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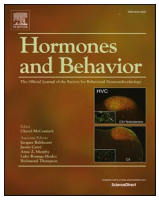

Dominance and aggressiveness are associated with vasotocin neuron numbers in a cooperatively breeding cichlid fish

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ABSTRACT

Within dominance hierarchies, individuals must interact in a rank-appropriate manner, thus behavior and its underlying neural mechanisms must change with social status. One such potential neural mechanism is arginine vasotocin (AVT), a nonapeptide which has been implicated in the regulation of dominance and aggression across vertebrate taxa. We investigated the relationship between social status, dominance-related behaviors, and vasotocin neuron counts in daffodil cichlids (*Neolamprologus pulcher*). Daffodil cichlids live in stable, mixed-sex, cooperatively breeding social groups that are organised into linear dominance hierarchies. Group members of both sexes exhibit complex behavioral repertoires which differ depending on their current social status. We recorded agonistic behaviors within groups of daffodil cichlids and correlated these with the number of AVT cells within the three distinct neuronal populations in the preoptic area of the brain, comparing across social status and sex. We found that parvocellular AVT neurons were more abundant in dominant individuals than subordinates. We also found that numbers of both parvocellular and magnocellular AVT neurons were positively associated with aggression in dominant individuals. AVT neuron counts were unrelated to submissive behavior in subordinate fish. Our data emphasise the role of AVT in modulating status and aggression in social vertebrates.

1. Introduction

Dominance hierarchies are a common feature of animal societies and have significant impacts on individuals' fitness and reproductive success (Sapolsky, 2005; Wingfield and Sapolsky, 2003; Zhou et al., 2018). Dominance is a relative measure and not an absolute property of individuals, and dominance related behaviors must be flexible in response to social context (Drews, 1993; Sapolsky, 2005). Over time, individuals are likely to occupy different ranks within their group due to growth, ageing, or attrition. Animals must thus perform the appropriate behaviors to maintain their current social rank and alter their behavior if opportunities to increase rank arise (Clutton-Brock et al., 2008), an ability known as social competence (Taborsky and Oliveira, 2012). It therefore follows that the neural and physiological mechanisms that regulate dominance-relevant behaviors must be flexible and change with social rank. Variations in endocrine state linked to social status have been widely documented (Bartolomucci et al., 2001; Maruska et al., 2022; Sapolsky, 1982).

Nonapeptides are a conserved class of neuropeptides among vertebrates and are recognised as key regulators of various physiological processes, including cardiovascular function, osmoregulation, and the stress response (Banerjee et al., 2017). Furthermore, nonapeptides play an important role in regulating social behaviors (Balment et al., 2006; Bass and Groberb, 2001; Godwin and Thompson, 2012; Goodson and Bass, 2001; Goodson et al., 2003; Thompson et al., 2006), including behaviors relevant to dominance interactions such as offensive aggression (Ferris and Delville, 1994), social avoidance (Thompson and Walton, 2004), and aggressive responses to perceived threat (De Dreu et al., 2010). Arginine vasotocin (AVT), the non-mammalian homologue of arginine vasopressin, is a nonapeptide which acts as both a neurotransmitter and neuromodulator in the central nervous system of fishes (Godwin and Thompson, 2012; Goodson and Thompson, 2010; Kulczykowska, 2008), birds (Goossens et al., 1977) and amphibians (Moore et al., 2005; Moore and Lowry, 1998). AVT is mainly produced by neurons in the preoptic area (POA) of the anterior hypothalamus, which project to brain areas including the ventral telencephalon, the ventral thalamus, and the mesencephalon (Huffman et al., 2012; Saito et al., 2004), as well as to the neurohypophysis, where AVT is released into the bloodstream to act peripherally (Godwin and Thompson, 2012). Social behavior involves a complex integration of sensory inputs from the

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environment, such as social information and seasonal cues, and internal stimuli related to the endocrine state (Wilczynski, 1992), with the sensorimotor and physiological processes necessary to generate an appropriate profile of complex, species- and sex-specific behaviors. AVT has emerged as a key component in coordinating these complex behavioral outputs across a wide range of vertebrate species (Goodson and Bass, 2001).

