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Social Status and Arginine Vasotocin Neuronal Phenotypes in a Cichlid Fish

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Arginine vasotocin · Nonapeptides · Social behaviour · Social dominance · Territoriality · Tilapia

Abstract
The nonapeptide arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin play a key role in the regulation of social behaviour across vertebrates. In teleost fishes, three AVT neuronal populations have been described in the preoptic area (POA): the parvocellular (pPOA), the magnocellular (mPOA) and the gigantocellular (gPOA). Neurons from each of these areas project both to the pituitary and to other brain regions, where AVT is supposed to regulate neural circuits underlying social behaviour. However, in the fish species studied so far, there is considerable variation in which AVT neuronal populations are involved in behavioural modulation and in the direction of the effect. In this study, the association between AVT neuronal phenotypes and social status was investigated in the Mozambique tilapia *(Oreochromis mossambicus)*. This species is an African female mouth-brooding cichlid fish in which males form breeding aggregations in which dominant males establish territories and subordinate males to act as floaters. With respect to sex differences in AVT neuronal phenotypes, females have a larger number of AVT neurons in the pPOA and mPOA. Within males, AVT appeared associated with social subordination, as indicated by the larger cell body areas of AVT neurons in mPOA and gPOA nuclei of non-territorial males. There were also positive correlations between submissive behaviour and the soma size of AVT cells in all three nuclei and AVT cell number in the mPOA. In summary, the results provide evidence for an involvement of AVT in the modulation of social behaviour in tilapia, but it was not possible to identify specific roles for specific AVT neuronal populations. The results presented here also contrast with those previously published for another cichlid species with a similar mating system, which highlights the species-specific nature of the pattern of association between AVT and social behaviour even within the same taxonomic family.

Introduction

The nonapeptides arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) play a major role as central neuromodulators involved in the expression of social behaviour in a wide range of vertebrates [Goodson, 2008; Goodson and Thompson, 2010]. However, both considerable conservation and diversity have been documented in the mechanisms through which these nonapeptides mediate social behaviour. Two ancestral AVT/AVP-immunoreactive (ir) cell groups (one magnocellular and one parvocellular, corresponding to the supraoptic and paraventricular nuclei

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in amniotes, respectively) have been consistently observed in the preoptic area (POA) and hypothalamus of all vertebrates [Goodson and Bass, 2001]. Cells from these nuclei project to the pituitary, where AVT/AVP stimulate adrenocorticotropin release (in the anterior lobe) or is itself released into the bloodstream (at the level of the posterior lobe) becoming a neurohormone that will act in peripheral target tissues to regulate body states, and to the midbrain tegmentum and hindbrain where AVT/AVP regulates autonomic functions and stereotypical species-specific action patterns (e.g. social withdrawal in goldfish [Thompson et al., 2008; Thompson and Walton, 2009]; for general reviews see Goodson and Bass [2001] and Goodson and Thompson [2010]). A third AVT/AVP-ir cell group located in the bed nucleus of the stria terminalis and projecting to the lateral septum and basal forebrain regions has evolved in tetrapods [Goodson and Bass, 2001; Goodson and Thompson, 2010]. This bed nucleus of the stria terminalis/lateral septum nucleus has been implicated in the regulation of more complex and flexible social behaviours, such as social recognition, aggression and affiliative behaviours, in mammals and birds [Donaldson and Young, 2008; Goodson et al., 2012].

In teleost fish, extra-preoptic/hypothalamic AVT cell groups are absent and therefore the question arises of how the primitive parvocellular and magnocellular AVT nuclei regulate complex social behaviours that are also present in this taxon. Interestingly, in many teleost species, the preoptic magnocellular nucleus appears further subdivided into three parts: the pars parvocellularis (pPOA), which is a more anterior and ventral group of smaller cells in larger numbers that extends from the parvocellular nucleus; the pars magnocellularis (mPOA), which is a group of larger cells that follows caudally and slightly more dorsally from the pPOA, and a group of larger and fewer multipolar cells which lie dorsal to the mPOA, termed the pars gigantocellularis (gPOA) [Bradford and Northcutt, 1983]. These regions correspond to the pPOA, mPOA and gPOA AVT-ir cell groups in teleost fishes. In some species, the parvocellular nucleus also has AVT-ir cells, which are usually considered part of the pPOA. Therefore, the pPOA AVT-ir cell cluster does not correspond to neuroanatomical boundaries. Despite the increasing evidence for a role of AVT on fish social behaviour coming both from descriptive studies that correlate AVT neuronal phenotypes with behaviour and from experiments that study the impact of AVT manipulations on behaviour, it is not yet clear which neuronal population(s) is (are) involved in the regulation of social behaviour [Godwin and Thompson, 2012]. For example, the association between AVT neuronal phenotypes and the expression of status-dependent social behaviour in fish does not appear to be conserved. In most teleost species studied so far, the expression of territorial behaviour and/or social dominance is associated with the higher number or size of AVT-ir cells in mPOA or gPOA, whereas non-territorial behaviour and/or social submission have been associated with the number or size of pPOA AVT-ir cells (e.g. zebrafish, *Danio rerio* [Larson et al., 2006]; African cichlid, *Astatotilapia burtoni* [Greenwood et al., 2008] and butterfly fishes [Dewan et al., 2008; Dewan and Tricas, 2011; Dewan et al., 2011]. However, in species with alternative reproductive tactics, where large and territorial and small and parasitic male phenotypes occur, the larger territorial phenotypes usually have less or smaller POA AVT-ir cells after correcting for the body size difference between the two male types, e.g. pPOA in peacock blenny, *Salaria pavo* [Grober et al., 2002], or all POA AVT nuclei (e.g. rock-pool blenny, *Parablennius sanguinolentus* [Miranda et al., 2003] and plainfin midshipman, *Porichtys notatus* [Foran and Bass, 1998]). Also, in the pupfish *(Cyprinodon nevadensis)*, where variation in aggressive behaviour has been documented between two allopatric populations, males from the more aggressive population have smaller pPOA AVT-ir cells than those from the less aggressive one [Lema, 2006]. An explanatory hypothesis for this diversity in the patterns of association between AVT and social dominance in fish is still missing.

