

Short communication

Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*

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Abstract

In vertebrates, circulating androgen levels are regulated by the hypothalamic-pituitary-gonadal (HPG) axis through which the brain controls the gonads via the pituitary. Androgen levels ultimately depend on factors including season, temperature, social circumstance, age, and other variables related to reproductive capacity and opportunity. Previous studies with an African cichlid fish, *Astatotilapia burtoni*, suggested that changes in both testosterone and 11-ketotestosterone (11-KT), an androgen specific to teleost fish, depend on male social status. Here we characterize circulating plasma concentrations of testosterone and 11-KT in socially dominant (territorial) and socially subordinate (non-territorial) males. Territorial males have significantly higher circulating levels of both forms of androgen, which is another defining difference between dominant and subordinate males in this species. These results underscore how internal and external cues related to reproduction are integrated at the level of the HPG axis.

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In all animals, the orchestration of external and internal factor governs reproductive success. Reproductive behavior and physiology can be regulated by environmental factors including season, temperature, and day length as well as by the social environment. This regulation influences many physiological variables including hormone levels and changes in the brain.

Androgens are of primary interest in brain–hormone interactions because of their relationship to reproduction, which ultimately describes the evolutionary fitness of an individual. Within a social context, it has been suggested that androgen secretion during episodic aggression can increase an individual's circulating androgen levels above a breeding season baseline [1]. According to the challenge hypothesis, dominant males in tournament species, which are polygynous and have low rates of paternal care and who constantly defend a territory by engaging in more aggressive bouts, will display

higher androgens levels than submissive males, who do not engage in territory disputes.

Importantly, several studies have correlated aggressive behavior with androgens in fish species. In the stoplight parrotfish (*Sparisoma viride*), for example, male intrusions into territories of established territorial males induce androgen levels to increase [2]. Also, in territorial male demoiselles (*Chromis dispilus*), the frequency of territorial interaction is correlated with androgen levels [3]. Among cichlids, St. Peter's fish (*Sarotherodon galilaeus*) display elevated androgen levels as well as increased male–male aggression in groups where the ratio of males to females is large as compared with groups with equal numbers of males and females or with a higher ratio of females to males. This correlation suggests that increased aggression depends on social context and is correlated with increased androgen level [4]. Interestingly, Hirschenhauser et al. [5] showed that the predictions of the challenge hypothesis concerning territory intrusions hold for 11-KT but not testosterone in five cichlid species (*Neolamprologus pulcher*, *Lamprologus callipterus*,

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Tropheus moorii, *Pseudosimochromis curvifrons*, and *Oreochromis mossambicus*) [5].

Astatotilapia burtoni, an African cichlid, is a good model system in which to test predictions put forth by the challenge hypothesis and supported by the correlation between androgens and aggression cited above. Male *A. burtoni* exist in one of two distinct social phenotypes: dominant (territorial, T) males which comprise approximately 10–30% of the population and subordinate (non-territorial, NT) males which make up the remaining 70–90% of the population [6]. *A. burtoni* live in a lek-like social system in shore pools of Lake Tanganyika, Africa. T males are brightly colored, large, reproductively capable and defend territories containing a food resource used to entice females to spawn with them. NT males are camouflaged, smaller, have regressed gonads, and school with females [7]. Importantly, males shift between these social states depending on their success in aggressive encounters [8]. Thus aggressive behavior plays a key role an individual's social rank, which in turn allows access to reproductive opportunity. Females are smaller than males and following spawning in the territory of a T male, brood the young in their mouths for ca. 2 weeks.

In order to understand if androgen patterns in *A. burtoni* follow those reported in other cichlid species and predicted by the challenge hypothesis, we measured androgen levels of males in both social states. Androgen levels have been correlated with dominance rank in some fish species, although the strength of this correlation depends on the social system of the species. At present, data exists for three cichlid species with respect to androgen level as a function of status. In another African cichlid, *Oreochromis mossambicus*, territorial males had significantly higher rates of androgen excretion as compared to non-territorial males [9]. However, in a cooperatively breeding cichlid, *Neolamprologus pulcher*, helper males ascend in status via a queuing system, and do not differ in their androgen excretion from territorial males [10] (see Table 1). Further, earlier observations on sword-tail fish showed that status-linked differences in androgen level did not exist in times of social stability [11]. These results suggest that the correlation between androgens and social status depends on the importance of aggressive competition for rank acquisition. Several salmonid species also show elevated androgen levels among dominant versus sub-

missive males including *Onchoryncus mykiss*, *Salmo trutta*, and *Salvelinus alpinus* [12, Table 1].

