

## Variability of GnRH secretion in two goby species with socially controlled alternative male mating tactics

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### Abstract

Male reproductive phenotypic plasticity related to environmental–social conditions is common among teleost fish. In several species, males adopt different mating tactics depending on their size, monopolizing mates when larger, while parasitizing dominant male spawns when smaller. Males performing alternative mating tactics are often characterized by a strong dimorphism in both primary and secondary reproductive traits. According to studies on sex-changing species and on species where only one male morph is reproductively active, male alternative phenotypes are expected to vary also in gonadotropin-releasing hormone (GnRH) neurons in forebrain preoptic area (POA). Here, we compared the intra- and inter-sexual variations in number and size of GnRH neurons, along with gonads and male accessory structure investment, in two goby species, the grass goby, *Zosterisessor ophiocephalus*, and the black goby, *Gobius niger*, characterized by male alternative mating phenotypes. In both species, older and larger males defend nests, court and perform parental care, while younger and smaller ones try to sneak territorial male spawning. We found that grass goby and black goby have different patterns of GnRH expression. Grass goby presents a clear intra-sexual dimorphism in GnRH expression, related to the occurrence of alternative mating tactics, while in the black goby, only inter-sexual differences are observed. The inter- and intra-specific variability in the GnRH neurons in these two goby species is discussed in light of the differences in migratory behavior, nest type, and mating system.

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### Introduction

Among vertebrates, teleost fish show an unparalleled variability of reproductive and social patterns with several species presenting extensive reproductive phenotype plasticity, ranging from seasonal variations related to the breeding season to sex/role change (Breder and Rosen, 1966; Demski, 1987; Gross, 1996; Taborsky, 2001). The influence of environmental/social interactions on the regulation of individual sexuality and on its morpho-physiological correlates have been demonstrated in several sequential hermaphrodites (Shapiro, 1984; Godwin et al., 1996; Bass and Grober, 2001) and also in some gonochoric species with distinct male phenotypes (Francis et al., 1993;

Immler et al., 2004; Scaggiante et al., 2004). The latter species, where single males exhibit during their life cycle different mating tactics, provide excellent models to understand the evolution and the proximate control of reproductive phenotype variability (Gross, 1996; Bass and Grober, 2001; Taborsky, 1994, 1998).

Alternative male mating tactics are characterized by the presence of two or more male phenotypes, differing in body size, coloration, ornamentation, serum hormone levels, and chemical signals (Taborsky, 1998; Locatello et al., 2003; Oliveira et al., 2001a,b,c,d). Dominant males invest in resource monopolization, either females directly or limited resources, whereas subordinate males parasitize the investment of dominant ones by attempting to fertilize the eggs spawned in their domain (Taborsky, 1994; 1998). Indeed dominant males, territorial/nesting individuals, are well known to invest

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primarily in territory defense, mate attraction, guarding, and often in parental care (Taborsky, 1998; Alonzo and Warner, 2000). On the other hand, parasitic males, defined as sneakers, streakers, or satellites, depending on their reproductive behavior, save investment in mate attraction, intra-sexual contests and paternal care and invest predominantly in sperm production, to cope with higher levels of sperm competition (Petersen and Warner, 1998; Taborsky, 1998, 2001). Alternative male phenotypes may be fixed for life (Brantley and Bass, 1994; Gross, 1996) or flexible, with transitions between reproductive morphs occurring in relation to the ontogenetic cycle and/or changes in environmental conditions (Taborsky, 1998, 2001). This male reproductive plasticity has been documented in several species belonging to families Blenniidae, Tripterygiidae, Gobiidae, Labridae, and Cichlidae where body and testis size, ejaculate expenditure, development of ornaments, and of gonadal accessory organs, vary according to the adopted tactic (de Jonge et al., 1989; Ruchon et al., 1995; Grober, 1998; Scaggiante et al., 1999; Mazzoldi, 1999; Neat, 2001; Rasotto and Mazzoldi, 2002; Oliveira et al., 2005). In some goby species, the switch from parasitic to dominant tactic has been experimentally achieved by modifying the social context of individuals, in terms of nest and female availability (Immler et al., 2004; Scaggiante et al., 2004).

Social factors are known to contribute to phenotypic variations inducing differences in the endocrine system (Dufty et al., 2002). In fish, differences between sexes, mating tactics, and reproductive conditions appear to correlate primarily with differences in brain modulation of gonadotropin-releasing hormone (GnRH) (Foran and Bass, 1999; Bass and Grober, 2001; Weltzien et al., 2004), a key peptide implicated in the neuroendocrine regulation of the hypothalamus–pituitary–gonadal (HPG) axis. Teleost brain contains at least two GnRH variants, but in the most common situation, three GnRH isoforms are detected (Lethimonier et al., 2004; Weltzien et al., 2004). The three GnRHs are expressed in the midbrain tegumentum (MT), in the terminal nerve ganglion (TN), and in the hypothalamic–preoptic area (POA) (Weltzien et al., 2004). Although the specific physiological role of each GnRH isoform is not yet fully understood, those expressed in TN and MT appear to be involved in coupling olfactory information with reproduction (Weltzien et al., 2004) and expression of social behavior (Ogawa et al., 2003) respectively. At variance, the GnRH form expressed in the POA seems to play a major role in inducing the release of gonadotropin hormones from the pituitary (Weltzien et al., 2004). Both seasonal and sex/status phenotypic variations have been correlated with variations of GnRH brain content or POA GnRH neurons number and/or size (Andersson et al., 2001; Bass and Grober, 2001). In several species, GnRH peaks prior or during the spawning seasons (Weltzien et al., 2004), and its increase is significantly due to the form expressed in the POA (Rodríguez et al., 2000; Andersson et al., 2001; Collins et al., 2001). These observations on the reproductive-related role of POA GnRH are supported by the concomitant increase of this isoform and of the gonadosomatic index (GSI) (Weltzien et al., 2004).

