

Electronic Thesis and Dissertation Repository

4-16-2012 12:00 AM

Effects of exogenous androgens on parental care behaviour in male bluegill sunfish (*Lepomis macrochirus*)

Chandra M.C. Rodgers
The University of Western Ontario

Supervisor
Dr. Bryan Neff
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
© Chandra M.C. Rodgers 2012

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Behavior and Ethology Commons](#)

Recommended Citation

Rodgers, Chandra M.C., "Effects of exogenous androgens on parental care behaviour in male bluegill sunfish (*Lepomis macrochirus*)" (2012). *Electronic Thesis and Dissertation Repository*. 504.
<https://ir.lib.uwo.ca/etd/504>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

**EFFECTS OF EXOGENOUS ANDROGENS ON PARENTAL CARE BEHAVIOUR
IN MALE BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*)**

(Spine title: Effects of androgens on paternal care in bluegill sunfish)

(Thesis format: Monograph)

by

Chandra M.C. Rodgers

Graduate Program in Biology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Chandra M.C. Rodgers 2012

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Examiners

Dr. Bryan Neff

Dr. Graham Thompson

Supervisory Committee

Dr. Louise Milligan

Dr. Scott MacDougall-Shackleton

Dr. Scott MacDougall-Shackleton

Dr. Jeremy McNeil

The thesis by

Chandra Mary Catherine Rodgers

entitled:

**Effects of exogenous androgens on parental care behaviour in
male bluegill sunfish (*Lepomis macrochirus*)**

is accepted in partial fulfillment of the
requirements for the degree of
Master of Science

Date

Chair of the Thesis Examination Board

Abstract

Research suggests an androgen mediated trade-off between nurturing and defensive behaviour during parental care. This research, however, comes from species with biparental care, where changes in behaviour of one parent can be compensated for by the other parent. I tested the validity of this trade-off by manipulating androgen levels in bluegill sunfish (*Lepomis macrochirus*), a species where males provide sole parental care. I implanted males with testosterone, 11-ketotestosterone or flutamide, an androgen receptor blocker, and tested their nurturing behaviour and aggressiveness towards a brood predator. Males implanted with 11-ketotestosterone were 64% more aggressive and 71% less nurturing than controls. In contrast, males implanted with flutamide were 7% less aggressive and 126% more nurturing. Males with elevated testosterone levels showed marginally higher aggression, but no reduction in nurturing behaviour. This study is among the first to confirm an androgen mediated trade-off in aggression and nurturing in a uniparental care system.

Keywords: Behaviour, bluegill sunfish, androgens, flutamide, parental care, aggression, nurturing, trade-off, Challenge Hypothesis

Co-Authorship Statement

Chandra Rodgers: Observed fish behaviours, assisted in performing radioimmunoassays, analyzed data and drafted the manuscripts.

Bryan Neff: Observed fish behaviours and provided input into experimental design and the manuscripts.

Rosemary Knapp: Collaborated in performing radioimmunoassays, and provided input into experimental design and the manuscripts.

Acknowledgments

Firstly, I thank my supervisor, Bryan Neff, for his sincere support throughout the past two and a half years. Bryan has played an active role in his participation in my project. His intelligent ideas and expertise in writing effectively have greatly shaped my writing skills, critical thinking, and have made my masters a valuable learning experience. Likewise, my collaborator, Rosemary Knapp, has been supportive of my project as a whole, from assisting in performing radioimmunoassays and brainstorming ideas for the discussion in manuscripts to sketching and scanning emergency pictures to me of how to take blood samples from a fish. A special thanks to my advisory committee, Jeremy McNeil and Scott MacDougall-Shackleton for helpful direction in shaping and guiding me through my thesis project. Jessica Van Zwol, Ross Breckels, Aimee Lee Houde, Shawn Garner, Adam Henkel, Nico Munoz, Ainsley Furlonger, Marie-Christine Bellemare, Michael Roubakha, Adriana Clapp and Cory Toth have assisted me in performing statistical analyses, provided valuable input on manuscripts and/or have actively helped me collect data in the field. Tim Hain has provided tremendous guidance to me through his statistical knowledge, his writing abilities and his eagerness to help or de-stress me in any way he could. Furthermore, I extend my sincere gratitude to Scott Colborne who has been one of my greatest supporters both academically and emotionally. These individuals have also made my journey through my masters not only fun, but filled with new learning experiences. My close friends, Taylor Martino, Dianne Vandepas, Courtney Rowe and Mallory Fraser, listened endlessly to me practicing presentations so much so that they could likely recite one on cue. I express immense appreciation to my family, Lee Anne Rodgers, Krista Rodgers and Danielle Rodgers who, to this day, do not entirely understand my project, but have listened to me rave about it for longer than I am sure they cared to. Finally, and foremost, I share this thesis with my father, James W. Rodgers, who was my strongest believer and carried me through both the good times and the bad.

Table of Contents

CERTIFICATE OF EXAMINATION	ii
Abstract	iii
Co-Authorship Statement.....	iv
Acknowledgments.....	v
Table of Contents	vi
List of Tables	viii
List of Figures	ix
List of Appendices	x
1 Chapter 1: Introduction	1
1.1 The relationship between hormones and behaviour.....	1
1.2 Hypothalamic-Pituitary-Gonadal axis	1
1.3 The Challenge Hypothesis	2
1.4 Trade-off within parental care: an extension of the Challenge Hypothesis.....	3
1.5 Bluegill sunfish: a uniparental study species	4
1.6 Research objective	6
2 Chapter 2: Materials and Methods	7
2.1 Treatment assignment and implants.....	7
2.2 Behavioural observations.....	8
2.3 Radioimmunoassay	9
2.4 Statistical analyses	11
3 Chapter 3: Results	13
3.1 Testosterone manipulations (2009).....	13
3.1.1 <i>Abandonment data</i>	13
3.1.2 <i>Hormone data</i>	13

3.1.3	<i>Behavioural data</i>	17
3.2	11-ketotestosterone and flutamide manipulations (2010).....	20
3.2.1	<i>Abandonment data</i>	20
3.2.2	<i>Hormone data</i>	20
3.2.3	<i>Behavioural data</i>	23
4	Chapter 4: Discussion	27
4.1	Testosterone manipulations (2009).....	27
4.2	11-ketotestosterone and flutamide manipulations (2010).....	29
4.3	Conclusions.....	31
5	References	33
6	Appendices.....	40
7	Curriculum Vitae.....	48

List of Tables

Table 1: Number of male bluegill sunfish (<i>Lepomis macrochirus</i>) that stayed and abandoned throughout the experiment	14
Table 2: Mean (\pm SD) hormone levels, bleed times, Fulton's condition factor, body length, body mass and egg scores of parental male bluegill (<i>Lepomis macrochirus</i>) with experimentally manipulated levels of testosterone	15
Table 3: Summary of repeated measures analysis of variance for hormone levels in parental male bluegill (<i>Lepomis macrochirus</i>) with experimentally manipulated levels of testosterone	16
Table 4: Mean (\pm SD) hormone levels, bleed times, Fulton's condition factor, body length, body mass and egg scores of parental male bluegill (<i>Lepomis macrochirus</i>) with experimentally manipulated levels of 11-ketotestosterone and flutamide.....	21
Table 5: Summary of repeated measures analysis of variance results for hormone levels in parental male bluegill (<i>Lepomis macrochirus</i>) with experimentally manipulated levels of 11-ketotestosterone and flutamide	22

List of Figures

- Figure 1.** Mean (\pm SE) number of aggressive and nurturing behaviours by male bluegill (*Lepomis macrochirus*) in response to experimentally manipulated levels of testosterone ... 18
- Figure 2.** Figure 2. Mean (\pm SE) change in circulating KT concentrations in male bluegill sunfish (*Lepomis macrochirus*) with manipulations of 11-ketotestosterone or flutamide 24
- Figure 3.** Mean (\pm SE) number of aggressive and nurturing behaviours by male bluegill (*Lepomis macrochirus*) in response to experimentally manipulated levels of 11-ketotestosterone and flutamide 25

List of Appendices

Appendix A: Nurturing and aggressive behaviours quantified during parental care in male bluegill (<i>Lepomis macrochirus</i>).....	40
Appendix B: Mean (\pm SD) nurturing and aggressive behaviours by parental male bluegill (<i>Lepomis macrochirus</i>) with manipulated levels of testosterone	41
Appendix C: Mean (\pm SD) nurturing and aggressive behaviours by parental male bluegill (<i>Lepomis macrochirus</i>) with manipulated levels of 11-ketotestosterone or fluatamide.....	42
Appendix D: Mean (\pm SD) PC1 values for nurturing and aggressive behaviours by parental male bluegill (<i>Lepomis macrochirus</i>).	43
Appendix E: Representative radioimmunoassay standard curve for plasma 11-ketotestosterone for my 2010 study	44
Appendix F: Representative radioimmunoassay standard curve for plasma testosterone for my 2010 study.....	45
Appendix G: Representative radioimmunoassay standard curve for plasma cortisol for my 2010 study.....	46
Appendix H: Ethics approval	47

1 Chapter 1: Introduction

1.1 The relationship between hormones and behaviour

While there has recently been increasing interest on the relationship between hormones and behaviour in a wide range of disciplines, there has been a long history of research focused on these interactions. Among the first to study hormone-behaviour relationships was A.A. Berthold, who showed that testosterone (T) affects the mating and aggressive behaviours of roosters, *Gallus gallus*. Upon castration of the immatures, the males did not develop to display typical rooster plumage, mating behaviour or aggression (Berthold 1849). However, when one of their testes was re-implanted, the birds developed into typical adults (for a review, see Nelson 2000). Since this early experiment, many endocrinologists have been interested in androgens, such as T, and their influences on behaviour (e.g. Rose and Holaday 1971; Silverin 1980; Eising and Groothuis 2003; Mutzel et al. 2011).

1.2 Hypothalamic-Pituitary-Gonadal axis

In vertebrates, T and its derivatives have been found to play a key role in the hormonal link to aggressive behaviours. For example, during the mating season male ring-tailed lemurs (*Lemur catta*) with higher T displayed more aggressive acts, such as biting, chasing and lunging, than males with lower T (Cavigelli and Pereira 2000). Furthermore, subordinate male cichlids (*Oreochromis moccambicus*) tend to have both lower 11-ketotestosterone (KT), the primary T derivative in fish, and exhibit lower aggression than dominant males (Oliveira et al. 1996). On the other hand, dominant male cichlids (*Neolamprologus pulcher*) have higher levels of KT and exhibit aggressive behaviours more frequently than subordinate males (Taves et al. 2009). These hormones are involved in the hypothalamic-pituitary-gonadal (HPG) axis, which is an important component underlying hormone-behaviour relationships in vertebrates. When stimuli, such as a

competitive encounter, signal the hypothalamus to release gonadotropin releasing hormone (GnRH), the GnRH in turn signals the anterior pituitary gland to produce lutenizing hormone (LH), which subsequently signals the testes to produce T (Nelson 2000). Testosterone and its derivatives can bind to receptors forming a complex and subsequently the complex can influence behaviour by either binding directly to genes and influencing transcription of those genes or by activating a secondary messenger to do so (Ikeuchi et al. 2001). An increase in the concentration of any of these hormones could increase the probability that individuals will behave in a certain way (e.g. compete or submit). However, this cycle cannot continue indefinitely and when the circumstances are appropriate, such as the removal or completion of a competitive encounter, there is a negative feedback loop where high concentrations of T signal back to the pituitary and hypothalamus to stop producing LH and GnRH, respectively, thus ending the production of T and its derivatives (Nelson 2000). In fish, T is either converted to KT by the enzyme 17- β -hydroxysteroid dehydrogenase or to estradiol by the enzyme aromatase, and these steroids bind to intercellular or intracellular receptors to have downstream influences on behaviour (Borg 1994; Steinman and Trainor 2010). Overall, the influence of the HPG axis is a key component behind the observed relationship between hormones and certain behaviours.

