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11-Ketotestosterone Inhibits the Alternative Mating Tactic in Sneaker Males of the Peacock Blenny, *Salaria pavo*

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Key Words
Fish · Alternative reproductive tactic · Androgens · 11-Ketotestosterone · Arginine vasotocin · Relative plasticity hypothesis

Abstract
In the peacock blenny, *Salaria pavo,* a species with courtship sex-role reversal, smaller, younger males mimic the courtship behavior and the nuptial coloration of females in order to get access to nests during spawning and to parasitize egg fertilization from nest-holder males. Later in their life, sneakers transform both morphologically and behaviorally into nest-holder males. In the present paper we investigate the activational role of 11-ketotestosterone (KT), the most potent androgen in most teleost species, to promote the switch between tactics in sneaker males of *S. pavo.* Sneakers were implanted either with KT or with control (i.e. castor oil) silastic implants. A week after implantation they were subjected to a set of behavioral tests and morphometric measurements. KT treatment promoted the differentiation of secondary sex characters, such as the anal glands, and inhibited the expression of female courtship behavior. KT-treated sneakers also showed a trend toward less frequent display of female nuptial coloration. There was no effect of KT treatment on the expression of typical nest-holder male behavior. Finally, there was no effect of KT treatment on the number or soma size of arginine vasotocin neurons in the preoptic area, which are often associated with the expression of vertebrate sexual behavior. Thus, KT seems to play a key role in mating tactic switching by inhibiting the expression of female courtship behavior and by promoting the development of male displaying traits (e.g. anal glands). The lack of a KT effect on behavior typical of nest-holding males and vasotocinergic preoptic neurons suggests that a longer time frame or other endocrine/social signals are needed for the initiation of these traits in males that are switching tactics.

Introduction

Androgens are known to be involved in the control of social behavior in vertebrates both in terms of organization and activation [Arnold and Breedlove, 1985]. In a number of teleost species the most potent androgen is 11-ketotestosterone (KT), which has been shown to be involved in the expression of male mating behavior [e.g. sticklebacks, *Gasterosteus aculeatus*, Borg, 1987; bluegill sunfish, *Lepomis macrochirus*, Kindler et al., 1991], the differentiation of male secondary sex characters [e.g. breeding colors in sockeye salmon, *Oncorhynchus nerka*, Idler et al., 1961; development of gonopodium in platyfish, *Xiphoporus maculatus*, Schreibman et al., 1986; kidney hypertrophy in *G. aculeatus*, Borg et al., 1993] and spermatogenesis [e.g.

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Schreibman et al., 1986; Kobayashi et al., 1991; Miura et al., 1992; for a general review of androgens in teleost fishes see Borg, 1994]. In some teleost species alternative mating tactics occur: (a) 'bourgeois' males that actively compete among themselves, investing in the acquisition of mates (e.g. by defending breeding territories); and (b) 'parasitic' males that exploit the investment of bourgeois males to get access to females and fertilize eggs [Taborsky, 1997]. In the species studied so far these alternative phenotypes differ in their circulating KT levels but no clear pattern for testosterone has been found [i.e. in 3 out of 6 published studies the T levels were higher in the bourgeois males, in 2/6 of the studies were higher in the parasitic males and in 1/6 of the published studies were equivalent between the two male mating types; Brantley et al., 1993a]. In a number of species from different families (i.e. Salmonidae: Atlantic salmon, *Salmo salar;* Centrarchidae: bluegill sunfish, *Lepomis macrochirus;* Scaridae: stoplight parrotfish, *Sparisoma viride;* Labridae: saddleback wrasse, *Thalassoma duperrey;* Batrachoididae: plainfin midshipman, *Porichthys notatus;* Cichlidae: Mozambique tilapia, *Oreochromis mossambicus;* Blenniidae: rock-pool blenny, *Parablennius sanguinolentus parvicornis*) bourgeois males have significantly higher levels of circulating KT than those adopting a parasitic tactic [i.e. sneakers, streakers or satellites; Brantley et al., 1993a; Oliveira et al., 1996, 2001a], suggesting that either KT plays a major role in the expression of the male bourgeois tactic, or that KT levels are highly responsive to the expression of the tactic itself. The fact that in some teleost species females have higher testosterone levels than males [e.g. winter flounder, *Pseudopleuronectes americanus,* Campbell et al., 1976; rainbow trout, *Oncorhynchus mykiss,* Scott et al., 1980], although KT is specific to males in most species, further supports the view that KT is probably involved in the expression of the bourgeois tactic as a whole or in the expression of some of its components (e.g. male secondary sex characters).