In *A. burtoni*, both testosterone and 11-KT have been linked to territorial behavior [13]. It has previously been shown that intramuscular injection of testosterone in *A. burtoni* T males significantly increases approach and attack toward other animals and intensifies coloration patterns [14]. Further, when *A. burtoni* T males are castrated to eliminate circulating androgens, GnRH1 containing neurons enlarge dramatically. Androgen replacement (either testosterone or 11-KT) returns these neurons to normal size [13]. This confirms the presence of feedback regulation of GnRH1 production by androgens typically found in vertebrates. Although Soma et al. [13] discovered androgen regulation of GnRH1 containing neurons in T males, the androgen levels of NT males have not yet been studied [13]. Here, we measured circulating levels of both testosterone and 11-KT in T and NT males to understand the interaction between androgens and social status. This discovery will illuminate the study of androgens with respect to aggression and social context.

1. Materials and methods

1.1. Subjects

Seventeen *A. burtoni* males (9 T's and 8 NT's) were used in this study. Subjects were originally derived from wild-caught stock in Lake Tanganyika, East Africa [5]. Prior to the experiment, fish were kept in aquaria under conditions similar to those of their natural environment (28 °C, pH 8 and a 12:12h light–dark cycle using full spectrum illumination). Fish were fed each morning ad libitum with cichlid pellets and flakes (AquaDine, healdsburg, CA). Animals were provided with gravel covering the floor of the aquaria and terra cotta pots were placed in each aquarium to facilitate establishment of territories.

Each community tank held 2–4 T males, 2–4 NT males, and 5–12 females. Animals were treated at all times in accordance with Stanford's Administrative Panel for Laboratory Animal Care (APLAC) approved animal treatment policies.

Each subject was observed for 3 min three times per week for at least 1 week prior to selection. Behavioral patterns characteristic of T or NT males were recorded, including, in order of importance, establishment and defense of a territory, coloration, and aggressive and sexual behavioral displays. Due to dynamic social conditions, T males often defend poorly defined or variable territories. Thus, to ensure that males were of full T status, only those males defending a discernable territory were considered territorial.

1.2. Plasma collection

Following subject identification, blood samples (between 50 and 100 µl) were taken from each male using heparinized capillary tube butterfly needles. Blood was collected from the first caudal artery within 4 min of removal from the tank, and centrifuged for 3 min at 13,000 rpm to separate plasma from red blood cells. Plasma was then isolated and stored at –80 °C until assayed. After blood collection, subjects were immediately returned to their community tanks.

Table 1
Androgen correlates of social status among cichlid fish

Cichlid species	11-KT	Testosterone	Author
<i>O. mossambicus</i>	D > S	D > S	[9]
<i>N. pulcher</i>	T = H	T = H	[10]
<i>A. burtoni</i>	T > NT	T > NT	

O. mossambicus and *A. burtoni* both show higher 11-KT and testosterone levels among territorial/dominant versus non-territorial/submissive males. The cooperative breeder, *N. pulcher* shows no difference between territorial and helper males in either 11-KT or testosterone levels.

1.3. Testosterone assay

Plasma testosterone concentration was measured using an ELISA (Assay designs Inc., testosterone correlate-EIA Kit, Ann Arbor, MI). Plasma samples were thawed and diluted in a 1:30 solution with assay buffer. The ELISA kit protocol was then strictly followed with the exclusion of heating the plate to 37 °C and placing it on a plate shaker during the second incubation instead of letting it sit at room temperature. In validation trials with *A. burtoni* plasma, this step was found to greatly reduce inter-well variability. Plates were read (Molecular Devices Vmax Microplate Reader) at 405 nm.

1.4. Keto-testosterone assay

Plasma 11-KT concentration was also measured using an ELISA (Cayman Chemical). A 10 µl sample of plasma from each subject was extracted twice using 200 µl diethyl ether. In validation trials with *A. burtoni* plasma, this step was found to greatly reduce inter-well variability and increase the detection capacity of the kit. The diethyl ether was then evaporated over nitrogen and samples were stored overnight in desiccation at −20 °C. Prior to the assay, samples were reconstituted with 300 µl assay buffer. Following these procedures, the kit protocol was strictly followed. Plates were read (Molecular Devices Vmax Microplate Reader) at 405 nm.