1.3 The Challenge Hypothesis

The 'Challenge Hypothesis' suggests that individuals with high levels of androgens show high levels of territorial aggression or defence when confronted with an intruder (Wingfield et al. 1990). For example, Hau et al. (2000) found that male spotted antbirds (*Hylophylax naevioides*) with experimentally increased androgen levels displayed a higher frequency of aggression toward a social challenger than control males, while those implanted with the androgen receptor blocker, flutamide, were less aggressive than controls. The hypothesis has been studied predominately in birds (e.g. dark-eyed juncos, *Junco hyemalis*: Cawthorn et al. 1998; cliff swallows, *Petrochelidon pyrrhonota*: Smith et al. 2005; house sparrows, *Passer domesticus*: Buchanan et al. 2010). However, there is support for the Challenge Hypothesis across a diverse range of taxa including reptiles

(e.g. tree-lizards, *Urosaurus ornatus*: Weiss and Moore 2004), insects (via their androgen equivalent, juvenile hormone, e.g. burying beetles, genus *Nicrophorus*: Scott 2006) and mammals (e.g. ring-tailed lemurs, *Lemur catta*: Cavigelli and Pereira 2000; California mice, *Peromyscus californicus*: Trainor and Marler 2001; Iberian wolves, *Canis lupus*: Barja et al. 2008) including humans (Archer 2006; Gettler et al. 2011). This research has contributed a great deal to our understanding of hormone-behaviour interactions.

1.4 Trade-off within parental care: an extension of the Challenge Hypothesis

There has also been increasing interest in the potential trade-off between aggression and nurturing behaviours during parental care. Building on the Challenge Hypothesis, it has been proposed that this trade-off is also mediated by changes in androgens. In an early study testing the effects of androgens on parental behaviours, Hegner and Wingfield (1987) implanted male house sparrows with exogenous T or with the androgen receptor blocker, flutamide. They found that T-implanted males performed more acts of nest defence but fewer acts of nurturing behaviours (i.e. feeding the young) than control males, while the inverse was seen in flutamide-implanted males. More recently, Schwagmeyer et al. (2005) also found a similar behavioural trade-off in T-implanted house sparrows. McGlothlin et al. (2007) found that, under natural conditions, male dark-eyed juncos with elevated GnRH (and subsequently increased T) displayed more territorial aggression towards an intruder and provided less food to their offspring than control males. These studies on birds, among others (e.g. Silverin 1980; Ketterson et al. 1992), provide early evidence that male parental behaviours may be affected by androgen levels.

The majority of studies examining the trade-off have done so in species with bi-parental care, but interpretation of the results from such studies could be problematic because changes in behaviour of the manipulated parent can be, and often are, compensated for by changes in behaviour of the partner. This trend of female compensation has been reported for several species including house sparrows (Hegner and Wingfield 1987; Schwagmeyer

et al. 2005) and dark-eyed juncos (Ketterson et al. 1992). In such systems, parental roles may be divided into one of defender and the other of nurturer. While the trade-off still tends to be found in species with bi-parental care and appears to be mediated by T, it is not clear if the observed trade-off in males is entirely due to increased androgens or only partially due to increased androgens, with compensation by the females driving the remaining effect. Examining the trade-off in a system where only one parent provides care would overcome this potential problem because the effects of androgens on aggression and nurturing cannot be driven or compensated for by another parent.

Another confounding variable in studies of the trade-off between aggression and nurturing is that mating behaviours often occur simultaneously with parental care. Studies have found an additional androgen-mediated trade-off between mating and nurturing behaviours (e.g. European starlings, *Sturnus vulgaris*: De Ridder et al. 2000; superb fairy-wrens, *Malurus cyaneus*: Peters 2002). In these cases, males with increased androgens performed fewer nurturing behaviours towards their offspring and instead sought out additional mating opportunities. However, it becomes difficult to investigate the effects of androgens on parental nurturing and aggressive behaviour if another androgen-mediated trade-off (that drives the male away from his nest) may be occurring simultaneously. Therefore, investigating the androgen-mediated trade-off during parental care in species where mating is discrete from parental care would also be beneficial in determining the relationship between androgens and parental care behaviours.

1.5 Bluegill sunfish: a uniparental study species

The bluegill sunfish (*Lepomis macrochirus*) is native to North America in freshwater lakes ranging from northern Mexico to southern Canada (Scott and Crossman 1973). I conducted this study in Lake Opinicon (44°34'N, 76°19'W), Ontario, Canada, where the bluegill mating system and general ecology has been studied for over the past 30 years (e.g. Colgan et al. 1979, Colborne et al. 2011). The mating system of bluegill is remarkable in that their reproductive life histories involve three male alternative reproductive tactics: parental, female mimic (satellite) and sneaker (Gross 1982). In Lake

Opinicon females sexually mature at 4 years of age while parental males, the largest of the morphs, do so at about 7 years (Gross 1982; Neff 2003; Neff and Knapp 2009). Parental males build nests, court females and provide sole parental care for the young. In contrast, cuckolder males (consisting of sneaker males and female mimics) do not build nests of their own or care for their offspring. These males mature precociously and steal fertilization opportunities from parental males. The smallest of the morphs, “sneaker” males, mature at the age of 2 years. Sneaker males exploit their size by hiding behind plants, rocks or debris near a nest until the female begins to release her eggs, at which point they dart into nests and release sperm (Gross 1982). Sneaker males that survive long enough will develop into “female mimics” at 4-5 years old (Neff 2003). Female mimics look and behave like female bluegill and their reproductive tactic is to squeeze between a mating parental male and female, deceiving the parental male that he is a second female in the nest. When the female releases her eggs, the female mimic immediately releases his sperm and darts away. Cuckolder males leave the parental male to care for both the parental’s offspring as well as the cuckolder males’ offspring (Gross 1982; Neff and Gross 2001).

In Lake Opinicon, mature parental males spawn with females from late May to early July in several discrete breeding bouts (Gross 1982). A group of parental males form a colony in the littoral zone of the lake, where there may be up to 300 males, each of whom build an individual nest (Cargnelli and Neff 2006). Nest building typically lasts one day before females arrive in groups. Females are promiscuous and release a small proportion of their eggs in several nests. A male and female will begin a spawning ritual where they circle the outer rim of the nest until the female dips on her side and releases a few of her eggs, which is quickly followed by the release of the male’s sperm. Spawning lasts one day, after which females return to the deeper waters of the lake to feed and replenish their energy. Parental males remain at the nests for another 7-10 days to provide care for the offspring (Scott and Crossman 1973; Magee et al. 2006; Neff and Knapp 2009). For the first 3 days (egg phase), a parental male must fan the eggs to increase oxygen availability and must remove moulding eggs from the nest (Côté and Gross 1993). However, males must also protect their offspring from brood predators by actively chasing and biting predators as well as performing other aggressive displays (Neff and Gross 2001; Neff

2003). Once the eggs hatch, the parental male no longer displays nurturing behaviours but continues to provide defence against brood predators until the young leave the nest 5-7 days later. A parental male will then return to deeper waters of the lake to replenish his energy supplies before possibly returning to the littoral zone for another breeding bout (Cargnelli and Neff 2006).

1.6 Research objective

Bluegill sunfish are an ideal candidate species for testing the trade-off between aggression and nurturing during parental care for multiple reasons. Firstly, the natural levels of androgens in bluegill over parental care periods have previously been documented. Under standard conditions T and KT levels in male bluegill are lower during high-investment parental care periods, compared to levels prior to and during spawning (Kindler et al. 1989; Magee et al. 2006). These data provide some support for the trade-off between aggression and nurturing in bluegill, suggesting that androgens are naturally high when males must be aggressive and compete for the best nest sites, but decrease during the egg phase of parental care when nurturing is most crucial to the survival of the young (Côté and Gross 1993). An additional benefit to studying the trade-off in bluegill is that parental male bluegill are the sole caretakers of the offspring and, furthermore, mating opportunities with the females terminates before care of the offspring begins. Therefore, using hormone manipulations, I investigated the role that androgens play on aggressive and nurturing behaviours during parental care in bluegill sunfish. I hypothesized that there would be an androgen mediated trade-off between nurturing and aggressive behaviours in male bluegill sunfish. Specifically, I predicted that male bluegill implanted with T or KT would be more aggressive towards a brood predator and less nurturing than control males, while males implanted with flutamide would be less aggressive towards a brood predator but more nurturing than controls.

2 Chapter 2: Materials and Methods

2.1 Treatment assignment and implants

I performed the testosterone manipulations between June 14 - 21, 2009. Using daily snorkeling surveys, swimmers located nests with spawning parental male and female bluegill. The day after spawning (day 0), a swimmer captured males on their nests using dip nets and brought them to a nearby boat, where I took initial blood samples (~300 μ L) from the caudal vein. Numbered tiles were placed on the shore side of each nest for fish identification and covers were placed on nests to protect the eggs from brood predators while parental males were being handled on the boat. Blood collection time (measured from the time the fish was caught until the needle was removed from the caudal vein) ranged from 59 to 240 seconds (average was 114 seconds). In total, 56 parental males were captured. I anesthetized the fish using clove oil, weighed them (to the nearest gram) and measured them for total length (to the nearest mm). Fulton's condition factor was later calculated $[(\text{mass}/\text{length}^3) \times 10^5]$ to estimate an individual's energetic state. This condition factor is positively correlated with an individual's stores of non-polar lipids, the main source of energy during periods of starvation (Neff and Cargnelli 2004). The number of eggs in each male's nest was estimated using Claussen's (1991) ratings of 1 (1-4900 eggs), 2 (4600-29,000 eggs), 3 (27,000-53,000 eggs), 4 (49,000-87,000 eggs) or 5 (82,000-113,000 eggs). Individuals were then assigned to one of four treatments. The males in the treated groups received a placebo, one testosterone implant (T1) or two testosterone implants (T2) which were placed in the abdominal cavity of the fish. Males in the fourth group (Control) were handled but had no surgery. Fish were collected randomly from the colony and treatment was assigned by rotation through the treatments.

I made testosterone implants by packing pure crystalline T propionate (Sigma Aldrich, Oakville, ON, Canada) into silastic tubing (Konigsburg Instruments, Pasadena, CA). Placebo implants were completely filled with silicone sealant. These implants were 10 mm in total length (1.47 mm i.d. and 1.96 mm o.d.) and were sealed with 1 mm silicone sealant on the ends.

Following implantation, I injected 50 μ L of antibiotic (oxytetracycline; Sigma-Aldrich, Oakville, ON, Canada) into the incision in the fish to prevent infection and sealed the wound with New Skin (Prestige Brand Holdings, Inc., NY, USA). Fish were placed in a bucket filled with lake water and allowed to recover for 5 minutes before placing them back on their nests where they typically resumed care within a few minutes.

I performed 11-ketotestosterone and flutamide manipulations between June 4 - 19, 2010 using the same methods as above, but with slight modifications. Ninety-eight males were captured on their nests and assigned to one of five groups: in the treated groups, males received either a placebo implant filled with castor oil, one flutamide implant, one 11-ketotestosterone implant (KT1) or two 11-ketotestosterone implants (KT2). Individuals assigned to the fifth group (Control) were handled but did not undergo surgery or receive an implant. Initial bleed time ranged from 35 to 120 seconds. I made placebo and KT implants with silastic tubing (1.47 mm i.d., 1.96 mm o.d.) and these implants were 7 mm in total length with 1 mm silicone sealant on the ends. I made the KT implants using crystalline 11-ketotestosterone (Steraloids, Newport, RI, United States) dissolved in ethanol and subsequently mixed into castor oil (concentration ~8 mg KT/mL oil). Flutamide implants were made by packing the interior of the tubing with crystalline flutamide (Sigma Aldrich, Oakville, ON, Canada). These implants were 8 mm in total length (1.47 mm i.d. and 1.96 mm o.d.) and were sealed with 1 mm silicone sealant on the ends. I calculated testosterone, flutamide and 11-ketotestosterone doses based on implant doses used in previous studies on fish and birds, while keeping average body mass in mind (e.g. Hegner and Wingfield 1987; Kindler et al. 1991; Ros et al. 2004; Yamaguchi et al. 2004; Yamaguchi et al. 2005; Alonso-Alvarez et al. 2007).

