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A test of the 'challenge hypothesis' in cichlid fish: simulated partner and territory intruder experiments

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In male birds, the responsiveness of androgens to sexual and territorial behaviour is predicted to vary with mating system and the degree of paternal investment ('challenge hypothesis', CH; Wingfield et al. 1990, *American Naturalist*, **136**, 829–846). The CH predicts a higher and longer lasting 'breeding baseline' androgen level in males of polygynous species with no or only short-term paternal investment than in males of monogamous species with a high degree of paternal investment. Since the applicability of the CH to nonavian vertebrates has been unclear, we experimentally tested its predictions in several cichlid fish (*Neolamprologus pulcher*, *Lamprologus callipterus*, *Tropheus moorii*, *Pseudosimochromis curvifrons* and *Oreochromis mossambicus*) using a simulated territorial intruder protocol. Androgens (11-ketotestosterone: 11-KT; testosterone: T) were measured from fish-holding water. In all species sampled, the 11-KT patterns confirmed the predictions of the CH originating from the avian literature, but T patterns did not. Males of all species sampled were highly responsive to territorial intrusions; however, the magnitude and duration of this response, that is, the rapid return to baseline 11-KT levels, could not clearly be explained by the degree of paternal care. 11-KT responses to interactions with ovulating females were observed in maternal mouthbrooders but not in biparental species (e.g. Lamprologini). At the interspecific level, androgen responsiveness was greater among males of monogamous species, as predicted, but also in species with more intense pair bonding (e.g. *Tropheus moorii*). Thus, this study confirms the predictions of the CH in cichlid fish at both the intraspecific and the interspecific levels.

Androgens are responsible for secondary sexual characters, spermatogenesis and the expression of male sexual behaviour. Conversely, androgen levels are also open to influences of the social environment. The social modulation of androgens, that is, interactions of androgens with both sexual and territorial behaviour, has been introduced to the field of behavioural endocrinology in a conceptual framework known as the 'challenge hypothesis' (Wingfield et al. 1990, 2000). This hypothesis has its origin in the avian literature and predicts large androgen responses to territorial challenges in males of monogamous species that invest in parental duties (paternal, biparental). In contrast, males of species with a high degree of polygyny and little or no paternal care are exposed to higher levels of social interactions and, therefore, are predicted to respond with only small androgen increases to additional territorial challenges. As a measure of the individual androgen responsiveness, Wingfield et al. (1990) introduced the concepts of the constitutive non-breeding androgen level (NB), the breeding baseline (BB; i.e. the average androgen level throughout all breeding phases) and the physiological maximum response (PhM)

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to a territorial challenge. From these measures, the androgen responsiveness, expressed as the ratio (PhM – NB)/(BB – NB), can be related to territorial challenges of individual males independently of individual nonbreeding baseline androgen levels. This ratio may be used not only at the intraspecific level, but also for comparisons of androgen responsiveness between species. However, predictions of the challenge hypothesis at the intraspecific level do not necessarily coincide with predictions at the interspecific level (Hirschenhauser et al. 2003). For example, a trade-off between paternal investment and high breeding levels of testosterone (T) is a common phenomenon within species (Silverin 1980; Rissman & Wingfield 1984; Wingfield 1984; Hegner & Wingfield 1987; Beletsky et al. 1989; Dittami et al. 1991; Ketterson & Nolan 1992; Wada et al. 1999; Wikelski et al. 1999; De Ridder et al. 2000; Hirschenhauser et al. 2000; Wingfield et al. 2000), but in a sample of 84 bird species the relation between paternal care and variation in androgen responsiveness was minor after controlling for phylogeny (Hirschenhauser et al. 2003). In contrast, the relation between avian mating systems and androgen responsiveness remained robust to phylogenetic control. Furthermore, in a literature survey of teleost fish, an effect of mating system on the evolution of androgen responsiveness was suggested, whereas the relation between paternal care and androgen responsiveness was not as clear (Oliveira et al. 2002).

In the present study, we experimentally tested the predictions of the challenge hypothesis in cichlid fish, a rapidly evolving group with a great variety of breeding systems (Trewavas 1983; Sturmbauer et al. 2002). To avoid phylogenetic bias, we compared androgen responsiveness (the dependent variable) in pairs of closely related species that differed in their prevalent mating system (the independent variable). This approach assumes that it is highly unlikely that multiple character state changes of the independent and dependent variables have occurred simultaneously during a relatively short evolutionary time span (Møller & Birkhead 1992). The two closely related pairs of species were the haplochromine cichlids *Neolamprologus pulcher* versus *Lamprologus callipterus* and *Tropheus moorii* versus *Pseudosimochromis curvifrons* (Table 1). To test the predictions of the challenge hypothesis in a standardized way, we established a 'simulated territorial intruder' protocol. To avoid repeated blood sampling, which in fish is limited by body size and would introduce handling stress as a spurious variable in the study, we measured steroids from fish-holding water (Hirschenhauser et al. 2002). Steroids are released into the water via urine and by diffusion via the gills (Scott & Liley 1994; Oliveira et al. 1996; Vermeirssen & Scott 1996; Greenwood et al. 2001). Measuring steroids from fish-holding water not only reduces handling to a minimum, but it also allows the monitoring of sequential hormonal changes from the same individual, even in small specimens. For each species, we assessed the constitutive nonbreeding androgen level (NB), the male's androgen response to the presence of and interaction with an ovulating female (BB), and the male's response to an additional challenge by a conspecific intruder male (PhM). At the intraspecific level we predicted effects of paternal care and of mating system on BB androgen levels (Oliveira et al. 2001a, 2002). To test for the interspecific predictions, we compared the androgen responsiveness (as defined by Wingfield et al. 1990) between species with different mating systems in a pairwise approach. We predicted an effect of mating system: that is, higher responsiveness in males of monogamous species than in polygynous males.

