

## Changes along the male reproductive axis in response to social context in a gonochoristic gobiid, *Zosterisessor ophiocephalus* (Teleostei, Gobiidae), with alternative mating tactics

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### Abstract

Sexual selection has given rise, in several taxa, to intrasexual variation in male phenotype. While evolutionary studies have provided explanations of the adaptive function of this dramatic male phenotypic diversity, the proximate control of its expression has still to be completely understood. Several observations, primarily from sex-changing species, indicated a major role of social interactions in reproductive axis regulation and consequently in the expression of alternative male phenotypes. Here we documented changes along the male reproductive axis in response to social context in a gonochoristic species, the grass goby *Zosterisessor ophiocephalus*, where fully functional alternative male mating tactics appear to be expressed as an ontogenetic gradient. In the grass goby, larger and older males dig a nest and perform parental care, while smaller males sneak fertilization during territorial male spawning. Territorial males are characterized by a higher number of gonadotropin-releasing hormone (GnRH) neurons in forebrain preoptic area, smaller testes, larger seminal vesicles, and viscous ejaculates that last longer and contain fewer sperm than those of sneakers. To experimentally investigate the role of social factors in inducing changes along the male reproductive axis, sneakers were tested in two different situations: nesting alone or with ripe females. Sneakers that mated and performed parental care showed dramatic changes in brain, reproductive apparatus morphology, and ejaculate traits. GnRH-immunoreactive cells in forebrain preoptic area increased in number, reaching values typical of wild-caught parental males. Testes size decreased while seminal vesicle size increased and ejaculates showed lower sperm densities. These results were discussed within the framework of the social transduction hypothesis, which predicts that social experience should mediate, through a cascade of internal processes, shifts between morphs throughout life.

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### Introduction

Over the past three decades, increasing numbers of studies have shown that sexual selection can induce a variety of adaptations at behavioral, morphological, endocrine, and neurochemical levels, not only between but also within the two sexes (Andersson, 1994; Foran and Bass, 1999; Gross, 1996). In particular, male competition for

mates may lead to the evolution of alternative reproductive strategies and tactics (Gross, 1996), with dominant males as territorial or nesting individuals who monopolize mates and subordinates that exploit this investment in mate attraction by parasitizing the dominant males (Taborsky, 1994, 1998). The expression of alternative male morphs arises from phenotypic diversity in behavior, morphology, physiology, and life history traits (Andersson, 1994; Gross, 1996). Among vertebrates, teleost fishes exhibit the widest range of male reproductive morphs, and the polymorphism in mating and parental behavior appears to be consistently linked to differences in testis size (Petersen and Warner, 1998;

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Taborsky, 1998), accessory reproductive structure size and function (Barni et al., 2001; de Jonge et al., 1989; Rasotto and Mazzoldi, 2002; Scaggiante et al., 1999), ejaculate characteristics (Neat, 2001; Rasotto and Mazzoldi, 2002; Scaggiante et al., 1999), serum hormone levels (Brantley et al., 1993b; Moore et al., 1998; Oliveira et al., 2001a,b,c,d), and neurochemistry (Goodson and Bass, 2001; Grober et al., 2002; Foran and Bass, 1999; Hoffman and Fernald, 2000).

Male phenotypic diversity may be fixed for life or flexible. In *Xiphophorus nigrensis*, male morphs are expressed as a fixed polymorphism that appears to be under the control of a single gene (Ryan et al., 1992). In the plainfin midshipman, *Porichthys notatus*, males also permanently adopt different life history strategies, as a result of diverging developmental trajectories in juveniles (Bass, 1992, 1996). On the other end of this continuum, the great majority of both hermaphroditic and gonochoristic species show flexible alternative reproductive tactics, with transitions between distinct male morphs occurring in relation to increasing age and/or changing environmental conditions (Taborsky, 2001). While various complementary explanations have been provided for the evolution and maintenance of different male morphs (Bass, 1998; Gross, 1996; Shermann, 1988), the proximate mechanisms that regulate both the timing and expression of the switch between alternative male phenotypes are not fully delineated.

In several fish species, growth and reproduction are influenced by social interactions (Hoffman et al., 1999; Schultz et al., 1991; Warner, 1984a). A major example of socially regulated reproduction is the African cichlid *Haplochromis burtoni*, in which gonad maturation is suppressed in juvenile and subordinate adult males in the presence of dominant adult males (Davis and Fernald, 1990; Francis et al., 1993). Dominant males show brighter coloration, larger testes, and larger gonadotropin-releasing hormone (GnRH) neurons in hypothalamic preoptic area (POA) than reproductively inactive males (Davis and Fernald, 1990). Switches from reproductively active to inactive male phenotypes occur in either direction in response to changes in an individual's social status (Francis et al., 1993; Hoffman et al., 1999). Social signals appear to regulate the male reproductive axis by influencing gene expression of the key regulatory neuropeptide GnRH (White et al., 2002).

Social factors are also known to shape the expression of sexual phenotypes in many sequential sex changing species (Godwin et al., 2003; Grober and Bass, 1991; Grober and Sunobe, 1996; Reavis and Grober, 1999; Shapiro, 1981; Warner and Swearer, 1991). In some protogynous hermaphrodites, defined as diandric, two reproductively active male morphs are present and these species exhibit both sex change and role change (from sneaker to courting male) (Warner, 1984b). These species include *Thalassoma bifasciatum*, an intensively studied wrasse where behavioral sex and/or role change to a dominant male phenotype is correlated with changes in color pattern, gonadal tissue,

hormone levels, and with an increase in GnRH cell numbers in forebrain POA (Godwin et al., 1996; Grober and Bass, 1991; Grober et al., 1991; Warner and Swearer, 1991).