The variability in GnRH expression has been investigated only in a few species exhibiting flexible male mating tactics (Elofsson et al., 1997; Grober and Bass, 1991; Foran and Bass, 1999). The pattern emerging from these studies, together with the information on the variability in serum hormone levels between male reproductive phenotypes (Oliveira et al., 2001a,b,c,d), indicates that, in presence of alternative male mating tactics, intra-sexual differences in POA GnRH expression should be expected. Indeed intra-sexual differences of POA GnRH producing cells have been observed in the anemone fish *Amphiprion melanopus*, the African cichlid, *Haplochromis burtoni*, and the bluehead wrasse *Thalassoma bifasciatum*, with dominant males showing an higher number/area of GnRH neurons than subordinate ones (Davis and Fernald, 1990; Grober and Bass, 1991; Elofsson et al., 1997). However, the male phenotypes of the studied species show conspicuous divergences in behavior, morphology, and physiology, as exemplified by *A. melanopus* and *H. burtoni*, where only one male morph is reproductively functional, while the other is hypogonadal (Francis et al., 1993; Fox et al., 1997; Elofsson et al., 1997; Foran and Bass, 1999). Consequently, the hypothesis that differences in male reproductive tactics are accompanied by different patterns of GnRH expression should be tested in species characterized by less marked differences between male phenotypes.

The Gobiidae, a family of demersal spawners, represents an excellent model to investigate the relationship between phenotype plasticity and GnRH expression, as flexible alternative male mating tactics are known to occur in several species (Cole, 1982; Magnhagen, 1992; Magnhagen and Kvarnemo, 1989; Forsgren et al., 1996b; Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000). In particular, in two temperate gobies, the grass goby *Zosterisessor ophiocephalus* (Pallas) and the black goby *Gobius niger* L., mating systems and alternative male mating tactics have been carefully characterized (Table 1). Both species present a polygynous mating system and large territorial males monopolize females, defend a nest, and provide egg parental care. By contrast, smaller and younger males adopt a parasitic behavior. Male traits associated with alternative mating tactics appear to differ with respect to age, external morphology, relative testis size, size, and function of accessory organs to the reproductive apparatus, and ejaculate performances (Scaggiante et al., 1999; Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002). Parental males show a larger body size, smaller testes, and larger seminal vesicles than opportunistic males (Scaggiante et al., 1999; Rasotto and Mazzoldi, 2002). Despite these similarities in the mode of reproduction, grass goby and black goby differ in seminal vesicle function, type of nest, degree of polygyny, and sperm competition intensity (Gandolfi et al., 1991; Mazzoldi, 1999; Mazzoldi et al., 2005).

In order to test if GnRH variability is consistently present in species with flexible alternative male mating tactics, we performed immuno-histochemical analyses of brain areas involved in the regulation of reproductive axis in both grass goby and black goby individuals. The number and the soma size of GnRH immunoreactive cells, in addition to body, gonad and

Table 1  
Summary of species-specific reproductive traits of grass goby and black goby

	Grass goby	Black goby
Mating system	Polygynous	Polygynous
Polygyny degree	High	Low
Sperm competition intensity	High	Low
Male mating tactics	Parental and sneaker males	Parental and sneaker males
Parental males traits	Large body size, old age, small testes, large seminal vesicles	Large body size, old age, small testes, large seminal vesicles
Parental males behavior	Females monopolization	Females monopolization
Nest type	Muddy burrow dug under seagrass	Stones, rocks or artificial objects
Nest size	Positively related to male body size	Not related to male body size
Sneakers traits	Small body size	Small body size
Sneakers behavior	Sneaking inside the nests of territorial males during pair spawns	Sneaking inside the nests of territorial males during pair spawns
Seminal vesicles function	Mucin production in parental males sperm storage in sneakers	Mucin production and sperm storage in both males

(Data from Gandolfi et al., 1991; Mazzoldi, 1999; Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000; Scaggiante et al., 1999; Rasotto and Mazzoldi, 2002).

accessory organs sizes, were recorded, across seasons, in females, parental males, and sneaker males.

## Materials and methods

### Study species

The grass goby, *Z. ophiocephalus*, is a large coastal fish inhabiting seagrass meadows in shallow brackish water. In the Venetian Lagoon (Northern Adriatic Sea, Italy), it reaches sexual maturity at 1 year of age, lives up to 3 years, and presents a conspicuous variability in the size of mature males: from 7.4 to 25 cm total length (De Girolamo, 1994). During the breeding season, lasting from March to June, individuals larger than 16 cm and 3 years old, dig and defend a nest under the seagrass rhizomes, 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, whose sizes positively correlate

with parental male body length (Mazzoldi et al., 2000). One to several females lay the eggs on the nest ceiling, while males release sperm by rubbing their genital papillae (Marconato et al., 1996). Egg deposition lasts on average 7–8 h (Scaggiante et al., 1999), and males perform parental care on eggs, cleaning, and fanning them until hatching (Gandolfi et al., 1991). Nesting males have small gonadosomatic index (GSI) (Scaggiante et al., 1999) and release sperm in trails, sticky bands of mucins in which sperm are embedded (Marconato et al., 1996; Scaggiante et al., 1999). Mucins are produced and stored by accessory gonadal structures called seminal vesicles, which are highly developed in territorial males, as demonstrated by the high value of their seminal vesicles somatic index (SVSI) (Scaggiante et al., 1999). Males smaller than 12.5 cm and 1 year old do not engage in nest construction and courtship but mate opportunistically by sneaking inside parental males nests (Mazzoldi et al., 2000). They present higher GSI and smaller SVSI values than nesting males (Scaggiante et al., 1999), their sperm trails are less abundant in mucins and have a higher sperm density (Mazzoldi et al., 2000). In opportunistic males, the major function of seminal vesicles is sperm storage with a minimal production of mucins (Scaggiante et al., 1999).

The black goby, *G. niger* is a coastal fish inhabiting sandy environments and frequently entering brackish lagoons and estuaries (Whitehead et al., 1986; Joyeux et al., 1991a,b, 1992; Pampoulie et al., 1999). In the Venetian Lagoon, it reaches a maximum total length of 16 cm, 4 years of age and sexual maturity at the first year of life (De Girolamo, 1994) with a polygynous mating system (Mazzoldi and Rasotto, 2002; Rasotto and Mazzoldi, 2002). Gobies have two dorsal fins, and in this species, the first one is elongated with a well-developed 4th ray (Gandolfi et al., 1991). The reproductive apparatus of black goby males present seminal vesicles and also a mesorchial gland, an organ associated with testis, producing sexual pheromones involved in female attraction (Colombo and Burighel, 1974; Colombo et al., 1980). The mesorchial gland is well developed in parental males but reduced in sneakers, making them pheromonally inconspicuous and consequently minimizing their chances to be detected by parental males (Locatello et al., 2003). During the breeding season, from June to August, males larger than 9 cm and 3–4 years old, nest under different types of hard substrates (Gandolfi et al., 1991; Mazzoldi and Rasotto, 2002). Females lay the eggs on nest surface, while males release sperm trails (Marconato et al., 1996). Eggs deposition lasts on average 5–6 h (Marconato et al., 1996), and males perform parental care, cleaning, and fanning embryos until hatching (Gandolfi et al., 1991). Territorial males are characterized by a high and elongated 4th ray of the first dorsal fin and a sexually dimorphic black coloration; they present smaller GSI and higher SVSI than opportunistic males (Mazzoldi and Rasotto, 2002). Sperm trails released by parental males contain few sperm and a large amount of mucins (Rasotto and Mazzoldi, 2002). On the other hand, opportunistic males, smaller than 8 cm and 1 year old, mimic the female morph by not presenting dorsal fin elongation and dark body coloration (Mazzoldi and Rasotto, 2002; Locatello et al., 2003). They were observed to congregate around the nests where spawning was occurring, parasitizing parental males spawns (Mazzoldi and Rasotto, 2002).