Here, we studied the association between AVT neuronal phenotypes and the expression of territorial behaviour in an African cichlid species (Mozambique tilapia, *Oreochromis mossambicus*) that exhibits a high level of behavioural flexibility with the expression of social status-dependent behaviour within males [Oliveira and Almada, 1998a]. In this species, males form dense breeding aggregations during the reproductive period in which two male types can be recognized: (1) Territorial (T) males establish and defend a territory centred on a spawning pit that they dig with their mouth; they exhibit a typical dark nuptial colouration and actively court females trying to lead them to their spawning pit. (2) Non-territorial (NT) males that hover around inside the breeding aggregation but lack a territory and adopt a pale silver colouration similar to that of females; NT males are frequently courted by T males and respond with typical female-like behaviour, which enables them to stay inside the breeding aggregations and to sneak fertilizations

[Oliveira and Almada, 1998a]. NT males are subordinate, but if an opportunity to establish a territory arises, they can rapidly establish a territory and display dominant behaviour [Oliveira and Almada, 1996, 1998b]. The high behavioural flexibility present in this species makes it an excellent model to investigate the association between different AVT neuronal phenotypes and social behaviour. Furthermore, given the information on the relationship between AVT neuronal phenotypes and social status already available for another cichlid species with a similar mating system [Greenwood et al., 2008], the results from this study will help to clarify if there is a conserved pattern of association between these neuropeptides and social behaviour or if it is species specific.

The specific goal of this study is to investigate if there is a specific AVT neuronal population associated with the expression of status-dependent behaviour (i.e. aggressive vs. submissive behaviour). For this purpose, the individual behaviour of males from a different social status (T vs. NT) was recorded and the AVT neuronal populations were characterized in the same individuals. The neuronal AVT phenotypes of females were also characterised, to be used as a reference group in the comparison between the two male types, since subordinate males are known to mimic female behaviour [Oliveira and Almada, 1998a]. Steroid hormones (androgens and cortisol), which may modulate eventual feedbacks of the social status on AVT neuronal phenotypes, were also studied.

Materials and Methods

Animals and Housing

Fish used in this study came from a stock of Mozambique tilapia held at the ISPA – Instituto Universitário (Lisbon, Portugal), which were maintained in stable social groups of 4 males and 5 females per group in glass tanks (120 × 40 × 50 cm, 240 litres). Tanks were supplied with a double filtering system (sand and external biofilter; Eheim) and constant aeration. The presence of substrate in the tanks allows for the nest construction by T males. Water quality was monitored on a weekly basis for nitrite (0.2–0.5 ppm), ammonia (<0.5 ppm; Pallintest kit®) and pH (6.0–6.2). Fish were kept at a temperature of 26 ± 2°C and a 12-hour light:12-hour dark photoperiod, and fed with commercial cichlid floating and sinking sticks. The social status of the males in each stock tank was monitored. Territorial status was determined by body colouration and the possession of a spawning pit on the substrate [Oliveira and Almada, 1996]. To avoid a potential source of confounding bias in the previous social status, only T males were selected from the stock tanks for this experiment.

Experimental Procedure

Six males (which were territorial in the home stock tank) and 4 females were removed from their stock tanks and introduced simultaneously in new experimental aquaria (120 × 40 × 50 cm, 240 litres; same housing conditions as those of stock tanks). These new experimental groups (n = 5 groups) remained undisturbed for 7–8 weeks in order to allow for a new social status to be established and for dominance hierarchies to stabilize. Thirteen males that maintained their social status (either T or NT) for at least 5 consecutive weeks (out of a maximum of 8 weeks) were removed from the experimental tanks and sampled [mean ± SD; T males: body weight = 56.61 ± 15.78 g, standard length (SL) = 11.99 ± 0.99 cm, n = 7; NT males: body weight = 42.09 ± 9.98 g, SL = 10.77 ± 0.95 cm, n = 6]. Four females (mean ± SD; body weight: 32.37 ± 6.03 g; SL: 10.10 ± 0.63 cm) were also sampled in order to provide a reference for the males. Immediately after removal from the experimental tanks, fish were anaesthetised, a blood sample was collected, and fish were then killed and brain tissue collected. These procedures are described in detail below.

Behaviour Sampling

Male social status, based on the dark nuptial colouration and the defence of a nest in the substrate, was monitored in the experimental tanks on a daily basis. After social status became stable, on the 1st or 2nd week after group formation, 2-min focal observations were performed once a week using a behavioural sampling with a continuous recording method [Martin and Bateson, 2007]. The ethogramme provided by Baerends and Baerends-van Roon [1950] was used to identify the relevant behavioural patterns, and male-male behavioural interactions were noted in order to measure male aggressive (i.e. bite and chasing) and submissive behaviour (i.e. escape). Female behaviour was not recorded, since this study was focused on the differences between T and NT males. However, ad libitum behavioural observations confirm previous descriptions of female behaviour in this species [Oliveira and Almada, 1998c] in that they stay in the water column and only come to the substrate when courted by T males.

