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Research Report

Arginine vasotocin neuronal phenotypes and their relationship to aggressive behavior in the territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*

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ABSTRACT

Intra and interspecific comparisons of arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin (AVP) demonstrate several relationships between these neuropeptides and aggression/dominance behaviors. Prior studies in coral reef butterflyfishes and other fishes indicate that features of AVT neurons in the gigantocellular preoptic area (gPOA) and axon varicosities within the ventral nucleus of the ventral telencephalon should have a positive relationship with aggressive behavior, whereas AVT-ir neuronal features in the parvocellular preoptic area (pPOA) should have a negative relationship. We measured the offensive aggression of wild caught territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*, in a simple lab paradigm that controlled for social context and variations in social stimuli. Offensive aggression did not follow a clear stereotyped pattern, but rather a complex sequence that includes five action patterns and two approach behaviors. We then used immunohistochemistry to test for associations between AVT immunoreactive features and projections with overall offensive aggression. Our results indicate that gPOA cell number was positively related to aggression while both the size and number of pPOA cells were negatively related to aggression. No association between aggression and the number of axon varicosities in the telencephalic region proposed to be associated with aggression was found. This study provides further support for the relationship between AVT neuronal features and aggression in fishes, and provides preliminary evidence that this relationship may relate to the motivation to produce aggressive behaviors in the immediate future.

1. Introduction

Arginine vasotocin (AVT) and its mammalian homolog, arginine vasopressin (AVP) influence numerous social behaviors through their action in the brain (Goodson and Bass, 2001). The influence of AVT / AVP on aggressive behavior is well documented (Backstrom and Winberg, 2009; Ferris, 2005; Lema and Nevitt, 2004; Semsar et al., 2001; Veenema and Neumann, 2008) but the relationship between these neuropeptides and aggression can be complex, occasionally contradictory, and is

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reported to be species-specific (Goodson, 2008; Goodson et al., 2009). For example, social, and physiological factors as well as differences in behavioral context likely contribute to differential AVT/AVP influences on behavior. Support for this hypothesis comes mainly from song birds in which aggression is influenced by AVT in a context and phenotypic-dependent manner (Kabelik et al., 2009; Goodson et al., 2009). Similarly within fishes, the relationship between AVT neuronal features and aggression is known to be influenced by environmental factors (Lema, 2006; Lema and Nevitt, 2004), whereas the effect of exogenous AVT on aggression is dependent upon social status (Semsar et al., 2001). Other factors such as seasonality and context also influence AVT mediated behavior (Perrone et al., 2010, Walton et al., 2010), while both environmental and social stress, sex, seasonality, social rank or status, and other steroids or neuromodulators can influence AVT neural expression (Gilchriest et al., 2000; Iwata et al., 2010; Larson et al., 2006; Lema, 2006; Maruska, 2009; Saito et al., 2003; Semsar et al., 2004). Since these relationships between AVT and aggression are dynamic, studies which relate AVT neuronal features to aggressive behavior should attempt to control for environmental and social factors.

Despite the myriad of influences on AVT's action, the relationships between its neuronal features and aggression within fishes are surprisingly conserved. Larger or more numerous AVT-ir cells or higher production of AVT within the gigantocellular preoptic area (gPOA) are often present in aggressive, dominant, or breeding individuals within a species (Greenwood et al., 2008; Larson et al., 2006; Maruska, 2009; Ota et al., 1999; Semsar and Godwin, 2003) as well as territorial or aggressive species (Dewan et al., 2008, 2011). Of note, a territorial subspecies of pupfish had smaller gPOA/mPOA cells compared to a non-territorial species but this may be due to differences in environmental conditions (Lema and Nevitt, 2004). In contrast, subordinate or non-aggressive individuals often have more numerous, larger AVT-ir neurons, or have higher AVT production within the parvocellular preoptic area (pPOA) (Greenwood et al., 2008; Grober et al., 2002; Larson et al., 2006). However, such differences in neuronal morphology were not found among non-aggressive compared to aggressive butterflyfish species (Dewan et al., 2008; 2011). The above studies compared established aggressive phenotypes within a species or across multiple species but did not link these neuronal differences directly to the behavior of individuals. Greenwood et al. (2008) described positive and negative correlations between gPOA and pPOA AVT mRNA expression and aggressive behaviors, respectively. Lema (2006) described a negative relationship between AVT-ir pPOA size and aggression in the rearing environment and a positive relationship between gPOA and rearing environment aggression in at least one species. In addition, Santangelo and Bass (2010) reported a negative relationship between the number of preoptic area fibers and aggression. While these three studies provided an important link between AVT neural expression and aggression, fishes in these studies were analyzed over several days/trials and the frequency of their behavior was averaged. Thus, it remains to be determined whether AVT neural features are related to the motivation to produce aggression during an immediate social encounter.

In fishes, the neural circuitry and regional action of AVT on aggressive behavior is unknown. The proposed neural circuit for AVP influenced aggressive and territorial behavior in birds and mammals involves the AVT/AVP-ir cells in the bed nucleus of the stria terminalis and medial amygdala that project to the lateral septum and additional AVP-ir cells that project to the anterior hypothalamus (Ferris et al., 1997; Goodson et al., 2009; Veenema and Neumann, 2008). In contrast, fishes have only three preoptic area and in some species one hypothalamic AVT-ir cell groups (Dewan et al., 2008; Moore and Lowry, 1998). The gPOA and mPOA cell groups are thought to be homologous to the supraoptic nucleus while the pPOA cell group is thought to be homologous to the paraventricular nucleus (Moore and Lowry, 1998). Thus, the neural circuitry of AVT influenced aggression must originate from different source cell populations than in either birds or mammals.