AVT-expressing neurons occur in three distinct populations: parvocellular, magnocellular and gigantocellular neurons, and throughout the course of vertebrate evolution, the neuroanatomical locations of these AVT populations and their projections have demonstrated a high degree of conservation (Goodson and Bass, 2001; Moore and Lowry, 1998). While analysis of vertebrate AVT receptor gene sequences has revealed a remarkable conservation of the core-ligand receptor interaction sites (Cho et al., 2007), species differences in social behavior have been associated with intra- or interspecific divergence in AVT neuronal properties, and the expression and distribution of AVT receptors (Almeida and Oliveira, 2015; Goodson and Bass, 2001; Greenwood et al., 2008; Larson et al., 2006; Lema et al., 2012). In fishes, AVT plays a role in mediating social interactions (Almeida and Oliveira, 2015; Dewan and Tricas, 2011; Kulczykowska and Kleszczyńska, 2014; Loveland and Fernald, 2017; Teles et al., 2016), and levels of AVT are associated with variation in both aggressive and submissive behaviors (Almeida et al., 2012; Kleszczyńska et al., 2012; Lema et al., 2015; Perrone and Silva, 2018). Investigations of the different AVT neuronal populations also suggest that they differ in how they regulate dominance and submissive behavior in fishes. In the African cichlid *Astatotilapia burtoni,* positive correlations between gigantocellular AVT expression and the frequency of aggressive and reproductive behaviors were observed in dominant males, and between parvocellular AVT expression and the frequency of fleeing behavior in subordinate males (Greenwood et al., 2008). The number of AVT neurons in the brain is related to the expression of status-dependent social behavior in several fish species. A greater quantity of magnocellular or gigantocellular AVT neurons often correlates with territorial behaviors and social dominance, whereas a larger number of parvocellular AVT neurons has been linked with submission (e.g. zebrafish, *Danio rerio* (Larson et al., 2006), butterfly fishes (Dewan et al., 2008; Dewan et al., 2011; Dewan and Tricas, 2011), pupfish (Lema, 2006)). However, most of the studies investigating the relationship between nonapeptides and social behaviors have examined fish species with relatively unstructured or transient social groups and/or single sex dominance hierarchies. Understanding the role that nonapeptides play in the regulation of social behavior and dominance in species with stable long term social groups and status structuring in both sexes can help to further illuminate the role of these molecules as a proximate substrate for social behavior.

The daffodil cichlid (*Neolamprologus pulcher*) is a cooperatively breeding freshwater fish native to the rocky littoral zone in Lake Tanganyika, East Africa (Balshine-Earn et al., 1998), which exhibits a complex dominance-based social system characterised by frequent social interactions (Balshine-Earn et al., 1998; Balshine et al., 2017; Bruintjes and Taborsky, 2008; Dey et al., 2013; Reddon et al., 2015; Wong and Balshine, 2011a). Daffodil cichlids live and breed in social groups consisting of a dominant breeding pair and 1–20 non-breeding adult subordinates of both sexes who assist the dominant pair in clearing the territory, defending against predators and competitors, and caring for the dominants' offspring (Balshine et al., 2001; Desjardins et al., 2008; Heg et al., 2005). Social status is determined primarily by body size (Reddon et al., 2011) and maintained through the use of aggressive behavior primarily directed by dominants and larger subordinates down the hierarchy, and by submissive behaviors primarily directed by subordinate fish up the hierarchy (Balshine-Earn et al., 1998; Heg et al., 2004; Heg and Taborsky, 2010; Wong and Balshine, 2011a). Vacancies in the dominance hierarchy regularly arise due to predation or dispersal events (Groenewoud et al., 2016; Jungwirth et al., 2023; O'Connor et al., 2015; Wong and Balshine, 2011b), and when these occur in dominant positions, individuals from both within and outside the group may compete for the newly available breeding position (Balshine-Earn et al., 1998; Fitzpatrick et al., 2008; O'Connor et al., 2015; Stiver et al., 2004; Wong and Balshine, 2011b). The contextual link between behavior and status (that is, aggressive when dominant and submissive when subordinate) may be mediated by AVT. For example, previous studies have shown that dominant daffodil cichlids have higher brain AVT gene expression than subordinates (Aubin-Horth et al., 2007), but also that subordinates have higher concentrations of free AVT in the brain compared to dominants (Reddon et al., 2015). Both studies were based on whole brain assays and therefore may obscure the different roles played by the parvocellular vs. magnocellular/gigantocellular cell populations observed in other species (Greenwood et al., 2008; Larson et al., 2006). Describing the AVT neuronal phenotypes in daffodil cichlids will help to clarify the role of AVT in mediating social status and the expression of dominance behaviors in daffodil cichlids, a highly social fish with dominance hierarchies involving both sexes.

