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Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish

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Neuropeptides have widespread modulatory effects on behaviour and physiology and are associated with phenotypic transitions in a variety of animals. Arginine vasotocin (AVT) is implicated in mediating alternative male phenotypes in teleost fish, but the direction of the association differs among species, with either higher or lower AVT related to more territorial behaviour in different fishes. To clarify the complex relationship between AVT and alternative phenotype, we evaluated AVT expression in an African cichlid in which social status is associated with divergent behaviour and physiology. We compared AVT mRNA expression between territorial and non-territorial (NT) males in both whole brains and microdissected anterior preoptic areas using transcription profiling, and in individual preoptic nuclei using *in situ* hybridization. These complementary methods revealed that in the posterior preoptic area (gigantocellular nucleus), territorial males exhibit higher levels of AVT expression than NT males. Conversely, in the anterior preoptic area (parvocellular nucleus), AVT expression is lower in territorial males than NT males. We further correlated AVT expression with behavioural and physiological characteristics of social status to gain insight into the divergent functions of individual AVT nuclei. Overall, our findings highlight a complex association between AVT and social behaviour.

Keywords: preoptic area; fish; vasotocin; social behaviour; dominance

1. INTRODUCTION

Animal life histories are often characterized by dramatic phenotypic transitions, such as metamorphosis, puberty, hibernation and migration, where physiology and behaviour change in a coordinated manner. These processes require the brain to integrate various internal and external signals to produce a suite of alterations in behaviour and physiology. Neuropeptides, as broad modulators of physiology and behaviour, have been attractive targets for research into factors that might coordinate widespread phenotypic change. Here, we focus on the neuropeptide arginine vasotocin (AVT) and its role in life-history changes using an African cichlid fish, *Astatotilapia burtoni*, as a model system.

Many teleost fish exhibit reproductive flexibility as adults, including changes in reproductive competence, reversible or permanent changes in gonadal sex or the adoption of distinct reproductive tactics within one sex (Ross 1990; Taborsky 1994). During reproductive transitions, transformations in the state of the gonads are often coupled with changes in appearance and behaviour and other aspects of physiology (Foran & Bass 1999; Bass & Grober 2001; Hofmann & Fernald 2001; Godwin *et al.* 2003; Hofmann 2003). Animals exhibiting these distinct physiological and behavioural profiles are described as displaying alternative phenotypes. Alternative phenotypes are typically associated with variation in social dominance or the control of a territory. Depending on the species, animals adopting a non-dominant phenotype may be reproductively active, such as males displaying the alternative reproductive tactics of satellite or sneak spawning, or can be in a non-reproductive state. In *A. burtoni*, males are found in one of two alternative phenotypes that are linked to both social and reproductive status. Subordinate non-territorial (NT) males have immature gonads containing low levels of mature sperm when compared with territorial (T) males, and they exhibit downregulation of the neural substrates that mediate reproduction (Fraley & Fernald 1982; Francis *et al.* 1993; White *et al.* 2002; Greenwood & Fernald 2004; Au *et al.* 2006). *Astatotilapia burtoni* NT males, unlike T males, only rarely perform courtship or reproductive behaviours and their performance of offensive aggression is suppressed (Fernald & Hirata 1977; White *et al.* 2002). T and NT males can change status rapidly depending on the social environment and will correspondingly change their behaviour and reproductive physiology (White *et al.* 2002;

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Table 1. Summary of previous studies of AVT expression differences as a function of alternative male phenotypes. (Arrows indicate whether a particular measure is higher or lower in territory holders compared with NT, sneaker or satellite males. A dash indicates that a particular population was not examined. For simplicity this table only includes studies that compared different male phenotypes within a single species. ISH, *in situ* hybridization and ICC, immunocytochemistry.)

		AVT population			
species	method	combined	parvocellular	magnocellular	gigantocellular
midshipman; Foran & Bass (1998)	ICC: cell no.	↓ territorial[a]	—	—	—
peacock blenny; Grober *et al.* (2002)[b]	ICC: cell size	—	↓ territorial	no difference	no difference
	ISH: grains/cell	↓ territorial	—	—	—
rock-pool blenny; Miranda *et al.* (2003)	ICC: cell size	—	↓ territorial[a]	↓ territorial[a]	↓ territorial[a]
salmon; Ota *et al.* (1999)[c]	ISH: intensity		↓ breeding	no difference	↑ breeding
zebrafish; Larson *et al.* (2006)[d]	ICC: cell no.	—	↓ dominant	↑ dominant	↑ dominant

[a] Only significant when corrected for body size.
[b] Peacock blenny males defend a territory but do not court.
[c] Authors compare breeding and non-breeding males, but do not describe behavioural differences.
[d] Zebrafish do not normally defend territories, but do form dominance relationships when paired in the laboratory. Parvocellular cells were not found in dominant fish and magnocellular/gigantocellular cells were not found in subordinate fish. Authors did not differentiate between magnocellular and gigantocellular cell populations.

Burmeister *et al.* 2005). In addition to differences in reproduction and aggression, T and NT males also differ in a wide variety of other phenotypic traits (see §4).

