

# Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the brain of the halfspotted goby

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## Abstract

Gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT) are critical regulators of reproductive behaviors that exhibit tremendous plasticity, but co-variation in discrete GnRH and AVT neuron populations among sex and season are only partially described in fishes. We used immunocytochemistry to examine sexual and temporal variations in neuron number and size in three GnRH and AVT cell groups in relation to reproductive activities in the halfspotted goby (*Asterropteryx semipunctata*). GnRH-immunoreactive (-ir) somata occur in the terminal nerve, preoptic area, and midbrain tegmentum, and AVT-ir somata within parvocellular, magnocellular, and gigantocellular regions of the preoptic area. Sex differences were found among all GnRH and AVT cell groups, but were time-period dependent. Seasonal variations also occurred in all GnRH and AVT cell groups, with coincident elevations most prominent in females during the peak- and non-spawning periods. Sex and temporal variability in neuropeptide-containing neurons are correlated with the goby's seasonally-transient reproductive physiology, social interactions, territoriality and parental care. Morphological examination of GnRH and AVT neuron subgroups within a single time period provides detailed information on their activities among sexes, whereas seasonal comparisons provide a fine temporal sequence to interpret the proximate control of reproduction and the evolution of social behavior.

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**Keywords:** *Asterropteryx semipunctata*; AVT; Brain; Goby; GnRH; Immunoreactive; Neuromodulator; Reproduction

**Abbreviations:** AC, anterior commissure; AP, area postrema; CC, cerebellar crest; CE, cerebellum; CR, crista cerebellaris; Dc, central zone of area dorsalis telencephali; Dl, lateral zone of area dorsalis telencephali; Dm, medial zone of area dorsalis telencephali; Dp, posterior zone of area dorsalis telencephali; EG, eminentia granularis; gPOA, gigantocellular preoptic area; G, nucleus glomerulosus; HYP, hypothalamus; IL, inferior lobe of hypothalamus; mPOA, magnocellular preoptic area; M, medulla; MLE, medial longitudinal fasciculus; Npt, nucleus posterior tuberis; OB, olfactory bulb; ON, optic nerve; pPOA, parvocellular preoptic area; PHT, preoptico-hypophyseal tract; PIT, pituitary; POA, preoptic area; PPv, nucleus preectalis periventricularis pars ventralis; PVL, periventricular cell layer; RI, nucleus reticularis inferioris; RL, nucleus reticularis lateralis; Rm, nucleus reticularis medius; Rs, nucleus reticularis superior; SAC, stratum album centrale; SC, spinal cord; SFGS, stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale; SGP, stratum griseum periventriculare; SV, saccus vasculosus; T, tectum; TEG, tegmentum; TEL, telencephalon; TL, torus longitudinalis; TLa, nucleus tori lateralis; TN, terminal nerve; TS, torus semicircularis; V, ventricle; VCe, valvula cerebelli; VL, vagal lobe; Vd, dorsal zone of area ventralis telencephali; Vv, ventral zone of area ventralis telencephali.

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## 1. Introduction

Sex and species-specific behaviors in vertebrates are regulated by multiple neurochemicals in the brain. The neuropeptide hormones gonadotropin-releasing hormone (GnRH) and arginine vasotocin/vasopressin (AVT/AVP) have wide distributions in the brain and are important regulators of social behaviors such as reproduction, aggression and parental care (Maney et al., 1997; Goodson and Bass, 2001; Semsar et al., 2001; Millar, 2003). GnRH and AVT/AVP behavioral regulation is mainly localized to specific forebrain regions such as the preoptic area (POA), bed nucleus of the stria terminalis, lateral septum, lateral amygdala, and medial amygdaloid nucleus (Sakuma and Suga, 1997; Goodson and Bass, 2001; Somoza et al., 2002). GnRH and AVT/AVP somata, fibers, and receptors are now known to vary in these regions according to sex, season, reproductive condition, behavioral state, social system, and species (Bass and Grober, 2001; Goodson and Bass, 2001;

Parhar et al., 2001; Keverne and Curley, 2004). While GnRH, AVT/AVP, and their receptors are conserved in structure across vertebrates, their action on behaviors are diverse and differ among closely related species. Thus to understand the evolution and proximate control of social behavior, it is necessary to determine neuroanatomical co-variation by sex and season among multiple neurochemical groups within a single species.

Wild fish populations show distinct annual reproductive cycles and associated behaviors that are regulated by GnRH, but information on the co-variation among all GnRH cell populations is incomplete. The majority of previous studies examine sex and seasonal differences in somata characters only in the POA GnRH cell group (reviewed by Bass and Grober, 2001). For example, terminal phase males of the protogynous bluehead wrasse (*Thalassoma bifasciatum*) and ballan wrasse (*Labrus berggylta*) have more POA GnRH cells than females or initial phase males (Grober et al., 1991; Elofsson et al., 1999). In species with dimorphic males and alternative reproductive tactics such as the plainfin midshipman (*Porichthys notatus*) and cichlid (*Astatotilapia burtoni*) dominant territorial males have larger POA GnRH cells than non-territorial males (Francis et al., 1993; Foran and Bass, 1999). The first study to compare sex differences among three GnRH cell populations of the gonochoristic monomorphic goldfish found larger POA GnRH cells in males compared to females (Parhar et al., 2001), but no sex differences in distribution, numbers, optical density of staining, or gross morphology for terminal nerve and midbrain GnRH cells (Parhar et al., 2001). However, that study did not assess possible seasonal variations between or within sexes. A recent comparative study on two goby species with alternative mating tactics showed intra- and intersexual variations in both the TN and POA cell groups that may be related to migratory behavior, nest type, or mating system differences between these species (Scaggiante et al., 2006), but they did not include the midbrain GnRH cells that are important modulators of reproductive behavior (Millar, 2003; Temple et al., 2003). Further studies of both sex and seasonal variation among all GnRH cell populations within and among related species are needed to examine functional correlates of behavior and reproduction.

Arginine vasotocin (and its mammalian homolog, arginine vasopressin) is produced in parvocellular and magnocellular neurons of the preoptic area and anterior hypothalamus, and implicated in the modulation of a variety of sex-typical and species-specific social and reproductive behaviors (see Goodson and Bass, 2001 for review). The abundance of AVT/AVP neurons in certain brain regions is often sexually dimorphic, seasonally variable and regulated by sex steroids (Dubois-Dauphin et al., 1987; Boyd and Moore, 1992; Ota et al., 1996, 1999a,b; Goodson and Bass, 2000, 2001; Moore et al., 2000; Panzica et al., 2001; DeVries and Panzica, 2006). Similar to GnRH, studies on AVT in fishes have concentrated on neuronal sexual dimorphisms in sex-changing species (e.g. wrasses) and those with alternative reproductive morphs (e.g. midshipman; blennies) (Foran and Bass, 1998; Reavis and Grober, 1999; Godwin et al., 2000; Grober et al., 2002; Miranda et al., 2003). In these species, it is often the dominant territorial males that have either larger or more abundant AVT cells compared to

subordinate non-territorial males or females. However, in the serial-reversible sex-changing marine goby (*Trimma okinawae*) females have larger AVT cells than males (Grober and Sunobe, 1996). Parhar et al. (2001) examined sexual dimorphisms in AVT neurons in the goldfish and found no difference in neuronal volume or cell optical staining density between sexually mature males and females. However, their study did not quantify differences among parvocellular (pPOA), magnocellular (mPOA), and gigantocellular (gPOA) preoptic area AVT-immunoreactive (-ir) nuclei, nor did it examine seasonal variations. Ota et al. (1996, 1999a,b) did demonstrate seasonal changes in vasotocin gene expression that were correlated with plasma steroid levels in masu salmon, but these studies concentrated on immature and pre-spawning individuals. Measures for AVT-ir cell numbers and size among cell groups, sexes, and season within single teleost fish species are needed to examine functional behavior and reproductive significance across taxa.

Both GnRH and AVT cell populations have prominent projections to midbrain and hindbrain regions, but their functions remain to be clearly defined. The extensive fiber distributions throughout the brain indicate these peptides function as neuromodulators, and several studies demonstrate that GnRH and AVT influence peripheral and central sensory and sensorimotor processing (Stell et al., 1987; Oka and Matsushima, 1993; Penna et al., 1992; Oka, 1992, 1997; Rose et al., 1995; Eisthen et al., 2000; Wirsig-Wiechmann, 2001; Rose and Moore, 2002; Maruska and Tricas, 2007). Reef fishes use a combination of sensory cues such as olfactory, visual, auditory, vestibular, and mechanosensory information during intra- and inter-specific social interactions to coordinate reproductive behaviors (see Myrberg and Fuiman, 2002 for review). Thus monitoring cell changes over reproductive and non-reproductive seasons can indicate the period of the reproductive cycle that AVT and GnRH differentially modulate sensory and sensorimotor systems, and provide the basis for predictions on the neuromodulatory potential of these important neuropeptides.