In some teleost species with alternative mating tactics, individuals first reproduce using one of the tactics and later in life switch to the alternative tactic and breed again [i.e. sequential mating tactics; e.g. *Pomatoschistus microps,* Magnhagen, 1992]. To our knowledge all the described examples in the literature of sequential tactic switching always occur from the parasitic to the bourgeois tactic. From the level of analysis of ultimate causation the sequential alternative mating tactics have been viewed as conditional strategies, in which the reproductive success of the individuals following each of the alternative tactics changes with age or size in such a way that it pays off to switch tactic at the point when the two functions cross [Gross, 1996]. From the level of analysis of proximate causes it is relevant to investigate which signal can inform the organism that it has achieved the tactic switching point. Sex steroids are good candidates for such a role as they are already involved in a number of mechanisms underlying the mode of reproduction of the individual such as investment in secondary sex characters and in gonadal tissue. As explained above, among the sex steroids KT is a good candidate for such a role.

The peacock blenny, *Salaria pavo,* is an intertidal blenniid species common in the rocky shores of the Mediterranean sea [Zander, 1986]. Males defend a nest in a crevice and only the male provides parental care. Each male receives eggs from more than one female in its nest and each female spawns batches of eggs in different nests [Fishelson, 1963; Patzner et al., 1986]. In Southern Portugal a population of *S. pavo* occurs in a sandy coastal lagoon (Ria Formosa) and presents a unique set of reproductive traits. Due to a shortage of suitable nesting substratum there is very intense competition among males for nest sites and as a result only large males are able to establish nests and nest-holder males seldom leave their nests, even to court females [Almada et al., 1994; Oliveira et al., 1999]. Thus, courtship sex-role reversal occurs with females playing an active role. Females court males by adopting a nuptial coloration and presenting their flanks to the nest-holder males at the entrance of the nest while flicking the pectoral fins and open-and-closing the mouth with rapid movements [Almada et al., 1995]. Smaller and younger males engage in sneaking and adopt female-like behaviors and nuptial coloration, a behavior that facilitates approaching and entering the nests during a spawning episode [Gonçalves et al., 1996]. Sneaker males have functional testes and sperm can be collected from their vas deferens [Gonçalves et al., 1996]. However, sneaker males lack or have only a vestigial testicular gland, an accessory structure to the gonad apparently involved in the sperm maturation in nest-holder males [Lahnsteiner and Patzner, 1990].

These differences in behavior and reproductive biology between nest-holders and sneakers have a neuroendocrine correlate in the arginine vasotocin (AVT) system in the forebrain preoptic area (POA). Although there are no differences in the number of AVT immunoreactive (AVT-ir) cells between the two male morphs, both male morphs have more AVT-ir cells than females and sneaker males have higher AVT-mRNA expression in the POA compared to nest-holders [George et al., 1999]. AVT is a neurohypophysial neuropeptide that has been implicated in the expression of reproductive behavior in all non-mammalian vertebrates [Moore, 1992]. Sexual dimorphism has been described in AVT neuronal systems in fish [e.g. Reavis and Grober,

1999], amphibians [e.g. Moore and Lowry, 1998] and birds [e.g. Viglietti-Panzica et al., 1992] and also in AVP homologue systems in mammals [e.g. Zhou et al., 1995; for a general review see Moore, 1992]. In teleost species with male alternative mating tactics intra-sexual differences in AVT containing neurons in the POA have also been described that parallel the differences found in mating behavior [e.g. Foran and Bass, 1998]. Similarly, in our study population of *S. pavo* courtship behavior is most vigorous in females and sneaker males and these two morphs exhibit higher levels of AVT mRNA expression relative to nest-holder males [George et al., 1999].

Otolith readings indicate that *S. pavo* born early in a spawning season can become reproductively mature and behave as sneaker males in the same spawning season, whereas other sneakers males were born in the previous breeding season [Gonçalves et al., 1996]. Mark-recapture in the field indicates that males that reproduced as sneakers in a given year can switch tactics and breed as nest-holders in the following years [D.M. Gonçalves and R.F. Oliveira, unpubl. data]. Thus, sequential alternative mating tactics are present in this species.

The present paper investigated the effect of KT on tactic switching in *S. pavo*. For this purpose sneaker males were implanted with silastic implants filled with KT dissolved in castor oil or with castor oil only (control) and their effects on mating behavior in captivity, forebrain neurochemistry and the differentiation of male secondary sex characters were investigated.

