Male and female cooperatively breeding fish provide support for the "Challenge Hypothesis"

Julie K. Desjardins,[a] **Mark R. Hazelden,**[a] **Glen J. Van der Kraak,**[b] **and Sigal Balshine**[a]
[a]Animal Behaviour Group, Department of Psychology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada, and [b]Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

The idea that territorial aggression is regulated by androgens and that aggression itself can modulate androgen levels is well established in males. In many species, females also display aggressive behavior, yet little work has been conducted on the effects of female aggression on hormone levels. In this study, we compared the effects of a simulated territory intrusion (a method for testing the Challenge Hypothesis) on males and females of the fish, *Neolamprologus pulcher*. This cichlid fish from Lake Tanganyika is a particularly useful species to examine sex differences in the behavioral mediation of hormones as breeding pairs remain in a territory year round and both sexes defend this territory against conspecific and heterospecific intruders. In our study, both sexes indeed aggressively defended their territory against a simulated territory intruder. In response to intruders, both males and females displayed elevated levels of circulating 11-ketotestosterone, but only females exhibited increases in testosterone. Neither aggressing male nor female fish showed changes in estradiol levels compared to control (nonaggressing) fish. Residents were more aggressive than the intruders and won most of the interactions. However, residents (or winners) did not show higher hormone levels than intruders (or losers). We suggest that aggression commonly modulates androgen levels in both male and female teleost fish. *Key words:* Cichlidae, estradiol, 11-ketotestosterone, Lake Tanganyika, *Neolamprologus pulcher*, sexual differences, territorial aggression, testosterone. *[Behav Ecol 17:149–154 (2006)]*

The explicit function of androgens as initiators and modulators of aggressive behavior is well known (reviewed by Nelson, 1994). Studies using castration have shown that the associated decreases in androgens dampen or eliminate social aggression in males of many species, while hormone replacement in castrated individuals restores or even increases aggressive behavior (Balthazart, 1983; Francis et al., 1992; Moore, 1998). Conversely, the idea that behavior, particularly reproductive aggression, influences hormone levels became clear through the research of John Wingfield and colleagues. The researchers showed that androgen levels fluctuate in birds in response to a territorial intrusion (song sparrows, *Melospiza melodia*: Wingfield, 1985; Wingfield et al., 1987), effectively linking observed aggressive behavior to a physiological response. The Challenge Hypothesis, proposed by Wingfield et al. (1990), postulates that male-male aggressive interactions will stimulate the production of androgens in males. Since the publication of this now classic paper, a plethora of research has emerged in a wide variety of species cementing our understanding of how aggressive interactions increase plasma, urinary, and fecal androgen levels (in birds: Wingfield et al., 1990; in fish: Francis et al., 1992, 1993; Hirschenhauser et al., 2004; Ros et al., 2003; in reptiles: Jessop et al., 1999; Klukowski and Nelson, 1998; Moore, 1998; in mammals: Cavigelli and Pereira, 2000; Goymann et al., 2003; Muller and Wrangham, 2004; but see Ostner and Kappeler, 2002).

Males of many species require high levels of androgens for territory defense, mate attraction, and reproduction but presumably need to depress androgen levels to ensure effective paternal care (Hegner and Wingfield, 1987; Silverin, 1980).

Wingfield et al. (1987, 1990) used the comparative approach to argue that male response to a territory challenge will depend on the mating system and the male's role in parental care. In socially monogamous species with high levels of paternal care, testosterone (T) levels would not be expected to be maintained at maximum physiological levels, and hence, such males are expected to respond to a territory intrusion by increasing T above baseline levels (Wingfield et al., 1990). In contrast, in polygamous species with low levels of paternal care, T is likely to be high throughout the breeding season as males continually court females and compete with other males. Hence, polygynous males are not expected to show elevations in T as a result of a territory intrusion or an aggressive interaction; T may already be at or near the maximum physiological levels. Creel et al. (1993) argue that participation in parental care does not dictate hormone modulation. They found that despite male participation in care, in Dwarf Mongoose (*Helogale parvula*), urinary androgens did not correlate with aggressive interactions. They argue that the regular and frequent male-male aggression and the social instability caused by the multiple matings by both males and females select for constantly high and relatively unflexible hormone levels.

Although the Challenge Hypothesis was initially proposed to explain how behavior modulates androgen levels in males, females can also be aggressive (Horne and Itzkowitz, 1995; Johnsson et al., 2001; Wingfield, 1994). Only three studies have examined female hormonal responses to a simulated territory intrusion (STI). Contrary to the predictions of the Challenge Hypothesis, female song sparrows (*M. melodia*) experiencing a territory intrusion had lower levels of circulating androgens than control females (Elekonich and Wingfield, 2000). Female California mice (*Peromyscus californicus*) showed no changes in T, estradiol (E2), or corticosterone levels in response to a STI (Davis and Marler, 2003). Evidence in support of the Challenge Hypothesis comes from studies with dunnocks (*Prunella modularis*) where females involved

in female-female aggression after the removal of males exhibited increases in fecal T levels compared to controls (Langmore et al., 2002).