METHODS

Study Animals

For the simulated territorial intruder experiments we selected pairs of closely related species with different mating systems (Table 1). Among the substrate-spawning Lamprologini we tested the monogamous and biparental *N. pulcher*, which lives in large groups with helpers of both sexes and different sizes (Taborsky & Limberger 1981; Balshine-Earn et al. 2001), and the polygynous and biparental snail-shell-breeding *L. callipterus* (Sato 1994). In both species, intense male–male competition has been observed: male *N. pulcher* helpers share in fertilizing the territorial pair's broods (Dierkes et al. 1999) and in *L. callipterus* the large territorial males not only have to defend their transportable spawning substrate (snail shells) against other nest males but are also regularly challenged by parasitic sneakers and dwarf males (Sato 1994; Taborsky 1994, 2001). Among the maternally mouthbrooding Tropheini we sampled the polygynous *T. moorii* with temporary pair formations (Wickler 1969; Nishida & Yanagisawa 1991) and the polygynous *P. curvifrons* with explosive lek breeding (Kuwamura 1987). These four species were sampled from a local stock bred at the Konrad Lorenz Institut für Vergleichende Verhaltensforschung in Vienna, Austria. Local housing routines were large mixed-species groups in a ringtank for *T. moorii* and *P. curvifrons* (two 2000-litre compartments with 30 individuals of both species), large family groups with up to 18 adult helpers for *N. pulcher* (Oliveira et al. 2003), and groups of up to four males and large female groups (up to 20 females) for *L. callipterus*. Whenever we isolated focal males we took the entire group out of the home tanks simultaneously, so that we could put them back again as a group after all focal males were sampled. All fish were kept at 13:11 h light:dark regime and were fed with standard tetramin flakes (four times daily) and with frozen daphnia and artemia larvae (twice daily).

We also included in the present comparisons a species of tilapia, the lek-breeding maternal mouthbrooder *Oreochromis mossambicus* (Trewavas 1983; Oliveira et al. 1996) from a stock bred at the Instituto de Psicología Aplicada in Lisbon, Portugal. These fish were housed as mixed-sex groups with up to 15 individuals. This species lacks a closely related counterpart to be compared with in this study. However, it is of interest because measuring steroids from fish-holding water has been validated in detail for this species in our laboratory (Ros et al. 2000; Hirschenhauser et al. 2002; and see below). Therefore, if the androgen response patterns from *O. mossambicus* yield biologically meaningful patterns in the experiments, we

Table 1. Mating systems and parental care of the sampled cichlid species (*N* = number of sampled focal males/control males) and mean body mass ± SEM of tested males

Species	Suprafamily	*N*	Body mass (g)	Mating system	Parental care
Neolamprologus pulcher	Lamprologini	12/7	15.3±1.4	Monogamous, territorial pair with helpers of all sizes and both sexes	SS, biparental
Lamprologus callipterus	Lamprologini	8/1	21.0±2.1	Polygynous, parasitic males	SS (snail shells), biparental
Tropheus moorii	Tropheini	13/7	14.5±1.7	Polygynous, temporary pair formation	MB, maternal
Pseudosimochromis curvifrons	Tropheini	13/6	25.1±2.0	Polygynous, exploding leks	MB, maternal
Oreochromis mossambicus	Tilapini	16/6	22.1±2.0	Polygynous, lek arenas, parasitic males (facultative)	MB, maternal

SS: substrate spawners; MB: mouthbrooders.

can assume our methods are also valid for the androgen measurements of the other species we sampled.

The experiments were done in tanks (100 × 40 cm and 40 cm high) at a water temperature of 26°C for haplochromine species and at 28°C for the tilapia. Food (tetramin flakes, daphnia and artemia larvae) was provided every morning; on experimental days, focal males were fed immediately after sampling.

Sampling Fish-holding Water

As a noninvasive alternative to repeated blood sampling from fish, we measured steroids from fish-holding water (Hirschenhauser et al. 2002; Oliveira et al. 2003). During all phases of the experiment, a sample container with a closed bottom (for collecting steroid samples) and a presentation container with an open bottom (for presenting intruders), both 20 × 20 cm and made of glass, were present inside the experimental tank (Fig. 1). Before the behavioural experiment (i.e. before the female was introduced and before the male intruder was presented), the sample container was filled with a constant volume of water (2 litres) in which the fish was placed for 1 h. No individual of any species tested showed extreme behavioural responses to being placed into presentation or sample containers. The water for sample containers always originated from a large 'pool water tank', which never held any fish and was freshly refilled whenever necessary, as well as each time we started testing a new species. To exclude contamination, we always washed sample containers and all materials used with methanol and distilled water before sampling. Immediately after water collection, particulate matter such as faeces was removed from the samples using filter paper. By means of a vacuum pump, the lipophilic compounds of the sample were then drawn through solid phase chromatography cartridges (Merck LiChrolut RP-18, 500 mg), which had been previously activated with 5 ml of methanol followed by 5 ml of distilled water. Thereafter, they were immediately stored at −20°C until further processing (Scott & Sorensen 1994). To avoid bias from diurnal hormone fluctuations (Oliveira et al. 2001b), sampling always took place at the same time of day (between 1400 and 1500 hours).