Despite the apparent relationship of the gPOA cell group and aggression in fishes (Dewan et al., 2008, 2011; Greenwood et al., 2008; Larson et al., 2006; Lema, 2006; Maruska, 2009; Semsar and Godwin, 2003) the specific sites of peptide release and functions remain unknown. AVT cells presumably from the rostral gPOA cell group have extensive connections with the telencephalon (Holmqvist and Ekström, 1995). Thus, one candidate site of action for gPOA AVT-ir cells is the ventral nucleus of the ventral telencephalon, thought to be homologous to the lateral septum of birds and mammals (Northcutt, 1995). The density of AVT-ir varicosities within this nucleus is highly predictive of individuals that belong to territorial/aggressive species of butterflyfish (Dewan et al., 2011). Further analyses of the fish AVT system, specifically within candidate telencephalic nuclei, are therefore needed to verify a relationship between the AVT features and aggressive behavior. If present, such relationships could be compared to AVT/AVP behavioral circuits identified previously in birds and mammals, which might provide broader insights into the evolution of AVT/AVP regulation of aggression across vertebrates.

The multiband (pebbled) butterflyfish, (*Chaetodon multicinctus*) is a monogamous corallivore in which pairs aggressively defend a permanent feeding territory from conspecifics and other food competitors (Tricas, 1985, 1989). In addition to territorial defense, both males and females display aggression toward individuals of the same sex for the purpose of mate guarding (Strang, 2005). The consistency of this species' territorial behavior is associated with AVT-ir neuronal features, which do not differ between sexes or across reproductive seasons (Dewan et al., 2008). In addition, similar to other fish species, the multiband butterflyfish has larger gPOA AVT-ir cells than other non-territorial butterflyfish species (Dewan et al., 2008; 2011). This species was also one of several territorial butterflyfishes, which were distinguished by higher AVT-ir varicosities within the ventral nucleus of the ventral telencephalon (Dewan et al., 2011). Thus, this species provides a good model to identify relationships between AVT neuronal features/telencephalic varicosities and specific aggressive behaviors. Based on previous research in butterflyfishes and other fishes, the gPOA AVT-ir neuronal features and varicosities within the ventral nucleus of the ventral telencephalon should have a positive relationship with aggressive behavior, while pPOA AVT-ir neuronal features a negative relationship.

2. Results

A total of 17 adult multiband butterflyfish (8 males and 9 females) were tested in this study. Males (BW: $\bar{x}$=22.7±8.6 SD g; SL: $\bar{x}$=81.4±8.3 SD mm) and females (BW: $\bar{x}$=25.6±7.3 SD g; SL: $\bar{x}$=83.7±7.3SD mm) did not differ in either body weight (p=0.47, df=13; Student's t-test) or standard length (p=0.56, df=14; Student's t-test).

All individuals exhibited aggressive behaviors directed toward their image reflected on the mirror (Table 1). A total of 1860 behaviors were performed during 401 response approaches by the 17 individuals. Tail slaps were the most common behavior performed, followed by bites and lateral displays (Table 1). The least common behaviors performed were directed turn and dorsal fin raise (Table 1). At the start of an interaction, individuals were more likely to approach the mirror using only their pectoral fins (slow approach 63.8%) than using their caudal fin (rapid approach, 36.2%) (Fig. 1). However, encounters that began with a rapid approach were most frequently followed by a tail slap (40.0%) whereas encounters that began with a slow approach were most frequently followed by a lateral display (41.4%). Bites only occasionally followed either approach behavior (rapid: 17.2%; slow: 11.7%) and most commonly followed a directed turn (28.4%) and preceded a tail slap (22.8%). Tail slap (35.2%), bite (48.9%) and lateral display (17.6%) were the three most frequently repeated behaviors (Fig. 1). The frequency of each aggressive behavior did not differ between male and females (Student's t-tests p>0.05) (Table 1).

The principal component analysis confirmed that the frequency of each aggressive behavior and the total time spent interacting with the mirror were good indicators of an individual's overall aggression level. The bivariate scatter plot showed a clear separation of highly aggressive and less aggressive individuals across the PC1 axis (Fig. 2). The analysis of covariance matrix indicated that the first two PC axes explain 72.5% of the variance among individuals (Table 2). PC1 explained 48.3% of the variance and had strong negative loading factors for the total time interacting with the mirror, and the frequency of bites and tails slaps (Table 2). PC2 explained 24.2% of the variance and had strong positive loading for the frequency of dorsal fin raises and directed turns, and a strong negative loading for the frequency of lateral displays (Table 2). These results provide support for the variates in PC1 to be used as an index of aggression.

There were several relationships between AVT neuronal features and the index of aggression. The number of gPOA cells had a positive relationship with the aggression index (p=0.036, stepwise linear regression) (Fig. 3; Table 3). The number and size of pPOA cells had negative relationships with the aggression index (p<0.05, stepwise linear regression) (Table 3). Neither the number of varicosities in the ventral nucleus of the ventral telencephalon (p=0.74) nor body size (BW: p=0.46; SL: p=0.36) were related to the aggression index in the stepwise linear regression analysis.