Table 2

Total length (TL), body weight (BW), gonadosomatic (GSI) and seminal vesicle somatic (SVSI) indexes in females, sneaker and parental males of the grass goby, *Z. ophiocephalus*, and the black goby, *G. niger*, during the reproductive (RS) and the non-reproductive seasons (NRS)

Species and reproductive phenotypes	N	TL (cm) range (mean ± SE)	BW (g) range (mean ± SE)	GSI range (mean ± SE)	SVSI range (mean ± SE)
Grass goby females RS	11	8.3–13.5 (10.88 ± 0.59)	5.37–22.52 (12.93 ± 1.94)	0.506–4.412 (2.842 ± 0.365)	–
Grass goby females NRS	7	10.5–15.6 (12.98 ± 0.70)	10.72–36.55 (23.02 ± 3.65)	0.444–0.634 (0.546 ± 0.026)	–
Grass goby sneakers RS	11	7.7–10.4 (9.05 ± 0.22)	4.44–10.12 (6.70 ± 0.53)	0.366–9.719 (4.662 ± 0.835)	0.087–0.338 (0.222 ± 0.024)
Grass goby sneakers NRS	7	9–12.4 (10.31 ± 0.42)	7.53–20.86 (11.52 ± 1.69)	0.177–3.332 (0.933 ± 0.408)	0.030–0.088 (0.059 ± 0.009)
Grass goby parental males RS	16	16.4–21.8 (19.02 ± 0.45)	39.66–95.16 (59.42 ± 4.02)	0.561–2.337 (1.187 ± 0.126)	0.514–1.811 (1.150 ± 0.106)
Grass goby parental males NRS	7	14.7–22.2 (20.11 ± 0.97)	31.25–110.6 (84.71 ± 10.29)	0.496–1.346 (0.977 ± 0.130)	0.035–0.363 (0.228 ± 0.041)
Black goby females RS	9	6.3–11 (8.84 ± 0.60)	2.79–15.31 (8.73 ± 1.62)	1.660–4.050 (2.842 ± 0.276)	–
Black goby females NRS	6	8.6–11.2 (9.93 ± 0.43)	7.38–18.13 (11.87 ± 1.61)	0.402–0.651 (0.498 ± 0.043)	–
Black goby sneakers RS	9	5.4–7.6 (6.4 ± 0.24)	1.85–5.34 (3.07 ± 0.41)	0.209–3.692 (2.482 ± 0.357)	0.063–0.870 (0.426 ± 0.087)
Black goby sneakers NRS	2	8.7–8.9 (8.8 ± 0.1)	7.20–8.22 (7.71 ± 0.51)	0.158–0.194 (0.176 ± 0.018)	0.014–0.061 (0.038 ± 0.024)
Black goby parental males RS	9	8–13.7 (11.07 ± 0.56)	5.68–23.38 (16.82 ± 1.89)	0.201–1.135 (0.655 ± 0.106)	0.113–0.780 (0.477 ± 0.069)
Black goby parental males NRS	6	8.2–14.2 (11.3 ± 0.81)	6.14–32.35 (17.78 ± 3.99)	0.061–0.166 (0.115 ± 0.016)	0.016–0.287 (0.072 ± 0.043)

### Fish collection

Fish used for this study were field collected from the Venetian Lagoon, Northern Adriatic Sea, Italy, under a scientific fishing permit from the Regione Veneto or provided by local professional fishers. Individuals were collected in 1999 and 2000, both during the reproductive (RS) and non-reproductive season (NRS). Grass goby was collected from April to June (RS,  $n = 38$ ) and from October to November (NRS,  $n = 21$ ). Black goby was collected from June to August (RS;  $n = 27$ ) and from October to November (NRS;  $n = 14$ ). For both species, animals were transported to the laboratory within 30 min after collection, sexed by their dimorphic genital papillae, being elongated and pointed in males, short and rounded in females (Gandolfi et al., 1991), and then sacrificed with an excess of MS222 anaesthetic. Total body length (TL) was measured to the nearest mm with digital calipers, and body weight (BW) was measured to the nearest mg. Gonads and seminal vesicles were separately removed and weighted to the nearest mg (GW and SVW), the gonadosomatic index ( $GSI = 100 \times GW/BW$ ) and seminal vesicle somatic index ( $SVSI = 100 \times SVW/BW$ ) were computed. For black goby, testis weight measurements included the mesorchial gland, that is connected to testis tissue and could not be separately weighted.

All fish were placed into three separate reproductive phenotypes: females (F), parental males (P), and sneaker males (S) according to sex and mating tactic, male morph was assessed by checking body size, presence/absence of sexual secondary traits, such as dorsal fin development, and, during the breeding season, ejaculate characteristics (Scaggiante et al., 1999; Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002; Table 2). Collection, housing, and experimental procedures were in accordance with institutional guidelines of the Italian Ministry of Education, University and Research (DL 116/92).

### Immunohistochemistry

Brains were fixed by immersion in Bouin's solution for 12 h, rinsed in 50% ethanol and stored at 4°C. All samples were processed within 1 to 2 weeks after fixation. Samples were embedded in paraffin (46–48°C melting point), sectioned transversally at 15  $\mu\text{m}$ , and mounted as three parallel series through the brain on chromoalum-coated slides. Sections were either reacted immediately or stored at 4°C. Following removal of paraffin and hydration, slides were processed as described 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 employed at a concentration of 1:1000. As control for antisera specificity, some brain sections ( $n = 6$  for each species) were processed omitting the primary antibody.

