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# Arginine Vasotocin Neuronal Phenotypes among Congeneric Territorial and Shoaling Reef Butterflyfishes: Species, Sex and Reproductive Season Comparisons

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Arginine vasotocin (AVT) and the homologous arginine vasopressin (AVP) neuropeptides are involved in the control of aggression, spacing behaviour and mating systems in vertebrates, but the function of AVT in the regulation of social behaviour among closely-related fish species needs further clarification. We used immunocytochemical techniques to test whether AVT neurones show species, sex or seasonal differences in two sympatric butterflyfish sister species: the territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*, and the shoaling polygamous milletseed butterflyfish, *Chaetodon miliaris*. The territorial species had larger AVT-immunoreactive (-ir) somata within the preoptic area, and higher AVT fibre densities within but not limited to the ventral telencephalon, medial and dorsal nucleus of the dorsal telencephalon, torus semicircularis, and tectum compared to the shoaling nonterritorial species. Furthermore, AVT-ir somata size and number did not differ among sexes or spawning periods in the territorial species, and showed only limited variation within the shoaling species. The distinct difference in AVT neuronal characteristics among species is likely to be independent of body size differences, and the lack of sex and seasonal variability is consistent with their divergent but stable social and mating systems. These phenotypic differences among species may be related to the influence of AVT on social spacing, aggression or monogamy, as reported for other fish, avian and mammalian models. The present study provides the first evidence for variation in vasotocin neural organisation in two congeneric and sympatric fish species with different social systems.

**Key words:** AVT, aggression, immunoreactive, social behaviour, teleost.

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Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) are produced in the vertebrate brain and form a prominent neuropeptide system that influences reproductive and social behaviours. When compared within or among closely-related species, patterns of neuronal organisation can provide functional relationships to explain sex, season or system level differences (1, 2). Intraspecies differences in the organisation of AVT and AVP neurones within specific regions of the brain are frequently associated with social behaviours (3, 4). Manipulation of endogenous AVT and AVP is known to affect aggression, mate choice, social recognition, and parental and sexual behaviour, often in a sex or species-dependent manner (3). In mammals, comparisons of the AVP system among species with different social systems are abundant, but similar interspecies comparisons of the non-mammalian AVT system are limited to birds and virtually unstudied in the highly speciose fishes. Thus, further studies are needed to test whether AVT neuronal organisation is associated with divergent social behaviours in fishes.

Variation in the neuronal organisation of AVT can differ among sexes and reproductive strategies in teleost fish that show diverse social or reproductive behaviours, but such studies are usually limited to a single species. In teleosts with alternative reproductive tactics, sneak spawning males generally have more AVT-immunoreactive (-ir) preoptic area (POA) cells per unit body weight than either territorial males such as in the Azorean rock-pool blenny (*Parablennius parvicornis)* (5) or territorial males and females such as in the plainfin midshipman (*Porichthys notatus*) (6). However, in the peacock blenny (*Salaria pavo*), both sneaker and territorial males have more AVT-ir cells than females (7). Territorial and initial phase male bluehead wrasse (*Thalassoma bifasciatum*) have higher levels of AVT mRNA within POA cells compared to females (8). However, only ter-

ritorial male bluehead wrasse have more AVT mRNA producing cells than females (8). Sex change in teleosts is also accompanied by alterations in the AVT system within the POA (9, 10). However, these variations may depend upon social context and are independent of gonads in at least one species, the bluehead wrasse (11). In addition, AVT neurones differ in the half-spotted goby (*Asterropteryx semipunctata*) across sex and reproductive season (12). These variations in the social behaviours of fish with different reproductive strategies can be related to AVT neuronal phenotype, but also highlight that patterns of AVT and behaviour are not fixed among species. Thus, to interpret the relationship between AVT and the evolution of social behaviours, it is important to identify potential intraspecific variation in neural organisation and reduce phylogenetic effects by comparison of closely-related species.

Comparisons of the AVT system among related fish species with different social systems are needed to test for the conserved function and action of AVT on vertebrate neural circuits that modulate social spacing, aggression and social systems. Only a single study (13) has compared AVT-ir regions among two closely-related fishes with different social behaviours. Differences in size but not number of POA AVT-ir cells were found among two pupfish subspecies. However, these subspecies were present in slightly different environments and were exposed to different osmoregulatory demands, which can have profound effects on the AVT system (14). Thus, further studies are needed to analyse whether the association between AVT/AVP neuronal features in the forebrain and exclusive home ranges, aggression and monogamy shown in other vertebrate taxa is also present in fishes.

Coral reef butterflyfishes (family *Chaetodontidae*, approximately 120 species) provide an excellent model system for comparative neuropeptide neuroanatomy related to behaviour. The diversity of social behaviour in closely-related butterflyfishes allows the comparison of AVT neuronal organisation with social behaviour because species within a single subgenus show different social spacing, mating systems and levels of aggression (15, 16). The social behaviour of these fishes is mediated by both visual and acoustic communication (17). The multiband (or pebbled) butterflyfish, *Chaetodon Exornator multicinctus*, is a monogamous corallivore in which mate pairs intensely defend a permanent feeding territory from other food competitors, particularly conspecifics (18). By contrast, the sympatric milletseed (or millet) butterflyfish, *Chaetodon Exornator miliaris* is a polygamous group spawning planktivorous species that aggregates in mixed-sex shoals (19) (Table 1). Individuals of this species display limited aggression and are not territorial.

**Table 1.** Taxonomic and Behavioural Comparison of the Two Species of Butterflyfish.

| | *Chaetodon multicinctus* | *Chaetodon miliaris* |
|---|---|---|
| Subgenus | *Exornator* | *Exornator* |
| Mating system | Monogamous | Polygamous |
| Social behaviour | Territorial | Shoaling |
| Feeding ecology | Corallivore | Planktivore |
| Habitat | Coral reefs | Coral reef water column |

The distinction in social spacing, mating systems and aggression in these two butterflyfishes may in part be related to the differences in organisation and temporal stability of their AVT systems. The present study aimed to test for species differences in AVT neural organisation, as well as intraspecific variation in AVT neurones across sex and reproductive season. The results obtained demonstrate interspecific variation in the AVT system because the territorial species has larger AVT somata in the POA, and greater AVT fibre densities in many brain regions compared to the shoaling species. These features did not vary across sexes or season in the territorial species, and showed only limited differences in the shoaling species. The present study is the first to report interspecific differences in AVT neuronal phenotypes among a territorial monogamous and closely-related shoaling polygamous fish species that occur in the same environment. The differences in the AVT system may contribute to their divergent but species-typical social behaviours, and provide a model system for future experiments on AVT control of fish social behaviour.

## Materials and methods

### Animal and tissue preparation

Individuals of each study species and sex were sampled during their spawning (April/May) and nonspawning (July) seasons. Adult *C. multicinctus* and *C. miliaris* were collected with barrier and hand nets from the west and north shore of Oahu in the afternoon, transported to the laboratory, held in flow-through aquaria overnight and perfused the next morning. Four *C. multicinctus* (two male and two female nonspawning) were held for an additional 24 h prior to perfusion but there was no difference in cell size or number for any cell group between these four fish and the other sample individuals (t-test, $P > 0.050$). To test for potential stress effects due to capture, transport and overnight holding on cell size and number, two additional adult *C. multicinctus* were collected by divers with barrier and hand nets on a shallow reef and then perfused on the boat within 4 min of capture. AVT cells in these fish did not differ in number or size from sampled individuals held overnight (95% confidence interval). Thus, we assume that all fishes were exposed to approximately equivalent post-capture stress and these did not affect cell measurement or count data. Adult male and female *C. multicinctus* with a standard length (SL) > 65 mm and *C. miliaris* with a SL > 90 mm were chosen based on previous work on sexual maturity and spawning seasonality of these populations (19, 20). Fish were anaesthetised with tricaine methanesulfonate (MS-222), measured for SL, total length and body weight (BW), and perfused transcardially with 0.9% heparinised saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Gonads were examined under a compound microscope at × 400 to verify sexual maturity (presence of mature spermatogonia or oocytes) and spawning seasonality (large gonad size and either mature sperm or hydrated/yolked oocytes). Brains were removed, postfixed in 4% paraformaldehyde in 0.1 M PB at 4 °C for 12–24 h, rinsed in 0.1 M PB, and cryoprotected overnight in 30% sucrose in 0.1 M PB prior to sectioning.

