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Arginine vasotocin neuronal phenotypes, telencephalic fiber varicosities, and social behavior in butterflyfishes (Chaetodontidae): Potential similarities to birds and mammals

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ABSTRACT

The neuropeptide arginine vasopressin (AVP) influences many social behaviors through its action in the forebrain of mammals. However, the function of the homologous arginine vasotocin (AVT) in the forebrain of fishes, specifically the telencephalon remains unresolved. We tested whether the density of AVT-immunoreactive (-ir) fiber varicosities, somata size or number of AVT-ir neuronal phenotypes within the forebrain were predictive of social behavior in reproductive males of seven species of butterflyfishes (family Chaetodontidae) in four phylogenetic clades. Similar to other fishes, the aggressive (often territorial) species in most cases had larger AVT-ir cells within the gigantocellular preoptic cell group. Linear discriminant function analyses demonstrated that the density of AVT-ir varicosities within homologous telencephalic nuclei to those important for social behavior in mammals and birds were predictive of aggressive behavior, social affiliations, and mating system. Of note, the density of AVT-ir varicosities within the ventral nucleus of the ventral telencephalon, thought to be homologous to the septum of other vertebrates, was the strongest predictor of aggressive behavior, social affiliation, and mating system. These results are consistent with the postulate that AVT within the telencephalon of fishes plays an important role in social behavior and may function in a similar manner to that of AVT / AVP in birds and mammals despite having cell populations solely within the preoptic area.

Arginine vasopressin (AVP) and its non-mammalian homolog arginine vasotocin (AVT) are neuropeptides that modulate vertebrate social behavior (Goodson and Bass, 2001). The relationship between the organization of the AVP system and social behavior in microtine rodents provided functional information for this neuropeptide and a foundation for research on affiliative behavior and social disorders (Hammock and Young, 2006). In non-mammalian AVT systems, comparisons of closely related species are limited to birds (e.g. Goodson et al., 2006) and virtually unstudied in the highly speciose and behaviorally diverse fishes (Dewan et al., 2008; Lema and Nevitt, 2004a). Thus, comparative studies in closely related species are needed to test whether the neuroanatomical organization and density of the AVT system are correlated and potentially influence fish social behavior. This is also necessary in order to ultimately determine whether these relationships in fishes are similar to those in other vertebrates.

The neural organization and function of the mammalian AVP system are well studied and provide a basis for predictions of AVT function in other vertebrates. Comparative studies in mammals show correlations with the density of AVP production, release sites or receptor properties and different social behaviors. Although sometimes based on relatively few species, these studies indicate that the density of AVP features within the bed nucleus of the stria terminalis or lateral septum were related to parental behavior (Bamshad et al., 1994; Bester-Meredith and Marler, 2003), aggression (Bester-Meredith et al., 1999), affiliation (Ho et al., 2010), and mating system (Insel et al., 1991). Additional studies provided for support for these hypotheses with either injection manipulations (parental behavior: Parker and Lee, 2001; Wang et al., 1994; and aggression, Ferris, 2005), or measures of AVP production (parental behavior: Wang et al., 2000 and aggression: Veenema et al.,

Abbreviations: BA, basal amygdala; BNST, bed nucleus of stria terminalis; Dc2, central part of the dorsal telencephalon, subdivision 2; Dd, dorsal part of the dorsal telencephalon; Dld, dorsal division of lateral part of the dorsal telencephalon; Dlv, ventral division of the lateral part of the dorsal telencephalon; Dm1, medial part of the dorsal telencephalon, subdivision 1; Dm2, medial part of the dorsal telencephalon, subdivision 2; Dp, posterior part of the dorsal telencephalon; gPOA, gigantocellular group of preoptic area; mPOA, magnocellular group of preoptic area; pPOA, parvocellular group of preoptic area; Vc, central nucleus of the ventral telencephalon; Vd, dorsal nucleus of the ventral telencephalon; Vi, intermediate nucleus of the ventral telencephalon; Vl, lateral nucleus of the ventral telencephalon; Vp, postcommissural nucleus of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; Vu, cuneate nucleus of the ventral telencephalon; Vv, ventral nucleus of the ventral telencephalon.

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Table 1
Proposed homologies for teleost brain regions.

Butterflyfish brain region	Presumed mammalian homolog
Dc	Dorsal pallium[6] (Neocortex)
Dd	Dorsal pallium[1] (Neocortex)
Dld	Dorsal pallium[1,6] (Neocortex)
	Hippocampus[2] (Archicortex)
	Medial pallium[4] (Archicortex)
Dlv	Medial pallium[1,3,4,6] (Archicortex)
	Hippocampus[2](Archicortex)
Dm	Dorsal pallium[6] (Neocortex)
	Pallial amygdala[1,2,3,4] (Paleocortex)
Dp	Lateral pallium[3] (Paleocortex)
Vc	Striatum[4,5]
Vd	Striatum[4,5,7,8]
Vi	Unknown
Vl	Septum[4,5,7]
	Olfactory tubercle[7]
Vp	Basal amygdala[7]
Vs	Basal amygdala[7], bed nucleus of stria terminalis[7]
Vu	Unknown
Vv	Septum[4,5], lateral septum[7]

1—Rodriguez et al., 2002; 2—Portavella et al., 2002; 3—Wullimann and Rink, 2002; 4—Northcutt, 2006; 5—Wullimann and Mueller, 2004; 6—Yamamoto et al., 2007; 7—Northcutt, 1995; 8—Braford, 1995.

2010). Alternatively, larger data sets with multiple species can also provide further information regarding these hypotheses (Turner et al., 2010). Thus if function is conserved, non-mammalian vertebrate social behavior should also covary with AVT features in the lateral septum and bed nucleus of the stria terminalis. In fact, intraseptal injections of AVT modulate aggressive behavior in birds (Goodson, 1998) while a comparison of five finch species yielded a relationship between the density of V1a binding sites in the septum and social group sizes (Goodson et al., 2006). In addition, AVT neurons within the bed nucleus of the stria terminalis of multiple species of finches increased their Fos expression upon exposure to a positive social stimuli (Goodson et al., 2009a). While AVT within the lateral septum and bed nucleus of the stria terminalis of birds may have some conserved function, no study to date has attempted to address whether AVT within homologous regions such as the ventral, supracommissural or lateral nuclei of the ventral telencephalon (Table 1) of fishes may also function in aggression, sociality or other relevant social behavior.

Butterflyfishes (family Chaetodontidae) are a good system to examine these relationships among wild populations. Species within this family exhibit diverse social behaviors (Hourigan, 1989) that can be mapped on a robust morphological and molecular phylogeny (Fessler and Westneat, 2007; Smith et al. 2003). In addition, descriptions of the aggressive behaviors, social affiliations, and mating systems, as well as the timing of their protracted spawning season, are well characterized and temporally stable (Hourigan, 1989; Ludwig, 1984; Ralston, 1981; Tricas and Hiramoto, 1989; Walsh 1987). The AVT system of butterflyfishes (and perciform fishes in general) consists of three main cells groups within the preoptic area (gigantocellular (gPOA), magnocellular (mPOA), or parvocellular (pPOA) preoptic groups) and one small cell group within the ventral hypothalamus (Dewan et al., 2008). The innervation pattern of each of these four cell groups has not been analyzed in butterflyfishes and is unclear in fishes in general; however, intracellular label of single mPOA AVT cells determined that this cell group projects towards the telencephalon, thalamus, and pituitary (Saito et al., 2004). While AVT-ir cells within gPOA and some within mPOA appear to project to the thalamus, periventricular pretectal nuclei, and optic tectum (Holmqvist and Ekström, 1995). In addition, rostral AVT cells presumably from the gPOA cell group have extensive connections with the telencephalon (Holmqvist and Ekström, 1995). The pPOA AVT cell group of butterflyfishes has only a single projection that appears to join the preoptico-hypophyseal tract towards the pituitary but this has not been analyzed in detail (Dewan et al., 2008). The gPOA and mPOA of fishes are thought to be homologous to the supraoptic nucleus while the pPOA is thought to be homologous to the paraventricular nucleus of mammals (Moore and Lowry, 1998). Recent work shows distinct differences in the size of the AVT-ir gPOA and mPOA groups and qualitative differences in telencephalic AVT-ir fiber projections among the paired, aggressive, monogamous multiband butterflyfish (*Chaetodon multicinctus)*, and a non-aggressive, shoaling, polygamous milletseed butterflyfish (*Chaetodon miliaris)* (Dewan et al., 2008). While there is a clear association with AVT and social behavior in these two closely related species, further work is needed to test the broader prediction that this correlation applies to a range of species. In addition, the site-specific role of AVT in the fish telencephalon remains untested as well as the potential for functional similarities with the AVP system of mammals.