We sought to characterise AVT neuronal phenotypes in daffodil cichlids of both sexes and of both dominant and subordinate social status by counting the number of AVT neurons in each of the three cell populations to look for correlations between neuron number, dominance, and agonistic behavior. We recorded the behavior of daffodil cichlids housed in laboratory social groups, and then quantified the number of AVT cells in each neuronal population in the POA. We predicted differences in the number of AVT cells between dominant and subordinate individuals. In particular, based on observations made in other fish species (Dewan et al., 2011; Greenwood et al., 2008; Larson et al., 2006), we expected dominants to have more magnocellular and/or gigantocellular neurons than subordinates, but fewer parvocellular neurons. We also predicted that, among dominant individuals, aggression levels would be positively correlated with the number of AVT neurons, particularly in the magnocellular and gigantocellular populations, whereas in subordinate individuals we predicted that submissive behaviors would be positively correlated with the number of parvocellular AVT neurons.

2. Materials and methods

2.1. Study animals

The research subjects were laboratory reared daffodil cichlids (*Neolamprologus pulcher*) descended from fish captured from Lake Tanganyika, near Kasakalawe point, Republic of Zambia. Prior to the experiment, all fish were kept in mixed-sex groups, at a density of approximately 50 fish per aquarium (105 × 43 × 40 cm, 180 L). Each stock aquarium was equipped with a heater, a thermometer, two powered filters, an air stone, and 3 cm of fine coral sand. Temperature was maintained at 27 ± 1 °C and water chemistry mimicked Lake Tanganyika. The fish were kept on a 12 h:12 h light:dark day cycle, with 15-min of gradual transition from light to dark to simulate sunrise and sunset. The aquaria were regularly checked for water quality parameters (pH, NO_2, NO_3, and NH_3) and weekly water changes were performed. Fish were fed to satiation daily with a mix of dried prepared cichlid foods (Tetra Werke, Germany).

2.2. Social groups

Appropriately sized fish were haphazardly selected from our stock tanks and moved into 90 L aquaria (53 × 43 × 38 cm) to form ten social groups. Each aquarium contained two foam filters, a heater, a thermometer, 2 cm of fine coral sand, and was furnished with 4 terracotta caves which could be used by the fish as breeding substrate as well as shelter, and two floating translucent green PET bottles attached to the aquaria near to the surface of the water, providing additional refuge for the fish. Social groups were kept under the same husbandry regime described above. Each social group included two dominant breeder fish

(male and female) and 6–7 subordinates. Within each group, the dominant breeder pair and the two largest subordinates ("subordinate 1" and "subordinate 2") were the focal subjects for this study. Sex of the dominant breeder fish was determined by examination of the genital papillae and confirmed post-mortem by dissection of the gonads. This was the first time these individuals had acted as dominant fish, but they had prior experience as subordinates in other social groups, as well as in the relatively socially undifferentiated stock aquaria. Many of the subordinates used in this study were too young to be sexed with certainty, and so to avoid any possible misattribution, we decided not to attempt to sex the subordinates. At group formation, the dominant breeding fish and focal subordinates were measured from the tip of the snout to the end of the caudal peduncle (standard length: dominant males (mean ± SE) = 5.29 ± 0.13 cm; dominant females = 4.86 ± 0.14 cm; subordinate 1 = 3.71 ± 0.08 cm; subordinate 2 = 3.48 ± 0.08 cm). Focal subordinates were chosen to be ~3-4 cm in standard length while focal dominants were > 4.5 cm with the dominant male approximately 10 % larger than the dominant female, mimicking natural social groups. To form the social groups, we introduced subordinate individuals into the experimental tanks 24 h before the breeding pairs. We carefully observed groups for the first week after group formation and checked for excess aggression (characterised by persistent chasing and biting behavior from the dominant directed at a single subordinate) or the social rejection of any group members. Two groups that rejected focal fish were dissolved and fish were returned to the stock aquaria, and then replacement groups were formed with new naïve individuals. Stable groups were housed together for at least one month prior to observation for this study and had all successfully spawned at least once (range: 1–2 spawning events). At the time of observation, all groups contained fry (<1 cm standard length) which did not interact with the focal individuals.