Before running quantitative analyses, slides were coded, and the observer was not aware of the fish species and reproductive phenotypes of the samples under observation. The mean area of GnRH immunoreactive (GnRH-ir) cell ( $\mu\text{m}^2$ ) was determined using a Leica DMLB microscope equipped with a three-color CCD video camera (24 bites/pixel) and an image processing software (Windows Microimage version 3.4). Areas were only measured for cells in sections where the nucleus and at least one neurite were present, avoiding the possibility that cells were re-counted in subsequent sections. All determinations of cell area were run twice by two different operators (MS and VL) obtaining the same qualitative results (Wilcoxon's test,  $n = 20$ ,  $P > 0.05$ ).

For six brains of both species, immunohistochemistry was performed on three parallel series of slides and the number of GnRH-ir cells in each series was summed to yield the total cell number. There were no significant differences in the total number of cells among the three alternate brain series (Friedman's ANOVA  $P > 0.05$  for both species and both brain areas). For this reason, the number of cells for brains with only one available series was tripled to obtain the same total cell number. Second and third series were used as controls for immunolabeling specificity (see above) or to run immunocytochemistry with different anti-GnRH antibodies. A series of anti-GnRH antibodies were used at different working dilutions. The chicken I, chicken II, salmon, sea bream, C-free, and LHRH RBX polyclonal antibodies did not react, and only LRH13 gave a positive reaction in the brain of these goby species and was used throughout.

### Statistical analyses

Descriptive analyses were reported as mean  $\pm$  standard error of the mean (SEM). Data were checked for normality distribution using a Shapiro–Wilks test and, if not normally distributed, they were log-transformed. Non-parametric analyses were applied since data could not be assumed to be normally distributed.

The effects of mating tactic, sex, and season of each reproductive phenotype on immunoreactive cell number and size were analyzed using analysis of variance (ANOVA), and individual differences were addressed using Least Significance Difference (LSD) or the Tukey HSD tests. Statistical analyses were done by using STATISTICA 6.0.

### Results

GnRH-ir cells and their associated neurites appeared densely stained. Immunoreactive cell bodies showed dark brown cytoplasm and an unstained nucleus and were observed solely in two brain areas: the ganglion of the terminal nerve (TN) and the preoptic area (POA). GnRH-ir fibers had a punctate-like appearance and were observed in several regions of the forebrain and midbrain. Qualitative differences in their distribution were not found among the three reproductive phenotypes and between the two species. The most anterior label was associated with the olfactory bulbs, where fibers branched throughout the bulbs in a homogeneous way. At the level of the TN, fine neurites radiated dorsally and laterally from the ganglion and then concentrated in the anterior commissure and throughout the POA, where they became thicker fibers. Fibers were abundant along the edges of the third ventricle and close to the ventral margin of the brain; the latter was likely the preoptic–hypophyseal pathway. GnRH-ir fibers become less dense toward the caudal extent of the POA. Smaller and thinner fibers were seen also in the optic tectum and in the neurohypophysis.

The immunoreactivity at pituitary levels is likely to be due to the presence of GnRH neuron terminals as POA GnRH neurons are known to innervate the pituitary in most teleosts (Yamamoto et al., 1998). The signal is low for both goby pituitaries, owing to a lower GnRH content at the nerve terminals than in somata (Tiwary et al., 2002), and thus a quantitative analysis was not possible.

### *Zosterisessor ophiocephalus*

We calculated GSI and SVSI indexes for all individuals used for the immunocytochemical analysis in both seasons (Table 2). Females and sneakers had average GSI values five times higher in RS than NRS, while parental males increased GSI values twice in RS. SVSI averaged values in RS, compared to NRS, increased four times in sneakers and almost six times in parental males.

### Terminal nerve (TN)

The ganglion of the terminal nerve appeared as a cluster of large neurons located caudally at the junction of the olfactory bulbs with the ventral telencephalon (Figs.

1A, B). Cells, located in a ventral position, had a typical oval shape with their larger axis usually lying parallel to the ventral tissue edge; their axons originated and oriented mainly dorsally. At the level of the TN ganglion, we found positive reaction in all the brains analyzed except for two parental males collected during the reproductive season.

*TN, inter-sexual differences in non-reproductive season*

Fish from the three reproductive phenotypes differed significantly in body size (ANOVA,  $n = 21$ ,  $F_{2,18} = 48.033$ ,  $P < 0.001$ ). Parental males were larger than females and sneakers, females were intermediate in size, and sneakers were the smallest individuals (LSD test: P–S  $P < 0.001$ , P–F