This study investigates the AVT system in reproductive males of seven Hawaiian butterflyfishes (Table 2): three monogamous paired aggressive species; multiband butterflyfish (*Chaetodon multicinctus*), oval butterflyfish (*Chaetodon lunulatus*), threadfin butterflyfish (*Chaetodon auriga*) in different clades, two polygamous shoaling non-aggressive species; milletseed butterflyfish (*Chaetodon miliaris*) and pennant butterflyfish (*Heniochus diphreutes*) in different clades, one aggressive haremic species; forceps fish (*Forcipiger flavissimus*), and one monogamous species; teardrop butterflyfish (*Chaetodon unimaculatus*) that occurs as solitary individuals, pairs or large groups and whose extent of aggression is not yet reported (Fessler and Westneat, 2007; Hourigan, 1989; Roberts and Ormond, 1992). Females were not used in this study for several reasons. First, species differences in social behavior are likely more robust in males, as monogamous males are more aggressive than females and defend territories that contain mates rather than food resources (Hourigan 1989; Tricas, 1989). Second, females of at least two of the species analyzed in the current study exhibit vast differences in gonad condition during the spawning season (Ralston, 1981; Tricas and Hiramoto, 1989) whereas all males used for this study had mature sperm and enlarged testes. Third, AVT research in male butterflyfishes provides a better comparison to mammalian AVP research, most frequently performed in male rodents. The results show strong correlative evidence for the involvement of AVT in the regulation of fish social behavior and preliminary evidence for a functional role potentially similar to that of AVT in birds and AVP in mammals.

Table 2
Taxonomic and behavioral comparisons of seven species of butterflyfish.

Species	BW (g)	Clade	Aggressive	Social grouping	Mating system
Pennant butterflyfish (*Heniochus diphreutes*)	41.8 ± 28.6	B	Non-aggressive[2]	Shoal[1]	Polygamous[5]
Forcepsfish (*Forcipiger flavissimus*)	54.5 ± 19.1	B	Aggressive[3]	Trio[3]	Haremic[3]
Pebbled butterflyfish (*Chaetodon multicinctus*)	25.2 ± 3.7	C2	Aggressive[2]	Pair[1]	Monogamous[1]
Milletseed butterflyfish (*Chaetodon miliaris*)	56.6 ± 10.9	C2	Non-aggressive[2]	Shoal[1]	Polygamous[1]
Teardrop butterflyfish (*Chaetodon unimaculatus*)	67.3 ± 14.2	C2	Unknown	Mixed[1]	Monogamous[6]
Oval butterflyfish (*Chaetodon lunulatus*)	54.3 ± 10.6	C3*	Aggressive[4]	Pair[4]	Monogamous[4]
Threadfin butterflyfish (*Chaetodon auriga*)	69.7 ± 6.8	C4	Aggressive[2]	Pair[1]	Monogamous[5]

Phylogenetic clades are from Fessler and Westneat, 2007. * *C. lunulatus* was not analyzed in Fessler and Westneat but *C. trafascilis*, a closely related species was placed within this clade. 1—Hourigan, 1989; 2—Roberts and Ormond, 1992; 3—Boyle and Tricas in prep; 4—Yabuta, 1999; 5—Whiteman and Cote, 2004; 6—Sancho et al., 2000;

Methods

Animal and tissue preparation

This study sampled males of seven butterflyfish species during their natural spawning seasons. AVT features are influenced by spawning season in certain fish species (Maruska, 2009; Maruska et al., 2007; Ohya and Hayashi, 2006), thus, all collections were restricted to one reproductive period. Previous studies show that Hawaiian butterflyfishes have protracted and uninterrupted spawning periods that begin during winter months of relatively low temperatures, increase during late winter and peak in the spring (Ludwig, 1984; Tricas and Hiramoto, 1989; Walsh 1987) and we confirmed active spawning by direct examination of gonadal tissues from all sampled individuals. Adult males were collected across a 17 day period in 2009 from 25 March to 5 May (pennant butterflyfish: 27 March–25 April; oval butterflyfish: 3–14 April; forceps fish: 3 April–5 May; threadfin butterflyfish: 4–14 April; teardrop butterflyfish: 5 April–5 May; pebbled butterflyfish: 17–22 April; milletseed butterflyfish: 17–30 April) with barrier and hand nets from the west and north shore of Oahu in the afternoon or early evening. After capture, fish were transported to the lab, held in flow-through fresh seawater aquaria for approximately 12–16 hours under identical conditions and perfused the next morning. This four hour time period is because fish were captured haphazardly but processed in a random order. Although stressors associated with capture and holding could influence the AVT system of these fishes, species did not differ in the order they were collected (one-way ANOVA, $p>0.05$) and each individual was exposed to roughly identical capture and holding stressors. In addition, previous work shows that AVT neuronal features do not differ between immediate and 12–16 hours post capture in the pebbled butterflyfish (Dewan et al., 2008). Thus, this study makes two reasonable assumptions in potential non-behavioral influences on AVT circuitry. The first is that the fish collected for each species experienced typical prior social interactions in their wild populations. All fish collected for this study were collected haphazardly from the wild population with no information on prior social encounters. The second assumption is that all individuals of all species have equivalent physiological responses to these stressors.

The reproductive status of individuals was confirmed by the presence of live sperm in the testes of males. Females of each species were also collected at the same time from the same locations to verify reproductive season in the local population. Adult pebbled butterflyfish with a standard length (SL)>65 mm and milletseed butterflyfish with a SL>90 mm were chosen based on previous work on sexual maturity of these populations (Ralston, 1981; Tricas and Hiramoto, 1989). The sexual maturity of all seven species was verified by gonadal analysis (see below). Fish collections occurred across a 2-month period of the spawning season in a couple of these species (Ralston, 1981; Tricas and Hiramoto, 1989). Despite this elongated period, the in-depth gonadal analysis (see below) of both sexes provides robust evidence that all males used in this study were in the same reproductive condition and were collected during their respective spawning season.

Fishes were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222), measured for SL, total length, and body weight (BW), and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains and gonads were removed, weighed, postfixed in 4% paraformaldehyde in 0.1 M PB at 4 °C for 12–24 hours, rinsed in 0.1 M PB, and cryoprotected overnight in 30% sucrose in 0.1 M PB prior to sectioning. Gonads of all species were embedded with Histoprep mounting media (Fisher Scientific) and sectioned at 20 μm with a cryostat. Sections were collected on chrom-alum-coated slides, dried at room temperature overnight, and stained with hematoxylin and eosin. Stained slides 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).

