Endocrine control of sexual behavior in sneaker males of the peacock blenny *Salaria pavo*: Effects of castration, aromatase inhibition, testosterone and estradiol

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Abstract

The effects of castration and sex steroid manipulations on the expression of sexual behavior were investigated in a small fish, the peacock blenny, *Salaria pavo*. In this species, large males defend nests and attract females while small "sneaker" males reproduce by imitating the female morphology and courtship behavior in order to approach nests during spawning events and parasitically fertilize eggs. Sneakers switch into nest holders in their second breeding season, thus displaying both male and female-like sexual behavior during their lifetime. We tested the effects of castration and of an aromatase inhibitor (Fadrozole, F), testosterone (T) or 17β-estradiol (E_2) implants on the expression of male and female-like behavior in sneakers. Sneakers were either sham-operated, castrated or castrated and implanted with vehicle, F, T+F or E_2+F. Seven days after the treatment, sneakers were placed in a tank with a nesting male, two ripe females and an available nest. Castrated fish had lower levels of circulating T and increased the time spent displaying female typical nuptial coloration. T implants had the opposite effect, inhibiting the expression of female-like behavior and coloration. E_2 implants had no significant effect on the display of sexual behavior but the frequency of aggressive displays decreased. The results agree with previous findings in sneakers of *S. pavo* that demonstrated an inhibition of female-like behavior by 11-ketotestosterone (11-KT). The reported increase in T and 11-KT production when sneakers change into nest holders may thus contribute to behaviorally defeminize sneakers. Contrarily, both T and E_2 failed to promote male-like behavior, suggesting that behavioral masculization during tactic switching depends on other neuroendocrine mechanisms or that the time length of the experiment was insufficient to induce male-like behavioral changes in sneakers.

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Keywords: Aromatase; Sex steroids; Reproductive behavior; *Salaria pavo*; Peacock blenny; Androgens; Estrogens; Implants; Alternative reproductive tactics; Fadrozole

Introduction

The influence of sex steroids on the control of sexual behavior has been investigated in detail in animal models from diverse taxa (e.g. rats, quails, lizards). From these studies, it has long been known that gonadal androgens administered to males promote male-typical sexual behavior. However, estrogen administration to males may also induce male typical sexual displays (e.g. Ball, 1937; Beach, 1942; Guhl, 1949). This paradoxical observation was the basis for the aromatase hypothesis (Naftolin et al., 1975), which suggests that the masculinizing effects of gonadal testosterone (T) on reproductive behavior are partially dependent on its local conversion to estradiol (E_2) by the enzyme aromatase in the brain. This hypothesis has been thoroughly corroborated by studies in mammals and birds where male sexual behavior was inhibited by blocking brain aromatase and reestablished by administering estrogens (e.g. Adkins et al., 1980; Vagell and McGinnis, 1997; for reviews see Baum, 2003; Ball and Balthazart, 2004).

Surprisingly, although fish have been extensively used to study endocrine mechanisms of sex determination and differentiation (review in Devlin and Nagahama, 2002), the role of the aromatization process on the regulation of fish sexual behavior has been poorly investigated. One reason for this is that the non-aromatisable androgen 11-ketotestosterone (11-

KT) is thought to be the most potent androgen in fish (Borg, 1994). 11-KT is the most abundant circulating androgen in most male teleosts and was shown to restore male sexual behavior in castrated sticklebacks and white perches (Borg, 1987; Salek et al., 2001), to increase male sexual displays in intact bluegill sunfish (Kindler et al., 1991) and to induce male sexual displays in gynogenetic carps (Kobayashi and Nakanishi, 1999). However, in other species the most abundant circulating androgen during reproductive periods is T (e.g. Kime and Groves, 1986; Oliveira et al., 2001b) and in the only study conducted so far investigating the effects of blocking aromatase in fish sexual behavior, two of three investigated male sexual displays in guppies were reduced by aromatase inhibition (Hallgren et al., 2006). Thus, for some fish species, T aromatization into E2 may also mediate the expression of male sexual displays. The largely ignored role of brain aromatization of androgens on fish sexual behavior is also surprising as teleosts present by far the highest levels of brain aromatase activity in vertebrates (Callard et al., 1990) and, in fish, aromatase occurs in brain areas involved in the control of reproductive behavior (Forlano et al., 2001; Menuet et al., 2003, 2005) and peaks during the species reproductive seasons (e.g. Gelinas et al., 1998; Gonzalez and Piferrer, 2002, 2003; Forlano and Bass, 2005). On the other hand, exogenous estrogen administration to fish has been shown to decrease male sexual behavior (e.g. Bayley et al., 2003; Bjerselius et al., 2001; Doyle and Lim, 2005; Oshima et al., 2003) and there are no published studies suggesting that estrogens promote male sexual behavior in fish. Thus, the role of high aromatase levels in brain areas typically associated with the control of sexual behavior is unclear and, more generally, the mechanisms of neuroendocrine control of sexual behavior in fish are still poorly understood.