Simulated Territorial Intruder Protocol

Males were socially isolated for a week before the experiment; all fish behaved calmly and did not suffer any adverse health effects. After this period (on the 'first day' of the experiment), each male was placed into the sample container to measure its nonbreeding baseline androgen level (NB, Fig. 1). On the second day, each male was confronted with an ovulating female (see below). After 4 h of male–female interactions, the breeding baseline androgen level (BB) was sampled from the focal male,

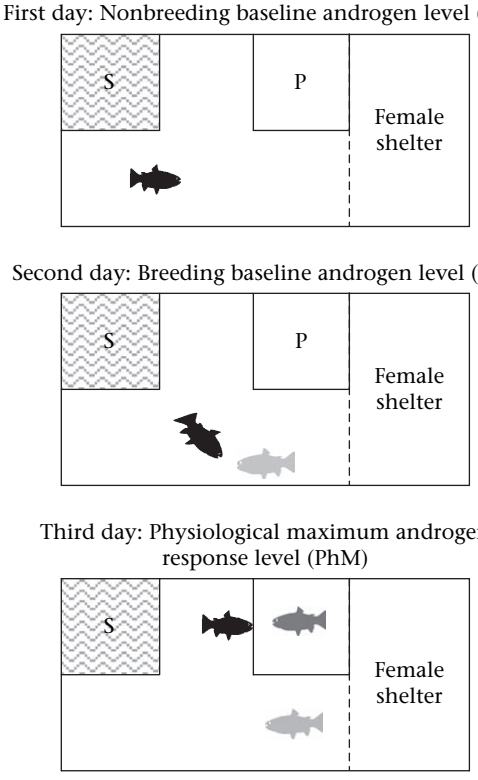

Figure 1. The experimental set-up during the 3 consecutive test days to assess the androgen responsiveness (PhM − NB)/(BB − NB) for each species. S: sampling container (bottom closed); P: presentation container (bottom open). Black: focal male; light grey: ovulating female; darker grey: intruder male.

which remained in visual contact with the female, as the sample container was inside the experimental tank. In the field, female mouthbrooders would leave the male's territory after a successful spawning. Because in the laboratory the males were observed to threaten the female after some hours of courting, we introduced a glass partition behind which the female remained until the next day (female shelter in Fig. 1). Water could flow underneath the partition, so visual–chemical contact between the male and the female continued, but physical injury was prevented.

On the third day, the focal male, with the female present, was confronted with an intruder male ('territorial challenge') which was placed inside the presentation container (Fig. 1) to prevent males fighting and injuring each other. After 1 h of interaction, the focal male was placed into the sample container to assess its physiological maximum androgen level (PhM). The males remained in visual contact throughout the sampling hour.

We collected a number of control samples. (1) We sampled the neutral 'pool water' from the empty tank whenever this was refilled. (2) A control male (NBc, BBc, PhMc and BBc0c) was always sampled simultaneously with the focal males. This control male went through the same procedures as test males did, but was not confronted with any conspecific (female or male) intruder. (3) The 'breeding baseline control' (BBc0): as the androgen response to territorial challenges is a short-term phenomenon, the hypothesis would predict that after removal of the challenger, the focal male's androgen levels should return to the breeding baseline. Therefore, on the fourth day, after the male had spent another night in visual–chemical contact with the female, we collected the BBc0 sample. This was not sampled for *O. mossambicus*.

The challenge hypothesis predicts variation in androgen responsiveness in relation to the mating system and the degree of paternal care at the interspecific level. Based on the experimentally induced variations in androgen levels, we attempted to test these predictions among the sampled cichlid species. To do this, we calculated (PhM − NB)/(BB − NB) (Wingfield et al. 1990) using the geometric means of all sampled individuals per test phase for each species. The androgen responsiveness of the five species sampled was then plotted by mating system.

Induced Ovulation

Ovulation was induced by treatment with LHRH (des-Gly^{10}[D-Ala^{6}]-Luteinizing Hormone-Releasing-Hormone-Ethylamide, Sigma-Aldrich L-4513, Sintra, Portugal; Donaldson & Hunter 1983; Canàrio & Scott 1990; Volkoff & Peter 1999) at a dosage of 70 μg LHRH/10 g of body weight in teleost Ringer solution as vehicle. This has been reported to induce ovulation after 48 h (Donaldson & Hunter 1983; Hirschenhauser et al. 2002). We injected each female intraperitoneally 48 h before the planned male–female observation. Ovulation was confirmed by inspection of the genital papilla swelling (Trewavas 1983) before we put the female in the experimental tank. The induction of ovulation using LHRH was approved by the Austrian national committee for the use of live animals in research. We observed no adverse effects caused by the LHRH treatment.

Hormone Assays

We collected 329 water samples from focal and control males during the experiments. From each sample we extracted the free steroid fraction and the corresponding glucuronides and sulphates following the procedures to measure steroids from fish urine described by Scott & Canàrio (1992) and Oliveira et al. (1996). We calculated the sum of all three fractions (free, sulphates and glucuronides), that is, total amounts of the steroid contained in each sample corrected for body mass and sampling volume (1 litre). We used radioimmunoassays to measure 11-ketotestosterone (11-KT), the major biologically active androgen in male teleosts (Kime 1993; Borg 1994), and testosterone (T). The details of the antibodies' cross-reactivities are given elsewhere (11-KT: Kime & Manning 1982; T: Scott et al. 1984). Intra-assay and interassay coefficients of variation were 8.2% and 11.6% for 11-KT and 7.5% and 12.4% for T, respectively.