Immunohistochemistry

Cryoprotected brains were embedded in Histoprep mounting media (Fisher Scientific) and sectioned at 24 μm with a cryostat in the coronal plane. Brain sections were collected on alternate chrom-alum-coated slides, dried at room temperature overnight, and stored at 4 °C until reacted or stained. All specimens were sectioned and reacted within 1 month of collection. One series of alternate brain sections was stained with 0.5% cresyl violet, dehydrated in an ethanol series, cleared in toluene, and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific). These slides provided detailed neuroanatomical boundaries necessary for the quantification of telencephalic AVT-ir fibers. The other series of alternate brain sections was immunoreacted for the AVT peptide and associated controls according to Dewan et al. (2008). This antibody (generously donated by Matthew Grober, Georgia State University) was produced by Synpep from AVT coupled to keyhole limpet hemocyanin and was used in several other studies (Dewan et al., 2008; Maruska, 2009; Maruska et al., 2007). The additional controls of preabsorption with the 8 μM of the carrier protein or isotocin resulted in no change in staining intensity. Each reaction consisted of a random assortment of individuals from different species in order to reduce any potential bias from slight differences in immunohistochemistry staining intensities.

Quantification

Each specimen was analyzed for the number and size of AVT-ir cells as well as the density of AVT-ir fiber varicosities in each telencephalic nucleus. AVT-ir soma was assigned to the gPOA, mPOA, or pPOA preoptic group based on neuroanatomical position, neuronal morphology, and size (Braford and Northcutt, 1983). Cell numbers of each preoptic group were determined under magnification at 400× with aid of a camera lucida. Cell profile area was computed from digital images of somata at 400× with Sigma Scan Pro 5.0 (SPSS, Inc.). 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 and the same region as prior AVT studies (Dewan et al., 2008; Maruska, 2009; Maruska et al., 2007). The number of AVT-ir fiber varicosities was determined in each telencephalic nucleus. AVT-ir fiber varicosities are axonal swellings and were quantified because they are putative locations for neuropeptide release (Whim and Lloyd, 1994), neuromodulation (Zoeller and Moore, 1986), and sites of synaptic contact (Sesack et al., 1998). Neuroanatomical boundaries of immunoreacted sections were determined with alternative series stained with cresyl violet and detailed descriptions of each telencephalic nucleus (Dewan and Tricas, *in review*). Subsequently, each nucleus on every section was carefully scanned by eye at 400× magnification and the number of AVT-ir varicosities was counted. This method may underestimate the potential neuromodulatory effect of AVT within a region as synaptic contact can occur within the neuropil (Caruncho et al., 1990) and the number of AVT varicosities was only counted within the boundaries of a nucleus. This method was employed because only a very small percentage of varicosities were located outside the boundary of telencephalic region and these varicosities could not be accurately assigned to a specific region. Thus, this conservative method did not influence the results of the study with the possible exception of Vl. In this nucleus, a small AVT-ir fiber tract frequently ran adjacent to the dorsal boundary of the somata but was not enumerated because there is no current evidence that this fiber tract makes synaptic contact with the somata of Vl. As these species differed in body size (see below), the total number of AVT-ir varicosities in each nucleus was corrected for volume. A high resolution digital picture was taken of every section of the telencephalon for each individual. The volume of each nucleus was determined from the same immunoreacted sections that AVT-ir varicosities were counted from using Sigma Scan Pro 5.0 (SPSS, Inc.).

We only analyzed the volume of nuclei that had a minimum of twenty AVT-ir varicosities in at least one individual. The volume was summed through the addition of alternative sections and multiplied by the thickness of the section.

Statistical analyses

Species comparisons of body size were compared with a Kruskal–Wallis test with Dunn's test for multiple comparisons as these data could not be normalized by transformation. AVT-ir cell number and cell size were compared across species with a one-way ANOVA and Tukey's test after a log transform that controlled for the false discovery rate (FDR) (Benjamini and Hochberg, 1995). We analyzed all telencephalic nuclei that had at least 20 varicosities or about 2 fibers in at least one individual. Thus, our analysis was limited to the dorsal (Vd), central (Vc), lateral (Vl), ventral (Vv), postcommissural (Vp), supracommissural (Vs), intermediate (Vi), and cuneate (Vu) nuclei of the ventral telencephalon. In the dorsal telencephalon, our analysis included all subdivisions of the medial (Dm1, Dm2, Dm3, and Dm4), central (Dc1 and Dc2), dorsal (Dd), and posterior (Dp) parts of dorsal telencephalon as well as the third subdivision of the ventral part of the lateral region of the dorsal telencephalon (Dlv3) and nucleus taenia (NT). In total these analyses included 18 of the 24 telencephalic nuclei. AVT-ir varicosities within the entopeduncular nucleus, and the first and second subdivisions of the ventral part, the dorsal part, medial part, and posterior part of the lateral region of the dorsal telencephalon were not analyzed as they did not contain the minimum number of AVT-ir varicosities.

The relationship between the density of AVT-ir varicosities and social behavior was determined by a linear discriminant analysis (LDA), a multivariate classification procedure (Engelman, 2000). This statistical test determines whether the density of AVT-ir varicosities can discriminate group membership (e.g. aggressive vs. non-aggressive groups) in any of the 18 telencephalic nuclei. Both forward and backward stepwise LDA were used to determine if predictive relationships exist between the density of AVT-ir varicosities (within a subset of telencephalic nuclei) and species level classifications of aggressive behavior, mating systems, social affiliations, or phylogenetic clades. The stepwise LDA procedure added at each step (forward stepping) the variable (density of AVT-ir varicosities within a specific brain region for all individuals) that best classified group membership (determined by the F-to-remove) and removed at each step (backward stepping) the variable (density of AVT-ir varicosities within a different brain region for all individuals) that contributed the least to this classification. This process is repeated until the addition (forward stepping) or removal (backward stepping) of a variable reduces the power of this statistical procedure to correctly classify group membership. This process results in a linear combination of a subset of the 18 nuclei that correctly classifies group membership if the LDA is statistically significant ($p<0.05$). The percentage of individuals that can be correctly classified into each group are then determined. We also report the jackknife classification which provides a more conservative classification of group membership. It should be noted that the teardrop butterflyfish was not included in analysis of aggression as this social behavior was not confirmed for this species. This LDA multivariate classification procedure yields the variables (density of AVT-ir varicosities within specific regions) that may contribute to group membership but is limited as it does not determine whether these variables actually differ between groups. Thus, the density of AVT-ir varicosities in brain regions that contributed to group membership (determined by LDA) were subsequently compared with a one-way ANOVA and Tukey's test controlling for the FDR (Benjamini and Hochberg, 1995). All statistical analyses were performed in Minitab with the exception of the LDA which was performed in MYSTAT 12.0.

To verify our LDA, we also examined whether body size differences among species (see below) influenced our data and interpretations. Since brain and body size do not appear to scale isometrically in these species (Dewan unpublished), it is possible that some other factor that relates to body size may influence AVT features such as hormone production, gonad size, dominance, or stress levels. Thus, an additional statistical test was performed to ensure that the results obtained from the LDA were based on species and likely not additional size-related factors. Several of our data sets did not meet the parallel slope assumption necessary for an ANCOVA, the preferred method of body size correction (Packard and Boardman, 1999). Therefore, we used a sheared principal component analysis (PCA) to assess for variation that was independent of body size differences between species. AVT-ir cell size, number and the density of varicosities within four nuclei predictive of social behavior (LDA analysis) were log transformed and analyzed with the sheared PCA algorithm (Dewan et al., 2008; Rohlf and Bookstein, 1987). In this analysis, variation in body size is present in PC1 and is removed by a "shear" or rotation that is applied to subsequent principal components. Thus, variables that have either high positive or negative loading factors in PC2 and PC3 explain the variation in the data after the confounding factor of body size is removed.

All experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Hawaii.