Small benthic marine fishes such as gobies (family Gobiidae) and blennies (family Blenniidae) are excellent for comparative studies on the action of neuropeptides on social behaviors and sensorimotor modulation because they have separate and distinct neuropeptide populations, show diverse social behaviors and use multiple sensory cues for social interactions. In addition, they are small, easily accessible in the wild and maintained in captive experiments, and are the subject of recent studies on GnRH and AVT systems (Grober and Sunobe, 1996; Reavis and Grober, 1999; Grober et al., 2002; Miranda et al., 2003; Scaggiante et al., 2004, 2006). These types of alternative model systems are essential for understanding the plasticity of neuropeptide effects in an organismal context (Hofmann, 2006). In this study we examine sex and seasonal variations in the GnRH and AVT neuronal systems in a wild population of the half-spotted goby (*Asterropteryx semipunctata*). This species has sex-specific and temporal reproductive activities associated with its polygamous social system, benthic courtship and spawning behavior, territoriality, and parental care (Privitera, 2002a,b). We show both sex and seasonal plasticity in GnRH and AVT neuronal systems, and discuss these findings in

relation to reproductive physiology and behavior of this species. Our concurrent examination of subgroups of these two neuropeptide populations across seasons and between sexes provides a detailed analysis of differences among males and females and the temporal changes during spawning and non-spawning periods. Further, our chronological comparisons emphasize the importance of fine scale temporal analyses to interpret the proximate control and evolution of reproduction and behavior.

## 2. Methods

### 2.1. Animals and tissue preparation

Adult halfspotted gobies (*Asterropteryx semipunctata*) (Perciformes: Gobiidae) were collected via hand net or hook and line from Kaneohe Bay, Oahu, transported to the lab, and immediately euthanized with an overdose of tricaine methanesulfonate (MS-222). Sexually mature adult males and females (standard length  $\geq 22$  mm) were collected from four separate time periods: Dec.–Jan. (minimal gonadal indices; non-spawn), Mar.–Apr. (pre-spawn), Jun.–Jul. (maximum gonadal indices; peak-spawn), and Sept.–Oct. (post-spawn), based on previous reproductive analyses of this population (Privitera, 2002a). Standard length (SL) and total length (TL) were measured to the nearest 0.5 mm, and total body weight (BW) to the nearest 0.1 g. Males of this species display two breeding tactics: the primary mode is characterized by large territorial males that engage in nest building and territory defense, courtship, and brood care, while the alternative mode is characterized by small non-territorial sneaker males (Privitera, 2002b). Sneaker males are smaller and often show yellow pigmentation on the caudal peduncle, similar to females (Privitera, 2002a,b). Only large territorial males and females were used in this study. Fish were sexed by examination of their sexually dimorphic urogenital papilla, and verified by examination of gonadal tissue under a compound microscope at 400 $\times$ . The brain was exposed by removal of the dorsal cranium, and the entire fish immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Fish were fixed for 1–5 days at 4°C, rinsed in 0.1 M PB, brains removed, and cryoprotected overnight in 30% sucrose in 0.1 M PB prior to sectioning. Collection and euthanization procedures were approved by the University of Hawaii IACUC.

### 2.2. Immunocytochemistry

Cryoprotected brains were embedded in Histoprep mounting media (Fisher Scientific) and sectioned in the sagittal or transverse plane at 24  $\mu$ m with a cryostat. Serial sections were collected onto chrom-alum-coated slides, dried flat overnight at room temperature, and stored at 4°C prior to immunocytochemistry.

Mounted brain sections were brought to room temperature (20–22°C), surrounded with a hydrophobic barrier (Immedge pen; Vector Laboratories), rinsed with 0.05 M phosphate buffered saline (PBS), blocked with 0.3% Triton-X 100 (Sigma) in PBS with 2% normal goat serum (NGS; Vector Laboratories) for 30 min, and incubated with primary antibody (1:5000 final concentration) overnight (14–16 h) in a sealed humidified

chamber. Brain tissue for quantification purposes was incubated with either anti-AVT (donated by Dr. Matthew Grober, Georgia State University, USA) or anti-GnRH 7CR-10 (donated by Dr. Nancy Sherwood, University of Victoria, BC, Canada). Anti-GnRH 7CR-10 is a broad-based polyclonal antibody that labels multiple forms of GnRH (see Forlano et al., 2000 for cross-reactivity data) and intensely labels somata in the terminal nerve (TN) ganglion, preoptic area (POA) and midbrain tegmentum (TEG) in the halfspotted goby. Primary antibody incubation was followed by a PBS wash (3  $\times$  10 min), incubation with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) with 2% NGS for 1 h, PBS wash (3  $\times$  10 min), quenching with 0.5–3% hydrogen peroxide in PBS for 10–15 min, PBS wash (3  $\times$  10 min), incubation with avidin–biotin–horseradish peroxidase complex (ABC Elite kit; Vector Laboratories) for 2 h, PBS wash (3  $\times$  10 min), and reacted with a diaminobenzidine (DAB) chromogen substrate kit with nickel chloride intensification (Vector Laboratories) for 3–6 min. Slides were then soaked in distilled water for 10 min to stop the reaction, counterstained with 0.1% methyl green or 0.5% cresyl violet acetate, dehydrated in an ethanol series (50%–100%), cleared in toluene, and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific).

Immunocytochemistry controls included: (1) omission of primary antisera, secondary antisera, ABC solution or DAB all resulted in no staining, (2) preabsorption of anti-AVT with 8  $\mu$ M AVT peptide (Sigma) eliminated all reaction product, (3) incubation with a mammalian GnRH antibody (635.5, donated by Jennes to I.S. Parhar) labeled TN, POA, and TEG somata and fibers, (4) preabsorption of a cGnRHIII-specific antiserum (Adams-100, donated by T. Adams, University of California at Davis; see Forlano et al., 2000 for crossreactivity data) with 8  $\mu$ M sGnRH labeled somata and fibers associated with the midbrain tegmentum only (and not the TN or POA), (5) incubation with a seabream specific GnRH antibody (ISPI, donated by Dr. Ishwar Parhar, Nippon Medical School, Tokyo, Japan) produced no staining at all in the goby brain, (6) incubation with anti-sGnRH (1668, donated by J. King, University of Cape Town Medical School, South Africa) labeled only TN somata and fibers, and (7) preabsorption of antiserum 7CR-10 with 8  $\mu$ M salmon or chicken II GnRH peptide (Bachem) reduced but did not eliminate reaction product. Brain sections were observed on a Zeiss Axioskop 2 microscope and images captured with an Optronics Macrofire digital camera. Line illustrations were made with a drawing tube on an Olympus BH2 microscope.

### 2.3. Quantification

Unbiased estimates of the number and size of GnRH and AVT cells were acquired from sagittal sections without knowledge of SL, BW, sex, or month collected. Counts for GnRH-ir cells were made for the terminal nerve ganglion, preoptic area, and midbrain tegmentum from sections reacted with anti-GnRH 7CR-10. Each AVT soma was assigned to either the parvocellular (pPOA), magnocellular (mPOA), or gigantocellular (gPOA) cell group based on neuroanatomical descriptions, somata morphology, and size (Braford and Northcutt, 1983). To assess whether somata

Table 1  
Correlation statistics and test for linear regression for gonadotropin-releasing hormone (GnRH) and arginine vasotocin (AVT) cell numbers and profile areas versus body length and weight in the halfspotted goby, *A. semipunctata*

	Standard length (mm)		Body weight (g)	
	$r^2$	$p$	$r^2$	$p$
<i>Males</i>				
Cell number — GnRH				
TEG, pre-spawn	0.64	0.03	0.71	0.02
POA, post-spawn	–	–	0.98	0.001
AVT				
pPOA, non-spawn	0.91	0.01	0.92	0.01
pPOA, post-spawn	0.74	0.01	0.67	0.02
Cell size — GnRH				
POA, pre-spawn	0.80	0.04	0.65	0.03
TEG, non-spawn	0.73	0.03	0.82	0.01
AVT				
gPOA, non-spawn	0.71	0.04	0.72	0.03
<i>Females</i>				
Cell number — GnRH				
POA, non-spawn	0.71	0.02	0.73	0.01
POA, peak-spawn	0.93	0.007	0.87	0.02
AVT				
pPOA, non-spawn	0.93	0.008	0.95	0.004
mPOA, non-spawn	0.98	0.001	0.99	0.001
mPOA, peak-spawn	0.66	0.05	–	–
gPOA, peak-spawn	0.72	0.03	–	–
Cell size — GnRH				
POA, non-spawn	0.73	0.01	0.74	0.01
POA, peak-spawn	0.80	0.04	–	–
TEG, post-spawn	0.91	0.05	0.90	0.05
AVT				
pPOA, non-spawn	0.93	0.008	0.91	0.01
pPOA, peak-spawn	0.94	0.03	0.90	0.05

Coefficient of determination,  $r^2$ , indicates the strength of the association between the neuron character and body size.  $p$  value indicates the probability that there is no relationship between neuropeptide neuron character and body size.

M, male; F, female. gPOA, gigantocellular preoptic area; mPOA, magnocellular preoptic area; pPOA, parvocellular preoptic area; POA, preoptic area; TEG, midbrain tegmentum. Only those correlations where  $p \leq 0.05$  are shown.

could be counted more than once in adjacent sections, ten randomly chosen cell diameters from each AVT and GnRH cell group for two fish were measured along the medial–lateral brain axis in transverse sections. GnRH somata in the TN were the largest immunoreactive cells ( $16.5 \pm 1.5 \mu\text{m}$  SE diameter) but were smaller than section thickness thus duplicate counts in serial sections were minimal. This was further confirmed by comparing cell counts made on serial versus alternate sections where counts were doubled, and there was no difference between the methods (Chi-square test,  $p > 0.05$ ). Also, there was no difference in the total number of cells counted between the two alternate sections (Chi-square test,  $p > 0.05$ ). These tests demonstrate that our method provided an unbiased estimate of cell number suitable for sex and seasonal comparisons.

