# Effects of Testosterone Exposure on Parental Care of African Convict Cichlids: A Test of the "Challenge Hypothesis"

By
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April, 2011




**Allegheny College**
**Department of Biology**

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.***

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Pledge

Approval by the Senior Thesis Committee:

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*Dr. Scott Wissinger*

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*Dr. Ron Mumme*

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## ABSTRACT

Animals that provide parental care face a trade-off between offspring defense and offspring care. The "challenge hypothesis" proposes that testosterone plays a modulating role in monogamous males' responses to such social stimuli. It predicts a trade-off between parental care and male-male aggression that depends on endogenous testosterone levels. Through endocrine-disrupting activity, exogenous testosterone might impact behavior. In this investigation, 17α-methyltestosterone was added to experimental tanks consisting of monogamous pairs of biparental, aggressive African convict cichlids, *Archocentrus nigrofasciatus*. Behavioral assays were conducted during offspring stages (egg, wriggler, fry) in the presence and absence of an intruder and presence and absence of exogenous testosterone. I hypothesized that exogenous testosterone would decrease parental care and increase aggression as predicted by the "challenge hypothesis." Males in the testosterone treatment exhibited a significant 1) increase in distance from the brood, 2) decrease in parental care behaviors, and 3) increase in aggressive behaviors in comparison to control-treated males. These results were mainly apparent during the egg stage of offspring development and only with an intruder present. Females exhibited no behavioral differences between the testosterone and control treatments. The behavioral responses of males are consistent with the "challenge hypothesis" and provide insight into potential effects of androgenic contamination on fish behavior.

# INTRODUCTION

Androgens, including testosterone, play a large role in vertebrate reproduction, including the development and maintenance of secondary sexual characteristics, spermatogenesis, and reproductive behavior in males (Nelson, 1994). Testosterone, in particular, accounts for the majority of sex differences in vertebrate neural structure by inhibiting or exacerbating cell death, modulating the formation and elimination of synapses, and overall masculinizing the brain (Morris et al., 2004). In many species, high levels of androgens are needed for successful territory defense, mate attraction, and reproductive success (Wingfield et al., 1990; Nelson, 1994). Particularly in social behaviors, androgens can serve an activational role, initiating aggressive and sexual behavior (Nelson, 1994; Gleason et al., 2009). These behaviors can influence androgen levels, suggesting a bidirectional relationship (Villars, 1983; Gleason et al., 2009).

## *Endogenous Testosterone, Parental Care, and the "Challenge Hypothesis"*

Androgen expression varies across development; thus, testosterone levels could increase or decrease depending upon behavioral context. The "challenge hypothesis" proposes a set of predictions for differing levels of male testosterone secretion. According to this hypothesis, testosterone levels increase during times of territorial defense and social instability, such as during rank establishment and mating. As a response to social stimuli, testosterone secretion regulates aggression and breeding strategies. Although higher testosterone levels can lead to benefits like increased aggression and thus increased acquisition of mates and breeding territory, the maintenance of high testosterone levels is costly in terms of survival and reproductive success (Wingfield et al., 1990; Duffy et al., 2000).

The "challenge hypothesis" predicts a trade-off between male-male aggressive behavior and parental care for monogamous males. Increased testosterone secretion is predicted to promote an increase in territorial aggression, and corresponding decrease in resources dedicated to parental care. Males spend increased time away from offspring and consequently reduce their investment in parental care. Decreased parental care can lead to a decrease in offspring viability and subsequently reproductive success (Wingfield et al., 1990; Duckworth, 2006). In some male birds, testosterone implants result in a dramatically reduced number of young per brood compared to controls (Silverin, 1980; Hegner & Wingfield, 1987). Testosterone levels are thus predicted to decrease during phases of parental care in monogamous males (Wingfield et al., 1990).

In addition to birds, the effects of endogenous androgen levels on parental care in fish have been studied in the context of the "challenge hypothesis" (Oliveira et al., 2002; Parikh et al., 2006). In many cichlid fish, the response of male androgen levels to simulated intruders supports the predictions of the "challenge hypothesis" (Hirschenhauser et al., 2004). In the plainfin midshipman fish, *Porichthys notatus*, males with nests containing embryos exhibit undetectable testosterone levels as compared to males with empty nests (Knapp et al., 1999). In the three-spined stickleback *Gasterosteus aculeatus*, males exhibit high circulating levels of 11-ketotestosterone if they are unable to spawn (Pall et al., 2002a). In male bluegill (*Lepomis macrochirus)*, testosterone levels are negatively related to paternity levels and positively related to defensive behavior (Neff & Knapp, 2009). Similarly, males of the Azorean Rock-Pool Blenny (*Parablennius sanguinolentus parvicornis*) exhibit a negative correlation between 11-ketotestosterone levels and parental behavior (Oliveira et al., 2001a).

While the "challenge hypothesis" explains the modulating role of testosterone in male fish, androgens can also play a role in aggressive female fish. Female cichlids (*Neolamprologus pulcher)* exhibit elevated levels of circulating 11-ketotestosterone and testosterone after territorial defense as predicted by the "challenge hypothesis" (Desjardins et al., 2006; Wingfield et al., 1990). Testosterone immersion increases agonistic behavior in the female cichlid *Aequidens pulcher* (Munro & Pitcher, 1985). In the context of parental care, captive brooding females of the black-chinned tilapia (*Sarotherodon melanotheron)* exhibit lower levels of androgens than prespawning females (Specker & Kishida, 2000). Androgen levels can affect the behaviors of aggressive female fish, depending on the behavioral and reproductive context, similar to that of aggressive male fish (Arnott & Elwood, 2009).

*Evidence for the Release and Accumulation of Exogenous Androgens in the Environment*

The release of endocrine-disrupting chemicals that mimic endogenous hormones is well documented, as are the impacts on prenatal or early postnatal endocrine development in wildlife and humans (Colborn et al., 1993; Wolff & Weston, 1997). In addition to a wide array of pesticides, such as tributyl tin, herbicides (Lyons, 1996; Sumpter, 2005), and the alkylphenolic group of chemicals (Soto et al., 1991; Sumpter, 2005), natural and synthetic vertebrate steroids are introduced into the environment on a consistent basis (Shore & Shemesh, 2003).

Most research on endocrine disruptors has focused on the effects of estrogen mimics (Purdom et al., 1994; Toppari et al., 1996; Gray et al., 1997; Barontoni et al., 2000), whereas the effects of testosterone mimics have received less attention (Kumar et al., 2008). As potential hormone mimics, environmental testosterone and estrogen share similar origins. Both are continuously produced and released into the environment from a variety of anthropogenic and natural sources (Fan et al., 2007), including the urine and feces of humans, livestock, and

wildlife (Shore et al., 1997; Shore & Shemesh, 2003). Raw and treated wastewater effluents from industry, hospital, and municipal sewage treatment plants and surface extracts exhibit both androgenic and estrogenic activity (Van Der Linden et al., 2008). Environmental androgens could thus be just as prevalent and pose as much of an environmental risk as estrogens and should be a matter of concern (Kumar et al., 2008).

After excretion, testosterone and other steroid hormones can accumulate and later be transported throughout the environment (Deksissa, 2008). Both ground water and surface water can become contaminated with testosterone through leaching from soil and baseflow (Shore et al., 2004), from wastewater treatment plants (Kirk et al., 2002), and from the application of sewage sludge to agricultural land (Aguayo et al., 2004). Although wastewater treatment systems kill pathogens and nutrients, they typically do not degrade hormones, allowing for the accumulation of steroids, including testosterone and associated ethinylestradiol, estrone, and estriol (Kirk et al., 2002; Shore et al., 2004). Rangeland grazing could lead to steroid contamination in agricultural watersheds that are used by cattle for drinking water (Kolodziej & Sedlak, 2007). Additionally, testosterone enters water sources as runoff from soil that has been amended with animal manure from concentrated animal feeding operations (CAFOs) (Shore et al., 2004; Deksissa, 2008).

In addition to their excretion of naturally-produced testosterone (Lange et al., 2002), farm animals are often treated with, and are thus likely to excrete, exogenous sources of hormones. Cattle producers, including those in the United States, Australia, Argentina, and Canada, use hormonal growth promoters that contain pharmaceutical-grade compounds possessing androgenic activity (Schiffer et al., 2001; Silence, 2004). These include testosterone (Le Guevel & Pakdel, 2001) and the synthetic androgen, 17β-trenbolone (TB), which is found in high

concentrations in waters receiving cattle feedlot effluent (Wilson et al., 2002). Such exogenous steroids and synthetic substances synchronize reproductive cycles and improve feed efficiency, and possibly contribute to the androgenic activity found in cattle feedlot effluents (Schiffer et al., 2001) and livestock manures (Lorenzen et al., 2004).

Manure-borne steroids are likely to be a major disturbance in aquatic environments. They are the most potent endocrine disrupting compounds besides industrial products (Jobling & Sumpter, 1993), and are heavily detected in soil and water used by farmers and producers (Deksissa, 2008). Although the United States Environmental Protection Agency (EPA) has tightened the regulation of CAFOs (U.S. EPA, 2008), environmentalists argue that livestock waste management practices are not providing adequate protection from these contaminants (Burkholder et al., 2006). With increasing use of these exogenous hormones and related farming practices, the potential for androgenic contamination from the application of animal manure to agricultural lands is significant (Lorenzen et al., 2005).

Once released into the environment, the mobility and persistence of testosterone is a major concern to environmental toxicologists. In soils of the midwestern United States, testosterone and other hormones largely associate with the sorbed phase, reach sorption equilibrium rapidly, and thus display an increased potential for surface-water contamination and a limited capacity to leach to ground water (Lee et al., 2003; Deksissa, 2008). In comparison to estrogenic hormones, testosterone displays more mobility because it is not as strongly absorbed to the soil matrix (Casey et al., 2004; Kim et al., 2006; Stumpe & Marschner, 2007).

Testosterone exhibits a short half-life under aerobic conditions (Lee et al., 2003), degrades four times more rapidly than estrogen (Casey et al., 2004), and mineralizes at a high rate in all soils (Stumpe & Marschner, 2007). The rate of testosterone biodegradation is

temperature dependent (Lorenzen et al., 2005), and affected by moisture content, aeration, antibiotics, and presence of viable microbial biomass (Dekissa, 2008).

Despite a relatively short half life, testosterone is continuously present in the environment (Deksissa, 2008), and accumulates in relatively high concentrations (e.g., 670 ng/g; Fisher et al., 2005). Testosterone is detectable in U.S. streams (200 ng/l; Kolpin et al., 2002); the Chesapeake Bay (5-10 ng/l; Dorabawila & Gupta, 2005), soil amended with poultry manure (260 ng/kg), and runoff water from grasslands amended with poultry manure (10-1,380 ng/kg; Finlay-Moore et al., 2000). Despite the evidence for the presence of both androgens and estrogen in the waters of the United States, the EPA does not yet require the monitoring of steroids in the water supply (Deksissa, 2008).

Exogenous testosterone contamination is not limited to the United States. Effluents from Swedish domestic sewage treatment plants contain a maximum concentration of 160 ng of dihydrotestosterone (DHT) equivalents/l (Svenson & Allard, 2005). Select components found in extracts of leachates from Swedish landfills interact with androgenic receptors, promoting a response in the β-galactosidase marker system (Svenson et al., 2005). In the United Kingdom, yeast-based androgen screens from sewage treatment effluents reveal androgenic activity in concentrations up to 635 ng DHT/l. Surface water, sediment pore water, and sediment particulate material solvent extracts of estuaries receiving such effluents display androgenic activity in concentrations ranging from 3 to 187 ng DHT/l (Thomas et al., 2002). In Israel, testosterone accumulation in the Upper Jordan Valley is known to wash out after major rain events, first as runoff from cattle pasture and fish pond effluent and later as discharge from the sulfous peat soil (Shore et al., 2004).