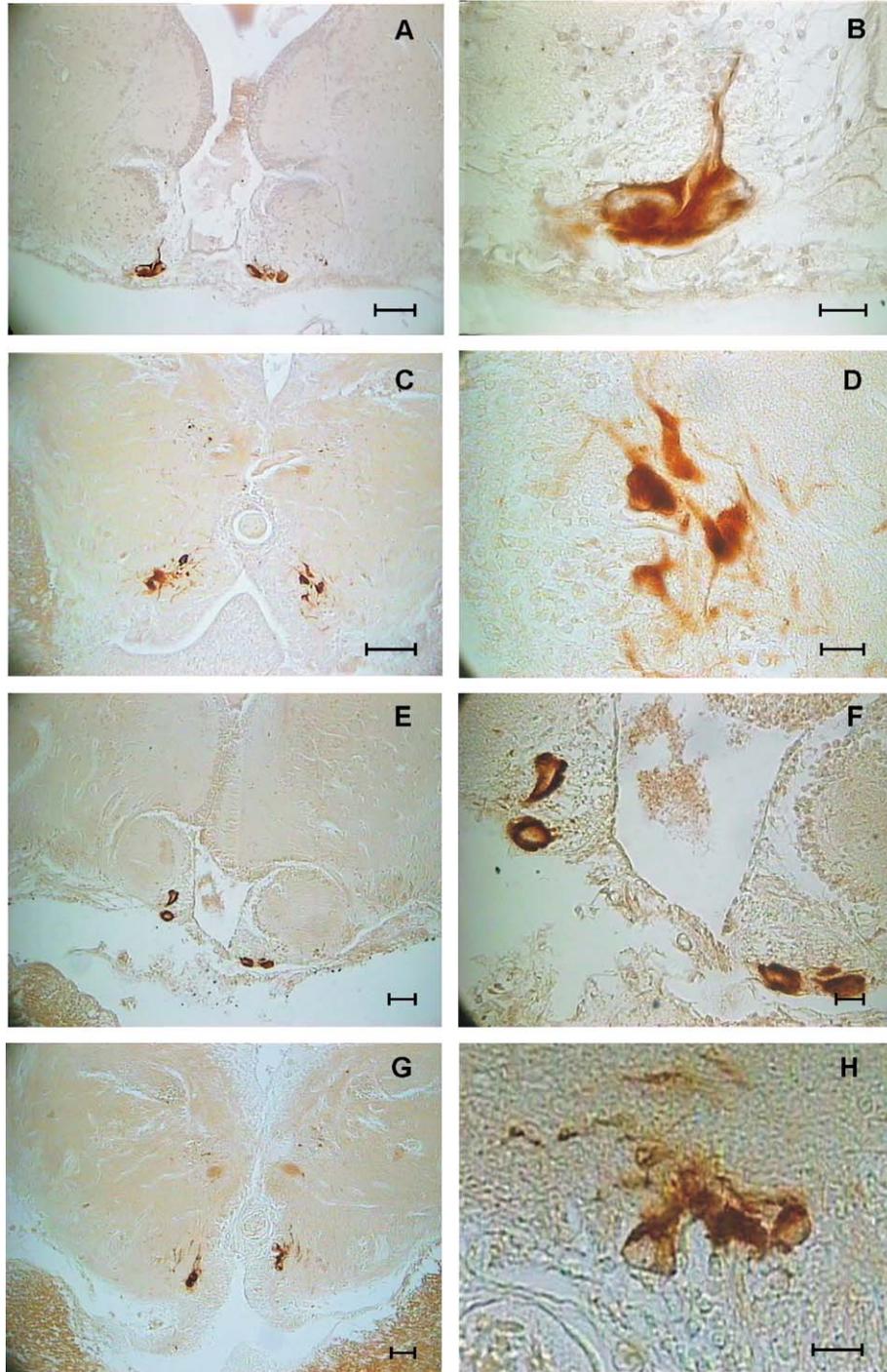


Fig. 1. Photomicrographs of brain transverse sections showing GnRH-ir cells in grass and black goby. (A and B) Two different magnifications of terminal nerve ganglion in grass goby (scale bar represent 100  $\mu\text{m}$  for A and 20  $\mu\text{m}$  for B). (C and D) Two different magnifications of preoptic area in grass goby (scale bar represent 100  $\mu\text{m}$  for C and 20  $\mu\text{m}$  for D). (E and F) Two different magnifications of terminal nerve ganglion in black goby (scale bar represent 50  $\mu\text{m}$  for E and 20  $\mu\text{m}$  for F). (G and H) Two different magnifications of preoptic area in female black goby (scale bar represent 50  $\mu\text{m}$  for G and 20  $\mu\text{m}$  for H).

$P < 0.001$ , F–S  $P = 0.019$ ). The mean number and size of GnRH-ir cells differed significantly among the three reproductive phenotypes (ANOVA,  $n = 21$ , cell number:  $F_{2,18} = 4.354$ ,  $P = 0.029$ ; cell size:  $F_{2,18} = 4.181$ ,  $P = 0.032$ ; Table 3), being more abundant and larger in parental males (LSD test, cell number: P–S  $P = 0.037$ , P–F  $P = 0.012$ , F–S  $P = 0.603$ ; cell size: P–S  $P = 0.010$ , P–F  $P = 0.100$ , F–S  $P = 0.271$ ).

#### TN, inter-sexual differences in reproductive season

Fish collected during the reproductive season differed significantly in body size (ANOVA,  $n = 38$ ,  $F_{2,35} = 145.348$ ,  $P < 0.001$ ); parental males were larger than females, which were larger than sneakers (HSD test: P–S  $P < 0.001$ , P–F  $P < 0.001$ , F–S  $P = 0.034$ ). The mean number of GnRH-ir cells did not differ between the reproductive phenotypes (ANOVA,  $n = 38$ ,  $F_{2,35} = 1.490$ ,  $P = 0.239$ ), while cell size was significantly different (ANOVA,  $n = 36$ ,  $F_{2,33} = 7.743$ ,  $P = 0.002$ ), with parental males characterized by the largest cells (HSD test: P–S  $P = 0.001$ , P–F  $P = 0.184$ , F–S  $P = 0.125$ ; Table 3).

#### TN, intra-sexual differences across seasons

##### Females

Females from the two different seasons showed different body size ( $t$  test:  $n = 18$ ,  $t_{16} = 2.264$ ,  $P = 0.038$ ), with individuals collected in autumn being larger than those collected during spring time. The mean number and size of GnRH-ir cells did not differ significantly across seasons ( $t$  test,  $n = 18$ , cell number:  $t_{16} = -0.611$ ,  $P = 0.550$ ; cell size:  $t_{16} = -1.827$ ,  $P = 0.086$ ).

##### Sneaker males

Sneakers had a larger body size in the samples collected during the non-breeding season ( $t$  test:  $n = 18$ ,  $t_{16} = 2.900$ ,  $P = 0.010$ ). We did not observe any difference between GnRH-ir cell number and size across the two collecting seasons ( $t$  test,  $n = 18$ , cell number:  $t_{16} = 1.458$ ,  $P = 0.164$ ; cell size:  $t_{16} = -1.433$ ,  $P = 0.171$ ).

#### Parental males

Territorial males did not show significant variations in body size between seasons ( $t$  test:  $n = 23$ ,  $t_{21} = 1.175$ ,  $P = 0.253e$ ). The GnRH-ir cell number differed significantly between NRS and RS samples, with a two-fold higher number in males collected during the autumn season ( $t$  test:  $n = 23$ ,  $t_{21} = 2.922$ ,  $P = 0.008$ ), while cell size did not show any difference ( $t$  test:  $n = 21$ ,  $t_{19} = -1.310$ ,  $P = 0.206$ ).

#### Preoptic area (POA)

GnRH-ir cells were found lateral to the dense clusters that form the parvocellular and magnocellular divisions of the POA. Cell bodies had a round shape and were bipolar and smaller than TN immunoreactive neurons (Figs. 1C, D). In this brain area, we did not find positive reaction in 2 sneakers out of 7 in NRS samples, in 2 females out of 11 in RS, and in 7 sneakers out of 11 in RS samples.