Cell size was determined from digital images of somata at  $400\times$  and cell profile area was calculated with Sigma Scan Pro 5.0 (SPSS, Inc.). For each fish, 4–10 randomly chosen AVT cells were measured in each POA region, while 2–10 GnRH cells were measured for each TN, POA and TEG region ( $< 10$  cells were used *only* when  $< 10$  cells were present within an

individual). For both GnRH and AVT somata, cell profile areas were only measured for cells with at least one neurite present, and measurements were taken within the same brain region among individual fish. Mean AVT cell profile area ( $\mu\text{m}^2$ ) differed (1-way ANOVA  $p < 0.001$ ; Tukey's test  $p \leq 0.05$ ) among pPOA, mPOA, and gPOA somata in the following order: pPOA  $<$  mPOA  $<$  gPOA, which served as a further character to distinguish these cell groups.

#### 2.4. Statistical analyses

Correlation statistics and linear regression tests were used to determine the effect of body size on cell number and size because our data did not meet the assumption of parallel slopes required for analysis of covariance. There were several significant relationships between cell characters and body size (see Table 1), and males were always larger than females within a given time period (Student's  $t$ -tests,  $p \leq 0.05$ ). Thus, we corrected the data for body size by dividing cell number and size by fish body weight (g). Differences in the number and size of GnRH and AVT-ir somata among sexes and seasons were determined with two-way analysis of variance (ANOVA) with subsequent Tukey's tests for pairwise multiple comparisons. In some cases, somata number and size data were log or square root transformed prior to ANOVA testing. Co-variation of the summed AVT and GnRH cell numbers across sex and season were also examined with a two-way analysis of variance and subsequent Tukey's tests for pairwise multiple comparisons. All statistical analyses were performed with SigmaStat 3.1 (Systat, Inc.).

### 3. Results

A total of 101 gobies (51 male; 50 female) were used for quantitative analyses in this study. Mean fish size was  $31.6 \pm 5.4$  SD mm SL (BW:  $1.2 \pm 0.60$  SD g). There was no difference in mean standard length or body weight among males or females caught during the four separate time periods (1-way ANOVA,  $p > 0.05$ ). Thus, any seasonal differences in cell numbers or size are not due to a size sample bias within a sex.

#### 3.1. Gonadotropin-releasing hormone neuronal system: distribution

GnRH-ir somata are located in three separate regions of the goby brain (Figs. 1–3). Terminal nerve cells form a discrete clustered nucleus located ventrally at the junction of the olfactory bulb and telencephalon (Figs. 1, 2A, 3A). There are also several scattered GnRH-ir somata located more rostral in the olfactory bulb at the junction of the olfactory nerve and olfactory bulb (Fig. 1). Terminal nerve cells have their maximum width along the rostro-caudal body axis with prominent projections from the olfactory bulb–telencephalon junction through the preoptic area (see Fig. 2A). Multiple antibody and preabsorption experiments show GnRH-ir axons that originate from the terminal nerve ganglion have major projections to forebrain regions such as the olfactory bulbs, telencephalon, preoptic area, thalamus, and hypothalamus. However, there are

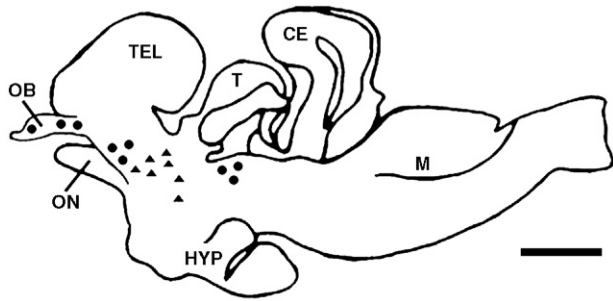


Fig. 1. Distribution of GnRH and AVT-immunoreactive somata in the brain of the halfspotted goby, *Asterropteryx semipunctata*. Schematic line drawing of sagittal section through the brain shows the location of GnRH-ir (dots) and AVT-ir (triangles) somata relative to the mid-sagittal plane. Three separate populations of GnRH-ir somata are located in the terminal nerve ganglion, preoptic area, and midbrain tegmentum. The parvocellular, magnocellular, and gigantocellular AVT-ir somata subgroups are located within the preoptic area (see text for descriptions). Scale bar = 1 mm.

also significant projections of GnRH-ir fibers from the TN cells in the optic nerve and retina, tectum, cerebellum, midbrain, and medulla.

GnRH-ir somata in the preoptic area are located in the region dorsal to the optic chiasm and parvocellular division of the POA (Fig. 1). These cells extend ventrally past the chiasm and pPOA (Figs. 2B, 3B). Preoptic area cells are scattered and do not form a tight discrete nucleus, but show prominent ventral projections in the preoptico-hypophyseal tract that course along the rostral edge of the hypothalamus to the pituitary.

GnRH-ir somata in the midbrain are located along the midline of the tegmentum below the fourth ventricle (Figs. 1, 2C, 3E). These cells are multi- or mono-polar with processes that project primarily to caudal brain regions such as the tegmentum, tectum, torus semicircularis, torus longitudinalis, cerebellum, medulla, and rostral spinal cord (as determined by application of multiple antibodies and preabsorption experiments).

The distribution of GnRH-ir axons is widespread in the goby brain. In the forebrain, GnRH-ir fibers are most prominent in the olfactory bulbs, central, dorsal, lateral and posterior zones of area dorsalis telencephali, area ventralis telencephali, preoptic area, hypophysis, inferior lobe of the hypothalamus, pretectal nuclei, and several thalamic nuclei (Fig. 3A–C). In the midbrain, most fibers occur in the tegmentum, torus semicircularis and

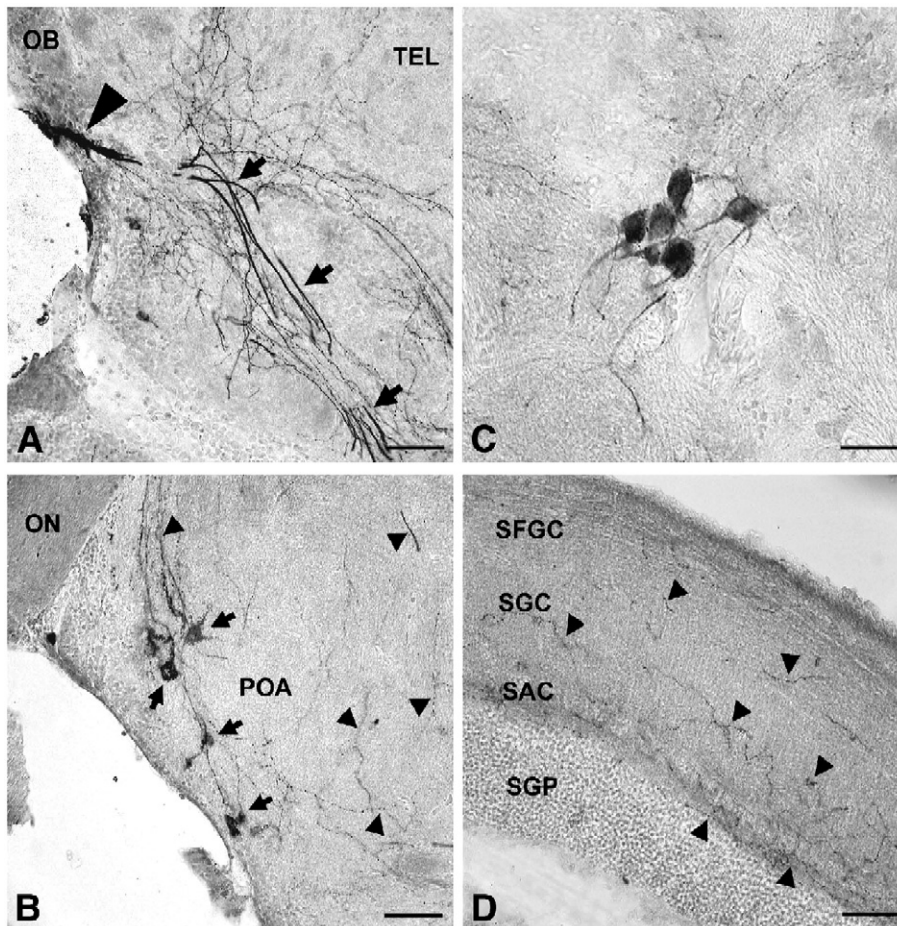


Fig. 2. Photomicrographs of GnRH-immunoreactive somata and fibers in the brain of the halfspotted goby, *Asterropteryx semipunctata*. (A) Sagittal section shows the cluster of large terminal nerve cells (arrow head) and thick immunoreactive tracts (arrows) at the olfactory bulb (OB) and rostral telencephalon (TEL). (B) Sagittal section in the preoptic area (POA) shows scattered fusiform GnRH-ir somata (arrows) and fibers (arrowheads). (C) Sagittal section of GnRH-ir somata in the midbrain tegmentum shows several multipolar cells. (D) Sagittal section shows numerous varicose GnRH-ir axons (arrowheads) in the stratum album centrale (SAC) and stratum griseum centrale (SGC), and scattered axons in the stratum fibrosum et griseum superficiale (SFGS) of the tectum. ON, optic nerve; SGP, stratum griseum periventriculare. Scale bars = 50  $\mu$ m (A–D).