2.2 Behavioural observations

Each male was observed by one of six swimmers for nurturing and aggressive behaviours between 0900 and 1700 EST. On days 1 and 2 after implantation, the number of caudal sweeps, pectoral fans and egg removal behaviours were tallied and summed as a measure of nurturing behaviour per fish. On days 3 and 4 after implantation, the number of bites,

opercular flares and lateral displays directed towards a brood predator – a 16 cm pumpkinseed sunfish (*Lepomis gibbosus*) in a plastic bag attached to a pole – were tallied and summed as a measure of aggressive behaviour. A description of individual nurturing and aggressive behaviours is provided in Appendix A. The number of nurturing behaviours were recorded for 15 minutes per day for the two days, providing a 30 minute tally for each fish. The number of aggressive behaviours were recorded for a 1 minute period per day for the two days for a total of a 2 minute tally per fish. Each day, the 1 minute presentation of the brood predator was broken into two 30 second periods, with a 30 second break between presentations (see Neff and Knapp 2009).

On day 6 after implantation, males were re-captured from their nests, and final blood samples were taken from the caudal vein to measure changes in hormone levels. I then weighed and measured the length of the males for final calculations of Fulton's condition factor. They were then euthanized using clove oil, and dissected to ensure implants had stayed in place. Range of bleed time for the final blood samples was 41 to 181 seconds in 2009 and 39 to 150 seconds in 2010. All blood samples were kept on ice until they were returned to the lab, where the plasma was separated and extracted using a centrifuge and was frozen at -20°C until hormone assays were conducted.

In 2010, I modified my behavioural observation methods, recording both nurturing and aggressive behaviours on days 1 and 2 after implant surgery. The purpose of this modification was to more accurately test the trade-off between parental nurturing and aggression simultaneously. The order of recording nurturing and aggressive behaviours was switched each day. Final blood samples, as well as measurements of length and weight of these fish were collected on day 3 after implant surgery, and fish were subsequently euthanized and dissected.

2.3 Radioimmunoassay

I determined plasma levels of KT, T and estradiol using radioimmunoassay (RIA) following extraction and chromatographic separation (Magee et al. 2006). From each

plasma sample, I used 100 μ L of plasma and added 2000 cpm of each titrated hormone to allow for correction for losses during extraction and chromatography. Hormones were extracted from the plasma using two 2 mL diethyl ether washes. The combined ether extraction was dried down under nitrogen, resuspended in 10% ethyl acetate in iso-octane and then run through diatomaceous earth-glycol columns. Collection of fractions of T, estradiol and KT was achieved by sequential elutions with 10%, 20% and 30% ethyl acetate in iso-octane, respectively. I dried down fractions under nitrogen, resuspended each in 500 μ L phosphate-buffered saline containing 0.1% gelatin and stored the fractions overnight at 4°C. The T antibody I used (Wien T-3003 from Research Diagnostics, Flanders, NJ, now Fitzgerald Industries, Acton, MA, USA) has high cross-reactivity with KT and could thus be used to assay both hormones. The estradiol antibody I used was from Biogenesis (7010–2650, Kingston, NH, USA). RIAs were conducted using two 200 μ L replicates of each sample and a charcoal-dextran solution in phosphate-buffered saline (without gelatin) was used to separate bound and unbound hormone fractions. Samples were counted on a Beckman Tri Carb scintillation counter.

In 2009, samples were run in two assays. Intra-assay coefficients of variation for T were 4.8% and 6.6%, for estradiol were 19.7% and 14.2%, and for KT were 9.3% and 13.7%. Inter-assay coefficients of variation were 3.5%, 5.3% and 9.2% for T, estradiol, and KT, respectively. In 2010, samples were run in three assays. Intra-assay coefficients of variation for T and KT ranged from 8.3-22.5% and 18.3-25.5%, respectively. Inter-assay coefficients of variation were 17.4% and 7.6% for T and KT, respectively.

Most (77%) of the initial estradiol levels in all treatment groups and 83% of the second levels of estradiol in placebo and control groups in 2009 were below the sensitivity of the assay (\sim 0.35 pg/tube; \sim 0.9 ng/mL plasma). Thus, these data were not included in statistical analyses. However, I present descriptive statistics for estradiol in the second blood samples from T-implanted groups because only 1 of these 23 (4.3%; from a T2 male) had non-detectable levels. For this male, I used the value calculated from the standard curve in my analyses as an estimate of his very low estradiol levels. Levels of all other hormones were detectable and were used in the analyses. All initial and final estradiol levels for 2010 were below the assay's level of detectability and thus are not

discussed further and were not analyzed statistically. All other hormones in 2010 were detectable.

Cortisol was extracted from a separate 100 μ L aliquot of plasma using diethyl ether washes as above and then assayed via RIA, but without prior chromatographic separation. The cortisol antibody used was purchased from Esoterix Endocrinology (F3-314, Calabasas Hills, CA). Cortisol levels in 2009 plasma samples were run in a single assay with an intra-assay coefficient of variation of 16.5%. Cortisol for 2010 samples were run in two assays with intra-assay coefficients of variation of 13.6% and 11.4% and an inter-assay coefficient of variation of 12.5%.

2.4 Statistical analyses

All data were analyzed in JMP version 4.0 (SAS Institute Inc.). The same analyses were performed for both years of data. First, I used a Chi-Square test to determine if the number of males that abandoned their nests was specific to any particular treatment group. Next, I used a MANOVA to determine if there were differences between males that stayed and males that abandoned in initial bleed time, body length, body mass, Fulton's condition factor, and egg score. If significance was found, t-tests were performed to specify where the differences lay between males that stayed and males that abandoned for each of these variables. For the males that stayed, I used a MANOVA to analyze differences among treatment groups in initial and final bleed time, body length, body mass, Fulton's condition factor, and egg score. If significance was found, I performed ANOVA's to specify where the differences lay among treatments within each of these variables. I then analyzed the relationship between bleed time and hormone levels using Pearson correlations.

I analyzed initial hormone levels among treatments using a MANOVA. I then performed three repeated measure ANOVAs (one for each hormone analyzed; T, KT and cortisol) to compare each of the hormone levels over time, across treatments and in the interaction between treatment and time. The repeated measure was the hormone concentration in the

first and second blood samples (i.e. before and after the implantation). Tukey's post-hoc tests specified differences among treatments. The change in KT and T over the time points in flutamide males were excluded from this analysis in 2010 because the hormone difference in these males does not assess the effectiveness of the KT implants.

Summed aggressive behaviours and summed nurturing behaviours were each analyzed for treatment differences using an ANOVA. I added observer as a random factor in these models. For all analyses, a Tukey's post-hoc test was used for pairwise comparisons. Originally, I included initial body length, body mass, Fulton's condition factor, and egg score as covariates for all analyses. In 2009, only initial Fulton's condition factor, body length and body mass had an effect on overall models and thus these covariates were included in the final 2009 analyses. However, in 2010 none of these factors had a significant effect and were thus excluded from the final analyses.

3 Chapter 3: Results

3.1 Testosterone manipulations (2009)

3.1.1 *Abandonment data*

Twenty-two males abandoned their nests before the experiment ended (see sample sizes for each treatment in Table 1), thus a full set of behavioural and hormonal data were collected from 34 males for hormone and behaviour analyses. The number of males that abandoned their nests did not differ significantly between treatments (Chi-Square: $\chi^2=0.66$, $df=3$, $p=0.88$; Table 1). There were also no significant differences in initial bleed time, body length, body mass, Fulton's condition factor or egg score between males that stayed versus males that abandoned their nests (MANOVA: $F_{4,48}=0.29$, $p=0.88$). For the males that remained for the duration of the experiment, there was no significant difference among treatments in the overall model of initial and final bleed time, body length, body mass, Fulton's condition factor and egg score (MANOVA: $F_{24,67}=1.32$, $p=0.18$; Table 2). Bleed time did not correlate with any hormone levels ($p>0.18$).

3.1.2 *Hormone data*

Mean (\pm SD) initial levels of T, KT, and cortisol for males that stayed throughout the experiment were 2.3 ± 1.7 ng/mL, 7.5 ± 4.9 ng/mL, and 23.6 ± 31.6 ng/mL, respectively, and did not differ significantly among treatments (MANOVA: $F_{6,44}=1.13$, $p=0.36$). There were no significant differences in levels of KT or cortisol among treatments, over time or in their interactions between treatment and time (Table 2, 3). However, as expected, there was a significant difference in T levels between the sampling dates, among treatment groups and there was a significant interaction in treatment \times time (Table 2, 3).

Table 1: The total number of male bluegill sunfish (*Lepomis macrochirus*) sampled, as well as number that abandoned or stayed on their nests until 4-7 days after spawning for each treatment group in Lake Opinicon at Queen's University Biological Station. In June 2009, treatments included males implanted with one placebo, one testosterone (T1), two testosterone (T2) or no implants (Control). In June 2010, treatments included males implanted with one placebo, one flutamide, one 11-ketotestosterone (KT1), two 11-ketotestosterone (KT2), or no implants (Control).

Year	Treatment	Total number of fish	Number abandoned	Number stayed
2009	Control	14	7	7
	Placebo	14	4	10
	T1	14	5	9
	T2	14	6	8
2010	Control	21	10	11
	Placebo	17	9	8
	Flutamide	17	8	9
	KT1	20	11	9
	KT2	23	13	10

Table 2: Means (\pm SD) for hormone levels, bleed times, Fulton's condition factor, body length, body mass and egg score of parental male bluegill (*Lepomis macrochirus*). Measurements were taken in June 2009 on Lake Opinicon the day after spawning (upper lines) and seven days after implantation (lower lines). Treatments included males implanted with a placebo, one testosterone implant (T1), two testosterone implants (T2) or none (Control).

Treatment	n	Bleed Time (sec)	Testosterone (ng/mL)	11-Ketotestosterone (ng/mL)	Estradiol (ng/mL)	Cortisol (ng/mL)	Body length (mm)	Body Mass (g)	Fulton's condition factor ($\text{g}/\text{mm}^3 \times 10^5$)	Egg Score
Control	7	94 \pm 37	2.3 \pm 0.5	9.8 \pm 5.7	ND	26.6 \pm 29.3	194 \pm 10	142 \pm 19	2.0 \pm 0.1	3 \pm 1
		93 \pm 29	2.4 \pm 2.2	7.0 \pm 6.6	ND	25.8 \pm 30.0	194 \pm 9	129 \pm 16	1.8 \pm 0.1	
Placebo	10	127 \pm 46	2.8 \pm 2.6	7.4 \pm 5.2	ND	9.9 \pm 13.8	203 \pm 6	150 \pm 12	1.8 \pm 0.1	3 \pm 1
		90 \pm 39	1.8 \pm 0.7	5.4 \pm 2.2	ND	51.5 \pm 62.3	202 \pm 6	143 \pm 12	1.8 \pm 0.1	
T1	9	121 \pm 54	2.2 \pm 1.3	7.2 \pm 5.0	ND	18.9 \pm 11.5	201 \pm 5	147 \pm 14	1.8 \pm 0.1	2 \pm 1
		95 \pm 46	93.4 \pm 57.5	8.1 \pm 5.8	7.6 \pm 11.2	39.8 \pm 35.5	199 \pm 6	139 \pm 16	1.8 \pm 0.1	
T2	8	120 \pm 50	1.6 \pm 1.2	5.7 \pm 3.7	ND	44.1 \pm 56.9	204 \pm 5	153 \pm 14	1.8 \pm 0.1	3 \pm 1
		75 \pm 23	125.1 \pm 26.5	7.7 \pm 8.4	12.4 \pm 13.2	8.6 \pm 7.4	201 \pm 7	145 \pm 14	1.8 \pm 0.1	

ND = non-detectable (see text for details).

Table 3: Summary of repeated measures analysis of variance for hormone levels in parental male bluegill (*Lepomis macrochirus*) in June 2009 in Lake Opinicon. Hormone levels were measured prior to and seven days after assignment to one of four treatments: implantation of a placebo, one testosterone or two testosterone implants or none (controls).