Blood Sampling and Steroid Radioimmunoassay

Each blood sample was collected from the caudal vein (using 1-ml syringes with 25-gauge/16-mm needles) under anaesthesia (MS-222; Pharmaq; 300–400 ppm). Blood sampling and the start of perfusion (see below) always took less than 4 min from the induction of anaesthesia, which prevents possible confounding of handling stress on cortisol levels [Foo and Lam, 1993]. Blood was centrifuged (10 min; 600 *g*) and the plasma was stored at –20°C until further processing. Plasma samples were denatured in a dilution of 1:10 in gelatine buffer [used in radioimmunoassay (RIA)] at 80°C for 60 min in a dry bath. Steroid concentrations were measured by RIA. Commercially available antibodies and marked hormones were used for cortisol (rabbit anti-cortisol, ref. 20-CR50; Interchim, Fitzgerald; 1,2,6,7–^{3}H cortisol; Amersham Biosciences, ref. TRK407-250UCI) and testosterone (rabbit anti-testosterone; Research Diagnostics Inc., ref. WLI-T3003; 1,2,6,7-^{3}H testosterone, Amersham Biosciences, ref. TRK402-250mCi) RIA. A custom-made antibody, kindly donated by D.E. Kime (the specificity table for this antibody has been published in Kime and Manning [1982], and tritiated 11-keto-testosterone produced in-house from marked cortisol [Kime and Manning, 1982], were used for the 11-keto-testosterone RIA. All samples were run in a single assay, and intra-assay variability was 6.3% for cortisol; 5.6% for testosterone, and 5.2% for 11-keto-testosterone.

Tissue Preparation

After blood sample collection, fish were returned to the anaesthetic solution until opercular movements ceased. They were then perfused transcardially with 0.9% heparinised 0.1 M phosphate-buffered saline solution (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS for 30 min. Brains were then removed, post-fixed in 4% paraformaldehyde for 1–2 h, transferred to 30% sucrose in 0.1 M PBS and kept at 4°C overnight.

Immunocytochemistry

Cryoprotected brains were embedded in Tissue-Tek® (optimal cutting temperature compound), frozen at –80°C and sectioned with the cryostat at 20 μm in the coronal plane. Two series of silane-treated glass slides (Sigma) were used to collect alternate brain sections which were stored at –80°C until further processing. One of the alternate series was immunoreacted following a protocol adapted from Grober et al. [1991]. On day 1, slides were incubated at room temperature and tissue sections were surrounded with a hydrophobic barrier (PAP pen; Sigma) covered with 0.1 M PBS (pH 7.4) for 7 min and blocked with 0.4% Triton® X-100 (Sigma) in PBS with 2.8% goat serum (Sigma) for 20 min followed by 3% H_2O_2 in 0.1 M PBS for endogenous peroxidase blockage. After two additional washes (7 min each) with 0.1 M PBS, the primary AVT antibody was applied to the mounted section at a final dilution of 1:10,000 and incubated overnight (20–24 h) in a sealed humidified chamber at 4°C. This AVT antibody (kindly donated by Dr. Matthew Grober, Georgia State University, USA) has already been used successfully in similar studies with different teleost species (e.g. butterfly fishes, *Chaetodon* spp. [Dewan et al., 2008]; halfspotted goby, *Asterropteryx semipunctata* [Maruska et al., 2007], and cleaner wrasse, *Labroides dimidiatus* [Mendonça et al., 2013]). On day 2, after two 0.1 M PBS washes (7 min each), mounted sections were incubated with the biotinylated goat anti-rabbit secondary antibody (KPL) for 30 min, followed by two additional washes (7 min each) and incubation with peroxidase-labelled streptavidin (KPL) for another 30 min. After two additional 0.1 M PB washes of 7 min, the slides were reacted with a diaminobenzidine chromogen peroxidase substrate kit (Vector Laboratories Inc.) for 3–6 min or until golden brown colouration was achieved (according to the manufacturer's instructions). The reaction was then stopped by immersing the slides in distilled water, followed by dehydration in an ethanol series (70, 85, 95, and 3 × 100%; 1 min each bath, with the exception of the last bath: 2 min) and clearance in xylol (Sigma; two baths: first 1 min and then 5 min). Finally, slides were coverslipped with Cytoseal 60 mounting media (Richard-Allan Scientific). The specificity of this antibody for AVT was assessed by investigation of non-specific labelling of the neurons in a second alternate series. The tissue was immunoreacted after pre-adsorbing the antibody with 8 μM AVT peptide (catalogue No. V0130; Sigma) overnight at 4°C. All the other steps remained the same. No stained neurons were observed, thus confirming the specificity of the antibody used. Other studies had already shown this antibody specificity for different species, including another cichlid species [Maruska et al., 2007; Dewan et al., 2008; Maruska, 2009; Mendonça et al., 2013].

Quantification

Cell counting and measurements were done following a blind protocol so that neither sex nor social status of the specimen was known during the quantification process. AVT-ir cell bodies were only detected in the POA, and, therefore, all quantifications were done in this region. Each AVT-ir cell was assigned to either the pPOA, mPOA or gPOA cell group based on neuroanatomical location, cell size and cell morphology (following Bradford and Northcutt [1983]). This nomenclature has been widely used in similar studies [Semsar and Godwin, 2003; Lema, 2006; Maruska, 2009; Mendonça et al., 2013]. Digital images were captured at a magnification of ×400 with a digital camera (Olympus C-2020 Z) attached to a microscope (Olympus BX50). After calibration for magnification, the cell profile was traced and the cell body area was automatically calculate using ImageJ software [Rasband, 2002]. Cell numbers were determined by visually counting the cells belonging to each cell group with the microscope. The criterion for counting a cell was the presence of a neurite attached to a discernible perimeter from the background, except for pPOA cells where the neurite was not visible in a few cells.

Data Analysis

Behavioural data did not conform to parametric assumptions, and therefore the comparisons of aggressive and submissive behaviour between T and NT males were performed using the non-parametric Mann-Whitney U test.