Few studies have examined hormonal fluctuations relating to aggression after a territory intrusion in fishes. This is surprising given that teleost fishes represent more than half of all known vertebrates. Cardwell and Liley (1991) were the first to demonstrate that established territorial male stoplight parrotfish (*Sparisoma viride*) exhibit elevated androgens after intrusions of other males. A laboratory study on four cichlid fish species showed that males exhibited elevated levels of 11-ketotestosterone (11-KT) after a territory intrusion (Hirschenhauser et al., 2004). Also, consistent with the Challenge Hypothesis, androgen levels in male fish have been shown to increase in relation to density (Pankhurst and Barnett, 1993; Ros et al., 2003) and dominance status (Cardwell et al., 1996; Elofsson et al., 2000; Liley and Kroon, 1995; Oliveira et al., 1996; Remage-Healy and Bass, 2005). However, not all laboratory experiments have shown the expected androgen relationship with social status (e.g., Cardwell et al., 1996; Hannes, 1986; Neat and Mayer, 1999), perhaps because creating natural conditions for relevant social interactions in the laboratory is extremely difficult. We attempted to alleviate this problem by using a much smaller fish than used in most previous research and a species that both habituates quickly and shows a full range of natural behaviors in the laboratory.

In our study, we examined the effects of aggressive interactions, in particular a STI, on levels of plasma steroid hormones in both male and female *Neolamprologus pulcher*, a cooperatively breeding cichlid fish from Lake Tanganyika. Breeding pairs of *N. pulcher* jointly defend a permanent territory, and both sexes care for the young (Balshine et al., 2001; Balshine-Earn et al., 1998). Individual resident fish were presented with a conspecific territory intruder, the two were allowed to interact for 20 min, and then blood samples were collected for hormone analysis. Three related steroid hormones were examined. T was measured because of its known importance in controlling aggression (Balthazart, 1983). 11-KT (a fish-specific androgen derived from T) was measured because it is thought to be the major active androgen in male teleosts (Borg, 1994; Kime, 1993). E2, also directly related to T, was measured as some research suggests that it influences aggression in both sexes when T is aromatized into E2 (Lisk and Nachtigall, 1988; Toda et al., 2001; Woodley and Moore, 2002). We predicted that both males and females would exhibit increases in T, 11-KT, and E2 as a result of an STI.

METHODS

Study animals and holding conditions

Tests of the Challenge Hypothesis were conducted between 16 October and 17 December 2003 using a colony of *N. pulcher* (a subspecies or sister species of *Neolamprologus brichardi*) held at McMaster University in Hamilton, Ontario, Canada. All fish used in this study were F1 or F2, originating from wild-caught breeders collected on the southern shores of Lake Tanganyika in 2001 and 2002. A total of 75 fish were selected from a stock tank, sexed, measured, and marked subcutaneously with a unique color mark of nontoxic acrylic paint to aid in identification. No adverse effects of the marking procedure were observed, and all fish recovered quickly. The fish averaged 52.6 ± 5.1 mm in standard length (± standard deviation) and were 4.6 ± 1.3 g in body weight (± standard deviation). Fish were housed (in sex- and size-matched trios) for 1 week in one of three equal compartments (300 × 260 × 160 mm) separated by opaque barriers within a 40-l tank. Fish (in each trio) were randomly assigned to the role of control, resident,

or intruder. The 6-day period of social isolation allowed each fish to habituate to its new environment and establish a territory within its own compartment. Six days of isolation also minimized any effects of prior social status while in the stock tank (Oliveira et al., 1996, 2001a). Each tank was equipped with an air-driven sponge filter. Each compartment contained gravel substrate 2 cm deep and a polyvinyl chloride tube (75-mm diam, 65-mm length) provided for shelter. Throughout the experiment, water temperatures in the tanks ranged from 26°C–28°C, a 13:11 h light:dark cycle was maintained, and fish were fed brine shrimp and bloodworms daily, ad libitum. In the afternoon of the sixth day, the STI (or the challenge) was conducted.

Simulated territory intruder protocol

The challenge test involved moving the intruder into the resident's territory and observing the ensuing interaction for 20 min. In order to control for the effects of netting and movement, the resident fish was also captured and then immediately replaced into it's own territory prior to the experiment. We conducted 12 female challenge trials and 13 male challenge trials. Focal observations on interacting fish (residents and intruders) were made simultaneously by two observers (one observer following each fish) sitting at a distance of 1.5 m from the tank, and each challenge test was also videotaped. To habituate the fish to the observers, observers sat in close proximity to the tank for 10 min prior to the trials. Trials were always run in the afternoon between 1300 and 1700 h to control for possible daily fluctuations in androgen levels. All aggressive (bites, chases, rams, puffed throats, and aggressive postures) and submissive (submissive displays and submissive postures) interactions were recorded (see Buchner et al., 2004, for details). The total number of displays of aggression was used to define an individual's overall value of aggression. The status of the "winner" of the contest was clearly determined by the amount of aggression displayed and the spatial dominance over the substrate and shelter within the territory. "Losers" typically provided a clear stereotyped sign of defeat or submission by retreating to the top corners of the tank and not entering the shelter. Winner and loser status was assigned at the end of each 20-min interaction.