### Immunocytochemistry

Cryoprotected brains were embedded in Histoprep mounting media (Fisher Scientific Co., Pittsburgh, PA, USA) and sectioned at 24 $\mu$m with a cryostat in the sagittal, horizontal, or transverse plane. Alternate brain sections were collected on chrom-alum-coated slides, dried at room temperature overnight, and stored at 4 °C until reacted. Slides were brought to room temper-

ature, surrounded with a hydrophobic barrier (Immedge pen; Vector Laboratories, Inc., Burlingame, CA, USA), rinsed with 0.05 M phosphate-buffered saline (PBS), and blocked with 0.3% Triton-X 100 (Sigma, St Louis, MO, USA) in PBS with 2% normal goat serum (NGS; Vector Laboratories) for 30 min. The primary AVT antibody [donated by Dr Matthew Grober, Georgia State University, USA and used in other fish species (12)] was applied to the mounted sections (1 : 5000 final concentration) and incubated overnight (14–16 h) at room temperature in a sealed humidified chamber. Mounted sections were subsequently rinsed in PBS, incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) with 2% NGS for 1 h, rinsed in PBS, quenched with 0.5–3% hydrogen peroxide in PBS for 10–15 min, incubated with avidin-biotin-horseradish peroxidase complex (ABC Elite Kit; Vector Laboratories) for 2 h, rinsed in PBS, and reacted with a diaminobenzidine (DAB) chromogen substrate kit with nickel chloride intensification (Vector Laboratories) for 5–6 min. Slides were then soaked in distilled water for 10 min to stop the reaction, counterstained with 0.1% methyl green, dehydrated in an ethanol series, cleared in toluene, and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific, Thermo Scientific, Waltham, MA, USA). The following immunocytochemical controls were also performed in parallel with experimental tissue: the omission of primary antisera, secondary antisera, ABC solution, or DAB, as well as preabsorption of anti-AVT with 8 $\mu$M AVT peptide (Sigma) all resulted in no staining. The possibility of cross-reactivity with isotocin was assessed by comparison of alternate sections, one incubated with plain AVT antibody and the other with AVT antibody preabsorbed with 8 $\mu$M isotocin peptide (Bachem, Torrance, CA, USA). There was no difference in the number of AVT-ir cells for any cell group counted on alternate sections reacted with plain anti-AVT and anti-AVT preabsorbed with isotocin [t-test; *C. multicinctus*: gigantocellular POA (gPOA), P = 0.839, magnocellular POA (mPOA), P = 0.864, parvocellular POA (pPOA), P = 0.919; *C. miliaris*: gPOA, P = 0.621, mPOA, P = 0.383, pPOA, P = 0.779]. Thus, we are confident that only AVT and not isotocin immunoreactive neurones were labelled in the present study.

## Quantification

Each AVT-ir soma was assigned to either the pPOA, mPOA or gPOA group based on neuroanatomical position, somata morphology, and size (21). To assess whether somata could be counted more than once in adjacent alternate sagittal sections, ten randomly chosen cell diameters from each cell group for two fish of each species were measured along the medial-lateral axis in transverse sections. In both *C. multicinctus* and *C. miliaris*, the largest cells (mean $\pm$ SE) occurred in the gPOA and were 20.0 $\pm$ 1.1 and 17.0 $\pm$ 2.1 $\mu$m in diameter, respectively. Thus, there was no duplication of cell counts made on alternate 24 $\mu$m sections. The size and number of AVT-ir somata were measured on sagittal sections without prior knowledge of sex, reproductive condition, SL, or BW. Cell numbers were determined under magnification at $\times$ 400 with aid of a camera lucida. Cell profile area was computed from digital images of somata at $\times$ 400 with Sigma Scan Pro 5.0 (SPSS Inc., Chicago, IL, USA). Ten randomly chosen AVT-ir cells from each cell group with at least one neurite present were measured from the same brain region across individuals. An additional cell group within the ventral tuberal hypothalamus was present in some individuals of both species but was not quantified due to inconsistent labelling.

Arginine vasotocin-immunoreactive fibre varicosities were counted in one sagittal section of the torus semicircularis and tectum in each individual from both species. AVT-ir fibre varicosities were quantified because they are putative areas of synaptic contact (22), and sites for neuropeptide release (23) and neuromodulation (24, 25). Two digital photos of each of these two regions were taken at $\times$ 400 (140 mm$^2$ field), and the number of varicosities visually tallied and then projection region area calculated with Sigma Scan Pro 5.0 software (SPSS Inc.). Care was taken to match these sections across individuals with the greatest accuracy possible using unambiguous neuroanatomical landmarks such as gross brain structures, fibre tracts and cell nuclei. The number of AVT-ir fibres was also determined from transverse sections in one female nonspawning individual from each species in several additional brain regions (dorsal and medial regions of the dorsal telencephalon, ventral telencephalon, olfactory bulbs, medial octavolateral nucleus, descending octaval nucleus, sensory region of vagal lobe, and the preoptico-hypophyseal tract).

## Statistical analysis

Neurone size, number and shape often vary among individuals as a function of body size (6). Thus, we examined whether differences in body size among species solely influence observed differences in our cell measurements. Several of our data sets did not meet the assumption of parallel slopes required for ANCOVA. Therefore, we used sheared principle component analysis (PCA) to assess for variation in cell number and size that were independent of inherent body size differences among the two species (26). Data observations were log transformed and analysed with the sheared PCA algorithm (26). In this analysis, variation in size, which is prominent in the PC1 axis, is removed by a 'shear' or rotation that is applied to PC2 (and other subsequent components) to remove that variation from the component that is correlated with variation in PC1 (e.g. primarily body size).

Differences in the number and size of AVT-ir somata and fibres across sex and season within and among species were determined with a three-way ANOVA followed by pairwise multiple comparisons (Tukey's test). In cases where assumptions of normality were not met, data were log transformed. We used linear regression and correlation analyses to examine for relationships among sex and season and measured cell variates for each species. All statistical analyses were performed with Minitab Release 14 (Minitab Inc., State College, PA, USA).

# Results

A total of 22 territorial *C. multicinctus* (SL: 79.5 $\pm$ 7.9 mm; BW: 21.2 $\pm$ 7.4 g) and 25 shoaling *C. miliaris* (SL: 98.4 $\pm$ 12.8 mm; BW: 39.9 $\pm$ 17.3 g) were analysed in this study. For *C. multicinctus*, we used six spawning (SL: 82.7 $\pm$ 8.2 mm; BW: 23.9 $\pm$ 8.4 g) and four nonspawning (SL: 75.8 $\pm$ 12.6 mm; BW: 19.2 $\pm$ 7.9 g) males, and six spawning (SL: 77.5 $\pm$ 8.9 mm; BW: 20.2 $\pm$ 7.6 g) and six nonspawning (SL: 74.8 $\pm$ 7.6 mm; BW: 17.6 $\pm$ 7.7 g) females. For *C. miliaris*, we analysed six spawning (SL: 100.8 $\pm$ 20.7 mm; BW: 44.3 $\pm$ 28.6 g) and six nonspawning (SL: 103.0 $\pm$ 11.4 mm; BW: 44.3 $\pm$ 14.9 g) males, and seven spawning (SL: 101.0 $\pm$ 10.3 mm; BW: 40.4 $\pm$ 13.4 g) and six nonspawning (SL: 94.3 $\pm$ 7.3 mm; BW: 30.6 $\pm$ 5.9 g) females. There was no difference across either sex or season in mean SL for either species (three-way ANOVA and Tukey's test: *C. multicinctus*: sex, P = 0.904; season, P = 0.629; *C. miliaris*: sex, P = 0.886; season, P = 0.984) or body weight (three-way ANOVA and Tukey's test: *C. multicinctus*: sex, P = 0.846; season, P = 0.687; *C. miliaris*: sex, P = 0.737; season, P = 0.968). Therefore, any differences in cell numbers or size are not due to a size sample bias within a sex or season. However, the territorial butterflyfish, *C. multicinctus*, is smaller in both weight and length compared to the shoaling species (three-way ANOVA: P < 0.001). Most of these body size differences are maintained when species are examined across both sex and season (three-way ANOVA and Tukey's test: BW: female spawn, P = 0.040; female nonspawn, P = 0.152; male spawn,