Materials and Methods

Origin and Maintenance of the Fish

Seventeen sneaker males (standard length: mean ± STD = 6.3 ± 0.6 cm, min = 4.9 cm, max = 7.2 cm) were captured at Culatra Island (36°59′N, 7°51′W; Ria Formosa Natural Park) during low tide and transported to the laboratory at ISPA (Lisbon) where they were kept in a community tank at room temperature (20 ± 2 °C) and natural photoperiod (14L:10D). Because not all males of this size class are sexually active and breeding as sneakers, we used two criteria to classify males as sneakers (i.e. small sexually active males lacking male secondary sex characters): (a) sperm collection by applying a slight pressure on the ventro-lateral surface of their flanks; (b) a posteriori gonad development was assessed. Fish were fed daily (except the day before the surgery and the day of the behavioral tests) with common cockles (*Cerastoderma* spp.). The experiments were conducted in June and July 1997 during the breeding season.

Steroid Implants

Implants were placed in the peritoneal cavity via a surgical incision at the base of the ventral fins. Silastic implants (1.47 mm inner diameter, 1.96 mm outer diameter, 10 mm length; Silicone, type A, Dow Corning) contained either 20 µl of 5 mg of KT (Sigma-Aldrich, Madrid, Spain) per ml of castor oil (KT-treated group, n = 9) or only the castor oil vehicle (control group, n = 8). The concentration used in the implants was calculated according to the dosage (amount of administered KT per body weight) used by Kobayashi et al. [1991]. The individuals were anaesthetized with MS222 (tricaine methanesulfonate, Sigma-Aldrich) for the surgical procedure and recovered with extra aeration in small tanks. They were then placed in separate tanks (15 × 30 × 30 cm) to avoid any risks of infection in the post-surgical period and to control for effects of past experience in the subsequent behavioral tests. Each tank had an artificial nest placed in its center. There was no mortality during or after the surgical procedure.

The experiment lasted seven days, a duration considered appropriate on the basis of experiments with similar implants in other species in which measurable effects were detected after 5–6 days [e.g. color change from the female to the male pattern in treated female *Thalassoma bifasciatum* 6 days post-treatment, Grober and Bass, 1991; differentiation of a male-like genital papilla in treated female *Lythrypnus dalli* 5 days after the treatment, Carlisle et al., 2000].

Due to the small size of the individuals used in the experiment it was not possible to collect blood to assay circulating levels of KT. In other studies with small fish that did not allow blood collection authors have used whole-body concentrations of steroids or gonadal levels to compare treatments [e.g. Siamese fighting fish, *Betta splendens*, Leitz, 1987; green swordtail, *Xiphophorus helleri*, Hannes, 1984]. Thus, we decided to assay KT gonadal levels of both experimental groups. For this purpose gonads were homogenized in 1 ml of marine teleost Ringer solution and the samples were extracted with butanol and then fractionated for free, glucuronidated and sulphated steroids as described by Scott and Canario [1992]. Extracts were dissolved in 0.1 *M* phosphate buffer containing 0.02 % (w/v) bovine serum albumin for radioimmunoassay (RIA). The KT anti-serum was generously provided by Dr. D.E. Kime (University of Sheffield, UK) and its characteristics reported by Kime and Manning [1982]. The limit of detection of the assay was 15 pg/gonad and the intra- and inter-assay coefficients of variation were 8.2% and 11.6%, respectively.

Behavioral Tests

On the eighth day after implantation individuals were subjected to a set of behavioral tests: a test of aggressiveness (response to own image in a mirror); a test of courtship behavior towards a female; and a test of mating tactic choice. The tests started at 9 a.m. and were always performed in this order with approximately 1 h between them.

The mirror test was conducted by placing a mirror against one of the lateral walls of the isolation tank and the reaction of the individual towards the mirror was recorded. The observation started when the subject first reacted to the presence of the mirror and lasted for 10 min. The displays and attacks directed towards the mirror and the time spent inside the nest were recorded.

The courtship test was intended to check whether treated sneakers could display male typical courtship behavior towards females. A female was introduced in the subject's tank and the behavior of the individual towards the female was recorded (i.e. agonistic displays and attacks directed to the female, courtship acts directed towards the female and time spent inside the nest). The observation period started when the first interaction occurred and lasted for 10 min. Because only ovulating females are receptive towards courting males and to standardize their receptivity, the females were injected intraperitoneally with an ovulatory dose of 200 µl saline containing 5 µg of des-Gly 10, [D-Ala 6]-luteinizing hormone releasing hormone ethylamide (Sigma) 48 h before their introduction in the tanks. The effectiveness of the

Table 1. Brief description of the behavioral and color patterns exhibited by *S. pavo*

