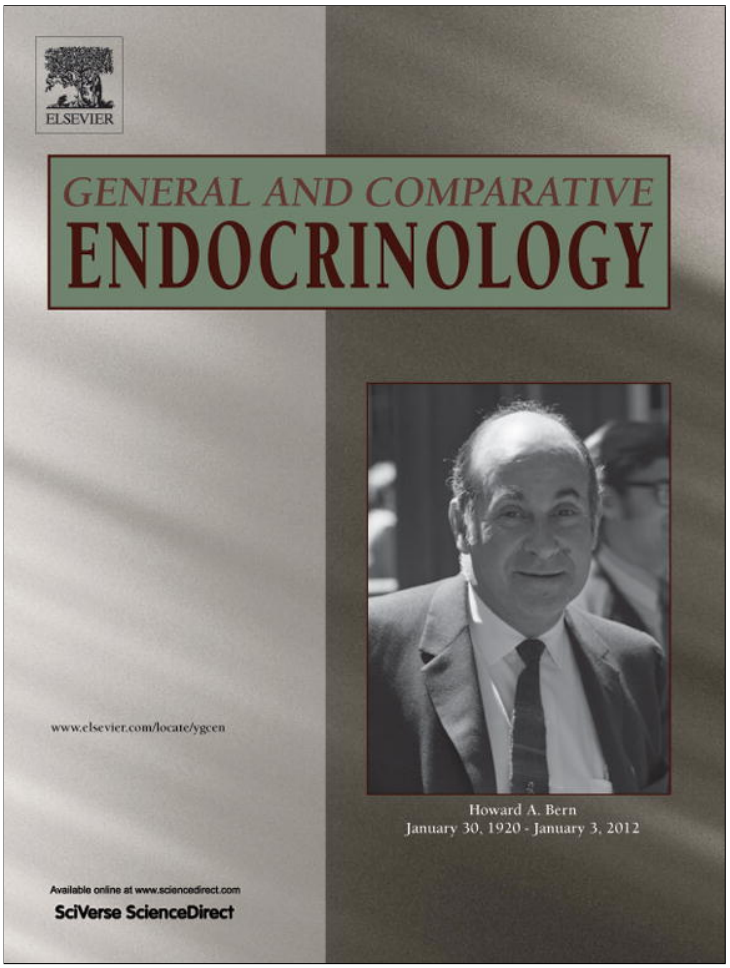

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Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*

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ABSTRACT

In non-mammalian vertebrates, the nonapeptide arginine-vasotocin (AVT) is involved in the regulation of social behavior related to reproduction and aggression. The cichlid fish *Cichlasoma dimerus* is a monogamous species with complex social hierarchies. Males are found in one of two basic alternative phenotypes: Non-territorial and territorial males. In this work we characterize the vasotocinergic system in males of *C. dimerus* in relation to social status with particular emphasis on the various putative sites of action of AVT across the hypothalamic–pituitary–gonad (HPG) axis, and its effects on reproductive and social behavior. The location and distribution of vasotocinergic neurons in the brain was studied, highlighting a morphometric analysis of AVT producing neurons in males of different social status. The effect of AVT on pituitary gonadotropin secretion was analyzed by single pituitary culture while expression of AVT in peripheral organs was studied by RT-PCR using specific primers. Finally, the role of AVT on testicular androgen release was assessed by *in vitro* incubation of testis. Results showed a positive effect of AVT on gonadotropin secretion, where β-LH showcased a triphasic response under increasing AVT concentration, while β-FSH's response was dose-dependent and directly proportional. AVT showed a positive and concentration-dependent effect over testicular androgens synthesis and secretion *in vitro*. Vasotocin expression was observed in testicular somatic tissue located in the interstitial compartment. Thus, the AVT system in *C. dimerus* appears to be of high complexity, with multiple sites of action in the hypothalamus–pituitary–gonadal axis.

1. Introduction

Social interactions affect critical aspects of animal life, and as such can regulate major phenotypic transitions, such as metamorphosis, puberty, hibernation and migration, where physiology and behavior change in a coordinated manner [19]. Neuropeptides, via interactions with other neurotransmitter systems within specific neural circuits, have emerged as central players in the regulation of social cognition, behavior and phenotypic transitions [19]. Neuropeptides may act as neurotransmitters, if released within synapses, or as neurohormones, activating receptors distant from the site of release [32]. Within the group of neuropeptides, a small family has caught particular attention due to its active role in the regulation of social behavior, as well as in various processes involved in reproductive physiology (see Godwin & Thompson [16] for review): the vasopressin–vasotocin peptide family.