With the increasing presence of synthetic androgens in aquatic systems, their effect is of increasing relevance to humans. Research suggests a link between some cancers and exposure to endocrine-disrupting chemicals (Wolff & Weston, 1997) as well as an association between environmental accumulation of steroids and younger ages of puberty among girls (Herman-Giddens et al., 1997) and a declining ratio of male births to female births (World Health Organization, 2002). These hormonal changes could lead to decreases in sperm counts, decreased male reproductive capacity, and several diseases (Chen et al., 2007; Roy & Pereira, 2005)

*Evidence for Exogenous Testosterone Effects on Fish Development and Behavior*

There is a growing body of evidence that exposure to exogenous androgens can have profound effects on the development and behavior of animals in the natural environment (Colborn et al., 1993). Specifically in fish, exogenous androgens can promote the development of male reproductive and secondary sexual characteristics (Oliveira et al., 2001b; Kraak et al., 2001; see review in Sumpter, 2005). Testosterone exposure induces sex-reversal and suppression of p450arom gene expression in the Japanese flounder (*Paralichthys olviaceus;* Kitano et al., 2000) and intersex gonads in newly-hatched Medaka (*Oryzias latipes;* Koger et al., 2000). Mozambique tilapia (*Oreochromis mossambicus)* exhibit increased gonado-somatic index, imposex, and enlarged male genital papilla in sewage-mixed Koraiyar water (Subramanian & Amutha, 2006). Masculinization of female fish is also observed in populations exposed to androgens in pulp and paper mill effluents (Howell et al., 1980; Parks et al., 2001; Larsson et al., 2000) and feedlot effluents (Orlando et al., 2004).

Whereas these developmental and reproductive effects are well documented, the behavioral effects of exogenous androgens are not as well studied in fish taxa. Literature reviews

suggest even low levels of exogenous steroids can affect breeding strategies and pre-spawning behaviors (Borg, 1994; Van Der Kraack et al., 2001). Androgen administration is shown to promote courtship and reproductive aggression in many fish (Smith, 1969; see review in Liley & Stacey, 1983; Pankhurst, 1995; Salek et. al., 2001). However, few studies address post-spawning effects of exogenous testosterone on aggression. In male fathead minnows (*Pimephales promelas),* androgen exposure results in increased aggressiveness and increased acquisition of nests in the presence of competitors (Martinovic et al., 2007).

Several studies note the effect of administered testosterone on fish parental care (Kramer, 1972; Kindler et al., 1991; Pall et al., 2002b; Desjardins et al., 2008). To my knowledge, literature regarding the effect of elevated testosterone levels on both direct parental care and anti-predator aggression is limited and involves only fish that exhibit exclusive paternal care. In testosterone-implanted males of Azorean rock-pool blenny *Parablennius parvicornis,* defense of large territories is incompatible with parental behavior (Ros et al., 2004). Treatment with an androgen antagonist induces decreased nest defense in male smallmouth bass (*Micropterus dolomieu*; Dey et al., 2010), but does not affect anti-predator defense in male bluegill (*Lepomis macrochirus;* Kindler et al., 1991). The effects of exogenous testosterone in fish species that exhibit both maternal and paternal brood defense and parental care could provide more insight into the relationship between androgens and the degree of parental investment than previous studies.

*Goals and Hypotheses of this Study*

The African convict cichlid, *Archocentrus nigrofasciatus* (hereafter, "convict cichlids"), is an appropriate model to study the effects of exogenous androgens on parental care and the "challenge hypothesis" because this species exhibits highly aggressive behavior (Arnott &

Elwood, 2009) and extended biparental care (Keenleyside, 1991). Males and females form discrete, monogamous pairs. Females lay eggs on the ceiling of small caves (Wisenden et al., 2008). Suitable spawning sites are an important resource as they establish territory, initiate competition, and influence parental roles in the mating system (Wisenden et al., 2008). Both parents guard the cave and repel potential predators, including conspecifics (Wisenden, 1995). However, females typically spend more time than males with offspring and provide direct care (mouthing the brood, fanning the brood) whereas males engage in territory defense (Keenleyside, 1991; Wisenden, 1995). Both sexes still display behavioral flexibility in parental roles (Itzkowitz et al., 2005), especially during decreased mate effort and mate absence (Itzkowitz et al., 2003).

In this study, I added 17α-methyltestosterone, a synthetic androgen, to experimental aquaria to mimic exposure to exogenous androgens in the environment. I hypothesized, based on the predictions of the "challenge hypothesis," that exposure to exogenous sources of testosterone should increase aggression at the expense of parental care. I examined the behavioral responses of convict cichlids in the absence and presence of an intruder to determine if androgen exposure potentially decreases parental care only in the context of predation. Specifically, I predicted the following effects of convict cichlid exposure to 17α-methyltestosterone-treated water after mating:

1) Males and females would spend more time farther from the brood in the absence and presence of an intruder as compared to controls;

2) Males and females would exhibit a decrease in parental care behaviors in the absence of a potential intruder, and in the presence of an intruder, also exhibit an increase in aggressive behaviors as compared to controls. Alternatively, if parental care is more important than territory

defense, males and females will remain close to the brood and exhibit high levels of parental care behaviors regardless of testosterone levels and the presence of an intruder; and

3) Offspring survival would be lower in testosterone treatments than in controls because potentially decreased parental care could lead to decreased offspring survival.

## MATERIALS AND METHODS

### *Experimental Animals*

Convict cichlids are a substrate-brooding species found in lakes and streams in Central America, ranging from Guatemala to northern Panama (Bussing, 1987). Females lay eggs on objects found on the substratum of aquariums (Wisenden, 1995). Offspring develop through three stages: egg (embryo), wriggler (free, non-swimming embryo), and fry (free-swimming larvae). The embryo stage and wriggler stage last three to four days while the fry stage can last up to six weeks (Wisenden, 1995).

### *Fish rearing*

Same-sex stock populations of unmated males and unmated females of convict cichlids were maintained in the aquatics laboratory in 50 L holding tanks. The fish were originally purchased from a commercial supplier and bred continuously. The holding tanks were kept as close to 26˚C as possible under an ambient light:dark cycle. Each tank contained clay flower pots, a power filter, and aquatic gravel. The fish were fed with commercial fish food twice daily.

### *Experimental Set-up*

Behavioral assays were conducted in 10-gallon aquaria in which one male was paired with one female convict cichlid. Seven pairs were exposed to a 17α-methyltestorone-ethanol solution and seven pairs were exposed to ethanol (total of 14 pairs). Two behavioral assays, one with an intruder and one without an intruder, were conducted at each offspring stage (egg,

wriggler, fry) for a total of six behavioral assays per mated pair (total of 90 trials). The location and behaviors of males and females were recorded in each trial (total of 180 observations).

*Behavioral Assay*

For each trial, a 10-gallon experimental tank, maintained at 28˚C in an environmental unit with a 15:9 hours light:dark cycle, was filled with 3 cm of aquatic gravel, a corner filter, and a clay flower pot (diameter 11 cm x height 10 cm) located 8 cm from one end of the tank. Each tank contained a perforated, translucent plastic barrier installed 8 cm away from the opposite end of the tank, measured from the center of the divider. This barrier divided the tank into a "resident" area for the breeding pair of convict cichlids and an "intruder" area for a selected predator (Fig. 1).

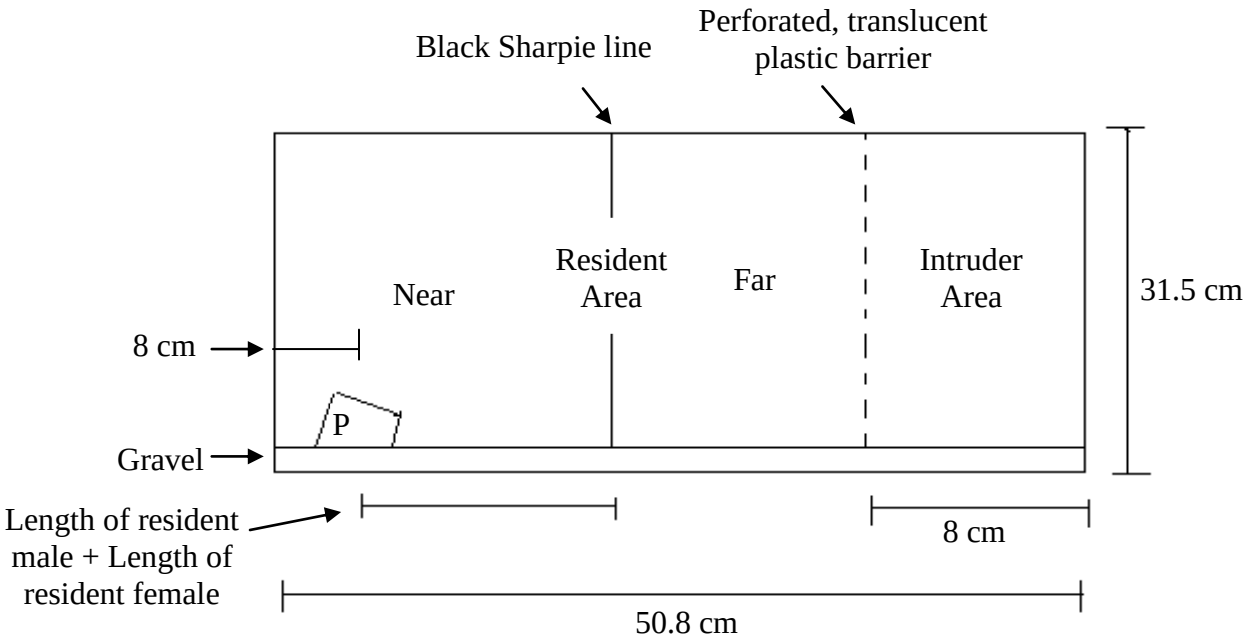



Fig. 1. Set-up of 10-gallon experimental tank used in behavioral assay. P = clay flower pot (diameter 11 cm x height 10 cm).

One male and one female from each respective stock population were measured and placed into the "resident" area of an experimental tank. Experimental tanks were placed next to neighboring tanks so paired fish were able to visually interact with neighboring pairs (Lavery,

1995), a process which helped to induce mating. Using a black Sharpie, a line was drawn along the side of the tank to indicate a distance, equal to the sum of the length of the resident male and resident female, from the center of the brood site toward the "intruder" area (Lavery, 1995). This further divided the resident area into an area "Near" to the brood and an area "Far" from the brood (Fig. 1).

Fish pairs were fed twice daily with commercial flake food and were given two weeks to form a resident pair and spawn. Additional fish were added to the intruder area before the laying of eggs to induce breeding. These fish were removed and opaque dividers were placed between neighboring tanks once eggs were laid. If the pair failed to lay eggs within the two-week period, the fish were returned to their respective stock tanks and a different male and different female were selected (Snekser & Itzkowitz, 2009).

Within twelve hours of the resident female laying eggs, the experimental tanks were treated either with 15 mg of 17α-methyltestosterone (Munro & Pitcher, 1985; Fitzpatrick et al., 1999; Sigma, St. Louis, MO, USA) dissolved in 7 ml of 95% ethanol (Wassermann & Afonso, 2003) or 7 ml of 95% ethanol (control). This solution was poured directly into the experimental tanks (Pandian & Sheela, 1995; Silarudee & Kongchum, 2008). The type of solution added, whether exogenous testosterone or control, was chosen at random in order to avoid potential seasonal effects.

In each experimental tank, the number of eggs present before all observations was recorded. The breeding pair was observed for two 20 minute trials, one in the absence of a potential intruder and one in the presence of a potential intruder, at each developmental stage (egg, wriggler, and fry). The order of exposure to a potential intruder, whether in the first or second trial, was chosen at random; however, the pattern remained consistent. If observations

were first made in the presence of an intruder during the egg stage, first trials at later developmental stages were made in the presence of an intruder.

In intruder treatments, a male convict cichlid was obtained from the stock population, measured, and placed into the "intruder" area of the experimental tank. After a two-minute acclimation period (Lavery, 1995), the position of the male and female relative to the brood was recorded every 30 seconds over the 20-minute trial (Table 1). In addition, parental care and aggressive behaviors of the male and female exhibited were recorded every 30 seconds over the 20-minute trial (Table 2). The intruder was removed and returned to the stock population after the trial. For trials in the absence of an intruder, a collecting net was dipped into the intruder side of the tank to imitate the placement of an intruder. After a two-minute acclimation period, the position of the male and the female relative to the brood (Table 1) and parental care and aggressive behaviors (Table 2) were again recorded every 30 seconds during the 20 minute trial.