Behavioral pattern	Description
Agonistic interactions	
Lateral display	The fish erects the dorsal, caudal and anal fins and distends the brachiostegal membrane showing the flank to the opponent
Attack	Rapid swim towards the opponent usually combined with biting and chasing
Courtship behaviors*	
Female courting	The female approaches the male beating the pectoral fins rapidly while performing high frequency respiratory movements. It often turns its belly to the male
Male courting	The male jerks his body laterally with intense but low frequency movements and distends the brachiostegal membrane
Nuptial coloration patterns*	
Female, neutral	Body color is uniform, varying from brown to grey dull
Female, nuptial	The female exhibits a conspicuous stripped pattern, mainly at the anterior portion of the body, with dark stripes contrasting with a light background
Male, neutral	Body color varies between brown and grey dull. The crest is bright yellow and a dark bar crossing the eyes runs from the tip of the crest to the throat
Male, nuptial	The throat becomes yellowish and the dark bar becomes darker, increasing its conspicuousness. Other dark bars, not visible in the neutral coloration, appear mainly in the anterior portion of the body

*Sneakers can exhibit both female and male courtship behaviors and female nuptial coloration.

LHRH treatment was assessed by testing females with a male before they were used in the mating tactic choice test. Only treated females that courted the male were used in the test.

The mating tactic choice test was intended to assess which mating behavior treated sneakers would choose when confronted with the possibility of establishing their own nest. After performing the two previously described tests subjects were transferred to a larger tank ($70 \times 30 \times 40$ cm) containing an established nest-holder male (in a nest with eggs), two receptive (i.e. LHRH-treated) females and an available empty artificial nest. The observation lasted for 1 h and the following variables describing the behavior of the subject fish were measured: time spent inside the empty nest; display of female nuptial courtship towards the nest-holder male; female-like courtship behavior towards the nest-holder male; male-like courtship behavior towards females; agonistic displays and attacks directed to and received from females; agonistic displays and attacks directed to and received from the nest-holder male (table 1) [a detailed description of the social behavior of *S. pavo* can be found in Fishelson, 1963; Patzner et al., 1986; and Almada et al., 1995]. The behavioral tests were videotaped with the time code using a Sony Hi8 HandyCam and subsequently analyzed using video analysis (Observer v. 5.0, Noldus Information Technology, Wageningen, The Netherlands).

Morphological Measurements and Brain Tissue Preparation

Measurements of external morphological traits were taken at two sampling points. First, when the individuals were implanted with the silastic tubes, and second immediately after the behavioral tests, 8 days after implantation. The following measurements were taken with the fish anaesthetized with MS-222: standard length (from the tip of the snout to the end of the caudal peduncle), head height (including the crest), body height (taken at the insertion of the pectoral fins), genital papillae length (distance between the anterior insertion of the papillae and its posterior tip measured on ventral view), first ray anal gland width (maximum distance between the left and the right contour of the glandular tissue of the first ray of the anal fin measured on ventral view), first ray anal gland length (distance between the anterior insertion of the glandular tissue of the first anal fin ray and its posterior tip measured on ventral view), and the second ray anal gland width (maximum distance between the left and the right contour of the glandular tissue of the second ray of the anal fin measured on ventral view). Crest size (CS) was measured as the ratio between head height and body height. Standard length and head and body height measurements were taken with a Vernier calliper to the nearest 0.1 mm. All the other measurements were taken with a calibrated micrometer on a stereomicroscope (Olympus SZ60), to the nearest 10 μm.

After the behavioral tests, and after the above mentioned morphological variables were measured, the individuals were perfused transcardially, first with a marine teleost Ringer solution, to clear the vasculature, followed by a solution of 4% paraformaldehyde in 0.1 *M* phosphate buffer, to fix the tissues. The brains were removed from the skull and postfixed overnight at 4 °C using the same fixative solution, and transferred to 0.1 *M* phosphate buffer for storage at 4 °C. Forty eight hours prior to processing, the brains were submerged in 30% sucrose in 0.1 *M* phosphate buffer until they became saturated. Twenty-four hours before sectioning, the brains were transferred to cryomatrix (Histo Prep, Fisher). Brains were sectioned frozen at 20 μm on a cryostat (Microm) and mounted on chromalum coated slides.

After the perfusion the gonads were extracted and the following measurements were also taken to the nearest 10 μm: gonad width (maximum distance between the left and the right edges of the right testis on ventral view), gonad length (maximum distance between the anterior and the posterior edges of the right testis on ventral view), testicular gland width (maximum distance between the left and the right edges of the right testicular gland on ventral view), testicular

Table 2. Differences in morphological traits between treatments

Variable	KT (n = 9)	Control (n = 8)	Mann-Whitney U test (Z value)
Body mass (g)	–0.23 ± 0.06	–0.17 ± 0.03	–0.57
Crest size (head height/body height)	0.17 ± 0.12	0.04 ± 0.03	–0.86
Genital papillae (µm)	0.83 ± 0.16	0.06 ± 0.06	–2.60 **
First ray anal gland width (µm)	7.0 ± 0.75	–0.86 ± 0.35	–3.50 ***
First ray anal gland length (µm)	2.44 ± 2.35	–7.13 ± 2.04	–2.26 *
Second ray anal gland width (µm)	5.55 ± 0.99	–0.75 ± 0.45	–3.41***
GSI	1.35 ± 0.18	0.89 ± 0.28	–0.96
TGI	0.23 ± 0.03	0.17 ± 0.06	–1.05