To underline the validity of the androgen measurements from water, we present some technical tests of the assay system using samples from male tilapia. Figure 2a shows the binding specificity of the antibodies used. To show that our hormone measures reflect systemic levels, we injected male tilapia ($N = 12$) with LHRH (50 μg/kg fish intraperitoneally; Silverstein et al. 1999) and collected individual water samples (1 litre in which the fish had been placed for 2 h) before and after the physiological challenge. These samples were expected to reveal baseline and response androgen levels (11-KT and T). As controls, male tilapia were injected with teleost Ringer solution ($N = 7$) and went through the same procedures as the LHRH-treated males. The water levels of both 11-KT and T were increased 2 h after the treatment, remained elevated for at least 32 h, and were higher in LHRH-treated males than in control males (Fig. 2b, c). Therefore, we are confident that the androgen measurements from fish-holding water reliably reflect systemic levels of both 11-KT and T.

Statistical Analyses

All probability tests are two tailed. The androgen data did not follow a normal distribution in all the species (Kolmogorov–Smirnov tests: *O. mossambicus*: $Z = 0.96$, $N = 66$, $P = 0.286$ and $Z = 1.86$, $N = 66$, $P = 0.002$; *N. pulcher*: $Z = 0.71$, $N = 76$, $P = 0.692$ and $Z = 1.15$, $N = 76$, $P = 0.143$; *L. callipterus*: $Z = 1.25$, $N = 35$, NS and $Z = 1.75$, $N = 35$, $P = 0.004$; *T. moorii*: $Z = 1.86$, $N = 80$, $P = 0.002$ and $Z = 1.71$, $N = 80$, $P = 0.006$; *P. curvifrons*: $Z = 1.66$, $N = 72$, $P = 0.008$ and $Z = 2.42$, $N = 72$, $P < 0.0001$; 11-KT and T, respectively). We therefore used nonparametric tests. For each species we used Wilcoxon signed-ranks tests to test for differences between androgen

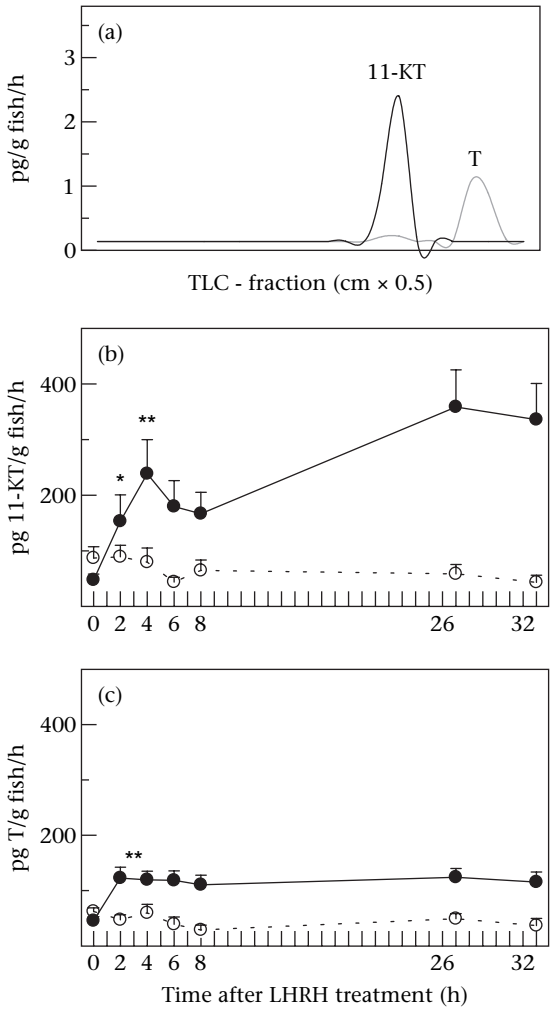

Figure 2. (a) Binding specificity of the 11-ketotestosterone (11-KT) and testosterone (T) antibodies used in the RIA of Tilapia-holding water. Water samples were separated by thin layer chromatography (TLC, silica gel). The mobile phases of these chromatograms were extracted, and all fractions tested for their immunoreactive binding to the 11-KT and T antibodies. (b) 11-KT levels in water holding male tilapia after LHRH (luteinizing hormone-releasing-hormone) treatment. Full lines: test males; dashed line: control males. (Wilcoxon signed-ranks test: 0 h versus 2 h: $T = 9$, $N = 12$, $P = 0.019$; 2 h versus 4 h: $T = 0$, $N = 12$, $P = 0.002$). Test versus control males at 32 h: Mann–Whitney U test: $U = 8$, $N_1 = 12$, $N_2 = 7$, $P = 0.003$). (c) T levels in water holding male tilapia after LHRH treatment. Full line: test males; dashed line: control males. 0 h versus 2 h: $T = 0$, $N = 12$, $P = 0.002$). Test versus control males at 32 h: $U = 12$, $N_1 = 12$, $N_2 = 7$, $P = 0.010$). (b) and (c) show means per sampling time $\pm$ SEM. Asterisks indicate significant differences between sampling times (*$P < 0.05$; **$P < 0.01$).

levels on consecutive test days for focal males. We adjusted the resulting probabilities to the number of multiple comparisons with identical data by using the post hoc Bonferroni correction. Friedman rank ANOVAs were used to check for overall effects of time and procedures on androgen patterns among the control males for each species.