The preoptic GnRH system, with its primary role in vertebrate reproduction and well-known sexual dimorphism and intrasexual polymorphism (Cooke et al., 1998; Fernald and White, 1999; Foran and Bass, 1999; Godwin et al., 2003; Parhar et al., 1995; Rissman et al., 1997; White et al., 2002), is becoming a major focus in understanding the mechanisms underlying socially regulated changes in endocrine and cytological function resulting in sex and/or role change in both hermaphroditic and gonochoristic species. The “social transduction hypothesis” (Grober, 1998) stating that social interactions determine the regulation of the hypothalamic–pituitary–gonadal (HPG) axis, and consequently the expression of male reproductive phenotypes, can be applied not only to hermaphroditic but also to gonochoristic species expressing alternative morphs (Oliveira et al., 2001d). Differences between species with fixed and flexible morphs may be based upon the limited vs. life-long ability of neuroendocrine mechanisms to respond to social experience (Oliveira et al., 2001d). Experimental studies, in sex or role changing species and in the gonochoristic African cichlid, strongly support this hypothesis (Grober and Bass, 1991; White et al., 2002). However, despite information on differences in serum hormone levels and neurochemistry activity in gobies and blennies with alternative male mating tactics (see Oliveira et al., 2001d), the responsiveness of neuroendocrine mechanisms to social regulation in a gonochoristic teleost with functional male morphs has not been examined experimentally.

In gonochoristic gobiids and blenniids, species with intrasexual functional polymorphism are common (gobiids: Cole, 1982; Magnhagen, 1992, 1998; Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000; blenniids: Neat, 2001; Oliveira et al., 2001a,c,d; Ruchon et al., 1995), and in some of them, many male traits have been fully characterized with regard to each reproductive tactic (Mazzoldi, 1999; Neat, 2001; Oliveira et al., 2001d; Rasotto and Mazzoldi, 2002). The grass goby, *Zosterisessor ophiocephalus* (Pallas, 1814), is an extensively studied species (Mazzoldi et al., 2000; Scaggiante et al., 1999) where a plastic response to social context might be expected since it is known that parasitic males are able to adjust sperm expenditure in relation to the number of competitors present at the same spawning event (Pilastro et al., 2002). The grass goby is a substrate spawner and males are known to release ejaculate in form of trails, bands of mucins in which sperm are embedded (Marconato et al., 1996). The mucins, secreted by an accessory gland defined as the seminal vesicles (Scaggiante et al., 1999), slowly dissolve in seawater and therefore release active sperm over a prolonged period of time (Marconato et al., 1996). During the breeding season, large dominant males excavate and defend a nest where they court females, mate, and perform paternal egg care (Gandolfi et al., 1991; Mazzoldi et al., 2000). Nests consist of a main large chamber, with multiple

entrances and size that positively correlate with parental male body length (Mazzoldi et al., 2000). Smaller mature males do not engage in nest construction and courtship but mate opportunistically by sneaking inside parental males nests (Mazzoldi et al., 2000). In addition to nesting and mating behavior, parental and sneaker males are characterized by several morphologically and functionally different traits (Table 1). Parental males are older and larger than sneakers (Mazzoldi et al., 2000). They typically show a smaller investment in sperm production, a much lower gonadosomatic index (i.e., gonad weight/body weight  $\times$  100) than sneaker males, but highly developed seminal vesicles secreting a large volume of mucins (Scaggiante et al., 1999). As a result of these differences in the morphology and secretory abilities of the reproductive apparatus, ejaculates released by parental and sneakers males perform differently. Indeed sperm trails of parental males, richer in mucin and containing a lower number of gametes, are opaque whitish, release sperm more constantly over time, and last longer than those of sneaker males that, being poorer in mucins, are bright white and release most of their sperm immediately (Mazzoldi et al., 2000; Scaggiante et al., 1999). Analyses of the forebrain preoptic area show that parental males have a higher number of GnRH neurons, when corrected for differences in body size, than sneaker males (Scaggiante, 2002).

For this well-known suite of behavioral, morphological, and functional traits that characterize the male reproductive phenotypes (Table 1) and for the rapid responsiveness to social interactions (Pilastro et al., 2002), the grass goby provides an excellent opportunity to test the role of social experience in mediating transformation between alternative reproductive morphs. To examine the role of social context in inducing changes along the male reproductive axis, we tested sneaker males in two distinct situations: nesting alone

and nesting with ripe females and fertilized eggs. We recorded several morph-typical traits such as (a) behavior, (b) ejaculate color and sperm trail density, (c) gonadosomatic and seminal vesicles somatic indices (GSI and SVSI), (d) seminal vesicle morphology, and (e) number and size of forebrain preoptic GnRH-immunoreactive cells.

Considering that, in the field, small males with both reproductive apparatus and ejaculate characteristics typical of sneakers have sometimes been found to occupy large nests (Mazzoldi et al., 2000), we expect sneakers nesting alone to display territorial behavior, but we do not expect the lack of a parental male to be a sufficient stimulus to induce morphological changes toward the parental male morph. The appearance of morphological and functional parental male traits is instead expected in sneakers exposed to ripe females and performing the full complement of reproductive behaviors, from courtship to egg care. We expect all experimental sneakers to display typical parental male behavior (e.g., territoriality and nest defense).