Because T males were significantly larger than both NT males and females (T males vs. females: $t = 3.39$; $p = 0.008$; T males vs. NT males: $t = 2.25$; $p = 0.046$; NT males vs. females: $t = 1.22$; $p = 0.258$), body size could be a confounding variable in the relationship between socio-sexual phenotype and cell number or cell size. Therefore, SL was used as a covariate in our analyses. Given that different cell nuclei within the POA cannot be seen as completely independent from each other, we used multivariate analysis of covariance (MANCOVA) with three dependent variables (pPOA, mPOA and gPOA), an independent categorical variable (socio-sexual phenotype: T male vs. NT male vs. female) and a covariate (SL). Cell numbers and cell body areas were not correlated and were analysed independently (i.e. one MANCOVA for each). Post hoc LSD tests were used to compare cell measurements between different socio-sexual phenotypes within each cell group. Planned comparisons were used to test for sex differences (T males + NT males vs. females).

One-way ANOVA was used to test the effects of socio-sexual phenotypes on circulating hormone (testosterone, 11-keto-testosterone and cortisol) levels. Differences between each socio-sexual phenotype were subsequently investigated using planned comparisons.

Putative relationships between behavioural, hormonal and AVT-ir cell variables were assessed using partial correlations, controlling for SL and the whole male data set (i.e. T and NT males together, $n = 13$). All tests are two tailed at a significance level of $p < 0.05$. All statistical analyses were run on the software package STATISTICA (version 10.0; StatSoft, Hamburg, Germany).

Results

Behaviour of T and NT Males

T males exhibited the nuptial dark colouration typical of breeding males of this species and mouth digging, and defended a nest in the substrate for at least 5 consecutive

weeks during the 8-week period of the study. Behavioural observations showed that only T males exhibited aggressive behaviour (Mann-Whitney U test: Z = 2.93, p = 0.003), and that escape behaviour was mainly exhibited by NT males (Mann-Whitney U test: Z = –2.93, p = 0.003; fig. 1). Thus, T males exhibited a dominant behavioural phenotype and NT males a subordinate one.

Brain Distribution of AVT-ir Neurons

AVT-ir cells were exclusively found in the POA. Three cellular populations of AVT-ir cells could be recognized based on cell size (repeated-measure ANOVA: $F_{2, 32}$ = 344.14, p < 0.0001; post hoc tests: gPOA > mPOA > pPOA at p < 0.05), cytoarchitecture and localization within the POA. Parvocellular cells are the smallest ones (T males = 38.1 ± 10.3 μm^2; NT males = 48.6 ± 9.4 μm^2, and females 41.4 ± 14.6 μm^2) and are mainly monopolar with some

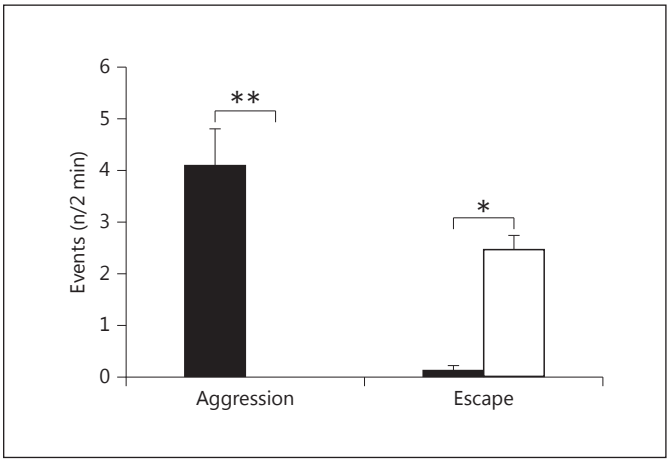
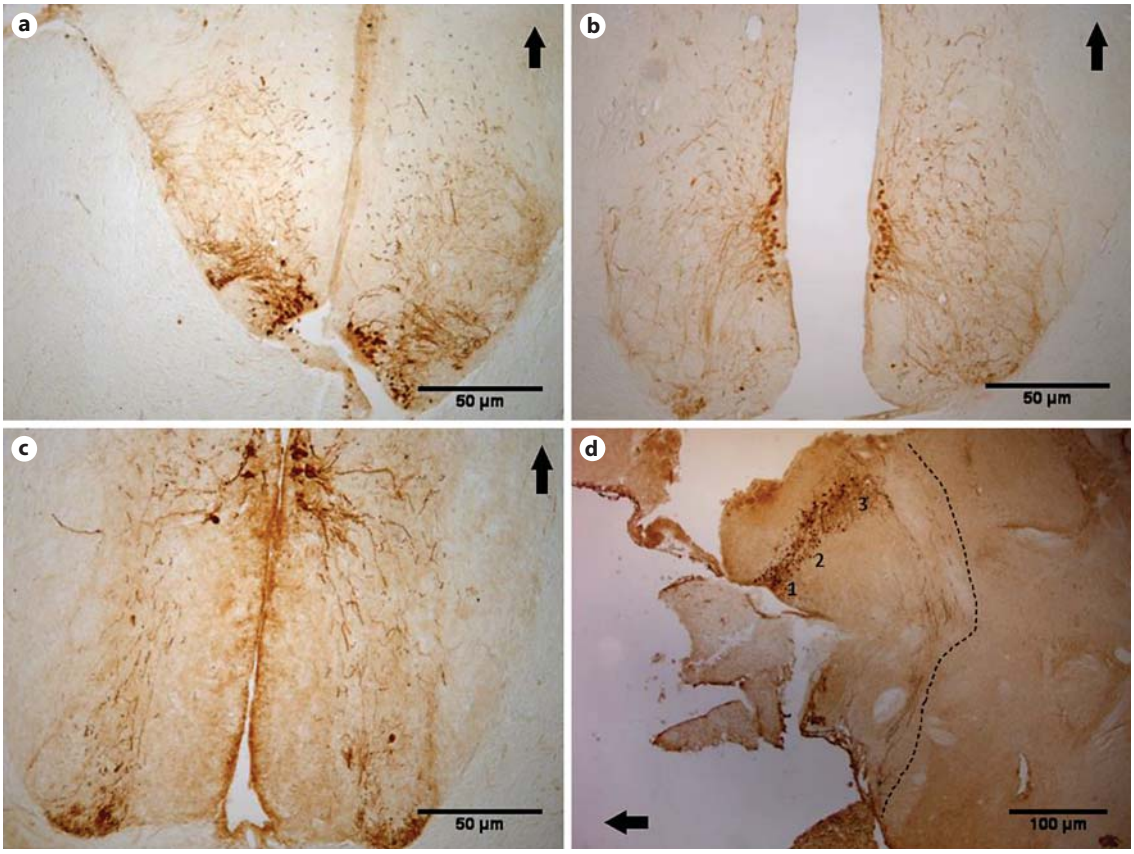