P = 0.327; male nonspawn, P = 0.031; SL: female spawn, P = 0.008; female nonspawn, P = 0.039; male spawn, P = 0.174; male nonspawn, P = 0.007). Despite these differences in body size, all data are presented without correction for body size because the assumption of parallel slopes required for ANCOVA, the preferred method of body size correction (27), was not met. We tested for body size effects that could account for species differences in AVT neurones in the PCA species comparison section described below.

## AVT neurone distributions

The location and distribution of AVT-ir cell bodies were similar in both butterflyfish species. AVT-ir somata formed a large band or arch within the POA that extended from the optic chiasm to the caudal POA and rostral midbrain (Fig. 1A,G). The pPOA cells were the most rostral and numerous, round or oval in shape, monopolar, and of small diameter (*C. miliaris*: 6.42 ± 0.9 μm; *C. multicinctus*: 7.01 ± 0.9 μm; Fig. 1E,K). The mPOA cells were immediately caudal, approximately twice the size of pPOA somata (*C. miliaris*: 9.34 ± 2.3 μm; *C. multicinctus*: 10.57 ± 1.7 μm) and were multi or monopolar (Fig. 1D,J). The gPOA cells were most caudal, 2.0—2.5-fold larger than mPOA cells (*C. miliaris*: 17.0 ± 2.1 μm; *C. multicinctus*: 20.0 ± 1.1 μm), located along a dorso-ventral band that extended from above the mPOA cell region in the caudal-ventral telencephalon ventrally into the rostral hypothalamus (Fig. 1C,I). These cells were multipolar with multidirectional processes that appeared to project towards caudal brain regions. An additional AVT-ir cell group was also present in the ventral tuberal hypothalamus of both species, located just caudal to the preoptico-hypophyseal tract near the ventral margin of the hypothalamus (Fig. 1F,L). These hypothalamic cells were monopolar, round or oval in shape, and had a small diameter (*C. miliaris*: 4.47 ± 0.9 μm; *C. multicinctus*: 4.97 ± 1.2 μm).

The regional distribution of AVT-ir fibres was also similar among butterflyfish species, but innervation density was greater in many brain regions outside of the preoptico-hypophyseal tract in the territorial *C. multicinctus* compared to the shoaling *C. miliaris* (Figs 1B,H and 2). The greatest concentration of AVT-ir axons occurred within the POA and formed a dense preoptico-hypophyseal tract that coursed ventro-laterally from the POA to the pituitary in both species (Fig. 1B,H). No fibres were observed in the olfactory bulbs, and few were found within regions of the dorsal telencephalon, thalamic nuclei and hypothalamus (Fig. 2A). The most abundant AVT projections in the forebrain were to the ventral telencephalon and POA (Fig. 2A–D). In the midbrain, AVT-ir projections were observed to the torus semicircularis and tegmentum, but densities differed profoundly among species (see below) (Fig. 2D–E). Only sparse immunoreactive fibres were found in the deep layers of the tectum (e.g. stratum album centrale) (Fig. 2B–E). Sparse AVT-ir fibres also occurred in both the valvula and corpus granular layer of the cerebellum. AVT-ir fibres projected through the midbrain to the medulla and spinal cord in a lateral rostro-caudal tract. In the hindbrain, AVT-ir axons were abundant in the ventral medulla and reticular formation, and some scattered fibres were found within octavolateralis nuclei (Fig. 2F–I). AVT-ir fibres were also associated with several motor nuclei in the hindbrain and varicose fibres appeared to make synaptic contacts with cell bodies in these regions, particularly in the vagal motor nucleus. Caudal to the fourth ventricle at the junction of the caudal medulla and rostral spinal cord, AVT-ir fibres formed a dense plexus of beaded fibres along the dorsal midline near the area postrema (Fig. 2H). Although both species had a similar distribution of fibres, the territorial butterflyfish had a higher density of AVT-ir fibres compared to the shoaling butterflyfish, particularly in extra-hypothalamic regions.

## Species comparisons

Results of the sheared PCA, which used a shear algorithm to assess for differences in AVT-ir cell size and number between the two species while controlling for variation in fish size, were consistent with the existence of cell size differences among these two species. Analysis of the covariance matrix of size-adjusted AVT-ir cell measurements (both body and cell measurements) indicated that the first three PCs explained approximately 84.1% of variance in the AVT-ir cell characters between the two species (Table 2). PC1 (which is not sheared in the analysis) included variation associated with fish body size differences, accounted for 47.7% of the total variation and showed strong positive loadings for cell size in all three AVT groups. By contrast, all subsequent axes present additional components from which the variation for size effects in PC1 are removed (by the statistical shear process). PC2 represents the first body size-corrected component that explained 26.8% of additional variation and had strong negative loadings for cell sizes in the three AVT-ir cell groups. PC3 explained 9.6% of additional body size-corrected variation with strong negative loading for pPOA size and strong positive loadings for pPOA cell number, and gPOA and mPOA cell size. The bivariate scatter plot of PC2 and PC3 (Fig. 3) shows a distinct separation of the two species based upon size-adjusted cell measurements on PC2. This analysis provided further confidence that some of the variation in AVT group cell size is most likely independent of the inherent body size differences in these two species.

A three-way ANOVA of cell group, species and season factors shows that variation in AVT-ir cell size was explained by species in all three cell groups with an interaction between species and season within mPOA (Table 3). Post-hoc multiple comparisons revealed that AVT-ir cell size in all cell groups of the territorial monogamous *C. multicinctus* differed from the shoaling polygamous *C. miliaris* (Table 4). Furthermore, when species were compared across sexes and seasons, these differences were maintained in the gPOA cell group with the exception of spawning males and in the mPOA cell group with the exception of spawning females (Fig. 4 and Table 4). However, the pPOA cell group did not differ between species when compared across sex and season (Fig. 4 and Table 4). By contrast, the variation in AVT-ir cell number was explained by the season factor for all three cell groups with an interaction between species and season in the gPOA cell group (Table 3). The number of AVT-ir cells did not differ between the two species for any cell group (Table 4). However, when species were compared across sexes and seasons, female nonspawning territorial butterflyfish had fewer