AVT has been linked to reproductive plasticity in a variety of teleosts that show distinctive types of alternative phenotypes, and we hypothesized that it may be associated with social status changes in *A. burtoni*. AVT and its mammalian homologue arginine vasopressin were originally identified for their role in the regulation of cardiovascular function, osmoregulation and stress hormone release, but have subsequently been recognized to also mediate social behaviours in many species, including fish (Goodson & Bass 2000*a*; Semsar *et al.* 2001; Salek *et al.* 2002), amphibians (Moore & Miller 1983; Moore 1992; Boyd 1994; Marler *et al.* 1995), birds (de Kloet *et al.* 1993; Maney *et al.* 1997; Goodson 1998*a*,*b*) and mammals (Ferris & Delville 1994; Albers & Bamshad 1999; Wang *et al.* 1999; Wynne-Edwards 2001).

Several lines of evidence implicate AVT in the generation of alternative phenotypes in fish. First, the manipulation of AVT levels affects behaviours that are exhibited predominantly by animals of one phenotype, such as courtship and aggression. AVT administration increases aggression and/or courtship in several species (Semsar *et al.* 2001; Salek *et al.* 2002; Carneiro *et al.* 2003; Santangelo & Bass 2006) and inhibits social behaviours in other species (Goodson & Bass 2000*a*; Bastian *et al.* 2001; Lema & Nevitt 2004*a*). The magnitude or direction of AVT's effect on behaviour can be different for animals of each phenotype, which lends further support for a role for AVT in the production of phenotype-specific behaviours (Goodson & Bass 2000*a*; Semsar *et al.* 2001).

Studies of AVT localization and expression have provided additional correlative evidence connecting AVT to reproductive plasticity, revealing that AVT expression varies in fish exhibiting distinct alternative phenotypes (see table 1). In some species, the courting, aggressive phenotype has more AVT mRNA expression (Godwin *et al.* 2000; Aubin-Horth *et al.* 2007), while in others, dominant, aggressive animals possess smaller AVT-immunoreactive neurons (Grober & Sunobe 1996; Grober *et al.* 2002; Miranda *et al.* 2003) or a lower density of AVT mRNA per cell (Grober *et al.* 2002) than NT males.

Teleost fish have the following three main subpopulations of AVT neurons: the parvocellular; magnocellular; and gigantocellular preoptic AVT nuclei. Although these nuclei have been associated with distinct physiological functions (e.g. parvocellular: stress hormone release (Olivereau & Olivereau 1990; Gilchriest *et al.* 2000); magnocellular: blood pressure, osmoregulation, response to acute stress (Hyodo & Urano 1991; Bond *et al.* 2007); gigantocellular: response to acute stress (Bond *et al.* 2007)), it is not known which population(s) contribute to behavioural modulation. Subsets of cells from each nucleus have projections to the pituitary, within the brain or to both targets (Schreibman & Halpern 1980; Holmqvist & Ekstrom 1995; Saito *et al.* 2004). Higher AVT levels in either the magnocellular or the gigantocellular population have been associated with territorial behaviour in several species. However, in other species territoriality is associated with lower AVT levels in the magnocellular, gigantocellular and/or parvocellular nucleus (table 1).

Thus, AVT is differentially expressed as a function of alternative phenotypes and modulates social behaviour in many fish species. However, the direction of the association between AVT expression and territorial behaviour, and which nucleus varies, does not appear to be conserved (reviewed in Foran & Bass 1999; Bass & Grober 2001; Goodson & Bass 2001). Some of the variability in AVT expression might be due to phenotypic differences unrelated to social behaviour, such as stress physiology (e.g. Lema & Nevitt 2004*b*). Alternatively, these variations may be explained by behavioural or other differences among species, such as differences in social organization (Goodson & Bass 2001). Territory holders from different species vary in their behavioural profiles, for example, in whether or not they perform courtship behaviour, and many of the previous studies of AVT expression focused specifically on species in which the alternative phenotypes were distinctive in some way from those previously examined. Critically, inconsistencies in methodology and anatomical attribution may also contribute to discrepant findings between studies. For example, the relationship between neuron size and number determined using immunohistochemistry and AVT

expression measured using *in situ* hybridization might not be straightforward (e.g. Semsar & Godwin 2003). Additionally, some previous studies only examined a single AVT population (Godwin *et al.* 2000), and there have been some differences in descriptions of anatomy across species (Larson *et al.* 2006).

We sought to evaluate AVT expression as a function of social dominance in all populations of AVT neurons, and to correlate this expression with several measures of each alternative phenotype. We used two complementary approaches to measure mRNA expression, which has a known association with peptide release (Leng *et al.* 1992), unlike measures such as cell size or number. We mined neural transcription profiles to determine AVT expression levels in both the whole brain and within microdissected anterior/medial preoptic areas. We further quantified AVT expression in anatomically distinct preoptic area AVT nuclei using *in situ* hybridization. To gain insight into AVT's potential role in producing alternative dominant and subordinate phenotypes, we correlated AVT expression levels with measurements of reproductive and stress physiology and levels of aggressive and courtship behaviour.