Fig. 1. Effects of territorial status [T males (black bar) vs. NT males (white bar)] on the aggressive and submissive behaviour of *O. mossambicus* males. ** p < 0.01 (Mann-Whitney U test).



Color version available online

Fig. 2. AVT-ir neurons in the brain of *O. mossambicus*. Coronal view (magnification ×100) of pPOA (**a**), mPOA (**b**) and gPOA (**c**) AVT cell nuclei (arrow indicates dorsal side). **d** Sagittal section (arrow indicates rostral side) through the POA showing AVT-ir fibres and cell bodies of parvocellular (1), magnocellular (2) and gigantocellular (3) neurons (dashed line delimits the diencephalic area).

occurrences without an obvious neurite. They start to appear at the more rostral part of the POA apposed to the third ventricle, and thus are the most rostral and ventral AVT-ir cell group (fig. 2b–d), and extend dorsocaudally until magnocelullar cells start to appear. Magnocellular cells have a soma that is approximately twice the size of that of parvocellular cells (T males = 64.1 ± 6.8 μm^2; NT males = 91.3 ± 11.7 μm^2, and females 79.5 ± 18.9 μm^2), occupy a medial location in the POA (fig. 2b) that extends caudally lying against the third ventricle and have one prominent axon projecting towards the pituitary (preoptico-hypophyseal tract). Finally, gigantocelullar cells have the largest cell bodies, which have an area that is more than double that of magnocelullar cells (T males = 191.6 ± 28.7 μm^2; NT males = 222.5 ± 30.1 μm^2, and females = 173.1 ± 17.1 μm^2), and are usually multipolar, having many surrounding fibres extending in/from different directions. However, it is clear that most fibres of this cell group project towards the pituitary (fig. 2). The gPOA cell group is located in a more dorsal position and was less numerous than either pPOA or mPOA AVT-ir cell groups (repeated-measure ANOVA: $F_{2, 32} = 111.30$, $p < 0.001$; post hoc tests: pPOA > mPOA > gPOA at $p < 0.05$). Fibres of the three neuronal AVT-ir groups seem to project in different directions (fig. 2).

Effect of Socio-Sexual Phenotype on Preoptic AVT-ir Cell Numbers

MANCOVA showed no effect of the socio-sexual phenotype (Wilks $\lambda = 0.45$, $F_{3, 11} = 1.78$, $p = 0.150$) and a marginally non-significant effect of SL (Wilks $\lambda = 0.53$, $F_{3, 11} = 3.20$, $p = 0.066$) on the number of AVT-ir neurons. The lack of multivariate outcome in respect to the socio-sexual phenotype was confirmed for the mPOA and gPOA cell groups ($F_{3, 13} = 1.73$, $p = 0.209$ and $F_{3, 13} = 1.60$, $p = 0.239$, respectively), but there was a marginally non-significant multivariate effect for pPOA ($F_{3, 13} = 3.32$, $p = 0.054$).

Post hoc analyses of the univariate outcomes adjusted for body size showed that T males have significantly fewer mPOA AVT-ir neurons than females (fig. 3a; table 1) and marginally non-significant fewer gPOA AVT-ir neurons than NT males (fig. 3a; table 1).

Overall sex differences were investigated using planned comparisons [(T males + NT males) vs. females] of the univariate outcomes adjusted for body size. These revealed that females have significantly more AVT pPOA cells and marginally non-significant more AVT mPOA cells than males (pPOA: $F_{1, 13} = 8.34$, $p = 0.013$; mPOA: $F_{1, 13} = 3.83$, $p = 0.072$, and gPOA: $F_{1, 13} = 0.43$, $p = 0.521$).