2. Results

T males have a mean circulating testosterone level of 288.4 ± 62.2 pg/µl (mean \pm S.E.M.) and a mean circulating level of 11-KT of 14.3 ± 4.6 pg/µl. In contrast, NT males have a mean circulating testosterone level of 59.1 ± 26.0 pg/µl and mean circulating 11-KT level of 1.7 ± 0.7 pg/µl (see Figs. 1 and 2). When these data were subjected to a one-way ANOVA, territorial males were found to have significantly higher levels of testosterone ($[F(1,23) = 9.557, p = 0.005]$) and 11-KT ($[F(1,21) = 7.596, p = 0.012]$) than non-territorial males.

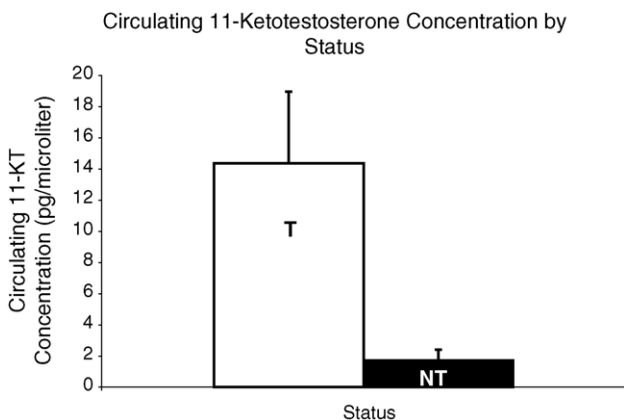


Fig. 1. Circulating levels of 11-KT by status. Territorial males have a mean circulating level of 11-KT of 14.3 ± 4.6 pg/µl. NT males have a mean circulating 11-KT level of 1.7 ± 0.7 pg/µl ($[F(1,21) = 7.596, p = 0.012]$).

Circulating Testosterone Concentration by Status

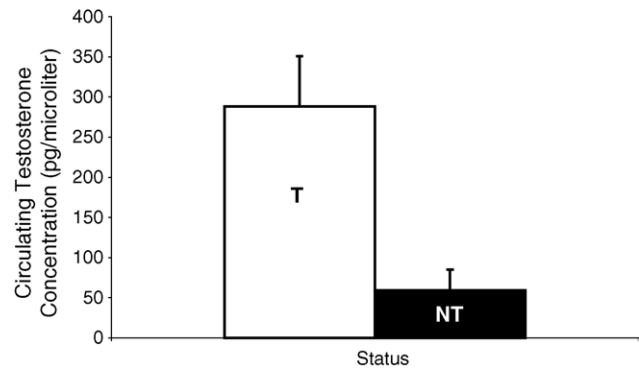


Fig. 2. Circulating levels of testosterone by status. Territorial males had a mean circulating testosterone level of 288.4 ± 62.2 pg/µl (mean \pm S.E.M.). NT males had a mean circulating testosterone level of 59.1 ± 26.0 pg/µl ($[F(1,23) = 9.557, p = 0.005]$).

3. Discussion

Our data show that, in *A. burtoni*, dominant (T) males have significantly higher circulating levels of both testosterone and 11-KT than do subordinate (NT) males. This is consistent with data from *O. mossambicus*, a cichlid species in which dominant males express higher androgen levels than submissive males [9]. In *A. burtoni*, in a stable social context, T males engage in aggressive behavior toward other members of the community in contrast to NT males, and also have access to reproductive opportunities, whereas, NT males do not. Further, our data support the observation in *A. burtoni* that intramuscular injection of testosterone can increase aggressive behavior among males [14].

This data is also in accordance with teleost data showing no androgen difference in cooperative breeders [10]. Because *A. burtoni* are polygynous and because rank ascent depends on aggressive behavior, we would expect that androgen level would be different between dominant and submissive males, whereas, in species that use queuing systems and where status acquisition does not depend on aggression (e.g., *N. pulcher*) we would expect there to be no difference in androgen expression between territorial and helper males.