## Materials and methods

Fish were field collected from the Venetian Lagoon, Northern Adriatic Sea, Italy, during their breeding season, from April 1999 to June 2000. Individuals were transported to the laboratory and sexed by their dimorphic genital papilla, being elongated and pointed in males, and short and rounded in females (Gandolfi et al., 1991). Fish were anaesthetized in a solution of 2% MS222 in seawater and total body length (TL) was measured to the nearest mm with digital calipers. Male morph was assessed by checking well-known polymorphic traits, including body size, sperm trail color, and sperm trail density (Mazzoldi et al., 2000;

Table 1  
Summary of sexually polymorphic traits in grass goby parental and sneaker males (Mazzoldi et al., 2000; Scaggiante, 2002; Scaggiante et al., 1999)

	Polymorphic traits	Parental males	Sneaker males
Life history	Total length (cm)	17.2–22.6	8.1–12.9
	Age	2–3 years	1–2 years
Behavior	Nest building	yes	no
	Parental care	yes	no
Reproductive apparatus development	Gonadosomatic index (GSI) mean $\pm$ SEM (range)	1.07 $\pm$ 0.12 (0.09–2.02)	3.45 $\pm$ 0.20 (0.08–8.60)
	Seminal vesicle somatic index (SVSI)	1.16 $\pm$ 0.09 (0.43–1.92)	0.27 $\pm$ 0.02 (0.01–1.08)
	Seminal vesicles mucin content	high	low
	Morphology of seminal vesicle chambers	large chambers walls of flat epithelium lumina entirely filled with secretion	small chambers walls of cylindrical epithelium lumina mainly filled with sperm
Ejaculate characteristics	Sperm trail color	opaque	white
	Sperm trail width (mm)	4.6 $\pm$ 0.3	1.5 $\pm$ 0.1
	Sperm trail longevity	24 h	11 h
	Total sperm number/trail ( $\times 10^5$ )	40.0 $\pm$ 21.0	219.0 $\pm$ 61.0
	Sperm trail density ( $\times 10^5$ ) (no. of sperm/mm <sup>2</sup> )	0.5 $\pm$ 0.2	9.4 $\pm$ 2.0
Forebrain characteristics in reproductive season	Mucin content	high	low
	Number of preoptic GnRH cells	51.56 $\pm$ 4.21	2.18 $\pm$ 1.15

Table 2  
Sample size, total length, and body weight of experimental sneakers

Male	<i>n</i>	Total length (cm) range (mean ± SE)	Body weight (g) range (mean ± SE)	
SA	6	9.2–12.1 (10.42 ± 0.43)	6.16–17.46 (11.66 ± 1.72)	
SF	TS	14	8.7–12.3 (10.66 ± 0.28)	5.14–19.02 (12.89 ± 1.04)
	SS	23	8.8–12.0 (10.38 ± 0.21)	6.00–20.00 (10.88 ± 0.77)

Abbreviations: SA = sneakers alone, SF = sneakers with females, TS = transformed sneakers, SS = stimulated sneakers. Data are reported as mean ± standard error of the mean (SEM).

Scaggiante et al., 1999). Artificial sperm trails (not different from natural trails, as demonstrated by Scaggiante et al., 1999) were collected by gently squeezing the male's belly onto a transparent acetate sheet, and sperm trail color was recorded according to criteria proposed by Scaggiante et al. (1999). To characterize mucin content, artificial trails were stained with periodic acid-Schiff (PAS) reaction for polysaccharide detection (Pearse, 1985). Sperm density, determined through the measure of trail length and width and an estimation of the total number of sperm embedded, was evaluated as per the methods established by Mazzoldi et al. (2000) and Scaggiante et al. (1999). Sneaker males were then housed in individual tanks (70 l) provided with a sandy bottom and with a PVC pipe for nesting, while females were kept in large group tanks (120 l). Water temperature was kept at 16–18°C and artificial light followed a cycle of 12 h of light and 12 h of dark. Fish were fed daily with fresh black mussels.

Acquisition of laboratory animals, housing, and experimental procedures have been conducted in accordance with the institutional guidelines of Italian Ministry of University and Research (MIUR, DL 116/92).

#### Change of tactic experiments

Forty-three sneakers, ranging in size from 8.3 to 12.2 mm TL, were tested in two different social conditions: isolated in the tank (sneakers alone, SA, *n* = 6), or together with a ripe female (sneakers with female, SF, *n* = 37). Before performing the experiments, two sperm trails from every sneaker male were obtained and trail color, mucin content, and sperm density were recorded.

A total of 112 ripe females, characterized by a round belly and a reddened genital papilla, were randomly chosen from the group tank and introduced into SF males tanks. When spawning occurred, females were removed at the end of egg deposition, lasting on average 7–8 h (Mazzoldi et al., 2000), and released back in the field. If spawning did not occur, females were removed when evidence of ripeness declined and were immediately replaced with a new ripe female. SF spawning males were left alone in tanks until egg hatching, and at the third day after egg deposition a sample of eggs (*n* = 50) were taken from the male's nest to check for fertilization success. The experiment ended after egg hatching, about 9 days postspawning (*n* = 14, average = 8.50 ± 0.34 days, range: 7–11). Nonspawning SF males were exposed to ripe females until a maximum of 30 days. SA sneakers were kept isolated in tanks for the same maximum number of days. At the end of the experiment, both SA and SF males were then anesthetized again with MS222 and additional ejaculates were collected to reevaluate sperm color and sperm density. All males were then killed with an excess of MS222 and their TL (to the nearest mm) and body weight (BW; to the nearest mg) were measured. Gonads and seminal vesicles were separately removed and weighed to the nearest mg (GW and SVW), and the gonadosomatic index (GSI = 100 × GW/BW) and seminal vesicles somatic index (SVSI = 100 × SVW/BW) were calculated. Seminal vesicles were processed for histological analysis and brains were removed and processed for immunohistochemical analyses.