Hormone assays

After the experiments, "test fish" were netted, weighed, measured (standard and total length), and anesthetized with a lethal dose of benzocaine (ethyl *p*-aminobenzoate, 1.0 μg/ml), and a blood sample was taken by caudal severance. The procedure, from the time the fish were disturbed by the researchers approaching the tank at the end of the trial, to the completion of blood sampling, took no longer than 3 min. Blood was collected in heparinized microcapillary tubes and spun at 8000 revolutions/min for 8 min, and the plasma was then removed and frozen at −20°C for later analysis of T, 11-KT, and E2. On average, 32 (± 8.2) μl of plasma was recovered from blood. Livers and gonads were removed, measured, and stored in ethanol. The liver is known to be an important energy storage organ, and the size of the liver has been related to social dominance rank (Fox et al., 1997).

We extracted steroids from plasma by shaking the aliquots with 5 ml of diethyl ether. After freezing the aqueous phase in an acetone/dry ice bath, the organic phase was decanted and dried. The dry extracts were resolubilized in assay buffer and frozen at −20°C for later assay. Extraction losses were independently monitored, and recoveries after a double extraction procedure were 88%. We used radioimmunoassays to measure T, 11-KT, and E2. We followed the radioimmunoassay

techniques to measure steroids from plasma described by McMaster et al. (1995). The reported values are the average concentrations calculated from duplicate aliquots whose values fell within the 5–95% binding limits of the appropriate standard curve after the linearization with a log-log transformation. Intraassay coefficients of variance did not exceed 12% for these assays, and the minimum detectable concentrations for the steroids in this study were 0.347 ng/ml for T, 0.082 ng/ml for 11-KT, and 0.074 ng/ml for E2. The assays for T, 11-KT, and E2 were validated for this species prior to the experiment. Serially diluted plasma pools produced displacement curves parallel to that of serially diluted T, 11-KT, and E2 standards (the regression lines did not differ significantly in slope; $p > .43$ for all three steroids).

Ethical note

The fish did not appear to be in distress during the trials, and behavior was closely and constantly monitored for any sign of excessive aggression. Trials would have been stopped if the interacting fish appeared particularly stressed or received any injury; however, in none of the trials were the animals overly aggressive, and no injuries were sustained. The procedures described for catching, tagging, and blood sampling the fish had been reviewed and approved by McMaster University's Animal Research Ethics Board (Animal Utilization Protocol # 03-09-54) and adhere to the animal handling guidelines specified by the Canadian Council for Animal Care.

Statistical analysis

All statistical analyses were performed using the STATVIEW package on a Macintosh computer. All reported tests were two tailed and corrected for ties. All data were tested for normality prior to statistical analysis using the Kolmogorov-Smirnov test, and none of the parameters violated the assumptions of parametric tests. Gonadosomatic index (GSI—a measure of gonadal investment controlled for body mass) was calculated as gonad mass/body mass × 100, and hepatosomatic index (HSI—a measure of liver size controlled for body mass) was calculated as liver mass/body mass × 100. To test for the effects of status (control, resident, or intruder) on hormone levels while controlling for the effect of tank environment, we used repeated-measures analyses of variance (ANOVAs). As there were no differences between resident and intruder fish in hormone level (see Did Aggression Levels Reflect Hormone Levels?), fish participating in an aggressive interaction (intruders and residents) were combined into a single group that we called test fish for some analyses. To test for the effect of participating in an aggressive interaction on hormone levels (control fish vs. test fish), we used two-way ANOVAs, followed by paired and unpaired t tests. To test for relationships between aggression, steroid hormones, and other physiological measures, Pearson's correlations and regression analyses were performed. We used post hoc Tukey-Kramer corrections to adjust significance levels in relation to the number of multiple comparisons performed. Variations in sample sizes for physiological variables reflect the loss of a few samples during hormonal analysis.

RESULTS

Were residents more aggressive than intruders?