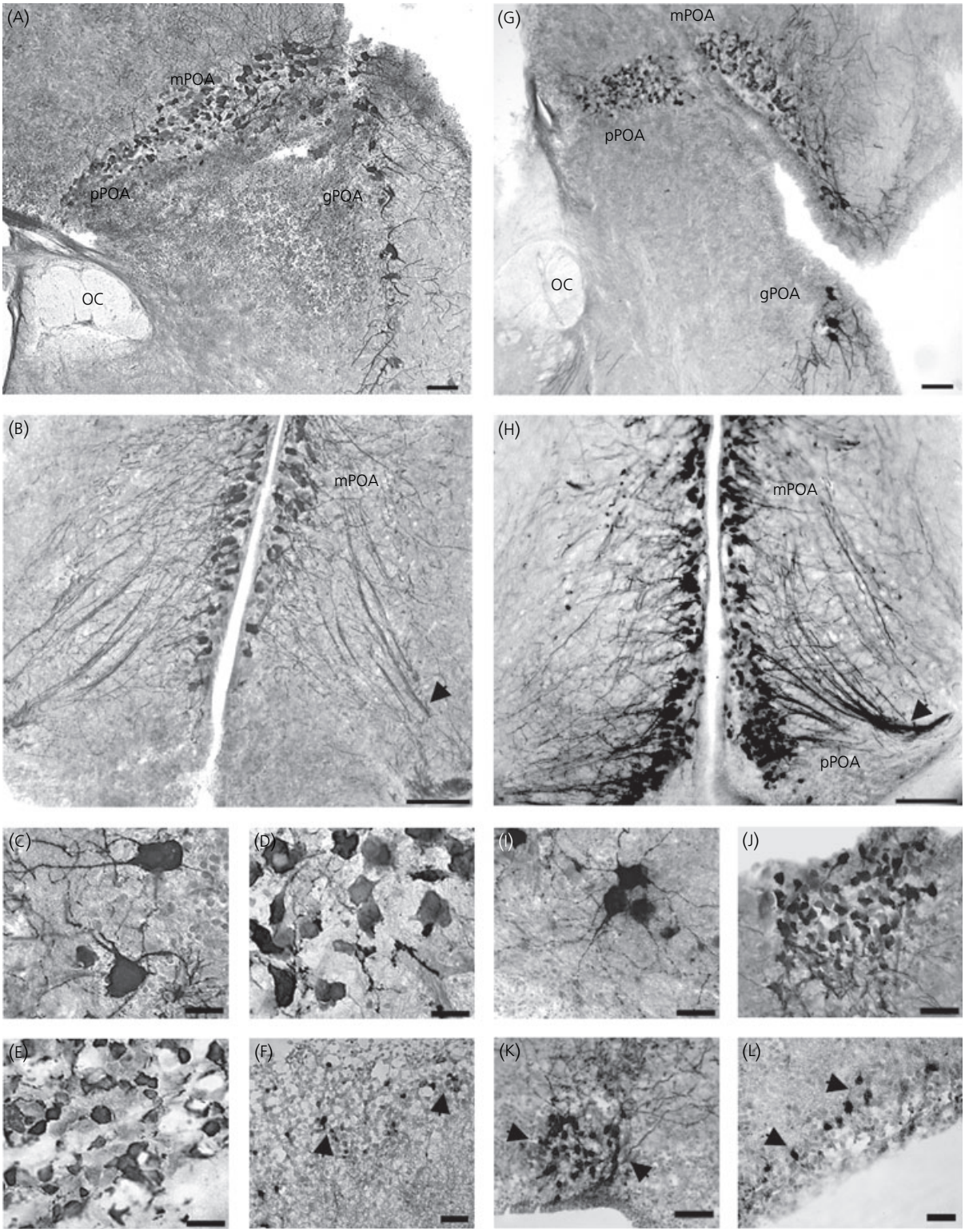



**Fig. 1.** Photomicrographs of arginine vasotocin (AVT)-immunoreactive (-ir) somata and fibres in the brain of territorial and shoaling congeneric butterflyfishes. Territorial monogamous *Chaetodon multicinctus* on the left (A–F) and shoaling *Chaetodon miliaris* on the right (G–L). Sagittal sections through the preoptic area of the territorial (A) and shoaling (G) butterflyfish show the relative position of parvocellular (pPOA), magnocellular (mPOA) and gigantocellular (gPOA) AVT-ir cell groups (rostral is up). Transverse section through brain of territorial (B) and shoaling (H) butterflyfish show similar fibre densities in the preotico-hypophyseal tract (arrows). High magnification of gPOA (C, I), mPOA (D, J), pPOA (E, K), and ventral tuberal hypothalamic cells (F, L) in territorial (C–F) and shoaling (I–L) butterflyfish. OC, optic chiasm. Scale bars = 100 $\mu$m (A, G); 50 $\mu$m (B, H); 20 $\mu$m (C–F, I–L).

gPOA cells than the shoaling species (Fig. 4 and Table 4). Furthermore, a seasonal increase in number of AVT-ir cells across all three cell groups was found in the nonspawning compared to the spawning season (three-way ANOVA and Tukey's test: gPOA, P = 0.003; mPOA, P = 0.006; pPOA, P = 0.042).

The densities of AVT-ir fibres in most brain regions were also greater in the territorial versus the shoaling species (Figs 2, 5 and 6). Several telencephalic and sensory processing brain regions were examined for species differences in AVT-ir fibre densities. In the telencephalon, this difference was evident in the dorsal and medial zones of the dorsal telencephalon and ventral telencephalon with a notable difference in the ventral nucleus of the ventral telencephalon (Figs 2A and 5A,B). Qualitatively, the territorial butterflyfish had approximately twice the density of fibres in the ventral telencephalon, × 1.5—2 the density in the medial nucleus of the dorsal telencephalon, and × 1.5—2 the density in the dorsal nucleus of the dorsal telencephalon. AVT-ir fibre density differences among species were found in the sensory processing region of the torus semicircularis and multimodal centre of the tectum (Figs 2B–E, 5C,D and 6), with more fibre varicosities observed in the territorial *C. multicinctus* (Fig. 6 and Table 4). However, in hindbrain regions such as the medial octavolateral nucleus, descending octavolateral nucleus, and sensory region of the vagal lobe, the density of fibres was low in each nucleus and qualitatively equivalent between the two species. Furthermore, the density of the AVT-ir preoptico-hypophyseal tract was also qualitatively similar among the two species.

### Sex and seasonal comparisons

#### *Chaetodon multicinctus*

There was no difference in AVT-ir somata number or size among sex or spawning season for the territorial monogamous butterflyfish (Table 4). However, there were several relationships between AVT-ir cell parameters and body size. Male spawning fish showed a positive relationship between log transformed BW and pPOA (slope = 179; $r^2$ = 0.66; P = 0.049), mPOA (slope = 364; $r^2$ = 0.74; P = 0.027) and gPOA cell sizes (slope = 1150; $r^2$ = 0.78; P = 0.019). Male spawning fish also showed a positive relationship between log SL and pPOA somata size (slope = 689; $r^2$ = 0.78; P = 0.020). Male nonspawning fish showed a positive relationship between log SL and number of mPOA cells (slope = 802; $r^2$ = 0.99; P = 0.005). There were no differences in the number of immunoreactive varicosities in the torus semicircularis or tectum across either sex or season (Table 4).