Here, the effects of castration and sex steroid manipulation on male and female-like displays were tested in the peacock blenny *Salaria pavo*. In this species, males are larger than females and have a pronounced head crest and an anal gland in the first two rays of the anal fin (Fishelson, 1963; Papaconstantinou, 1979; Patzner et al., 1986). Males defend nests in crevices or holes and, in the studied population, a shortage of nest sites promotes a strong male-male competition for nests (Almada et al., 1994, 1995). Females compete for the access to nesting males and take the initiative in courtship. Females court males by quickly beating the pectoral fins and open-and-closing the mouth in synchrony while displaying a typical nuptial coloration, which consists of an alternated pattern of vertical dark and light bars in the head and anterior portion of the body (Almada et al., 1995). Nesting males assume a passive role during courtship but occasionally will also court females from the nest with intense but low-frequency jerking movements of the anterior portion of the body (Patzner et al., 1986). Small "sneaker" males are unable to acquire nests and reproduce by imitating the female's morphology, courtship displays and nuptial coloration in order to approach and deceive nesting males and parasitically fertilize eggs (Gonçalves et al., 1996). When compared with size-matched females, small sneakers are equally courted and attacked by nesting males suggesting that their female-mimicry is efficient (Gonçalves et al., 2005).

Sneaker males develop secondary sexual characters and may reproduce as nesting males in their second breeding season (T. Fagundes, D. Gonçalves and R.F. Oliveira, unpublished data). Thus, at least some males perform female-like displays in their first breeding season and male-like displays in the following reproductive seasons. Sneakers have lower circulating and gonadal levels of both T and 11-KT than nesting males, and T is the most abundant androgen in the two morphs both in the plasma (D. Gonçalves and R.F. Oliveira, unpublished data) and in the gonads (Oliveira et al., 2001a). In sneakers, 11-KT implants inhibit female-like displays and promote the development of male secondary sexual characters but it does not induce male-like sexual behavior (Oliveira et al., 2001d).

In this experiment, the effects of castration and of administration to castrated sneakers of an aromatase blocker, T or E2 on male and female-like sexual displays were tested.

Methods

Animals

During the breeding season (June and July 2005), males, females and sneakers were collected at Culatra Island (36°59′N, 7°51′W, Algarve, Southern Portugal) during morning low tide and transported to a field station located in the island. In the field, sneakers were classified as such if they lacked or had vestigial male typical secondary sexual characters (head crest, anal gland and male-like coloration) and sperm could be easily extruded from their *vas deferens* by gently pressing the abdomen (Gonçalves et al., 1996). In relation to nesting males, sneakers have proportionally larger gonads that lack a testicular gland (Gonçalves et al., 1996) and this criterion was also used to exclude potential non-sneakers from the experiment during the surgical procedure. Animals were kept in 20 l tanks under natural photoperiod (14L; 10E) and temperature (24±2 °C) and fed with frozen cockles until testing. Nesting males were kept together with females and provided with abundant nest sites so that they were defending eggs before the experiment. Sneakers and females used in the experiment were kept in separate tanks.

Reagents

The aromatase inhibitor Fadrozole (F) was gently donated by Novartis Pharma AG (Basel, Switzerland). T and E2 were purchased from Steraloids (Newport, USA) and the tritiated water and androstenedione used in the radiometric procedure purchased from PerkinElmer (Oporto, Portugal). All other reagents were purchased from Sigma-Aldrich (Madrid, Spain).

Castration and implant procedure

After being transported to the field station, sneakers were anesthetized with a light dosage of MS222 (tricaine methanesulfonate, dilution 1:10,000), measured and weighed. To assess treatment effects on male-like typical characters, the head and body height were measured to provide an indicator of head crest development (head height/body height) and the genital papilla and the anal gland development were classified in an ordinal scale from 0 (not developed) to 9 (fully developed). Sneakers were recovered in a small tank with full aeration and transferred to a provisional container provided with shelters in abundance until the surgical procedure. Silastic implants (internal diameter =1.47 mm, external diameter =1.96 mm) were then prepared and their length adjusted to the sneaker's weight in order to keep the implant concentration equal for all fish. The implant concentration was based on previous studies in fish (Kobayashi et al., 1991; Oliveira et al., 2001c; Modesto and Canário, 2003; Ros et al., 2004) and was of 100 µg per gram of fish for T and for the aromatase blocker Fadrozole (F) and of 1 µg per gram of fish for E2. Steroids and F were thoroughly dissolved in castor oil. A total of 54 sneakers were randomly assigned to one of six treatments: (1) Sham; (2) castrated (Cast); (3) castrated controls (C; castor oil