3. Discussion

This study demonstrates several relationships between AVT-ir neuronal features and aggression in both male and female multiband butterflyfish. Specifically, aggression showed a positive relationship to the number of AVT-ir gPOA cells while both the size and number of AVT-ir pPOA cells had a negative relationship. These findings agree with previous intraspecific studies in which aggressive/territorial fish usually showed larger, more numerous somata, or higher AVT production levels in gPOA AVT-ir cell group (Greenwood et al., 2008; Larson et al., 2006; Maruska, 2009; Semsar and Godwin, 2003) while non-aggressive/non-territorial fish had larger, more numerous somata, or higher AVT production in pPOA AVT-ir cell group (Greenwood et al., 2008; Grober et al., 2002; Larson et al., 2006; Miranda et al., 2003). These results also provide support for previous interspecies comparisons in which aggressive species have larger gPOA cells than non-aggressive species (Dewan et al., 2008; 2011). Similar to other studies, (Greenwood et al., 2008; Lema, 2006; Santangelo and Bass, 2010) the current study provides preliminary evidence for a link between the preoptic AVT system and aggressive behavior. Thus, these studies may indicate that the observed individual differences in potential AVT production (inferred by neuronal phenotypes) are factors that may influence level of aggression during an immediate social encounter. Although this hypothesis is relatively well supported when differences in social behavior, phylogeny, and methodology are considered, little is known about the context, circuitry, synaptic interactions or neural encoding of these cells and the mechanism by which they influence aggression.

Despite its robust relationship with aggression in fishes, the projection sites of the AVT-ir gPOA cell group remain undefined. AVT-ir gPOA cells most likely innervate multiple brain regions

Table 1 – Frequency of approach and aggressive behaviors of the multiband butterflyfish, *Chaetodon multicinctus*.

Behavior	Description	Total ($\bar{x}$ ± SD)	Male ($\bar{x}$ ± SD)	Female ($\bar{x}$ ± SD)
Rapid approach	Rapid swim towards mirror with the use of caudal fin	14.1±20.1	14.9±19.9	14.2±21.5
Slow approach	Swim towards mirror with the use of pectoral fins	15.0±18.16	19.6±26.8	11.4±6.9
Bite	Touches mouth to mirror	21.5±16.3	22.3±16.7	20.9±16.9
Tail slap	Caudal fin rapidly moved towards mirror	30.3±30.9	38.9±35.0	22.7±26.6
Directed turn	Rapid turn to face mirror	7.6±6.0	7.8±3.6	7.4±7.8
Dorsal fin raise	Dorsal fin is erected	6.4±6.3	4.0±3.7	8.6±7.6
Lateral display	Parallel to mirror motionless and frequently with caudal dorsal fin and anal fin erected.	20.0±30.3	30.1±46.0	11.0±6.5

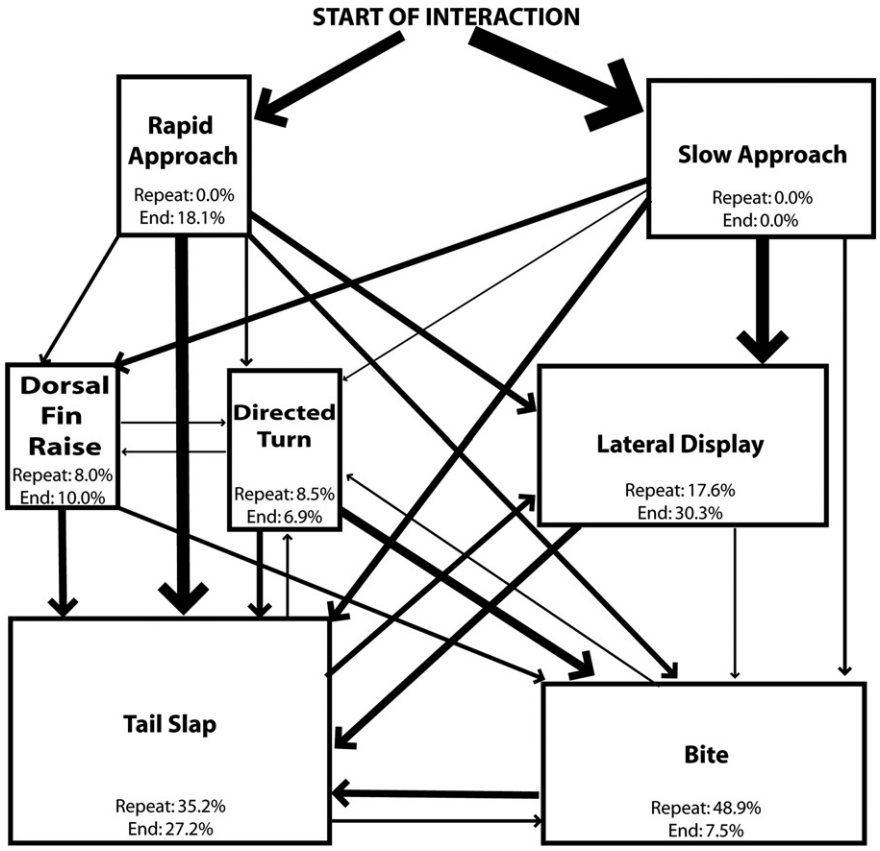

Fig. 1 – Analysis of aggressive behavior in the multiband butterflyfish, *Chaetodon multicinctus*. The figure presents a first-order Markov chain analysis of 1,860 behavioral patterns during 401 encounters across 17 individuals. The size of the boxes around the behavior represents the relative frequency of occurrence of a particular behavior. Sequence of behavior is depicted by arrows. Thickness of the arrow indicates the probability of a particular transition. Repeat represents the probability of the behavior being performed in succession. End represents the probability of being the terminal behavior in an encounter.