#### Behavioral observations

Males were randomly chosen for observations of species-typical behaviors (Mazzoldi et al., 2000). Behavioral observations were conducted prespawning (4 days, *n* = 17), during spawning (1 day, *n* = 4), and postspawning (4 days, *n* = 4) twice a day for 20 min each. During the spawning day, data were recorded for 20 min every hour, starting from female introduction in the tank until the end of egg deposition. We recorded spacing, aggression, courtship, and spawning behavior displayed by males as follows: (i) time spent inside or outside the nest (seconds); (ii) time spent in fin-sweeping (seconds); (iii) the number of female stimulations, which includes pursuing and biting the female; (iv) the number of times the male rubs his

Table 3  
Behaviors recorded in transformed sneakers during spawning and postspawning phases as seconds on 20 min of observations

Phase	<i>n</i>	Time inside the nest (s)	Female stimulations (s)	No. of genital papilla rubs	Rub lasting (s)	Mouth opening (s)
Spawning	4	50.79 ± 21.73	16.12 ± 3.67	3.37 ± 0.67	6.89 ± 1.22	0.76 ± 0.43
Phase	<i>n</i>	Time inside the nest (s)	Egg checking (s)	Egg fanning (s)	Rub lasting (s)	
Postspawning	4	623.97 ± 109.79	53.00 ± 17.15	448.44 ± 82.43	4.56 ± 1.00	

Behaviors are expressed in seconds per 20 min of observation time. Data are reported as mean ± standard error of the mean (SEM).

urogenital papilla on the nest surface, a display indicating sperm trail release (Marconato et al., 1996); (v) the duration of each rub (seconds); (vi) time spent for mouth openings, a display related to acoustic communication (Malavasi et al., 2003); (vii) time spent fanning the eggs in the nest (seconds); and (viii) time spent checking the eggs with the mouth (seconds).

### Histology

Seminal vesicles were fixed in Bouin's solution, embedded in paraffin, and 7  $\mu\text{m}$  sections were mounted on slides and stained with hematoxylin and eosin. Sections were analyzed for morphological traits known to differ between parental and sneaker males (Scaggiante et al., 1999; Table 1): (i) thickness of chamber walls (thin–thick); (ii) presence of sperm in chamber lumina (yes–no); and (iii) secretion of mucin (present–absent).

### Immunohistochemistry

Brains were immersion fixed in Bouin's solution for 12 h, rinsed in 50% ethanol, and stored at 4°C. Samples were embedded in paraffin, sectioned transversally at 15  $\mu\text{m}$ , and mounted as three parallel series through the brain on chromoalum-coated slides. Following deparaffinization and hydration, slides were treated using the methods outlined by Grober and Bass (1991), except for the following details: (a) a Streptavidin–Biotin Kit (KPL) was used and (b) a monoclonal antibody against GnRH (Park and Wakabayashi, 1986) was used at a concentration of 1:1000. As control for antiserum specificity, sections from different brains ( $n = 6$ ) were processed omitting the primary antibody.

Before running quantitative analyses, all slides were coded so that researchers were blind to the fish's experimental group. To test for differences in our quantification technique, measurements were duplicated by two different authors (MS and VL), who obtained the same qualitative results (Wilcoxon's test:  $n = 12$ ,  $P > 0.2$  for all brain areas). For six animals, immunocytochemistry was run on three parallel series of slides and the number of GnRH cells in each series was summed to yield the total cell number for those brains. There were no significant differences in the number of cells among the three alternate brain series (Friedman's ANOVA:  $P > 0.7$  for both TN and POA). For this reason, the number of cells for brains with only one available series was tripled to obtain the total cell number. Second and third series were used to conduct controls for immunolabeling specificity (see above) or to run immunocytochemistry with different GnRH antibodies. We applied different GnRH antibodies at different working dilutions: chicken I, chicken II, salmon, sea bream, C-free, and LHRH RBX polyclonal antibody, but only LRH13 had positive reaction in the brain of this goby.

The mean area ( $\mu\text{m}^2$ ) of GnRH ir-cells was determined using a Leica DMLB microscope equipped with a three-

CCD color video camera (24 bites/pixel) and image-processing software (Windows Microimage 3.4). Areas were only measured for cells in sections where the nucleus and one neurite were both present, avoiding the possibility that cells were recounted in subsequent sections.