2. MATERIAL AND METHODS

Subjects were from a laboratory population derived from a wild-caught stock of *A. burtoni* (formerly called *Haplochromis burtoni*; Fernald & Hirata 1977). Fish were housed in approximately 120 l aquaria at either Harvard University (transcription profiling) or Stanford University (*in situ* hybridization). Fish in both locations were maintained in aquaria at pH 8.0 and 28°C. Lights were on between 08.00 and 20.00, with an additional 10 min of dim light at dark/light transitions. Fish were fed cichlid flakes and pellets each morning (Aquadine, Healdsburg, CA, and Tetra, Blacksburg, VA).

(a) *Comparison of AVT expression using transcription profiling*

We mined the datasets obtained from two separate transcription profiling experiments, where either whole brains or microdissected anterior/medial preoptic areas from T and NT males were subjected to microarray analysis (Larkins-Ford *et al.* in preparation; Renn *et al.* submitted). For both experiments, two to three weeks after setting up community tanks of 8–10 males and females each, repeated focal observations were performed over a three week period to identify territorial and NT males. Stable T and NT males were chosen according to standard criteria (e.g. Fernald & Hirata 1977; Francis *et al.* 1993). The brains were removed and mRNA from either whole brains or microdissected preoptic areas was isolated and subjected to transcription profiling to compare AVT mRNA expression. Further details of transcription profiling methods are described in electronic supplementary material.

(b) *Comparison of AVT expression using* in situ *hybridization*

Six T and six NT males from community tanks that had maintained a stable status for at least three weeks were moved into tanks consisting of single pairs of T and NT males. Fish were placed into social pairs in order to obtain the maximal reproductive and behavioural suppression of NT males (Fox *et al.* 1997), and to ensure that the opportunities for behavioural interactions were as similar as possible for each male. Tanks ($25\times45\times60$ cm) were divided into two compartments with a transparent, perforated barrier. One T male, one NT male and five females were added to each half of the tank. Neighbouring T males were able to interact through the barrier. Fish were housed in social pairs for 5–7 days; no statistical differences emerged from the length of time in social pairs. Behavioural observations were performed between 12.30 and 13.30 for 2 days before tissue collection. Fish were watched for 3 min each and the performance of several behaviours was recorded: chases and bites towards other fish, threat displays towards other fish, reproductive behaviours (courting females and territory building) and fleeing. Both T and NT males exhibited chases, bites, threat displays and reproductive behaviours, although the levels shown by NT males were typically low (see §3). Only NT males displayed fleeing. The frequency of each behaviour was averaged across observations.

Blood drawing and tissue collection were completed between 15.30 and 16.30. All fish in one tank were caught and blood from the caudal vein was drawn into heparinized butterfly needles within 4 min of capture (Fox *et al.* 1997). The weight and standard length were recorded, then fish were decapitated and their brains were removed, embedded in OCT compound (Sakura Finetek, Torrance, CA), rapidly frozen and stored at −80°C until use. Gonads were dissected and weighed to assess reproductive state using the gonadal somatic index: GSI = gonad weight/body weight × 100.

The methods for *in situ* hybridization and subsequent image analysis are presented in detail in electronic supplementary material. Briefly, following radioactive *in situ* hybridization and autoradiography, we quantified AVT expression by counting the number of silver grains from a portion of each AVT-expressing population, as follows: the parvocellular preoptic nucleus, the magnocellular and gigantocellular portions of the magnocellular preoptic nucleus, and the ventral portion of the lateral tuberal nucleus of the hypothalamus. For the gigantocellular population, where cells were clearly distinct from one another, we also recorded the total number of cells expressing AVT. The slides for several fish (one NT male and two T males) did not have labelled gigantocellular cells. This is probably a result of the fact that AVT-expressing gigantocellular cells are sparsely distributed and few in number, and also that the *in situ* hybridization included only one of three alternate series of brain sections. In one NT subject, the slide containing the hypothalamic population was inadvertently omitted from the *in situ* hybridization.

Blood was collected and centrifuged and plasma was placed in a fresh tube and stored at −80°C until use in enzyme-linked immunoassays (Assay Designs, Ann Arbor, MI) for cortisol and testosterone, a major androgen in this species (Francis *et al.* 1993; Parikh *et al.* 2006; Trainor & Hofmann 2006). All samples were run in triplicate in a single assay for each hormone. The coefficient of variation was 2.1 and 2.9% for cortisol and testosterone, respectively. One NT male was not included in the testosterone assay owing to insufficient plasma.

All statistical analyses were performed in SPSS (Chicago, IL). We used an ANOVA to test for effects of social status on AVT expression and behavioural and physiological measurements. We used Pearson's correlations to describe relationships between variables. After employing a false discovery rate p value threshold (Benjamini & Hochberg 1995), only one correlation remained statistically significant (see §3). However, given our small sample size and the non-independence of these correlational tests, a correction for multiple testing may increase the chance of making a type II error. Therefore, we have reported all exact p values in the text, but have explicitly stated which correlations retain significance. Linear regression lines were added to scatter plots of correlations in order to facilitate the comprehension of the results.

3. RESULTS

AVT mRNA expression levels in whole brains of T and NT males were compared using transcription profiling (Renn *et al.* submitted). AVT expression was significantly higher in T males compared with NT males by approximately twofold ($PP=0.9999$; figure 1).