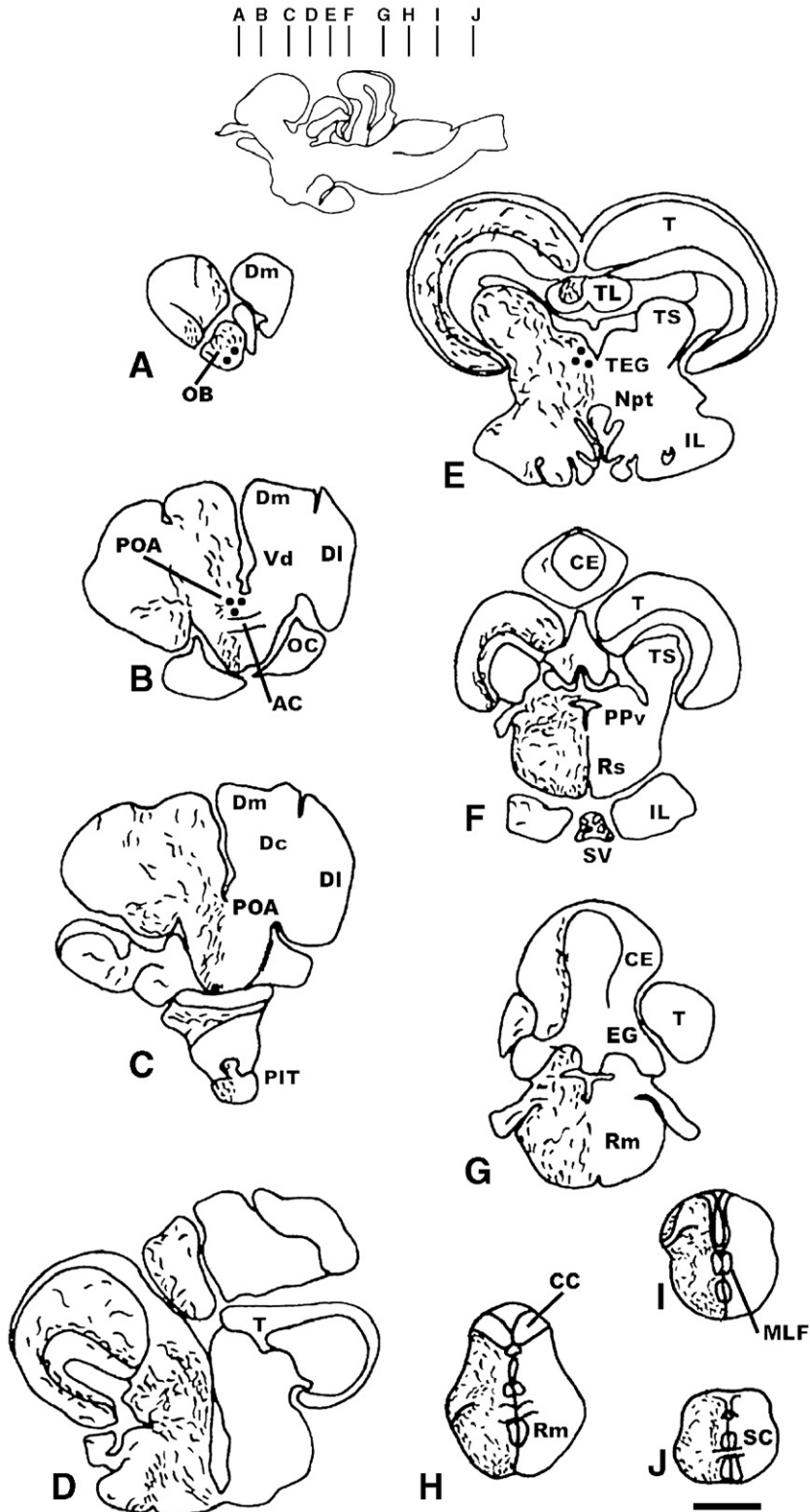


Fig. 3. Distribution of GnRH-immunoreactive neurons in the brain of the halfspotted goby, *Asterropteryx semipunctata*. Camera lucida drawings of transverse sections illustrate the locations of GnRH-ir somata (dots) and fibers (lines). No distinction is made between fibers that originate from the terminal nerve, preoptic area, or midbrain tegmentum in this figure. Inset shows a schematic sagittal brain with the approximate locations of each cross section. Scale bar=1 mm.

torus longitudinalis (Fig. 3D–F). There are prominent projections to the central tectal zone (stratum griseum centrale) and in the region above the periventricular cell layer (stratum album

centrale), but only sparse fibers in superficial layers of the tectum (Figs. 2D, 3D–F). In the caudal brain, fibers are scattered in the cerebellum (valvula and corpus) near the purkinje cell layer and

in the granular layer, in the eminentia granularis, and are abundant in the vagal lobes, octavolateralis nuclei, motor nuclei, ventral medulla within nuclei of the reticular formation, and rostral spinal cord (Fig. 3G–J).

### 3.2. Gonadotropin-releasing hormone neuronal system: sex and seasonal comparisons

There were several relationships between GnRH cell characters and body size (log SL and log BW) in the goby (Table 1). The majority of relationships were found in the POA cell group, which showed positive relationships with body size. The relationship between TEG cell number and body size was negative for pre-spawning males, while there was a positive relationship between TEG cell size and body size for non-spawning males and post-spawning females. We found no relationship between any TN GnRH cell character and body size.

GnRH cell populations in the goby showed both sex and seasonal variations. When corrected for body size, sex differences were found within all cell groups during certain seasonal periods and females always had proportionally more or larger cells than males (Fig. 4). Female gobies had proportionally more and larger cells in the TN and TEG only during peak- and

non-spawn, and in the POA during peak, post- and non-spawn periods (2-way ANOVA,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ).

There were also several seasonal differences in all three GnRH cell groups. In the TN, females had fewer cells during the pre-spawn period compared to both peak- and non-spawn times, and males had more cells during non-spawn compared to the peak-spawn period (2-way ANOVA,  $p = 0.008$ ; Tukey's test,  $p \leq 0.05$ ). Males also had larger cells during the non-spawn period compared to all other times (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ) (Fig. 4). Similarly, females had larger TN cells during the non-spawn period compared to pre- and post-spawning, and during peak-spawn compared to pre- and post-spawn times (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ) (Fig. 4).

In the POA, male gobies had fewer cells during post-spawn compared to peak- and non-spawn periods and more cells during non-spawn compared to the pre-spawn time (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ). Females also had more cells during non-spawn compared to pre- and post-spawn periods (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ). Further, males had smaller POA cells during post-spawn compared to peak- and non-spawn periods (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ). Females had larger POA cells during non-spawn compared to post- and pre-spawn times, and peak-spawn compared to the pre-spawn period (2-way ANOVA,

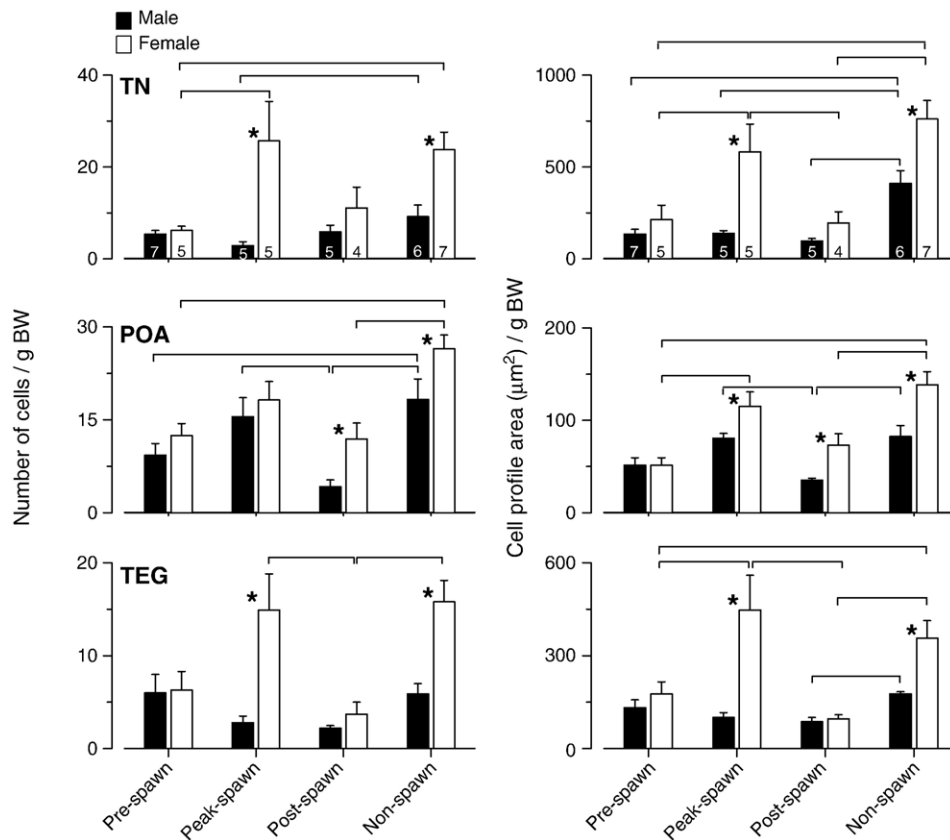


Fig. 4. Cell number and profile area of GnRH-immunoreactive somata within terminal nerve, preoptic area, and midbrain tegmentum groups across sex and spawning season in the halfspotted goby, *Asterropteryx semipunctata*. Sex differences were observed in all three cell groups during certain time periods where females always (open bars) had more or larger cells compared to males (solid bars). There were also seasonal differences in all GnRH cell groups for both males and females. Bars show mean  $\pm$  SEM cell number and size. Numbers indicate sample size for each group. Cell numbers and areas were adjusted per g body weight (g BW). \* indicate sex differences within a period and lines with tick marks link periods that differ within a single sex (2-way ANOVA,  $p \leq 0.05$ ; Tukey's test,  $p \leq 0.05$ ).