implanted); (4) castrated+F (F); (5) castrated+T+F (T+F); (6) castrated+E$_2$+F (E$_2$+F) (Table 1). In the T+F treatment, Fadrozole was co-administered with T in order to block its synthesis into E$_2$. To control for possible secondary effects of F administration, F was also co-administered with E$_2$. Approximately 8 h after the measuring procedure fish were again anesthetized for the surgical procedure. A 1 cm abdominal cut was made in the abdomen and, with the exception of fish from the Sham group where testicles were manipulated with blunted tweezers but left intact, all animals were castrated. Fish from the Sham and Cast groups received no implant. In the remaining animals the implant was inserted in the peritoneal cavity. The wound was sutured with silk line (Ethicon, Lisbon, Portugal). Fish were recovered as previously described and kept in 20 l tanks, separated by treatment, until being tested. The standard length of fish from the different treatments did not differ (one-way ANOVA: $F_{5,48}=1.6$, $P=0.15$).

Behavioral test

Seven days after being implanted, sneakers were tested in a mating tactic choice setup previously described by Oliveira et al. (2001d). This period was considered sufficient for steroid-induced behavioral changes to take place as in a previous experiment with *S. pavo* steroid implants that were shown to induce behavioral changes in sneakers after 7 days (Oliveira et al., 2001d). The test intended to assess which of the two male reproductive tactics, sneaking or adopting a nest, sneakers would choose when presented with ripe females and a nesting male, and to allow the quantification of female and male-like displays in sneakers. The test tank was 70×30×40 cm and contained an established nest-holder male in a nest with eggs and an available empty nest. The sneaker was introduced in the tank together with two ripe females and the following variables were recorded for the sneaker: time spent inside the empty nest, frequency of female-like displays towards the nesting male, frequency of male-like displays towards females, time spent displaying the female-like nuptial coloration and frequency of agonistic attacks performed towards females. The observation lasted 1 h and behavioral variables were quantified using JWatcher software v0.9 (Blumstein et al., 2000). Observers did not know before hand to which experimental treatment the focal animal belonged. A total of 8 different nesting males were used and females were never used twice.

Tissue collection and hormone analysis

Immediately after being tested, sneakers were anesthetized as previously described. Blood was collected from their caudal vein, centrifuged and kept at −20 °C for hormone analyses by radioimmunoassay (RIA). Due to the small size of these fish, in a few animals it was not possible to collect enough blood (25 μl) for the RIA (see Table 3 for *N* values). Blood plasma was stored at −20 °C until steroid extraction. Free steroid fraction was extracted using previously described methodology (Canário and Scott, 1989; Scott and Canário, 1992). Steroid residues were redissolved in phosphate buffer 0.1 M, pH 7.6, containing gelatine (1 g/l) and stored again at −20 °C until assayed for 11-KT, T and E$_2$. The antibody used for the 11-KT assay was kindly donated by D.E. Kime and the corresponding specificity table was published in Kime and Manning (1982). The E$_2$ antibody (reference: 20-ER06) was purchased from Interchim (Montluçon, France) and the cross-reactivity measured by the supplier varied between 1% for estrone and 0% for androstenedione. The T antibody (reference: RDI-TRK2T2) was purchased from Research Diagnostics

Table 1
Description of the six experimental groups used in the experiment

Treatment	Castration	Implant	*N*
Sham	No	No implant	7
Cast	Yes	No implant	10
C	Yes	Vehicle (castor oil)	7
F	Yes	Fadrozole	9
T+F	Yes	Testosterone+Fadrozole	11
E$_2$+F	Yes	17β-estradiol+Fadrozole	10

Fish from Cast and C treatments were pooled together in another group, Cast2, to increase statistical power in the comparisons with the remaining groups.

Inc. (Concord, USA) and the cross-reactivity measured by the supplier varied between 16% for 5-alpha-dehydrotestosterone and <0.01% for E$_2$. For each hormone, circulating plasma levels from all animals were measured within the same assay. Intra-assay coefficients of variation for the T, 11-KT and E$_2$ assays were 9.5%, 12.3% and 5.5%, respectively.