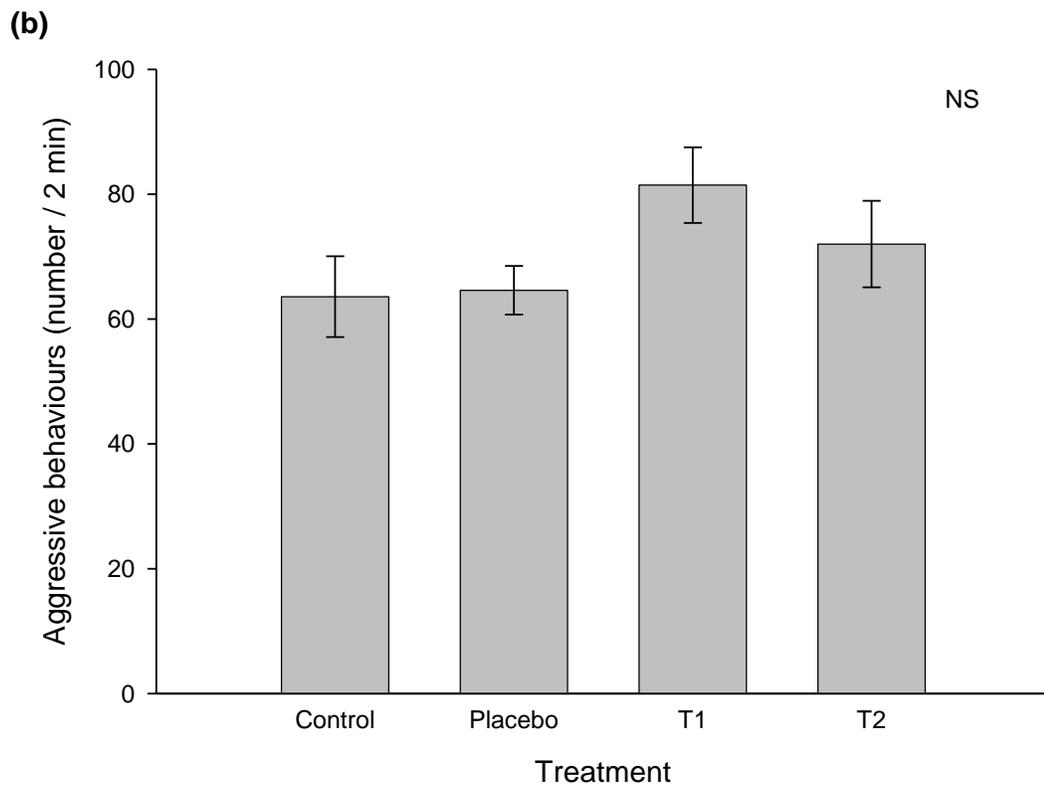
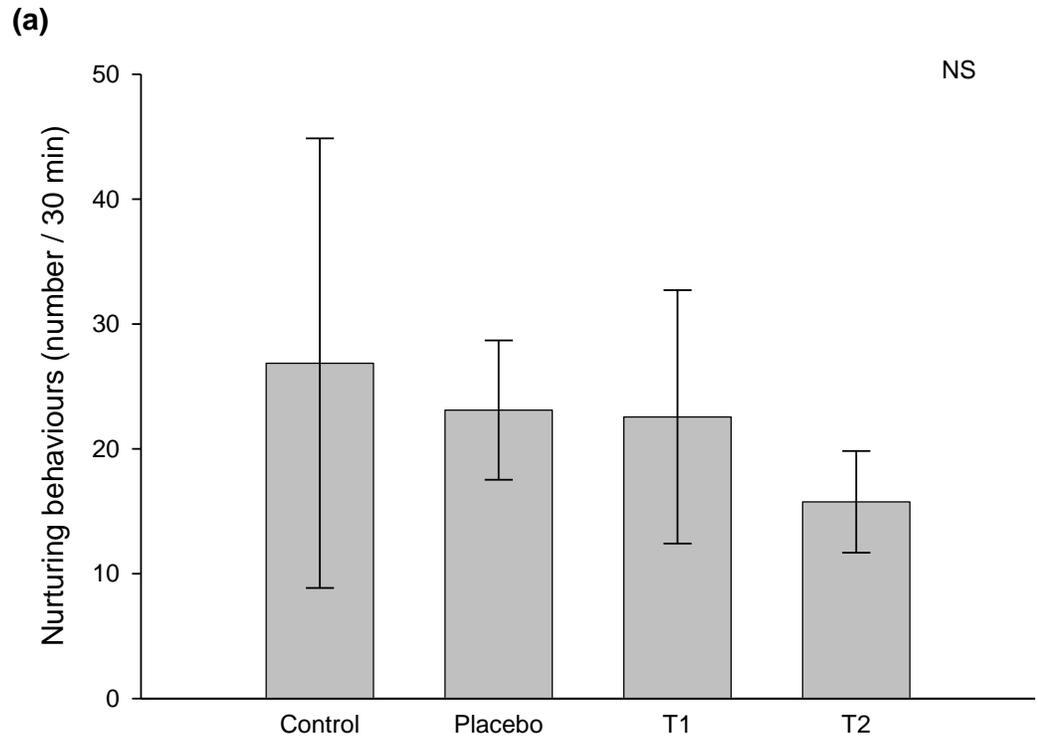
Hormone	Variable	df	F	P
Testosterone	Treatment	3,30	31.23	<0.001
	Time	1,30	91.84	<0.001
	Treatment × Time	3,30	32.63	<0.001
KT	Treatment	3,30	0.40	0.75
	Time	1,30	0.13	0.72
	Treatment × Time	3,30	0.76	0.52
Cortisol	Treatment	3,22	0.07	0.98
	Time	1,22	0.36	0.55
	Treatment × Time	3,22	2.03	0.14

In particular, T1 and T2 males had a higher T six days after implantation than control ($p < 0.001$ for both) and placebo males ($p < 0.001$ for both). Typical mean levels of hormones in bluegill at the onset of care are 1-8 ng/mL for T, 5-15 ng/mL for KT and 25-150 ng/mL for cortisol (Kindler et al. 1989; Magee et al. 2006). Thus, the elevated T levels in my study were about 30-fold higher than is typical of nesting parental males and about 20-fold higher than levels seen in parental males in the days immediately prior to spawning (Kindler et al. 1989; Magee et al. 2006). However, natural T levels in bluegill have been reported as high as 55 ng/mL (Kindler et al. 1989) which is only 2-fold lower than the levels I induced.

3.1.3 *Behavioural data*

The number of nurturing behaviours did not differ significantly among treatment groups (ANCOVA: $F_{3,20}=0.44$, $p=0.73$; Figure 1a). Total aggressive behaviours differed marginally among treatments, with T1 males being most aggressive, but the difference was not significant (ANCOVA: $F_{3,25}=2.58$, $p=0.07$; Figure 1b). Means for each nurturing and aggressive behaviour recorded are summarized in Appendices B and C.

Figure 1. Parental behaviour displayed by male bluegill (*Lepomis macrochirus*) in response to experimentally manipulated levels of testosterone in June 2009 in Lake Opinicon. Shown are means (\pm SE) for (a) nurturing behaviours and (b) aggressive behaviours displayed during parental care. Nurturing behaviours comprised caudal sweeps, pectoral fanning and egg removal. Aggressive behaviours comprised biting, opercular flares and lateral displays directed towards the predator. Treatments include males given no implant (Control, n=7), a placebo (n=10), one testosterone implant (T1, n=9) or two testosterone implants (T2, n=8).



3.2 11-ketotestosterone and flutamide manipulations (2010)

3.2.1 *Abandonment data*

Fifty-one males abandoned their nests before the experiment ended (Table 1), therefore, a complete set of data were collected from 47 males which were used in behaviour and hormone analyses. There was no significant difference in the number of males from each treatment group that abandoned their nests (Chi-Square: $\chi^2=0.29$, $df=4$, $p=0.99$; Table 1). There was a significant difference between males that stayed and males that abandoned in the overall model of initial measures of bleed time, body length, body mass, Fulton's condition factor and egg score (MANOVA: $F_{4,91}=3.17$, $p=0.02$). Males that abandoned were marginally shorter in length ($t=1.79$, $df=96$, $p=0.08$), in better body condition ($t=1.83$, $df=96$, $p=0.07$), had lower egg scores ($t=1.74$, $df=96$, $p=0.09$) and took longer to bleed ($t=1.86$, $df=96$, $p=0.07$). The direction of these variables may be more common among males that abandon within a breeding bout and they may instead return for a later bout in the season (e.g. Magee et al. 2006). Among the males that stayed throughout the experiment, there were no differences in initial or final bleed time, body length, body mass, Fulton's condition factor or in egg score among treatment groups (MANOVA: $F_{34,131}=0.80$, $p=0.76$; Table 4). Bleed time did not correlate with any of the hormone levels ($p>0.09$ for all).

3.2.2 *Hormone data*

Mean initial hormone levels (\pm SD) for males that remained throughout the experiment were 2.0 ± 1.2 , 12.8 ± 9.6 and 11.9 ± 13.6 ng/mL for T, KT and cortisol respectively. Initial hormone levels did not differ significantly among treatments (MANOVA: $F_{8,82}=1.68$, $p=0.12$). Overall, mean T levels were significantly higher on day 3 than on day 1, but there was no difference among treatments, nor was there an interaction between treatment and time, however, T tended to be higher on day 3 in KT1 males compared to controls (Tables 4, 5). There was no significant difference in KT levels among treatments, between the sampled time points or in the interaction between treatment and time. The changes in KT levels between the days sampled were higher in

Table 4: Means (\pm SD) for hormone levels, bleed times, Fulton's condition factor, body length, body mass and egg score of parental male bluegill (*Lepomis macrochirus*). Measurements were taken in June 2010 from a population in Lake Opinicon the day after spawning (upper lines) and three days after implantation (lower lines). Treatments included males implanted with a placebo, flutamide, one 11-ketotestosterone implant (KT1), two 11-ketotestosterone implants (KT2) or none (Control).

Treatment	n	Bleed time (sec)	11-Ketotestosterone (ng/mL)	Testosterone (ng/mL)	Cortisol (ng/mL)	Body length (mm)	Body mass (g)	Fulton's condition factor (g/mm ³ × 10 ⁵)	Egg Score
Control	11	59 ± 14	14.3 ± 10.0	2.1 ± 1.3	13.5 ± 13.4	197 ± 8	145 ± 17	1.9 ± 0.2	2 ± 1
		77 ± 27	7.7 ± 6.3	2.0 ± 1.6	33.7 ± 52.4	196 ± 9	140 ± 16	1.9 ± 0.1	
Placebo	8	65 ± 17	17.4 ± 13.6	1.9 ± 1.4	9.6 ± 7.2	206 ± 10	167 ± 25	1.9 ± 0.1	2 ± 1
		73 ± 25	12.2 ± 7.9	2.9 ± 2.7	11.5 ± 11.7	204 ± 10	159 ± 22	1.9 ± 0.1	
Flutamide	9	65 ± 28	14.3 ± 11.2	1.9 ± 0.4	19.5 ± 21.5	199 ± 7	155 ± 18	2.0 ± 0.1	2 ± 1
		73 ± 30	16.9 ± 13.6	3.2 ± 2.2	14.1 ± 13.9	198 ± 8	149 ± 15	1.9 ± 0.1	
KT1	9	64 ± 22	7.8 ± 5.2	1.7 ± 1.6	11.5 ± 11.3	206 ± 13	165 ± 32	1.9 ± 0.2	3 ± 1
		72 ± 25	12.4 ± 7.9	4.6 ± 3.2	7.5 ± 8.0	206 ± 14	163 ± 33	1.9 ± 0.1	
KT2	10	63 ± 18	10.5 ± 5.2	2.3 ± 1.0	4.6 ± 7.1	199 ± 12	155 ± 32	1.9 ± 0.1	2 ± 1
		69 ± 18	11.0 ± 6.2	2.9 ± 2.7	6.2 ± 6.5	198 ± 13	148 ± 31	1.9 ± 0.1	

Table 5: Summary of repeated measures analysis of variance for hormone levels in parental male bluegill (*Lepomis macrochirus*) in June 2010 in Lake Opinicon. Hormone levels were measured prior to and three days after assignment to one of four treatments: placebo, flutamide, one 11-ketotestosterone implant, two 11-ketotestosterone implants or none (Controls).