In a separate transcription profiling study, we examined the gene expression in tissue from the anterior and medial preoptic area that was isolated using laser microdissection (Larkins-Ford *et al.* in preparation). The brain region collected included the entire preoptic area with the exception of the gigantocellular portion of the magnocellular preoptic nucleus (see electronic supplementary material). In contrast to the whole-brain result, AVT expression in microdissected preoptic samples was significantly higher in NT males ($PP=0.9982$; figure 1).

To understand the discrepancy between the whole brain and preoptic area-specific AVT expression profiles, we measured AVT expression in distinct anatomical nuclei using *in situ* hybridization. As expected from the work on other fish species (Goossens *et al.* 1977; Schreibman & Halpern 1980; Van den Dungen *et al.* 1982; Batten *et al.* 1990; Goodson & Bass 2000*b*), AVT-expressing neurons were present in three regions of the preoptic area, as follows: the parvocellular preoptic nucleus and the magnocellular and gigantocellular portions of the magnocellular preoptic nucleus (figure 2). These three populations exhibited high levels of AVT expression, with prominent silver grain clustering detectible after 6 hours of exposure to nuclear emulsion. In addition to the preoptic populations, we identified AVT-expressing neurons in one other population: the lateral tuberal nucleus of the hypothalamus, which was also previously described in the midshipman (Goodson & Bass 2000*b*; Goodson *et al.* 2003). The expression level in this population was much lower than in the preoptic populations. After 6 hours of autoradiographic exposure, hypothalamic expression was undetectable, but was obvious after seven days of exposure (figure 2).

For each AVT population, we quantified the number of silver grains to assess AVT mRNA expression as a function of social status. AVT expression within the parvocellular nucleus was significantly higher in NT males ($F_{1,10}=6.5$; $p=0.029$; figure 3). By contrast, AVT expression in the gigantocellular nucleus was significantly higher in T males ($F_{1,7}=15.8$; $p=0.005$; figure 3). This increase in gigantocellular AVT expression was associated with the observation of more AVT expressing cells within the gigantocellular nucleus of T males (NT: $n=5$, 3.5 ± 0.9 cells; T: $n=4$, 7.5 ± 0.9 cells; $F_{1.7}=9.8$; $p=0.017$). Expression levels in the magnocellular preoptic population and the hypothalamus were independent of social status ($p>0.7$ for both; figure 3).

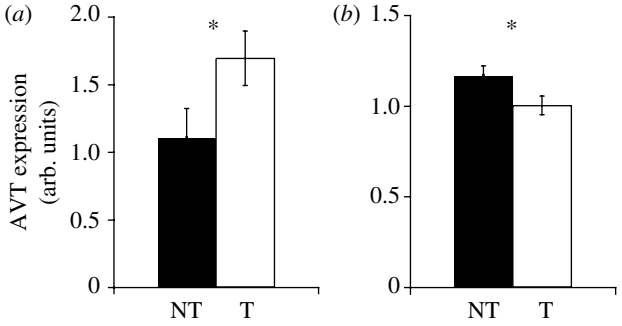

Figure 1. AVT expression in the whole brain and anterior preoptic region varies with social dominance. AVT mRNA expression as a function of social status in (*a*) the whole brain and (*b*) the anterior preoptic area (excluding the gigantocellular portion) is plotted as mean $\pm$s.e.m. T males are shown in open bars and NT males in filled bars. $^{*}PP>0.99$.

To determine how AVT expression in these populations might relate to phenotypic differences between T and NT males, we asked whether silver grain counts in each nucleus covaried with quantitative behavioural and physiological measurements in individual T and NT males. As expected from previous work, T and NT males housed together differed significantly in their type and amount of aggressive and courtship behaviours (data not shown, all $p<0.01$), although some NT males did exhibit low levels of these behaviours. We examined the covariation of AVT expression with behaviour across all T and NT males and within status. In both T and NT males, AVT expression in the parvocellular nucleus was negatively correlated with the production of territorial and reproductive behaviours, and was strongly positively correlated with the tendency of NT males to flee from T males (figure 4, electronic supplementary material, table 1). AVT expression in the gigantocellular population was positively correlated with the production of agonistic and reproductive behaviours (figure 4, electronic supplementary material, table 1). Most of these correlations were not significant when examined in only males of one status, with the exception of agonistic behaviour in NT males, which was strongly positively correlated with gigantocellular AVT expression (figure 4). There was also a significant correlation between gigantocellular AVT expression and agonistic behaviours in T males; however, this was in the opposite direction ($R=-0.978$; $p<0.03$; $n=4$). Following the application of a false discovery rate threshold for multiple tests (see §2), only the correlation between fleeing and parvocellular AVT expression retained statistical significance.