Results

A total of 40 individuals were analyzed. We used six males each of threadfin butterflyfish (BW: 69.7 ± 6.8 g; SL: 122.0 ± 4.5 mm), teardrop butterflyfish (BW: 67.3 ± 14.2 g; SL: 115.2 ± 7.4 mm), milletseed butterflyfish (BW: 56.6 ± 10.9 g; SL: 111.7 ± 7.5 mm), forceps fish (BW: 54.5 ± 19.1 g; SL: 138.3 ± 12.6 mm), and pebbled butterflyfish (BW: 25.2 ± 3.7 g; SL: 86.3 ± 5.7 mm). We used five male oval butterflyfish (BW: 54.3 ± 10.6 g; SL: 110.0 ± 6.5 mm) and pennant butterflyfish (BW: 41.8 ± 28.6 g; SL: 92.0 ± 22.5 mm). There were species differences in both standard length (Kruskal–Wallis $p<0.001$) and body weight (Kruskal–Wallis $p=0.006$). Both threadfin and teardrop butterflyfishes were larger in body weight than the pebbled butterflyfish (Kruskal–Wallis and Dunn's test $p<0.05$). In addition, the forceps fish were larger in standard length compared to either pebbled or pennant butterflyfishes (Kruskal–Wallis and Dunn's test $p<0.05$). Despite these species differences in body weight and length, AVT-ir cell data could not be corrected for body size because the assumption of parallel slopes necessary for an ANCOVA, the preferred method of correction (Packard and Boardman, 1999) was not met. However, we tested for body size differences that could account for species differences in the sheared PCA analysis below.

AVT-ir neuronal phenotypes

AVT-ir cell size within the mPOA and pPOA did not differ across species ($p>0.05$, one-way ANOVA with FDR). However, AVT-ir gPOA cell size differed across species ($p<0.001$, one-way ANOVA with FDR) with an overall trend of monogamous aggressive species having larger AVT-ir cells compared to polygamous non-aggressive species (Fig. 1). Oval butterflyfish had larger AVT-ir gpOA cells compared to all other species ($p < 0.001$, one-way ANOVA and Tukey's test). Teardrop butterflyfish had larger AVT-ir gPOA cells compared to pennant butterflyfish ($p<0.001$, one-way ANOVA and Tukey's test), milletseed butterflyfish ($p<0.001$, one-way ANOVA and Tukey's test), threadfin butterflyfish ($p=0.006$, one-way ANOVA and Tukey's test), and forceps fish ($p=0.008$, one-way ANOVA and Tukey's test). Lastly, pebbled butterflyfish had larger AVT-ir gPOA cells compared to milletseed butterflyfish ($p=0.030$, one-way ANOVA and Tukey's test) and pennant butterflyfish ($p=0.008$, one-way ANOVA and Tukey's test). This analysis provides some evidence that monogamous aggressive species have larger AVT-ir gPOA cells.

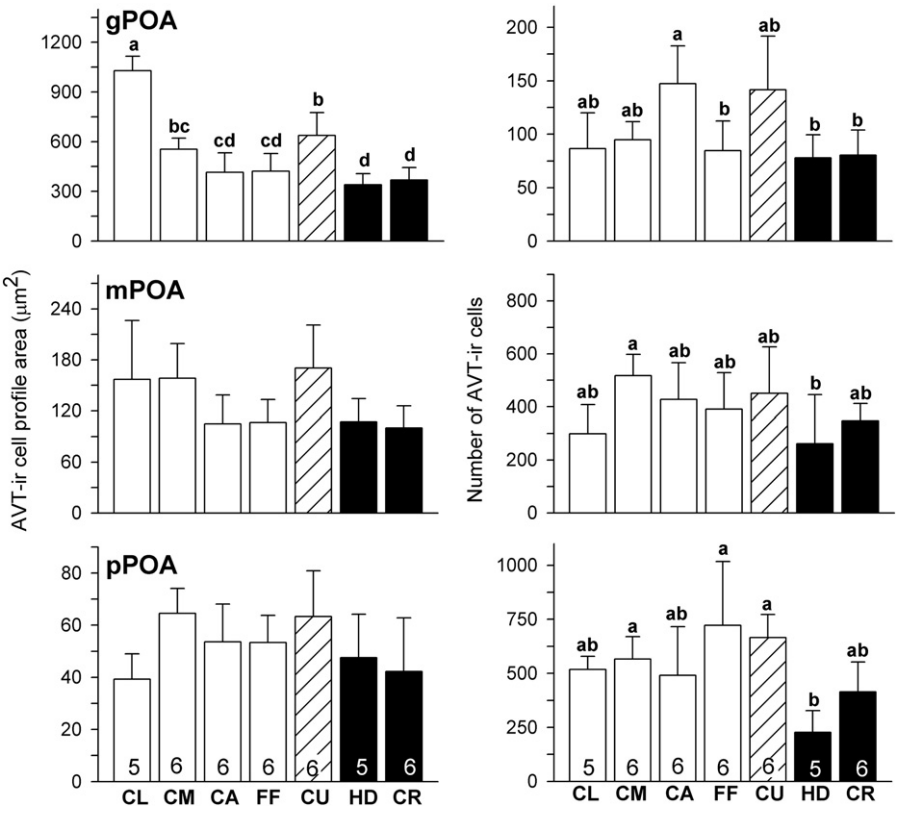

Fig. 1. Species differences in the size and number of AVT-ir cell groups. A pattern of larger gPOA cells exists for territorial aggressive (open bars) compared to gregarious non-aggressive (solid bars) species. Three aggressive monogamous paired species = CL, oval butterflyfish (*Chaetodon lunulatus*); CM, pebbled butterflyfish (*C. multicinctus*) and CA, threadfin butterflyfish (*C. auriga*). Two non-aggressive, polygamous, grouped species = CR, milletseed butterflyfish (*C. miliaris*) and HD, pennant butterflyfish (*Heniochus diphreutes*). One aggressive, haremic species = FF, forceps fish (*F. flavissimus*). One monogamous species with mixed social affiliations for which no published data on aggression or territorial behavior exist (hatched bars) = CU, teardrop butterflyfish (*C. unimaculatus*). Letters indicate species differences (One-way ANOVA with false discovery rate and Tukey's test $p<0.05$). Numbers indicate sample size.

The number of AVT-ir cells in the gPOA differed among species ($p<0.001$, one-way ANOVA with FDR) (Fig. 1). Threadfin butterflyfish had more gPOA AVT-ir cell than milletseed butterflyfish ($p=0.014$, one-way ANOVA and Tukey's test), forceps fish ($p=0.026$, one-way ANOVA and Tukey's test), and pennant butterflyfish ($p=0.010$, one-way ANOVA and Tukey's test) AVT-ir cell number within the mPOA also differed among species ($p=0.048$, one-way ANOVA with FDR) (Fig. 1), where multiband butterflyfish had more mPOA cells than pennant butterflyfish ($p = 0.035$, one-way ANOVA and Tukey's test). The number of AVT-ir pPOA cells differed among species ($p<0.001$, one-way ANOVA with FDR), where pennant butterflyfish had fewer pPOA AVT-ir cells than forcepsfish ($p < 0.001$, one-way ANOVA and Tukey's test), teardrop butterflyfish ($p=0.002$, one-way ANOVA and Tukey's test), and pebbled butterflyfish ($p = 0.034$, one-way ANOVA and Tukey's test) (Fig. 1). These analyses showed limited species differences in the number of AVT-ir cells across all three cell groups but did not have a clear relationship with social behavior.