The morphological variables recorded in the first day were again measured to assess variation in the development of male-like characters between treatments. The brain was extracted, immediately kept in liquid nitrogen and later transferred to a −80 °C fridge until the radiometric procedure used to measure brain aromatase activity. The abdominal cavity was carefully inspected to check for the implant presence and for the development of gonadal tissue. No fish lost the implant, and no castrated fish had any visible gonadal tissue.

During the experimental procedure the "ASAB guidelines for the use of animals in research" were applied.

Aromatase activity assay

A radiometric assay modified from Gonzalez and Piferrer (2002) was used to measure aromatase activity in order to confirm that F significantly reduced brain aromatase activity. This assay measures aromatase activity from the sterospecific loss of 1β-^{3}H from the tritiated substrate to water during the aromatization reaction (Lephart and Simpson, 1991). Briefly, brains were homogenized in 400 μl of ice-cold phosphate buffer 0.05 M, pH 7.4, containing 0.25 M sucrose and 0.01 M dithiothreitol with a Teflon homogenizer. Homogenates were split in 100 μl aliquots and frozen at −80 °C until the radiometric procedure. 150 nM [1β-^{3}H]-androstenedione was first preincubated at 37 °C for 1 h with a NADPH generating system, consisting of 1 mM NADP, 10 mM glucose-6-phosphate and 2 IU/ml glucose-6-phosphate dehydrogenase. A 100 μl brain homogenate aliquot was then incubated at 30 °C for 30 min with 200 μl of the hormone/NADPH solution and 100 μl of phosphate buffer. The reaction was terminated by placing tubes in ice and adding 200 μl of trichloroacetic acid. Samples were centrifuged and the supernatant extracted with chloroform. The aqueous phase was washed with dextran (0.05%)-activated charcoal (0.5%) and 400 μl of the supernatant was counted in a liquid scintillation counter. The protein pellet was dissolved with 0.2 N of NaOH and the protein content determined with the Lowry procedure (Lowry et al., 1951). Aromatase activity was expressed as pmol/mg protein/hour.

Statistics

Differences between groups were compared with two-tailed parametric tests. When necessary, data were log or square root transformed to comply with heteroscedasticity and normality assumptions.

The morphological and behavioral effects of castration and hormonal manipulation were assessed by comparing the Cast2, Sham, F, T+F and E$_2$+F groups with one-way ANOVAs. When differences were significant ($P<0.05$), Dunnett post hoc tests were applied to test for specific differences between the control group, Cast2 and the remaining groups.

Results

Implant validation

First, to test if the implant procedure per se had a significant effect on the behavior or morphology of sneakers, animals from the Cast (castrated and not implanted) and C (castrated and implanted with vehicle) treatments were compared with unpaired *t*-tests. Fish from these two groups did not differ in circulating levels of T, 11-KT or E$_2$ ($t<1.5$, $df=15$, $P>0.15$) and no difference in any of the behavioral ($t<1.2$, $df=15$, $P>0.25$) or morphological ($t<1.4$, $df=15$, $P>0.16$) variables was found, suggesting that the implant per se did not induce any significant change. Animals from the Cast and C groups were

thus pooled together in a second control group, Cast2, in order to increase statistical power.

To validate the use of the aromatase blocker, F, differences in brain aromatase activity between animals that received (F, T+F and E_2+F) and did not receive (Sham, Cast2) F were tested. Brain aromatase activity was approximately 3.5-fold lower in animals that received F when compared with animals that did not receive F (unpaired t-test, $t=5.1$, $df=41$, $P<0.001$, Table 2). No difference in brain aromatase activity was recorded between F, T+F and E_2+F implanted animals (one-way ANOVA, $F_{2,17}=0.0$, $P=0.99$) or between Sham and Cast2 animals (unpaired t-test, $t=0.8$, $df=21$, $P=0.44$) (Table 2).