Table 1. Observed positions of resident males and females.

| Position[a] | Definition |
|---|---|
| Brood | Fish positioned $\leq$ 1 body length from center of brood |
| Near | Fish positioned > 1 but $\leq$ 2 body lengths away from center of brood (Fig. 1) |
| Far | Fish positioned > 2 body lengths away from center of brood (Fig. 1) |

[a]Described in Lavery, 1995

Table 2. Observed parental care and aggressive behaviors of resident males and females.

| Parental Care Behavior[a] | Definition |
|---|---|
| Fanning brood | Fish positioned within one body length of brood. Caudal fin and body undulate while pectoral fins move alternately over brood |
| Mouthing brood | Fish nips at embryo and removes debris |
| Mouthing substrate | Fish touches gravel, picks up gravel and deposits in same position, or picks up gravel and moves with mouth |
| Fin-digging | Fish stirs substrate by moving the pectoral fins forward while simultaneously beating the caudal fin |
| Fry retrieval | Fish picks up stray larva with mouth and returns larva to the center of the brood |
| Guarding | Fish positioned motionless anywhere in tank with attention on observer |
| Observing | Fish visually survey brood while within one body length of center of brood |
| **Aggressive Behavior** | **Definition** |
| Frontal display | Fish faces intruder area while extending its unpaired fins and lowering its branchiostegals |
| Lateral display[b] | Fish presents its side to intruder area while extending its unpaired fins and lowering its branchiostegals |
| Bites | Open-mouthed contacts with barrier or other resident fish |
| Tail beats[c] | Fish beats its tail and water is pushed in direction of intruder area |

[a]Described in Keenleyside 1991
[b]Described in Itzkowitz et al. 2001
[c]Described in Enquist et al. 1990

The first and second observational trials at the egg and wriggler stages were conducted within the first 24 hours after the laying of eggs and emergence of wrigglers, respectively. The first and second observational trials at the fry stage were conducted on day 2 of the fry stage (Lavery, 1995). All observations were taken in either a morning interval, 8:00 am to 11:00 am, or an evening interval, 5:00 pm and 8:00 pm. After all trials, the total number of surviving fry in each experimental tank was recorded.

*Statistical Analyses*

The distance from the brood was first calculated as a mean of the observed positions during each trial. Each position was assigned a value: “Brood” was 1, “Near” was 2, and “Far” was 3. The number of times the resident fish was found at each position was multiplied by the

value, and an average position was determined. The values for distance from the brood thus ranged between 1 and 3, with increasing values corresponding to increasing distance from the brood. These values were then analyzed using 3-way analysis of variance (ANOVA) and subsequent protected two- and one- way ANOVA (Day & Quinn, 1989) to disentangle the main and interactive effects of developmental stage (egg, wriggler, fry), intruder (present, absent), and testosterone (present, absent).

The observed behaviors were divided into two groups: parental care and aggression. Parental care consisted of the total number of parental care behaviors (fanning the brood, mouthing the brood, mouthing the substrate, fin digging, fry retrieval, guarding, observing) in each trial. Aggression consisted of the total number of aggressive behaviors (frontal display, lateral display, bites, tail beats) in each trial. Parental care and aggression were then analyzed using 3-way ANOVA and subsequent protected two- and one-way ANOVAs as described above.

The difference between the length of resident males and females and the differences between resident fish length and intruder length in each trial were analyzed using paired t-tests. The relationship between fish size (length of resident fish, length of intruder, relative length of resident male to intruder male) and distance from the brood, parental care, and aggression of resident fish was analyzed using linear regression.

The relationships between offspring characteristics (number of eggs, number of fry, percentage of offspring survival) and distance from the brood, parental care, and aggression of resident fish were analyzed using linear regression. An unpaired t-test was used to analyze the percentage of offspring survival between the testosterone and no-testosterone treatments.

All statistical analyses were conducted using StatView.

## RESULTS

Behavioral responses were grouped into three aggregates: distance from the brood, parental care, and aggression. The main and interactive effects of developmental stage (egg, wriggler, fry), intruder (presence, absence), and testosterone (presence, absence) are first presented for each behavioral response. Males and females differed significantly in each behavioral response, thus males and females were analyzed separately. A summary of these effects is provided at the end of the section.

The next section describes the differences between resident male and female length and resident fish and intruder length as well as the relationships between fish size (length of resident fish, length of intruder, relative length of resident male to intruder male) and parental behaviors. A summary of these relationships is provided at the end of the section.

The final section describes the relationships between offspring characteristics (number of eggs, number of fry) and parental behaviors, and conversely between parental behaviors and offspring survival. The effect of testosterone treatment on offspring survival is further described.

*Effects of Offspring Stage, Intruder, and Testosterone on Parental Behavior*

Distance from the Brood. Across all trials, males were located farther from the brood than females (one-way ANOVA main effect of sex $F_{1,162} = 122.4$; $P < 0$ .0001). Across offspring development, male distance from the brood decreased (three-way ANOVA main effect of stage $F_{2,70} = 5.9$; $P = 0.004$; Fig. 2). Males were found farther from the brood in the presence of an intruder than in the absence of an intruder (three-way ANOVA main effect of intruder $F_{1,70} = 52.2$; $P < 0.0001$) at every offspring stage (two-way ANOVA main effect of intruder at each stage; egg: $F_{1,24} = 28.6$; $P < 0.0001$; wriggler: $F_{1,24} = 43.7$; $P < 0.0001$; fry: $F_{1,22} = 4.7$; $P = 0.042$; Fig. 2). A stage x intruder interaction was not found (three-way ANOVA $P > 0.05$).

Testosterone-treated males were found farther from the brood than control-treated males (three-way ANOVA main effect of testosterone $F_{1,70} = 4.8$; $P = 0.03$; Fig. 2). Further analysis revealed this effect was apparent in the presence of an intruder (two-way ANOVA main effect of testosterone $F_{2,35} = 3.9$; $P = 0.029$), but only at the egg stage (one-way ANOVA main effect of testosterone $F_{1,19} = 4.5$; $P = 0.047$) and not at the wriggler and fry stages (one-way ANOVA main effect of testosterone, both $P > 0.05$; Fig. 3). In contrast, testosterone did not have an effect on male distance from the brood in the absence of an intruder (two-way ANOVA main effect of testosterone $F_{2,35} = 4.0$; $P = 0.3$). Neither an intruder x testosterone interaction nor a stage x testosterone interaction was found (three-way ANOVA, both $P > 0.05$).

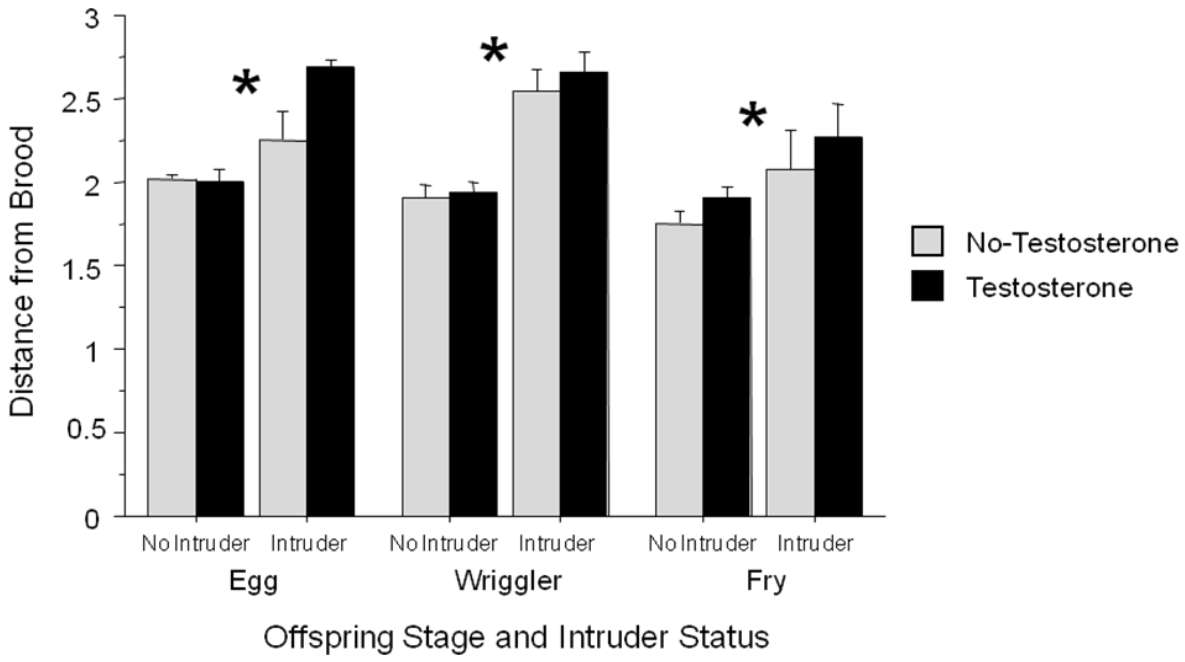



Fig. 2. Mean (± 1 S.E.) distance from the brood of resident males per trial. Asterisks (*) indicate a significant difference in distance from the brood between the no intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

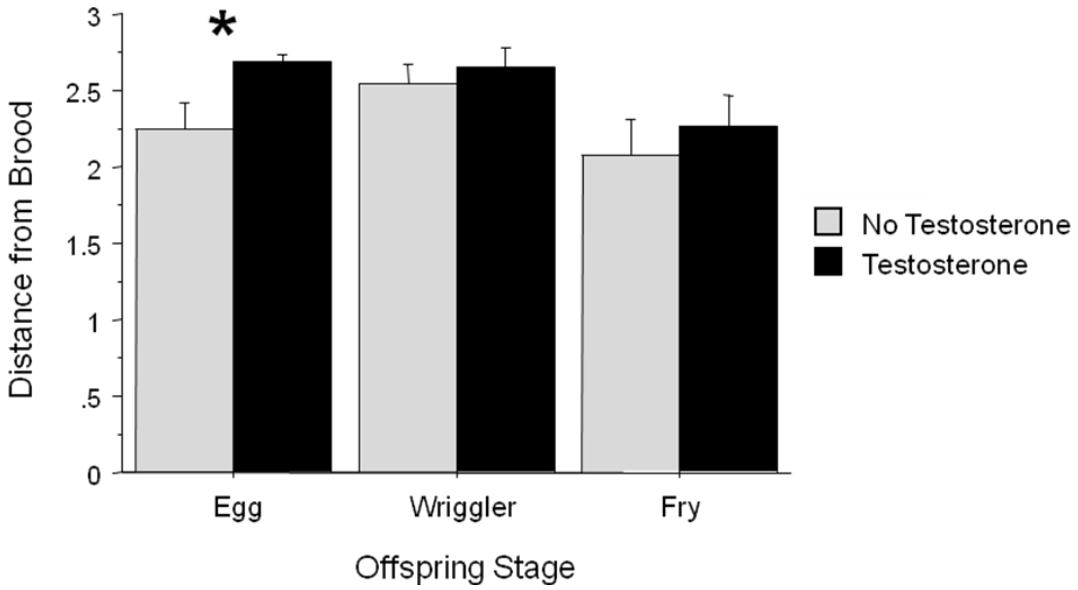



Fig. 3. Mean (± 1 S.E.) distance from brood of resident males per trial in the presence of an intruder. Asterisks (*) indicate a significant difference in distance from the brood between the no-testosterone and testosterone treatments within an offspring stage after ANOVA ($P < 0.05$).

Female distance from the brood increased as offspring developed from eggs into fry (three-way ANOVA main effect of stage $F_{2,70} = 6.3$; $P = 0.003$; Fig. 4). Further analysis revealed that this effect was apparent without an intruder (two-way ANOVA main effect of stage $F_{2,35} = 10.1$; $P = 0.0003$) and not with an intruder (two-way ANOVA main effect of stage $F_{2,35} = 1.9$; $P = 0.17$). Females were found farther from the brood when an intruder was present than when an intruder was absent (three-way ANOVA main effect of intruder $F_{1,70} = 8.7$; $P = 0.004$), but only at the egg stage (two-way ANOVA main effect of intruder $F_{1,26} = 4.8$; $P = 0.04$), and not at the wriggler and fry stages (two-way ANOVA main effect of intruder; both $P > 0.05$; Fig. 4). A stage x intruder interaction was not found (three-way ANOVA $P > 0.05$).

Female distance from the brood did not differ between the testosterone and no-testosterone treatments (three-way ANOVA main effect of testosterone $F_{1,70} = 0.6$; $P = 0.4$; Fig. 4). Neither a stage x testosterone interaction nor an intruder x testosterone interaction was found (three-way ANOVA, both $P > 0.05$).