AVT-ir telencephalic varicosities

The distribution of AVT-ir varicosities in the telencephalon was similar in all seven species with AVT-ir fibers present in 21 of the 24 telencephalic nuclei (see above). The step-wise LDA which used the density of AVT-ir varicosities to predict aggression delineated two brain regions (Vv and Dm1) that were able to classify 93% (89% jackknife classification) of the individuals that belong to each social grouping ($p<0.001$) (Table 3). Of these two brain regions, only Vv differed in the density of AVT-ir varicosities between aggressive and non-aggressive species (Fig. 2) (Vv, $p<0.001$; Dm1, $p=0.683$; one-way ANOVA and Tukey's test). These analyses showed that a high density of AVT-ir varicosities within the Vv is indicative of an individual that belongs to an aggressive species.

The density of AVT-ir varicosities within the Vv, Dm1, and Vu classified 78% (73% jackknife classification) of the individuals that belong to each mating system (step-wise LDA $p<0.001$) (Table 4). The Vv and Vu differed in the density of AVT-ir varicosities according to social system ($p<0.001$, one-way ANOVA). Specifically, monogamous species had a higher density of AVT-ir varicosities in the both Vv and Vu than haremic (Vu, $p=0.042$; Vv, $p=0.016$, one-way ANOVA and Tukey's test) and polygamous species (Vu, $p=0.016$; Vv, $p<0.001$, one-way ANOVA and Tukey's test). The Dm1 did not differ between

Table 3
Linear discriminant analysis (LDA) using neuroanatomical variables of AVT-ir varicosities/nuclei volume to predict aggression. Wilk's lambda 0.583; $df=2,37$; $p<0.001$.

Neuroanatomical variable	*F*-to-remove	Group means of AVT-ir varicosities/nuclei volume (cm^3)	
		Aggressive	Non-aggressive
Vv *	26.135	1145.008	191.037
Dm1	7.258	339.285	229.285
	Total	Aggressive	Non-aggressive
Correct classifications (%)	93	93	91
Jackknife classifications (%)	88	93	73

The density of AVT-ir varicosities within the ventral nucleus of the ventral telencephalon (Vv) and medial region 1 of the dorsal telencephalon (Dm1) is predictive of aggressive/territorial species. Only Vv differed between territorial and non-territorial species (one-way ANOVA with false discovery rate and Tukey's test). Linear discriminant analysis with jackknife stepwise analysis. Dm1—medial region 1 of dorsal telencephalon; Vv—ventral nucleus of the ventral telencephalon. * Comparisons across groups with $p<0.05$.

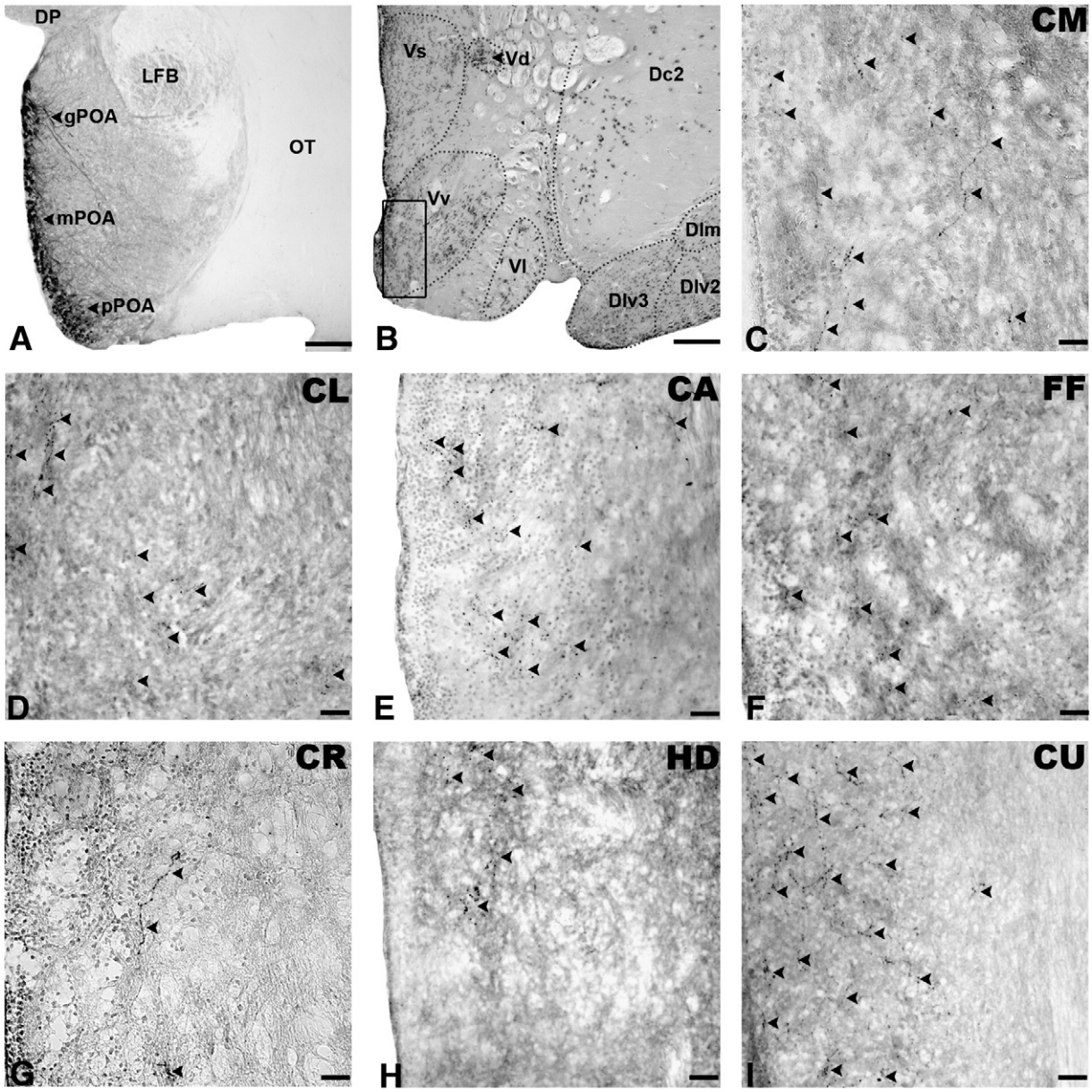

Fig. 2. A. Transverse section through the brain of oval butterflyfish *(Chaetodon lunulatus)* that shows the relative position of parvocellular (pPOA), magnocellular (mPOA), gigantocellular (gPOA) AVT-ir cells (dorsal is up). B. Nissl stained section through the brain of *C. multicinctus* that shows the relative position of the ventral nucleus of the ventral telencephalon (Vv). Box marks the relative location of C-I. The density AVT-ir varicosities within the Vv of aggressive species (C–F) is higher than non-aggressive species (G–H). I. An additional species, teardrop butterflyfish (CU) in which no information is available about their aggressive behavior has a very high density of AVT-ir varicosities within the Vv. CL, oval butterflyfish (*C. lunulatus*); CM, pebbled butterflyfish (*C. multicinctus*); and CA, threadfin butterflyfish (*C. auriga*); FF, forcepsfish (*F. flavissimus*); CR, milletseed butterflyfish (*C. miliaris*) and HD, pennant butterflyfish (*Heniochus diphreutes*). Scale bars = 100 μm (A–B); 20 μm (C–I).

species ($p = 0.484$, one-way ANOVA). These analyses provided some evidence that the densities of AVT-ir varicosities within the Vv and Vu were related to mating system as monogamous individuals had higher levels in both nuclei.