Residents were more aggressive than intruders (two-way ANOVA: $F = 17.320$, $p = .0001$, Figure 1), and resident status accurately predicted victory in the aggressive interaction (chi-
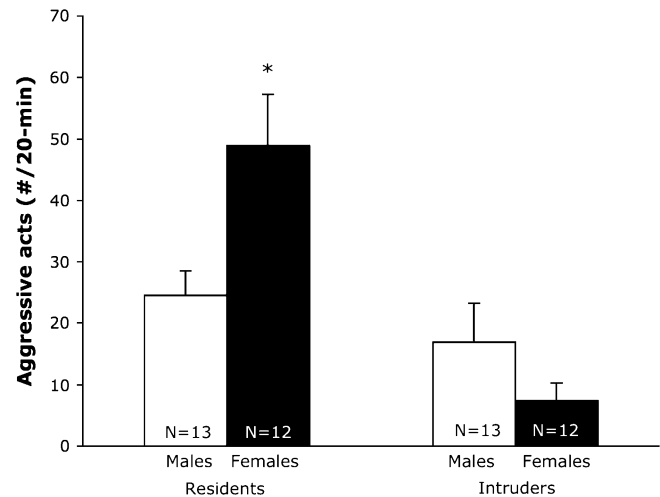

Figure 1
Mean (± standard error) number of aggressive acts displayed during a 20-min interaction of both male and female residents and intruders. The asterisk indicates a statistical difference at the $\alpha = 0.05$ level of a two-tailed paired t test.

square test: $\chi^2 = 11.6$, $p < .001$). The level of aggression depended on both the sex and the status (resident or intruder) of the fish (sex × status: $F = 8.185$, $p = .006$). Post hoc tests revealed that female residents were more aggressive than male residents (unpaired t test: $t = 2.688$, $p = .01$, Figure 1), but male and female intruders were equally aggressive ($t = -1.250$, $p = .22$).

Did steroid levels differ by status (resident vs. intruder) or by the outcome (winner vs. loser) of the contest?

Despite the differences in aggression levels, circulating steroid levels did not vary with status (resident or intruder) of the fish. Residents and intruders showed similar levels of T (two-way ANOVA: $F = 0.001$, $p = .98$), 11-KT ($F = 0.044$, $p = .83$), and E2 ($F = 2.955$, $p = .09$). Also, the outcome (winner or loser) did not predict steroid hormone levels. Winners and losers did not differ significantly in T ($F = 0.009$, $p = .93$), 11-KT ($F = 0.01$, $p = .91$), or E2 ($F = 2.254$, $p = .10$).

Did aggression levels reflect hormone levels?

Overall aggression levels (irrespective of sex or resident/intruder status) did not correlate with T, 11-KT, or E2. When the sexes were analyzed separately, male aggression correlated with T (Pearson's correlation: $r^2 = .209$, $p = .05$) but not with 11-KT or E2, while female aggression did not correlate with any steroid measured. With respect to the status of individual fish, aggression by residents was correlated with levels of T ($r^2 = .255$, $p = .02$) but not 11-KT or E2 levels. However, in intruders, aggression did not correlate with any steroid hormone. When we examined winners and losers separately, we found that aggression displayed by winners and losers was not related to T, 11-KT, or E2.

Was there aggression-induced modulation of steroid hormone levels?

Controls had lower levels of T and 11-KT compared to residents and intruders (repeated-measures ANOVA—T: $F = 3.644$, $p = .04$; 11-KT: $F = 3.919$, $p = .03$) but did not exhibit lower levels of E2 ($F = 1.632$, $p = .21$).

We found that test fish (residents and intruders combined) had higher T (two-way ANOVA: $F = 11.851$, $p = .0001$) and 11-KT ($F = 5.233$, $p = .026$) but not E2 ($F = 0.860$, $p = .36$) relative to controls. Post hoc tests revealed that test females had higher T levels compared to controls, but males did not show this difference (unpaired t tests—females: $t = -3.87$, $p = .02$; males: $t = -0.292$, $p = .78$; Figure 2a). Test fish (residents and intruders) of both sexes had higher levels of 11-KT (males: $t = -3.083$, $p = .01$; females: $t = -2.297$, $p = .05$; Figure 2b). There were no differences between control and test fish (males and females) in E2 levels (males: $t = -0.179$, $p = .86$; females: $t = 0.736$, $p = .48$; Figure 2c).

Did the levels of the various steroids measured correlate with one another?

T levels correlated with 11-KT (Pearson's correlation: $r^2 = .267$, $p = .0009$) and with E2 ($r^2 = .121$, $p = .03$), but E2 levels did not correlate with the levels of 11-KT ($r^2 = .003$, $p = .76$).

Did other physiological measures give any indication of hormone or aggression levels?

In males, the HSI (a measure of liver investment controlled for body mass) was negatively related to aggression ($p = .03$). In females, this was not the case. In both sexes, HSI was not related to any hormone levels. In males, the GSI (a measure of gonad size compared to body mass) was not related to aggression or hormone levels. However, in females, a nonsignificant negative correlation was observed between GSI and E2 levels ($p = .06$), but neither aggression nor T or 11-KT levels were related to GSI in females.

DISCUSSION

Resident fish (males and females) exposed to a simulated territory intruder behaved aggressively toward the intruder and had higher levels of 11-KT compared to control fish. In fact, both the interacting fish, residents and intruders (test fish), behaved aggressively and showed higher levels of circulating 11-KT; only female test fish also showed higher levels of T. Interestingly, females displayed higher levels of both aggression and androgens (T and 11-KT) in response to the territory challenge. These results are of particular interest because many researchers assume that males are more aggressive than females and that the behavioral modulation of androgens in females is negligible. The notion that behavior modulates androgens in females could have profound impact on the study of female reproductive physiology, maternal care, and female mate choice.

