or higher specialization of B-cell lineages may instead contribute to differences in how immunochallenegs affect androgens, yet the general structure of the fish immune system is remarkably similar to that of other vertebrates (Magadan et al. 2015). It remains premature to say with certainty what differences between fishes and other vertebrates may account for the apparent lower effect sizes in response to immunochallenges.

It has been suggested that a species' mean circulating androgen concentration may affect the relationship between androgens and immunity (Roberts et al. 2004) because species that produce high concentrations of androgens may need to lower their circulating androgen

concentration more than species with low androgen concentrations to facilitate an immune response. I explored this hypothesis by analyzing the relationship between the effect size of immunochallenges on testosterone and the mean testosterone concentration of control animals in each study included in my meta-analysis. There were an insufficient number of studies to perform a similar analysis for circulating 11-KT concentrations. I predicted that study animals with higher mean testosterone concentrations at the time of the immunochallenge would demonstrate larger effect sizes, but I found no relationship between these two measures, suggesting that the effect of immunochallenges on testosterone is independent of testosterone concentration. The absence of a relationship between absolute circulating testosterone concentration and the response to an immunochallenge may occur because androgen effects depend in large part on androgen receptor densities, which also differ across taxa. For example, two subspecies of dark-eyed junco (*Junco hyemalis*) that differ in their aggression were found to also differ in androgen receptor expression but not in circulating testosterone concentration (Burns et al. 2013). Furthermore, spotted antbirds (*Hylophylax naevioides*) have been found to maintain a steady circulating testosterone concentration throughout the year, but the aggressive behaviour associated with breeding instead corresponds to an increase in androgen receptor expression (Canoine et al. 2007). These findings suggest that animals have a secondary means to regulate the downstream effects of androgens. The lack of a relationship found between circulating androgen concentration and the effect size of immunochallenges may be due to this additional level of regulation and complexity in endocrine system function. Examining the relationship between mean androgen concentrations and the effect of immunochallenges while controlling for variation in androgen receptor density would serve as an important test of the hypothesis that mean circulating androgen concentration affects the response following an immunochallenge.

The third prediction that arises from the posited immunosuppressive effects of androgens, that experimentally elevated androgens will suppress immunity, has received a great deal of attention. Indeed, some of the strongest support for testosterone being immunosuppressive comes from studies that experimentally raised circulating testosterone concentrations and then observed decreased immunity (Foo et al. 2016). Amongst the smaller collection of studies that have manipulated 11-KT, some authors found an immunosuppressive effect of

experimental 11-KT elevation (Watanuki et al. 2002; Kurtz et al. 2007), whereas other authors found no effect (Law et al. 2001; Ros et al. 2006b). In my study, I was interested in whether natural variation in androgen concentration would relate to variation in immunity, so I chose not to experimentally manipulate 11-KT concentrations. An 11-KT manipulation study would be useful to address the prediction that I did not test: namely, that experimentally elevated concentrations of this androgen will suppress immunity. The four 11-KT manipulation studies carried out to date have suggested that experimental elevations of this androgen are immunosuppressive. However, more 11-KT studies are needed before the overall effects of experimental 11-KT elevations can be analyzed to the same depth as previous analyses of testosterone in other taxa.

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. It took many studies to conclude that testosterone manipulations are immunosuppressive, with this effect supported by a recent meta-analysis (Foo et al. 2016), but not an earlier meta-analysis (Roberts et al. 2004). While fewer studies were required in order to show that immunochallenges lower testosterone concentrations (Boonekamp et al. 2008), my meta-analysis detected a weaker effect size of immunochallenges on androgens in fishes, which challenges the notion that immunochallenges lead to decreased androgen concentrations in all taxa. Overall, even for the two predictions of the immunosuppressive effect of androgens that have received support in the testosterone literature, there is considerable variation in the strength of the evidence across studies. This variation may be due to differences in study designs, although it is likely that at least some of the variation is due to biological differences in the species being studied. The likelihood of detecting a negative relationship between androgens and immunity may depend on the life history of the species being examined. For example, many species produce androgens during their breeding season, but the traits and behaviours being mediated by these androgens may vary in their costliness to the organism. In this framework, the androgens themselves are not the cost, but the traits and behaviours associated with the androgens may still be costly. One mechanism through which this ‘cost’ could be incurred is through the elevation of circulating corticosteroid concentration (Rantala et al. 2012). Testosterone and