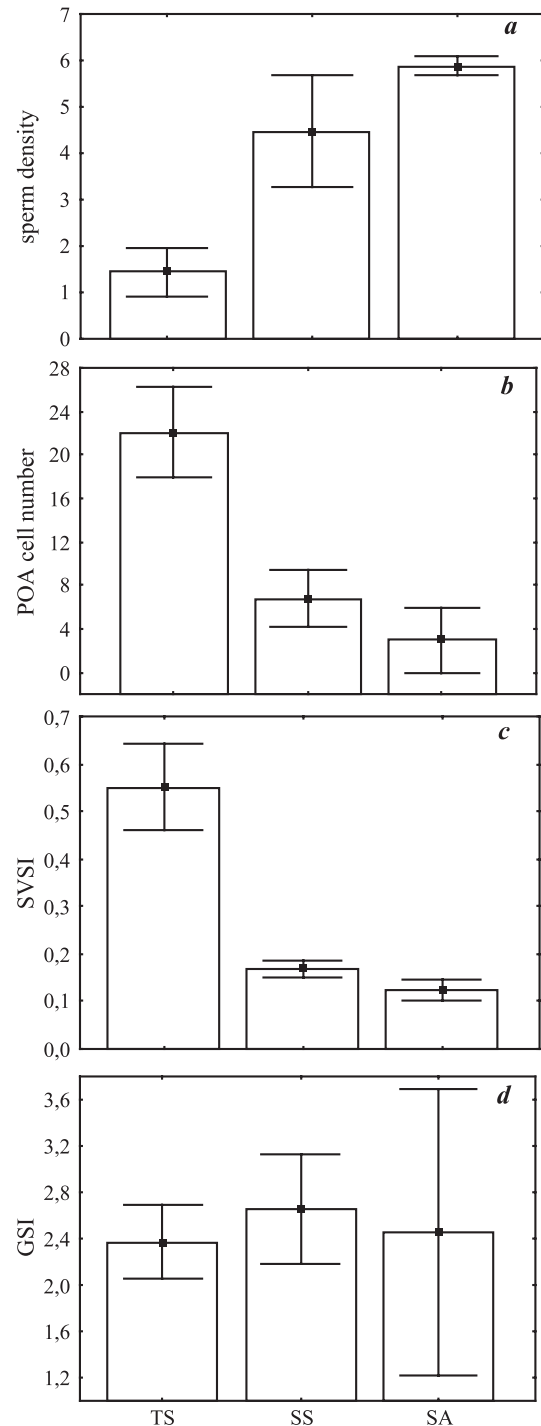


Fig. 1. Differences in (a) ejaculate sperm density (no. of sperm  $\times 10^5/\text{mm}^2$ ) at the end of the experiment ( $n$ : TS = 13, SS = 11, SA = 2), (b) number of GnRH-immunoreactive cells in the preoptic area, (c) SVSI values, and (d) GSI values in experimental sneaker males (for these three figures  $n$ : TS = 14, SS = 23, SA = 6). TS = transformed sneakers, SS = stimulated sneakers, SA = sneakers alone. Data are expressed as the mean  $\pm$  SEM.

### Statistical analyses

Descriptive analyses were reported as mean  $\pm$  standard error of the mean (SEM). We tested all data distributions for normality using a Shapiro–Wilks test and performed non-parametric analyses when the assumption of normality was violated even after appropriate data transformation. For behavioral data recorded during consecutive days or hours, we computed the average value. Statistical analyses were done by using STATISTICA 6.0.

### Results

Fourteen out of 37 SF males spawned in tanks and exhibited egg care until hatching (called transformed sneakers: TS; Table 2). The other 23 SF males did not spawn (called stimulated sneakers, SS), despite exhibiting

typical courting behavior. Total length of the three sneaker experimental groups (SA, TS, and SS) was not significantly different.

Pre-spawning behavioral observations showed significant differences between SA, SS, and TS in time spent inside or outside the nest (Kruskal–Wallis ANOVA:  $df = 2$ ,  $\chi^2 = 8.25$ ,  $P = 0.016$ ) and time spent fin-sweeping (Kruskal–Wallis ANOVA:  $df = 2$ ,  $\chi^2 = 11.26$ ,  $P = 0.004$ ), with SA males spending more time inside the nest than the other two groups and TS fish displaying less tail sweeping behavior than the other experimental males. After female introduction in the tank, all experimental sneakers immediately displayed courtship behavior. They stimulated females to enter the nest and were observed to rub their urogenital papilla onto the nest surface for sperm trail release. Among SF males, only TS successfully spawned and expressed those behaviors related to parental care (Table 3). Sneakers who spawned in captivity obtained an average fertilization