#### POA, inter-sexual differences in non-reproductive season

The mean number and size of GnRH-ir cells differed significantly among the three categories of animals (ANOVA, cell number:  $n = 21$ ,  $F_{2,18} = 7.409$ ,  $P = 0.004$ ; cell size:  $n = 19$ ,  $F_{2,16} = 14.897$ ,  $P < 0.001$ ; Table 3), being more abundant and larger in parental males (LSD test, cell number: P–S  $P = 0.001$ , P–F  $P = 0.200$ , F–S  $P = 0.024$ ; HSD test, cell size: P–S  $P < 0.001$ , P–F  $P = 0.004$ , F–S  $P = 0.225$ ).

#### POA, inter-sexual differences in reproductive season

Fish collected during the RS differed significantly in the mean number and size of GnRH-ir cells (ANOVA, cell number:  $n = 38$ ,  $F_{2,35} = 44.505$ ,  $P < 0.001$ ; cell size:  $n = 29$ ,  $F_{2,26} = 13.238$ ,  $P < 0.001$ ; Table 3). Parental males had more abundant and larger cells, while females and sneakers did not differ from each other (HSD test, cell number: P–S  $P < 0.001$ , P–F  $P < 0.001$ , F–S  $P = 0.054$ ; cell size: P–S  $P = 0.012$ , P–F  $P = 0.003$ , F–S  $P = 0.805$ ).

Table 3  
GnRH-ir cell number and size in the terminal nerve (TN) and preoptic area (POA) of the grass goby, *Z. ophiocephalus*, (RS = reproductive season, NRS = non-reproductive season)

Reproductive phenotypes	Brain area	<i>N</i>	Mean cell no. $\pm$ SE	Cell number range	<i>N</i>	Mean cell size ( $\mu\text{m}^2$ ) $\pm$ SE	Cell size range
Females RS	TN	11	22.91 $\pm$ 2.78	12–39	11	555.28 $\pm$ 55.23	365.133–876.583
Females NRS	TN	7	20.57 $\pm$ 1.90	15–30	7	409.894 $\pm$ 48.340	262.385–576.195
Sneakers RS	TN	11	18.00 $\pm$ 2.10	12–36	11	384.387 $\pm$ 31.765	210.633–587.077
Sneakers NRS	TN	7	22.29 $\pm$ 1.58	18–30	7	317.403 $\pm$ 30.336	236.509–462.367
Parental males RS	TN	16	17.10 $\pm$ 2.48	0–33	14	699.836 $\pm$ 68.753	405.025–1198.318
Parental males NRS	TN	7	29.57 $\pm$ 3.10	18–45	7	551.217 $\pm$ 81.799	346.614–962.035
Females RS	POA	11	16.63 $\pm$ 4.79	0–54	9	99.608 $\pm$ 12.205	46.328–151.539
Females NRS	POA	7	36.00 $\pm$ 2.62	30–45	7	86.330 $\pm$ 7.044	50.038–110.262
Sneakers RS	POA	11	2.18 $\pm$ 1.15	0–12	4	71.778 $\pm$ 17.745	27.780–104.783
Sneakers NRS	POA	7	14.57 $\pm$ 6.53	0–48	5	58.329 $\pm$ 5.575	47.760–78.648
Parental males RS	POA	16	51.56 $\pm$ 4.21	15–81	16	209.674 $\pm$ 19.009	106.719–357.066
Parental males NRS	POA	7	47.57 $\pm$ 8.00	18–87	7	142.726 $\pm$ 15.057	96.648–198.248

(TN = terminal nerve, POA = preoptic area).

*POA, intra-sexual differences**Females*

GnRH-ir cell number differed significantly between the two seasons, reaching higher values in autumn samples (*t* test:  $n = 18$ ,  $t_{16} = 3.020$ ,  $P = 0.008$ ). Cell size did not show any difference between collecting seasons (*t* test:  $n = 16$ ,  $t_{14} = -0.871$ ,  $P = 0.398$ ).

*Sneaker males*

We did not observe any difference in GnRH-ir cell size across seasons (*t* test:  $n = 9$ ,  $t_{7} t_{16} = -0.800$ ,  $P = 0.450$ ), while the mean cell number in sneakers collected in autumn was higher (*t* test:  $n = 18$ ,  $t_{16} = 2.330$ ,  $P = 0.033$ ).

*Parental males*

Territorial males did not show any difference in GnRH-ir cell number between RS and NRS (*t* test:  $n = 23$ ,  $t_{21} = -0.484$ ,  $P = 0.633$ ). The mean cell size differed significantly, being twice as large in reproductive season samples (*t* test:  $n = 23$ ,  $t_{21} = -2.182$ ,  $P = 0.041$ ).

*Gobius niger*

We calculated GSI and SVSI indexes for all individuals used in the immunocytochemical analysis (Table 2). Females and parental males had mean GSI values five times higher in RS than NRS, while sneakers showed 14-fold increased GSI values in RS. SVSI averaged values in RS, compared to NRS, increased six-fold in parental males and 11-fold in sneakers.

*Terminal nerve (TN)*

The ganglion of the terminal nerve had the same morphological characteristics and distribution as in the grass goby (Figs. 1E, F). Within this ganglion, a positive reaction was found only in a small number of individuals. For NRS samples, we found ir-cells in 3 females out of 6, 4 parental males out of 6 and 1 sneaker out of 2. In RS samples, we found ir-cells in 7 female samples out of 9, 5 parental male samples out of 9, and 7 sneaker samples out of 9.

*TN, inter-sexual differences in non-reproductive season*

Fish from the three reproductive phenotypes presented homogeneous total lengths (Kruskal–Wallis test:  $n = 14$ ,  $df = 2$ ,  $\chi^2 = 5.333$ ,  $P = 0.070$ ). Parental males were reported

to be significantly larger than females and sneakers (Mazzoldi and Rasotto, 2002), but this divergence was not observed here probably due to the limited number of individuals used for the analysis. The mean number and size of GnRH-ir cells did not differ significantly among the three reproductive phenotypes (Kruskal–Wallis test, cell number:  $n = 14$ ,  $df = 2$ ,  $\chi^2 = 0$ ,  $P = 1$ ; cell size:  $n = 8$ ,  $df = 2$ ,  $\chi^2 = 2.333$ ,  $P = 0.311$ ; Table 4).