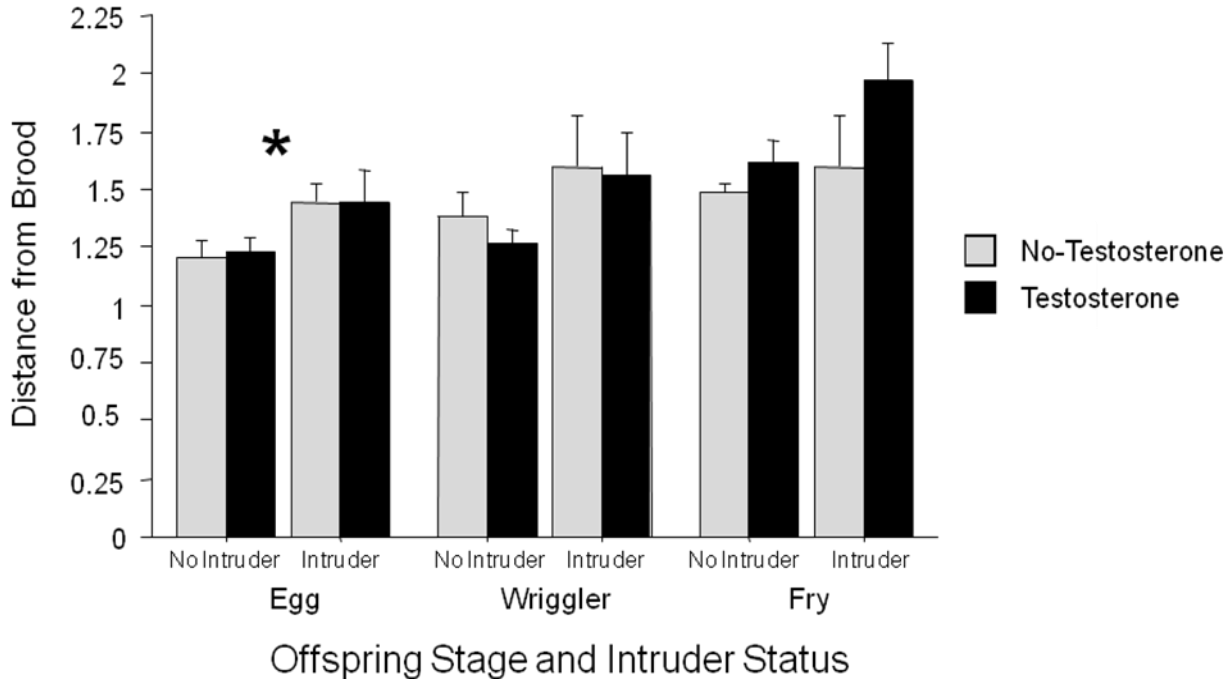



Fig. 4. Mean (± 1 S.E.) distance from the brood of resident females per trial. Asterisks (*) indicate a significant difference in distance from the brood between the no intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

Parental Care. Across all trials, males provided less parental care than females (one-way ANOVA main effect of sex $F_{1, 162} = 39.3$; $P < 0.0001$). Males provided less parental care in the presence of an intruder than the absence of an intruder (three-way ANOVA main effect of intruder $F_{1, 70} = 136.7$; $P < 0.0001$) at every offspring stage (two-way ANOVA main effect of intruder at each stage; egg: $F_{1,24} = 16.6$; $P = 0.0004$; wriggler: $F_{1,24} = 45.6$; $P < 0.0001$; fry: $F_{1,22} = 115.1$; $P < 0.0001$; Fig. 5).

Three-way analysis revealed no change in the level of male parental care as offspring developed from eggs to wrigglers to fry (three-way ANOVA main effect of intruder $F_{2,70} = 0.9$; $P = 0.43$). In the presence of an intruder, two-way analysis revealed that male parental care increased across offspring development (two-way ANOVA main effect of stage $F_{2, 35} = 4.9$; $P = 0.013$; Fig. 5). In contrast, in the absence of an intruder, two-way analysis revealed no change in male parental care across offspring development (two-way ANOVA main effect of stage $F_{1, 35} =$

0.6; $P = 0.6$), thus explaining the intruder x stage interaction (three-way ANOVA $F_{2, 70} = 4.0$; $P = 0.02$).

Testosterone-treated males provided less parental care than control-treated males (three-way ANOVA main effect of testosterone $F_{1, 70} = 4.0$; $P = 0.023$; Fig. 5). This effect was apparent in the presence of an intruder (two-way ANOVA main effect of testosterone $F_{1,35} = 9.7$; $P = 0.004$), but only at the egg stage (one-way ANOVA main effect of testosterone $F_{1, 19} = 7.8$; $P = 0.011$) and not at the wriggler and fry stages (one-way ANOVA main effect of testosterone, both $P > 0.05$; Fig. 6). In contrast, in the absence of an intruder, the level of parental care did not differ between testosterone-treated males and control-treated males (two-way ANOVA main effect of testosterone $F_{1, 35} = 0.9$; $P = 0.36$). Neither an intruder x testosterone interaction nor a stage x testosterone interaction was found (three-way ANOVA, both $P > 0.05$).

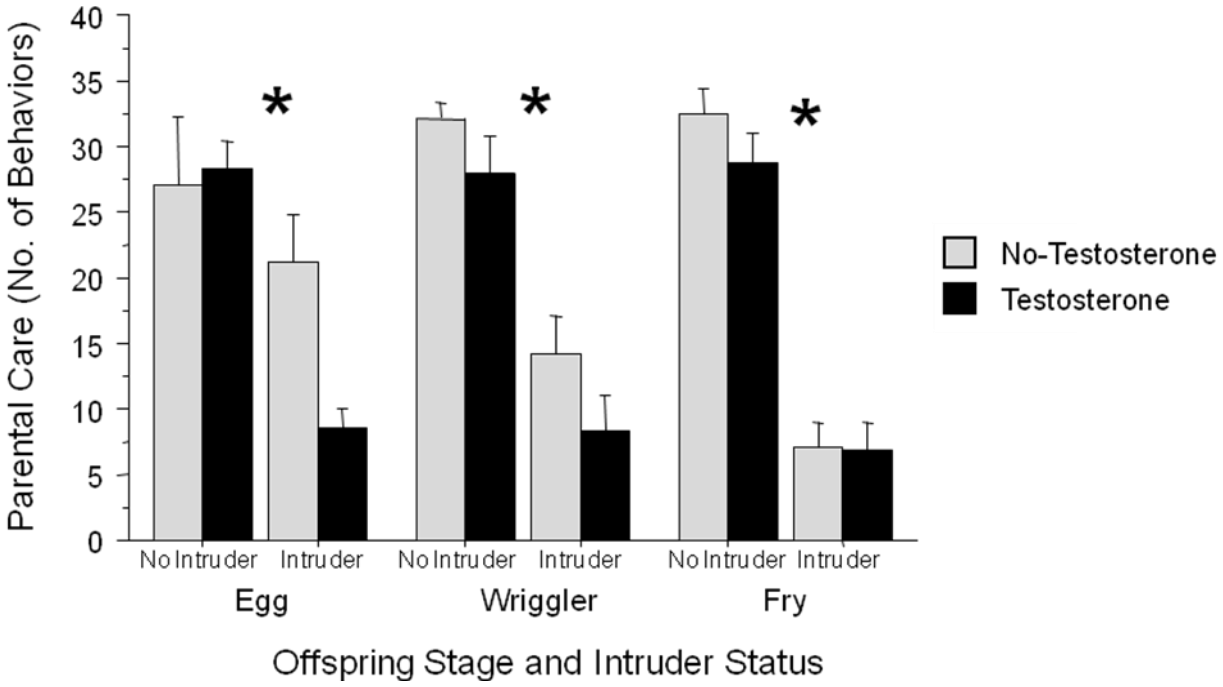



Fig. 5. Mean (± 1 S.E.) number of resident male parental care behaviors per trial. Asterisks (*) indicate a significant difference in parental care between the no-intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

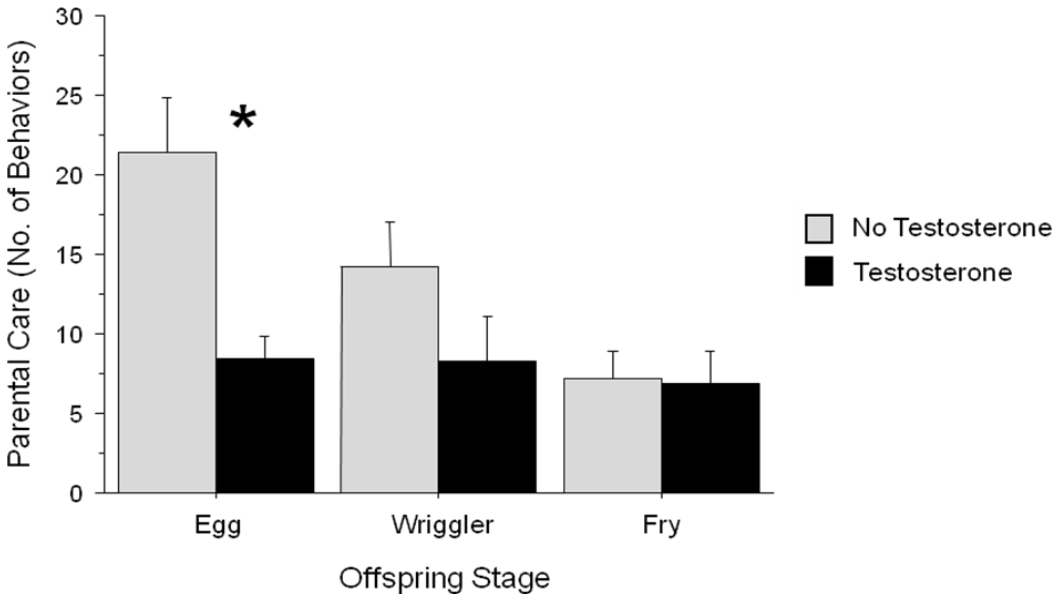



Fig. 6. Mean (± 1 S.E.) number of resident male parental care behaviors per trial in the presence of an intruder. Asterisks (*) indicate a significant difference in parental care between the no-testosterone and testosterone treatments within an offspring stage after ANOVA ($P < 0.05$).

The level of female parental care decreased across offspring development (three-way ANOVA main effect of stage $F_{2,70} = 24.2$; $P < 0.0001$; Fig. 7). This effect was more apparent with an intruder (two-way ANOVA main effect of stage $F_{2,38} = 20.5$; $P < 0.0001$) than without an intruder (two-way ANOVA main effect of stage $F_{2,38} = 4.0$; $P = 0.03$), thus accounting for the stage x intruder interaction (three-way ANOVA $F_{2, 70} = 8.8$; $P = 0.0004$; Fig. 7). Females provided less parental care in the presence of an intruder than in the absence of an intruder (three-way ANOVA main effect of intruder $F_{1, 70} = 60$; $P = 0.0004$) at every offspring stage (two-way ANOVA main effect of intruder at each stage; egg: $F_{1,26} = 9.9$; $P = 0.004$; wriggler: $F_{1,26} = 8.9$; $P = 0.006$; fry: $F_{1,24} = 48.6$; $P < 0.0001$; Fig. 7).

The level of female parental care did not differ between the testosterone and no-testosterone treatments (three-way ANOVA main effect of testosterone $F_{1, 70} = 1.6$; $P = 0.21$; Fig. 7). Neither a stage x testosterone interaction nor an intruder x testosterone interaction was found (three-way ANOVA two-way interactions, both $P > 0.05$).

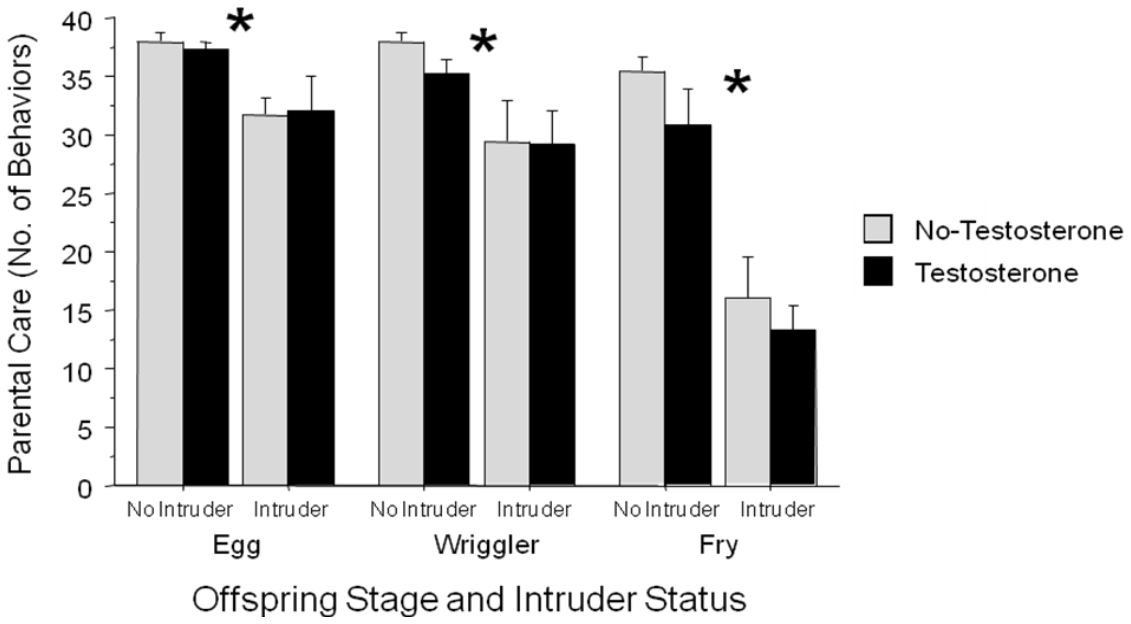



Fig. 7. Mean (± 1 S.E.) number of resident female parental care behaviors per trial. Asterisks (*) indicate a significant difference in parental care between no intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

Aggression. Males were more aggressive than females across all trials (one-way ANOVA main effect of sex $F_{1,162} = 14.1$; $P = 0.0002$). The level of male aggression was greater with an intruder than without an intruder (three-way ANOVA main effect of intruder $F_{1,70} = 390.9$; $P < 0.0001$) at every offspring stage (two-way ANOVA main effect of intruder; egg: $F_{1,24} = 84.4$; $P < 0.0001$; wriggler: $F_{1,24} = 115.1$; $P < 0.0001$; fry: $F_{1,22} = 221.3$; $P < 0.0001$; Fig. 8).