2.3. Experimental procedures

We observed each group for four 30-min periods over the course of a week, for a total of 120-min per group. Each 30-min observation was carried out between 10:00–18:00, on different days. Observations were taken by a stationary experimenter seated approximately 1.5 m from the front of the aquarium. A 10-min initial habituation period prior to each observation allowed the fish to acclimate to the presence of the observer. We recorded all interactions between each of the four focal group members (dominant male, dominant female, subordinate 1, subordinate 2) and behaviors directed by them towards the smaller, non-focal subordinates. The behaviors recorded as aggressive included: chases, rams, bites, aggressive postures, and frontal displays. The behaviors recorded as submissive were head up postures and tail quiver displays. For a detailed description of these agonistic behaviors, see Manara et al. (2023). We then calculated the mean number of aggressive behaviors displayed to other social group members per hour for each dominant fish, and the number of submissive behaviors per aggression received for each focal subordinate fish.

After the final observation, the four focal fish in each group were removed from the aquaria and euthanised by immersion in a 300 mg/L solution of the anaesthetic tricaine methanesulfonate (MS222; Sigma-Aldrich, Dorset, UK) buffered to pH 7.4. Once opercular movement ceased, fish were decapitated and brains dissected and placed in a solution of 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at 4 °C for overnight fixation, followed by 24 h cryoprotection in 30 % sucrose in PBS at 4 °C. Brains were then submerged in moulds of OCT compound and snap-frozen in dry ice-chilled hexane, before being sectioned with a Leica CM3050s Cryostat. 30 μm coronal sections were thaw mounted on Superfrost Plus Gold slides (Fisher Scientific, Loughborough, UK), then stored at -20 °C prior to immunohistochemistry.

2.4. Immunohistochemistry process and antibody validation

Slides were thawed and air-dried for 15-min before being outlined with a hydrophobic pen, then washed three times in PBS for five minutes. Nonspecific binding was blocked by incubating sections for 1 h in blocking buffer of 2 % normal goat serum (Vector Laboratories, Peterborough, UK) in PBST (PBS +0.1 % Triton X-100). Sections were then incubated overnight at 4 °C in 1:1000 polyclonal rabbit anti-arginine vasopressin primary antibody (α-AVP; #20069, ImmunoStar, Hudson WI, USA) in blocking buffer. The following day, slides were washed three times in PBS for five minutes and then incubated for 1 h in a 1:1000 solution of Alexa Fluor™ 555 labelled goat anti-rabbit secondary antibody (#ab150078, Abcam, Cambridge, UK) in blocking buffer. Slides were washed three times in PBS, once in PBST, and then briefly dipped in de-ionized water to remove salts. After mounting with Fluoroshield + DAPI mounting medium (Abcam), slides were sealed with nail varnish and then stored at 4 °C in the dark prior to imaging. All incubation steps were performed in a moist chamber to prevent sections drying out.

The α-AVP antibody has previously been used in teleost fish (Marsh et al., 2006; Subhedar et al., 2008) and we validated it for binding specificity against AVT and the closely related nonapeptide isotocin (IT; the teleost fish homologue of oxytocin) in daffodil cichlids before running our study. We pre-incubated 1:1000 α-AVP antibody with either an excess (50 μM) of AVT protein (Bachem, St Helens, UK), an excess (47 μM) of IT protein (Bachem) or an excess of both AVT and IT. We then ran 4 parallel series comparing the staining seen with these three pre-incubated α-AVP solutions with a 1:1000 α-AVP-only solution, following the immunohistochemistry steps outlined above. Typical AVT staining in the POA was observed with the α-AVP-only and the IT pre-incubation solutions but was blocked with the AVT and the AVT + IT pre-incubation solutions, indicating α-AVP antibody was specific for AVT in daffodil cichlids and did not cross-react with IT (Fig. S1).