*TN, inter-sexual differences in reproductive season*

Individuals collected during the reproductive season differed significantly in body size (ANOVA,  $n = 27$ :  $F_{2,24} = 22.519$ ,  $P < 0.001$ ) with parental males being larger than females and females being significantly larger than sneakers (LSD test: P–S  $P < 0.001$ , P–F  $P = 0.004$ , F–S  $P = 0.002$ ). Despite this body size difference, we did not find any significant variation in ir-GnRH cell number and size among the three reproductive phenotypes (ANOVA, cell number:  $n = 27$ ,  $F_{2,24} = 1.239$ ,  $P = 0.308$ ; cell size:  $n = 19$ ,  $F_{2,16} = 3.515$ ,  $P = 0.054$ ; Table 4).

*TN, intra-sexual differences**Females*

Females collected across seasons showed a similar body size (*t* test:  $n = 15$ ,  $df = 13$ ,  $t = -1.331$ ,  $P = 0.206$ ). The mean number and size of GnRH-ir cells did not differ between seasons (*t* test, cell number:  $n = 15$ ,  $df = 13$ ,  $t = 1.437$ ,  $P = 0.174$ ; cell size:  $n = 10$ ,  $t = 0.047$ ,  $P = 0.964$ ).

*Sneaker males*

Sneakers collected during the NRS showed a larger body size (Mann–Whitney *U* test:  $n = 11$ ,  $U = 0$ ,  $P = 0.034$ ). We did not observe any difference between GnRH-ir number in the two collecting seasons (Mann–Whitney *U* test:  $n = 11$ ,  $U = 9$ ,  $P = 1$ ). Having only one positive individual in the non-reproductive season data set, we could not apply any statistical test to the cell size number variable.

*Parental males*

Parental males did not show significant differences in body size (*t* test:  $n = 15$ ,  $df = 13$ ,  $t = -0.246$ ,  $P = 0.809$ ) and GnRH-ir cell number and size (*t* test, cell number:  $n = 15$ ,  $df = 13$ ,  $t = -0.270$ ,  $P = 0.792$ ; cell size:  $n = 9$ ,  $df = 7$ ,  $t = 0.979$ ,  $P = 0.360$ ) across seasons.

Table 4

GnRH-ir cell number and size in the terminal nerve of the black goby, *Gobius niger* (RS = reproductive season, NRS = non reproductive season)

Reproductive phenotypes	Season	<i>N</i>	Mean cell no. $\pm$ SE	Cell no. range	<i>N</i>	Mean cell size ( $\mu\text{m}^2$ ) $\pm$ SE	Cell size range
Females	RS	9	18 $\pm$ 4.3	0–33	7	359.52 $\pm$ 27.26	237.77–447.32
Females	NRS	6	9 $\pm$ 4.1	0–21	3	357.11 $\pm$ 47.86	292.56–450.59
Sneakers	RS	9	14.66 $\pm$ 3.06	0–27	7	295.47 $\pm$ 27.22	178.27–403.86
Sneakers	NRS	2	12 $\pm$ 12	0–24	1	366.67	366.67
Parental males	RS	9	10 $\pm$ 3.35	0–24	5	404.87 $\pm$ 32.06	311.05–512.12
Parental males	NRS	6	11.5 $\pm$ 4.61	0–30	4	364.42 $\pm$ 22.4	299.13–401.06

### Preoptic area (POA)

We obtained very limited results and for the POA and for this reason, the application of any statistical test was impossible. We found GnRH-ir cells only in females collected during the RS. Immunoreactive cells, when present, had the same location and morphology as in the grass goby brains (Figs. 1G, H).

Most of the females (6 of 9) collected during the breeding season showed GnRH positive cells (cell number:  $n = 9$ , range = 0–45, mean =  $16.66 \pm 5.10$ ; cell size:  $n = 6$ , range =  $76.50$ – $181.93 \mu\text{m}^2$ , mean =  $134.24 \pm 16.40 \mu\text{m}^2$ ), while we did not find any ir-cells in all the females sampled during the non-breeding season ( $n = 6$ ). Immunoreactive cells were never observed in parental ( $n = 15$ ) and sneaker males ( $n = 11$ ).

## Discussion

### *Zosterisessor ophiocephalus*

Individuals used for immunohistochemical analysis presented GSI and SVSI values consistent with those reported as typical for females and males adopting alternative reproductive tactics in this species (Scaggiante et al., 1999; Mazzoldi et al., 2000). Indeed, among males, GSI values were higher in sneakers than in territorial males, while SVSI indexes showed an opposite pattern. As expected, all individuals collected outside the breeding season were hypogonadal and reproductively inactive. Only two brain areas presented GnRH-ir neurons: the terminal nerve ganglion (TN) and the preoptic area (POA).

Within the TN, GnRH-ir neurons are found all year round, and in female and sneaker males, their number and size remain unchanged across seasons. Among parental males, the number of TN GnRH neurons is two times larger in the autumn than in the spring. Considering that grass gobies breed from March to June, this result suggests that GnRH produced in the TN is involved in modulating functions other than reproduction. The TN GnRH neurons have never been reported to vary among individuals, at both inter- and intra-sexual levels (Halpern-Sebold et al., 1986; Grober et al., 1994; Foran and Bass, 1999), and it has been proposed that it may play a role in olfaction (Eisthen et al., 2000; Weltzien et al., 2004). The possible influence of GnRH in olfactory cell activity could be a key to understand the observed seasonal variation of this neuropeptide in grass goby parental males. Olfaction is well developed in this species, and it is likely to be involved in the migratory behavior and digging activity performed by larger males in autumn. Indeed, while smaller males and females use empty nest or natural holes as winter shelter, parental males migrate from nesting locations to more protected areas of the lagoon, where they dig a hole, much smaller than a nest, where they spend the winter period in a quiescent status (Gandolfi et al., 1991).