Values (mean ± SD) for body mass, crest size, genital papillae and anal gland variables refer to increments between pre- and post-treatment values.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

gland length (maximum distance between the anterior and the posterior edges of the right testicular gland on ventral view). Based on these measurements a testicular gland index (TGI), the relative ventral area occupied by the testicular gland in the gonad, was calculated by dividing the testicular gland area by the total gonadal area (i.e. testis), assuming an ellipsoidal shape for both the testicular gland and the testis.

Body and gonad weights were also taken to the nearest mg using an electronic balance, based on which a gonadosomatic index was computed (GSI = gonad weight/body weight × 100).

AVT Immunocytochemistry

Following air-drying, the slides were treated using the methods outlined in Grober and Bass [1991], except that (1) a Streptavidin-Biotin Kit (KPL) was used and (2) a polyclonal antibody against arginine vasotocin (courtesy of Lieve Moons, Zoological Institute, Belgium) was used at a concentration of 1:1000. Overnight preabsorption of the antiserum with 1 µg/ml synthetic AVT (Sigma) eliminated all immunostaining.

All slides were coded so that the individuals who quantified the size of cells did not know the identity of the fish to which each slide corresponded. Cell size was measured by capturing images and tracing the outside margin of the soma using NIH Image 1.55 (W. Rasband, NIH, Bethesda, Md., USA). After calibrating for magnification, the imaging program provided a measure of the area of each cell. For each fish in the study we measured all preoptic cells that exhibited a nucleus and at least one neurite. The dorsoventral and rostrocaudal locations of cell groups in conjunction with cell area was used to distinguish among parvocellular (rostral, ventral and small), magnocellular (more caudal, dorsal and larger), or gigantocellular (most caudal, dorsal and largest) components of the preoptic area [nomenclature from Braford and Northcutt, 1983].

Statistical Analyses and Sample Sizes

All statistical procedures were performed using the software package Statistica for Windows v. 5.0 (Statsoft Inc., 1995). Samples were kept to a minimum as this species is classified as vulnerable in Portugal [I.C.N., 1993]. The vulnerable status of this species in Portugal is not due to the fact that it is not common, but because this population exhibits a particular mating system not described for the rocky shore populations of the Mediterranean sea, with the occurrence of nest-aggregations, lack of nest-holder territoriality, courtship sex-reversal and the occurrence of sneaker males [Almada et al., 1995; Gonçalves et al., 1996]. Thus the present study is part of a larger project on the reproductive biology of this population. Due to the small sample size (9 KT-treated and 8 control individuals) non-parametric tests were used. Smaller sample sizes in the behavioral tests (i.e. n = 7 for the KT-treated group) reflect the fact that time constraints prevented all individuals from being tested.

Results

Gonadal Androgens

Gonads of KT-treated individuals contained more KT than controls (KT-treated n = 8, mean ± SEM = 920.9 ± 169.2 pg g^{-1} of tissue; control: n = 8; mean ± SEM = 304.5 ± 91.0 pg g^{-1} of tissue; Mann-Whitney U test: Z = –2.63, p = 0.009).

Morphology

KT implants induced a significant increase in both the length of the genital papillae and the length and width of the anal glands relative to control implants (see table 2). KT treatment had no effect on body mass, crest size, GSI or TGI (table 2).

Behavior

In the mirror test there were no significant differences between treatments in the frequency of agonistic displays (i.e. lateral display) directed towards the mirror (Mann-Whitney U test: Z = –0.34, p = 0.73; KT-treated: mean ± SEM = 2.86 ± 1.90, n = 7; control: mean ± SEM = 3.63 ± 2.02, n = 8) but only control individuals (3 out of 8)

Table 3. Behavioral differences between treatments in the mating tactic choice test

Variable	KT (n = 7)	Control (n = 8)	Mann-Whitney U test (Z value)
Proportion of individuals that displayed female courtship	14.2% (1/7)	87.5% (7/8)	p = 0.014 §
Proportion of individuals that displayed female nuptial coloration	28.6% (2/7)	75% (6/8)	p = 0.09 §
Frequency of female courtship behavior (acts 10 min^{-1})	0.14 ± 0.14	2.13 ± 0.51	–2.82 **
Frequency of female nuptial coloration (acts 10 min^{-1})	0.29 ± 0.18	0.75 ± 0.25	–1.36
Frequency of approaches to the nest-holders nest (acts 10 min^{-1})	1.29 ± 0.52	2.75 ± 0.65	–1.61
Time spent inside own nest (s)	218.5 ± 152.2	210.1 ± 139.7	–0.14
Agonistic behavior towards females (acts h^{-1})	2.33 ± 1.28	2.71 ± 1.15	–0.66
Agonistic behavior received from females (acts h^{-1})	0.83 ± 0.65	2.57 ± 1.94	–0.49
Agonistic behavior received from nest-holder (acts h^{-1})	5.16 ± 2.52	2.42 ± 0.72	–0.65