$p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ) (Fig. 4). When POA cell number and size data are not corrected for body size, males have larger and more abundant cells during the peak-spawning period compared to females, males have more and larger POA cells during peak-spawn compared to pre- and post-spawn periods, and more cells during non-spawn compared to post-spawn (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ) but there are no seasonal differences in GnRH cell number or size in females (2-way ANOVAs,  $p > 0.05$ ).

In the TEG, females had more and larger cells during non-spawn and peak-spawn compared to post-spawn (2-way ANOVA,  $p = 0.008$ ; Tukey's test,  $p \leq 0.05$ ). Females also had larger cells during peak-spawn compared to pre- and post-spawn periods, and larger cells during non-spawn compared to pre- and post-spawn times (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ). Males also had larger TEG cells during non-spawn compared to the post-spawn period (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p = 0.03$ ) (Fig. 4).

### 3.3. Arginine vasotocin neuronal system: distribution

AVT-ir somata form a large band or arch within the POA that extends from the optic chiasm to the caudal preoptic area and

rostral midbrain (Figs. 1, 5, 6). The pPOA cells are the most rostral and numerous, round or oval in shape, mono-polar, and of small diameter (Figs. 5, 6). The mPOA cells are immediately caudal, approximately twice the size of pPOA somata, and are multi- or mono-polar (Figs. 5, 6). The gPOA cells are most caudal, 2–2.5 times larger than the mPOA cells, located along a dorso-ventral band that extends from above the mPOA cell region in the caudal-ventral telencephalon ventrally into the rostral hypothalamus (Figs. 5, 6). These cells are multi-polar with multi-directional processes that project extensively towards caudal brain regions (Fig. 5D).

The greatest concentration of AVT-ir axons occurs within the POA and forms a dense preoptico-hypophyseal tract that courses ventro-lateral from the preoptic area to the pituitary (Fig. 5B). No fibers were observed in the olfactory bulbs, and few were found within all regions of the area dorsalis telencephali, thalamic nuclei and hypothalamus (Fig. 6A–C). The most abundant AVT projections in the forebrain are to the area ventralis telencephali and POA (Fig. 6A–C). In the midbrain, AVT-ir axons are most abundant in the torus semicircularis and tegmentum (Fig. 6D–E). Only sparse immunoreactive fibers are found in the deep layers of the tectum (e.g. stratum album centrale), but they occur in the same region of dense GnRH-ir projections

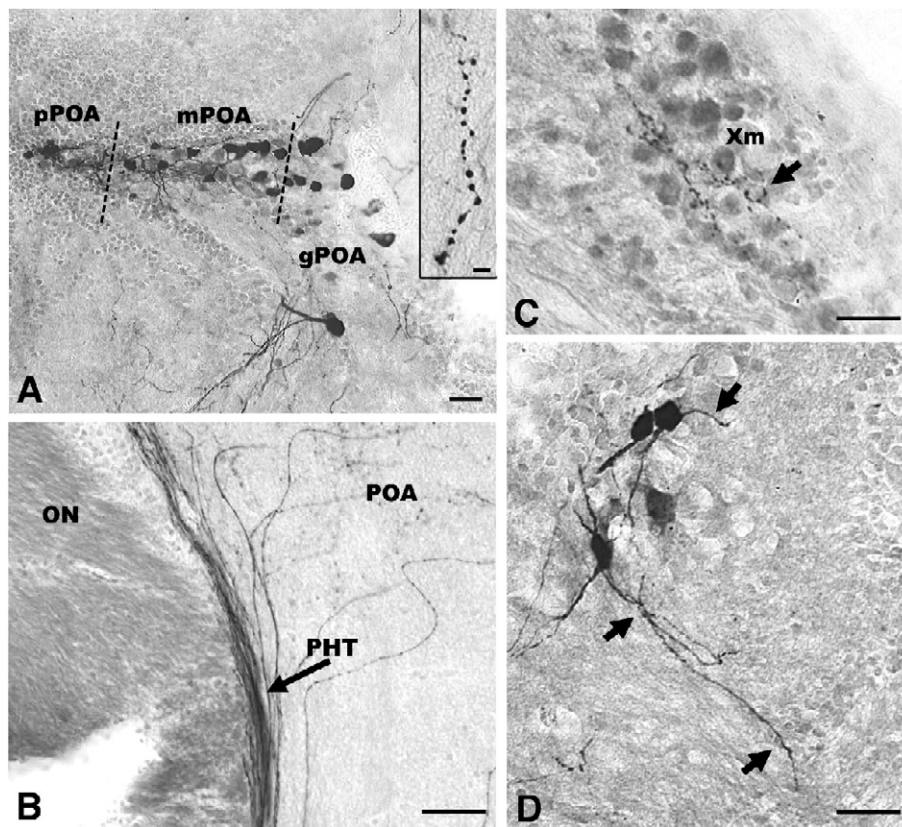


Fig. 5. Photomicrographs of AVT-immunoreactive somata and fibers in the brain of the halfspotted goby, *Asterropteryx semipunctata*. (A) Sagittal section through the preoptic area shows the relative location of the parvocellular (pPOA), magnocellular (mPOA) and gigantocellular (gPOA) cell groups. Dashed lines indicate the approximate divisions between the three areas. Inset: example of axon with large varicosities in the preoptic area. (B) Sagittal section through the preoptic area shows beaded fibers (arrow) of the thick preoptico-hypophyseal tract (PHT) that projects through the preoptic area (POA) along the rostral diencephalon to the pituitary. (C) Beaded AVT-ir axons make putative synaptic contacts (arrow) on motor neurons in the motor nucleus of nerve X (Xm) in the hindbrain. (D) AVT-ir gigantocellular somata are multipolar and often project towards caudal brain regions (arrows). Rostral is to the left for all figures. ON, optic nerve. Scale bars = 50  $\mu\text{m}$  (A); 5  $\mu\text{m}$  (inset in A); 20  $\mu\text{m}$  (B–E).