Within the POA area, GnRH-ir cell number and size were significantly higher in parental males during the breeding season. In several fish species, an increase in the POA GnRH cell number and/or size, paralleled by and increase in GSI, was shown to occur prior to or during the breeding season

(Andersson et al., 2001; Holland et al., 2001; Tiwary et al., 2002). Also in the grass goby, the GSI and the male SVSI values reach their highest values during the reproductive season. However, only parental males show a significant increase in POA GnRH expression, suggesting that GnRH variability is primarily linked to the reproductive investment in nesting and performing parental care. Moreover, parental males during the reproductive season have higher SVSI, but lower GSI, than sneakers. A similar pattern of GnRH expression and reproductive organ development has been documented in other species where alternative male reproductive phenotypes are present. Indeed in *Porichthys notatus*, territorial males show larger GnRH-ir neurons, smaller testes and more developed accessory structures than opportunistic males (Barni et al., 2001; Bass, 1992; Grober et al., 1994). Considering that, despite the lack of a significant seasonal variation in GnRH expression, sneaker males show higher GSI than parental males, the GnRH increase appears to have more influence on secondary sexual traits and behavior than on gonad development. This is supported also by the results of experimentally induced changes of tactic in the grass goby (Scaggiante et al., 2004). Indeed sneakers, when exposed to ripe females in the absence of territorial males, mate, perform parental care and show significant changes in POA GnRH neuron number, ejaculate characteristics, and SVSI, while both their body and testis size remain within the range of sneakers.

### *Gobius niger*

The GSI and SVSI of all the individuals used for immunohistochemistry were similar to those reported previously for females, parental and sneaker males of this species (Rasotto and Mazzoldi, 2002). Parental males had smaller testes and larger seminal vesicles than opportunistic ones and, as expected, both GSI and SVSI for samples collected during reproductive season were higher than those of individuals outside the breeding season. In the black goby, both inside and outside the reproductive season, the anti-GnRH monoclonal antibody showed positive reaction in the same brain areas as in the grass goby. Within TN, the GnRH-ir neurons, regardless of sex, male mating tactic, and season, did not vary as in other teleost species (Foran and Bass, 1999). This result could further support the hypothesis that TN GnRH plays multiple neuro-modulatory roles, not necessarily in relation to reproduction (Eisthen et al., 2000; Weltzien et al., 2004).

At POA levels, GnRH-ir neurons appeared active only in females collected during the breeding season. In parental males, sneakers, and in females outside the breeding period, the POA always lacked GnRH-ir neurons. Considering that, in females collected during the same time periods, we observed GnRH-ir neurons in the TN and POA, and GnRH-ir fibers distributed throughout the forebrain and midbrain, it seems unlikely that the lack of immunostaining within the POA of males was artifactual. The expression of POA GnRH in females during the breeding season can be related to gonad development and ovulation, as reported also in other teleost species (Andersson et al., 2001; Holland et al., 2001; Tiwary

et al., 2002), while the lack of GnRH activity in males appears more puzzling. The presence of a specific black goby male GnRH form cannot be excluded, but, to the best of our knowledge, the intra-specific occurrence of sexually dimorphic GnRH forms has never been reported. The observed pattern might be the consequence of a balance between synthesis and utilization of GnRH resulting in a level of peptide in POA cells undetectable by our methods.

However, the major outcome of these results is the lack of variability in GnRH expression in relation to alternative mating tactics. Similar to the other species with alternative male mating tactics where intra-sexual variability in GnRH expression has been documented (Foran and Bass, 1999), in the black goby male reproductive phenotypes also diverge in both primary and secondary sex traits and mating behavior (Locatello et al., 2003; Mazzoldi and Rasotto, 2002; Rasotto and Mazzoldi, 2002). Experiments, inducing a switch of tactics in black goby sneaker males, established behavioral, morphological, and physiological changes toward typical parental male phenotype (Immler et al., 2004). An ongoing study on the brain of changing tactic sneakers is confirming the lack of GnRH-positive reaction at POA levels (Scaggiante, unpublished data).

#### *General comparative remarks*

Grass goby and black goby, both showing alternative male reproductive phenotypes, did not show a similar pattern in GnRH brain system development. Indeed, while the grass goby showed a clear intra-sexual dimorphism in GnRH expression, the black goby showed only inter-sexual differences. Grass goby and black goby, despite sharing many ecological and life history traits, are known to differ in the degree of polygyny, the intensity of sperm competition (Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000), the type of nest (Gandolfi et al., 1991), and seminal vesicle function (Mazzoldi, 1999). Indeed, grass goby parental males dig, in muddy bottoms, a multi-chambered burrow whose sizes are significantly related to male body size, while black goby males nest under different types of available hard substrata (Gandolfi et al., 1991; Mazzoldi et al., 2000) and, consequently, they are not involved in nest construction and maintenance. The degree of polygyny and the sperm competition intensity, on the basis of a comparative analysis of number of egg clutches/nest, GSI values and number of sneaking attempts observed in the field (Mazzoldi and Rasotto, 2002; Mazzoldi et al., 2000), was higher in grass goby than in black goby. Differences in seminal vesicle function observed among males adopting alternative mating tactics (Scaggiante et al., 1999) appeared very well pronounced in grass goby but not in black goby males (Mazzoldi, 1999). In the grass goby parental males, these accessory organs secrete mucins, while they store sperm in sneakers ones (Scaggiante et al., 1999). Instead, in all black goby males, seminal vesicles perform both functions and only quantitative differences diversify the two male phenotypes, with mucin production prevailing in parental males and sperm

storage in sneaker ones (Rasotto and Mazzoldi, 2002). In addition to ejaculate performances, mucins produced by grass goby parental males appear to play a role also in nest construction and maintenance, keeping compact the muddy substrate where burrows are dug. In this species, nest size and condition appear to strongly affect egg number and development. Consequently, male reproductive success in the grass goby is highly related to parental male size and condition (Mazzoldi, 1999; Mazzoldi et al., 2000). On the contrary, in the black goby, the male reproductive success is mainly constrained by nest availability and sneaker density appears to be more influenced by environmental factors than by individual age and/or size (Mazzoldi, 1999; Mazzoldi and Rasotto, 2002). The different factors influencing nesting might account for the observed differences in both GnRH expression and degree of divergence between male morphs in these two goby species. Indeed grass goby parental males have more additional costs to meet than black goby males for expressing a typical parental morph, and these costs could be modulated by a higher GnRH level.

Previous studies on species presenting alternative male mating phenotypes, regardless to the fact that they are flexible or fixed for life, consistently showed that differences in male reproductive tactics were paralleled by variability in GnRH expression (Elofsson et al., 1999; Grober and Bass, 1991; Foran and Bass, 1999). The present results on grass goby and black goby, with the latter lacking intra-specific differences in male GnRH, support the hypothesis, based on androgens and forebrain arginine-vasotocin (AVT) levels, that the expression of alternative tactics is not under the control of a single endocrine system (Oliveira et al., 2005).

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