The density of AVT-ir varicosities within the Dm1, Vv, Vp, Vu correctly classified 76% (76% jackknife classification) of the individuals that belong to each social grouping (step-wise LDA, $p<0.001$) (Table 5). The Vv differed in the density of AVT-ir varicosities according to social affiliations ($p<0.001$, one-way ANOVA). Specifically, mixed species had higher densities than grouped, trio, or paired species ($p<0.001$, one-way ANOVA and Tukey's test). Paired species also had more AVT-ir varicosities within this nucleus than group species ($p = 0.006$, one-way ANOVA and Tukey's test). Trio species did not differ from either paired ($p = 0.140$) or grouped species ($p = 0.915$, one-way ANOVA and Tukey's test). The density of AVT-ir varicosities within Dm1, Vp and Vu also differed between species according to social affiliation (Dm1 and Vp: $p<0.001$; Vu: $p = 0.003$, one-way ANOVA) as mixed species in these three nuclei had higher densities than grouped, trio, or paired species ($p<0.05$, one-way ANOVA and Tukey's test). Paired species had also higher densities of AVT-ir varicosities within the Vp compared to grouped species ($p = 0.030$, one-way ANOVA and Tukey's test). These analyses indicated that high densities of AVT-ir varicosities within the Vv, Vp, Vu, and Dm1 were related to either the flexible social grouping or another yet unknown factor specific to the teardrop butterflyfish. In addition, as most monogamous species are also paired, it is no surprise that the high density of AVT-ir varicosities within the Vv was also indicative of paired individuals.

Table 4
Linear discriminant analysis (LDA) using neuroanatomical variables of AVT-ir varicosities/nuclei volume to predict mating systems. Wilk's lambda 0.453; $df = 6, 70$; $p < 0.001$.

Neuroanatomical variable	F-to-remove	Group means of AVT-ir varicosities/nuclei volume (cm^3)		
		Monogamous	Haremic	Polygamous
Vv*	11.240	1270.377	664.427	191.037
Dm1	4.330	567.165	37.804	339.285
Vu*	2.681	3076.596	212.589	862.382
	Total	Monogamous	Haremic	Polygamous
Correct classifications (%)	78	87	0	100
Jackknife classifications (%)	73	78	0	100

The density of AVT-ir varicosities within the ventral nucleus of the ventral telencephalon (Vv) and medial region 1 of the dorsal telencephalon (Dm1) is predictive of species with specific mating systems. The density of AVT-ir varicosities within Vv and Vu differed between mating systems (one-way ANOVA with false discovery rate and Tukey's test). Linear discriminant analysis with jackknife stepwise analysis. Dm1—medial region 1 of dorsal telencephalon; Vu—cuneate nucleus of the ventral telencephalon; Vv—ventral nucleus of the ventral telencephalon. * Comparisons across groups with $p < 0.05$.

Table 5
Linear discriminant analysis (LDA) using neuroanatomical variables of AVT-ir varicosities/nuclei volume to predict social affiliations. Wilk's Lambda 0.162; $df = 12, 103$; $p < 0.001$.

Neuroanatomical variable	*F*-to-remove	Group means of AVT-ir varicosities/nuclei volume (cm^3)			
		Pair	Trio	Group	Mixed
Dm1*	10.541	169.754	37.804	339.285	1693.164
Vv*	5.126	962.003	664.427	191.037	2144.103
Vp*	4.985	1253.869	647.933	323.641	2650.206
Vu*	4.623	2744.069	212.589	862.382	4018.755
	Total	Pair	Trio	Group	Mixed
Correct classifications (%)	76	87	0	100	67
Jackknife classifications (%)	76	87	0	100	67

The density of AVT-ir varicosities within the medial region 1 of dorsal telencephalon (Dm1), postcommissural nucleus of the ventral telencephalon (Vp), cuneate nucleus of the ventral telencephalon (Vu), and ventral nucleus of the ventral telencephalon (Vv). All nuclei differed across species with different social affiliations. Linear discriminant analysis with jackknife stepwise analysis. *Comparisons across groups with $p < 0.05$ one-way ANOVA with false discovery rate and Tukey's test.

The stepwise LDA which used the density of AVT-ir varicosities to predict phylogenetic clade delineated six brain regions (Vu, Dm1, Vs, Dm3, Dm2, Vp) that correctly classified 73% (73% jackknife classification) of the species according to phylogeny ($p < 0.001$) (Table 6). The Vu differed in the density of AVT-ir varicosities according to phylogeny ($p = 0.002$, Kruskal–Wallis). The fourth *Chaetodon* clade had higher densities of AVT-ir varicosities in the Vu compared to bannerfish clade ($p < 0.05$, Kruskal–Wallis and Dunn's test). The Dm3 differed in the density of AVT-ir varicosities according to phylogeny ($p = 0.009$, Kruskal–Wallis) as the second *Chaetodon* clade had higher densities of AVT-ir varicosities compared to bannerfish clade ($p < 0.05$, Kruskal–Wallis and Dunn's test). The Dm1, Dm2, Vs, and Vp did not differ in the density of AVT-ir varicosities according to phylogeny ($p > 0.05$, Kruskal–Wallis). These analyses showed that phylogenetic relatedness does have a weak association with the density of AVT-ir varicosities in particular nuclei but no relationship exists between nuclei with potential AVT influenced social relevance such as the Vv.

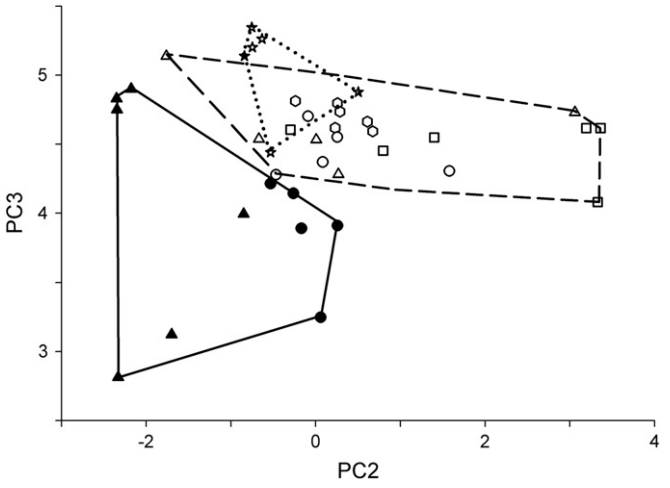

Fig. 3. Bivariate scatter plot from results of the sheared principle component analysis of arginine vasotocin (AVT)-immunoreactive (-ir) cell size and number and the density of varicosities within the Vv, Vs, Vu, and Dm1 across seven species of butterflyfishes. Aggressive species (open symbols and dashed line) and non-aggressive species (solid symbols and solid line) are separated on both PC2 and PC3. No published data on aggressive or territorial behavior are available for teardrop butterflyfish (☆ and dotted line). Note that principle component (PC) 2 axis was heavily loaded by the density of AVT-ir varicosities in Dm1, Vv, and Vu after correction for inherent body size variation among species (Table 7). PC3 axis was heavily loaded by the density of AVT-ir varicosities in Vv, Vs, Vu and cell number parameters after correction for inherent body size variation among species., threadfin butterflyfish; ○, oval butterflyfish; △, pebbled butterflyfish; ▲, milletseed butterflyfish; ☆ teardrop butterflyfish; □, forcepsfish; ●, pennant butterflyfish.

The sheared PCA analysis further indicated that the relationship between social behavior and features of the AVT system was not due to species differences in body size. This analysis showed a clear separation of aggressive and non-aggressive species within the PC2 and PC3 scatterplot (Fig. 3). The analysis of covariance matrix of these adjusted cell and varicosity variables indicated that these two PC axes explain 31.6% of the variance among individuals (Table 7). PC2 presents the first body size-corrected component and showed

Table 6
Linear discriminant analysis (LDA) using neuroanatomical variables of AVT-ir varicosities / nuclei volume to predict phylogenetic relationships. Wilk's lambda 0.143; $df = 18, 88$; $p < 0.001$.