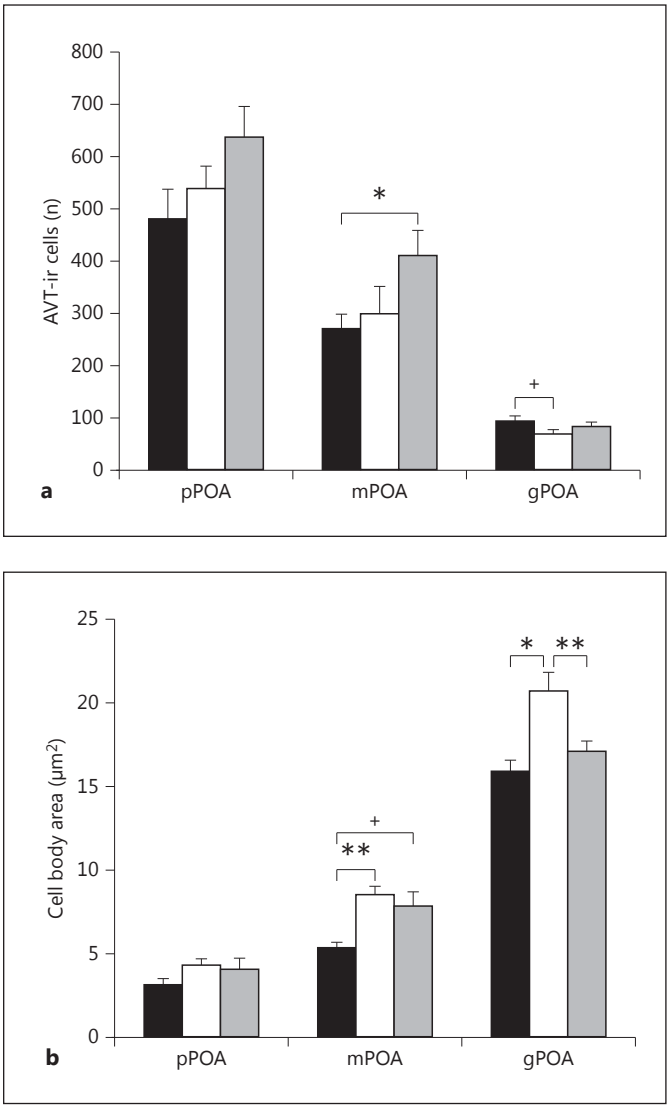


Fig. 3. Mean cell number (**a**) and cell body area (**b**) for the three AVT-ir neuronal populations in the pPOA, mPOA and gPOA of T males (black bar), NT males (white bar) and females (grey bar) of *O. mossambicus*. Means + SEM. * $p < 0.05$; ** $p < 0.01$; $^{+}$ $0.05 < p < 0.10$.

Effect of the Socio-Sexual Phenotype on Preoptic AVT-ir Cell Body Areas

MANCOVA showed a marginally non-significant effect of the socio-sexual phenotype (Wilks $\lambda = 0.35$, $F_{3, 11} = 2.51$, $p = 0.053$) and no effect of SL (Wilks $\lambda = 0.65$, $F_{3, 11} = 1.99$, $p = 0.173$) on cell body area of the AVT-ir neurons. The socio-sexual phenotype affected mPOA and gPOA, but not pPOA AVT-ir cell body areas (pPOA: $F_{3, 13} = 1.03$, $p = 0.410$; mPOA: $F_{3, 13} = 5.16$, $p = 0.015$, and gPOA: $F_{3, 13} = 5.81$, $p = 0.009$).

Table 1. Results of post hoc comparisons (p values for Fisher's LSD tests) of numbers of cells (above the diagonal of each POA division matrix) and cell body area (below the diagonal of each POA division matrix) of each AVT-ir cell group (pPOA, mPOA and gPOA) among the three socio-sexual phenotypes (T male vs. NT male vs. female)

		T male	NT male	Female
pPOA	T male	–	0.642	0.106
	NT male	0.189	–	0.387
	Female	0.641	0.477	–
mPOA	T male	–	0.629	0.044*
	NT male	0.002**	–	0.107
	Female	0.070[a]	0.169	–
gPOA	T male	–	0.076[a]	0.465
	NT male	0.034*	–	0.369
	Female	0.230	0.006**	–

* $p < 0.05$; ** $p < 0.01$.
[a] A marginally non-significant correlation ($p < 0.10$).

Post hoc analyses of the univariate outcomes adjusted for body size showed that NT males have significantly larger mPOA AVT-ir neurons than T males (fig. 3b; table 1) and significantly larger gPOA AVT-ir neurons than either T males or females (fig. 3b; table 1). Moreover, T males also have marginally non-significant smaller cell bodies of AVT-ir neurons than females (fig. 3b; table 1).

Finally, sex differences [planned comparisons for (T males + NT males) vs. females] were not found in the cell body area of AVT-ir cells across cell groups (pPOA: $F_{1, 13} = 0.22$, $p = 0.650$; mPOA: $F_{1, 13} = 0.047$, $p = 0.83$, and gPOA: $F_{1, 13} = 0.64$, $p = 0.439$).

Effect of the Socio-Sexual Phenotype on Circulating Hormone Levels

There was a main effect of the socio-sexual phenotype on circulating testosterone ($F_{2, 14} = 10.14$, $p = 0.002$) and 11-keto-testosterone ($F_{2, 14} = 11.15$, $p = 0.001$) levels. Post hoc tests (Fisher's LSD) showed that both male phenotypes have significantly higher androgen levels than females (testosterone: T male > female, $p = 0.001$; NT male > female, $p = 0.036$; 11-keto-testosterone: T male > female, $p = 0.001$; NT male > female, $p = 0.004$; fig. 4a, b). Plasma levels of testosterone, but not 11-keto-testosterone, are higher in T males than in NT males (testosterone: T male > NT male, $p = 0.034$; 11-keto-testosterone: T male = NT male, $p = 0.225$; fig. 4a, b). No main effect was found on cortisol plasma levels across different socio-sexual phenotypes ($F_{1, 14} = 1.09$, $p = 0.364$).