§ These p values refer to test of differences between two proportions (test of proportions, Statsoft, Inc., 1995). **p < 0.01

attacked their own image on the mirror (Mann-Whitney U test: Z = –1.78, p = 0.08; KT-treated: mean ± SEM = 0, n = 7; control: mean ± SEM = 1.88 ± 1.47, n = 8). None of the tested individuals spent any time inside the artificial nests provided during the mirror trials.

In the female presentation test KT-treated individuals interacted more with the females. There was a trend for KT-treated sneakers to exhibit more lateral displays towards females (Mann-Whitney U test: Z = –1.93, p = 0.053; KT-treated: mean ± SEM = 2.42 ± 1.25, n = 7; control: mean ± SEM = 0.13 ± 0.13, n = 8) and they had a lower latency to attack (Mann-Whitney U test: Z = –1.99, p = 0.047; KT-treated: mean ± SEM = 94.3 ± 31.12 s , n = 7; control: mean ± SEM = 542.1 ± 144.3 s, n = 8). Also, a larger proportion of KT-treated individuals (7 out of 7) attacked the females when compared to the control group (4 out of 8) (test of proportions: n = 15, p = 0.047; Statsoft Inc., 1995). However, there were no significant differences between the two treatments in the frequency of attacks towards the females (Mann-Whitney U test: Z = –1.29, p = 0.20; KT-treated: mean ± SEM = 25.3 ± 9.7, n = 7; control: mean ± SEM = 14.6 ± 6.1, n = 8). There were also no significant differences between groups regarding the time spent inside the nest (Mann-Whitney U test: Z = –0.94, p = 0.35; KT-treated: mean ± SEM = 0 s, n = 7; control: mean ± SEM = 0.61 ± 0.61 s, n = 8). Furthermore, no sneaker was observed to court the females with either the male or the female courtship behavior and no female entered the nests.

In the mating tactic choice test a significantly smaller proportion (1 out of 7) of KT-treated males courted the nest-holder males using typical female courtship behavior than control males (7 out of 8; see table 3). In addition, there was a trend for a larger proportion of control males to display the female nuptial coloration towards nest-holder males (6 out of 8) than in the KT-treated males (2 out of 7; see table 3). The frequency of female-like courtship behavior by sneakers was significantly higher in the control group than in the KT treatment (table 3). Moreover, during the 1 hr observation trials of the 15 tested sneakers only one sneaker from the control group managed to enter the nest of the nest-holder (for 32.6 s in one occasion and for 8.1 s in another). There were no differences in the other behavioral traits measured between the two treatments (table 3).

Table 4. Differences in AVT-ir neuron size (mean ± SD) and number between treatments

Variable	KT (n = 5)	Control (n = 6)	Mann-Whitney U (p value)
Parvocellular neurons			
Soma size (μm^2)	28.2 ± 16.5	22.2 ± 13.4	0.53
Cell number	19.7 ± 15.2	37.2 ± 37.6	0.32
Magnocellular neurons			
Soma size (μm^2)	50.6 ± 9.1	42.8 ± 26.0	0.51
Cell number	13.8 ± 8.2	25.8 ± 17.2	0.16
Gigantocellular neurons			
Soma size (μm^2)	67.9 ± 21.7	91.0 ± 29.3	0.17
Cell number	20.0 ±13.9	10.8 ± 7.6	0.22
All AVT-ir neurons			
Soma size (μm^2)	55.0 ± 10.5	51.1 ± 12.0	0.59
Cell number	73.8 ± 53.4	53.5 ± 30.3	0.44

Forebrain Neurochemistry: AVT Immunoreactive Neurons

In the forebrain preoptic area (POA) cell bodies and their neurites were labeled with DAB (brown stain) in the

magnocellular, gigantocellular and parvocellular regions indicating binding of the AVT antibody. Because the standard length of the fish in the two treatments did not differ significantly (Mann-Whitney U test: $z = -0.24$, $p = 0.81$, KT-treated: mean ± SEM = 6.1 ± 0.22, n = 6; control: mean ± SEM = 6.2 ± 0.27, n = 6) the absolute results (i.e. not corrected for body size or body mass) are presented in table 4. For all three populations of AVT-ir cells in the POA (i.e. parvocellular, magnocellular and gigantocellular), neither the number of cells nor their soma size differed significantly between treatments (table 4).