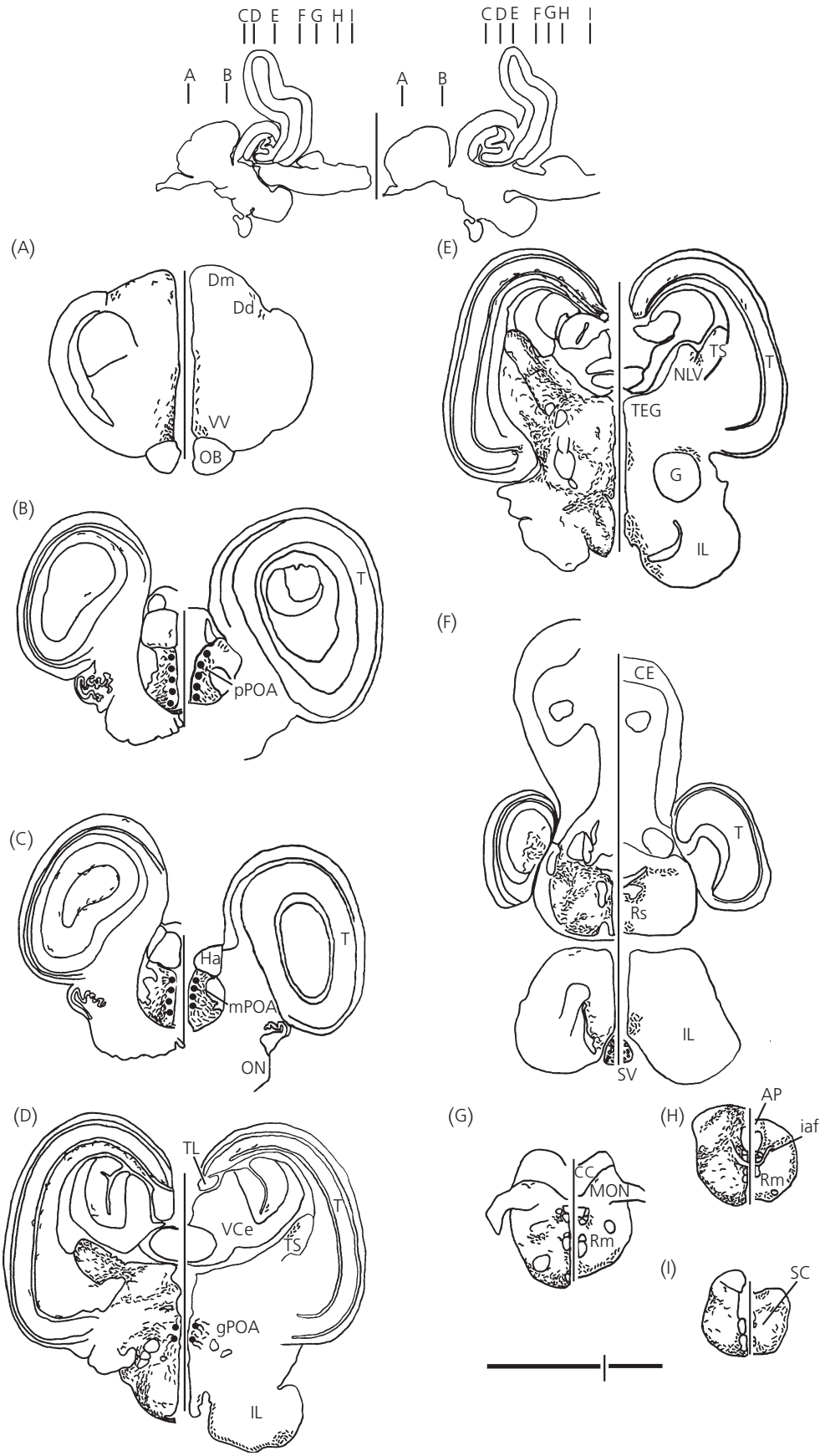
#### *Chaetodon miliaris*

There was only a single difference in AVT-ir somata number or size within this shoaling polygamous species (Table 4). Females in the nonspawning season had more gPOA cells compared to the spawning season (Fig. 4 and Table 4). There were also several relationships between AVT-ir somata and body size in the shoaling butterflyfish. Female nonspawning fish had a positive relationship between gPOA cell size and log BW (slope = 770; $r^2$ = 0.70; P = 0.038). Male nonspawning fish had positive relationships between pPOA cell size and log SL (slope = 137; $r^2$ = 0.70; P = 0.037) and log BW (slope = 45; $r^2$ = 0.67; P = 0.046). There were no differences in the number of varicosities in the torus semicircularis or tectum across either sex or season (Table 4).

## Discussion

We have demonstrated interspecific differences in the size of AVT-ir cells and their fibre projections among two closely-related butterflyfishes with distinctly different social behaviours. Interspecies comparisons show that regardless of sex or season, the territorial monogamous *C. multicinctus* has larger AVT-ir somata and higher fibre varicosity densities than the shoaling polygamous *C. miliaris*. Within species, there were neither sex nor seasonal differences in the territorial species and only limited variation in the shoaling species. This is the first study to show a difference in AVT neuronal features between two closely-related fish species that live within the same environment but have distinctly different social behaviours. The functional significance of these intra- and interspecies differences remains untested for this group, but is discussed below in terms of its potential relevance to the social behaviour, ecology and physiology of these two closely-related and sympatric fishes.

The observed difference in AVT-ir somata size between the monogamous and polygamous butterflyfishes may reflect the variation in their social behaviour. Both species examined are sympatric and exposed to the same environmental conditions such as salinity, temperature and light. Thus, the different features of their AVT systems may be associated with behavioural functions rather than osmoregulation or other physical environmental stressors, although this assumption remains to be confirmed. The larger AVT-ir somata in *C. multicinctus* may relate to its extreme aggressive behaviour, territoriality, mate association and/or monogamy. These social behaviours are in contrast to the polygamous *C. miliaris*, which forms large social shoals in the water column with relatively little aggression observed among individuals (18). In other fishes, AVT influences these social behaviours and may relate directly to a species' sociality, territoriality or possibly their social environment. Central AVT injection inhibits social approach behaviours in male goldfish and it was suggested that the level of endogenous AVT might modulate sociality in that species (28). AVT also alters aggressive behaviours in several fish species (29—33). The diversity of responses reported indicates that AVT actions may depend upon experimental behaviour paradigms, method of administration dose or delivery, social state of the animal or species-specific differences. Although we have not yet completed experiments on the action of exogenous AVT on butterflyfish aggressive behaviour, increased AVT gene expression is associated with aggressive territorial behaviour in the cichlid (*Neolamprologus pulcher*) and bluehead wrasse (8, 34). Although the larger AVT-ir somata of the territorial monogamous butterflyfish may reflect changes in peptide production, accumulation, or release that may be required for maintenance or modulation of their social behaviours, additional studies are needed to test these hypotheses in butterflyfishes.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The species level differences in AVT-ir cell size (Tables 3 and 4) were not maintained in all categories when species were compared by sex and season (Fig. 4). This statistical result is most likely due to the combined effects of low sample size/high individual variability and species-specific sex or seasonal fluctuations in behaviour or stress. Furthermore, despite a species level difference in pPOA cell size, no difference was detected when species were compared by sex and season. Parvocellular cell size is negatively correlated with stressful environmental and rearing situations in pupfish (35), and chronic stress decreases vasotocin gene transcript activity in the parvocellular region of the rainbow trout (36). Thus, the species level differences in pPOA cell size when compared by sex and season may be masked by the effects of post-capture stress, although this remains to be confirmed.

The results of our interspecific comparison of AVT-ir neurone phenotypes differs from those found for the only other species comparison in fishes. Subspecies of pupfish in different environments with diverse social behaviours also showed variation in size but not number of mPOA and pPOA AVT-ir cells, with smaller cells in the territorial species (13). The nonterritorial pupfish lives in a highly variable environment with large fluctuations in temperature and salinity. Therefore, larger cell sizes may also be due to differences in osmoregulatory demands or stress. This is supported by rearing experiments in the same subspecies of pupfish that demonstrate changes in cell size and number due to variations in salinity and temperature (35). Thus, the apparent discrepancy between the butterflyfish and pupfish systems may be due to differences in AVT-mediated physiological functions between the species, and indicates that comparative AVT studies focused solely on social behaviour in fishes may be best conducted under constant environmental conditions.

Arginine vasotocin-immunoreactive fibre densities differed between congeners in higher midbrain sensory processing regions such as the torus semicircularis (auditory and lateral line processing centre) and the tectum (visual processing and multimodal integration centre). Therefore, it is possible that AVT also modulates auditory, lateral line and/or visual sensory information. AVT was shown to influence both visual processing and vocal-acoustic behaviours in several vertebrate taxa (3, 37). Furthermore, the number of fibres present in hindbrain sensory processing regions, such as the afferent region of the vagal lobe, medial octavolateral nucleus (lateral line) and descending octaval nucleus (auditory), were approximately equivalent between the two species. Thus, any species differences in AVT modulation of sensory systems might occur in higher brain regions. However, it should be noted that the presence of varicose

**Table 2.** Sheared Principle Component Analysis for Arginine Vasotocin Cell Size and Number in the Territorial *Chaetodon multicinctus* and Shoaling *Chaetodon miliaris* Butterflyfishes.