Why do female *N. pulcher* have higher levels of T than males?

In most vertebrates, males have higher levels of T (but see Creel et al., 1993; Davis and Marler, 2003; Wingfield, 1994). Traditionally, androgens (especially T) have been considered "male hormones" and important in regulating male aggression. However, in a number of fish species, females have higher T (Borg, 1994; Hirschenhauser et al., 2004). Whether T or any other androgen regulates or modulates female aggression (competition for territories, competition for mates, or the defense of young) is a highly debated issue (Davis and Marler, 2003; Langmore et al., 2002; Wingfield, 1994). Typically, males are considered to be the more aggressive sex with respect to territorial behavior or competition for mates (Cardwell et al., 1996; Hirschenhauser et al., 2004; Oliveira et al., 2001b; Trivers, 1972). However, females (even in non-sex-role–reversed species) can also be extremely aggressive (see Floody,
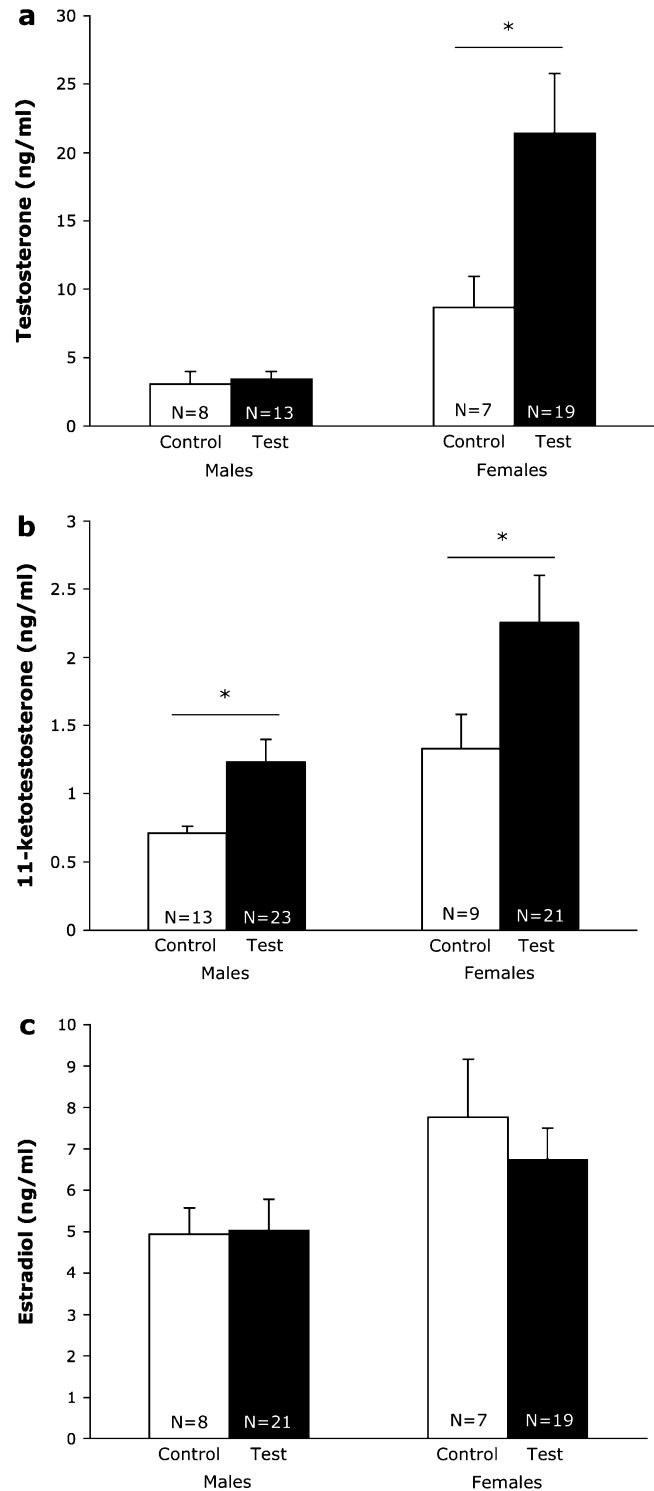
Figure 2
Mean (± standard error) of plasma T (a), 11-KT (b), and E2 (c) in nanogram per milliliters of control fish and test fish. Control fish did not experience an interaction with another fish, whereas test fish were allowed to interact for a 20-min period. The asterisks indicate a statistical difference at the $\alpha = 0.05$ level of a two-tailed paired t test.

1983; Horne and Itzkowitz, 1995; Johnsen, 1998) and can have significant quantities of circulating T (Wingfield, 1994).