Hormone	Variable	df	F	P
Testosterone	Treatment	3,34	0.81	0.50
	Time	1,34	6.88	0.01
	Treatment × Time	3,34	2.56	0.07
KT	Treatment	3,34	1.01	0.40
	Time	1,34	0.91	0.35
	Treatment × Time	3,34	2.26	0.10
Cortisol	Treatment	4,42	2.50	0.06
	Time	1,42	0.33	0.57
	Treatment × Time	4,42	1.08	0.38

KT-implanted males than in control and placebo groups, however, these differences were not significant (Tables 4, 5, Figure 2). Cortisol levels were not significantly different over time and there was no significant interaction between treatment and time, however, control males had marginally higher cortisol levels in general than KT2 males.

It is important to note that the final levels of KT are similar among the treatments, but the direction of change between sampling points is interesting: while control groups had decreased levels of KT over the three days (average C: -6.60 ng/mL, P: -5.20 ng/mL), all experimental groups had increased levels of KT (average KT1: +4.66 ng/mL, KT2: +0.48 ng/mL, F: +2.58 ng/mL; Table 4, Figure 2).

3.2.3 *Behavioural data*

There was a significant difference in the number of nurturing behaviours performed among treatment groups (ANOVA: $F_{4,34}=9.0$, $p<0.001$; Figure 3a). The frequency of nurturing behaviours was significantly less in KT2 males than control males ($p=0.01$). KT1 males performed less nurturing behaviours than control males, but this difference was not significant ($p=0.14$). Males treated with flutamide performed significantly more nurturing behaviours compared to all other groups ($p<0.02$). All other comparisons were not significantly different ($p>0.26$).

There was also a significant difference in aggression among treatment groups (ANOVA: $F_{4,37}=3.2$, $p=0.02$; Figure 3b). Specifically, males treated with two KT implants (KT2 group) performed more aggressive behaviours than flutamide, control and placebo groups ($p=0.01$ for all). All other comparisons were not significantly different ($p>0.21$).

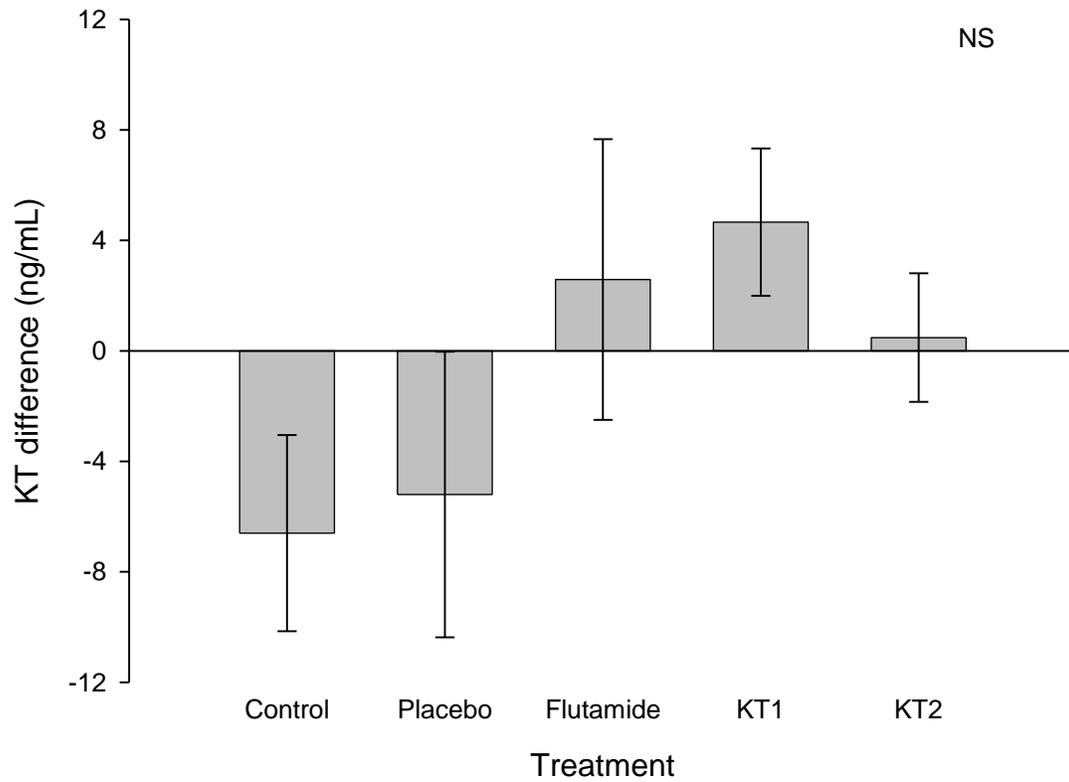
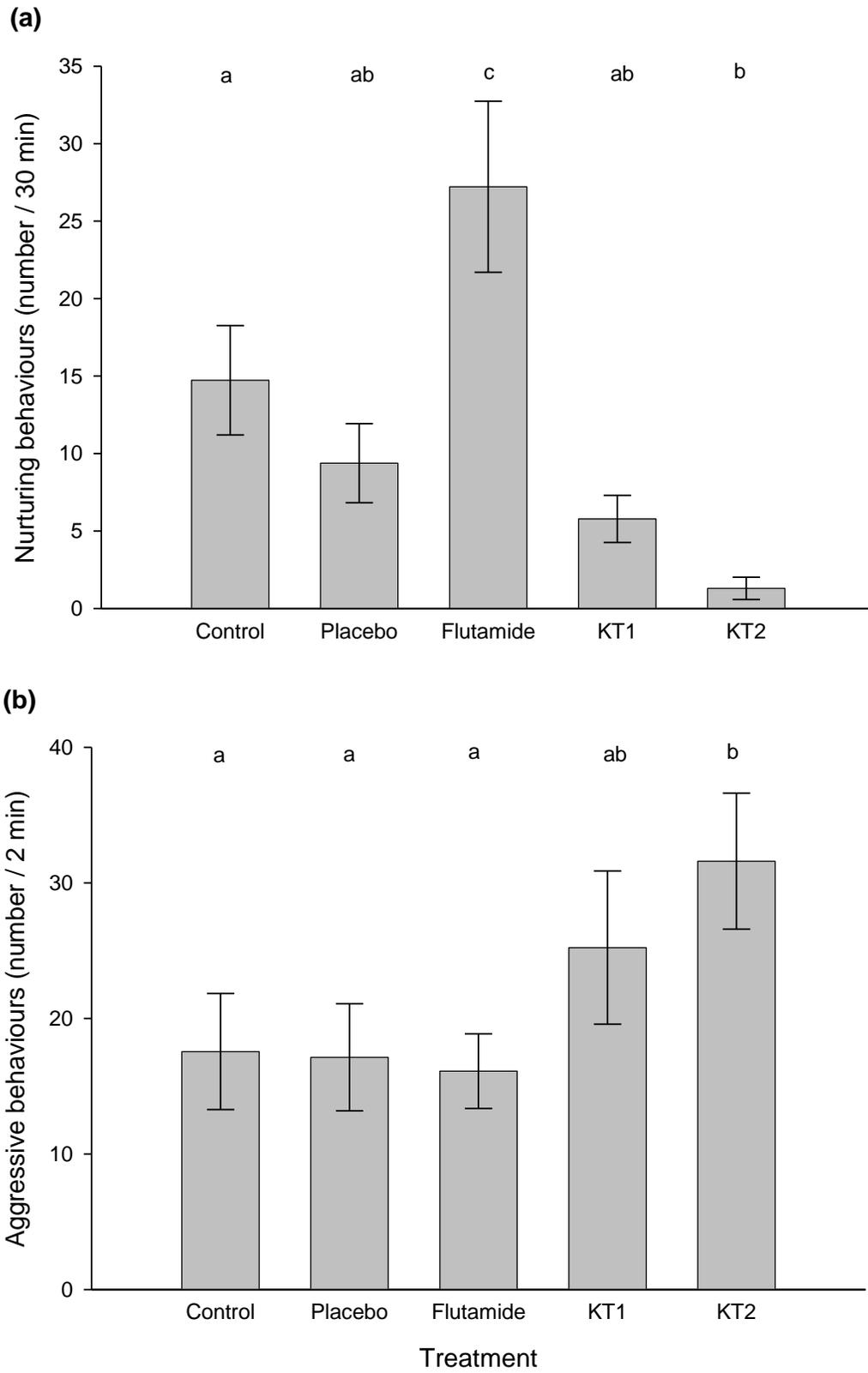


Figure 2. Mean (\pm SE) change in circulating KT concentrations in male bluegill sunfish (*Lepomis macrochirus*) in Lake Opinicon in June 2010. Hormone levels were collected before implantation and 3 days after implantation of a placebo (n=8), a flutamide (n=9), one 11-ketotestosterone implant (KT1, n=9), two 11-ketotestosterone implants (KT2, n=10) or no implant (Control, n=11).

Figure 3. Parental care behaviours displayed in response to experimentally manipulated levels of 11-ketotestosterone and flutamide in male bluegill (*Lepomis macrochirus*) in June 2010 in Lake Opinicon. Shown are means (\pm SE) for (a) nurturing behaviours and (b) aggressive behaviours. Nurturing behaviours comprised caudal sweeps, pectoral fanning and egg removal. Aggressive behaviours comprised biting at the brood predator, opercular flares and lateral displays directed towards the predator. Treatments included males given a placebo (n=8), a flutamide implant (n=9), one 11-ketotestosterone implant (KT1, n=9), two 11-ketotestosterone implants (KT2, n=10) or none (Control, n=11). Bars with different letters are significantly different from each other.



4 Chapter 4: Discussion

4.1 Testosterone manipulations (2009)

The data from 2009 reveals no significant differences in the frequency of aggressive or nurturing behaviour following increases in T. This could be due to a number of factors, such as the pharmacologically-high dose of T or small sample size. However, the trend for aggression in T-implanted males was still present and in the direction predicted. These trends are consistent with data from a number of other vertebrates. A positive relationship between androgen levels and aggression has been found in humans (Archer 2006), chimpanzees (*Pan troglodytes schweinfurthii*: Muller and Wrangham 2004), ring-tailed lemurs (*Lemur catta*: Cavigelli and Pereira 2000), lizards (*Anolis carolinensis*: Greenberg and Crews 1990) and some other fish species (see Oliveira et al. 2002 for a review; *Parablennius parvicornis*: Ros et al. 2004). Thus, a growing body of evidence indicates that T plays a part in mediating aggression in a broad range of animals.

If the T manipulations were responsible for the increased levels of aggression, then my results suggest that the mechanism by which T can influence aggression is via direct action of T and its receptors or via indirect action where T is converted to estradiol (see Table 2). Several studies in birds and rodents suggest that T does not influence aggression directly, but rather via its metabolism to estradiol in the brain (e.g. Hau et al. 2000; Soma et al. 2000; Silverin et al. 2004). For example, male California mice (*Peromyscus californicus*) treated with an inhibitor of aromatase, the enzyme that converts T to estradiol, displayed a lower frequency of aggressive behaviours towards an intruder than did control males (Trainor et al. 2006). Without having blocked the conversion of T to estradiol, I could not determine the specific mechanism by which T-implantation increased levels of aggression in my T1 fish.

The fact that T1 males were more aggressive than T2 males could be the result of an inverted-U-shaped dose response curve commonly seen in hormone manipulation studies (Hews and Moore 1997; Adkins-Regan 2005). For example, rats placed in an escapable

shock situation and administered various doses of corticosterone displayed an inverted-U-shaped response in learned helplessness behaviour: in the low and high dose treatments, rats exhibited few attempts to escape, whereas rats given a moderate dose maintained high escape attempts and success (Kademian et al. 2005). Similarly, while low or high exogenous prolactin can induce egg fanning in cichlid fish, moderate doses can inhibit the behaviour (Blüm and Fiedler 1965). Nonetheless, caution is warranted in interpreting my results because both of the T-implanted groups had T levels considerably above those that parental males can produce endogenously (Kindler et al. 1989; Magee et al. 2006). Despite this caveat, my 2009 data do indicate some role of T in mediating aggressive behaviour during parental care and suggest that an inverted-U-shaped dose response may be in effect. Future studies using implants that generate high physiological levels of T will be important to fully understand T's behavioural effects in the context of paternal care.