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| Fish length | −0.123 | 0.040 | −0.073 |
| gPOA # | −0.078 | 0.075 | −0.228 |
| mPOA # | 0.109 | 0.017 | 0.026 |
| pPOA # | 0.082 | 0.032 | **0.343** |
| gPOA size | **0.475** | **−0.146** | **0.370** |
| mPOA size | **0.676** | **−0.209** | **0.300** |
| pPOA size | **0.528** | **−0.152** | **−0.808** |
| Percent variation explained | 47.7% | 26.8% | 9.6% |

Principle component (PC) 1 indicates factors that explain variations associated with size. PC2 and PC3 represent factors that explain variation among species of body size-corrected cell measurements. Eigenvectors with the strongest loadings are in bold. Note that PC2 had heavy loading for cell size after corrections for measurements associated with variate size. gPOA, gigantocellular; mPOA, magnocellular; pPOA, parvocellular cell groups of preoptic area.

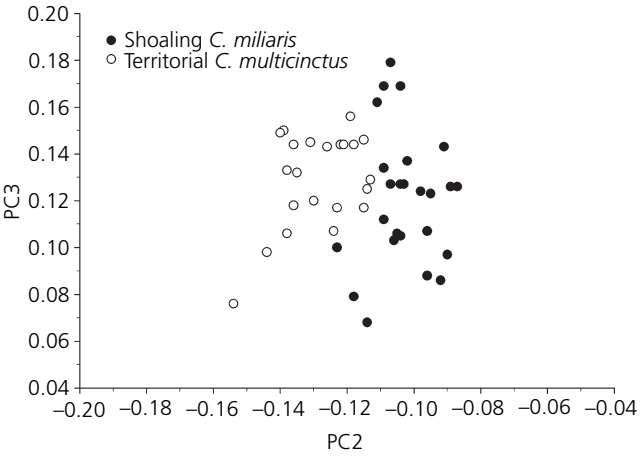


**Fig. 3.** Bivariate scatter plot from results of the sheared principle component analysis of arginine vasotocin (AVT)-immunoreactive (-ir) cell size and number in the shoaling *Chaetodon miliaris* (closed circles) and territorial *Chaetodon multicinctus* (open circles) butterflyfishes. Note that principle component (PC) 2 shows best separation of the two species. This axis was heavily loaded by AVT cell size after correction for inherent body size variation among species.

**Fig. 2.** Distribution of arginine vasotocin (AVT)-immunoreactive (-ir) neurones in the territorial (*Chaetodon multicinctus)* and shoaling (*Chaetodon miliaris)* butterflyfish brains. Left and right sides show the territorial and shoaling species, respectively. Camera lucida drawings of transverse sections through the brain show the location of AVT-ir somata (dots) and fibres (lines). The inset shows a schematic sagittal brain with the approximate location of each cross section indicated for each species. The territorial species has a higher density of fibres in most brain regions except the preoptico-hypophyseal tract and caudal brain. Differences in brain shape are due to variations in angle of section between the two brains. Scale bars = 1 mm. AP, area postrema; CC, cerebellar crest; CE, cerebellum; Dd, dorsal zone of the area dorsal telencephalon; Dm, medial zone of area dorsal telencephalon; G, glomerular nucleus; Ha, habenula; iaf, internal arcuate fibres; IL, inferior lobe of hypothalamus; MON, medial octavolateral nucleus; NLV, nucleus lateralis valvulae; OB, olfactory bulb; ON, optic nerve; gPOA, gigantocellular region of preoptic area; mPOA, magnocellular region of preoptic area; pPOA, parvocellular region of preoptic area; Rm, nucleus reticularis medius; Rs, nucleus reticularis superior; SC, spinal cord; SV, saccus vasculosus; T, tectum; TEG, tegmentum; TL, torus longitudinalis; TS, torus semicircularis; VCe, valvula cerebelli; Vv, ventral nucleus of ventral telencephalon.

**Table 3.** ANOVA of Arginine Vasotocin-Immunoreactive Cell Number and Size in the Forebrain Preoptic Area and Fibre Numbers in the Torus Semicircularis and Tectum of the Territorial Monogamous *Chaetodon multicinctus* and the Shoaling Polygamous *Chaetodon miliaris* Butterflyfish.

| | Cell number | | | | | | Cell size | | | | | | Fibres | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | gPOA | | mPOA | | pPOA | | gPOA | | mPOA | | pPOA | | TS | | T | |
| Source | F | P-value | F | P-value | F | P-value | F | P-value | F | P-value | F | P-value | F | P-value | F | P-value |
| Sex | 0.08 | 0.778 | 0.04 | 0.841 | 0.00 | 0.964 | 1.44 | 0.237 | 1.20 | 0.281 | 0.69 | 0.410 | 0.22 | 0.644 | 0.67 | 0.420 |
| Season | 10.05 | **0.003** | 8.37 | **0.006** | 4.40 | **0.042** | 0.48 | 0.492 | 0.28 | 0.598 | 0.21 | 0.652 | 0.23 | 0.636 | 0.00 | 0.948 |
| Species | 1.41 | 0.242 | 0.70 | 0.408 | 0.11 | 0.737 | 54.56 | **0.001** | 82.86 | **0.001** | 17.05 | **0.001** | 412.30 | **0.001** | 89.07 | **0.001** |
| Sex × Season | 0.34 | 0.560 | 0.62 | 0.435 | 0.27 | 0.606 | 0.32 | 0.575 | 0.00 | 0.950 | 0.09 | 0.767 | 0.01 | 0.919 | 0.07 | 0.798 |
| Sex × Species | 1.03 | 0.316 | 1.10 | 0.302 | 0.08 | 0.776 | 0.13 | 0.716 | 1.32 | 0.258 | 2.17 | 0.149 | 0.16 | 0.688 | 0.18 | 0.673 |
| Season × Species | 10.47 | **0.002** | 0.04 | 0.852 | 0.00 | 0.987 | 2.45 | 0.125 | 6.87 | **0.012** | 0.83 | 0.367 | 0.16 | 0.690 | 1.32 | 0.259 |
| Sex × Season × Species | 3.70 | 0.062 | 0.22 | 0.641 | 0.62 | 0.437 | 0.07 | 0.789 | 0.27 | 0.609 | 2.58 | 0.116 | 0.01 | 0.910 | 0.00 | 0.962 |

The seasonal factor explained differences in cell number while the species factor explained differences in cell size and fibres. Data used in this analysis are shown in Figs 4 and 6. gPOA cell size and number and mPOA cell number were log transformed prior to analysis. gPOA, gigantocellular; mPOA, magnocellular; pPOA, parvocellular cell groups of preoptic area; TS, torus semicircularis; T, tectum. F-statistic and P-values are from three-way ANOVA. Bold values indicate $P < 0.05$.