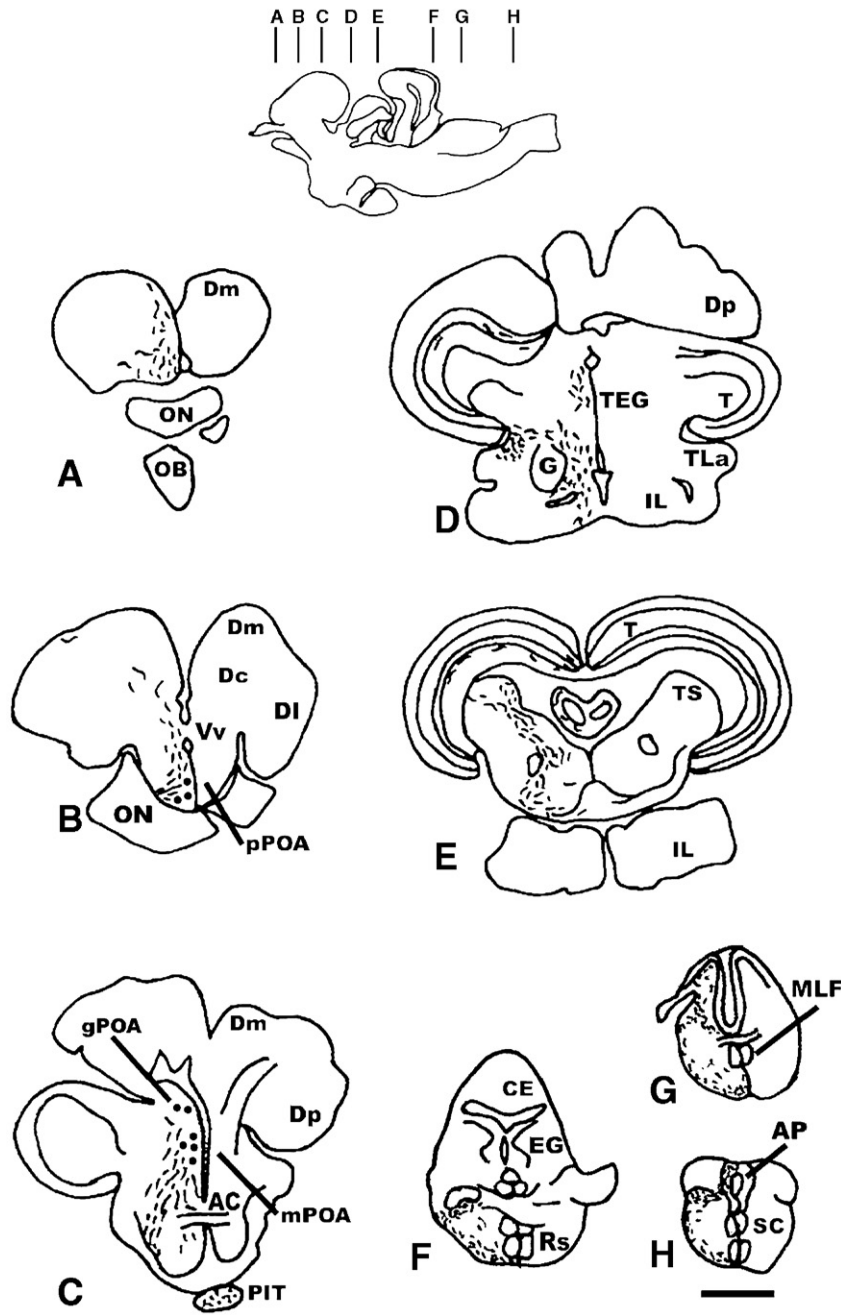


Fig. 6. Distribution of AVT-immunoreactive neurons in the brain of the halfspotted goby, *Asterropteryx semipunctata*. Camera lucida drawings of transverse sections through the brain show the locations of AVT-ir somata (dots) and fibers (lines). Inset shows a schematic sagittal brain with the approximate locations of each cross section indicated. Scale bar=1 mm.

(Fig. 6D–E). Sparse AVT-ir fibers occur in both the valvula and corpus granular layer of the cerebellum. AVT-ir fibers project through the midbrain to the medulla and spinal cord in a lateral rostro-caudal tract. In the caudal brain, AVT-ir axons are abundant in the ventral medulla and reticular formation, and some scattered fibers found within octavolateralis nuclei (Fig. 6F–G). AVT-ir fibers are also associated with several motor nuclei (glossopharyngeal and vagal) in the hindbrain and beaded fibers appear to make synaptic contacts with motor neurons in these regions (Figs. 5C, 6). Caudal to the fourth ventricle at the junction of the caudal medulla and rostral spinal cord, the lateral

AVT-ir tract courses medial and dorsal to form a dense plexus of beaded fibers along the dorsal midline near the area postrema (Fig. 6H).

#### 3.4. Arginine vasotocin neuronal system: sex and seasonal comparisons

Several relationships between AVT cell number and profile area with body size (log SL and log BW) were found in the goby (Table 1). The majority occurred in the pPOA cell group of females. In the pPOA, there was a positive relationship between

cell number and body size for females during non-spawn, males during post-spawn, and a negative relationship for males during non-spawn periods. There was a positive relationship between pPOA cell profile area and body size for non-spawning females but a negative relationship for peak-spawning females. A positive relationship exists between the total number of mPOA cells and body size only for females caught during non- and peak-spawn time periods. In the gPOA, the number of cells increased with increasing body size for peak-spawning females and non-spawning males.

Sex and seasonal differences were also identified in the goby AVT cell populations. When corrected for body size, sex differences were found within all cell groups during certain seasonal periods (most commonly during peak- and non-spawn periods) and females always had proportionally more or larger cells than males (Fig. 7). In the pPOA, females had proportionally more and larger cells during non-spawn and larger cells during peak- and post-spawn periods compared to males (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ). In the mPOA, females had proportionally more and larger cells during peak- and non-spawn, and larger cells during post-spawn periods compared to males (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ). In the gPOA, females had proportionally more and larger cells during non-spawn and larger cells during post-spawn compared to males (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ) (Fig. 7).

Females also showed more seasonal changes in AVT cell number and size compared to males when data are corrected for body size (Fig. 7). In both the pPOA and mPOA, females had more cells during non-spawn compared to pre- and post-spawn periods. Females also had larger pPOA cells during non-spawn and peak-spawn compared to the pre-spawn period, and larger mPOA cells during non-spawn compared to the pre-spawn time (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ). Males showed only a single seasonal difference in the mPOA where they had larger cells during non-spawn compared to the pre-spawn time (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p = 0.03$ ). In the gPOA, females had more cells during peak-spawn compared to pre- and post-spawn periods, more cells during non-spawn compared to pre- and post-spawn periods, and fewer cells during pre-spawn compared to all other times (2-way ANOVAs,  $p < 0.05$ ; Tukey's test,  $p \leq 0.05$ ). In males, seasonal variations were restricted to larger gPOA cells during non-spawn compared to pre- and post-spawn (2-way ANOVAs,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ) but not the peak-spawn period (Fig. 7).

### 3.5. Co-variation in AVT and GnRH

In order to examine the seasonal co-variation among AVT and GnRH within the goby brain, the total number of immunoreactive somata was summed for each peptide and plotted by

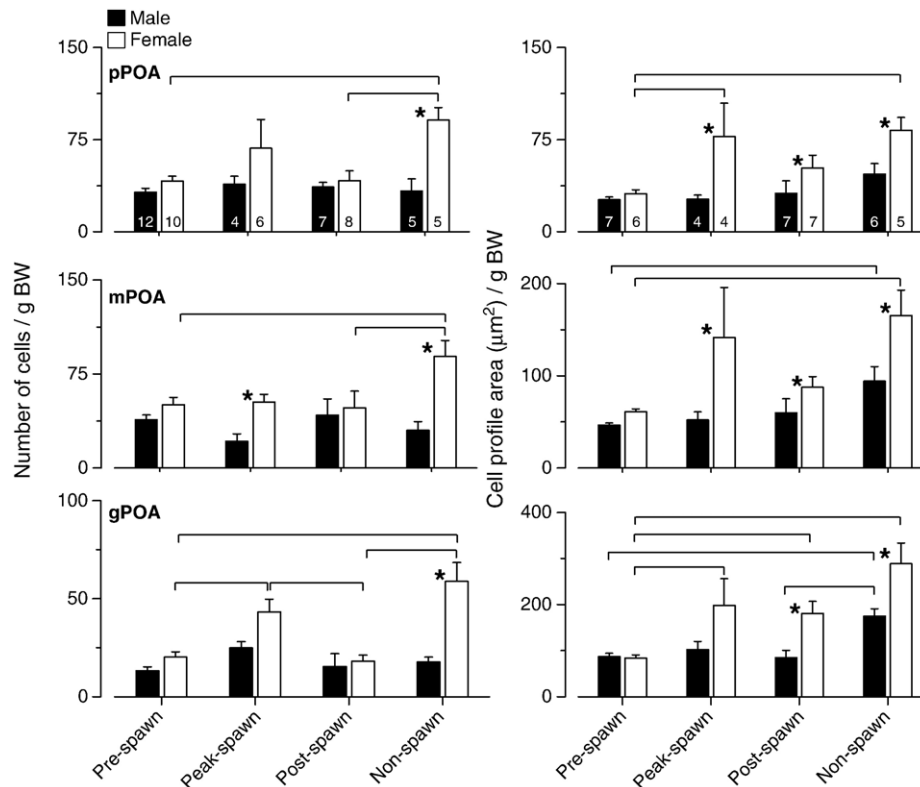


Fig. 7. Cell number and profile area of AVT-immunoreactive somata within parvocellular, magnocellular, and gigantocellular preoptic nuclei across sex and spawning season in the halfspotted goby, *Asterropteryx semipunctata*. There are differences in cell numbers between males (solid bars) and females (open bars) in all cell groups, but the majority of seasonal differences occurred in females. Bars show mean  $\pm$  SEM cell number and size. Numbers indicate sample size for each group. Cell numbers and areas were adjusted per g body weight (g BW). \* indicate sex differences within a period and lines with tick marks link periods that differ within a single sex (2-way ANOVA,  $p \leq 0.05$ ; Tukey's test,  $p \leq 0.05$ ).