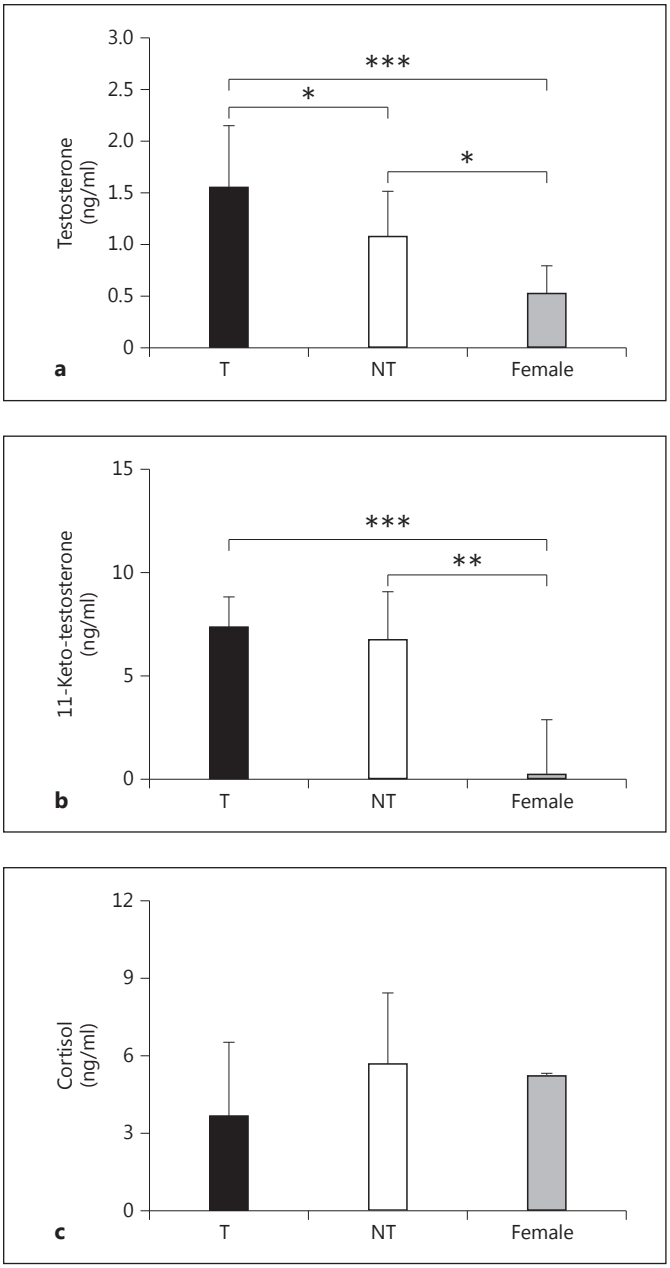


Fig. 4. Mean testosterone (**a**), 11-keto-testosterone (**b**) and cortisol (**c**) plasma levels of T male, NT male and female *O. mossambicus*. Means + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Association between Male Social Status, Hormone Levels and AVT Neuronal Phenotypes

The number of AVT-ir cells was negatively correlated with aggression in the pPOA (table 2). The cell body area of AVT-ir cells was positively correlated with escape behaviour in mPOA and gPOA. There were also marginally

Table 2. Partial correlations, controlling for SL, between characteristics (cell number and cell body area) of AVT-ir neurons, dominance behaviour, hormone level and kidney (mesonephros) weight for T and NT males (n = 13, except for kidney weight, where n = 10)

	Cell number			Cell body area		
	pPOA	mPOA	gPOA	pPOA	mPOA	gPOA
Aggression	–0.598*	–0.145	0.297	–0.522[a]	–0.539[a]	–0.390
Escape	0.413	0.411	0.025	0.495	0.700**	0.814***
Testosterone	0.021	0.076	–0.061	0.060	–0.325	–0.347
11-Keto-testosterone	–0.090	–0.110	0.042	0.020	0.035	0.005
Cortisol	0.429	–0.319	–0.499[a]	–0.022	0.127	–0.238

* $p < 0.05$; ** $p < 0.01$; *** 0.001;
[a] A marginally non-significant correlation ($p < 0.10$).

non-significant ($0.05 > p > 0.10$) negative correlations between the cell body area of AVT-ir cells in pPOA and mPOA (table 2).

Although there was no significant correlation between any measurement of AVT cells and sex steroids, there was a marginally non-significant negative correlation between the number of gPOA AVT-ir cells and cortisol levels (table 2).

Discussion

The vasotocin system of the Mozambique tilapia is restricted to the POA where parvocellular, magnocellular and gigantocellular AVT-ir cells can be recognized forming three distinct populations (pPOA, mPOA and gPOA) that follow a distribution previously described for other teleost [Goodson and Bass, 2000; Miranda et al., 2003; Lema and Nevitt, 2004; Maruska et al., 2007; Dewan et al., 2008; Maruska, 2009; Mendonça et al., 2013].

Here, a sex difference (i.e. comparison between T + NT males vs. females) was observed in the number of pPOA and mPOA cells but neither in the number of gPOA cells nor in the soma size of any of the three cell populations. The pattern of sexual dimorphism in the vasotocin system is diverse among teleost fish, with known cases of sex differences being present or absent in all POA regions (e.g. halfspotted goby [Maruska et al., 2007] and goldfish, *Carassius auratus* [Parhar et al., 2001], respectively). However, negative results in sex differences should be taken with caution since there are also marked seasonal effects (e.g. halfspotted goby [Maruska et al., 2007] and Hawaiian sergeant fish [Maruska, 2009]), and reproduction is known to influence the AVT system differently in the two sexes, and any of these two factors may mask the effect [Ota et al., 1996, 1999]. In this study, both sexes were kept in reproductive conditions and were actively breeding.