RESULTS

Androgen Patterns Within Species

The interaction with an ovulating female evoked 11-KT increases from the nonbreeding to the breeding baseline in *O. mossambicus* and both Tropheini (Fig. 3). However, in both Lamprologini the 11-KT response to the presence of, and the interaction with, an ovulating female was not significant (Fig. 3), even though active courting interactions were observed and, for example, some females of *L. callipterus* deposited eggs in the snail shell provided during the experiment. In addition, female *T. moorii* and *O. mossambicus* were found with eggs in their mouths at the end of the experiment. In contrast, in none of the sampled species did the males respond with significant T increases from nonbreeding to breeding baseline levels (Fig. 4).

In males of all five species, the predicted 11-KT response to the challenge with a territorial intruder was observed, that is an increase from the breeding baseline to the physiological maximum level (Fig. 3). Again, this response was not observed with T (Fig. 4). Because the response to a territorial intruder should be short term, the focal males' 11-KT levels were predicted to return to breeding baseline levels after removal of the intruder male. In *N. pulcher* and *P. curvifrons*, 11-KT levels decreased significantly, whereas in *L. callipterus* this difference was only marginally significant and in *T. moorii* it was not significant (Fig. 3). With regard to T, the decrease was significant only in males of *P. curvifrons* (Fig. 4).

Among the control males of all sampled species neither the 11-KT nor the T patterns differed between the 4 experimental days (Figs 3 and 4). A similar analysis of the control androgen patterns is missing for *L. callipterus*, as only one control individual was available. The steroid content of the 'pool water' was negligibly low ($N = 9$ refillings; $\overline{X} \pm \text{SEM} = 64.29 \pm 9.05$ pg 11-KT and 149.67 ± 37.37 pg T; which corresponds to 3.10 ± 0.44 pg 11-KT/g fish and 7.22 ± 1.80 pg T/g fish, if corrected for the average body mass of all fish).

Interspecific Patterns of Androgen Responsiveness

As predicted, among the Lamprologini the monogamous *N. pulcher* males had higher rates of responsiveness than the closely related polygynous *L. callipterus*, and among the Tropheini rates were higher for the *T. moorii* males, which are polygynous with temporary pair formation, than for the lekking *P. curvifrons* (Fig. 5a). With regard to parental care, both Lamprologini were biparental and both Tropheini were maternal (Table 1). Males of the two biparental species seemed to have higher rates than males of species with exclusive maternal care (Fig. 5b). However, in addition to the very low sample size, we cannot exclude a phylogenetic bias, since we are comparing two Lamprologini with two Tropheini species.

Oreochromis mossambicus lacks a closely related counterpart in this study. Nevertheless, the androgen responsiveness of *O. mossambicus* males was the lowest observed in