Since the pioneering studies in the 1990s [20], arginine-vasotocin (AVT) has been linked to reproductive plasticity in a variety of teleosts fishes. First, the manipulation of AVT levels affects behaviors that are exhibited predominantly by animals of one phenotype, such as courtship and aggression [4,30,49,50]. Second, AVT is differentially expressed as a function of alternative phenotypes and modulates social behavior in many fish species (*Astatotilapia burtoni*, [19]; *Salaria pavo*, [21]; *Danio rerio*, [28]).

Within the teleost brain, AVT is mainly and consistently expressed in neurons from one of three cellular subpopulations in the preoptic area (POA): parvocellular (pPOA); magnocellular (mPOA); and gigantocellular (gPOA) (see Goodson [17] for review). These groups of cells differ in morphology, size, position, and axonal target sites, and each has been associated with distinct physiological and behavioral functions (see Balment et al. [4] for review). In several species, higher AVT expression levels and/or soma size in the mPOA and/or gPOA have been associated with territorial behavior (*A. burtoni*, [19]; *D. rerio*, [28]; *Amphiprion ocellaris* [25]), while other species show the opposite pattern, with lower AVT levels in the mPOA, gPOA and/or pPOA nucleus associated to

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territoriality (*S. pavo* [21], *Parablennius parvicornis* [36], *Porichthys notatus* [18]). Thus, although a relationship between AVT and alternative phenotypes in teleosts is clear, the details of that relationship appear to be species specific.

C. dimerus (Heckel 1840) is a South American cichlid that adapts easily to aquarium conditions where it spawns throughout the year. Males are found in one of two basic alternative phenotypes that are linked to both social and reproductive status. Non-territorial (NT) males have dark ground coloration and lack immediate access to reproduction when compared with territorial (T) brightly colored males, that actively defend a territory. Males exhibit dominance of the linear type (A > B > C > D···) where larger males generally occupy reproductive-territorial positions as opposed to smaller males, socially neglected access to reproduction. Aggressive displays include biting, mouth holding, chasing, fin erection, while submissive displays include behaviors such as escape and fin retraction [2]. We hypothesize that AVT in male *C. dimerus* is differentially expressed as a function of social status, modulating aggressive behavior, and consequently access to a territory and ultimately reproductive success.

The aim of this study was to characterize the vasotocinergic system in males of *C. dimerus* in relation to social status with particular emphasis on the various putative sites of action of AVT across the hypothalamic–pituitary–gonad (HPG) axis, and its effects on reproductive and social behavior.

2. Materials and methods

2.1. Animals

C. dimerus were caught using fishing nets in Esteros del Riachuelo: 27°35′S; 58°45′W (Corrientes, Argentina), housed in aquaria under conditions mimicking their natural habitat and allowed to acclimate for at least 1 month before starting the experiments. Temperature (25 ± 1) °C and lighting (14:10 light:dark cycle with full spectrum illumination) were strictly regulated. A layer of gravel (~4 cm) covered the bottom of the aquaria. Natural aquatic plants and stones were placed in the aquaria which fish used to delimit their territories, along with flat slabs where fish lay their eggs. Animals were fed to satiation every morning with cichlid pellets (Tetra). Appropriate actions were taken to minimize pain or discomfort of the animals, and the experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations. Procedures are in compliance with the Guide for Care and Use of Laboratory Animals (eighth ed., 2011, National Academy Press, Washington, 220pp.). In some experiments, when potentially dangerous interactions were observed, the animals were separated to avoid injuries.

All males (n = 45) used in this study came from communal tanks where they had established themselves as T males along with a smaller T female. The territorial pairs were later isolated to facilitate sex recognition.