While increases in T may have mediated increases in the frequency of aggressive behaviour, they did not decrease the frequency of nurturing behaviours as predicted. However, as noted above, the T manipulations also led to an increase in estradiol levels, a hormone that has also been found to play a role in nurturing behaviours. For example, exposure to 17 α -ethinyl estradiol increased the time male sand gobies (*Pomatoschistus minutus*) spent fanning their eggs compared to controls (Saaristo et al. 2009). Thus, the higher estradiol levels in my T-implanted fish could have compensated for the predicted T-induced decrease in nurturing behaviours, thus yielding no apparent difference in nurturing behaviour. In future studies, it would be important to utilize an aromatase inhibitor or androgen receptor blocker to tease apart the roles of estradiol and androgens in mediating nurturing and aggressive behaviours during paternal care in bluegill.

It is also possible that nurturing and aggressive behaviour during parental care are mediated by a more complex network of hormones and peptides. Arginine vasopressin and oxytocin, for example, both have been implicated in nurturing and aggressive behaviour (reviewed by van Anders et al. 2011). Aggression shown during parental care in defence of the young is associated with increases in arginine vasopressin, and nurturing behaviour in general is linked to increases in oxytocin levels, both of which in

turn appear to facilitate social bonding (e.g. Stribley and Carter 1999; also see review by Keverne and Curley 2004). Furthermore, because LH and GnRH were not measured in this study, but are directly involved in the feedback loop of the HPG axis, I cannot rule out the possible impact these hormones have on aggression. However, in fishes, KT is the primary androgen and thus KT, as opposed to T, may more directly inhibit nurturing behaviour. For example, in the rock-pool blenny (*Parablennius parvicornis*), exposure to KT decreased the frequency of males' egg fanning behaviour (Oliveira et al. 2001). In addition, in bluegill, KT levels are lowest during the egg phase of paternal care when fanning and other offspring nurturing behaviours are most frequent (Magee et al. 2006), and thus this candidate hormone was selected for further analysis in the June 2010 bluegill breeding season.

4.2 11-ketotestosterone and flutamide manipulations (2010)

My results from this study provide compelling support for a trade-off between parental aggression and nurturing in a fish and supports that this trade-off is mediated at least in part by KT. As a group, males implanted with KT displayed 64% more aggressive behaviours and 71% fewer nurturing behaviours than control groups. In contrast, males implanted with the androgen receptor blocker, flutamide, displayed 7% fewer aggressive behaviours and 126% more nurturing behaviours than controls. As in the present study, Kindler et al. (1991) investigated aggression and nurturing in nesting bluegill but did not find an overall effect of KT or T. However, those authors recorded rim circling (continuous swimming around the edge of the nest) as their measure of nurturing behaviour but it is unclear if this behaviour is a territorial or nurturing response (Colgan et al. 1979). Furthermore, the authors used a plastic model of a conspecific bluegill to assess aggression whereas I used a live potential predator, which may have represented a more realistic threat to the nesting male bluegill. These discrepancies in methods may explain the apparently disparate results. Nevertheless, my study adds to growing literature supporting a trade-off between nurturing and aggression during parental care.

While the implants I used were long lasting (Lynn et al. 2009), changes in circulating androgen levels may rapidly lead to changes in behaviour. Implants of this sort typically release steady pulses of the hormone within 24 hours of implant placement (Lynn et al. 2009). However, Yamaguchi et al. (2004) used KT implants that were similar to mine in seabream fish (*Pagrus major*) and found that KT levels were initially high but dropped three days after implantation, likely due to negative feedback. I conducted my behavioural observations on days 1 and 2 after implantation, but took final blood samples on day 3. Thus, it is possible that KT levels were higher in my KT-implanted males during the behavioural observation period, but had declined by day 3 when the post-implant blood samples were taken. As such, when taking blood samples, I may have missed the peak when KT levels were highest. Even so, while the difference in KT was not significantly different among treatments as measured between day 0 and day 3, the direction of change in KT was opposite in the KT-implanted males compared to both control groups. Mean KT levels increased in KT-implanted groups whereas mean KT levels decreased in the control groups, as is typical for natural KT levels in bluegill over the egg stage of care (Magee et al. 2006). Taken together, this study supports a role for KT in inhibiting nurturing behaviour and eliciting aggressive behaviour during parental care in male bluegill.

However, nurturing and aggressive behaviours may show different sensitivities to circulating androgen levels. Flutamide-implanted males displayed more nurturing behaviours than any other group, but contrary to my expectations, they did not show significantly less aggression than the control groups. A possible explanation for this result is that the dose of flutamide I used may have blocked enough androgen receptors to have an effect on downstream influences on nurturing behaviour, but was insufficient to have an effect on downstream influences on aggression. Indeed, a study on house sparrows, which weigh only about a fifth as much as the bluegill used in my studies, used double my dose of flutamide (Hegner and Wingfield 1987). Those authors found that there was both an increase in nurturing behaviours and a decrease in aggression in flutamide-implanted males compared to controls. Thus, it is possible that the two types of behaviours have different thresholds of androgen sensitivity. Alternatively, the lack of an effect on aggression when androgen receptors are blocked could be explained by the

‘essential parental care’ hypothesis (Lynn 2008), describing that, regardless of androgen levels, a minimum amount of care or defence may be required to ensure the survival of the offspring or ensure the efforts of the parent do not go to waste. My data suggests that the trade-off between nurturing and aggression is indeed complex but is at least partially linked to androgen-bound receptor complexes in the brain.

Despite the observed effects of androgens on parental behaviours, the specific genomic targets of androgen-receptor complexes that influence the parental care trade-off are unknown. In stickleback fish, it has been found that territorial and dominance types of aggression are heritable but the specific genes affecting the behaviours were not identified (Baker 1994). Knockout experiments in mice have identified 36 genes that affect aggression (including the androgen receptor gene; reviewed by Maxon and Canastar 2003), however studies on other taxa are rare. Genes that affect nurturing behaviour in mice may include *FosB*, *SPRY1* and *Rad* (Kuroda et al. 2008), however, like aggression, studies identifying the genes that affect nurturing behaviour in other taxa have been rare. Thus, while the trade-off between parental aggression and nurturing has been observed for decades, the genomic mechanism behind the trade-off remains unknown.

4.3 Conclusions

Taken together, my data suggest that manipulating T levels influenced parental behaviours in that males with elevated T display marginally more aggressive behaviours than control males, but there is no difference in the frequency that these males display nurturing behaviours. The data suggest that androgens may play a role in aggression but a number of confounding factors limit the conclusiveness that can be drawn. However, through manipulations of KT levels, I found that males implanted with KT performed more acts of aggression and less acts of nurturing in parental care than controls, suggesting that androgens do play a role in a parental care trade-off.

In my T manipulation study, because my manipulations of T also elevated circulating estradiol levels, likely via the steroidogenic enzyme aromatase, I could not conclude that

the effects I observed in that study were mediated by changes in T levels and thus via androgen receptors. The results of my 2010 study, investigating the effects of KT and flutamide on nurturing and aggression, do show changes in parental care behaviour while estradiol levels remained low. These recent results not only rule out estradiol as a mediator of the behaviour observed but also demonstrating that at least some of the nurturing and aggressive behavioural effects I observed were mediated via androgen receptors.

The effectiveness of hormone implants in a new study species, such as bluegill, are difficult to predict, however, I suggest some ways for future studies to improve on the methods I used here. Firstly, I strongly recommend that future studies include a dose response curve of the implants used, either via daily blood samples of the implanted study species or via implants in saline solution (e.g. Kindler et al. 1991), to ensure the implants are in fact releasing the target hormone. Furthermore, to tease apart the role of T and KT in parental nurturing and aggression, T manipulations in bluegill should use a lower dose to induce high physiological levels of the androgen. Specifically, implants should be filled with approximately 1 mm of testosterone propionate (which cannot convert directly to KT and thus would measure only the behavioural response to T). This dose would likely result in a T concentration within the physiological range of bluegill (the range for initial T samples in this study was between 0.09 ng/mL and 13.09 ng/mL).

These two studies are among the first to investigate the trade-off between nurturing and aggression during parental care in a uniparental care system, and provide support for the idea that the trade-off in parental behaviour in fish is mediated at least partially by circulating androgen levels. Understanding the physiological mechanisms underlying such a trade-off is valuable for furthering our knowledge about how hormone-behaviour relationships contribute to an individual's reproductive fitness. Additionally, these findings indicate that exploration of the mechanistic details of how androgens influence paternal behaviour would be a very productive area for future research.

5 References

- Adkins-Regan, E. 2005. Mating, fighting, parenting and signaling. p. 34-41. *In* Hormones and Animal Social Behavior. J.R Krebs and T. Clutton-Brock [eds.] Princeton University Press., Princeton, New Jersey.
- Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O. and Sorci, G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proc. R. Soc. London, Ser. B Biol. Sci.* **274**: 819—825.
- Archer, J. 2006. Testosterone and human aggression: an evaluation of the challenge hypothesis. *Neurosci. Biobehav. Rev.* **30**: 319—345.
- Baker, T.C.M. 1994. Genetics correlations and the control of behavior, exemplified by aggressiveness in sticklebacks. *Adv. Stud. Behav.* **23**: 135—171.
- Barja, I., Silvan, G. and Illera, J.C. 2008. Relationships between sex and stress hormone levels in feces and marking behavior in a wild population of Iberian wolves (*Canis lupus signatus*). *J. Chem. Ecol.* **34**: 697—701.
- Berthold, A.A. 1849. Transplantation of testes. Trans. by D. P. Quiring, 1944, *Bull. Hist. Med.* **16**: 399—401.
- Blüm, V. and Fiedler, K. 1965. Hormonal control of reproductive behavior in some Cichlid fish. *Gen. Comp. Endocr.* **5**: 186—196.
- Borg, B. 1994. Androgens in teleost fishes. *Comp. Biochem. Physiol.* **109C**: 219—245.
- Buchanan, K.L. Evans, M.R., Robert, M.L., Rowe, L. and Goldsmith, A.R. 2010. Does testosterone determine dominance in the house sparrow, *Passer domesticus*? *J. Avian Biol.* **41**: 445—451.
- Cargnelli, L.M. and Neff, B.D. 2006. Condition-dependent nesting in bluegill sunfish *Lepomis macrochirus*. *J. Anim. Ecol.* **75**: 627—633.
- Cavigelli, S.A. and Pereira, M.E. 2000. Mating season aggression and fecal testosterone levels in male ring-tailed lemurs (*Lemur catta*). *Horm. Behav.* **37**: 246—255.
- Cawthorn, J.M., Morris, D.L., Ketterson, E.D. and Nolan, V. 1998. Influence of experimentally elevated testosterone on nest defence in dark-eyed juncos. *Anim. Behav.* **56**: 617—621.

- Claussen, J.E. 1991. Annual variation in the reproductive activity of a bluegill population: effect of clutch size and temperature (MSc thesis). Toronto: University of Toronto.
- Colborne, S.F., Bellemare, M.C. Peres-Neto, P.R. and Neff, B.D. 2011. Morphological and swim performance variation among reproductive tactics of bluegill sunfish (*Lepomis macrochirus*). *Can. J. Fish. Aquat. Sci.*, **68**: 1808—1810.
- Colgan, P.W., Nowell, W.A., Gross M.R. and Grant, J.W. 1979. Aggressive habituation and rim circling in the social organization of bluegill sunfish (*Lepomis macrochirus*). *Environ. Biol. Fish.* **4**: 29—36.
- Côté, I.M. and Gross, M.R. 1993. Reduced disease in offspring: a benefit of coloniality in sunfish. *Behav. Ecol. Sociobiol.* **33**: 269—274.
- De Ridder, E.D. Pinxten, R. and Eens, M. 2000. Experimental evidence of a testosterone-induced shift from paternal to mating behaviour in a facultatively polygamous songbird. *Behav. Ecol. Sociobiol.* **49**: 24—30.
- Eising, C.M. and Groothuis, T.G.G. 2003. Yolk androgens and begging behaviour in black-headed gull chicks: an experimental field study. *Anim. Behav.* **66**: 1027—1034.
- Gettler, L.T., McDade, T.W., Feranil, A.B. and Kuzawa, C.W. 2011. Longitudinal evidence that fatherhood decreases testosterone in human males. *PNAS.* **108**: 16194—16199.
- Greenberg, N. and Crews, D. 1990. Endocrine and behavioral responses to aggression and social dominance in the green anole lizard, *Anolis carolinensis*. *Gen. Comp. Endocr.* **77**: 246—255.
- Gross, M.R. 1982. Sneakers, satellites and parentals: Polymorphic mating strategies in North American sunfish. *Z. Tierpsychol.* **60**: 1—26.
- Hau, M., Wikelski, M., Soma, K.K. and Wingfield, J.C. 2000. Testosterone and year-round territorial aggression in a tropical bird. *Gen. Comp. Endocr.* **117**: 20—33.
- Hegner, R.E. and Wingfield, J.C. 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. *Auk* **104**: 462—469.