Neuroanatomical variable	*F*-to-remove	Group means of AVT-ir varicosities/nuclei volume (cm^3)			
		Bannerfish	Chaetodon 2	Chaetodon 3	Chaetodon 4
Vu*	14.196	374.479	1743.795	3255.339	4956.527
Dm1	11.445	85.482	749.408	269.027	204.831
Vs	3.538	194.661	538.281	366.573	663.978
Dm3*	3.490	0.924	7.089	1.356	1.008
Dm2	2.704	130.981	214.934	83.469	78.141
Vp	2.687	493.286	1232.767	1622.948	1488.994
	Total	Bannerfish	Chaetodon 2	Chaetodon 3	Chaetodon 4
Correct classifications (%)	73	82	72	40	83
Jackknife classifications (%)	73	82	72	40	83

The density of AVT-ir varicosities within the. medial region 1 of dorsal telencephalon (Dm1), medial region 2 of the dorsal telencephalon (Dm2), medial region 3 of the dorsal telencephalon (Dm3), postcommissural region of the ventral telencephalon (Vp), and cuneate nucleus of the ventral telencephalon (Vu). The Vu and Dm3 differed between species in different phylogenetic clades. Linear discriminant analysis with jackknife stepwise analysis *Comparisons across groups with $p < 0.05$ one-way ANOVA with false discovery rate and Tukey's test.

Table 7
Sheared principle component analysis for arginine vasotocin cell size, number and density of varicosities in four telencephalic nuclei across seven butterflyfish species.

	PC1	PC2	PC3
Vv varicosities	0.185	**0.096**	**0.646**
Vs varicosities	0.183	0.024	**0.656**
Vu varicosities	0.894	**0.351**	**−0.267**
Dm1 varicosities	0.362	**−0.930**	−0.011
gPOA size	0.017	0.016	0.061
mPOA size	0.017	0.008	0.071
pPOA size	0.005	0.011	0.094
gPOA number	0.019	−0.002	**0.133**
mPOA number	−0.004	−0.011	**0.133**
pPOA number	−0.001	0.033	**0.161**
Percent variance explained	65.74	27.89	3.66

Principle component (PC) 1 indicates factors that explain variations associated with body size. The first three principal components explain 96.30% of the variation. PC2 and PC3 represent factors that explain variation among species for corrected cell measurements. Eigenvectors with the strongest loadings are in bold. Note that PC2 had heavy loading for the density of varicosities within the Vv, Vu, and Dm1. PC3 had heavy loading for the density of varicosities within Vv, Vs, Vu and less for gPOA, mPOA, and pPOA cell number. Dm1, subdivision 1 of the medial division of the dorsal telencephalon; gPOA, gigantocellular; mPOA, magnocellular; pPOA, parvocellular cell groups of preoptic area; Vs, supracommissural nucleus of ventral telencephalon; Vv, ventral nucleus of the ventral telencephalon; Vu, cuneate nucleus of the ventral telencephalon.

positive loadings for varicosity density within Vv and Vu and a strong negative loading for Dm1 varicosity density. Although the proportion of total variation explained by PC3 was much lower, it showed a strong positive loading for the density of varicosities within Vv and Vs, a moderate loading for cell number in all AVT groups, and a negative loading for the density of varicosities within Vu. These results further support the relationship between butterflyfish social behavior and varicosity density in Vv, Vs, Vu and Dm1, and to a lesser extent cell number.

Discussion

This study found several relationships between social behavior and the density of AVT-ir varicosities in homologous regions that predict social functions of AVP in mammals, particularly within the septal (Vv) homolog. Furthermore, limited species differences in AVT-ir somata features also exist. These AVT features are not static variables and are potentially influenced by prior social experiences, stress, hormones, and other physiological factors (Balment et al., 2006; Bass and Grober, 2001; Goodson and Bass, 2001). Although these factors likely increase intraspecies variability in AVT features and thereby only weakened the described relationships with social behavior, the contribution of these factors to interspecies differences in AVT features not related to social behavior are still unresolved. Although the function of these correlative relationships remains untested, it is discussed below in terms of its potential relevance to fish social behavior and similarity to birds and mammals.

The action of AVT within the telencephalon of fishes remains unclear because most functional studies in fishes are limited to intraperitoneal or intramuscular injections of AVT. Although these studies demonstrate a strong effect of AVT on behaviors such as aggression (Lema and Nevitt, 2004b; Santangelo and Bass, 2006; Semsar et al., 2001), courtship and spawning reflex (Carneiro et al., 2003; Pickford and Strecker, 1977; Semsar et al., 2001), and social communication (Bastian et al., 2001) the sites of action are not confirmed. The site of action is important because AVT can influence many physiological processes in fishes such as stress through its actions in the pituitary and kidneys, metabolism through influence on the hypothalamic–pituitary–interrenal axis, cardiovascular function in the branchial vasculature and gills, osmoregulation in both the kidneys and gills, steroid hormone levels in ovarian tissue and oocyte hydration, and contractions in smooth muscle tissues of the gastrointenstinal tract, urinary bladder, and gallbladder (Balment et al., 2006; Conklin et al., 1999; Singh and Joy, 2009; 2010). Intracerebroventricular injections frequently exhibit more consistent and robust changes in social behavior, such as courtship (Salek et al., 2002) or aggression (Backström and Winberg, 2009) but only eliminate potential peripheral influences of AVT on behavior and do not directly implicate a specific area or areas responsible for the behavior. The central mechanisms of AVT influenced behavior with known sites of action in fishes include AVT-mediated inhibition of social approach through its influence on substance P neurons in the hindbrain of the goldfish (Thompson et al., 2008) and vocal motor actions on the paralemniscal midbrain tegmentum and preoptic area/anterior hypothalamus of the midshipman that precisely mimic natural vocalizations (Goodson and Bass, 2000a;b). Thus, despite many important and intricate experiments, the role of AVT within the telencephalon of fishes is still unclear. However, Goodson and Bass (2000b) reported that the microinjection of AVT within the ventral telencephalon of midshipman had no effect on vocal motor response. The current study provides correlative evidence that AVT within the telencephalon of fishes is involved in the expression of distinct social behaviors.

The density of AVT-ir varicosities within the butterflyfish Vv was related to aggression, mating system and social affiliations. This region is considered homologous to the septum (Table 1). The regulation of mammalian aggression is thought to involve AVP-ir neurons from the bed nucleus of the stria terminalis and medial amygdala that project to the lateral septum (Veenema and Neumann, 2008). Similarly, the LDA and sheared PCA analyses provide correlative evidence that AVT may have a similar function within the Vv in butterflyfishes. However, the sources of these extra-hypothalamic projections in fishes are from AVT-ir preoptic area cells and possibly the ventral hypothalamus (Dewan et al. 2008) as no homologous telencephalic AVT-ir cell groups are known. Although AVT varicosity density is strongly predictive of social behavior, further studies of AVT transcription, release, and receptor expression are needed to fully characterize this system.

The influence of AVP within the lateral septum on aggressive behavior remains unclear. Correlation and manipulation studies on AVP and aggression frequently conflict when multiple species are compared (Veenema and Neumann, 2008). These indicate that septal vasopressin may regulate context-specific aggression that depends upon the integration of social environment or state of the animal. Within fishes, the influence of AVT on aggressive behavior also frequently conflicts (Backström and Winberg, 2009; Lema and Nevitt, 2004b; Santangelo and Bass, 2006; Semsar et al., 2001). A clear relationship is complicated by the use of different injection sites, behavioral paradigms, and species with different social behaviors. Thus the conflicting effects of AVT on aggression in fishes may be due to differences in context (Goodson et al., 2009b), the site of action (Veenema et al., 2010), or other factors such as reproductive condition (Walton et al., 2010). Although the action of AVT within the Vv of butterflyfishes is unknown, the robust relationship between the density of AVT-ir varicosities or putative release sites and aggression indicates a possible similar role to AVP in the rodent septum. Both AVP release and receptor density within the lateral septum were positively correlated with aggression in two rodent species (Bester-Meredith et al., 1999; Veenema et al., 2010). In addition, the infusion of AVP in the lateral septum increased aggression in castrated rodents (Koolhaas et al., 1991). However, a negative correlation between septal AVP fiber density and aggression occurred in selected strains of mice and wild rats (Compaan et al., 1993; Everts et al., 1997). Although the proximate mechanisms remain to be determined, a robust relationship between features of the AVT/AVP system within homologous nuclei and aggression exists in these phylogenetically distant lineages and highlights that AVT within the telencephalon of fishes may have social relevance.