Discussion

The effectiveness of the implants can be demonstrated by two facts. First, there was an increase in the genital papilla length in KT-treated sneakers, a structure known to be androgen dependent in a number of teleost species [Levy and Aronson, 1955; Oliveira and Almada, 1998; Carlisle et al., 2000]. Second, KT-treated sneakers showed higher levels of gonadal KT compared to controls. Although endogenous KT should be expected to show higher levels in the gonads than elsewhere, as this is where it is produced, the difference observed in gonadal KT levels between the experimental and control groups is most likely a reflection of a difference in circulating levels. Moreover, it has been shown that gonadal levels of KT are correlated with circulating levels in *S. pavo* [Oliveira et al., 2001b]. The gonadal concentration of KT in nest-holders is 5.3 ± 0.6 ng ml^{-1} in the testis and 41.5 ± 7.4 ng ml^{-1} in the testicular gland [Oliveira et al., 2001b]. Considering that the testicular gland comprises 12.6% of the nest-holders gonad [Oliveira et al., 2001b] the weighted mean gonadal KT concentration in nest-holders should be 9.86 ng ml^{-1}, which is very similar to the levels measured in the KT-treated sneakers whole gonads (i.e. 9.2 ± 1.7 ng ml^{-1}). Thus, it is plausible to assume that the KT-treated group experienced an increase in circulating KT levels during the time course of the experiment within the range of the KT levels present in nest-holders.

A week after being implanted, KT-treated individuals developed anal glands in their anal fins and decreased their female-like courtship behavior. However, they did not court females with male-like courtship behavior and when an opportunity was given to establish their own nest they failed to do so. These results indicate that a one-week KT treatment inhibited the expression of the female mimicking tactic in sneaker males of *S. pavo*, but it did not promote a complete switch to the male nest-holding tactic.

The secondary sex characters (SSC) of male *S. pavo* consist mainly of a head crest, two sex-pheromone producing anal glands, localized in the first two rays of the anal fin [Laumen et al., 1974], and a typical male coloration pattern [Fishelson, 1963; Zander, 1975]. Sneaker males lack all these traits [Gonçalves et al., 1996; Ruchon et al., 1995]. In the current study the development of two of these male sexual traits in treated sneakers was monitored: the anal glands and the head crest. A week of KT treatment was effective in promoting the development of anal glands, but not the head crest. An activational role of androgens in the differentiation of male sexual traits in parasitic males is not always clear from the literature. In the plainfin midshipman *(Porichthys notatus)* both KT and testosterone propionate induce an increase in sonic muscle weight, a trait involved in the production of a humming sound, which is a mate attraction vocalization typical of bourgeois males [Brantley et al., 1993b]. In contrast, silastic implants of 11-ketoandrostenedione, which increases plasma KT, failed to induce the differentiation of SSC in immature parr of the Atlantic salmon [i.e. breeding colors and hooked jaw; Rydevik, 1988]. The lack of response of the head crest to KT treatment could be a result of the short time course of the experiment or, alternatively, head crest development might not be androgen dependent, as previously shown for the nuchal hump in cichlids [Bleick, 1975]. Further experiments with longer KT treatments are needed to clarify this point. These were not conducted in the current study as the number of sneaker males that we could use was kept to a minimum due to the vulnerable status of the species [I.C.N., 1993].

In *S. pavo* nest-holder males have relatively smaller gonads (i.e. lower GSI) compared to sneaker males [Gonçalves et al., 1996]. Moreover, nest-holder males also possess a testicular gland that may be involved in sperm maturation and/or the production of mucopolysaccarides [Lahnsteiner and Patzner, 1990; Marconato et al., 1996]. The absence of this accessory structure from sneaker males could indicate differences in sperm characteristics or sperm delivery between the two male morphs. Because there was no effect of KT treatment both in GSI and TGI it can be hypothesized that the experiment time-course was too short to allow the re-organization of the gonadal tissue in androgen treated sneakers, or that stimulation by other factors (e.g. gonadotropin) is required for their development.