- Hews, D.K. and Moore, M.C. 1997. Hormones and sex specific traits: critical questions. p. 277-292. *In* Parasites and Pathogens: Effects on host hormones and behaviour. N.E. Beckage [ed.]. Chapman & Hall. New York.
- Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. 2001. Two subtypes of androgen and progesterone receptors in fish testes. *Comp. Biochem and Physiol.* **129B**: 449—455.
- Kademian, S.M.E., Bignante, A.E., Lardone, P., McEwen, B.S. and Volosin, M. 2005. Biphasic effects of adrenal steroids on learned helplessness behavior induced by inescapable shock. *Neuropsychopharmacol.* **30**: 58—66.
- Ketterson, E.D., Nolan, V. Jr., Wolf, L. and Ziegenfus, C. 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on behavior and correlates of fitness in the Dark-eyed Junco (*Junco hyemalis*). *Am. Nat.* **140**: 980—999.
- Keverne, E.B. and Curley, J.P. 2004. Vasopressin, oxytocin and social behaviour. *Curr. Opin. Neurobiol.* **14**: 777—783.
- Kindler, P.M., Philipp, D.P., Gross, M.R. and Bahr, J.M. 1989. Serum 11-ketotestosterone and testosterone concentrations associated with reproduction in male bluegill (*Lepomis macrochirus*: Centrarchidae). *Gen. Comp. Endocrinol.* **75**: 446—453.
- Kindler, P.M., Bahr, J.M. and Phillips, D.P. 1991. The effects of exogenous 11-ketotestosterone, testosterone, and cyproterone acetate on prespawning and parental care behaviors of male bluegill. *Horm. Behav.* **25**: 410—423.
- Kuroda, K.O., Meaney, M.J., Uetani, N. and Kato, T. 2008. Neurobehavioral basis of the impaired nurturing in mice lacking the immediate early gene *FosB*. *Brain Res.* **1211**: 57—71.
- Lynn, S.E. 2008. Behavioural insensitivity to testosterone: Why and how does testosterone alter paternal and aggressive behaviour in some avian species but not others? *Gen. Comp. Endocr.* **157**: 233—240.
- Lynn, S.E., Prince, L.E., Schook, D.M and Moore, I.T. 2009. Supplementary testosterone inhibits parental care in a tropically breeding sparrow, *Zonotrichia capensis*. *Physiol. Biochem. Zool.* **82**: 699—708.

- Magee, S.E., Neff, B.D. and Knapp, R. 2006. Circulating concentrations of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. *Horm. Behav.* **49**: 598—609.
- Maxon, S.C. and Canastar, A. 2003. Conceptual and methodological issues in the genetics of mouse agonistic behavior. *Horm. Behav.* **44**: 258—262.
- McGlothlin, J.W., Jawor, J.M. and Ketterson, E.D. 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. *Am. Nat.* **170**: 864—875.
- Muller, M.N. and Wrangham, R.W. 2004. Testosterone, dominance and aggression in wild chimpanzees: a test of the challenge hypothesis. *Anim. Behav.* **67**: 113—123.
- Mutzel, A., Kempenaers, B., Laucht, S., Dingemanse, N.J. and Dale, J. 2011. Circulating testosterone levels do not affect exploration in house sparrows: observational and experimental tests. *Anim. Behav.* **81**: 731—739.
- Neff, B.D. and Gross, M. 2001. Dynamic adjustment of parental care in response to perceived paternity. *Proc. R. Soc. Lond. B-Biol. Sci.* **268**: 1559—1565.
- Neff, B.D. 2003. Decisions about parental care in response to perceived paternity. *Nature* **422**: 716—719.
- Neff, B.D. and Cargnelli, L.M. 2004. Relationships between condition factors, parasite load and paternity in bluegill sunfish, *Lepomis macrochirus*. *Environ. Biol. Fish.* **71**: 297—304.
- Neff, B.D. and Knapp, R. 2009. Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill. *Horm. Behav.* **56**: 239—245.
- Nelson, R.J. 2000. An introduction to behavioral endocrinology, 2nd ed. Sinauer Associates Inc., Sunderland Massachusetts.
- Oliveira, R.F., Almada, V.C. and Cario, A.V.M. 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish, *Oreochromis moccambicus*. *Horm. Behav.* **30**: 2-12.
- Oliveira, R.F., Canário, A.V.M. and Grober, M.S. 2001. Endocrine correlates of male polymorphism and alternative reproductive tactics in the Azorean rock-pool

- blenny, *Parablennius sanguinolentus parvicornis*. Gen. Comp. Endocr. **121**: 278—288.
- Oliveira, R.F., Hirschenhauser, K., Carneiro, L.A. and Canario, A.V.M. 2002. Social modulation of androgen levels in male teleost fish. Comp. Biochem. Physiol. **132**: 203—215.
- Peters, A. 2002. Testosterone and the trade-off between mating and paternal effort in extrapair-mating superb fairy-wrens. Anim. Behav. **64**: 103—112.
- Ros, A.F.H., Brintjes, R., Santos, R.S., Canário, A.V.M. and Oliveira, R.F. 2004. The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis*. Horm. Behav. **46**: 491—497.
- Rose R.M. and Holaday, J.W. 1971. Plasma testosterone, dominance rank and aggressive behaviour in male rhesus monkeys. Nature **231**: 366—368.
- Saaristo, M., Craft, J.A., Lehtonen, K.K., Bjork, H. and Lindstrom, K. 2009. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17 alpha-ethinyl estradiol, an endocrine disruptor. Horm. Behav. **55**: 530—537.
- Schwagmeyer, P.L., Schwabl, H.G. and Mock, D.W. 2005. Dynamics of biparental care in house sparrows: hormonal manipulations of paternal contributions. Anim. Behav. **69**: 481—488.
- Scott, M.P. 2006. Resource defense and juvenile hormone: The “challenge hypothesis” extended to insects. Horm. Behav. **49**: 276—281.
- Scott, W.B. and Crossman, E.J. 1973. Freshwater fishes of Canada. Fish. Res. Board Can. 184.
- Silverin, B. 1980. Effect of long-acting testosterone treatment on free-living pied flycatchers, *Ficedula hypoleuca*, during the breeding period. Anim. Behav. **28**: 906—912.
- Silverin, B., Baillien, M. and Balthazart, J. 2004. Territorial aggression, circulating levels of testosterone and brain aromatase activity in free-living pied flycatchers. Horm. Behav. **45**: 225—234.

- Smith, L.C., Raouf, S.A., Brown, M.B., Wingfield, J.C. and Brown, C.R. 2005. Testosterone and group size in cliff swallows: testing the "challenge hypothesis" in a colonial bird. *Horm. Behav.* **47**: 76—82.
- Soma, K.K., Tramontin, A.D. and Wingfield, J.C. 2000. Oestrogen regulates male aggression in the non-breeding season. *Proc. R. Soc. Lond. B.* **267**: 1089—1096.
- Steinman, M.Q. and Trainor, B.C. 2010. Rapid Effects of Steroid Hormones on Animal Behavior [online]. *Nature Education Knowledge*, **1**: 1. Available from <http://www.nature.com/scitable/knowledge/library/rapid-effects-of-steroid-hormones-on-animal-15104619> [accessed 20 December 2011].
- Stribley, J.M. and Carter, C.S. 1999. Developmental exposure to vasopressin increases aggression in adult prairie voles. *P. Natl. Acad. Sci. USA.* **96**: 12601—12604.
- Taves, M.D., Desjardins, J.K., Mishra, S. and Balshine, S. 2009. Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). *Gen. Comp. Endocr.* **161**: 202-207.
- Trainor, B.C. and Marler, C.A. 2001. Testosterone, paternal behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*). *Horm. Behav.* **40**: 32—42.
- Trainor, B.C., Greiwe, K.M. and Nelson, R.J. 2006. Individual differences in estrogen receptor α in select brain nuclei are associated with individual differences in aggression. *Horm. Behav.* **50**: 338—345.
- van Anders, S.M., Goldey, K.L. and Kuo, P.X. 2011. The steroid/peptide theory of social bonds: Integrating testosterone and peptide responses for classifying social behavioral contexts. *Psychoneuroendocrino.* **36**: 1265—1275.
- Weiss, S.L. and Moore, M.C. 2004. Activation of aggressive behaviour by progesterone and testosterone in male tree lizards, *Urosaurus ornatus*. *Gen. Comp. Endocr.* **136**: 282—288.
- Wingfield, J.C., Hegner, R.E., Dufty, A.M. and Ball, G.F. 1990. The "Challenge Hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Am. Nat.* **136**: 829—846.

- Yamaguchi, S., Kagawa, H., Gen, K., Okuzawa, K. and Matsuyama, M. 2004. Silicone implants for delivery of estradiol-17 β and 11-ketotestosterone to red seabream, *Pagrus major*. *Aquaculture*. **239**: 485—496.
- Yamaguchi, S., Gen, K., Okuzawa, K., Kumakura, N., Matsuyama, M. and Kagawa, H. 2005. Effects of 11-ketotestosterone and gonadotropin releasing hormone on follicle-stimulating hormone and luteinizing hormone gene expression in castrated and sham-operated male red seabream, *Pagrus major*. *Fisheries Sci.* **71**: 1049—1058.

6 Appendices

Appendix A: Behaviours quantified during paternal care in bluegill (*Lepomis macrochirus*)

Type of Behaviour	Behaviour Observed	Description of Behaviour
Nurturing	Pectoral fanning	Rapid synchronous movement of the pectoral fins outwards from the side of the body
	Caudal sweep	Movement of the caudal fin from side to side at a 45° angle from the nest
	Egg removal	Removing moulding eggs from the nest with the mouth
Aggressive	Biting	Nipping at the predator with the mouth and teeth
	Opercular flare	Extending the opercula laterally while facing the predator
	Lateral display	Presenting body lengthwise to the predator

Appendix B: Mean (\pm SD) number of individual nurturing and aggressive behaviours by parental male bluegill (*Lepomis macrochirus*) in June 2009 in Lake Opinicon. Males were implanted with a placebo, one testosterone implant (T1), two testosterone implants (T2) or no implant (Control). Nurturing behaviours were recorded over 30 minutes and aggressive behaviours toward a potential nest predator were recorded over 2 minutes (see text for details).

Treatment	n	Nurturing behaviour			Aggression toward predator		
		Caudal sweeps	Pectoral fanning	Egg removal	Bites	Opercular flares	Lateral displays
Control	7	13 \pm 32	8 \pm 16	6 \pm 5	46 \pm 16	11 \pm 8	7 \pm 3
Placebo	10	1 \pm 2	14 \pm 11	9 \pm 7	50 \pm 10	9 \pm 4	5 \pm 3
T1	9	8 \pm 23	11 \pm 13	4 \pm 4	61 \pm 12	10 \pm 9	11 \pm 4
T2	8	2 \pm 3	9 \pm 7	5 \pm 5	52 \pm 14	12 \pm 7	8 \pm 4

Appendix C: Mean (\pm SD) number of individual nurturing and aggressive parental behaviours on the first and second days after spawning (egg stage) by parental male bluegill (*Lepomis macrochirus*) in June 2010 in Lake Opinicon. Treatments include males implanted with a placebo, flutamide, an 11-ketotestosterone implant (KT1), two 11-ketotestosterone implants (KT2) or no implant (Control). Nurturing behaviours were recorded over 30 min and aggressive behaviours toward a potential nest predator were recorded over 2 min (see text for details).