The steroid implant procedure produced the expected effects (Table 3). Fish from the E_2+F treatment had significantly higher levels of circulating E_2 (unpaired t-test, $t=2.7$, $df=20$, $P=0.013$) but not of T ($t=1.4$, $df=25$, $P=0.172$) or 11-KT ($t=0.4$, $df=25$, $P=0.671$) than Cast2 fish (Table 3). Likewise, T+F implanted animals had significantly higher levels of circulating T ($t=5.0$, $df=21$, $P<0.001$) but not of E_2 ($t=0.9$, $df=20$, $P=0.335$) than animals from the Cast2 treatment. However, fish implanted with T+F also had significantly higher levels of 11-KT than Cast2 fish ($t=3.9$, $df=21$, $P<0.001$). The T+F implant resulted in an average T circulating plasma level of 28 ng/ml, close to the maximum physiological level so far measured for *S. pavo* males collected in the field (36 ng/ml, D. Gonçalves and R. F. Oliveira, unpublished data). Three of the eleven T+F implanted fish had T values above 36 ng/ml. The levels of 11-KT resulting from the T+F implants (maximum, 9.0 ng/ml) were all below the physiological maximum measured for the species (60.0 ng/ml, R. F. Oliveira, unpublished data). A comparison between the Sham and Cast2 treatments revealed that castration reduced the circulating levels of the three hormones, but significantly so only for T (E_2, $t=1.6$, $df=16$, $P=0.137$; T, $t=2.2$, $df=16$, $P=0.041$; 11-KT, $t=1.9$, $df=20$, $P=0.078$, Table 3).

Behavioral effects

Groups differed significantly in the frequency of female-like displays towards the nesting male ($F_{4,49}=6.$, $P<0.001$; Fig. 1). T+F fish performed significantly less displays than Cast2

Table 2
Brain aromatase activity measured in the five experimental groups at the end of the experiment

Treatment	Brain aromatase activity (pmol/mg protein/h)
Groups without Fadrozole	
Sham ($N=7$)	2.83±0.45
Cast2 ($N=16$)	2.33±0.43
Mean±S.E.	2.53±0.31
Groups with Fadrozole	
F ($N=7$)	0.71±0.20
T+F ($N=7$)	0.78±0.32
E_2+F ($N=6$)	0.63±0.11
Mean±S.E.	0.71±0.13

The number of animals from each group used in the assay is shown in brackets. Values are mean±S.E. of mean.

Table 3
Circulating levels of T, 11-KT and E_2 measured in the experimental groups at the end of the experiment

Treatment	E_2 (ng/ml)	T (ng/ml)	11-KT (ng/ml)
Sham ($N=6$)	0.90±0.42	3.01±2.26*	2.66±0.85
Cast2 ($N=12$)	0.27±0.20	0.06±0.01	1.11±0.37
F ($N=6$)	0.55±0.40	0.09±0.03	0.64±0.26
T+F ($N=10$)	0.57±0.24	28.48±6.48*	4.20±0.75*
E_2+F ($N=10$)	3.09±1.11*	0.16±0.07	0.94±0.22

The number of animals from each group used in the radioimmunoassay is shown in brackets. Values are mean±S.E. of mean. * Indicates significant differences ($P<0.05$) for the Cast2 treatment.

animals ($P<0.001$). The remaining groups did not differ from controls ($P>0.13$). Differences were also recorded for the time spent displaying the female-like nuptial coloration ($F_{4,49}=3.9$, $P<0.01$; Fig. 1). T+F ($P=0.01$) and Sham ($P=0.04$) animals spent significantly less time displaying the female coloration in relation to Cast2 fish (remaining groups, $P>0.99$). Finally, the frequency of agonistic attacks performed towards females also differed ($F_{4,49}=3.2$, $P=0.02$; Fig. 1). E_2+F treated fish
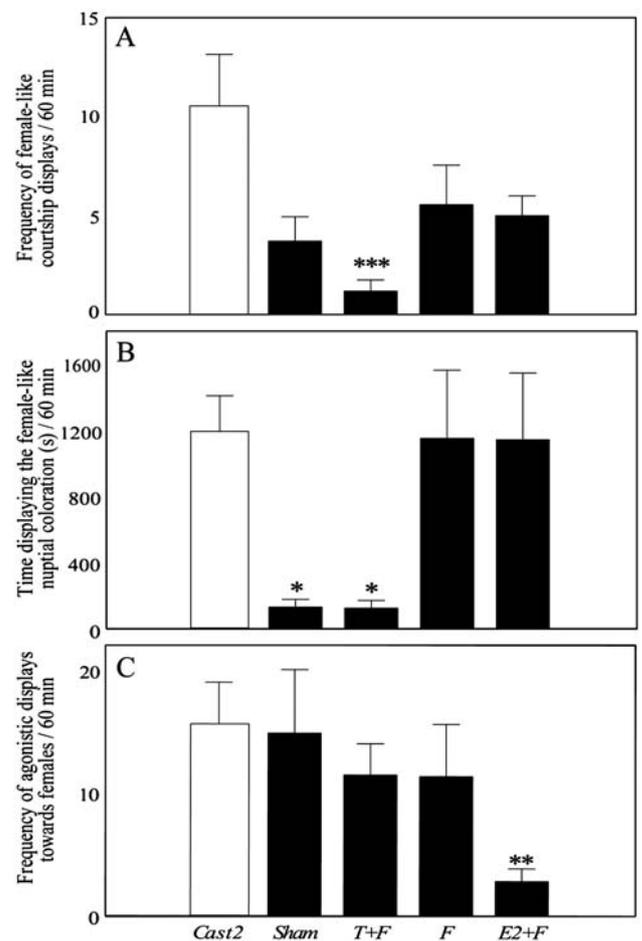