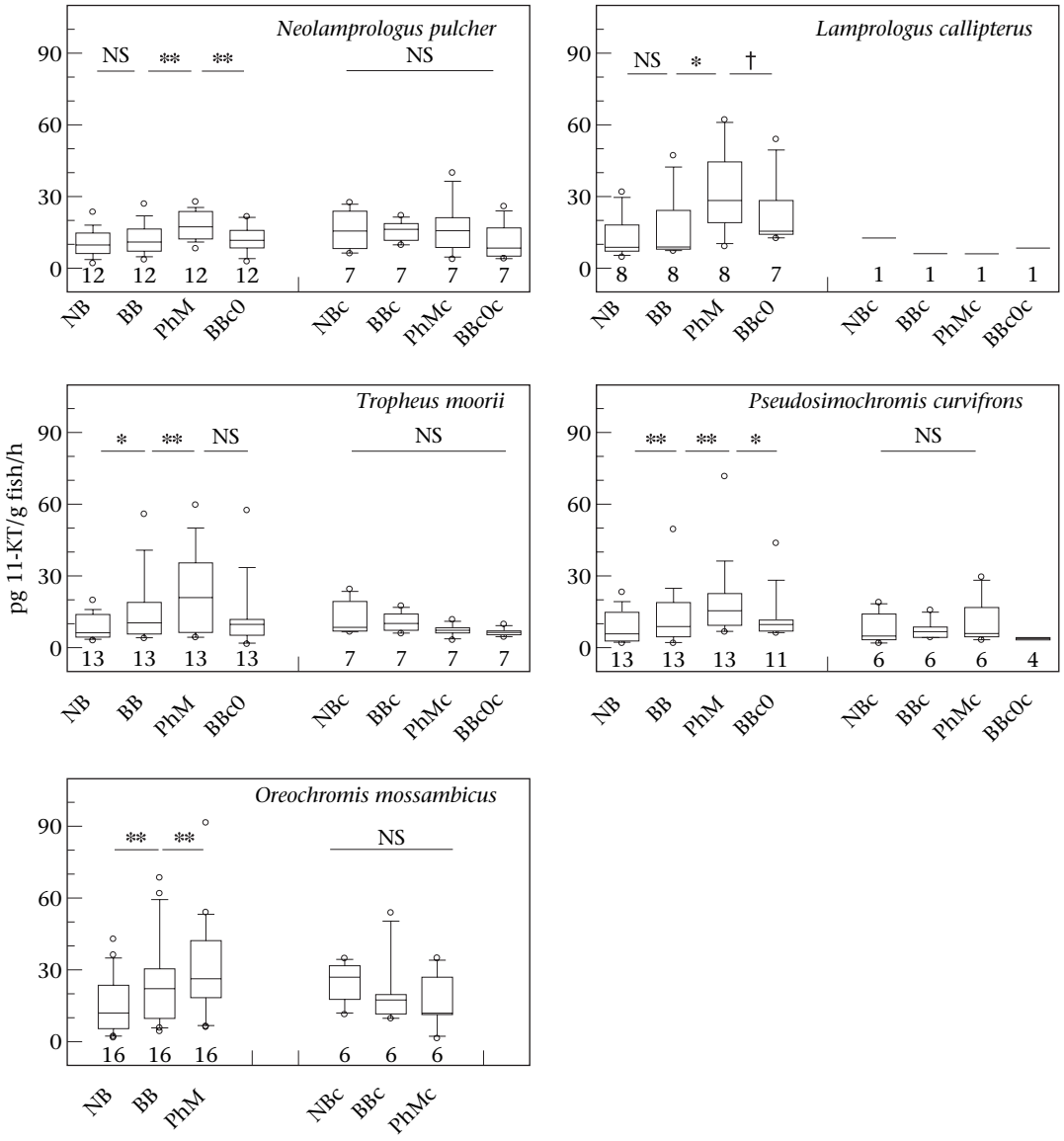

Figure 3. 11-Ketotestosterone (11-KT) patterns during the experiments. NB: nonbreeding baseline; BB: breeding baseline; PhM: physiological maximum in response to the intruder male; BBc0: breeding baseline control level after removal of the intruder male. NBc, BBc, PhMc, BBc0c: control males went through the same sampling procedures, but were not confronted with any conspecific (female or male) intruder. Geometric means are presented with the 25th and 75th percentiles, error bars show the 10th and 90th percentiles, and open dots are outliers. Numbers indicate sample sizes. All probabilities were corrected for multiple comparisons (Bonferroni). $^{\dagger}P = 0.054$; $*P < 0.05$; $**P < 0.01$. Wilcoxon signed-ranks tests: NB versus BB: *N. pulcher*: $T = 25$, NS; *L. callipterus*: $T = 10$, NS; *T. moorii*: $T = 5$, $P = 0.015$; *P. curvifrons*: $T = 6$, $P = 0.009$; *O. mossambicus*: $T = 6$, $P = 0.002$. BB versus PhM: *N. pulcher*: $T = 0$, $P = 0.006$; *L. callipterus*: $T = 0$, $P = 0.036$; *T. moorii*: $T = 3$, $P = 0.009$; *P. curvifrons*: $T = 3$, $P = 0.009$; *O. mossambicus*: $T = 16$, $P = 0.014$. PhM versus BBc0: *N. pulcher*: $T = 0$, $P = 0.006$; *L. callipterus*: $T = 0$, $P = 0.054$; *T. moorii*: $T = 15$, NS; *P. curvifrons*: $T = 2$, $P = 0.018$. 11-KT patterns among the control males Friedman rank ANOVAs: *N. pulcher*: $\chi^2_3 = 1.80$, NS; *T. moorii*: $\chi^2_3 = 6.94$, NS; *P. curvifrons*: $\chi^2_2 = 1.0$, NS; *O. mossambicus*: $\chi^2_2 = 5.33$, NS).

our sample, which is consistent with the predictions for a lek-breeding species with maternal brood care (Fig. 5).

DISCUSSION

Androgen Patterns Within Species

The 11-KT patterns resulting from our experiments in five cichlid species (Fig. 3) clearly met the intraspecific predictions originating from the avian literature (Wingfield et al. 1990, 2000; Hirschenhauser et al. 2003). However, the patterns for T did not (Fig. 4). This complements a large body of literature documenting that, for teleosts, 11-KT is the behaviourally relevant androgen rather than T (Kime 1993). 11-KT is found in higher levels in males than in females, whereas this is not usually the case for T, and 11-KT is generally more effective than T in stimulating secondary sexual characters, reproductive behaviour and spermatogenesis (Borg 1994). Furthermore,