**Table 4.** Statistical Comparisons of Arginine Vasotocin-Immunoreactive Cell Number and Size in the Forebrain Preoptic Area and Fibres in the Torus Semicircularis and Tectum of the Territorial Monogamous *Chaetodon multicinctus* and the Shoaling Polygamous *Chaetodon miliaris* Butterflyfish.

| | Cell number | | | Cell size | | | Fibres | |
|---|---|---|---|---|---|---|---|---|
| | gPOA | mPOA | pPOA | gPOA | mPOA | pPOA | TS | T |
| *Species* | 0.242 | 0.408 | 0.738 | **< 0.001** | **< 0.001** | **< 0.001** | **< 0.001** | **< 0.001** |
| Female spawn | 0.766 | 0.999 | 0.999 | **0.033** | 0.221 | 0.999 | **< 0.001** | **0.003** |
| Male spawn | 0.999 | 0.991 | 0.999 | 0.186 | **0.005** | 0.055 | **< 0.001** | **< 0.001** |
| Female nonspawn | **0.012** | 0.999 | 0.999 | **0.001** | **< 0.001** | 0.187 | **< 0.001** | **0.003** |
| Male nonspawn | 0.997 | 0.973 | 0.998 | **0.003** | **< 0.001** | 0.294 | **< 0.001** | **< 0.001** |
| *Chaetodon multicinctus* | | | | | | | | |
| Male versus female spawn | 0.999 | 0.999 | 0.961 | 0.999 | 0.873 | 0.977 | 0.999 | 0.996 |
| Male versus female nonspawn | 0.926 | 0.977 | 0.996 | 0.999 | 0.988 | 0.998 | 0.999 | 0.999 |
| Spawn versus nonspawn males | 0.999 | 0.616 | 0.777 | 0.999 | 0.897 | 0.999 | 0.999 | 0.999 |
| Spawn versus nonspawn females | 0.996 | 0.995 | 0.999 | 0.999 | 0.618 | 0.723 | 0.999 | 0.999 |
| *Chaetodon miliaris* | | | | | | | | |
| Male versus female spawn | 0.980 | 0.990 | 0.999 | 0.911 | 0.999 | 0.572 | 0.999 | 0.999 |
| Male versus female nonspawn | 0.713 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.995 |
| Spawn versus nonspawn males | 0.501 | 0.664 | 0.979 | 0.775 | 0.994 | 0.999 | 0.999 | 0.999 |
| Spawn versus nonspawn females | **0.001** | 0.821 | 0.907 | 0.995 | 0.857 | 0.982 | 0.999 | 0.994 |

The territorial species has larger cells in most categories of the three arginine vasotocin-immunoreactive cell groups and more fibres. Data used in this analysis are shown in Figs 4 and 6. gPOA cell size and number and mPOA cell number were log transformed prior to analysis. gPOA, gigantocellular; mPOA, magnocellular; pPOA, parvocellular cell groups of preoptic area; TS, torus semicircularis; T, tectum. P-values are from three-way ANOVA and post-hoc Tukey's tests. Comparisons were tested both for the species combined and separated by sex and season as well as sex and seasonal differences within a particular species. Bold values indicate $P < 0.05$.

axons alone does not directly translate into differential peptide activity, and similarities in fibre densities do not preclude species differences in AVT modulation. Intraspecific communication involves acoustic and visual displays in both of our study species, and acoustic communication may be necessary to maintain pair bonds in the territorial monogamous species (17). The increased density of AVT-ir fibres within the torus semicircularis and tectum of the territorial butterflyfish may reflect an enhanced neuromodulatory function of acoustic and visual processing compared to the shoaling species. However, future studies should quantify AVT-ir fibres and receptors in multiple butterflyfish species, and test for physiological and behavioural sensory neuromodulation in these species.

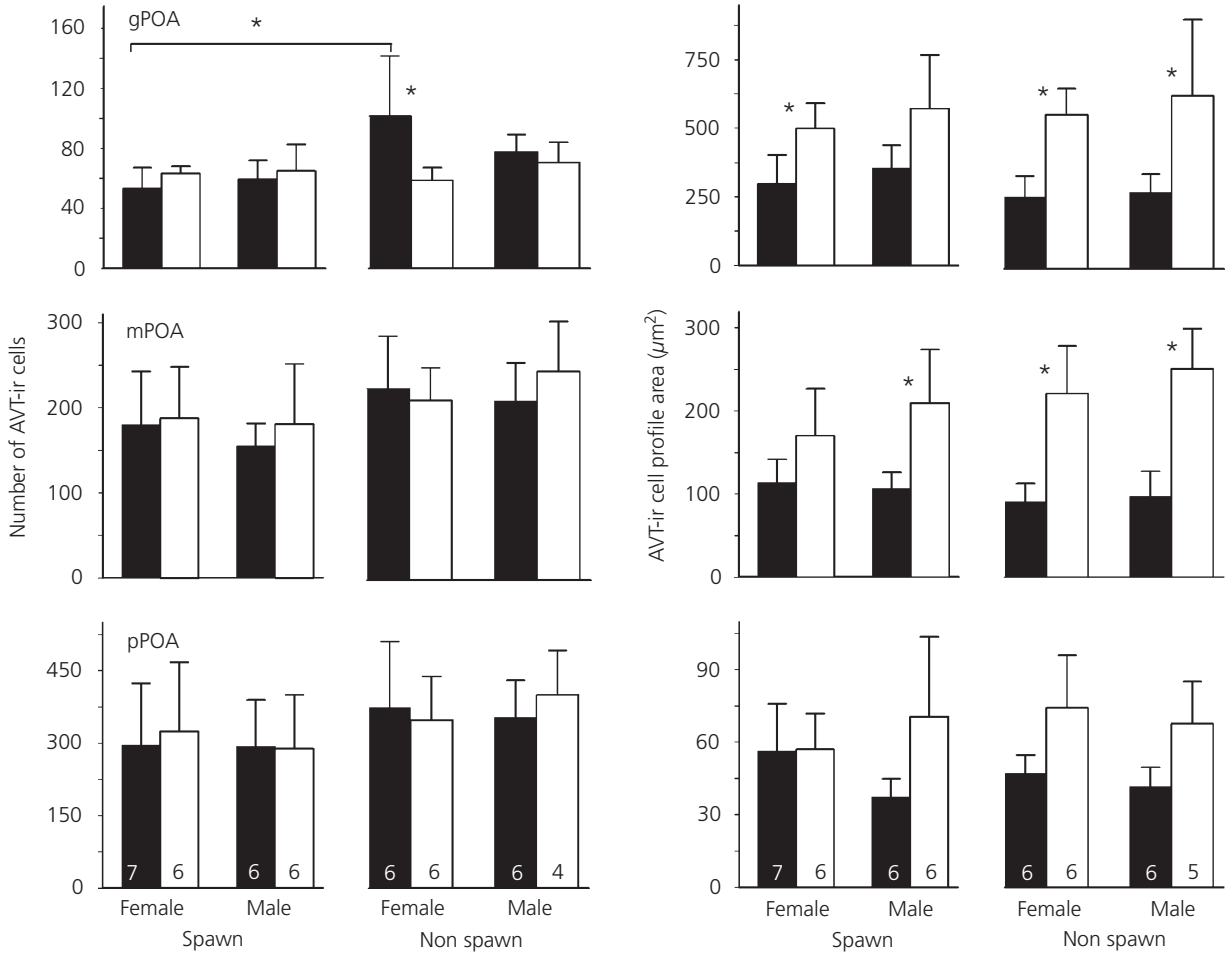


**Fig. 4.** Sex and seasonal differences in somata number and size of arginine vasotocin (AVT)-immunoreactive (-ir) cell groups in the monogamous territorial *Chaetodon multicinctus*, and shoaling polygamous *Chaetodon miliaris* butterflyfishes. The territorial species (open bars) had larger gigantocellular (gPOA), magnocellular (mPOA) cells compared to the shoaling species (closed bars) with the exception of gPOA cell group in spawning males and the mPOA cell group in spawning females. However, the territorial species had fewer gPOA cells in spawning females compared to the shoaling species. No differences in the number or size of AVT-ir cell groups in the territorial species were detected. Within the shoaling species, nonspawning females had more gPOA cells compared to spawning females. Bars show the original mean ± SD. Numbers indicate total number of animals analysed in each group. *Species, sex, or seasonal differences (three-way ANOVA and Tukey's test, $P < 0.05$). gPOA cell size and number and mPOA cell number were log transformed prior to analysis.

The present study found neither sex nor seasonal variations in any AVT-ir cell group in the territorial monogamous butterflyfish. This is similar to goldfish in which no sex differences or influence of sex steroids were found in AVT-ir neurones (38). However, these results differ from several other studies that demonstrate AVT-ir cell phenotype differences across social strategies or sexes (3, 5, 6). The behaviour of adult territorial butterflyfish is similar for both males and females, and remains relatively unchanged as the monogamous pair aggressively defends a permanent feeding territory throughout the year (18). Furthermore, unlike most species examined to date, the territorial butterflyfish does not exhibit any known alternative reproductive strategies. Thus, the lack of sex or seasonal variations in AVT-ir neurones in the monogamous species may reflect this species' temporally constant social behaviour. However, the absence of sex and seasonal differences in its AVT system at the cellular level does not preclude the presence of variations in postsynaptic mechanisms, AVT mRNA production, or AVT receptor expression and distribution.