Fig. 1. Behavioral variables recorded for castrated (Cast2) and sham-operated (*Sham*) fish and for 17β-estradiol (E_2+F), Fadrozole (F), and testosterone (T+F) implanted fish. (A) frequency of female-like courtship displays directed towards the nesting male; (B) Time spent displaying the female-like nuptial coloration; (C) frequency of agonistic displays directed towards females. Differences to the Cast2 group are marked with *($P<0.05$), **($P<0.01$), or ***($P<0.001$).
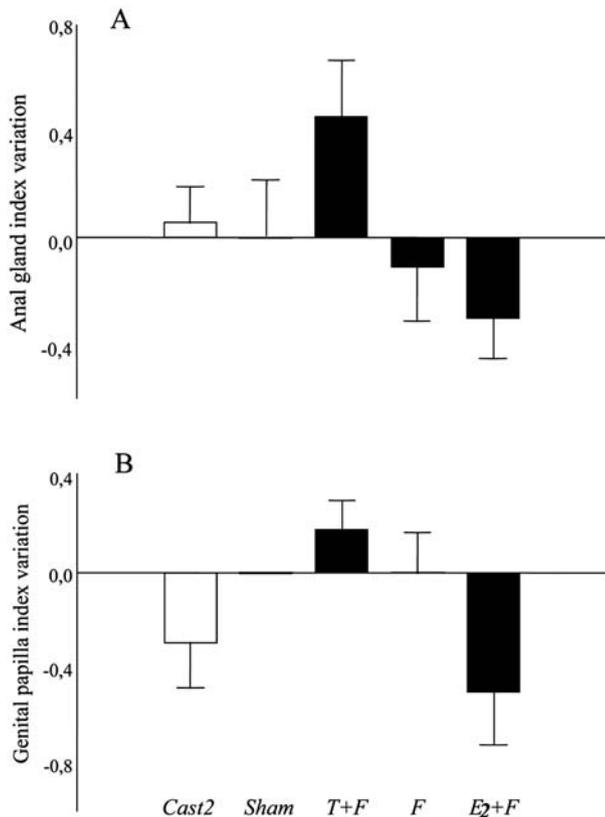
Fig. 2. Variation in (A) anal gland and (B) genital papilla development from the beginning to the end of the experiment in castrated (Cast2) and sham-operated (Sham) fish and in 17β-estradiol (E_2+F), Fadrozole (F) and testosterone (T+F) implanted fish.

performed less agonistic attacks than Cast2 animals ($P < 0.01$), while no difference was recorded for the remaining groups ($P > 0.83$). No difference between groups was recorded for the time spent inside the available nest ($F_{4,49} = 0.8$, $P = 0.52$). In all treatments, sneakers never performed nesting male-like courtship displays towards females.

Morphological effects

The variation in standard length and head crest from the beginning to the end of the experiment was similar between treatments (standard length, $F_{4,49} = 0.7$, $P = 0.56$; head crest, $F_{4,49} = 0.5$, $P = 0.76$). The variation in the anal gland and genital papilla index was marginally non-significant between groups (anal gland, $F_{4,49} = 2.4$, $P = 0.06$; genital papilla, $F_{4,49} = 2.2$, $P = 0.08$; Fig. 2). T+F animals had the most positive developments of these two traits while E_2+F fish had the most negative variation (Fig. 2).