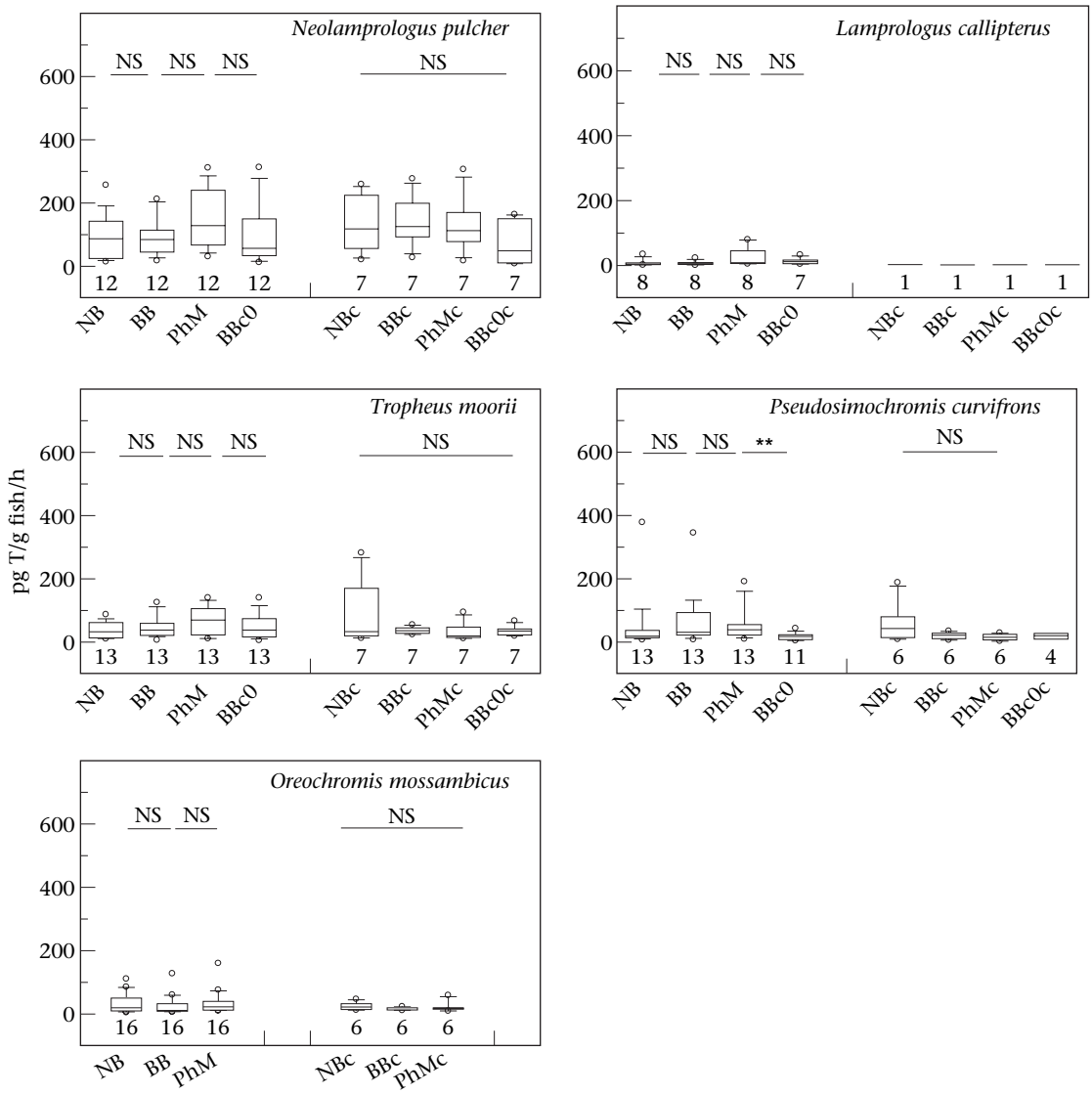

Figure 4. Testosterone (T) patterns during the experiments. For details see the legend of Fig. 3. Wilcoxon signed-ranks tests, NB versus BB: *N. pulcher*: $T = 38$, NS; *L. callipterus*: $T = 18$, NS; *T. moorii*: $T = 27$, NS; *P. curvifrons*: $T = 28$, NS; *O. mossambicus*: $T = 67$, NS. BB versus PhM: *N. pulcher*: $T = 15$, NS; *L. callipterus*: $T = 3$, NS; *T. moorii*: $T = 15$, NS; *P. curvifrons*: $T = 46$, NS; *O. mossambicus*: $T = 44$, NS. PhM versus BBc0: *N. pulcher*: $T = 20$, NS; *L. callipterus*: $T = 9$, NS; *T. moorii*: $T = 19$, NS; *P. curvifrons*: $T = 1$, $P = 0.009$; control males: Friedman rank ANOVAs: *N. pulcher*: $\chi^2_3 = 5.57$, NS; *T. moorii*: $\chi^2_3 = 0.77$, NS; *P. curvifrons*: $\chi^2_2 = 5.33$, NS; *O. mossambicus*: $\chi^2_2 = 0.33$, NS).

the androgen patterns determined from fish-holding water after both physiological and behavioural challenges demonstrate that using this noninvasive method may yield biologically meaningful data.

In all species tested, the males responded with significant 11-KT increases to the challenge by a territorial intruder. On the other hand, 11-KT responses to the interaction with an ovulating female were not consistently observed. Among both species of Lamprologini (*N. pulcher* and *L. callipterus*), the responses to the interaction with females were not significant, but in both, a day after the clear response to the territorial intruder, 11-KT levels had returned to the low BBc0 (although in *L. callipterus* this difference was only marginally significant; Fig. 3). This pattern was different for the two species of Tropheini, which responded with a significant increase in 11-KT from NB to BB in response to the female and with a further increase in response to the intruder. In some of the *T. moorii* males, 11-KT levels had not returned to the BB levels on the next day, whereas in the *P. curvifrons* males it clearly did so (Fig. 3). Both Tropheini are polygynous with no paternal contribution, in which case the challenge hypothesis would predict high levels of BB androgen levels over a longer period. For the biparental, monogamous *N. pulcher* and the biparental, polygynous *L. callipterus* the challenge hypothesis would predict low BB, but the males should remain responsive to intruders. Therefore, these data are consistent with our expectation of an effect of paternal investment on the observed androgen response patterns at the intraspecific level. The observed lack of a significant androgen response to an ovulating female among male Lamprologini may be explained by