2.5. Imaging and counting cells

Sections were imaged with a Leica LMD6 fluorescent microscope at 20× magnification using RHO (541-551 nm absorption peak) and DAPI (340-380 nm absorption peak) fluorescence filters. Neurons were discriminated by manually identifying the cell type based on soma size, shape, and localisation (Maruska, 2009). Parvocellular cells (Fig. S2a) were identified as densely packed small monopolar neurons located in the anterior and ventral region of the POA, magnocellular cells (Fig. S2b) as mid-sized neurons (approximately twice the size of the parvocellular cells) in the posterior and dorsal region throughout the POA, and gigantocellular cells (Fig. S2c) as large multipolar neurons (approximately twice the size of the magnocellular cells) in the posterior and dorsal region of the POA. All AVT cell types in the POA were counted manually, and final cell counts were divided by the number of sections to obtain mean cell counts per section for each cell type in each fish.

Due to loss of some sections during tissue processing, one dominant female and one subordinate individual were not quantified, while for another subordinate we gathered data only for gigantocellular neurons.

2.6. Statistical analysis

We checked the effect of body size on AVT neuron counts by fitting separate linear mixed models (LMMs) for dominant males, dominant females, and subordinates, with AVT neuron number as response variable, and standard length and neuron type as fixed factors. For the dominant male and dominant female models, we included social group as a random factor, while for the model for subordinates we included fish ID nested within social group as a random factor. The fixed factors were evaluated using likelihood ratio tests, and there was no effect of standard length on AVT number for any rank (all $p > 0.270$). As

dominance rank is confounded with body size in daffodil cichlids, we therefore did not include body size in our models analysing associations between dominant and subordinate behavior of our focal fish, and the number of AVT neurons.

We evaluated the effect of dominance status on AVT cell counts by fitting LMMs separately for each neuron type, with neuron number as the response variable, dominance rank as a fixed factor, and social group as a random factor. We tested whether AVT cell counts predicted dominant aggressive behavior by fitting separate LMMs for each AVT neuron type with aggressive behaviors per hour as the response variable, sex and neuron number as fixed factors, and social group as a random factor. We used a model simplification approach and dropped non-significant interactions between the fixed factors to obtain minimal adequate models. We tested whether AVT cell counts predicted subordinate submissive behaviors by fitting separate LMMs for each AVT neuron type with submissive behavior per aggression received as the response variable, neuron number as the fixed factor, and social group as a random factor.

All analyses were run with R v4.3.2 (R-Core-Team, 2023) and RStudio v2023.09.1 + 494 (Posit Team, 2023), using the packages "lme4" (Bates et al., 2015) to fit LMMs and "performance" (Lüdecke et al., 2021) to check model fit. Likelihood ratio tests were used to determine the effect of interactions and fixed factors in all models. Where necessary, response variables were log or square root transformed to meet model assumptions. Data and code for all analyses are available at Zenodo (https://doi.org/10.5281/zenodo.11485185).

2.7. Ethical note

Animal housing, handling, and study protocols were approved by the Liverpool John Moores University Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018–4) and adhered to the guidelines of the Animal Behavior Society and the Association for the Study of Animal Behavior. All fish were closely monitored for social exclusion or signs of injury. All observations were drawn from stable social groups showing typical levels of agonism for daffodil cichlids (Balshine et al., 2017).

3. Results

3.1. Dominance status and AVT cell counts

The number of parvocellular AVT neurons ($\chi^2 = 12.194$, $p < 0.001$) was higher among dominant fish (Fig. 1), but not the number of magnocellular neurons ($\chi^2 = 2.375$, $p = 0.123$) or gigantocellular neurons ($\chi^2 = 2.141$, $p = 0.143$).