We next asked whether basal levels of the stress hormone cortisol were correlated with AVT expression. Mean cortisol level did not differ as a function of social status (NT: $n=6$, 26 ± 6 ng ml^{-1}; T: $n=6$, 37 ± 13 ng ml^{-1}; $p>0.1$), which is consistent with a previous study that used a dyadic behavioural paradigm (Fox *et al.* 1997). AVT expression in the hypothalamus was significantly positively correlated with cortisol; however, this

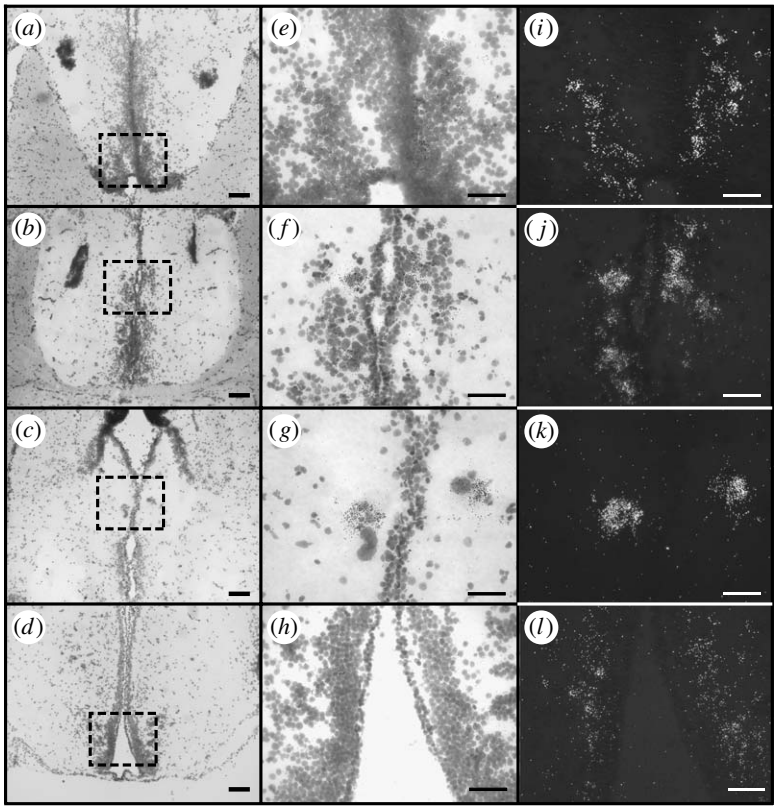

Figure 2. Distribution of AVT mRNA expressing populations in the preoptic area and hypothalamus. (*a*,*e*,*i*) AVT expression in the parvocellular population. (*b*,*f*,*j*) AVT expression in the magnocellular population. (*c*,*g*,*k*) AVT expression in the gigantocellular population. (*d*,*h*,*l*) AVT expression in the hypothalamic population. (*a–d*) low-power brightfield images of the four populations of AVT neurons. (*e–h*) higher power images of the region boxed in (*a–d*), respectively. (*i–l*) darkfield images corresponding to the same area as (*e–h*), respectively. Scale bars in (*a–d*) are 100 μm and in (*e–l*) are 50 μm.

correlation was due to a single subject with extreme values (electronic supplementary material, table 1). None of the regions in the preoptic area showed a significant relationship between AVT expression and cortisol (electronic supplementary material, table 1).

We further examined correlations with measures of reproductive physiology: gonad size (GSI) and circulating testosterone. On average, T males had higher GSI (T: $n=6$, 0.46 ± 0.05; NT: $n=6$, 0.38 ± 0.02) and higher testosterone levels (T: $n=6$, 113 ± 34 ng ml^{-1}; NT: $n=5$, 61 ± 22 ng ml^{-1}) than NT males; however, in contrast to previous studies of *A. burtoni*, these values were not significantly different ($p>0.1$ for both). This appears to partially reflect T males in the present study having smaller gonad size and lower testosterone than T males in previous studies (Francis *et al.* 1993; White *et al.* 2002; Parikh *et al.* 2006; Trainor & Hofmann 2006), although the behaviour of males in our study was typical of T males and quite distinct from NT males (see above). This difference may have resulted from methodological differences between the studies, for example, the use of different housing conditions (community tanks *versus* social pairs). Though testosterone levels did not covary with AVT expression, GSI was significantly positively correlated with the number of silver grains in the gigantocellular nucleus (figure 4; electronic supplementary material, table 1).

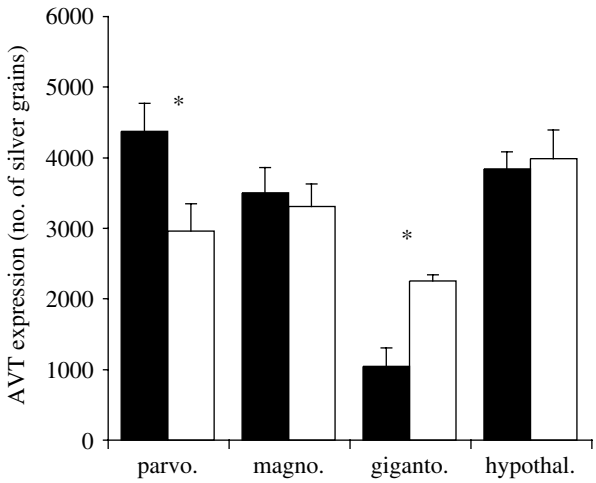

Figure 3. AVT expression levels *in situ* vary with social dominance. Data are plotted as mean ±s.e.m. T males are shown in open bars and NT males in filled bars. Statistical significance at $p<0.05$ is indicated with an asterisk. The number of subjects for each nucleus is as follows: for the parvocellular and magnocellular populations, T ($n=6$), NT ($n=6$); for the gigantocellular population, T ($n=4$), NT ($n=5$); for the hypothalamus, T ($n=6$), NT ($n=5$). Expression levels in the hypothalamic population cannot be directly compared with those in the preoptic nuclei due to differences in exposure time (see §3).