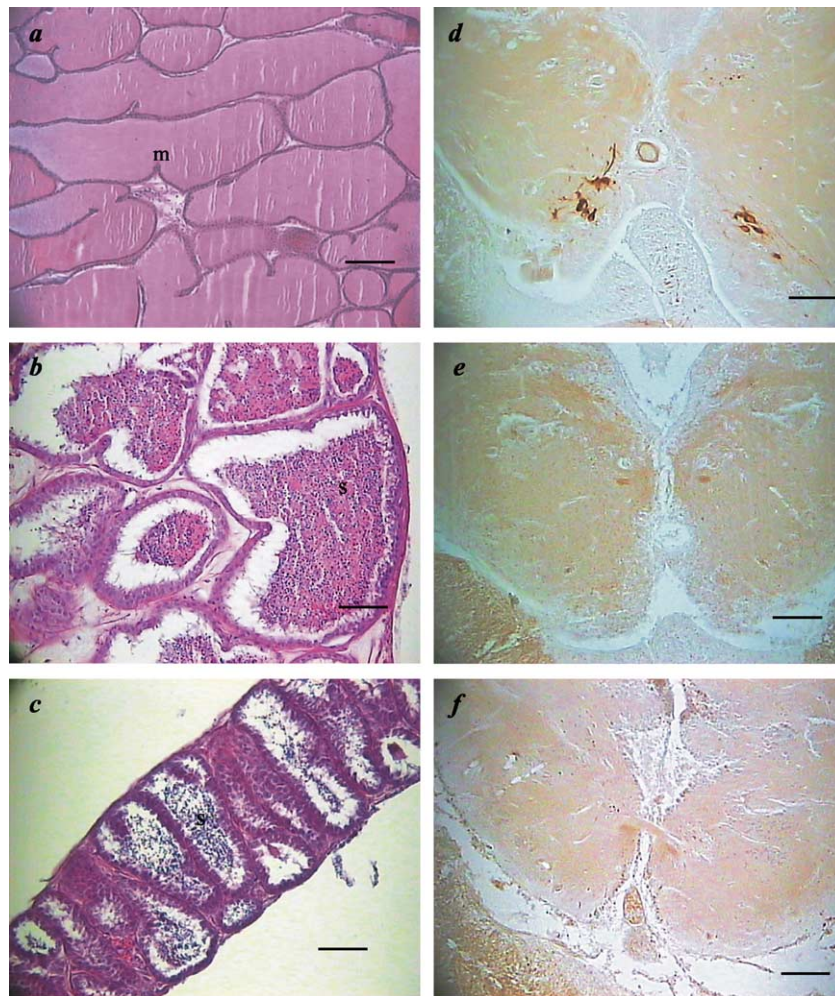


Fig. 2. Differences in seminal vesicles histology and preoptic GnRH immunohistochemistry observed among the three groups of experimental sneakers ( $n$ : TS = 14, SS = 23, SA = 6). Photomicrographs of transverse sections of seminal vesicles (a–c) and of brains at the level of preoptic area (d–f) are reported for TS (transformed sneakers), SS (stimulated sneakers), and SA (sneakers alone) males. In TS, seminal vesicles are organized in chambers with lumina filled with a homogeneous mucous secretion (m). In contrast, SS and SA present sneaker-typical vesicles with lumina mainly filled with sperm (s). These data are paralleled by the presence of more abundant GnRH-ir cells in TS fish while few cells were observed in SS and SA males (no cells in most sections). Scale bars represent 50  $\mu$ m for a–c and 100  $\mu$ m for d–f.

success of  $97.3 \pm 1.2\%$  ( $n = 14$ , range: 84–100%) with an average fertilized egg number of  $48.8 \pm 0.6$  out of 50.

Before performing social manipulation trials, all experimental sneakers released white sperm trails with an average ejaculate sperm density of  $9.2 \pm 1.1 \times 10^5$  sperm/mm<sup>2</sup> ( $n = 43$ , range  $0.94\text{--}31.95 \times 10^5$ ), and sperm density was not significantly different among the SA, TS, and SS groups (SA mean value =  $10.31 \pm 3.01 \times 10^5$  sperm/mm<sup>2</sup>; TS mean value =  $8.39 \pm 1.97 \times 10^5$  sperm/mm<sup>2</sup>; SS mean value =  $9.40 \pm 1.54 \times 10^5$  sperm/mm<sup>2</sup>). After the change of tactic experiments, TS ( $n = 13$ ) released ejaculates with an homogeneous opaque whitish color and with a reduced sperm density (Fig. 1a), while SS ( $n = 11$ ) and SA ( $n = 2$ ) males, after their own experimental procedures, still produced bright white trails with high sperm density ejaculates (Fig. 1a), although in a reduced number of individuals (48% of SS and 33% of SA). The observed decrease in sperm density for TS fish was significant (paired  $t$  test:  $df = 12$ ,  $t = 3.706$ ,  $P = 0.003$ ), whereas sperm density in SS did not show significant changes in response to the experimental treatments (no statistical test could be applied for SA). Moreover, before social manipulations, all experimental sneakers released bright white sperm trails with a low mucin content, as shown by a weak PAS staining reaction, while after captivity only TS fish produced opaque whitish ejaculates rich in mucin, strongly reacting to PAS.

The SVSI values of experimental groups differed significantly [TS:  $n = 14$ ; SS:  $n = 23$ , SA:  $n = 6$ ; ANOVA:  $F(2,40) = 17.684$ ,  $P < 0.001$ ; HSD test, SS–SA  $P > 0.8$ , in all the other cases  $P < 0.001$ ]. TS males showed a significantly higher index value than SS and SA groups (Fig. 1c). In contrast, GSI values exhibited a modest but

nonsignificant variation among the three sneaker experimental groups (Fig. 1d).

Histological analyses showed that TS seminal vesicles lost typical morphological and secretory traits of sneaker males (Scaggiante et al., 1999), presenting extended chambers with thin walls of cubic epithelial cells and lumina filled with mucin secretion (Fig. 2a). On the other hand, 57% of the SS group presented regressed vesicles, and the remaining SS plus all SA had seminal vesicles with reduced chambers characterized by thick walls of cylindrical cells and lumina mainly filled with sperm (Figs. 2b and c).

GnRH-ir cells were located in two distinct brain areas: the ganglion of the terminal nerve (TN; Fig. 3a) and the preoptic area (POA; Fig. 3b). Number and area of TN GnRH-ir cells did not change significantly between experimental sneakers. The number of GnRH-ir cells, but not their size, was significantly greater in the POA of TS fish than that of all other experimental sneaker groups [TS:  $n = 14$ ; SS:  $n = 23$ ; SA:  $n = 6$ ; ANOVA: cell number,  $F(2,40) = 7.333$ ,  $P = 0.002$ ; HSD test: in all the cases but SS–SA,  $P < 0.02$ ; Figs. 1b and 2d–f].