Studies in birds and mammals have also linked AVT/AVP to other potential functions in the lateral septum that are consistent with butterflyfish social behavior. In birds, the vasotocin circuit within the

BNST and lateral septum is proposed to function in sociality and mate competition aggression (Goodson et al., 2009a,b) whereas AVP within the lateral septum of mammals is also linked to social recognition (Bielsky et al., 2005; Landgraf et al., 2003). The current study provides support for this hypothesis as monogamous pair bonded species had higher densities of AVT-ir varicosities within the Vv than haremic or polygamous species. Thus, AVT within the Vv of these butterflyfishes could enhance the social recognition of mates and potential intruders (Yabuta, 2002), or function in aggression related to the mate guarding behavior of males (Fricke, 1986). Of note, the teardrop butterflyfish, which is found as single individuals, pairs or in larger groups, had the highest number of AVT-ir varicosities within this region. The teardrop butterflyfish is socially very aggressive in the field (F. Cox, personal communication) but neither occurs exclusively in pairs nor defends exclusive feeding territories. Thus, high levels of AVT in the Vv may be related to aggression over mate competition or dominance hierarchies yet to be described for this species. In addition, the teardrop butterflyfish is socially monogamous and spawns in pairs (Sancho et al., 2000); therefore, a heightened level of mate competition for spawning partners (and mate defense) may exist during congregation in large groups. Although AVT inhibits social approach in the goldfish, this behavior occurs via a hindbrain circuit (Thompson et al., 2008), which is likely independent of actions within the Vv and other telencephalic nuclei. However, the microinjection of AVT within the ventral telencephalon of midshipman had no effect on a vocal motor response important for reproduction (Goodson and Bass, 2000b). Thus while the proximate mechanisms of action are currently unknown, these findings are consistent with the proposal that AVT within the Vv is likely involved in fish aggressive behavior or social recognition and may be similar to that of septal AVT /AVP in birds or mammals.

The function of AVP within the septum of mammals is also related to stress and hormone levels (Landgraf et al., 2003; Wang and De Vries, 1993). AVT-ir features within fishes is influenced by reproductive season (Dewan et al., 2008; Maruska, 2009; Maruska et al., 2007), spawning (Ohya and Hayashi, 2006) or sexual maturation (Guiry et al., 2010). However, species differences due to the experimental protocol are unlikely as all individuals were captured during their peak reproductive period (assessed in a similar manner to Dewan et al., 2008; Maruska, 2009; and Maruska et al., 2007), and processed in a consistent fashion. However, further studies are needed to determine if these butterflyfishes naturally differ in their stress responses or hormonal profiles and if these differences are related to the density of AVT-ir varicosities.

The Vl of fishes is also a possible homolog of the mammalian septum but did not demonstrate any association with social behavior. The Vl was the only region in which the majority of AVT-ir fibers did not project within boundaries of the nucleus but rather passed within the neuropil adjacent to this region. Although synaptic contact can occur within the neuropil (Caruncho et al., 1990), these varicosities were not counted because it is not yet known whether these varicosities make synaptic contact with Vl or another nucleus. Thus, the number of AVT-ir varicosities measured within the Vl may not reflect the potential neuromodulatory function of potential regional release. In addition, varicose fibers in this region were qualitatively more numerous in aggressive monogamous than non-aggressive polygamous species. Thus, Vl may also function in context specific aggressive or affiliative behavior similar to the septum of birds or mammals.

The linear combination of features that predicted social affiliation behavior also included the Vp, Vu, and the Dm1 regions. The density of AVT varicosities within the Vp, which is homologous to basal portions of the amygdala (Table 1), may have relevance to social behavior but is not robustly associated with AVP/AVT influenced social behavior in other vertebrates. This nucleus is closely associated with Vs within the butterflyfish brain and shares a partial homology to basal portions of the amygdala and BNST (Table 1). AVT / AVP-ir cells within the BNST of birds and mammals are related to social behavior as they are activated by positive social stimuli (Goodson and Wang, 2006; Ho et al., 2010) and are likely involved in affiliative behavior (Young and Wang, 2004). However, the lack of a homologous BNST AVT-ir cell population in fishes may partly explain the absence of this region from the LDA analysis of social affiliations. However, the Vs of butterflyfish did have strong loading factors in a sheared PCA that separated aggressive from non-aggressive species. Thus, at this time the relationship between AVT-ir varicosities and social behavior in these regions is unclear. The Vu is a putative new brain region described in butterflyfishes with no yet identified homology (Dewan and Tricas, *in review*). In addition, since multiple regions of the dorsal telencephalon of fishes including Dm1 are thought to be homologous to the neocortex or paleocortex of mammals, the relevance of AVT within Dm1 or Vu remains to be determined. Thus, further work is needed to determine if the action of AVT within these regions of the telencephalon may affect social behavior.

This study assessed the relationship between AVT and behavior in multiple brain regions across a wide butterflyfish phylogeny. For example, the bannerfish clade, which includes the basal chaetodontids, had fewer AVT-ir varicosities than would be expected based on their social behaviors. The evolutionary and functional significance for this difference is unknown and highlights the importance of a strong phylogeny in multiple species comparisons. Since the inclusion of these species only weakens the LDA analyses of social behavior within the *Chaetodo*n genus, the data presented within this study are quite robust.

The limited species differences in gPOA AVT-ir cell group may also reflect aggressive behaviors. This overall relationship is supported in other fishes as aggression, dominant or breeding individuals or species have more numerous, larger, or more AVT mRNA in this cell population (Dewan et al., 2008; Greenwood et al., 2008; Larson et al., 2006; Maruska, 2009; Ota et al., 1999; Semsar and Godwin 2003). However, this relationship was not strongly supported in the present study when data were corrected for body size with a sheared PCA. In addition, the density of AVT-ir varicosities was more predictive of social behavior than cell parameters. Even though actions of the gPOA cell group in fishes may influence aggression or dominance, further studies are needed to determine the control and expression mechanisms that underlie this relationship.

The release of AVT from the paraventricular nucleus, a region homologous to the pPOA cell group of fishes (Moore and Lowry, 1998) is proposed to inhibit aggression (Goodson et al., 2009b). This hypothesis was not supported by either the size or number of butterflyfish pPOA cells but does not preclude possible differences in the rate of peptide release or production. In addition, most of the support for this hypothesis in fishes comes from within species analysis (Greenwood et al., 2008; Grober et al., 2002; Larson et al., 2006; Miranda et al., 2003). In this way, individual variations in aggressive behavior within a butterflyfish species may better reflect differences in pPOA AVT-ir cellular features. Thus, at this time there is no evidence that the pPOA cell group is related to aggressive behavior in butterflyfishes but future within species analyses that control for prior stress may provide support that hypothesis.

In summary, this study demonstrates a robust relationship between telencephalic AVT varicosities and social behavior in butterflyfishes. This relationship, particularly within the septal homolog of butterflyfishes, is consistent with the function of septal AVT/AVP in birds and mammals despite the fact that this innervation originates from different cell populations.

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