Status acquisition often occurs during times of social instability, which exist in the field and have been simulated in the laboratory [8,15,16]. During social instability, such changes in social hierarchy are necessarily coupled with an increase in aggressive behavior, as some males who were previously NT typically increase aggressive behavior to acquire territories while males who were previously T often struggle to maintain territories.

The challenge hypothesis is a useful framework for analyzing how stable androgen levels might arise after a period of social instability. In a stable social situation where status is highly dependent on aggressive encounters, one might expect to find higher androgen levels in dominant males who engage in more aggressive bouts in defense of a territory. But

in times of social instability when aggression is increased in all members of a community as discussed above, the challenge hypothesis would predict that an individual's androgen levels would rise and then either fall or increase to the respective level of his later established status. When this prediction was tested, indeed, the correlation of androgen level with social status does appear after a social stabilization [8].

As noted, *A. burtoni* live in a lek-like social system, and thus, two distinct social states exist among males: T and NT. Further, males can and do shift between these two states given social opportunity. When NT males ascend in status, their behavior changes immediately as they challenge other males aggressively and display T coloration including an eyebar within minutes (Burmeister et al., submitted). The aggressive behavior displayed by NT males ascending in status is posited by the challenge hypothesis, and shown empirically in the research reviewed above to be correlated with androgen output. It is possible that androgen feedback from aggressive encounters, especially winning, contributes to the increased circulating androgens levels as males ascend in social status.

However, the reproductive axis of non-territorial males is effectively shut down, and, as we show here, under stable social circumstances, NT males have very low androgen levels. Further, the physiological changes reflecting maturity of a T male take approximately 1 week to complete during social ascent [7]. If the challenge hypothesis were to hold in this species, this would mean that an alternate source of androgens must be available to non-territorial males over a short time scale (e.g., days) corresponding to this initial aggressive behavior.

In song birds, neural steroid metabolism has been implicated in non-breeding season aggression [17]. If this were the case in *A. burtoni*, brain aromatase reactions might produce the androgens that are expected to be coupled with the expression of the aggressive behavior necessary for status ascent long before their HPG axis has fully matured. Another possibility is that there are alternate organ systems that could deliver androgens. For example, following castration in *A. burtoni*, Soma et al. [13] showed that testosterone levels do not immediately decrease significantly in T males, suggesting stimulation of other sources such as the interrenal gland or liver might transiently produce this androgen [13]. It will be interesting to discover whether neurosteroids or other glands support transiently increased testosterone levels associated with aggressive behavior. Importantly, here, we have shown that these factors do not act to increase a submissive male's androgen levels at baseline. We only posit here that they may be involved in increasing his androgen levels during times of increased aggression in response to social opportunity.

Because the challenge hypothesis was proposed for avian model systems with distinct breeding seasons, it is possible that it would not apply to *A. burtoni*. In an equatorial species of sparrow, *Zonotrichia capensis*, with an extended breeding season, the challenge hypothesis did not predict androgen

response to simulated territory intrusions [18]. Similarly, *A. burtoni*, breeds year-round in its natural habitat at the equator suggesting that it may not support this hypothesis.

However, the challenge hypothesis has been tested in other cichlid models, and does hold for 11-KT in polygynous species (*Neolamprologus pulcher*, *Lamprologus callipterus*, *Tropheus moorii*, *Pseudosimochromis curvifrons*, and *Oreochromis mossambicus*) [5]. However, we have found here that both 11-KT and testosterone are significantly higher in T males than NT males. Our results as well as other literature indicate 11-KT as the behaviorally active androgen in teleosts, suggesting that the differences arising in 11-KT as the social situation stabilizes may be attributable to the challenge hypothesis [19]. However, the difference in testosterone level between these social states may be an artifact of the regressed HPG axis of NT males. The possibility remains, given testosterone injection data cited above [14] that both 11-KT and testosterone are behaviorally active androgens in *A. burtoni* and both act according to the challenge hypothesis.

Our data show androgen differences in socially determined behavioral states and suggest that social information taken together with endogenous endocrine cues contribute to social status. But how do community dynamics interact with internal physiological variables in a population? There are several possible mechanisms that could underlie these results. For example, both androgens and their receptors could play a role in the physiological and behavioral changes during a shift in social status. Future experiments will examine androgen receptor expression patterns as a function of social status to understand the connection between circulating androgen levels and status differences. By manipulating the social context and behavior of *A. burtoni* it may be possible to unravel the causal relationship between androgen levels, social status and social stability.

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