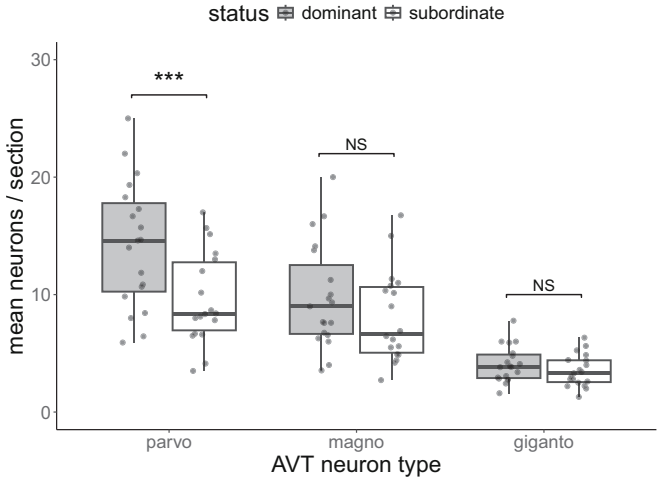

Fig. 1. Mean numbers of neurons per section for parvocellular, magnocellular and gigantocellular AVT neurons in dominant (grey bars) and subordinate (white bars) daffodil cichlids. Dominant fish had more parvocellular AVT neurons than subordinate fish.

3.2. AVT neuron numbers and aggressive behavior

There were no significant interacting effects of sex and AVT neuron number on aggressive behavior, and so these interactions were dropped from the models. Aggressive behavior was positively predicted by the number of parvocellular ($\chi^2 = 5.214$, $p = 0.022$) and magnocellular neurons ($\chi^2 = 6.814$, $p = 0.009$), but not by the number of gigantocellular neurons ($\chi^2 = 0.700$, $p = 0.403$; Fig. 2). There was a significant effect of sex on aggression in all three models (parvocellular: $\chi^2 = 9.788$, $p = 0.002$; magnocellular: $\chi^2 = 9.609$, p = 0.002; gigantocellular: $\chi^2 = 8.642$, $p = 0.003$) as males displayed higher levels of aggression than females.

3.3. AVT neuron numbers and submissive behavior

Submissive behavior was not influenced by AVT neuron number as we did not find any effects of the mean number of parvocellular ($\chi^2 = 1.877$, $p = 0.171$), magnocellular ($\chi^2 = 1.304$, $p = 0.254$) or gigantocellular neurons ($\chi^2 = 0.148$, $p = 0.701$) on the number of submission signals shown per aggression received (Fig. S3).

4. Discussion

We evaluated the status-dependent differences in arginine vasotocin (AVT) neuron numbers in the cooperatively breeding daffodil cichlid, *Neolamprologus pulcher*. We also sought to examine the relationship between dominance-related agonistic behavior and AVT neuron numbers. Based on work in other fish species, we predicted that dominant daffodil cichlids would have more AVT cells compared to subordinate fish, especially among the magno- and gigantocellular neuronal populations. Additionally, we predicted that within the dominant fish, more aggressive individuals would have a greater number and AVT magno- and gigantocellular neurons. We also anticipated that submissive behavior in subordinate individuals would show a positive correlation with parvocellular neuron number.

We found that the dominants had more parvocellular neurons compared to subordinates. Rather than social status per se, this difference could have been driven by differences in age between subordinates and dominants, as dominant fish are generally older. However age correlates with body size in this indeterminately growing species, and we did not find any relationship between body size and AVT neuron counts, arguing against an effect of age. Differences in reproductive status between dominants and subordinates could also drive differences in AVT cell counts given the role of AVT in the in the reproductive axis of fishes (Ramallo et al., 2012) and the fact that subordinate daffodil cichlids may be reproductively suppressed (Fitzpatrick et al., 2006), although low to moderate levels of subordinate reproduction are observed in the wild (Hellmann et al., 2015a). Changes in reproductive physiology and behavior typically accompany changes in status in social species with high reproductive skew, so it is difficult to disentangle the effects of reproductive state from social status (Maruska et al., 2022). Aggressive behaviors in the dominants were positively correlated with the parvo- and magnocellular neuron counts, and sex influenced the behaviors of the fish, with males being more aggressive than females. We found no relationships between submissive behaviors and AVT neuron counts in the subordinate fish.