In *N. pulcher*, both males and females defend the territory and need to maintain their position in a dominance hierarchy

(Balshine et al., 2001; Balshine-Earn et al., 1998). Although males are larger than females, the magnitude of the difference is small compared to many other cichlids (Barlow, 2000). These factors suggest that males and females should, in fact, have fairly similar androgen levels, so why were females' levels so much higher? Although males and females show behavioral similarity in the form of aggressive and parental behavior, in the field, males and females differ in the frequency or rate of these behaviors; females (both breeders and helpers) perform more defense behavior (Balshine et al., 2001; Stiver et al., 2005) and direct brood care (Taborsky and Grantner, 1998; Stiver et al., 2005). Given that greater aggression is generally associated with increased androgen levels (Wingfield, 1984), higher androgen levels displayed by *N. pulcher* females may have been selected for to facilitate their higher rates of defense of young and social aggression (however, see Goymann et al., 2004).

Why are females more responsive to a territory challenge than males?

Higher female responsiveness to the behavioral challenge may lie with the different mating strategies available to males and females. Male *N. pulcher* often breed with several females at one time (Limberger, 1983). In 2004, in our study site in Zambia, 80% of the males in the population held multiple territories and frequently attempted to take over additional territories from neighboring males (personal observation). As a result, territorial aggression was the norm, and males may be maintaining their circulating androgens at or near their physiological maximum. It may also be likely that males and females have different physiological maxima in both T and 11-KT. This may explain why males did not respond to the challenge to the extent that females did in our study.

Why did residents and intruders have similar hormone levels?

As expected, residents won contests more frequently than intruders (retaining their territory and shelter in 21 of 25 encounters). Interestingly, female residents always won and, in general, were more aggressive than male residents. Based on other behavioral physiological studies that have shown dominants having higher plasma androgens (Cardwell et al., 1996; Francis et al., 1992; Oliveira et al., 1996; Trainor et al., 2004), we expected that the winners, or fish that performed more aggressive behaviors, would have higher concentrations of steroids compared to losers. Most behavioral endocrinologists using the STI paradigm have not examined changes in steroid levels in intruders as these are often restrained animals (in cages) unable to respond appropriately. Moreover, there were other important differences between previous studies and our study that may explain the conflicting results. First, all test fish in our study were very closely size and sex matched (the mean body size difference between aggressing fish was 2 mm in standard length and ranged from 0 to 4 mm), implying that there was good reason to fight. Second, both fish were given a chance to behave in a territorial manner for 6 days directly prior to the challenge test, and both displayed vigorous aggressive displays and postures during the trial. Third, test fish in our study were sacrificed within 3 min of the end of the 20-min aggressive interaction, and hence, it is likely that the physiological response to defeat had not had time to take effect. The 20-min period was found, in a pilot study, to be the minimum time required for the establishment of a clear winner and loser. Had we provided a longer latency period between the establishment of dominance and the collection of samples, we may have seen the differences between winners and losers reported in other studies.

What is the role of estrogens and other hormones?

Estrogens have a large number of activational roles in birds and mammals regulating both female sexual behavior and maturation as well as maintaining proper functioning of the reproductive system (Balthazart, 1983; Nelson, 1994). E2 has been suggested as a likely candidate to control female aggression because in birds, E2 is high when females pair with males (a period of frequent social interaction and instability, see Wingfield, 1994; Wingfield and Farner, 1978, for reviews). Given the evidence on the association between estrogens and behavior, it is surprising that so few studies have examined candidate steroids other than T or its androgen derivatives as regulators of female aggression. In our study, we did not find a difference in E2 between aggressing and nonaggressing individuals. The question of the role of E2 in female aggression and how females are able to quickly switch aggression "on and off" in the appropriate context remain largely unexplained. Androgens and estrogens are not the only hormones that are likely to be socially modulated. There is considerable evidence that adrenal hormones such as corticosterone, progesterone, and cortisol (typically related to stress response) are also closely linked to dominance status and social environment (Overli et al., 1999; Woodley and Moore, 2002). Given the size constraint of our study species, we were unable to quantify all candidate hormones, but it is likely that stress related hormones such as cortisol are involved in aggressive responses in *N. pulcher.*

Our results represent the first experimental manipulation of aggression to explore male and female hormone levels in a single species of fish. We provide the first example of a hormonal change after an increase in aggression in female fish. In addition, this study demonstrates the need to examine sex steroid levels in males and females of the same species to fully understand sex differences in behavioral modulation of hormones.

REFERENCES

Balshine S, Leach B, Neat F, Reid H, Taborsky M, Werner N, 2001. Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). Behav Ecol Sociobiol 50:134–140.

Balshine-Earn S, Neat FC, Reid H, Taborsky M, 1998. Paying to stay or paying to breed? Field evidence for direct benefits of helping behaviour in a cooperatively breeding fish. Behav Ecol 9:432–438.

Balthazart J, 1983. Hormonal correlates of behavior. Avian Biol 70:221–365.

Barlow GW, 2000. The cichlid fishes: nature's grand experiment in evolution. Cambridge, Massachusetts: Perseus.

Borg B, 1994. Androgens in teleost fishes. Comp Biochem Physiol C Toxicol Pharmacol 109:219–245.