corticosterone have been shown to correlate in several species (e.g. Poiani et al. 2000; Evans et al. 2000; Sockman & Schwabl 2001; but see Astheimer et al. 2000), and the evidence linking corticosteroids to immunosuppression is strong (e.g. Munck et al. 1984; Apanius 1998). The idea of corticosteroids being the mediators of the immunosuppressive effects of androgens is supported by the finding that androgens do not appear to exert their immunosuppressive effects directly by binding to androgen receptors (Owen-Ashley et al. 2004). Comparing the effects of androgen and corticosteroid manipulations on immunity, as well as measuring the response of circulating corticosteroid concentration to an immunochallenge would both be interesting experiments for determining whether elevated corticosteroids are the means by which androgens exert their immunosuppression. Furthermore, it needs to be determined whether 11-KT demonstrates the same relationship with corticosteroids as testosterone. Differences in how testosterone and 11-KT relate to corticosteroids may be the key to explaining why these two androgens appear to demonstrate different relationships with immunity. The ICHH depends on the immunosuppressive effect of androgens as a means of explaining how the honest signalling of androgen-mediated traits is maintained across a wide range of taxa. If testosterone is immunosuppressive but other androgens that facilitate reproductive success, such as 11-KT, are not found to be immunosuppressive, this finding will necessitate a re-evaluation of the ICHH, as the hypothesis would be unable to explain what constrains 11-KT concentration during breeding. It is therefore critical that androgens other than testosterone, such as 11-KT, continue to receive attention in the context of the ICHH, as it remains to be determined whether Folstad & Karter's (1992) hypothesis applies to all species that use androgens during reproduction.

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Appendices

Appendix A: Details for how effect sizes and mean circulating androgen concentrations in control-treatment animals were calculated for each study included in my immunochallenge meta-analysis.

Barthelemy et al. 2004	Effect size from Boonekamp et al. 2008, testosterone data from Figure 1
Greiner et al. 2010	Sample size and p value from Wilcoxon signed-ranks test (Z = -4.27), testosterone concentration directly from paper
Hales et al. 2000	Effect size from Boonekamp et al. 2008, testosterone data from Figure 1A
He et al. 2000	Effect size from Boonekamp et al. 2008, unable to obtain mean testosterone concentration in control animals. Attempted correspondence with author, no reply.
Isseroff et al. 1986	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
Kasilima et al. 2005	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper (Table 2)
Mutayoba et al. 1997	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
O'Bryan et al. 2000	Effect size from Boonekamp et al. 2008, testosterone data from Figure 1C
Rogovin et al. 2015	Sample size and p value from t test (t = 0.91; Table 1). Mean testosterone across low and high immunoreactivity groups was calculated from means and sample sizes provided in Table 1.
Weil et al. 2006	Effect size from Boonekamp et al. 2008, testosterone data from Figure 2A
Boltz et al. 2004	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
Boltz et al. 2007	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
Casagrande & Groothuis 2011	Sample size and p value from ANOVA (F = 21.77; Table 1). Testosterone concentration directly from paper (Appendix, Table 3)
DeVaney et al. 1977	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
Garamszegi et al. 2004	Effect size from Boonekamp et al. 2008, testosterone concentration directly from paper
Muller et al. 2013	Effect size from Boonekamp et al. 2008, circulating testosterone concentration was not measured
Verhulst et al. 1999	Sample size and p value from AIC critical ratio statistic (C.R. = -2.7). Testosterone concentration directly from paper (Table 1)

Lister et al. 2002	Sample size and p value from ANOVA (no test statistic reported). Circulating testosterone concentration was not measured.
Ros et al. 2012	Sample size and p value from ANOVA ($F = 0.003$). Testosterone and 11-ketotestosterone concentrations determined via correspondence with author.
Vainikka et al. 2006	Sample size and p value from ANCOVA (no test statistic reported). Testosterone data from Figure 1.

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Issuer Account No. / N° de compte du délivreur de permis	10000846

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	Mr. Neff	Bryan	D

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University of Western Ontario

Mailing address of Licencee / Adresse postale du titulaire du permis	Street Name & No./PO Box/RR#/Gen. Del./ N° rue/C.P./R.R./poste restante		
	1151 Richmond Street		
	City/Town/Municipality / Ville/village/municipalité	Province/State / Province/État	Postal Code/Zip Code / Code Postal/Zip
	London	ON	N6A 5B7

to collect the species, size and quantites of fish from the waters as set out below.
Pour faire la collecte des espèces suivantes (stade et nombre indiqués ci-dessous):

Species / Espèces	Eggs / Oeuf X	Juvenile / Fretin X	Adults / Adulte X	Numbers / Nombre	Name of Waterbody / Nom de l'étendue d'eau
Bluegill			X	400	Lake Opinicon

Yes/Oui ☐ Additional species/Waterbody list attached / Liste d'espèces/d'étendue d'eau additionnelles ci-jointe

Purpose of collection / But de la collecte	Studying mating systems in fish

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FW0032 (04/00)

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