Among fishes, there is a general pattern for greater AVT expression in dominant individuals compared to subordinates (Maruska et al., 2022; Perrone and Silva, 2016; Solomon-Lane et al., 2022), but the details vary depending on species (Kleszczyńska et al., 2012; Winberg and Sneddon, 2022). For example, In *A. burtoni*, an African cichlid species with a lek based mating system, territorial males exhibit higher expression of AVT

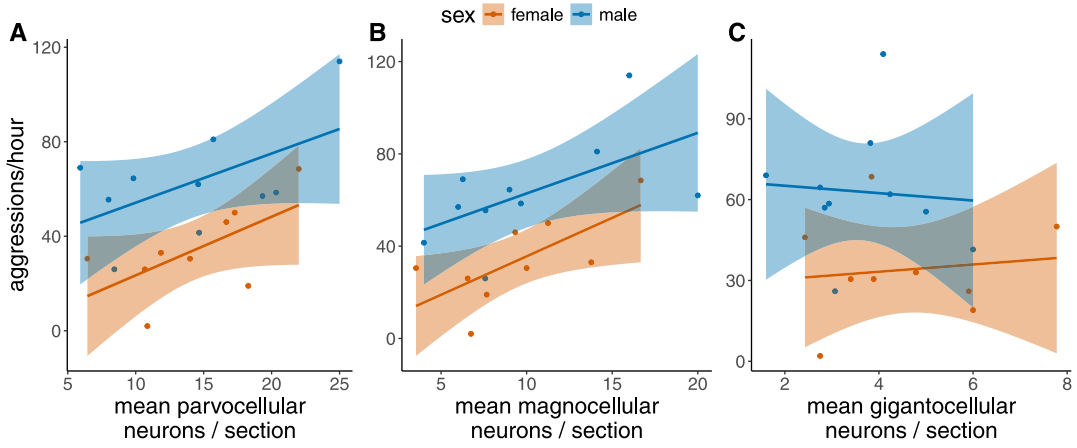

Fig. 2. The relationships between sex and the mean number of (A) parvocellular, (B) magnocellular and (C) gigantocellular AVT neurons on the aggressive behavior of dominant daffodil cichlids. There was a positive association between AVT neuron numbers and aggression levels in the parvocellular and magnocellular populations, and aggression levels were higher among males than females. Solid lines indicate the line of best fit for males and females with 95 % confidence intervals represented by the shaded area around each line.

mRNA in the gigantocellular neurons than non-territorial males (Greenwood et al., 2008). Within established zebrafish dyads, dominant individuals have more numerous magnocellular AVT neurons than subordinates, while subordinates have more numerous parvocellular AVT cells (Larson et al., 2006). Conversely, in Mozambique tilapia (*Oreochromis mossambicus*), subordinate males have a higher number of gigantocellular AVT cells compared to territorial males (Almeida and Oliveira, 2015). Previous studies on daffodil cichlids have found that dominant individuals had higher whole brain AVT gene expression compared to subordinates (Aubin-Horth et al., 2007) and our results are consistent with this finding. In contrast, another study found that subordinate daffodil cichlids had higher levels of bioavailable AVT (i.e., free AVT molecules, available to bind to nonapeptide receptors) in their brains than dominants (Reddon et al., 2015). More recently, a study of daffodil cichlids in the wild showed AVT gene expression in the whole POA was lower in subordinate than dominant fish (Culbert et al., 2024). Measures of gene expression may not always directly correlate with concentrations of the free nonapeptide because of the multiple steps between mRNA production and the end products (Greenbaum et al., 2003; Maier et al., 2009; Vogel and Marcotte, 2012). Variation in gene expression may also correspond to variation in AVT synthesis, while the amount of available peptide may be driven more by differences in storage (Aubin-Horth et al., 2007; Greenwood et al., 2008; Reddon et al., 2015). For instance, dominant and subordinate fish may vary in the extent to which AVT is released into the periphery versus being retained in the brain (Almeida et al., 2012; Reddon et al., 2015). In daffodil cichlids, as in many other cooperatively breeding species (Creel, 2001), dominant individuals show higher cortisol levels than subordinates (Buchner et al., 2004; Culbert et al., 2021; Mileva et al., 2009) which contrasts with the lek breeding *A. burtoni*, in which subordinate fish have higher cortisol levels (Fox et al., 1997). The parvocellular AVT neuron population is involved in the regulation of cortisol release through its actions on the hypothalamic-pituitary-interrenal axis (Balment et al., 2006). Therefore, differential circulating cortisol levels between dominants and subordinates across social systems could partly explain the species differences in parvocellular AVT when comparing dominant to subordinate individuals.