Buchner AS, Sloman KA, Balshine S, 2004. The physiological effects of social status in the cooperatively breeding cichlid *Neolamprologus pulcher.* J Fish Biol 4:1080–1095.

Cardwell JR, Liley NR, 1991. Androgen control of social status in males of a wild population of stoplight parrotfish, *Sparisoma viride* (Scaridae). Horm Behav 25:1–18.

Cardwell JR, Sorensen PW, Van der Kraak GJ, Liley NR, 1996. Effect of dominance status on sex hormone levels in laboratory and wild-spawning male trout. Gen Comp Endocrinol 101:333–341.

Cavigelli SA, Pereira ME, 2000. Mating season aggression and fecal testosterone levels in male ring-tailed lemurs (*Lemur catta*). Horm Behav 37:246–255.

Creel S, Wildt DE, Monfort SL, 1993. Aggression, reproduction and androgens in wild dwarf mongooses: a test of the challenge hypothesis. Am Nat 141:816–825.

Davis ES, Marler CA, 2003. The progesterone challenge: steroid hormone changes following a simulated territorial intrusion in female *Peromyscus californicus.* Horm Behav 44:185–198.

Elekonich MM, Wingfield JC, 2000. Seasonality and hormonal control of territorial aggression in female song sparrows (Passeriformes: Emberizidae: *Melospiza melodia*). Ethology 106:493–510.

Elofsson UO, Mayer I, Damsgard B, Winberg D, 2000. Intermale competition in sexually mature arctic charr: effects on brain monoamines, endocrine stress responses, sex hormone levels and behavior. Gen Comp Endocrinol 118:450–460.

Floody OR, 1983. Cuts between the septum and preoptic area increase ultrasound production, lordosis, and body weight in female hamsters. Physiol Behav 54:383–392.

Fox HE, While SA, Kao MHF, Fernald RD, 1997. Stress and dominance in a social fish. J Neurosci 17:6463–6469.

Francis RC, Jacobson B, Wingfield JC, Fernald RD, 1992. Castration lowers aggression but not social dominance in male *Haplochromis burtoni* (Cichlidae). Ethology 90:247–255.

Francis RC, Soma K, Fernald RD, 1993. Social regulation of the brain pituitary-gonadal axis. Proc Natl Acad Sci U S A 6:7794–7798.

Goymann W, East ML, Hofer H, 2003. Defense of females, but not social status, predicts plasma androgen levels in male spotted hyenas. Physiol Biochem Zool 76:586–593.

Goymann W, Wittenzellner A, Wingfield JC, 2004. Competing females and caring males. Polyandry and sex-role reversal in African black coucals, *Centropus grillii*. Ethology 110:1–17.

Hannes RP, 1986. Blood and whole-body androgen levels of male swordtails correlated with aggression measures in a standard-opponent test. Aggress Behav 12:249–254.

Hegner RE, Wingfield JC, 1987. Effects of experimental manipulations of testosterone levels on parental investment and breeding success in male house sparrows. Auk 104:462–469.

Hirschenhauser K, Taborsky M, Oliveira T, Canario AVM, Oliveira RF, 2004. A test of the 'challenge hypothesis' in cichlid fish: simulated partner and territory intruder experiments. Anim Behav 8:741–750.

Horne E, Itzkowitz M, 1995. Behaviour of the female beaugregory damselfish. J Fish Biol 46:457–461.

Jessop TS, FitzSimmons NN, Limpus CJ, Whittier JM, 1999. Interactions between behaviour and plasma steroids within the scramble mating system of the promiscuous green turtle, *Chelonia mydas*. Horm Behav 36:86–97.

Johnsen TS, 1998. Behavioural correlates of testosterone and seasonal changes of steroids in red-winged blackbirds. Anim Behav 55:957–965.

Johnsson JI, Sernland E, Blixt M, 2001. Sex-specific aggression and antipredator behaviour in young brown trout. Ethology 107:587–600.

Kime DE, 1993. Classical and nonclassical reproductive steroids in fish. Rev Fish Biol Fish 3:160–180.

Klukowski M, Nelson CE, 1998. The challenge hypothesis and seasonal changes in aggression and steroids in male northern fence lizards (*Sceloporus undulatus hyacinthinus*). Horm Behav 33:197–204.

Langmore NE, Cockrem JF, Candy EJ, 2002. Competition for male reproductive investment elevates testosterone levels in female dunnocks, *Prunella modularis*. Proc R Soc Lond B 269:2473–2478.

Liley NR, Kroon FJ, 1995. Male dominance, plasma hormone concentrations and availability of milt in male rainbow trout (*Oncorhynchus mykiss*). Can J Zool 73:826–836.

Limberger D, 1983. Pairs and harems in a cichlid fish, Lamprologus brichardi. J Comp Ethol 62:115–144.

Lisk RD, Nachtigall MJ, 1988. Estrogen regulation of agonistic and proceptive responses in the golden hamster. Horm Behav 22:35–48.