An effect of the male social status on the AVT neuronal phenotype was also observed in this study. NT males had AVT-ir neurons with larger cell bodies than T males in the mPOA and gPOA cell nuclei, and there was also a marginally non-significant effect for T males to have more gPOA AVT-ir cells than NT males. Furthermore, the pattern of correlations between AVT-ir cell measures and male social behaviour showed a positive association between the overall increased activity of the vasotocin system and the expression of submissive behaviour, as indicated by the positive correlations between escape behaviour and the number of cells in the mPOA, and between escape behaviour and the cell body size in all three AVT neuronal populations. Conversely, the expression of aggressive behaviour was associated with a lower activity of the vasotocin system, as indicated by negative correlations between aggressive behaviour and the cell body area of pPOA and mPOA AVT cell populations, and between aggressive behaviour and the number of AVT-ir pPOA cells. Together, these results suggest an association between the subordinate social status with an overall increased AVT activity, in particular for mPOA and gPOA nuclei. This association of the subordinate social status with increased vasotocin activity is also in accordance with recently published data on nonapeptide levels for this species, indicating higher concentrations of AVT in the pituitary of NT males than T males [Almeida et al., 2012]. Considering that AVT neurons from all three POA

regions project to the pituitary in this species, the immunocytochemical evidence presented in this paper matches nicely the results of peptide quantification published previously [Almeida et al., 2012].

In the scope of this comparison between immunocytochemical and quantitative peptide evidence, it is also worth mentioning that the presence of extra-hypophyseal projections in all AVT POA cell populations matches the occurrence of detectable levels of the AVT peptide in all brain regions assessed by HPLC [Almeida et al., 2012], and together these two sets of data confirm the role of AVT as a widespread neuromodulator in the central nervous system. Finally, it should be noted that despite the very high levels of the AVT peptide previously measured in the olfactory bulbs suggestive of local production, we have not identified any AVT-ir cells in the olfactory bulbs in this study. An intrinsic vasopressin (AVP) system made of a large population of interneurons has been recently described in the rat olfactory bulb, which is involved in social recognition [Tobin et al., 2010]. Interestingly, previous studies using traditional immunocytochemical techniques failed to localise these interneurons, which only became visible in a transgenic rat line enhanced with green fluorescent protein targeted at AVP secretory vesicles [Ueta et al., 2005]. Similarly, it is possible that our current technique is not able to detect a similar system in the fish olfactory bulb. As the abovementioned comparisons between HPLC AVT peptide quantification and immunocytochemistry characterisation of AVT-ir cells in tilapia illustrate, the use of multiple and complementary techniques will help to clarify the relationship between the vasotocin system and social behaviour.

The occurrence of within-species covariation in the activity of the vasotocin system and the expression of social dominance has already been described for another African cichlid species (*A. burtoni* [Greenwood et al., 2008]). Despite the fact that Mozambique tilapia and *A. burtoni* are phylogenetically close to each other and share a similar mating system (i.e. polygamy, exclusive female parental care and male breeding territoriality), the data presented here contrast sharply with those available for *A. burtoni* [Greenwood et al., 2008]. While in the Mozambique tilapia NT males have larger AVT-ir mPOA and gPOA cells than T males, and these are correlated with the expression of submissive behaviour, *A. burtoni* T males have higher gPOA AVT mRNA levels and these are correlated with aggressive behaviour, whereas NT males have higher pPOA levels and these are correlated with submissive behaviour [Greenwood et al., 2008]. Thus, the proposed specialisation of AVT populations on different aspects of social behaviour for *A. burtoni*, such as an involvement of gPOA in reproductive behaviour, including territoriality, and pPOA in social stress [Greenwood et al., 2008], does not seem to apply to *O. mossambicus*. However, these contrasting results can also be due to methodological differences between the two studies, since one used immunocytochemistry and the other in situ hybridization techniques. In a previous study with the peacock blenny, it has been shown that these two techniques may produce different results in terms of the number and size of AVT cells [Grober et al., 2002]. Therefore, despite the association between AVT neuronal phenotypes and the expression of social behaviour found in both species, comparative studies using the same technique are needed before a final picture of the AVT-behaviour relationship in cichlids can be achieved.

Interspecific variation in the direction of the association between AVT/AVP and aggressive behaviour has also been observed in other vertebrate taxa. For example, among mammals, increased AVP activity has been associated with increased aggression in Syrian hamsters, California mice and prairie voles, whereas a negative association has been observed in mice and rats that have been artificially selected for short-attack latencies [Pagani et al., 2013]. Moreover, within the same individual, brain regional differences in opposite directions have been described, such as aggressiveness being associated with an increase in AVP in the lateral septum and a decrease in the bed nucleus of the stria terminalis in Wistar rats [Veenema et al., 2010]. Therefore, the observed patterns of AVT-social dominance do not seem to be either evolutionarily conserved or dependent on an identified ecological factor (e.g. mating system). Therefore, an explanatory hypothesis for the diversity in these patterns of peptide-social dominance associations is still missing.

No significant correlations were found between any of the AVT cell measures and the circulating levels of any of the steroid hormones measured (testosterone, 11-ketotestosterone and cortisol). However, a marginally nonsignificant (i.e. $0.05 < p < 0.10$) negative correlation was identified between cortisol levels and the number of AVT-ir cells in the gPOA nuclei, which might suggest an association between the stress axis and the gPOA AVT nucleus. Therefore, given the small sample size used in this study, further research should address this possible relationship.

In summary, this paper describes a pattern of association between the AVT neuronal phenotype and the male

social status that contrasts with what has been described previously for another cichlid fish with a similar mating system. This result highlights the need for higher phylogenetic data coverage of the association between AVT/AVP neuronal phenotypes and social dominance [Dewan et al., 2011]. So far, comparative studies have been based on a reduced number of paired comparisons between closely related species. The diversity illustrated here between phylogenetically closely related species that share the same mating system warrant the study of increased numbers of species representing the relevant phylogenetic and ecological groups. Therefore, it is still important to increase the number of species for which this type of data become available in order to improve the knowledge on the relationship between AVT/AVP phenotypes and social dominance.

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