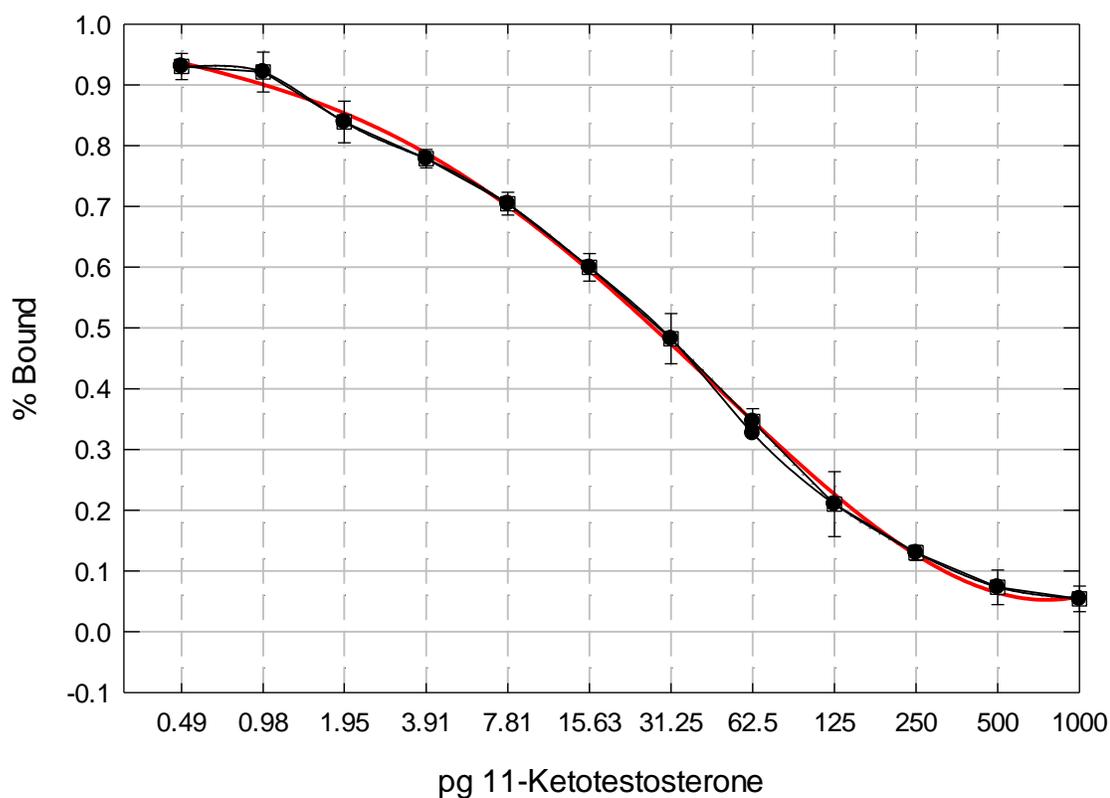
Treatment	n	Nurturing behaviour			Aggression toward predator		
		Caudal sweeps	Pectoral fanning	Egg removal	Bites	Opercular flares	Lateral displays
Control	11	2 \pm 4	4 \pm 5	9 \pm 13	9 \pm 12	5 \pm 4	3 \pm 2
Placebo	8	3 \pm 5	1 \pm 1	5 \pm 4	6 \pm 7	7 \pm 5	5 \pm 4
Flutamide	9	11 \pm 16	5 \pm 5	11 \pm 8	4 \pm 3	9 \pm 5	3 \pm 2
KT1	9	1 \pm 3	1 \pm 1	3 \pm 4	10 \pm 9	11 \pm 10	4 \pm 3
KT2	10	0 \pm 0	1 \pm 1	1 \pm 2	14 \pm 10	12 \pm 8	7 \pm 4

Appendix D: Mean (\pm SD) PC1 values for nurturing and aggressive behaviours by parental male bluegill (*Lepomis macrochirus*) implanted with nothing (Control), a placebo, one testosterone implant (T1), two testosterone implants (T2), flutamide, one 11-ketotestosterone implant (KT1) or two 11-ketotestosterone implants (KT2) for 2009 and 2010 study years.

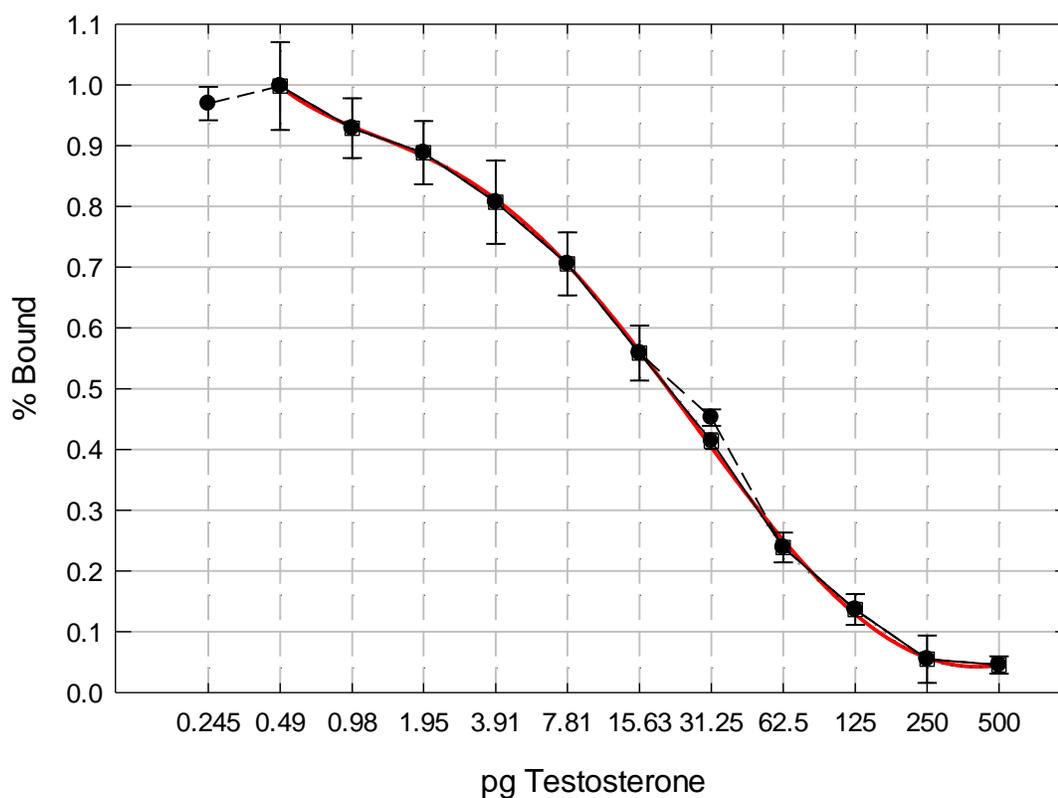
Year	Treatment	Nurturing behaviour	Aggressive behaviour
2009	Control	0.07 \pm 2.07	-0.57 \pm 1.28
	Placebo	0.18 \pm 1.10	-0.61 \pm 0.79
	T1	-0.10 \pm 1.33	0.96 \pm 0.84
	T2	-0.34 \pm 0.70	0.04 \pm 1.34
	ANOVA	$F_{3,30}=0.46$, $p=0.71$	$F_{3,30}=3.53$, $p=0.03$
2010	Control	0.12 \pm 1.02	-0.45 \pm 1.07
	Placebo	-0.24 \pm 0.47	-0.29 \pm 1.19
	Flutamide	1.11 \pm 2.26	-0.50 \pm 0.81
	KT1	-0.37 \pm 0.46	0.26 \pm 1.42
	KT2	-0.62 \pm 0.09	0.94 \pm 1.45
	ANOVA	$F_{4,42}=3.23$, $p=0.02$	$F_{4,42}=2.50$, $p=0.06$

Note: The first principal axis was used for each year and was composed of positive loadings for each behaviour. In 2009 the loadings were: nurturing behaviour: caudal sweeps = 0.591, pectoral fanning = 0.594, egg removal = 0.545, EigenValue = 1.62 and this captured 58% of the variance; aggression: biting = 0.708, lateral displays = 0.705, opercular flare = 0.039, EigenValue = 1.40 and this captured 49% of the variance. In 2010 the loadings were: nurturing behaviour: caudal sweeps = 0.696, pectoral fanning = 0.699, egg removal = -0.166, EigenValue = 1.55 and this captured 52% of the variance; aggression: biting = 0.565, lateral displays = 0.639, opercular flare = 0.522, EigenValue = 1.66 and this captured 55% of the variance.

Appendix E: Representative radioimmunoassay standard curve for determining plasma concentrations of 11-ketotestosterone in male bluegill sunfish (*Lepomis macrochirus*) implanted with a placebo, flutamide, one 11-ketotestosterone implant, two 11-ketotestosterone implants or no implants (Control). Hormone measurements were taken the day after spawning and 3 days after implantation in June 2010 from a population in Lake Opinicon.



Appendix F: Representative radioimmunoassay standard curve for determining plasma concentrations of testosterone in male bluegill sunfish (*Lepomis macrochirus*) implanted with a placebo, flutamide, one 11-ketotestosterone implant, two 11-ketotestosterone implants or no implants (Control). Hormone measurements were taken the day after spawning and 3 days after implantation in June 2010 from a population in Lake Opinicon.



Appendix G: Representative radioimmunoassay standard curve for determining plasma concentrations of cortisol in male bluegill sunfish (*Lepomis macrochirus*) implanted with a placebo, flutamide, one 11-ketotestosterone implant, two 11-ketotestosterone implants or no implants (Control). Hormone measurements were taken the day after spawning and 3 days after implantation in June 2010 from a population in Lake Opinicon.

Appendix H: Ethics approval



May 26, 2010

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2014

Dear Dr. Neff:

Your Animal Use Protocol form entitled:
 Behavioural and Molecular Ecology of Fishes
 Funding Agency NSERC Discovery Grant - R3244A04; NSERC Grant - R3244A16

has been approved by the University Council on Animal Care. This approval is valid from **May 26, 2010 to May 31, 2011**. The protocol number for this project is **#2010-214 which replaces #2006-062-05 which has expired..**

1. This number must be indicated when ordering animals for this project.
 2. Animals for other projects may not be ordered under this number.
 3. If no number appears please contact this office when grant approval is received.
- If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	4 Year Total Numbers Estimated as Required	List All Strain(s)	Age / Weight
Other	800	Sunfish - Bluegill (<i>Lepomis macrochirus</i>); Pumpkinseed (<i>L. gibbosus</i>)	0-11 years
Other	800	Guppies (<i>Poecilia reticulata</i>)	0-2 years
Other	800	Salmonids - Chinook (<i>Oncorhynchus tshawytscha</i>); Atlantic salmon (<i>Salmo salar</i>); Brown trout (<i>S. trutta</i>); Rainbow trout (<i>O. mykiss</i>)	0-6 years

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - B. Neff, S. Garner; W. Lagerwerf; S. Waring, J. O'Donnell (Queen's)

The University of Western Ontario
 Animal Use Subcommittee / University Council on Animal Care
 Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
 PH: 519-661-2111 ext. 86770 • FL 519-661-2028 • www.uwo.ca / animal

7 Curriculum Vitae

Name: Chandra M.C. Rodgers

Post-secondary Education and Degrees: University of Western Ontario
London, Ontario, Canada
2005-2009 B.Sc.H

University of Western Ontario
London, Ontario, Canada
2009-Present M.Sc. Candidate

Related Work Experience Teaching Assistant
University of Western Ontario
2009-2012

Undergraduate field assistant
University of Western Ontario
May 2009- June 2009

Publications:

Rodgers, CMC, BD Neff and R Knapp. 2012. Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology (accepted)*.

Rodgers, CMC, BD Neff and R Knapp. 2012. An androgen mediated trade-off between nurturing and aggressive paternal care in bluegill sunfish (*Lepomis macrochirus*). *Hormones and Behavior (in preparation)*.

Conferences:

Oral presentation at the Ecological and Evolutionary Ethology of Fishes conference, University of Windsor, June, 2012.

Oral presentation at the Biology Graduate Research Forum, University of Western Ontario, October, 2010.

Poster presentation at the Queen's University Biological Station open house, June, 2010.