Male aggression increased across offspring development (three-way ANOVA main effect of stage $F_{2,70} = 9.1$; $P = 0.0003$; Fig. 8). Two-way analysis showed that this effect was apparent in the presence of an intruder (two-way ANOVA main effect of stage $F_{2,35} = 8.9$; $P = 0.0007$), but revealed no change in male aggression across offspring development in the absence of an intruder (two-way ANOVA main effect of stage $F_{2,35} = 0.1$; $P = 0.53$), thus explaining the stage x intruder interaction (three-way ANOVA $F_{2,70} = 5.2$; $P = 0.008$).

The level of male aggression was greater in the testosterone treatment than no-testosterone treatment (three-way ANOVA main effect of testosterone $F_{1,70} = 13.581$; $P = 0.0004$; Fig. 8). This effect was apparent in the presence of an intruder (two-way ANOVA main effect of testosterone $F_{1,35} = 10.3$; $P = 0.049$), but only at the egg stage (one-way ANOVA main effect of testosterone $F_{1,19} = 9.4$; $P = 0.006$) and not at the wriggler and fry stages (one-way ANOVA main effect of testosterone, both $P > 0.05$; Fig. 9), thus explaining the stage x testosterone interactions (three-way ANOVA $F_{2,70} = 5.2$; $P = 0.008$; two-way ANOVA $F_{2,35} = 3.3$; $P = 0.049$). In contrast, two-way analysis did not reveal a testosterone effect on male aggression in the absence of an intruder (two-way ANOVA main effect of testosterone $F_{1,35} = 3.3$; $P = 0.078$), thus explaining the marginally non-significant intruder by testosterone interaction (three-way ANOVA $F_{1,70} = 3.9$; $P = 0.0505$).

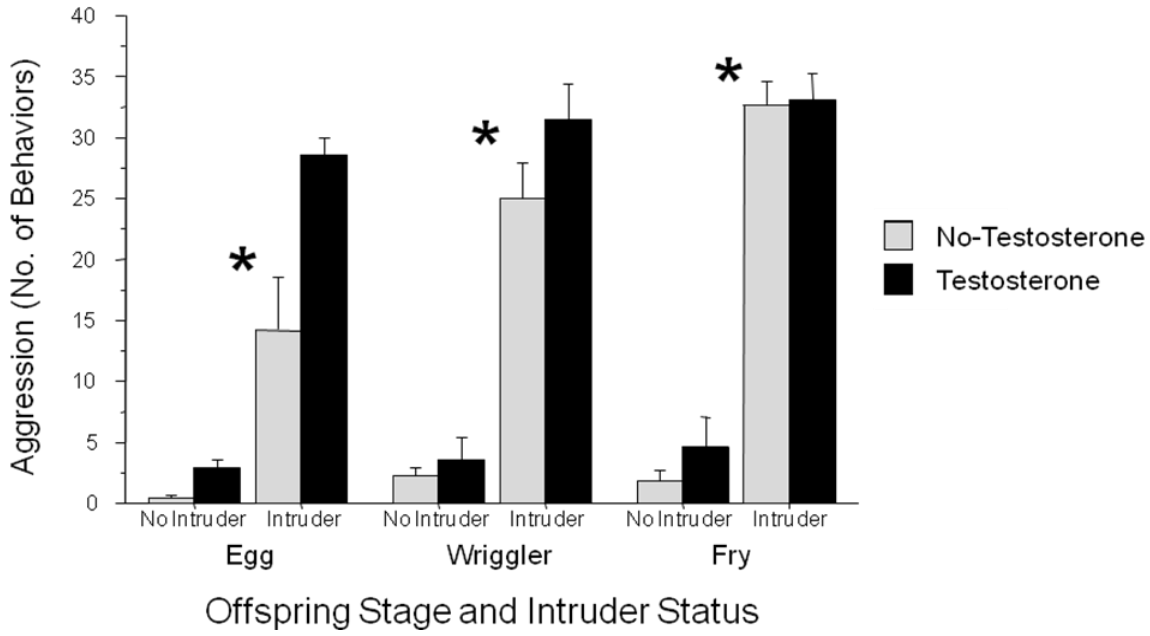



Fig. 8. Mean (± 1 S.E.) number of resident male aggressive behaviors per trial. Asterisks (*) indicate a significant difference in aggression between no intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

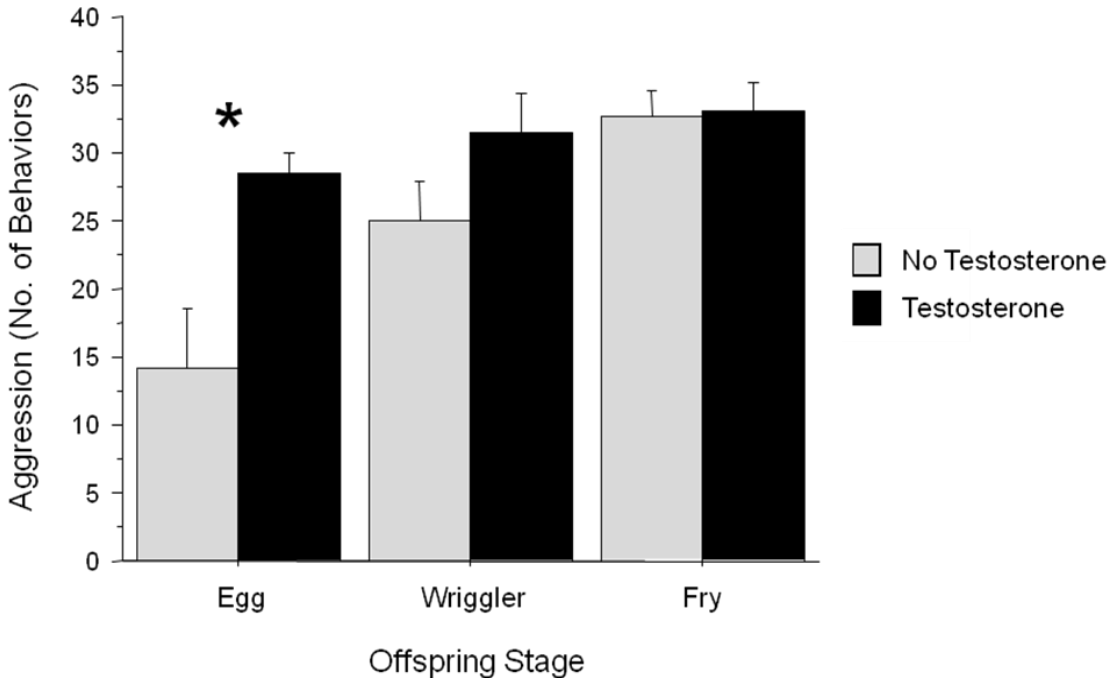



Fig. 9. Mean (± 1 S.E.) number of resident male aggressive behaviors per trial in the presence of an intruder. Asterisks (*) indicate a significant difference in aggression between the no-testosterone and testosterone treatments within an offspring stage after ANOVA ($P < 0.05$).

Females were more aggressive in the presence of an intruder than in the absence of an intruder (three-way ANOVA main effect of intruder $F_{1,70} = 97.9$; $P < 0.0001$) at every offspring stage (two-way ANOVA main effect of intruder at each stage; egg: $F_{1,26} = 13.9$; $P = 0.0009$; wriggler: $F_{1,26} = 17.2$; $P = 0.0003$; fry: $F_{1,24} = 81.6$; $P < 0.0001$; Fig. 10). Female aggression increased across offspring development (three-way ANOVA main effect of stage $F_{2,70} = 24.1$; $P < 0.0001$; Fig. 10). This increase was apparent in the presence of an intruder (two-way ANOVA main effect of stage $F_{2,35} = 21.9$; $P < 0.0001$). In contrast, two-way analysis revealed no change in female aggression across developmental stages in the absence of an intruder (one-way ANOVA main effect of stage $F_{2,35} = 2.4$; $P = 0.10$), thus explaining the stage x intruder interaction (three-way ANOVA $F_{2,70} = 13.7$; $P < 0.0001$).

The level of female aggression did not differ between the testosterone treatment and no-testosterone treatments (three-way ANOVA main effect of testosterone $F_{1,70} = 0.9$; $P = 0.36$; Fig.

10). Neither a stage x testosterone interaction nor an intruder x testosterone interaction was found (three-way ANOVA, both $P > 0.05$).

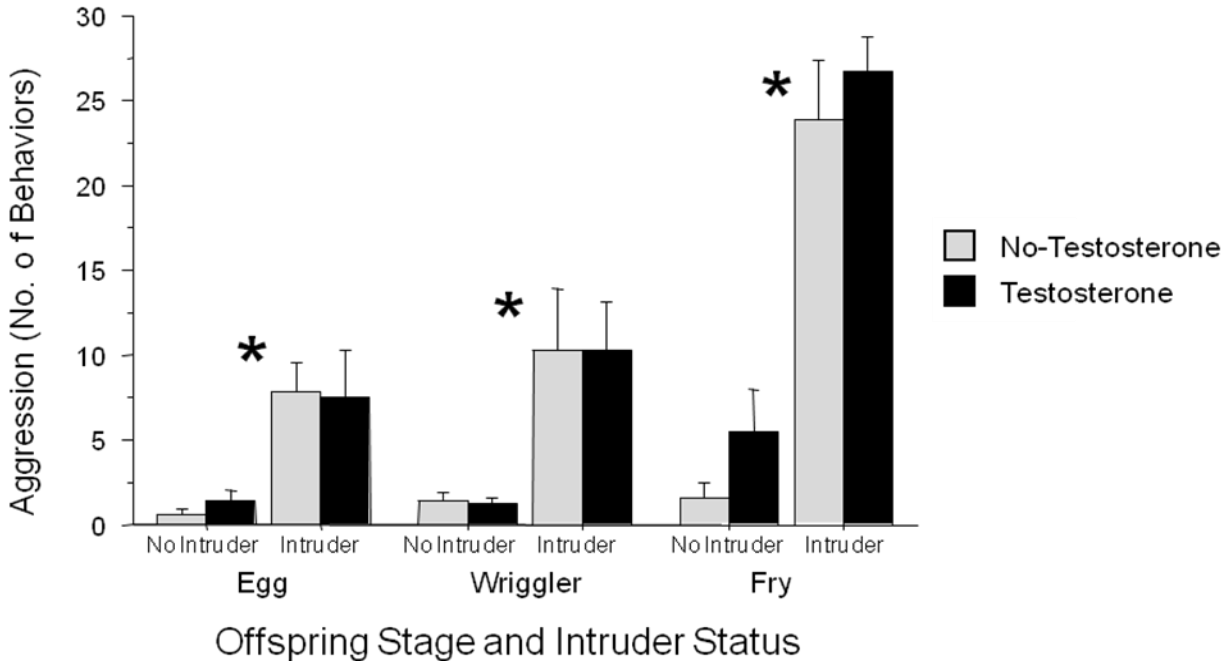



Fig. 10. Mean (± 1 S.E.) number of resident female aggressive behaviors per trial. Asterisks (*) indicate a significant difference in aggression between no intruder and intruder treatments within an offspring stage after ANOVA ($P < 0.05$).