Although AVT has been linked to the modulation of agonistic behaviors in several teleosts (Backström and Winberg, 2017), the role of AVT in controlling behavior can differ among species and social contexts (Silva and Pandolfi, 2019; Teles et al., 2016). In *A. burtoni*, dominant individuals exhibit increased magnocellular AVT activation after engaging in aggressive behaviors (Loveland and Fernald, 2017), suggesting a positive association between aggression and AVT signalling. Similarly, in beaugregory damselfish (*Stegastes leucostictus*), the injection of AVT or a vasotocin receptor antagonist respectively increased and decreased aggression levels (Santangelo and Bass, 2006). In contrast, juvenile rainbow trout (*Onchorhynchus mykiss*) treated with AVT were more likely to lose fights for social dominance, indicating a potential inhibitory role of AVT on aggression in some species (Backström and Winberg, 2009). In our study, dominant daffodil cichlids exhibited a positive correlation between aggressive behavior and the number of parvo- and magnocellular AVT cells.

We did not find any correlation between AVT cell counts and the expression of submissive behaviors in subordinates. These results are consistent with a previous study on this species (Reddon et al., 2015) which did not find a relationship between submissive behavior in subordinates and whole brain free AVT. In contrast, in *A. burtoni*, there is a correlation between submissive behaviors in subordinates and parvocellular AVT mRNA levels (Greenwood et al., 2008). The submissive repertoire of *A. burtoni* is relatively simple, consisting of only fleeing behavior, whereas submissive behavior in the daffodil cichlid includes multiple submission signals and postures (Manara et al., 2023) in addition to fleeing behavior, potentially reflective of the greater social complexity of daffodil cichlid groups. Additional neuroendocrine mechanisms may have been recruited in support of this diversified submissive repertoire in daffodil cichlids compared to species with a more restricted submissive repertoire. The closely related nonapeptide isotocin (IT), the teleost homologue of oxytocin, has been implicated in the production of submissive behaviors in daffodil cichlids (Hellmann et al., 2015b; O'Connor et al., 2016; Reddon et al., 2012), however, none of these previous studies have characterised number of isotocin neurons, but rather have involved pharmacological manipulations, or whole brain measurements of IT gene expression. Characterising the number of IT neurons in daffodil cichlids would be a fruitful area for future work. It is also worth noting that AVT and IT can bind to each other's receptors, so behavioral effects apparently mediated by one of these nonapeptides may also be influenced by binding of the other (Lyu et al., 2021).

Consistent with status differences in AVT, this nonapeptide seems to play a role in regulating social ascension in fishes (Huffman et al., 2015; Semsar et al., 2001). For example, although whole brain AVT expression does not significantly differ between dominants and subordinates in *A. burtoni*, AVT and V1A receptor expression is elevated in ascending males, indicating a role for AVT during the transition to dominance. Blocking vasotocin receptors in ascending males shifts their behavior from aggression to courtship, suggesting that AVT may regulate specific behavioral systems involved in or affected by the transition between social ranks (Huffman et al., 2015). Daffodil cichlids pass through

subordinate status before becoming dominant, and therefore must be able to express both phenotypes over their lifespans. Subordinate individuals must inhibit aggressive behaviors and show submission towards those ranked above them in the hierarchy (Arnold and Taborsky, 2010; Dey et al., 2013; Reddon et al., 2019; Reyes-Contreras et al., 2023). Future work should aim to characterise how AVT neuronal phenotypes change with shifts in social status, and to determine whether these are presaged by differential activation of AVT neurons during social status transitions (Pouso et al., 2024).

In conclusion, we sought to elucidate the role of AVT in mediating social status and the expression of agonistic behaviors by measuring AVT cell numbers in the daffodil cichlid, comparing these across rank and sex, and looking for correlations with dominance-relevant agonistic behavior. Daffodil cichlids are an emerging model system in the study of social behavior, and our study helps to better understand the mechanistic underpinnings of their social organisation. Our findings show that in daffodil cichlids, AVT is closely connected to status in the hierarchy and to aggressive behavior. Our results support an important role for AVT in regulating aggression and dominance across species.

CRediT authorship contribution statement

Tommaso Ruberto: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **William T. Swaney:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Adam R. Reddon:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2025.105677.

Data availability

Data and code for all analyses are available at Zenodo (https://doi.org/10.5281/zenodo.11485185).

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