## Discussion

Our results document the rapid appearance of a suite of traits typical of parental males, including mating and parental behavior, preoptic GnRH cell number, seminal vesicles investment and function, ejaculate sperm density, and testes investment, in opportunistic sneaker males exposed to specific social contexts. This appears to be the first demonstration of socially induced transformations

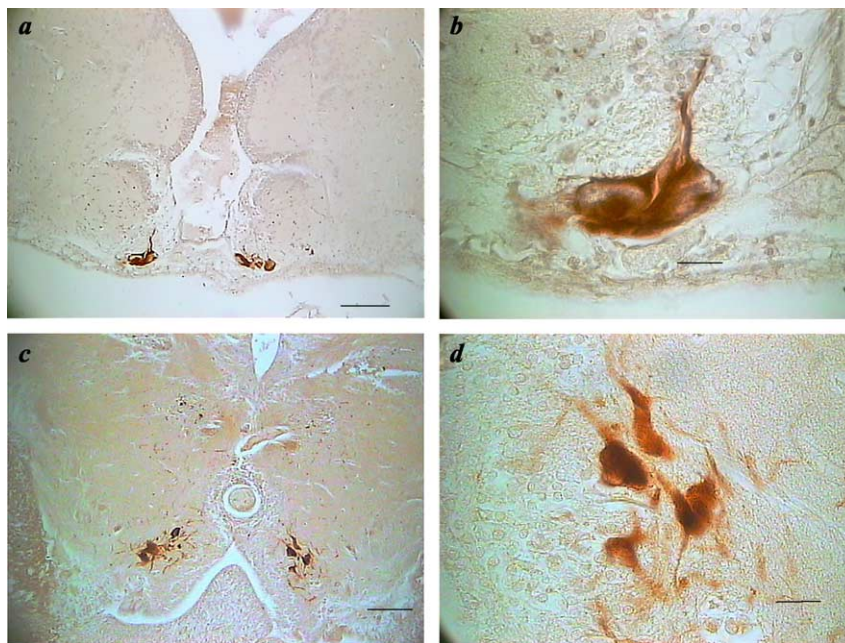


Fig. 3. Photomicrographs of transverse sections from a grass goby individual showing GnRH-immunoreactive cells in the ganglion of terminal nerve (a and b) and in preoptic area (c and d). Note the low background staining, intense cytoplasmic immunoreactivity, and unstained nuclei. Scale bars represent 100  $\mu\text{m}$  for a and c, and 20  $\mu\text{m}$  for b and d.

among reproductive phenotypes, in a gonochoristic species with two distinct, fully functional male alternative mating morphs that are sequentially expressed over the life cycle (Mazzoldi et al., 2000). Similar changes have been observed in a cichlid fish (White et al., 2002), wherein social interactions are able to influence GnRH gene expression. However, although males in this species can reversibly switch between dominant and subordinate morphs, the subordinate males are hypogonadal and thus are not a functional reproductive morph (Hoffman et al., 1999). The change of tactic shown by grass goby sneakers resembles the socially controlled mechanisms of sex or role change observed in hermaphroditic species and similarly involves rearrangements of behavior, brain, gonads, and secondary sexual characters (Foran and Bass, 1999; Godwin et al., 2003; Grober, 1998).

In our experiments, morphological or functional changes toward typical parental males traits were observed only in those experimental males that experienced spawning and egg care (TS), whereas sneakers housed alone with a nest (SA) and those exposed to a nest site and ripe females but did not spawn (SS) maintained typical sneaker characteristics (Table 4). The only behaviors expressed in the prespawning phase were as follows: movement inside and outside the nest, a behavior expressing spacing and territorial patrolling; and fin-sweeping, a behavior related to nest preparation. Unexpectedly, prespawning behaviors were expressed differently among experimental sneaker groups, a result that seemed to be related primarily to the limited number of study fishes and to the likely effect of differential subjective degree of acclimatization in tank. On the other hand, courtship behavior was performed by all experimental sneakers exposed to ripe females, as was egg care among those spawning in tanks. Interestingly, sneakers

spawning and taking care of eggs in tanks, with no other male competitors, expressed the full set of behaviors recorded for typical parental males spawning in the same artificial conditions (Mazzoldi et al., 2000). These data suggested that transformed sneakers did not differ from typical parental males with respect to mating and parental care; moreover, they showed an overexpression of these behaviors because compared to parental males, TS fish spent more time in female stimulation and increased their production of genital papilla rubs (Scaggiante, 2002).

Experimental sneakers who spawned in captivity (TS) obtained a fertilization rate similar to that observed for typical parental males spawning in aquaria (average rate = 96%, data from Scaggiante, 2002), demonstrating an equal capability to fertilize eggs for established parental males and our TS males. After mating and egg care, TS fish released sperm trails with characteristics significantly changed toward typical parental male conditions, with opaque whitish color, remarkable abundance of mucin content, and a significant reduction in sperm density. On the contrary, these same morphological traits in SS and SA ejaculates remained unchanged, maintaining values in the range typical of sneaker males (Tables 1 and 4).

Regarding the reproductive apparatus, major modifications were observed in seminal vesicle morphology. In SA and SS fish, vesicles were not regressed and exhibited sneaker-typical histology (Scaggiante et al., 1999), with small chambers lined with thick walls of cylindrical epithelium and internal lumina mainly filled with sperm. On the other hand, TS fish showed totally different vesicle morphology, with large chambers, walls of flat epithelium, and lumina filled with mucin secretion, typical of parental male seminal vesicles (Scaggiante et al., 1999) (Table 4). A small but not significant change in testis investment was

Table 4

Summary of sexually polymorphic traits in grass goby experimental sneaker males compared to typical parental males (PM)