(Holmqvist and Ekström, 1995). The innervation of multiple distant brain regions by a single AVT-ir cell (Saito et al., 2004) adds an additional level of complexity as evidence in mammals indicates that local dendritic release of AVP and oxytocin can be at least partially independent of somatic activity (Ludwig, 1998). Further, it is unclear whether all the cells within the gPOA innervate the same regions or rather subsets of neurons innervate different brain regions. The later possibility may exist as only a small subset of AVT-ir gPOA cells innervate the pituitary and a small rostral subset of AVT-ir gPOA cells was determined to innervate the dorsal telencephalon of Atlantic salmon (*Salmo salar*) (Holmqvist and Ekström, 1995). While further neuroanatomical studies are needed to develop detailed hypotheses, intra and interspecies comparisons as well as correlations with behavior can provide preliminary evidence for the functional role of AVT-ir gPOA cells.

Greenwood et al. (2008) hypothesized that the gPOA AVT-ir cells influence aggressive and courtship neural circuits directly through connections with extrapituitary regions and indirectly through connections with pituitary and the eventual production of gonadal steroids. AVT-ir fibers have extensive extra-pituitary innervation in butterflyfishes (Dewan et al., 2008). One nucleus that could be part of a neural circuit of aggression in butterflyfishes is the ventral nucleus of the ventral telencephalon (Vv). This nucleus is putatively homologous to a mammalian region (lateral septum) that is part of aggression circuits (Northcutt, 1995) and the density of AVT-ir varicosities within this region is predictive of aggressive species across several butterflyfish clades (Dewan et al., 2011). While these results provide support for the direct modulation of aggressive circuits, the current study did not find any relationships between AVT-ir varicosities within this region and aggressive behavior. Thus, AVT-ir varicosities within these regions may be related to some other aspect of social behavior or aggression not measured in the current protocol such as territorial or defensive aggression, mate guarding, or sociality. Alternatively, the density of AVT-ir varicosities may not relate to behavior but to some other neural mechanism common to territorial butterflyfishes. However, it is also possible that aggressive individuals may have a naturally higher density of AVT-ir varicosities and release more AVT within these regions during agonistic behavior, a hypothesis that cannot be tested using standard immunohistochemistry. Similarly, the negative relationship between number of AVT-ir preoptic fibers and aggression in another fish species was tentatively

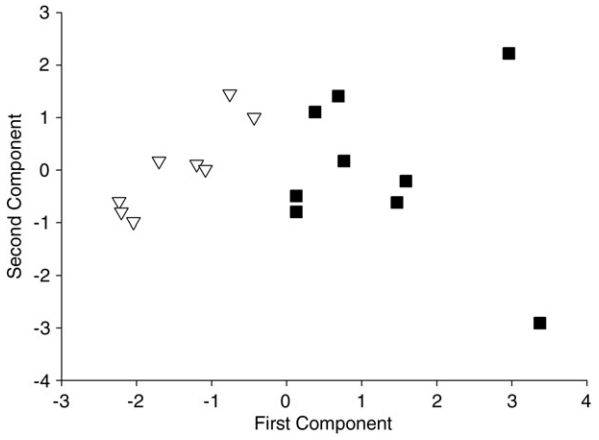

Fig. 2 – Bivariate scatter plot of the results of a principle component analysis focused on aggressive behaviors in the territorial multiband butterflyfish, *Chaetodon multicinctus*. This analysis incorporated the frequency of each of the five aggressive behaviors as well as the total time spent interacting with the mirror. In order to demonstrate the validity of using the first principal component as an aggression index, individuals were divided into the two roughly equal groups (8 and 9): aggressive individuals (solid square symbols) and less-aggressive individuals (open triangle symbols). These designations were defined based on the total number of aggressive behaviors. However, the total time interacting with the mirror was also a strong indicator of this designation (16 of the 17 individuals). These groups are defined solely to visually demonstrate the validity of the first principal component as an aggression index for this species. The categories of aggressive and non-aggressive fish are not used in any other analyses of the relationship between arginine vasotocin and aggressive behavior.

proposed to be due to increased AVT secretion (Santangelo and Bass 2010). These two studies highlight the need for further studies, which employ microdialysis probes within specific regions of the fish brain to measure AVT release during aggressive encounters similar to that in rodents (Veenema et al., 2010).

Table 2 – Principle component analysis of aggressive behavior in the territorial multiband butterflyfish, *Chaetodon multicinctus*.

	PC1	PC2
Interaction time	−0.521	−0.025
Bite	−0.495	0.156
Tail slap	−0.495	−0.188
Directed turn	−0.272	0.504
Lateral display	−0.375	−0.545
Dorsal fin raise	−0.155	0.623

Principal components (PC) 1 and 2 represents factors that explain variation among aggressive behaviors.