4. DISCUSSION

Variation in AVT neurochemistry is associated with the expression of dominant and subordinate phenotypes in a variety of fish species, but the details of this relationship depend on the species. Here we used independent, complementary methods to reveal that higher AVT mRNA expression is associated with both dominant and subordinate status in a single species, which may

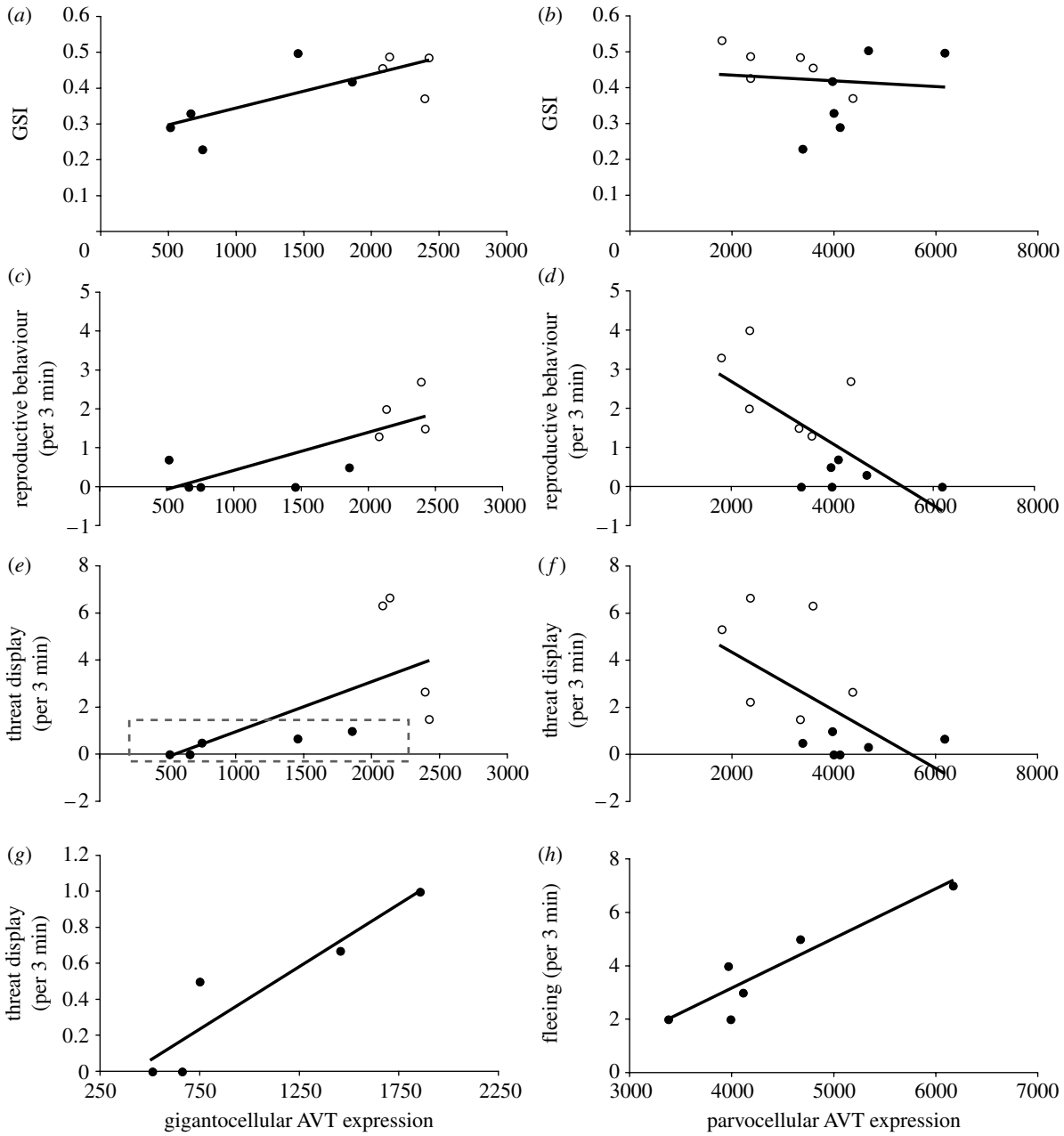

Figure 4. AVT expression is correlated with behaviour and reproductive physiology. Covariation between AVT expression (number of silver grains) in the gigantocellular or parvocellular preoptic area nucleus with (*a*,*b*) gonad size (GSI), (*c*,*d*) reproductive behaviour, and (*e*,*f*) threat displays for both T (unfilled circles) and NT (filled circles) males. (*g*,*h*) Correlation between AVT expression and (g) aggressive behaviour and (*h*) defensive behaviour within NT males only. The plot presented in (*g*) is an expanded view of the boxed portion of (*e*). Correlation statistics are as follows: (*a*) $R=0.745$, $p=0.021$, (*b*) $R=-0.097$, $p=0.76$, (*c*) $R=0.774$, $p=0.014$, (*d*) $R=-0.682$, $p=0.014$, (*e*) $R=0.626$, $p=0.071$, (*f*) $R=-0.558$, $p=0.044$, (*g*) $R=0.925$, $p=0.024$, (*h*) $R=0.985$, $p=0.003$.

provide insight into earlier results. We show that it will be critical to measure AVT expression within individual nuclei in additional species and to link this expression to multiple phenotypes in order to gain a complete picture of the relationship between AVT and alternative male phenotype.