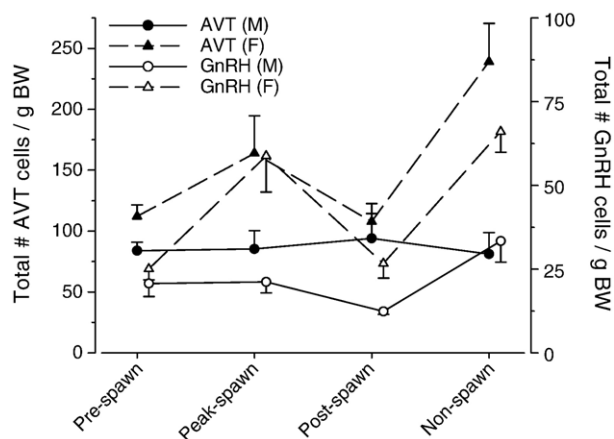


Fig. 8. Co-variation in total number of AVT and GnRH cells across spawning season in the halfspotted goby, *Asterropteryx semipunctata*. In male gobies, AVT cell numbers (closed circles) do not change seasonally, but GnRH cell numbers (open circles) are lower during post-spawn compared to the non-spawn period. In comparison, females show coincident increases in both AVT (closed triangles) and GnRH (open triangles) cell numbers during the non-spawning period compared to pre- and post-spawn times, while GnRH cell numbers (but not AVT) are also greater during this same period (see text for explanation). Data are plotted as the mean  $\pm$  SEM per g body weight for all AVT (pPOA, mPOA, gPOA) and GnRH (TN, POA, TEG) cell groups summed for both males and females. Female symbols are offset to the right of male symbols and error bars are shown in only one direction for clarity.

sex across reproductive season in Fig. 8. In male gobies, total AVT cell numbers did not change seasonally and GnRH cell numbers during the non-spawn period were greater than post-spawn (2-way ANOVAs,  $p < 0.001$ ; Tukey's test,  $p = 0.001$ ), but not pre- or peak-spawn periods. In females, total AVT and GnRH cell numbers were both higher during non-spawn compared to pre- and post-spawn periods (2-way ANOVAs,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ), but not compared to the peak-spawn time. In addition, total GnRH cell numbers in females were greater during the peak-spawn time compared to pre- and post-spawn periods (2-way ANOVA,  $p < 0.001$ ; Tukey's test,  $p \leq 0.05$ ), but AVT cell numbers during peak-spawn were not elevated above pre- and post-spawn numbers.

#### 4. Discussion

This study demonstrates sex and seasonal differences in discrete cell groups of gonadotropin-releasing hormone and arginine vasotocin-immunoreactive neurons in the brain of a single perciform fish species. Sex differences were found among all GnRH and AVT subgroups, but were time-period dependent. Seasonal variations also occurred in both peptides, with coincident elevations most prominent in females during peak- and non-spawning periods. Morphological comparisons of peptide subgroup somata within a single time period provide detailed insight into their different but coincident activities. Furthermore, serial comparisons across time emphasize the relevance of fine scale temporal analyses to help understand the plasticity of hormone effects among sexes, the proximate control of reproduction and the evolution of social behavior.

#### 4.1. Gonadotropin-releasing hormone neuronal system

Most perciform fishes contain three different molecular variants of GnRH in separate regions of the brain: sGnRH (GnRH3) in the olfactory bulb, terminal nerve, and telencephalon; sbGnRH (GnRH1) in the preoptic area; and cGnRHII (GnRH2) in the midbrain tegmentum, that are also hypothesized to be functionally divergent (see Amano et al., 1997 for review). However, recent studies in some perciform fishes that used more specific labeling techniques show sGnRH or sbGnRH-containing neurons distributed along a continuum from the terminal nerve and nucleus olfactive (NOR) region through the ventral forebrain and preoptic area to the anterior hypothalamus (Gonzalez-Martinez et al., 2001, 2002; Mohamed et al., 2005; Pandolfi et al., 2005; Mohamed and Khan, 2006). It was suggested that all perciform fishes show these scattered forebrain GnRH somata, but perciform fishes are not a monophyletic group (Elmerot et al., 2002) and many families of derived fishes remain to be examined for multiple GnRH variants. While the exact forms of GnRH within any species cannot be determined without sequence or molecular analyses, our immunocytochemical examination in the halfspotted goby indicates that sGnRH occurs in the terminal nerve and cGnRHII in the midbrain tegmentum, but we were unable to determine the variant present in the POA. Future molecular studies are required to definitively determine the GnRH variant responsible for primary gonadotropin control in this and other goby species.

Several studies indicate that the TN GnRH system may be involved in initiation of ontogenetic and seasonal physiological events related to development, reproduction and behavior (Amano et al., 1997; Yamamoto et al., 1997; Parhar et al., 2001). For example, lesion of the TN GnRH somata in male gouramis impairs nest building behavior (initial courtship), but does not affect other reproductive behaviors (Yamamoto et al., 1997). A correlation between TN GnRH neuronal numbers or sGnRH peptide levels in the brain and gonadosomatic index in several fishes also indicates some TN involvement in reproductive function (Parhar et al., 2001; Andersson et al., 2001; Du et al., 2005). Seasonal reproduction in the polygamous goby includes behaviors such as male–male aggression, maintenance of a breeding territory, preparation and defense of nest sites, and mate attraction (Privitera, 2002b), which may be associated with the observed temporal variability in TN cell characteristics. GnRH-ir axons from the TN ganglia also project to the retina and peripheral olfactory structures, where peptide release modulates visual and olfactory processing in some species (Stell et al., 1987; Nevitt et al., 1995; Eisthen et al., 2000; Wirsig-Wiechmann, 2001; Wirsig-Wiechmann and Oka, 2002; Maruska and Tricas, 2007). Thus the more numerous and larger TN cells observed in female gobies during peak-spawning may locally elevate GnRH peptide levels in specific brain regions to facilitate sensory-mediated reproductive behaviors such as mate localization, mate choice, and spawning. GnRH from the large TN cells in male and female gobies during the non-spawning period may also modulate visual or olfactory cues during the initiation of courtship and spawning. An increase in the number of TN GnRH-ir cells was also found in parental males of the grass goby (*Zosterisessor ophiocephalus*) during the non-reproductive season

(Scaggiante et al., 2006) and was suggested to influence olfactory processing associated with seasonal migratory and digging behavior in that species (Scaggiante et al., 2006). Further, peptide levels of sGnRH from the TN were low during spawning and high during the regressed phase in the red seabream (*Pagrus major*) (Senthilkumaran et al., 1999). Thus the TN GnRH system may function in a neuromodulatory capacity that is required at the onset of specific reproductive behaviors on both a short term (single spawning event) and long term (seasonal) basis.

Numerous studies demonstrate plasticity in POA GnRH cells that are related to social status, sex, reproductive condition, and sex reversal (see Foran and Bass, 1999; Bass and Grober, 2001 for reviews). One common feature in studies on seasonal variations in GnRH content within the brain and pituitary is the consistent correlation of POA sbGnRH with gonadal development and steroidogenesis (Senthilkumaran et al., 1999; Rodríguez et al., 2000; Holland et al., 2001; Andersson et al., 2001; Collins et al., 2001; Okuzawa et al., 2003; Du et al., 2005). We also observed both sex and seasonal variations in cell number and size within POA GnRH somata of the goby. Similar to other studies, male gobies (not corrected for body size) had larger and more abundant POA GnRH cells compared to females within the peak-spawning season, and there was an increase in the number and size of POA GnRH cells in males sampled during the peak-spawning season compared to those collected in preceding and following periods, which corresponds to changes in gonad size within this population (Privitera, 2002b). These fluctuations in POA GnRH cell number and size may be regulated by androgen hormones produced by the gonads, or socially determined as shown for the cichlid (Francis et al., 1993; White et al., 2002). In addition, greater POA GnRH-ir cell numbers and sizes observed in parental male grass gobies (*Z. ophiocephalus*) during the breeding season indicates POA GnRH variability may be linked to investments associated with nesting and parental care in males (Scaggiante et al., 2006). However, when halfspotted goby data were corrected for body size, females had proportionally more or larger POA cells per g body weight compared to males, and also showed seasonal fluctuations in number and size. Variability in POA GnRH somata number and size in females may be related to gonad development, steroid cycling, ovarian histology and ovulation as shown in other fishes (Andersson et al., 2001; Okuzawa et al., 2003), or environmental factors such as photoperiod and water temperature (Okuzawa et al., 2003).

Seasonal variations in cGnRHII levels were demonstrated in the brain of several fish species with molecular or chromatographic and immunological techniques (Senthilkumaran et al., 1999; Andersson et al., 2001; Okuzawa et al., 2003; Du et al., 2005). However, to our knowledge, this is the first study to demonstrate natural seasonal variations in the number and size of GnRH-immunoreactive cells within the midbrain tegmentum of a fish. Female gobies had smaller TEG cells during the post-spawning period compared to peak- and non-spawn periods, and fewer cells during post-spawn compared to the peak- and non-spawn periods. This reduced amount of midbrain GnRH following the spawning season in the goby is similar to the musk shrew that shows decreased numbers of midbrain GnRH-

ir cells 40 h post-mating only in females that had ovulated (Dellovade et al., 1995). Thus the midbrain cells could be regulated by the negative feedback effects of high levels of steroids present after ovulation. However, previous studies in fishes indicate that the midbrain TEG cell population may (Montero et al., 1995; Kitahashi et al., 2005) or may not (Dubois et al., 1998; Soga et al., 1998; Parhar et al., 1996, 2000, 2001; Amano et al., 2004) be a target for feedback effects of sex steroids. Other studies in musk shrews also demonstrate increased numbers of cGnRHII-ir cells and fibers in food-restricted females, likely caused by accumulation of peptide within neurons due to inhibition of peptide release (Temple et al., 2003). If this relationship between metabolism and cGnRHII is conserved across divergent taxa, then it is possible that the elevated levels of GnRH in the goby midbrain during the non-spawning season are due to reduced nutrition or food availability during winter months. Thus the variation in TEG cell number and size may be affected by gonadal activity or non-gonadal influences such as environmental, behavioral, or other physiological attributes, but requires further study.