There was no effect of the androgen treatment on either the number of AVT-ir cells or their soma size in the different cell populations of the POA. This result is in agreement with the fact that in wild-captured individuals there were also no differences in absolute cell numbers or soma size between nest-holders and sneakers [George et al., 1999]. If

corrected for standard length, sneaker males have higher cell numbers per body size than nest-holders, which indicates that they achieve the same number of AVT-ir neurons in the POA at an earlier age and with a smaller body size. In other teleost species with alternative mating tactics intrasexual differences in AVT-ir neurons in the POA have been described. In the plainfin midshipman *(Porichthys notatus)* parasitic males [i.e. type II males *sensu* Bass, 1996] have smaller and more numerous AVT-ir cells per body mass than bourgeois males [i.e. type I males *sensu* Bass, 1996; Foran and Bass, 1998]. In the bluehead wrasse *(Thalassoma bifasciatum)* terminal phase males [i.e. bourgeois males *sensu* Taborsky, 1997] have more and larger AVT-ir cells and show higher AVT-mRNA expression per cell than initial males [i.e. parasitic tactic *sensu* Taborsky, 1997; McIntyre, 1998]. Moreover, treatment of bluehead wrasse with KT had a significant effect on SSC (the blue head coloration), but did not significantly alter AVT-ir POA cell number or size [McIntyre, 1998], similar to the results of the present study. However, there was a significant effect of social context on AVT mRNA expression in bluehead wrasse, suggesting that AVT might be more sensitive to extended changes in social interactions rather than gonadal steroid hormones [McIntyre, 1998]. Support for this idea comes from a field study on bluehead wrasse that showed rapid changes in AVT mRNA expression in the POA following removal of the dominant fish from social groups [Godwin et al., 2000]. These changes in brain function precede any changes in circulating levels of gonadal steroids. In sum, these studies suggest that AVT in fish is probably regulated more by social interactions than by gonadal steroids, and this is consistent with the results of the present study. Although the functional significance of these differences between alternative sexual morphs is not well established [for a review see Foran and Bass, 1999] it is clearly associated with the expression of 'morph' typical behavior [Foran and Bass, 1999]. An alternative possibility that should not be excluded is that potential differences in AVT synthesis and/or release are not reflected in AVT-ir neuron number of soma size but could be seen in mRNA AVT expression. In fact, it has been shown that nest-holders have significantly lower expression of AVT mRNA than sneakers [George et al., 1999]. Unfortunately logistic problems prevented us from running the in situ hybridizations to collect this data.

Several aspects of the behavior of sneaker males were affected by the KT treatment. There was a non-significant trend for KT-treated sneakers to be less aggressive in the mirror test, suggesting that mirror-elicited aggression in *S. pavo* sneakers is not KT dependent. This result contrasts with the established role of androgens in the control of agonistic behavior in teleost fishes [Villars, 1983]. However, different agonistic motivational systems can be recognized which do not have necessarily to share the same proximate mechanisms [Brain, 1981], and most cases of established effects of androgens on agonistic behavior refer to the defense of breeding territories or to dominance in male-male competition for access to mates [Liley and Stacey, 1983; Borg, 1994]. Thus, the failure of KT to induce agonistic behaviors in this study can be viewed as a further example that androgens might not be causal agents of aggressiveness per se and that the relationship between androgens and aggression could depend to a great extent on context.

KT treatment inhibited the expression of female-typical courtship behavior in sneakers but failed to promote the expression of male-typical reproductive behavior, such as male–like courtship and nest establishment. It could be argued that the lack of effect on the expression of male-typical behaviors in KT-treated males would be due to KT-treated sneakers having KT circulating levels below an activational threshold level. However, the gonadal KT levels of nest-holders from the field are similar to those of KT-treated sneakers (see above) suggesting that circulating levels should not differ significantly between these two groups. Thus, the lack of induction of male behavior in sneakers by KT implants could be explained by one of the following hypotheses: (1) there might be two distinct and independent mechanisms for generating male vs. female typical mating behavior in this species, (2) sneaker males of *S. pavo* cannot simultaneously activate the mechanisms for homo- and heterotypical sex behaviors, and (3) KT inhibits the expression of female-typical behavior. The first two explanations are ruled out by the fact that large sneaker males in the field can switch in a matter of few minutes between female-like courtship behavior directed towards nesting males and male courtship behavior directed towards females [D.M. Gonçalves, pers. observ.]. These observations are in accordance with the finding that female KT-treated goldfish *(Carassius auratus)* are able to exhibit the full repertoire of male-typical reproductive behavior [Stacey and Kobayashi, 1996], whereas goldfish males treated with prostaglandins can express female-typical behavior [Stacey and Kyle, 1983], suggesting a potential for bisexuality in adult teleosts. The third hypothesis is the more parsimonious explanation and would have a parallel in the process of sex differentiation in mammals in which some androgens have a defeminization effect whereas others have a masculinization effect [e.g. McEwen, 1987].

In sum, the dose and treatment period used in this study did not induce the behavior typical of nest-holder males nor changes in AVT POA cell chemistry, suggesting that these changes might be context-dependent. Further studies are needed to establish whether a longer period of KT action, other hormones or prolonged exposure to appropriate social interactions are effective in promoting the expression of male-typical behavior in sneaker males.

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