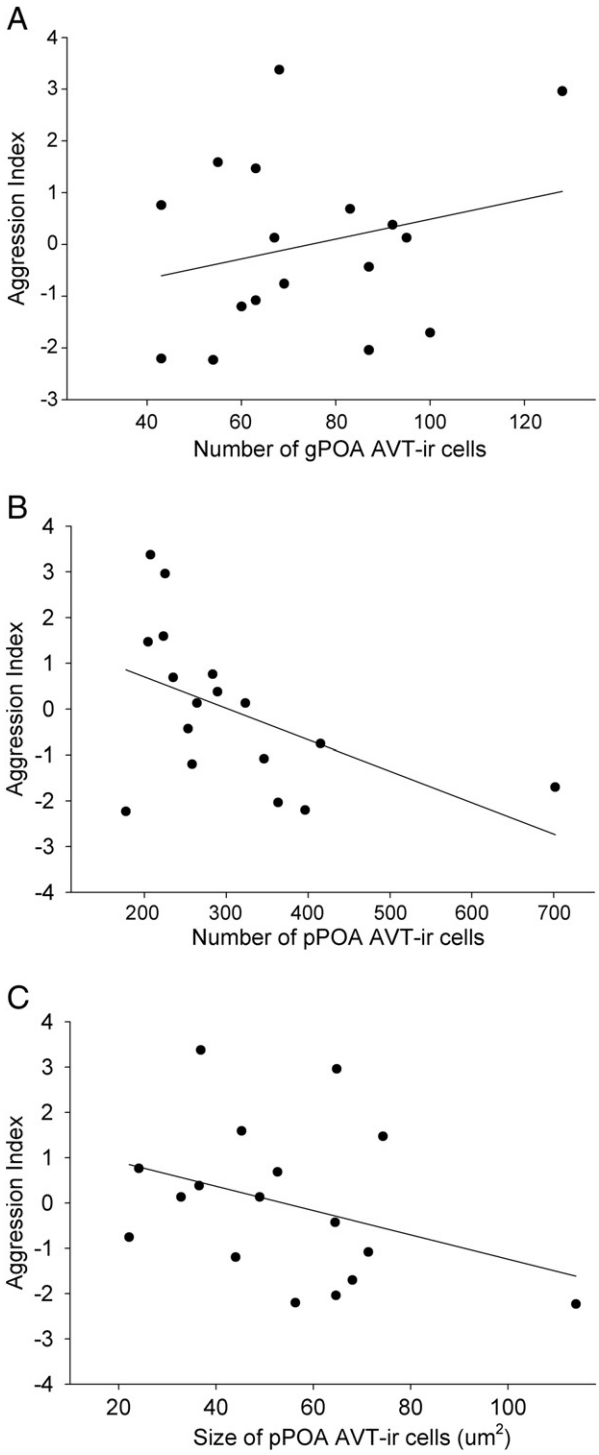

Fig. 3 – Regression analyses of AVT-ir neuronal phenotypes and the aggressive index in the multiband butterflyfish, *Chaetodon multicinctus*. Aggressive index is the first principal component of a principal component analysis of all aggressive behaviors. A. The number of AVT-ir gPOA cells has a positive relationship with the aggression index. B–C. Both the number and size of AVT-ir pPOA cells has a negative relationship with the aggression index. See Table 3 for statistical details. Data are shown for 17 test subjects.

Table 3 – Relationship between AVT-ir neuronal features and varicosities within the ventral nucleus of the ventral telencephalon and aggressive index in the multiband butterflyfish, *Chaetodon multicinctus*.

Variable	Coefficient	β value	T value	p Value	S	r^2	r^2 adj.
gPOA #	0.033	0.395	2.36	0.036	1.2	60.7	50.9
pPOA #	−0.008	−0.603	−3.05	0.010			
pPOA size	−0.038	−0.442	−2.76	0.017			

Stepwise multiple linear regression with alpha level of 0.15. Aggressive index was defined as the first component of the principal component analysis, which incorporated the frequency of all aggressive behaviors and the time spent interacting with the mirror.

The apparent association between aggression and the gPOA cell group could also be due to indirect mechanisms such as complex interactions with other neuropeptides or gonadal hormones that directly modulate behavior. Androgens modulate aggression in fishes (e.g. Munro and Pitcher, 1985) but may also influence the AVT system. The size of AVT-ir gPOA cells in fishes was influenced by castration and the development of testes (Semsar and Godwin, 2003) but not by androgen implants (Parhar et al., 2001; Semsar and Godwin, 2003). In addition, AVT immunoreactivity within the tree lizard (*Urosaurus ornatus*) was related to testosterone levels but not territorial aggression (Kabelik et al., 2008). Thus, the androgen sensitivity of the AVT/AVP systems (Goodson and Bass, 2001), raises the possibility that the observed relationship between AVT-ir neuronal phenotypes and aggression is due solely to the action of androgens. At least in the current study, this is unlikely for several reasons. First, both the size and number of AVT-ir cells within the multiband butterflyfish did not differ with either sex or reproductive season (Dewan et al., 2008). Second, both sexes display consistent aggression year-round despite large fluctuations in gonad size, and presumably androgen levels (Tricas and Hiramoto, 1989). Lastly, the aggressive interaction with a mirror did not modulate androgen levels in the cichlid (*Oreochromis mossambicus*) (Oliveira et al., 2005) although this needs to be confirmed in the multiband butterflyfish. It is also unlikely that AVT influences aggression solely through an increase in androgen production (at least in mature testes) (Rodríguez and Specker, 1991; Salek et al., 2002). However, AVT may influence aggression through interactions with serotonin. Within mammals, the action of AVP within the anterior hypothalamus-preoptic area is likely mediated by serotonin (Nelson and Chiavegatto, 2001). Similarly, a selective serotonin re-uptake inhibitor modulates aggression and alters AVT production in all preoptic area cells of the bluehead wrasse (Semsar et al., 2004). Dopamine, histamines, nitric oxide, somatostatin, substance P, and other neuromodulators likely influence aggression and could also potentially influence or be influenced by AVT (Filby et al., 2010; Nelson and Chiavegatto, 2001; Thompson et al., 2008). Thus, while the exact mechanism of AVT's involvement on aggression in fishes is still unclear, one important mechanism may originate from AVT production in the gPOA cell group.