McMaster ME, Munkittrick KR, Jardine JJ, Robinson RD, Van Der Kraak GJ, 1995. Protocol for measuring in vitro steroid production by fish gonadal tissue. Can Tech Rep Fisheries Aquat Sci. Burlington, Ontario: Fisheries and Oceans Canada.

Moore MC, 1998. Testosterone control of territorial behaviour: tonic release implants fully restore seasonal, short-term aggressive responses in free-living castrated lizards. Gen Comp Endocrinol 70:450–459.

Muller MN, Wrangham RW, 2004. Dominance, aggression and testosterone in wild chimpanzees: a test of the 'challenge hypothesis'. Anim Behav 67:113–123.

Neat FC, Mayer I, 1999. Plasma concentrations of sex steroids and fighting in male *Tilapia zillii*. J Fish Biol 54:695–697.

Nelson RJ, 1994. An introduction to behavioral endocrinology. Saunderland: Sinauer Association.

Oliveira RF, Almada VC, Canario AVM, 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus*. Horm Behav 30:2–12.

Oliveira RF, Almada VC, Gonçalves EJ, Forsgren E, Canario AVM, 2001a. Androgen levels and social interactions in breeding males of the peacock blenny. J Fish Biol 58:897–908.

Oliveira RF, Lopes M, Carneiro LA, Canario AVM, 2001b. Watching fights raises fish hormone levels. Nature 409:475.

Ostner J, Kappeler PM, 2002. Seasonal variation and social correlates of androgen excretion in male redfronted lemurs (*Eulemur fulvus rufus*). Behav Ecol Sociobiol 52:485–495.

Overli O, Pottinger TG, Carrick TR, Overli E, Winberg S, 1999. Brain monoaminergic activity in rainbow trout selected for high and low stress responsiveness. Brain Behav Evol 54:263–275.

Pankhurst NW, Barnett CW, 1993. Relationship of population density, territorial interaction and plasma levels of gonadal steroids in spawning male demoiselles *Chromis dispilus* (Pisces: Pomacentridae). Gen Comp Endocrinol 90:168–176.

Remage-Healy L, Bass AH, 2005. Rapid elevations in both steroid hormones and vocal signaling during playback challenge: a field experiment in gulf toadfish. Horm Behav 47:297–305.

Ros AH, Canario AVM, Couto E, Zeilstra I, Oliveira RF, 2003. Endocrine correlates of intra-specific variation in the mating system of the St. Peter's Fish (*Sarotherodon galilaeus*). Horm Behav 44:365–373.

Silverin B, 1980. Effects of long-lasting testosterone treatment on free-living pied flycatchers, *Fecedula hypoleuca*, during the breeding season. Anim Behav 218:906–912.

Stiver KA, Dierkes P, Taborsky M, Gibbs HL, Balshine S, 2005. Relatedness and helping in fish: examining theoretical predictions. Proc R Soc Lond B 272:1593–1599.

Taborsky M, Grantner A, 1998. Behavioural time-energy budgets of cooperatively breeding *Neolamprologus pulcher* (Pisces: Cichlidae). Anim Behav 56:1375–1382.

Toda K, Saibara T, Okada T, Onishi S, Shizuta Y, 2001. A loss of aggressive behaviour and its reinstatement by oestrogen in mice lacking the aromatase gene (Cyp19). J Endocrinol 168:217–220.

Trainor BC, Bird IM, Marler CA, 2004. Opposing hormonal mechanisms of aggression revealed through short-lived testosterone manipulations and multiple winning experiences. Horm Behav 45:115–121.

Trivers RL, 1972. Parental investment and sexual selection. In: Sexual selection and the descent of man (Campbell B, ed). Chicago: Aldine, pp. 136–179.

Wingfield JC, 1984. Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. I. Temporal organization of the breeding cycle. Gen Comp Endocrinol 56:406–416.

Wingfield JC, 1985. Short-term changes in plasma levels of hormones during establishment and defense of a breeding territory in male song sparrows, *Melospiza melodia*. Horm Behav 19:174–187.

Wingfield JC, 1994. Hormone-behavior interactions and mating systems in male and female birds. In: The differences between the sexes (Short RV, Balaban E, eds). Cambridge: Cambridge University Press; 303–330.

Wingfield JC, Ball GF, Dufty AM Jr, Hegner RE, Ramenofsky M, 1987. Testosterone and aggression in birds. Am Sci 75:602–608.

Wingfield JC, Farner DS, 1978. Endocrinology of a natural breeding population of white crowned sparrows (*Zonotrichia leucophrys pugetensis*). Physiol Zool 51:188–205.

Wingfield JC, Hegner RE, Dufty AM Jr, Ball GF, 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems and breeding strategies. Am Nat 136:829–846.

Woodley SK, Moore MC, 2002. Plasma corticosterone response to an acute stressor varies according to reproductive condition in female tree lizards (*Urosaurus ornatus*). Gen Comp Endocrinol 128:143–148.