Discussion

Female-like behavior

In relation to sham-operated fish, castrated sneakers had lower circulating levels of T and spent more time displaying the female-like nuptial coloration. Contrarily, T treated fish

performed less female-like courtship displays and spent less time exhibiting the nuptial coloration. These results strongly suggest that T, or one of its metabolites, inhibits female-like displays in sneakers. Because T was co-administered with the aromatase blocker, F, inhibition of female-like behavior was probably not a result of local aromatization of T into E_2. Moreover, E_2 implants did not suppress female-like displays. Another T metabolite, 11-KT, may have been responsible for the inhibition of female-like displays in T implanted fish. In this experiment, the metabolic pathway synthesizing 11-KT from T was not manipulated, and T implanted fish exhibited an increase in 11-KT circulating levels. In a previous study using a similar experimental methodology, 11-KT inhibited female-like displays in sneakers of *S. pavo* (Oliveira et al., 2001d) and in species with alternative reproductive tactics 11-KT, but not T, has been suggested to be involved in tactic switching (see Brantley et al., 1993; Oliveira, 2006). Nesting males of *S. pavo* have naturally higher circulating levels of both T and 11-KT than sneakers and this difference is higher for 11-KT (D. Gonçalves and R.F. Oliveira, unpublished data). Thus, either both androgens inhibit female-like behavior in *S. pavo* or 11-KT metabolized from T was responsible for the suppression of female-like behavior in this experiment. Androgen inhibition of female-like displays in sneakers of *S. pavo* is contradictory to what has been found in another teleost. In the plainfin midshipman, type I males build nests and court females while type II males cuckold the nests of type I males (Brantley and Bass, 1994). Although type II males have lower levels of circulating 11-KT than type I males (Brantley et al., 1993), 11-KT administration to type II males paradoxically promoted cuckoldry (Lee and Bass, 2005). Also in female goldfish, both T and 11-KT failed to suppress female sexual behavior (Kobayashi and Stacey, 1993; Stacey and Kobayashi, 1996). The interpretation of these contradictory results is still unclear, but may relate to the fact that alternative reproductive tactics are sequential in the peacock blenny. When changing into nesting males, sneakers of *S. pavo* will need to both masculinize and defeminize their sexual displays. Contrarily, both in type II males of the plainfin midshipman and in female goldfish the sexual tactic is fixed, and the neural circuits involved in the expression of female-like or female behavior may be more dependent on early hormonal organizational effects and less influenced by hormonal manipulation in adults (see Arnold and Breedlove, 1985; Moore, 1991; Moore et al., 1998).

Castrated sneakers were able to perform female-like behavior, suggesting that these displays are relatively independent of gonadal steroids. This finding is similar to what has been described for goldfish where ovariectomy did not suppress female sexual behavior (Kobayashi and Stacey, 1993). The fact that castration increased female-like behavior and T implants had the opposite effect suggests that there is a compromise between gonadal androgen production and the expression of female-like behavior in sneakers. Gonadal androgen production, mainly 11-KT, is necessary for sperm differentiation and maturation (reviewed in Nagahama, 1994) and our results suggest that an increase in androgen production will suppress female-like displays. Nesting males have a testicular gland that

sneakers lack, thought to be the main source of gonadal androgens (Oliveira et al., 2001a; Reinboth and Becker, 1986). Even if only testis tissue is considered (i.e. excluding the testicular gland) T and 11-KT concentrations are, respectively, ten and five-fold higher in nesting males testicular tissue when compared to sneakers (Oliveira et al., 2001a). Thus, development of the testicular gland and an increase in androgen production by testicular tissue during tactic switching will increase androgen production and this in turn will probably be responsible for the suppression of female-like behavior.

Nesting male behavior

The hormonal manipulations failed to induce typical nesting male behavior in sneakers. Sneakers did not court females or adopt the available nest for significant periods of time in any of the treatments. Thus, neither T nor E_2 promoted nesting male-like behavior in sneakers. In a previous experiment on *S. pavo*, 11-KT implants also failed to promote nesting male-like behavior in sneakers (Oliveira et al., 2001d). Similar results were found for the rock-pool blenny, where both methyltestosterone (MT) and 11-KT implants failed to promote nesting male sexual displays in satellite males (Oliveira et al., 2001c). Also in type II males of the plainfin midshipman 11-KT did not induce type I male sexual behavior (Lee and Bass, 2005). According to the "relative plasticity hypothesis" proposed under the framework of alternative reproductive tactics, hormonal manipulations in adults should be effective in promoting tactic switching in species with plastic tactics but not in species with fixed tactics (Moore, 1991; Moore et al., 1998). The plainfin midshipman presents fixed tactics and thus fits in this prediction. However, in disagreement with this prediction, in both the rock-pool and the peacock blenny the tactics are sequential and hormonal manipulations failed to induce tactic switching. Moreover, 11-KT has been shown to effectively promote male sexual behavior in gynogenetic crussian carps and in female goldfish (Kobayashi and Nakanishi, 1999; Stacey and Kobayashi, 1996). Thus, even in an all-female species and in females of a species with fixed sexual roles, androgens have been shown to effectively promote male sexual behavior. The potential for hormone manipulations to promote male sexual displays in female-like males of *S. pavo* that naturally switch into nesting males should thus be much higher than in the previous examples. Several hypotheses may explain this unexpected result:

1. The time lapse of the experiment was not sufficient for behavioral masculinization. Hormonal manipulations lasted 7 days and this period may not be sufficient for potential hormonal-induced changes involved in nesting male-like displays. In the abovementioned studies for crussian carps and goldfish, hormonal manipulation lasted between 3.5 and 5 months. In the study by Oliveira et al. (2001d), 11-KT effectively promoted the development of nesting male morphological traits in sneakers after 7 days (see below) but not a behavioral switch. Nevertheless, induction of male-like behavior in female gobies could be induced after 10–18 days of hormonal treatment with MT (Murphy and

Stacey, 2002) and male behavior in castrated male white perch was restored with 11-KT after 3 weeks (Salek et al., 2001). Also, complete female to male behavioral sex change can be socially induced in a goby species within 5 days (Grober and Sunobe, 1996). Likely, different hormone-dependent male traits differ in their temporal response to hormone manipulations and this may vary across species;

2. Nesting male behavior is not facilitated by either E_2, T or 11-KT in *S. pavo*. This is an unlikely explanation because at least the two androgens are significantly elevated in nesting males when compared to both females and sneakers (D. Gonçalves and R. F. Oliveira, unpublished data; no data available for E_2) and have been demonstrated to promote male-sexual displays in many fish species (for reviews see Borg, 1994; Liley and Stacey, 1983). Nevertheless, in a sex-changing wrasse it was demonstrated that female to male behavioral sex change can be induced in animals that had their gonads removed suggesting that gonadal sex steroids do not play a major role in the induction of male sexual displays in that species (Godwin et al., 1996; for a review see Grober, 1998);

3. Behavioral masculinization depends not only on an increase in circulating sex steroids but also on changes in other components of the steroid pathway. These may include changes in steroid binding globulins, steroid receptors, steroid receptor co-factors or steroidogenic enzymes. Thus, in our experiment, the experimentally induced increase in steroid circulating levels may have been insufficient, per se, to promote male-like behavior. If this is the case, transitional males, i.e. sneakers that are already undergoing the change into nesting males, should be more responsive to the same hormonal treatments.

The only significant effect of E_2 administration was a decrease in the frequency of aggressive behavior. This has been previously demonstrated in other fish species where exogenous estrogen administration decreased aggressive displays (e.g. Munro and Pitcher, 1985; Bell, 2001). Also, in a sex changing goby, rapid increases in aggression correlate with a decrease in brain aromatase activity, suggesting that a decrease in E_2 synthesis (or a higher availability of T) facilitates aggressive behavior (Black et al., 2005). The fact that E_2 implants altered aggressive displays shows that this hormone had a significant physiological effect in our experiment. The lack of an effect of E_2 regarding sexual behavior may indicate different temporal dynamics in the response to this hormone by brain circuits mediating sexual displays or that E_2 does not significantly affect sexual displays in sneakers. Nevertheless, because sneakers do not naturally display nesting male sexual behavior, the possibility remains that, as suggested for mammals, birds and also guppies (Hallgren et al., 2006), E_2 synthesis from T plays a role in the control of male sexual behavior in *S. pavo*.

Secondary male sexual traits

Hormone manipulations, namely T implants, were ineffective in inducing the development of nesting male secondary

sexual characters. This contrasts with the previous study by Oliveira et al. (2001d) where 11-KT administration to sneakers promoted the development of the genital papilla and anal gland within 1 week. 11-KT has been suggested to be the most effective androgen promoting the differentiation of male secondary sexual characters (see Oliveira, 2006) and thus differences between these two studies may relate to the differential role of T and 11-KT in male trait differentiation.

Concluding remarks

Gonadal steroids do not seem to promote female-like sexual displays in sneakers of *S. pavo* as castrated animals were able to display the full suite of female-like behaviors. Moreover, blocking aromatase or administering T or E_2 did not increase female-like displays further suggesting that the activation of these behaviors do not rely on sex steroids. Contrarily, exogenous androgen administration reduced female-like behavior and this is a likely mechanism for behavioral defemination of sexual displays during tactic switching in this species. The endocrine mechanism promoting masculinization of sexual displays during tactic switching in *S. pavo* is for now unclear as T, 11-KT and E_2 failed to induce nesting male-like displays in sneakers. Similar experiments in nesting males and future studies with longer-lasting hormonal manipulations in sneakers should contribute to elucidating this issue.

Acknowledgments

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