The pPOA cell group consists of a ventral cluster of small cells with one neurite (Dewan et al., 2008) that may be related to stress or subordinate behaviors (Greenwood et al., 2008). The current study found a negative relationship between the number and size of AVT-ir pPOA cells and aggression. In the African cichlid, AVT mRNA expression within this cell group had a negative relationship with dominant behavior and a positive relationship with subordinate behavior (Greenwood et al., 2008). Also the amount of aggression in the rearing environment was negatively related to AVT pPOA size in the Death Valley pupfish (Lema, 2006). Despite very different behavioral paradigms, these three studies indicate that higher production of AVT in the pPOA cell group is likely involved in the inhibition of aggression/dominance through either indirect or direct mechanisms. The complete innervation pattern of the pPOA AVT-ir cell group is unknown. However, since these somata have only one neurite in butterflyfishes (Dewan et al., 2008) and primarily project to the pituitary (Holmqvist and Ekström, 1995), an indirect influence on behavior is likely.

The influence of AVT on stress hormones could be one indirect mechanism that decreases aggressive or dominant behavior. AVT neurons innervate the corticotropin cells of the pituitary (Batten et al., 1990) and could therefore influence cortisol secretion from the interrenal glands. In fact, pPOA cells have higher AVT mRNA expression following acute confinement stress (Balment et al., 2006; Gilchriest et al., 2000). While potential environmental stressors such as changes in salinity influences pPOA cells in the Death Valley pupfish (Lema, 2006), it is only the subspecies of pupfish that lives within a stable environment that showed an increase in the size of pPOA cells in response to a potential environmental stressor (Lema, 2006). Thus, AVT-ir pPOA cells may only be responsive to non-chronic stressors, a hypothesis that is supported by repeated confinement stress in the rainbow trout (Gilchriest et al., 2000).

A separate mechanism for the inhibition of aggression could be AVT's influence on hindbrain substance P. In the goldfish, AVT inhibits social approach through the actions of substance P in a complex peripheral feedback loop and hindbrain circuit (Thompson et al., 2008). AVP projections to the hindbrain occur via the paraventricular nucleus of mammals (Sawchenko and Swanson, 1982), which is thought to be homologous to the pPOA cell group of fishes (Moore and Lowry, 1998). Further tract tracing, immunohistochemical, and behavioral experiments are needed to clarify the possible role of the pPOA cell group on the influence of aggressive behaviors.

Environmental, social, and physiological factors can alter the production of AVT/AVP and thereby potentially influence an individual's behavioral response. The interpretation of AVT's role in aggressive behavior can be dependent upon the context of the social interaction (Goodson et al., 2009; Kabelik et al., 2009). Thus, although the relationship between AVT-ir neuronal phenotypes and aggression appears to be relatively consistent within fishes, the context of the social interaction tested within the current study must be considered.

Environmental factors can influence AVT-ir neuronal phenotypes and potentially fish behavior. For example, alterations in salinity and/or temperature influenced AVT-ir neuronal phenotypes and aggression in the Death Valley pupfish (Lema, 2006). In fact, a fluctuating environment is likely one factor for the larger magnocellular preoptic cells (mPOA/gPOA) in the

non-territorial and less aggressive subspecies of pupfish (Lema and Nevitt, 2004; Lema, 2006). Environmental factors such as temperature and salinity are temporally very stable on the equatorial coral reefs on which these butterflyfish live. Thus in both the current study that used a constant flow through seawater system and in the wild, individual differences in AVT-ir neuronal phenotypes and aggression are likely independent of these environmental factors. However, the removal of these fish from their natural habitat and placement in to an artificial tank environment could result in various stressors that might influence AVT-ir neuronal phenotypes or behavior. Although acute confinement stress altered AVT production in pPOA cells (Gilchriest et al., 2000), prolonged confinement stress (3 weeks) did not change AVT-ir neuronal phenotypes in a closely related butterflyfish species (Dewan and Tricas, in prep). Further, all fish in the current study experienced very similar stress due to capture and holding. Thus, differences in environmental stress are not likely the primary causal factor for the observed relationship between the pPOA cell group and aggression. However individual differences in the sensitivity to stress cannot at this time be ruled out.

Social factors could also affect the observed relationship between aggression and AVT-ir neuronal phenotypes. AVT-ir neuronal phenotypes and AVT's action on behavior can differ with social context (Chu et al., 1998; Goodson et al., 2009; Kabelik et al., 2009; Perrone et al., 2010) and social status (Greenwood et al., 2008; Iwata et al., 2010; Semsar and Godwin, 2003). However, these factors are likely mitigated in the current study due to the experimental design and the test species. One advantage of a mirror stimulus is that it provides a simplified interaction in which some factors (e.g. size, behavior and social status of intruder) are controlled across test subjects. In addition, aspects of the natural social behavior of the multiband butterflyfish may also diminish the effect social factors present in other fish AVT studies. Aggression in this species (males and females) is directed at conspecifics and other food competitors for the purpose of territorial defense and mate guarding (Tricas, 1989; Strang, 2005). This behavior occurs year round and is independent of territory quality (Tricas, 1989). In addition, there is no evidence for a social hierarchy across these monogamous pairs. Although body size in this species was correlated with territory food abundance (Tricas, 1989), there was no relationship between body size and our aggression index in the current study. Thus, to our knowledge there are no social factors within this species that are primarily responsible for the observed relationship between AVT features and aggression.