Summary of Behavioral Responses. The following tables summarize the differences between sexes in behavioral responses (Table 3) and the main and interactive effects of offspring stage, intruder, and testosterone on behavioral responses of resident males (Table 4) and resident females (Table 5).

Table 3. Differences between resident males and females in behavioral responses. Two asterisks (**) indicate $P < 0.005$ after ANOVA.

| | Behavioral Responses | | |
|---|---|---|---|
| | Mean Distance from Brood | Parental Care | Aggression |
| Males vs. Females | ** Males > Females | ** Males < Females | ** Males > Females |

Table 4. Summary of three-way ANOVA main and interactive effects on male behavioral responses. One asterisk (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.005$, and N.S. indicates $P > 0.05$ after ANOVA.

| | Behavioral Response | | |
|---|---|---|---|
| | Distance from the Brood | Parental Care | Aggression |
| Main Effects | | | |
| Offspring Stage | ** decrease | N.S. | ** increase |
| Intruder | ** increase at every offspring stage | ** decrease at every offspring stage | ** increase at every offspring stage |
| Testosterone | * increase only at egg stage with intruder | * decrease only at egg stage with intruder | * increase only at egg stage with intruder |
| Interactions | | | |
| Stage x Intruder | N.S. | *decrease with intruder<br>N.S. without intruder | * increase with intruder<br>N.S. without intruder |
| Stage x Testosterone | N.S. | N.S. | N.S. (marginally) |
| Intruder x Testosterone | N.S. | N.S. | N.S. |

Table 5. Summary of three-way ANOVA main and interactive effects on female behavioral responses. One asterisk (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.005$, and N.S. indicates $P > 0.05$ after ANOVA.

| | Response Variables | | |
|---|---|---|---|
| | Distance from the Brood | Parental Care | Aggression |
| Main Effects | | | |
| Offspring Stage | ** increase without intruder | ** decrease | ** increase |
| Intruder | ** increase only at egg stage | ** decrease at every offspring stage | ** increase at every offspring stage |
| Testosterone | N.S. | N.S. | N.S. |
| Interactions | | | |
| Stage x Intruder | N.S. | ** decrease with intruder<br>* decrease without intruder | ** increase with intruder<br>N.S. without intruder |
| Stage x Testosterone | N.S. | N.S. | N.S. |
| Intruder x Testosterone | N.S. | N.S. | N.S. |

*Relationships Between Relative Fish Size and Parental Behaviors*

Length of Resident Fish. Males were larger than females within resident pairs (paired t-test; T = 5.3; $P = 0.0001$). Distance from the brood, parental care and aggression of resident males did not vary with the length of resident males (all $P > 0.05$). Distance from the brood, parental care, and aggression of resident females did not vary with the length of resident females (all $P > 0.05$).

Length of Intruder. Resident males were larger (paired t-test; T = 4.3; $P < 0.0001$) and resident females were smaller (paired t-test; T = - 4.015; $P = 0.0003$) than intruder males. The distance resident fish were found from the brood was not related to the length of the intruder ($F_{1,81} = 1.5$; $P = 0.23$). However, resident fish parental care decreased ($F_{1,81} = 4.6$; $P = 0.035$) and resident fish aggression increased ($F_{1,81} = 5.8$; $P = 0.02$) as intruder length increased. Further analysis revealed these relationships in resident males only. Thus, the distance resident males were found from the brood ($F_{1,40} = 0.5$; $P = 0.49$) was not related to intruder length, but resident male parental care decreased ($F_{1,40} = 17.6$; $P = 0.0002$; Fig. 11a) and resident male aggression increased ($F_{1,40} = 15.8$; $P = 0.0003$; Fig. 11b) with increasing intruder length. Female parental behaviors did not vary with intruder length (all $P > 0.05$).

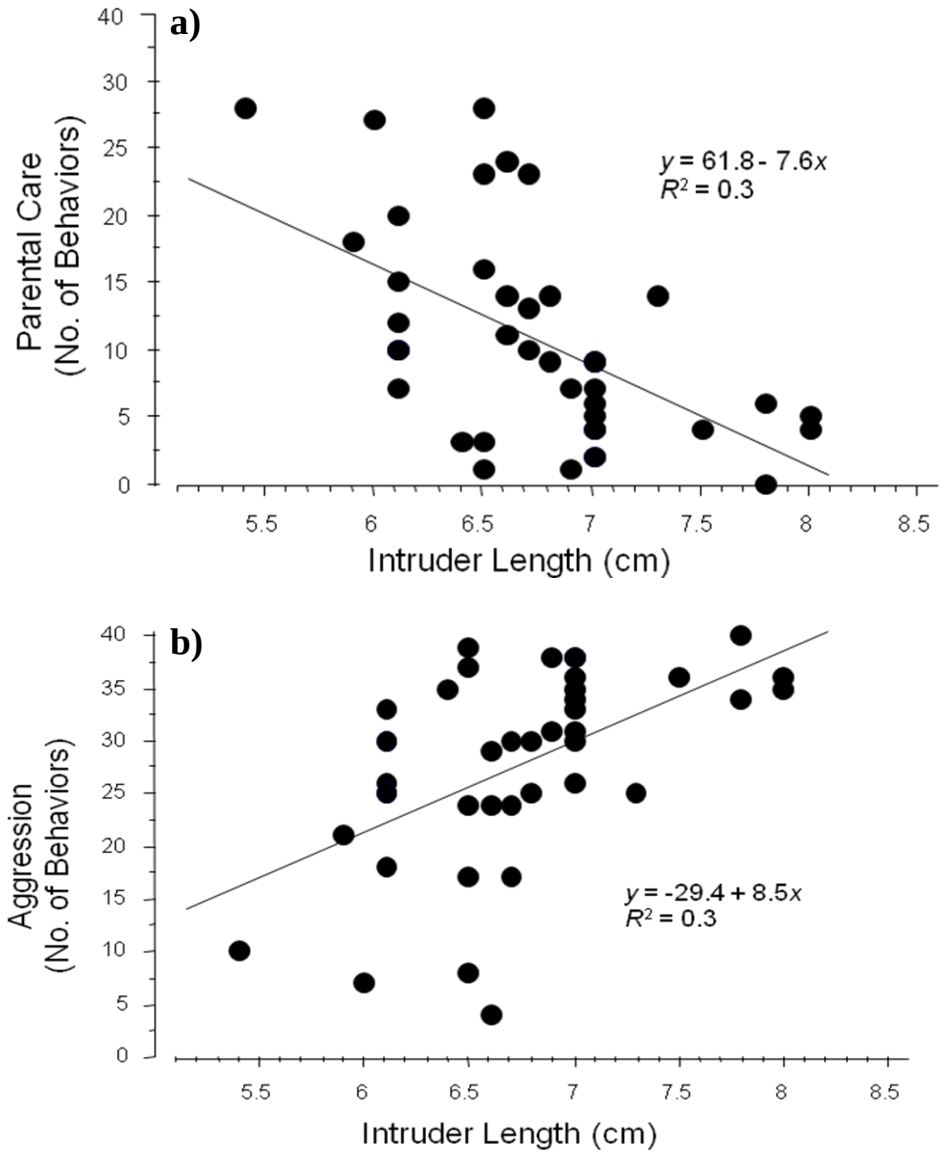



Fig. 11. Relationship between intruder length (cm) and the number of resident male a) parental care behaviors and b) aggressive behaviors. Each data point represents the number of behaviors observed in one trial with an intruder.

Relative Length of Resident Male to Intruder Male. To further examine the relationship between intruder length and resident male behavior, I examined the relationship between the *relative* length of resident males to intruder males and resident male behavior. The distance that resident males were found from the brood was not related to the relative size of resident males to intruder males ($P > 0.05$). However, resident male parental care increased ($P = 0.011$; Fig. 12a) and

resident male aggression decreased ($P$ = 0.004; Fig. 12b) as the relative size of resident males to intruder males increased.

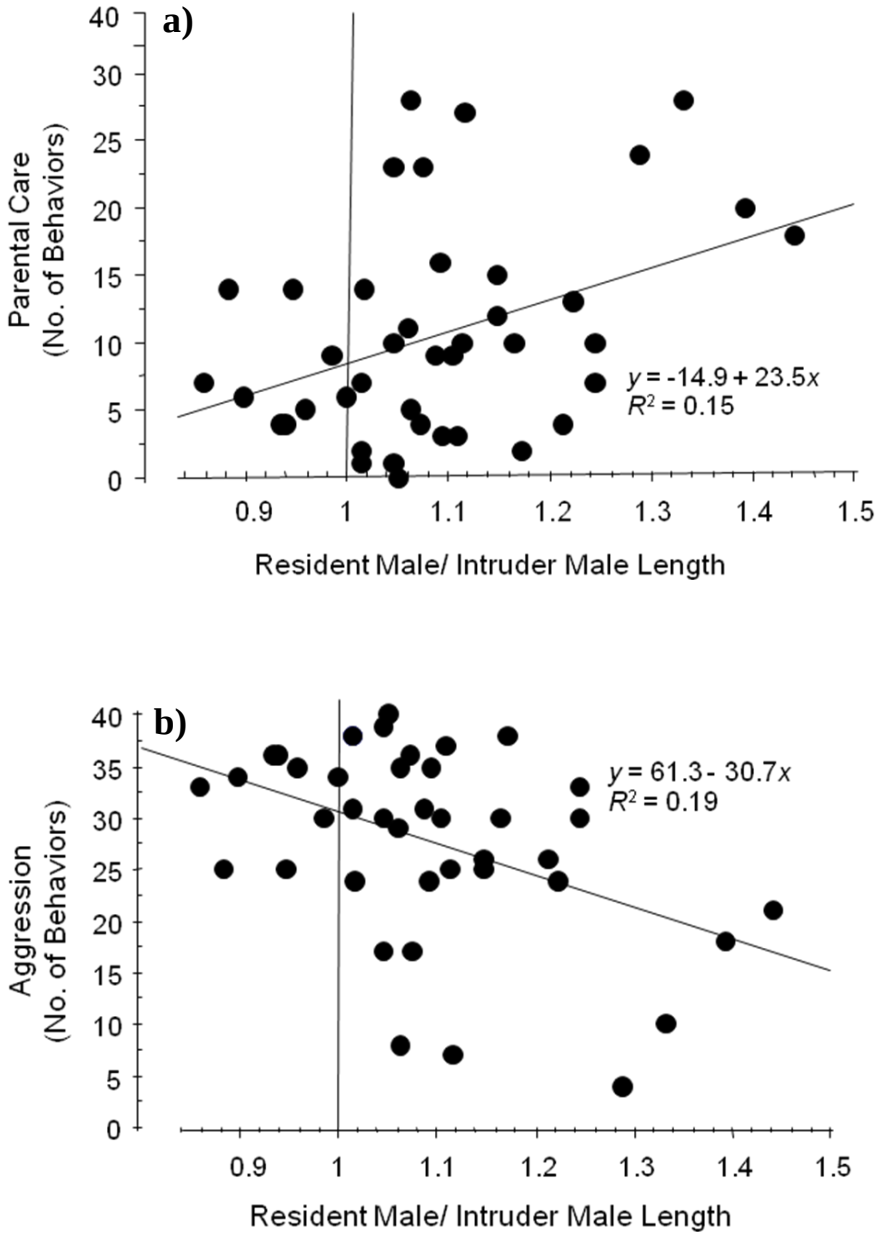



Fig. 12. The relationship between the relative length of resident males to intruder males and the number of resident male a) parental care behaviors and b) aggressive behaviors. Each data point represents the number of behaviors observed in one trial with an intruder. Vertical lines indicate the point at which resident male length equaled intruder male length.

Summary. The following table summarizes the relationships between the lengths of resident and intruder fish and parental behaviors (Table 6). Since most variables did not affect males and females differently, one table was used to summarize the data for both sexes.

Table 6. The relationships between absolute and relative resident and intruder fish length and resident fish behavioral responses. One asterisk (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.005$, and N.S. indicates $P > 0.05$ after ANOVA

| | Behavioral Responses | | |
|---|---|---|---|
| Fish Length | Distance from the Brood | Parental Care | Aggression |
| Length of Resident Male | N.S. | N.S. | N.S. |
| Length of Resident Female | N.S. | N.S. | N.S. |
| Length of Intruder | N.S. | * negative relationship<br>*Males<br>N.S. Females | * positive relationship<br>*Males<br>N.S. Females |
| Relative Length of Resident Male to Intruder | N.S. | * positive relationship (male only analysis) | ** negative relationship (male only analysis) |

*Relationships Between Offspring Characteristics and Parental Behaviors*

Number of Eggs. The distance resident fish were found from the brood, the level of parental care, and the level of aggression of resident fish did not vary with the number of eggs in a brood (all $P > 0.05$). Resident male behaviors were not related to the number of eggs in a brood (all $P > 0.05$), and resident female behaviors were not related to the number of eggs in a brood (all $P > 0.05$).