Within the anterior preoptic area, NT males had significantly higher AVT expression than T males when compared using transcription profiling. Based on our *in situ* hybridization data, we attribute this difference to the parvocellular preoptic nucleus. By contrast, when whole brain AVT expression was measured using transcription profiling, T males had higher AVT levels than did NT males. The *in situ* hybridization results indicate that the whole brain expression pattern is reflective of differences within the gigantocellular preoptic population. There were no statistically significant differences in AVT expression as a function of social status in the only two remaining AVT nuclei, as follows: the magnocellular preoptic population and the lateral tuberal nucleus of the hypothalamus, the latter of which showed very low AVT expression levels in general. We did not detect other AVT-expressing regions anywhere else in the brain, despite the use of a highly sensitive radioactive *in situ* hybridization procedure.

Although the results obtained using transcription profiling and *in situ* hybridization vary in the same direction, they do not appear to vary with the same magnitude. More specifically, attempted summation of

the *in situ* hybridization data across AVT nuclei to mimic a whole brain measurement would appear to result in no difference between T and NT males, which is incongruent with the twofold difference obtained using the whole brain microarray data. There are several explanations for this apparent inconsistency. First, the *in situ* hybridization procedure was designed to sample a subsection of each nucleus, not to obtain a total expression level. It is likely that the two studies would be more consistent if the total expression were measured *in situ*; however, we chose a sampling method in order to be confident in the anatomical separation of each nucleus. Second, it is not clear how levels of expression measured using these two methods are related. Another cause of the lack of proportionality between the experiments may be in the behavioural paradigm employed. For the transcription profiling study, fish were housed in community tanks (which results in higher levels of territorial defence behaviours in T males), but were in social pairs for the *in situ* hybridization study. Future work will be required to determine whether the social setting impacts AVT expression levels, although housing condition does influence other physiological factors (Fox *et al.* 1997).

We investigated several aspects of physiology and behaviour to understand how AVT expression is associated with the distinct phenotypes of T and NT males. In *A. burtoni*, a multitude of phenotypic changes are associated with a transition in social status, including appearance and behaviour (Fernald 1977; Fernald & Hirata 1977), reproductive physiology (Fraley & Fernald 1982; Davis & Fernald 1990; Francis *et al.* 1993; White *et al.* 2002; Greenwood & Fernald 2004; Au *et al.* 2006), stress physiology (Fox *et al.* 1997), growth rate and somatostatin function (Hofmann *et al.* 1999; Hofmann & Fernald 2000; Trainor & Hofmann 2007) and gene expression (Burmeister *et al.* 2007; Renn *et al.* submitted). We suggest that the parvocellular and gigantocellular AVT nuclei regulate distinct aspects of a subset of these, and possibly additional unknown, phenotypic differences as a function of alternative male phenotype. In the following paragraphs, we discuss possible explanations for our correlational data and present a model, which we hope will guide future experiments that will test these hypotheses directly.

In the gigantocellular AVT population, we hypothesize that higher AVT is related to the dramatic increase in the production of aggressive and courtship behaviours and/or upregulated reproductive physiology in T males. AVT expression within the gigantocellular population was significantly correlated with aggressive and reproductive behaviours. Even within NT males, which exhibited low amounts of agonistic behaviours in general, the production of agonistic behaviours was associated with higher AVT expression in the gigantocellular nucleus. There was also a significant correlation between gigantocellular AVT expression and gonad size. Strikingly, NT males with higher values of gigantocellular AVT expression had gonads that were indistinguishable in size from those of T males (figure 4). This result is consistent with literature from many different species, demonstrating an interconnected relationship between reproductive physiology and AVT (Foran & Bass 1999; Bass & Grober 2001; Goodson & Bass 2001; Semsar & Godwin 2003; De Vries & Panzica 2006; Marsh *et al.* 2006; Aubin-Horth *et al.* 2007). Our data are consistent with both of the aforementioned hypotheses, which state that AVT is associated with either upregulated behaviour or reproduction or both, and the direction of any causal relationship between these factors remains uncertain.