While this study provides further evidence for a link between aggression and AVT-ir neuronal phenotypes in fishes, several questions remain unanswered. First, what is the role of social context in AVT influenced aggression? The current study analyzed the relationship between AVT-ir neuronal phenotypes and aggression in simplified test paradigm that attempted to control for social context. Future experiments that test this relationship in different (natural) social contexts are needed to fully characterize the role of AVT on aggression. Second, little is known about the neural mechanisms and circuits of AVT influenced aggression in fishes. Results of the current study appear to indicate that AVT-ir cells within the preoptic area are part of this neural circuit of aggression and are related to the output of behavior in an immediate social encounter. However, almost nothing is known about the site of action, release mechanisms, stimulation and potential neuromodulators of these cells. Lastly, what is the role of AVT in the output of behaviors involved with aggression? Aggression is not a singular behavior, rather a sequence of different action patterns and behaviors that are used in different contexts (e.g. the defense of a territory or mate, or as a predator deterrent). The ethogram of the current study indicates some commonality of particular patterns of aggression within this species. However, these patterns were too complex to statistically compare to the AVT system without much larger sample sizes and more controlled experiments. Future experiments which measure AVT release within a specific nucleus before, during, and after a single interaction would be necessary for these comparisons. Such experiments could provide information on whether AVT increases the motivation to perform aggressive behaviors or the likelihood of a particular series of aggressive action patterns. In addition, it should be noted that this study measured only offensive aggression and does not preclude additional relationships between AVT features and defensive, mate guarding, or territorial aggression.

4. Conclusions

The present study provides experimental support (while controlling for context) for the relationship between AVT neuronal features and aggressive behavior hypothesized in prior intra and interspecies AVT comparisons. The amount of aggression in a single interaction with a mirror was positively related to the number of AVT gPOA cells and negatively related to both AVT-ir pPOA size and number. Our simplified experimental paradigm eliminated several confounding factors and provided a simple measure of aggressive motivation and behavior that could be correlated with neuronal features of the AVT system. These results indicate that individual differences in potential AVT production (inferred by neuronal phenotypes) are one factor that may influence levels of aggression produced during an immediate social encounter. Although the neural mechanism is not yet defined, AVT produced within the preoptic area may influence neural circuits of aggression or stress axes through different pathways. Future integrative experiments are needed to determine the mechanisms by which each AVT cell group influences aggressive behavior. AVT-ir varicosities in the ventral nucleus of the ventral telencephalon which was predictive of territoriality in a previous study (Dewan et al., 2011) was not related to offensive aggression as measured in the current study. Thus, AVT within this region may relate to some other behaviors not measured in the current study such as territorial aggression, mate guarding or sociality.

5. Experimental procedures

Adult male and female multiband butterflyfish were collected from the west and south shores of Oahu (HI, USA) with hand and barrier nets. At the time of collection, these fish appeared

to maintain a territory and be part of a monogamous pair. These fish were transported back to the Hawaii Institute of Marine Biology and placed into flow-through seawater aquaria under artificial lights maintained at a 12 hour light/dark cycle and allowed to acclimate for 24 hours. As these fish were acclimated in isolation, each individual was separated from their mated pair for roughly an equivalent period. Fish were fed brine shrimp 3–5 hours after introduction into the tank to counteract potential differences in behavior due to satiation. The 20 gallon aquaria (30"×12"×12") were fitted with a large mirror (12"×12") and an opaque Plexiglas sheet (12"×12") to temporarily hide a stimulus mirror from view.

The mirror was used to visually evoke behaviors from test subjects instead of a conspecific in order to eliminate the potential confounding factor of intruder behavior, to present the subject with a size-matched stimulus and to measure offensive aggression. Preliminary experiments with a conspecific determined that the aggression of the resident was highly influenced by the behavior of the intruder and that all fish were highly responsive to the mirror. Fish do not recognize their own image and attack a stimulus mirror as if it were an intruder (Rowland, 1999). This technique was used successfully in many prior fish aggression studies (e.g. Clotfelter et al., 2007; Gonçalves-de-Freitas and Mariguela, 2006; Ros et al., 2006) and is reported to produce highly reproducible aggression levels (Ruzzante, 1992). While mirror stimuli have many advantages, it also has several limitations. The main limitation is the mirror does not provide the same sensory feedback as a natural interaction. Clearly, a mirror only provides a visual stimulus and lacks the appropriate acoustic and hydrodynamic stimuli, which are both involved in natural agonistic interactions in the multiband butterflyfish (Tricas et al., 2006). In addition, interactions with a mirror prevent the androgen surges normally present in escalated encounters (Oliveira et al., 2005). Despite these limitations, we chose a mirror stimulus in order to determine in a standardize fashion the relationship between AVT and the motivation to perform a specific aggressive behavior. Due to this focus, the results of this study do not provide any direct information on the relationship of AVT with the establishment of dominance or physiological changes due to aggressive interactions.