		SA	SS	TS	PM
Behaviors	Spawning	no	no	yes	yes
	Egg care	no	no	yes	yes
Reproductive apparatus	GSI	2.46 ± 0.86	2.65 ± 0.44	2.37 ± 0.56	1.07 ± 0.12
	SVSI	0.13 ± 0.08	0.17 ± 0.04	0.55 ± 0.05	1.16 ± 0.09
	Seminal vesicles	low	low	high	high
	Seminal vesicles mucin content morphology	small chambers walls of cylindrical epithelium lumina mainly filled with sperm	small chambers walls of cylindrical epithelium lumina mainly filled with sperm	large chambers walls of flat epithelium lumina filled with mucin secretion	large chambers walls of flat epithelium lumina filled with mucin secretion
Ejaculate characteristics	Sperm trail color	white	white	opaque	opaque
	Sperm trail density (×10 <sup>5</sup> , no. of sperm/mm <sup>2</sup> )	5.87 ± 0.20	4.46 ± 1.21	1.45 ± 0.52	0.5 ± 0.2
	Mucin content	low	low	high	high
Forebrain characteristics in reproductive season	Number of preoptic GnRH cells	3.00 ± 5.34	6.78 ± 2.73	22.07 ± 3.50	51.56 ± 4.21
	Size of preoptic GnRH cells (μm <sup>2</sup> )	81.205 ± 26.420	52.924 ± 9.341	81.557 ± 7.328	209.674 ± 19.009

Abbreviations: SA = sneakers alone, SS = stimulated sneakers, TS = transformed sneakers, PM = parental males. Data are reported as mean ± standard error of the mean (SEM). Data for PM derived from Mazzoldi et al., 2000; Scaggiante et al., 1999; Scaggiante et al., submitted.

recorded, with GSI of transformed sneakers closer to values typical of parental males. On the contrary, SVSI values maintained sneaker-typical values in SA and SS fish, while TS males made a significant investment in seminal vesicles, consistent with their transformation to the parental or territorial reproductive morph (Table 4). These observed modifications in qualitative and quantitative aspects of the reproductive apparatus cannot be ascribed to life in captivity since GSI and SVSI of all experimental fish were within the range of values reported for field collected males (Mazzoldi et al., 2000; Scaggiante et al., 1999).

The TS trait, in addition to parental care, that perfectly overlapped with what has been reported for wild parental males was the number of preoptic GnRH-ir cells (Table 4). In TS fish, we recorded a very strong increase in preoptic cell number, reaching average values more similar to typical parental male, while SS and SA fish maintained a number of GnRH-ir cells still very reduced, as typical of wild sneakers. On the contrary, POA GnRH-ir cell area of TS fish did not vary and remained in the range typical of sneakers, as for SA and SS fish (Scaggiante, 2002). Both number and area of TN GnRH-ir cells did not change among experimental sneakers, in agreement with what has been observed in wild caught males, where TN neuron traits appear constant regardless of male phenotype (Scaggiante, 2002).

The present results are in agreement with field observations for grass goby males, showing that individuals with the age, reproductive apparatus, and ejaculate traits typical of sneakers can occasionally occupy spawning nests (Mazzoldi et al., 2000). Moreover, our results demonstrate that nest defense and female courtship alone, even in the absence of a dominant or parental male, are behaviors that can be displayed also by sneaker males before the modification of most morph-typical traits, without significantly affecting sneaker morphology. Similarly, in the hermaphrodite *T. bifasciatum* (Godwin et al., 1996), male dominant behavior can be fully expressed before the appearance of functional male testes on the process attaining social dominance and sex change. The dissociation of behavioral display and reproductive morphological modifications has been suggested to indicate that these two processes may be regulated by mechanisms involving not only the HPG axis but also hypothalamic–pituitary–adrenal axis (Perry and Grober, 2003).

The behavioral, morphological, and physiological modifications that occurred in TS sneakers were shifted toward parental male typical traits, suggesting that mating and parental care performances appeared to be the most relevant stimuli in regulating a complete reproductive morph switch. The present results provide compelling evidence that social interactions can drive changes in behavior and reorganization of the reproductive axis (Reavis and Grober, 1999; White et al., 2002) in order to function as a new sexual phenotype (Oliveira et al., 2001d). Based upon the so-called “social transduction mechanism,” social context is able to affect neuroendocrine mechanisms, which in turn through

sex hormone release can regulate the expression of secondary sexual traits, reproductive behavior, and gonadal development, thereby shaping the expression of sexual polymorphism (Grober, 1998; Oliveira et al., 2001d). In species with flexible morphs, like the grass goby, social environment may mediate shifts between reproductive morphs throughout life, indicating a life-long responsiveness to social regulation (Oliveira et al., 2001d). Our results support the hypothesis that the transformation induced between male morphs was a result of the recent social interactions experienced by study fish. The same hypothesis suggests that the full compliment of reproductive behaviors should be affected by variation in the male reproductive axis. However, in our study species, some parental male-typical behaviors were displayed by stimulated sneakers, experimental fish where no morphological or physiological switch toward the parental morph was recorded. This seems to indicate that some behaviors, such as territory defense and female courtship, can be displayed in sneaker males before the modification of most morph-typical traits.

In fish species, like the grass goby, with flexible reproductive morphs characterized by a suite of well-known traits, reproductive tactic shifts may be mediated by a rapid response to change in social context, with switching occurring as an opportunistic response of individuals to the probability of a fitness benefit in a particular social–environmental situation (Gross, 1996; Taborsky, 1998). Following this view, animals appear to be sensitive to a variety of social cues when evaluating transitions between sexual morphs, thus decreasing the number of unsuccessful transformations and thereby increasing overall reproductive success.

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