In the parvocellular preoptic AVT population, we suggest the following two main hypotheses, which are not mutually exclusive: that AVT is involved in generating aspects of subordinate behaviour characteristic of NT males and/or that AVT expression varies depending on the activation of the stress axis, which typically depends on social status. Evidence supporting the first hypothesis is that AVT expression in the parvocellular nucleus covaried strongly with fleeing behaviour in NT males. One interpretation of this relationship is that AVT might be involved in the regulation of defensive, fleeing behaviour or, alternatively, parvocellular AVT may serve to inhibit dominance behaviour towards other males. A possibility not explored in the current study is that parvocellular AVT may facilitate affiliative behaviour, such as schooling. Work in other species has demonstrated a role for vasopressin/vasotocin in mediating social affiliation (Goodson & Bass 2001; Thompson & Walton 2004; Lim & Young 2006). Manipulating levels of AVT in T and NT males may help to distinguish between these possibilities.

Instead of a direct link to behaviour, higher levels of parvocellular AVT expression in NT males may be related to the stress response. Evidence from other fish species has shown that the parvocellular AVT population is involved in the regulation of cortisol release via the actions of AVT on the hypothalamic–pituitary–interrenal axis (Olivereau & Olivereau 1990; Gilchriest *et al.* 2000; Balment *et al.* 2006). Previous work in *A. burtoni* has shown that under stable community housing conditions, NT males have significantly higher basal cortisol levels than do T males (Fox *et al.* 1997), making it important to explore such an explanation. However, the fish in our study were housed in social pairs, resulting in similar levels of basal cortisol among T and NT males, consistent with previous results (Fox *et al.* 1997). Moreover, basal cortisol levels did not correlate with parvocellular AVT expression. It is possible, however, that differences in the stress axis may not be reflected in a single measurement of basal cortisol levels. Also, since these fish were housed in social pairs for only several days, it may be that the variation in AVT expression that we saw is predictive of differences in the stress axis (including circulating cortisol) that may emerge with time as the community stabilizes.

In summary, our results reveal a complex relationship between AVT expression and social status within a single species. We have developed a model for how different AVT populations might act to regulate aspects of dominant and subordinate status in *A. burtoni* (figure 5). This model is based, in part, on known projections of AVT neurons (Schreibman & Halpern 1980; Holmqvist & Ekstrom 1995; Saito *et al.* 2004), along with data on AVT receptor distribution in the pituitary (Moons *et al.* 1989). We expect that this model will allow for the generation of testable hypotheses for how AVT mediates social behaviour in *A. burtoni*. This model may also be applicable to other fish species, such as the zebrafish and masu salmon (see table 1; Ota *et al.* 1999; Larson *et al.* 2006). However, the relationship of AVT expression to

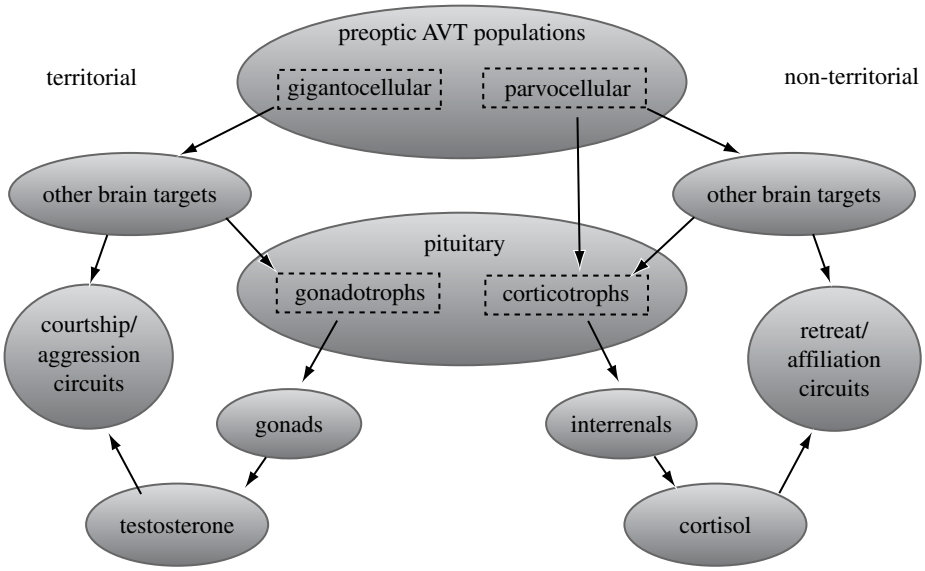

Figure 5. A model of the actions of AVT on alternative phenotypes in *A. burtoni*.

territoriality in *A. burtoni* differs from other species, including the midshipman and two species of blennies (table 1). We suggest that the inconsistent relationship between territoriality and AVT expression in different teleosts probably reflects the many and varied functions of this neuroendocrine pathway on social behaviour and other phenotypes, all of which can vary considerably among species. Males of territorial and NT classes from different species exhibit distinctive combinations of traits, several of which may be regulated by AVT. We suggest that it will be informative to look thoroughly at many individual traits, rather than phenotypic classes, which may be associated with or affected by AVT in additional species. In addition, future studies that selectively manipulate subsets of the AVT circuit will be essential in order to gain a thorough appreciation of how this complex neuromodulatory pathway shapes behaviour and physiology.

Fish were treated in accordance with the Institutional Animal Care and Use Committee regulations at both Harvard and Stanford Universities. The research adhered to the Association for the Study of Animal Behaviour/Animal Behaviour Society Guidelines for the Use of Animals in Research.

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