All fish were tested between 8:30 and 11:00 AM to control for any circadian periodicity in aggressive behavior. A digital video camera on a tripod was placed in front of the tank to record behaviors (30 frames per sec) after the opaque barrier was carefully removed without disturbing the test fish. Behavioral interactions with the reflected image on the mirror were recorded for 25 minutes.

5.1. *Aggressive behaviors*

Several preliminary experiments were run to assess this behavioral paradigm. These experiments were used to develop an ethogram of aggressive behavior in this species (Table 1). This analysis yielded 5 different stereotyped action patterns (bite, tail slap, directed turn, lateral display, and dorsal fin raise) and two approach patterns (slow and rapid approach). All fish performed each one of the stereotyped behaviors at least once after the exposure to mirror stimulus with the exception of one individual that did not perform any dorsal fin raises. In order to determine whether aggressive behaviors occur in a stereotyped order (see below), the start and end of an interaction with the mirror stimulus was defined. The start of the interaction was defined as the approach pattern that the individual performed to move within 3 body lengths of the mirror. The interaction deemed finished if the individual moved more than three body lengths away from the mirror or no behaviors occurred for 5 seconds. The frequency of each behavior as well as the time spent interacting with the mirror were analyzed with a principal component analysis (PCA) using Minitab. The resulting first principal component for each individual was used as an index of aggression. The bivariate scatter plot of the first and second principal component indicates a clear separation of highly aggressive and less aggressive individuals on the first principal component (Fig. 2). The use of an aggressive index has several advantages. First, aggression is not a singular behavior but consists of several action patterns. The PCA analysis provides an index of the overall aggressive level. Second, the ethogram indicates these aggressive behaviors are not isolated interactions but part of a larger response epoch. Thus the index of aggression is a better reflection of the overall aggressive level as compared to the frequency of a specific behavior.

5.2. *Immunohistochemistry*

Test subjects were immediately removed from aquaria after behavioral trials and anesthetized with 100 mg/L of tricaine methanesulfonate (MS-222). Anesthetized fish were measured for standard and total length, and body weight (BW) and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. All fish were perfused with fixative within 4–7 minutes after the conclusion of the behavior experiment. Brains were removed, postfixed in the same fixative at 4 °C for 2 hours, rinsed in 0.1 M phosphate buffer and cryoprotected in 30% sucrose in 0.1 M phosphate buffer overnight. Cryoprotected brains were embedded in Histoprep and sectioned at 24 μm in a transverse plane. Alternative sections were collected on chrom-alum-coated slides, dried at room temperature for 2 hours, and stored at 4 °C until processing. All brains were processed within 2 weeks of behavioral encounter. 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. These slides provided detailed neuroanatomical boundaries necessary for the quantification of AVT-ir fibers within the ventral nucleus of the ventral telencephalon. The other series of alternate brain sections was immunoreacted for the AVT peptide and associated controls according to Dewan et al. (2008).

5.3. *Analysis*

The digital video of each behavior trial was downloaded to a computer and analyzed frame by frame using GOM Player without the use of behavioral software. Videos were scored for the number of each aggressive behavior and the order they

were performed without any prior knowledge of the AVT features. A single order Markov chain analysis (Uncert Software) tested for non-random associations between behavioral patterns in order to determine if a stereotyped order of behavior occurs.

Each individual was analyzed for number and size of AVT-ir cells as well as the number of AVT-ir fiber varicosities within the ventral nucleus of the ventral telencephalon. AVT-ir somata were assigned to either gigantocellular (gPOA), magnocellular (mPOA), or parvocellular (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.). Twenty 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 as other studies (Dewan et al., 2011, 2008; Maruska 2009; Maruska et al., 2007). AVT-ir fiber varicosities 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). The number of AVT-ir varicosities within the ventral nucleus of the ventral telencephalon was analyzed since a comparison of eight butterflyfishes yielded this region as highly predictive of territorial/aggressive species (Dewan et al., 2011). With assistance from the alternative series stained with cresyl violet, exact neuroanatomical boundaries were determined on immunoreacted slides. Subsequently, on every section this nucleus was carefully visually scanned at 400× magnification and the number of AVT-ir varicosities was visually counted.

The relationship between the aggressive index and AVT neuronal features (size and number of gPOA, mPOA, and pPOA) and varicosities within the Vv was examined by stepwise multiple linear regression with an alpha level of 0.15 using Minitab. An alpha level of 0.15–0.20 is recommended for the stopping criteria of this type of statistical test (Lee and Koval, 1997). However, it should be noted that if the stopping criterion is set to an alpha of 0.05, the exact same results were obtained.

A few outliers are present in the relationships between AVT neuronal features and the index aggression. Specifically, one data point in both the number and size of pPOA cells, as well as one data point in the measure of gPOA number. These data extremes were included in the analysis for three reasons. First, these data were re-measured and found not to be due to experimental error. Second, each extreme point is from a different individual. Lastly, these cell parameters are within the normal range of the wild population as measured in previous studies (Dewan et al., 2008, 2011). The exclusion of solely these four data points decreased the p value and increased the coefficient in both the number of pPOA cells (coefficient: −0.0167; T-value: −4.57; $p=0.001$) and the size of the pPOA cells (coefficient: −0.041; T-value: −2.61; $p=0.016$). However, the relationship between the gPOA and the index of aggression was no longer statistically significant ($p>0.05$). All experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Hawaii.

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