Number of Fry. The distance resident fish were found from the brood, resident fish parental care, and resident fish aggression was not related to the number of fry in a brood (all $P > 0.05$). Resident male behaviors were not related to the number of fry in a brood (all $P > 0.05$), and resident female behaviors were not related to the number of fry in a brood (all $P > 0.05$).

Percentage Survival of Offspring. The percentage survival of offpsring was not related to parental distance from the brood, resident fish parental care, or resident fish aggression ($P >$

0.05). The percentage of offspring survival did not differ between the testosterone and no-testosterone treatments (unpaired T-test; T = -1.771; $P = 0.10$).

## DISCUSSION

Testosterone exposure elicited different behavioral responses in male and female convict cichlids. Testosterone-treated males, in the presence of an intruder, were located farther from the brood, provided decreased levels of parental care, and exhibited increased levels of aggression in comparison to males in the control treatment. These behaviors were most apparent during the egg stage of offspring development. Females did not exhibit such differences. Thus, only my results for male convict cichlid behavior are consistent with the predictions of the "challenge hypothesis." I will first discuss how sex-specific roles in parental care could have influenced this sex-specific testosterone effect, and then examine how stage-specific parental investment could have influenced the observed stage-specific effect of testosterone. The role of the intruder in the trade-off between convict cichlid parental care and aggression will further be discussed. I will synthesize these results in the context of the "challenge hypothesis," adding to the framework of diverse parental care systems. Lastly, the environmental consequences of exogenous testosterone on fish behavior will be addressed in relation to my findings.

### *Convict Cichlid Parental Care Strategies and Sex-Specific Testosterone Effect*

In this study, I found that male convict cichlids were located farther from the brood, provided less parental care, and exhibited more aggression than female convict cichlids across all trials. These sex-specific behavioral responses are likely influenced by the division and flexibility of parental care duties between substrate-brooding parents. The parental care strategy of convict cichlids could have thus influenced the observed sex-specific testosterone effect because of the different responsibilities of resident males and females.

In natural populations of convict cichlids (Keenleyside et al., 1990; Alonzo et al., 2001), parental care duties are often divided by sex; i.e. females engage in brood maintenance, mouthing and fanning the brood, whereas males engage in brood defense and repel intruders (Barlow, 1984). These sex-specific parental behaviors lead to sex-specific parental locations. One parent, usually the female, remains close to the brood while the other parent, usually the male, is free-swimming (Keenleyside, 1991). Not only was this pattern found in my study, but similar results are observed in convict cichlids in experimental aquaria (Itzkowitz et al., 2003; Itzkowitz et al., 2005; Snekser et al., 2011).

This parental care strategy is enforced by reciprocal pressures between males and females of resident pairs. Female convict cichlids limit the time of males with offspring (Itzkowitz et al., 2003) and consequently have unlimited access to the brood (Wisenden, 1995). Males could be more influential than females in enforcing the separation of brood maintenance and brood defense (Itzkowitz et al., 2003). In experimental aquaria, males are more aggressive toward female residents located farther from the brood than those located closer to offspring (Itzkowitz et al., 2003). In open habitats, males limit the time females spend away from the brood by aggressively chasing them back toward their young (Wisenden, 1995). As observed in my study, females were thus pressured to provide brood maintenance and males were pressured to patrol the territory.

The link between location and behavior was made further apparent in my study through recorded parental distance from the brood. Males were not only found farther from the brood, but also provided less parental care than females, suggesting that distance from the brood and levels of parental care are negatively related. In field populations, male movement toward the brood is similarly attributed to increased male parental care (Snekser et al., 2011). The locations of

parents can also influence parental care strategies within a specific behavior (Keenleyside, 1991). For example, females retrieve wrigglers that are displaced close to the nest, whereas males retrieve wrigglers that are displaced farther from the nest (Snekser & Itzkowitz, 2009).

The role of male convict cichlids during parental care could have influenced the sex-specific testosterone effect found in my study. Because brood defense is an inherently aggressive behavior (Barlow, 1984; Gross & Sargent, 1985), increased testosterone levels and related aggression could be more beneficial for males than females. As the free-swimming parent (Keenleyside, 1991), males are also more likely than females to encounter potential mates and invest in new broods (Keenleyside, 1991; Snekser et al., 2011). Males are consequently at a decreased loss in the case of brood failure (Wisenden et al., 2008). The reduced cost of increased male aggression relative to female aggression in terms of parental care could have influenced testosterone-treated males but not females to act more aggressively.

Parental care duties of convict cichlids are still flexible despite this sex-typical division of labor. Males and females can adopt the parental care responsibilities of their mates if circumstances require (Itzkowitz et al., 2001; Itzkowitz et al., 2003). This tactic is considered an evolutionary stable strategy to a maximum point that is lower than full compensation, depending on the effort provided by each parent (Houston & Davies, 1985). Specifically, if one parent decreases parental effort, the other parent increases its effort to a degree, minimizing but never fully compensating for the parental care loss (Harrison et al., 2009). Other vertebrates exhibit this strategy as well (O'Neal et al., 2008).

Female convict cichlids are the more flexible partner of the resident pair in adopting parental duties; i.e., females assist in male duties more often than males assist in female duties (Itzkowitz et al., 2005). Thus, brood defense during the egg stage is not exclusively male-biased

(Keenleyside, 1991). Females are less aggressive than males during the egg and wriggler stages in only two out of nine comparisons of parental roles (see review in Keenleyside, 1991). After brood loss, widowed males leave offspring more often than widowed females, suggesting males are less able than females to assume complete responsibility of parental care (Itzkowitz et al., 2001). Males, however, are still capable of adopting female parental duties (Wisenden, 1995; Itzkowitz et al., 2001), and will spend an increased time with offspring when females are occupied chasing an intruder (Itzkowitz et al., 2005).

The greater flexibility of females compared to males could have influenced the sex-specific testosterone effect in my study. Testosterone might have affected male behavioral responses because females can compensate for the decrease in parental care better than males. If females would have exhibited such a decrease in parental care, males might not have compensated to the same extent. Broods are thus more likely to succeed with decreased paternal effort than decreased maternal effort (Wisenden, 2008). The trade-off between increased aggression and offspring survival is seemingly less costly to reproductive success in males than females. This parental care strategy could have contributed to the observed testosterone effect in males only.

*Offspring Stage Influences Aggression, Parental Care, and Testosterone Effects*

With an intruder present, I found that males and females exhibited an increase in aggression and decrease in parental care across offspring stages. I also found that female convict cichlids moved farther from the brood whereas male convict cichlids moved closer to the brood during offspring development. These behavioral responses suggest that parental care strategies in convict cichlids are modified according to the demands of offspring (Snekser et al., 2011). Because these demands differ with offspring age, it is likely that testosterone treatment elicits

different levels of aggressive and parental care behaviors in males with younger offspring than older offspring.

Stage-specific parental behavior is typical of many animals (Sargent & Gross, 1985) and can be explained by increases in the reproductive value and vulnerability of older offspring (Barash, 1975; Harvey & Greenwood, 1978). The differences in male and female contributions to offspring are a significant determinant of the level of parental investment and could also lead to stage-specific parental behaviors (Trivers, 1972). These stage-specific changes in parental care and aggression could have thus influenced the stage-specific effect of exogenous testosterone in male convict cichlids of my study.

Brood Defense Increases Across Offspring Development. I found that both males and females were more aggressive with older offspring than younger offspring in the presence of an intruder. This increase is found in natural populations of cichlids (Alonzo et al., 2001; McKaye & Murry, 2008), and in males and females of experimental aquaria (Lavery & Colgan, 1991). In fish, anti-predator defense is an inherently agonistic behavior and is thought to have evolved from territory defense (Gross & Sargent, 1985). An increase in aggression, as an aspect of brood defense, across offspring development is predicted by the vulnerability hypothesis (Harvey & Greenwood, 1978) and reproductive hypothesis (Barash, 1975)

The vulnerability hypothesis states that brood defense increases with offspring age because older offspring are often more conspicuous targets for predators (Harvey & Greenwood, 1978). Convict cichlid juveniles are immobile as eggs and wrigglers (Keenleyside et al., 1990), but as free-swimming fry, are no longer confined to the brood site. Predation is considered to be most intense during this stage of development (McKaye & Barlow, 1976). The resident pair

could have therefore increased aggressive defenses more during the fry stage than earlier stages to occupy intruders and protect young.

The increase I found in aggression of both sexes across offspring development also supports the reproductive value hypothesis (Snekser et al., 2011), which states brood defense increases across offspring development because older offspring are of greater reproductive value and have a higher probability of surviving to breeding age (Barash, 1975; Montgomerie & Weatherhead, 1988; Tryjanowski & Golawski, 2004). Because fry are the closest to reaching adulthood among offspring stages, they are consequently of greatest reproductive value to parents (Snekser et al., 2011). This often leads parents to endure costly risks when defending fry (Lavery & Colgan, 1991; Snekser et al., 2011). Male and female convict cichlids in this study could have been more aggressive during the fry stage than earlier stages because increased defense would lead to greater benefits for their personal reproductive fitness.

The decreased reproductive value of younger offspring might account for the stage-specific testosterone effect found in my study (Barash, 1975). Testosterone-treated males could have traveled farther from the brood, exhibited less parental care, and exhibited more aggression during the egg stage than later stages because offspring must still survive high periods of predation before they contribute to parental fitness. In cichlids, males consequently abandon young more during the egg stage than later stages (McKaye & Barlow, 1976; Snekser et al., 2011). This suggests that the cost of increased aggression in males is lowest at the egg stage because these offspring contribute the least to parental fitness, prompting testosterone-treated males to allocate parental care resources toward aggression.

Offspring Demands Cause Shifts in Parental Investment. I found that male convict cichlids increased parental care whereas female convict cichlids decreased parental care as offspring

developed. These opposing trends could be a result of shifts in the level of male and female parental investment during offspring development. Males and females invest different levels of energy and resources in parental care depending on the age of the offspring (Keenleyside, 1991; Snekser et al., 2011), and these differences could have contributed to both the sex- and stage-specific effect of testosterone found in my study.

The biparental care of convict cichlids is uncommon in teleosts, and its evolution is not completely understood (Gross & Sargent, 1985; Keenleyside 1991). In terms of initial reproductive investment, cichlid sperm are not as energetically expensive to produce as eggs. A male often fertilizes a female's entire batch of eggs at little cost (Turner, 1993). Male reproductive success is seemingly dependent on female reproductive success, whereas female reproductive success is dependent upon producing eggs (Trivers, 1972; Snekser et al., 2011). When broods are experimentally removed, female convict cichlids maintain a constant level of investment in searching the abandoned area for young, whereas males spend an increased time away from the abandoned brood site (Wisenden et al., 2008). The higher parental investment of females relative to males in brood development could thus play a large role in the sex-specific effect of exogenous testosterone found in convict cichlids of my study.

The levels of male and female parental investment in convict cichlids become increasingly equal across offspring development. During the egg and wriggler stages, female cichlids are more involved in parental care than males (Keenleyside, 1991; Snekser et al., 2011). During the fry stage however, the importance of parental duties shifts from brood maintenance to brood defense (Keenleyside, 1991). Increased brood defense leads to increased male parental investment since males engage in this duty most often. However, females participate in brood defense as well (Keenleyside, 1991), and thus levels of parental investment during the fry stage

are more equal than at earlier offspring stages (Keenleyside et al., 1991; Snekser et al., 2011). This pattern is reflected in my results; female distance from the brood increased whereas male distance from the brood simultaneously decreased across offspring development.

The difference in initial parental investment between male and female convict cichlids could have contributed to the stage-specific testosterone effect in resident males of my study. Since males dedicate the least amount of resources immediately after fertilization (Trivers, 1972; Keenleyside, 1991), the devotion of parental care effort toward other interests during early offspring stages is not as energetically costly as during later offspring stages. Females provide much of the necessary parental care at the egg stage, suggesting that male parental care is not essential for brood success at early periods of offspring development (Snekser et al., 2011). Thus, testosterone could have elicited decreased male parental care only at the egg stage because reproductive success is not as dependent on male parental investment during early offspring stages as later stages.