The shoaling polygamous *C. miliaris* displayed only limited differences in AVT-ir neurones among sex or season. The limited number of differences may also be explained by their consistent shoaling behaviour throughout the year (19). Females had more gPOA AVT-ir somata in the nonspawning season compared to the spawning season. Similarly, a goby species showed greater numbers and larger sizes of gPOA AVT-ir somata during the nonspawn period compared to pre- and post-spawn times (12). The pre- and post-spawn periods were not tested in the butterflyfish and the presence of additional intraspecies differences may not have been detected. Gigantocellular preoptic AVT-ir cell number and size are also correlated with aggressive and dominant behaviours in zebrafish and bluehead wrasse (11, 39), respectively. Therefore, differences in the number of AVT-ir gPOA cells in the shoaling butterflyfish could reflect a change in behaviour or physiology related to spawning. The abundance of AVT/AVP in the brain can also be regulated by sex steroids and glucocorticoids and therefore the above differences

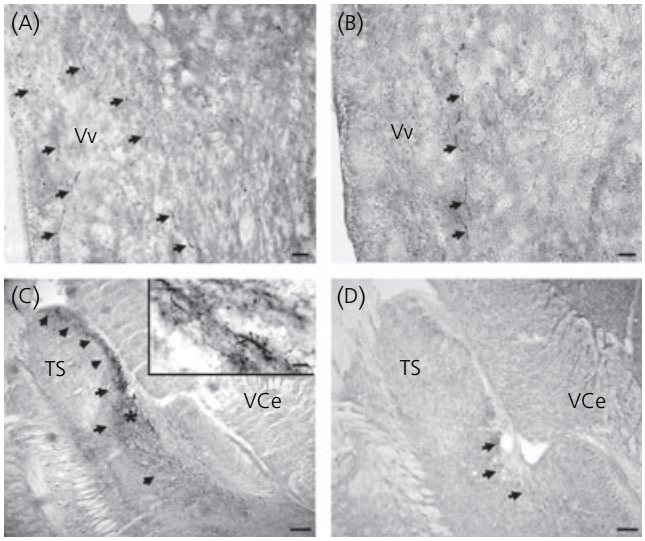


**Fig. 5.** Arginine vasotocin (AVT)-immunoreactive (-ir) axons in the forebrain ventral nucleus of the ventral telencephalon and midbrain torus semicircularis of the territorial *Chaetodon multicinctus* and shoaling *Chaetodon miliaris* butterflyfishes. Transverse sections through the ventral nucleus of the ventral telencephalon (Vv) and torus semicircularis (TS) show abundant AVT-ir varicose fibres (arrows) in the territorial species (A, C), but comparatively few fibres (arrows) in the shoaling species (B, D) within comparable brain regions. The inset in (C) shows higher magnification of AVT-ir fibres in the TS from the region indicated by the asterisk. VCe, valvula cerebelli. Scale bars = 20 $\mu$m (A, B), 50 $\mu$m (C, D), 10 $\mu$m (inset).

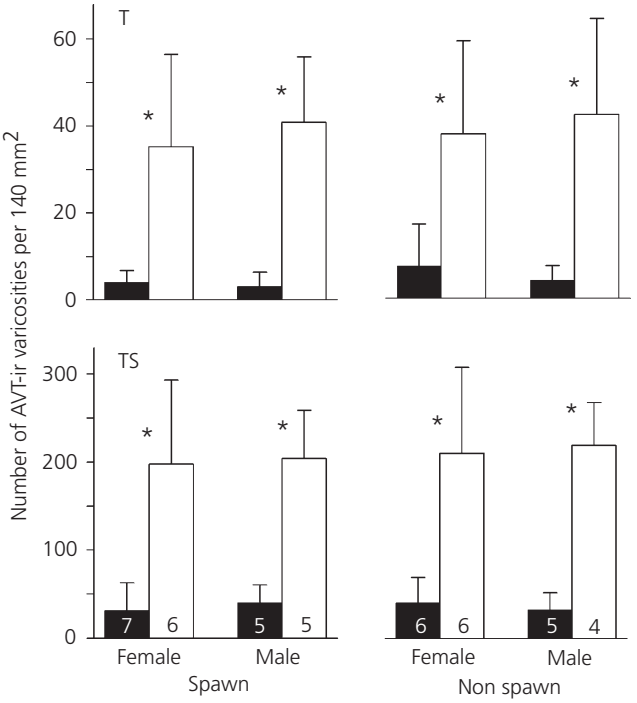


**Fig. 6.** Sex, season and species differences in the number of arginine vasotocin (AVT) fibre varicosities in the torus semicircularis and tectum of the territorial monogamous *Chaetodon multicinctus* and the shoaling polygamous *Chaetodon miliaris* butterflyfishes. The territorial species (open bars) has a greater density of AVT-immunoreactive (-ir) varicosities in both the torus semicircularis (TS) and tectum (T) compared to the shoaling species (closed bars), but there were no sex or season differences within each species. Bars show the mean ± SD. Numbers indicate total number of animals analysed in each group. *Species differences (three-way ANOVA and Tukey's test, $P < 0.05$).

may be solely influenced by seasonal differences in hormones (3, 40, 41). Further experiments are needed to determine the function and regulation of each AVT-ir cell group in fishes.

Nonspawning fishes regardless of sex or species had higher numbers of AVT-ir cells across all three cell groups compared to spawning individuals. By contrast, the medaka (*Oryzias latipes*) showed a decrease in the number of AVT-ir cells after spawning, but differences were likely concentrated in the pPOA and changed on a much shorter temporal scale (42). The seasonal increase in the number of AVT-ir cells in the butterflyfishes may be due to seasonal fluctuations in reproductive hormones, behaviour, or physiology. Further behavioural and physiological studies are needed to determine the exact function of this seasonal enhancement in the number of AVT-ir cells.

A function for the species level differences in the butterflyfish AVT-ir system is untested, but correlations between the number of AVP/AVT-ir cells in the bed nucleus of stria terminalis and exclusive home ranges in rodents and birds (3, 43) may indicate an analogous role for AVT projections in butterflyfishes. Butterflyfishes do not have AVT-ir somata in these brain regions, but show AVT-ir fibres projections from the POA to regions of the ventral telencephalon, which may be homologous to the bed nucleus of the stria terminalis of tetrapods (44). The more dense AVT-ir fibre projections in the territorial monogamous butterflyfish is similar to a study in mice in which an aggressive monogamous species had a greater density of fibres compared to a promiscuous species (45). However, this neural correlation was not well supported in voles, which had only minor and inconsistent differences in AVP-ir fibre densities between monogamous and polygamous species (46). Studies indicate that in mammals the AVP system is primarily modified by receptor distribution rather than peptide projection pathways, especially in relation to mating strategy. Thus, analyses of AVT-ir neuronal phenotypes, projections to sub-regions of the ventral telencephalon, and expression of AVT receptors in additional species with similar and divergent social behaviours are needed to provide further comparative support for a causal relationship between the AVT system and social behaviours in butterflyfishes.

In summary, the present study is the first to demonstrate differences in AVT-ir cell size and fibre densities between two teleost congeners with different social systems that inhabit the same environment. The larger AVT-ir cells and denser AVT-ir fibre projections of the territorial monogamous species may be related to their social spacing, aggression, or other behaviours associated with their monogamous mating system. Future integrative and comparative studies are needed to confirm behavioural consequences of these species level differences in the AVT system and determine the extent of similarities to other vertebrate models of AVP/AVT influenced social and reproductive behaviours.

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