Alternatively, midbrain GnRH-ir cells are thought not to be directly involved in the brain–pituitary–gonad axis, but rather have a neuromodulatory function to regulate reproductive behaviors and motor and sensory processing (Amano et al., 1997; Rosen et al., 1997; Oka, 1997; Forlano et al., 2000; Maruska and Tricas, 2007). Female gobies had more and larger cells during peak-spawning, and increased GnRH peptide in sensory and motor control regions during this time may facilitate detection of cues to detect readiness, coordinate synchronous spawning between mates, and improve territory guarding during the period of reproductive receptivity. Peptide levels of cGnRHII in the brain of the turbot (*Scophthalmus maximus*) also increased throughout the spawning period concomitant with increases in oocyte diameter in females (Andersson et al., 2001) and indicate a role in reproductive behavior similar to that shown for goldfish spawning (Volkoff and Peter, 2000). However, it is unknown whether changes in cell size and number correlate with variations in peptide synthesis, accumulation or release in the goby and other fish species. Thus further studies are needed to examine the functional consequence of changes in GnRH cell number and size, whether they are correlated with actual peptide levels, and what factors regulate these changes *in vivo*.

#### 4.2. Arginine vasotocin neuronal system

A context-dependent relationship exists between aggression and the AVT system in fishes, but varies considerably among species (Semsar et al., 2001; Lema and Nevitt, 2004a,b; Larson et al., 2006; Santangelo and Bass, 2006). Seasonal variations in large parental male halfspotted gobies were primarily restricted to the presence of larger gPOA cells during non-spawning compared to other periods. Most previous studies show that AVT increases aggression in fishes, but other studies in territorial species show that AVT causes decreased aggressive behavior (see Goodson and Bass, 2001 for review; Semsar et al., 2001; Lema and Nevitt, 2004b). Male gobies aggressively

defend breeding and shelter territories throughout the year, but there is a distinct decline in mating during the winter non-spawn period (Privitera, 2002b), a time when male gPOA AVT cells are largest. Thus if aggressive behaviors were modulated by AVT as in other territorial species, this transient seasonal increase in AVT production may function to reduce aggression during the period when females are not reproductively active and breeding territory or egg clutches are not defended. However, it is important to recognize that the proximate causal mechanisms for aggression related to breeding versus parental care and nest defense may be different. In contrast to the short-term effects of AVT on aggression, vocalization, and sexual behavior shown in other fishes (Macey et al., 1974; Goodson and Bass, 2000; Semsar et al., 2001; Lema and Nevitt, 2004b; Thompson and Walton, 2004), the relatively constant number and size of AVT neurons in male gobies may promote long-term territorial behavior and help maintain a protracted breeding season. This may also be true for other fishes with protracted breeding, but requires future examination of AVT neurons in fish species with different lengths of breeding season.

The constant AVT somata number and size in male gobies throughout the breeding season may also facilitate sensory-mediated reproductive behaviors. For example, AVT was shown to inhibit male goldfish social approach responses to the visual stimuli of female conspecific, while the V1 receptor antagonist and isotocin stimulated this behavior (Thompson and Walton, 2004). While the exact mechanism is unknown, that experiment demonstrates that endogenous AVT is associated with sensory-mediated sociality in fishes. However, the absence of other seasonal differences in the male goby AVT system at the cellular level also does not preclude the presence of variations in postsynaptic mechanisms, AVT mRNA production, or AVT receptor expression and distribution as shown for other taxa (Dubois-Dauphin et al., 1991; Delville and Ferris, 1995; Boyd, 1997; Semsar et al., 2004).

In contrast to the relatively constant temporal feature of AVT neurons throughout the reproductive season in males, female gobies demonstrate more pronounced seasonal changes in AVT neuron populations that may be related to changes in peptide production and release. Most notable, females showed a trend for more and larger AVT neurons during both peak- and non-spawn periods across all three cell groups. This indicates that AVT neurons may be related to physiological status associated with egg development, ovulation, and steroid production in females. Elevated levels of brain AVT are coincident with active reproduction in other fish species (Ota et al., 1999a,b; Gozdowska et al., 2006), and recent studies in the medaka show more AVT cells in pre-spawning compared to post-spawning females (Ohya and Hayashi, 2006). The large numerous AVT cells in the female goby brain during peak-spawning are associated with high gonadosomatic index, percentage of females with ripe oocytes, and female reproductive condition (Privitera, 2002b). In contrast, the observed increase in AVT cell number and size in females during the non-spawn period is associated with reduced mating and ovaries that contain primarily immature, ripening, and spent oocytes (Privitera, 2002b). However, the fact that the peak- and non-spawn periods are also associated

with greater numbers and larger GnRH-ir neurons in female gobies supports the idea that these neuropeptides have some relation to temporal reproductive physiology and behavior, or neuromodulatory function. Alternatively, elevated AVT during the non-spawning season in females may function to regulate aggression as discussed above for males, or may be related to some other physiological or behavioral process regulated by AVT in fishes such as circadian rhythms, osmoregulation, cardiovascular function, metabolism, or stress response (Balment et al., 2006).

The majority of seasonal differences in the goby occurred in the gPOA group with fewer changes in mPOA and pPOA. The gPOA cell group is found only in teleost fishes and may project both to the pituitary and extrapituitary regions in the fish brain (Holmqvist and Ekstrom, 1995; Saito et al., 2004). There is also evidence for differential regulation of the three AVT cell groups in fishes. For example, AVT-ir somata in the gPOA were larger in castrated dominant terminal-phase male bluehead wrasse compared to intact animals, while there were no changes in pPOA or mPOA cell groups (Semsar and Godwin, 2003). Further, seasonal changes in vasotocin hybridization signals in masu salmon were more predominant in the mPOA and gPOA compared to the pPOA (Ota et al., 1999b). Thus, the gPOA cell group likely plays an important role in temporal physiological or behavioral processes possibly related to the polygamous social system, territoriality, benthic spawning behavior, or male parental care present in the goby. Future studies are needed to test the hypothesis that pPOA, mPOA, and gPOA AVT cell populations serve different functions in fishes.

#### 4.3. Co-occurrence and co-variation of GnRH and AVT

Similar to the few species examined thus far, there is considerable anatomical overlap between GnRH and AVT within the goby brain that may indicate interactions between these two neuropeptide systems (see Foran and Bass, 1999; Bass and Grober, 2001 for reviews). Neurophysiological studies in the rainbow trout show that both sGnRH (GnRH3) and cGnRHII (GnRH2) facilitate the electrical activity of POA AVT neurons (Saito et al., 2003). This indicates that both terminal nerve and midbrain GnRH may modulate AVT neurons that regulate reproductive behaviors. Further, chicken GnRH I and AVT co-occur and are co-localized within single neurons in the preoptic area of the chicken, which indicates AVT may be involved in the regulation of GnRH release (or vice versa) and reproductive function in birds (D'Hondt et al., 2000). GnRH-ir and AVT-ir varicose fibers project to common regions in the goby that include the preoptic area, hypothalamus, pituitary, thalamus and pallial regions in the forebrain, the tegmentum, deep layers of the tectum, and torus semicircularis in the midbrain, and motor nuclei and sensory processing regions in the hindbrain. Thus both peptides may modulate visual, acoustic or lateral line stimuli and sensorimotor processing during social interactions as proposed by the *sensorimotor processing hypothesis* (Moore and Rose, 2002; Rose and Moore, 2002) and refined in the *sensory neuromodulation hypothesis* (Maruska and Tricas, 2007). However, further studies are

needed to determine the short and long term effects of GnRH release on AVT neurons (and vice versa), and the physiological processes and behaviors regulated by these neuropeptides in this and other species.

Seasonal and sex variations in GnRH and AVT neuron populations in the present study may also be explained by interactions between the two peptides or similar regulatory mechanisms, especially in females that show trends for coincident elevations in both peptides during the peak- and non-spawn periods and reductions during pre- and post-spawn times. This coincident cyclicality may represent seasonal synthesis and accumulation of peptide within somata (peak-, non-spawn), followed by release of peptide (pre-, post-spawn) to specific brain regions or general circulation for regulation of temporal reproductive physiology and behaviors, as suggested for photoperiodic control of GnRH in rodents (El Qandil et al., 2005). In contrast to females, male gobies showed relatively little seasonal variation in total numbers of both AVT and GnRH somata, with the exception of elevated numbers of GnRH somata during non-spawn compared to the post-spawn period. These distinctions indicate possible differential function and regulation of these neuropeptides among males and females. However, these variations may also result from interactions with other hormones or transmitters, physiological state, and environmental conditions. Comparison of the summed temporal variations in both peptides (Fig. 8) to those separated by different subgroups (Figs. 4, 7) illustrates the value of this detailed analysis within a single species to highlight which cells undergo putative changes in peptide synthesis, accumulation, and release. Further, had we only examined somata number and size during peak and non-spawning periods, much of the adult seasonal neuronal plasticity of these systems would be obscured. The expression of complex behaviors is under the control of multiple neural, sensory and endocrine systems. Different cell groups of both GnRH and AVT likely serve divergent functions, and quantification of cell characters within each subgroup across a fine temporal scale is necessary to understand the combined actions of these neuropeptides *in vivo*. Future studies should also address the physiological consequences of seasonal and sex variations to better understand the neuroendocrine actions on and evolution of reproductive and social behaviors.

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