*Role of Intruder in Trade-off Between Parental Care and Aggression*

In my study, I found that the presence of an intruder led to an increase in resident fish distance from the brood, decrease in resident fish parental care, and an increase in resident fish aggression. The observed increase in aggression and corresponding decrease in parental care in experimental aquaria suggested that the intruder was a threat to territory and offspring. Resident pairs were forced to divide time and parental care resources between the brood and intruder (Trivers, 1972). This allocation provides insight into the trade-off between parental care and aggressive behaviors of individual animals. For example, convict cichlids that participate more in brood provisioning are less aggressive toward novel fish than males that concentrate more on brood defense (Budaev et al., 1999).

Parental care strategies appear to depend on the presence of an intruder (Itzkowitz et al., 2001). In the absence of an intruder, parents will adjust parental care roles to ensure that one parent is present at the brood (Itzkowitz et al., 2001). However, in the presence of an intruder, male and female convict cichlids change location and parental care duties only in support of their mates; i.e. males will provide brood maintenance only if the female is present at the offspring and females will attack intruders only to aid males in brood defense (Itzkowitz et al., 2001). In my study, both males and females were found farther from the brood and were more aggressive when an intruder was present. Because males were located farther from the brood than females regardless of an intruder, these changes suggest that resident females engaged in brood defense only in support of resident males. The subordinate role of female convict cichlids in offspring defense could have further influenced the male-specific testosterone effect found in this study.

Parental care and aggression in convict cichlids could further depend on the size of resident fish (Itzkowitz et al., 2005; Awata & Kohda, 2004). In my study, males were significantly larger (total length) than females as is typical of pairs in nature (Wisenden, 1995). This size advantage is suggested to facilitate male's role in aggressive behaviors (Itzkowitz & Draud, 1992; Keeley & Grant, 1993) and influence parental care strategies. Convict cichlid pairs with large males encounter fewer intruders than those with small males (Gagliardi-Seeley & Itzkowitz, 2006), suggesting a size advantage in territory defense. In contrast to these studies, I found that parental care and aggressive behaviors of resident fish were not related to the size of the resident male or female.

In my study, it appeared that the size of the intruder was an important determinant in the trade-off between parental care and aggression. Resident females were smaller than the intruders, and neither female aggression nor parental care was related to intruder size. In Texas cichlids

(*Herichthys cyanoguttatum)*, reproductive condition is more important than body size in female-female aggression (Draud et al., 2004). Given that females in my study had already acquired the necessary resources for the production and maintenance of eggs, they could have responded less aggressively to territorial intrusions (Draud et al., 2004). In contrast, I found that as intruder length increased, male parental care decreased and aggression increased. The parental behaviors of males thus appeared to be more responsive than females to intruder characteristics. This increased responsiveness could have led to a pronounced trade-off between aggression and parental care in testosterone-treated males but not females.

Interestingly, I found that as the size of resident males increased relative to that of the intruder, male aggression decreased and parental care increased in contrast to previous studies (Itzkowitz et al., 2005). One explanation for this observed decline in aggression is that large resident males do not view intruders to be a significant threat. They invest in parental care instead of anti-predator aggression. Larger males might also attack less frequently but with more aggression than smaller males, as observed in Texas cichlids (*Hericthys cyanoguttatum;* Draud et al., 2004). Thus, the number of aggressive behaviors might not be the best measure of total aggression (Keenleyside, 1991). In my study, it appeared that relative size to the intruder rather than absolute size of resident males was more important in determining how resident males negotiated the trade-off between brood care and brood defense.

*The "Challenge Hypothesis:"Exogenous Testosterone and Parental Care*

Although there has been considerable research on the effects of *endogenous* testosterone levels on parental care (see review in Hirschenhauser & Oliveira, 2006), the effects of *exogenous* testosterone in vertebrates is less well-studied. Exogenous hormone manipulation could be more

useful than endogenous hormone responses in deciphering causal relationships in reproductive and parental care behaviors in animals (Wingfield et al., 1990).

Endogenous hormone responses during parental care should depend on the type of mating system (monogamous vs. polygamous) according to the "challenge hypothesis" (Wingfield et al., 1990). However, meta-analysis reveals that the effects of exogenous testosterone could depend more on the level of parental investment than on effort spent finding additional mates (see review in Hirschenhauser & Oliveira, 2006). For example, in the great tit (*Parus major)*, the more involved a male is in parental care, the less likely exogenous androgens will suppress that paternal care (Van Duyse et al., 2000). In my study, exogenous hormone manipulation was useful not only in determining the trade-off between parental care and aggression within sexes, but also provided insight into the relationship between maternal and paternal investment in fish that exhibit biparental care.

The predictions of the "challenge hypothesis" originate from observations of monogamous male birds (Wingfield et al., 1990), and have since been extended to a variety of animals (Townsend et al., 1991; Clark & Galef, 1999; Storey et al., 2006). The animal behavior observed in response to exogenous androgens in these studies is often species-specific (see review in Hirschenhauser & Oliveira, 2006; Storey et al., 2006). The effects of exogenous testosterone on parental care of females and males in avian and non-avian species provides a broad test of the "challenge hypothesis" that goes beyond results for any taxa. My findings will further add to this body of literature.

Exogenous Androgens and Female Behavior. There is less evidence for the role of exogenous testosterone on female than male parental care behavior. Exogenous testosterone reduces reproductive and parental care in female birds, specifically in red-winged blackbirds, (Searcy,

1988), spotless starlings, (Veiga et al., 2004), and dark-eyed juncos (Zysling et al., 2006). It is suggested that the phenotypic effect of exogenous testosterone might be more similar for male and female birds than previously thought (Zysling et al., 2006).

In contrast, the literature for non-avian taxa does not support an inhibitory effect of exogenous androgens on female parental care. In young human females, testosterone administration is correlated with heightened activation of the thalamocingulate region, insula, and the cerebellum in response to the cry of an infant, thus upregulating parental care (Bos et al., 2010). Studies involving female fish suggest that lower testosterone levels are not essential during parental care phases (Oliveira et al., 2002). In female blue gourami, *Trichogaster trichopterus* (Pisces, Belontiidae), methyltestosterone implantation facilitates an increase in parental care behaviors when tending to a large brood (Kramer, 1972).

Exogenous Androgens and Male Behavior. In male birds, elevated testosterone levels decrease reproductive success (Silverin, 1980; Hegner & Wingfield, 1987), increase sexual behavior (Ketterson & Nolan, 1992), and increase territorial aggression (Chandler et al., 1994), providing fundamental support for the trade-off predicted by the "challenge hypothesis" (Wingfield et al., 1990). The negative effects of exogenous testosterone on other measures of male parental care, including spatial activity (Moss et al., 2004), territory vigilance (Peters, 2002), and paternal incubation (Oring et al., 1989; De Ridder et al., 2000; Alonso-Alvarez, 2001; Van Roo, 2004), have also been studied.

There are only a few studies involving the effects of exogenous androgens in nonavian species (see review in Hirschenhauser & Oliveira, 2006). The effects of elevated testosterone levels on parental care have been examined in mammals and amphibians, and these studies are less consistent than those conducted with birds. For example, male Mongolian gerbils (*Meriones*

*unguiculatus),* that were gonadectomized and then implanted with testosterone, spent less time in contact with, huddling over, and licking pups than gonadectomized males without testosterone (Clark & Galef, 1999). However, in male neotropical frogs *Eleutherodactylus,* exogenous androgens do not suppress parental care (Townsend et al., 1991), and in biparental California mice (*Peromyscus californicus),* exogenous testosterone maintains high levels of paternal care with no effect on aggression in castrated males (Trainor & Marler, 2001).

In teleost fishes, most studies focus on either parental care or aggressive behaviors, and typically endogenous androgen levels drop during parental care (Oliveira et al., 2002), consistent with the "challenge hypothesis" (Wingfield et al., 1990). However, in the cooperative-breeding cichlid (*Neolamprologus pulcher),* high androgen levels increase parental care (Desjardins et al., 2006; Desjardins et al., 2008). In three-spined sticklebacks (*Gasterosteus aculeatus),* fanning behavior does not differ among castrated males, castrated males treated with 11-ketotestosterone, and sham-operated males (Pall et al., 2002b). In other fish species, 11-ketotestosterone treatment similarly does not suppress parental behaviors (Kindler et al., 1991; Ros et al., 2004; Dey et al., 2010). In contrast to studies of endogenous androgen levels, these studies suggest that the behavioral effects of exogenous androgens on fish parental care are not consistent with the "challenge hypothesis."

The trade-off between aggressive behaviors and parental care as an effect of exogenous testosterone has only been examined in fish species that exhibit exclusive paternal care. In the Azorean rock-pool blenny (*Parablennius parvicornis),* males implanted with 11-ketotesoterone are more aggressive and occupy a larger territory than control males, but the defense of such large territory is incompatible with parental behavior (Ros et al., 2004). In male bluegill (*Lepomis macrochirus*), cyproterone acetate, an androgen antagonist, and other androgens induce

no such effect on anti-predator aggression (Kindler et al., 1991). However, in the smallmouth bass (*Micropterus dolomieus),* males treated with cyproterone acetate exhibit a decreased level of nest defense toward a simulated intruder (Dey et al., 2010).

Exogenous Androgen and Convict Cichlids Behavior in This Study. In my study, males exposed to exogenous testosterone were found farther from the brood, provided less parental care, and were more aggressive than controls, suggesting that elevated testosterone levels elicited aggressive behaviors and suppressed parental care behaviors. These behavioral responses are consistent with the predictions of the "challenge hypothesis." However, exogenous testosterone treatment did not have an effect on offspring survival, and offspring survival was not related to levels of parental care or aggression. Thus, my results for male convict cichlids do not fully support the predictions of the "challenge hypothesis" (Silverin, 1980; Hegner & Wingfield, 1984). The absence of tangible predation in my study could have affected resident fish parental care behavior and increased offspring survival relative to that of natural habitats treated with exogenous testosterone.

Previous studies that have examined the effect of exogenous androgens on parental care and aggressive behaviors have used fish that exhibit exclusive paternal care (Kindler et al., 1991, Ros et al., 2004; Dey et al., 2010). The dependence on paternal care behaviors in such species likely influences the effects of exogenous androgens differently than paternal care behaviors in fish with biparental care (Hirschenhauser & Oliveira, 2006). This study provided unique evidence of how flexibility within such pairs could lead to different behavioral outcomes for each partner. The parental care strategy, influence of offspring age, and risk of predation, independently and interactively influenced convict cichlid parental care and aggression, contributing to an observed trade-off between parental care and elevated testosterone in males

only, specifically during early stages of offspring development. Whether or not the patterns I observed also occur in natural settings will require additional study.

*Potential Consequences of Testosterone Contamination in Natural Fish Populations*

Testosterone is frequently detected in water utilized by humans (Deksissa, 2008). Whereas as the excretion of androgens from natural sources (Fan et al., 2007), such as humans and wildlife (Shore et al., 1997), is unavoidable, the presence of endocrine-disrupting chemicals in agriculture (Sumpter, 2005), wastewater treatment effluents (Shore et al., 2004), and manure-borne steroids (Jobling & Sumpter, 1993), could influence the levels of exogenous testosterone found in environments (Deksissa, 2008). It will thus be of increasing importance to study the potential impacts of exogenous hormones on vulnerable populations, including aquatic organisms. These deleterious impacts already include the masculinization of female fish (Spies et al., 1988), as observed in the sewage-contaminated waters of South Wales, Australia (Batty & Lim, 1999) and several other environments (Howell et al., 1980; Larsson et al., 2000; Parks et al., 2001; Subramian & Amutha 2006; Martinovec et al., 2007).

While such studies focus on the developmental effects of exogenous testosterone, the behavioral effects could be just as important for the persistence of populations. If convict cichlids exposed to testosterone in field populations behave similarly to that observed in this study, the harm in reproductive success and future breeding strategies could have consequences for population viability. An increase in aggression and decrease in parental care could lead to increased brood failure as a result of increased predation and decreased parental investment. If such consequences are applied to the entire fish population, the increase in brood failure could not only lead to a decreased fish population, but could significantly disturb the dynamics of parental care. In this regard, future studies could benefit from these results by exploring related

social contexts, such as mate acquisition, and futher expanding upon the known effects of exogenous androgens on fish behavior.

Such future studies involving the effect of exogenous testosterone on parental care should take into account the fate of androgens in experimental aquaria and their effect on endogenous testosterone levels (Trainor & Marler, 2002; Bos et al., 2010) as well as the quantification of parental care and aggression in closed habitats. The effects of exogenous 11-ketotestosterone, the dominant androgen in teleost fish (Borg, 1994; Hirschenhauser et al., 2004), rather than testosterone (Wingfield et al., 1990), could further elaborate on the effects of exogenous androgens on fish biparental care.

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