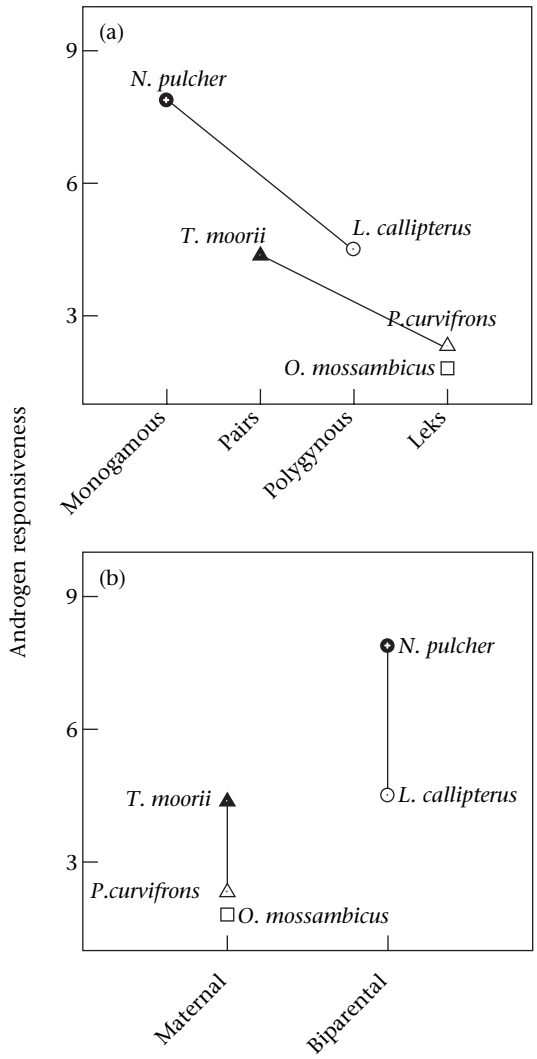

Figure 5. Interspecific patterns of the androgen responsiveness (see text for calculation) in all species sampled plotted by (a) mating system and (b) parental care. 11-KT geometric means of all sampled individuals per test phase were used for each species.

their contribution to the care of eggs and young, which naturally involves (at least a temporary) pair formation. Even though our set-up involved only 4 h of pair formation and interaction, but no brood care phases, there was no increase in BB androgen levels in the biparental Lamprologini.

Monogamous and paternal males may still remain highly responsive throughout the breeding phases, but they are predicted to return to the low BB androgen levels quickly in order not to interfere with the expression of paternal care. Our results provide two inconsistencies with this expectation: first, the only marginally significant difference between PhM and BBc0 levels in the biparental *L. callipterus* and, second, the clear decrease from PhM to BBc0 levels in the nonpaternal *P. curvifrons* males (Fig. 3). Explanations for these inconsistencies remain speculative. The lack of a significant result in *L. callipterus* may be the result of the small sample size. However, the unexpected return to low BB levels in *P. curvifrons* lacks an explanation.

The degree of paternal care may not be the only modulator of magnitude and duration of the androgen response to intruders. Alternative explanations for differences in the sensitivity to male or female challenges may include specific ecological constraints and life histories, which remain to be elucidated in field studies. Nevertheless, the observed variation in 11-KT response to interaction with females is in accordance with results from a literature survey on 59 teleost species in which the interspecific patterns of androgen responsiveness in relation to mating system were associated systematically with variation in BB levels rather than in PhM levels (Oliveira et al. 2001a). Further evidence for a systematic variation in response to females with the parental care pattern comes from a meta-analysis of published data from 55 vertebrate species of different taxa, where the effect of the interaction with females on androgen levels was largest among males of species with only maternal care, and decreased the more paternal care was provided (K. Hirschenhauser & R. F. Oliveira, unpublished data).

Interspecific Patterns of Androgen Responsiveness

This study allowed us to compare patterns of androgen responsiveness in two pairs of closely related haplochromine cichlids that do not share the same mating system. Among the (biparental) Lamprologini, responsiveness was higher in the monogamous *N. pulcher* that in polygynous *L. callipterus*, and among the Tropheini it was higher in the polygynous but temporary pair-forming *T. moorii* than in the lekking *P. curvifrons*. These results suggest that among cichlids androgen responsiveness may not only show larger rates in males of monogamous species, but also vary with pair bonding. In the Tropheini, the species with some pair bonding showed higher rates (i.e. *T. moorii*, Fig. 5a). Despite the absence of a closely phylogenetically related counterpart, *O. mossambicus* had the lowest rates in our sample, giving further support to the predictions of the challenge hypothesis for a lekking species with no paternal investment.

Relatively few studies have tested the predictions of the challenge hypothesis in nonavian vertebrate species. Among reptiles the predicted low androgen responsiveness to (staged) male–male agonistic encounters for promiscuous species with no paternal care has been observed in most cases (Thompson & Moore 1992; Schuett et al. 1996; Klukowski & Nelson 1998; but see Greenberg & Crews 1990). The results of amphibian (reviewed in Houck & Woodley 1995), teleost (Pankhurst & Barnett 1993; Oliveira et al. 1996, 2001b, 2002; but see Cardwell & Liley 1991; Ros et al. 2003) and mammalian (Cavigelli & Pereira 2000; Nunes et al. 2000; but see Creel et al. 1993; Lynch et al. 2002) studies, however, have been equivocal. The heterogeneous conclusions of these studies may be explained by methodological differences and the lack of a uniform test protocol. However, the challenge hypothesis combines the roles of territorial aggression, mating and paternal behaviour in modulating androgen responsiveness. At the interspecific level, tests of the predicted effects of mating system and the degree of paternal care on

responsiveness are limited to taxa with sufficient variation in their social systems. The prevalence of promiscuity and maternal care in mammals, for example, has so far prevented quantitative tests in this group. In contrast, sufficient variance in social systems may be explored among fish (Oliveira et al. 2001a, 2002) and birds (Hirschenhauser et al. 2003). In both taxa, the effect of mating system on responsiveness has been confirmed, whereas the effects of paternal investment have turned out to be more complex. The present study adds experimental evidence for the modulation of androgen responsiveness by mating and paternal behaviour at both the intraspecifc and interspecific levels. This supports the validity of the challenge hypothesis in nonavian vertebrates. Further quantitative examination of the androgen patterns predicted by this hypothesis is needed for other vertebrates, however.

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