2.2. Immunohistochemical localization of AVT neurons

Three adult male fish were anesthetized with 0.1% benzocaine and killed by decapitation. Their brains – with the pituitary attached - were fixed in Bouin's solution for 18 h at room temperature. Samples were dehydrated and embedded in Paraplast (Fisherbrand, Fisher, Wash). For immunohistochemistry (IHC), brains were completely sectioned coronally at 15-μm intervals and each brain section was mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher, Wash). The following procedures were carried out at room temperature. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4), treated for 30 min with PBS containing 5% nonfat dry milk, incubated with a 1:500 dilution of primary rabbit antiserum (rabbits were injected with an AVT Synpep coupled to Keyhole Limpet Hemocyanin) for 16 h at 4 °C, washed in PBS, and finally incubated for 45 min with a biotinylated anti-rabbit IgG (Sigma–Aldrich) diluted 1:600. Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (STRP–HRP) (Dako) diluted 1:800 and visualized with 0.1% 3,3′-diaminobenzidine (DAB) in TRIS buffer (pH 7.6) and 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, mounted, examined with a NIKON microphot FX microscope and digitally photographed (Coolpix 4500, Nikon). To confirm the specificity of the immunostaining, control sections were incubated with the primary antisera pre-adsorbed with an excess of AVT (1 μg/ml) (Sigma–Aldrich Arg8]-vasotocin acetate salt) (Fig. 1a). The possibility of cross-reactivity with isotocin was assessed by comparison of alternate sections, one incubated with plain AVT antibody and the other with AVT antibody pre-adsorbed with isotocin peptide (6 μg/ml) (Chem Impex, Intl., Wood Dale, IL) (kindly provided by Dr. Gustavo Somoza). There was no difference in the number of labeled somata between alternate sections reacted with plain anti-AVT and anti-AVT pre-adsorbed with isotocin (Student's t-tests, $p > 0.05$), thus demonstrating no cross-reactivity of the AVT antibody with isotocin-producing neurons (Supplementary Fig. 1). To avoid false positives caused by the immunohistochemistry itself, replacement of primary antisera with PBS was also performed. For the precise location of the AVT cell nuclei and their projections, we relied on the detailed atlases of two other Perciform species, *Dicentrarchus labrax* [8,9] and *A. burtoni* [12], and on previous studies of GnRH neurons localization in this species [40,41].

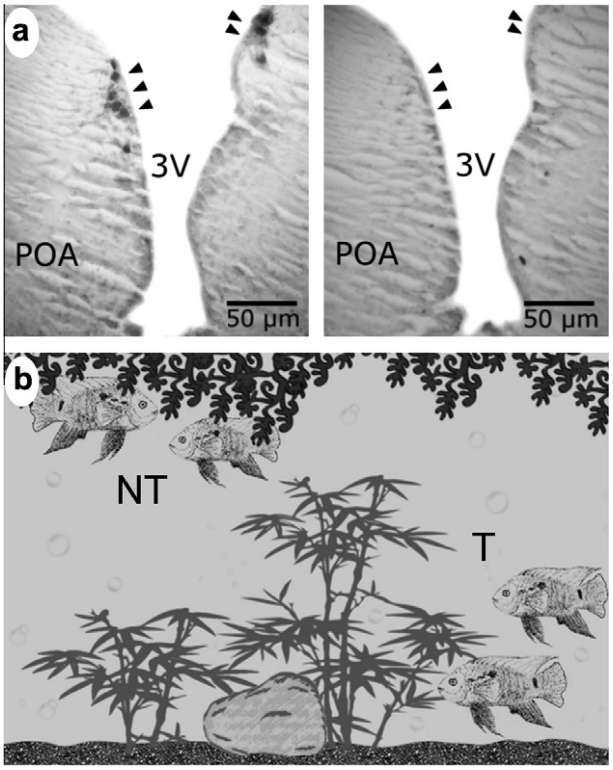

Fig. 1. (a) AVT antiserum specificity was tested by pre-adsorption with an excess of antigen (right) vs antiserum alone (left) on successive 15 μm sections. Arrow heads indicate AVT-ir cells' position (left) and its absence on the pre-adsorbed immediately successive section (right). 3 V: third ventricle; POA: preoptic area. (b) Schematic drawing showing typical fish pairs position once social hierarchy had been established. The territorial pair (T) actively defended a territory near the substrate, delimited by plants and rocks. Non-territorial individuals (NT) occupied higher areas of the aquarium near the surface, hidden between floating plants. Scale bar: in image.

2.3. Morphometry of AVT-ir cell bodies in territorial vs non-territorial males

Four groups of two adult fish pairs (8 males and 8 females) were used in this study. Standard length (SL) and body weight (BW) (expressed as mean ± standard error) were 9.9 ± 0.5 cm and 44.8 ± 4.7 g for males and 7.8 ± 0.4 cm and 26.3 ± 4.3 g for females. All pairs used came from communal tanks of 150 l, housing 8 fishes, where they had established themselves as a territorial pair. After 3–7 days of hierarchy stability, the territorial male and female were isolated from the communal tank, and later placed simultaneously with another territorial pair in a 53 l observation tank. Conditions remained unchanged until the clear establishment of the social hierarchy [2]. At that moment, non-territorial individuals spent most of their time in the upper layers of the aquarium, for the most part hidden between floating plants, while the territorial pair actively defended a territory near the substrate, generally delimited by plants or rocks (Fig. 1b).

Animals were collected with hand nets, anesthetized with 0.1% benzocaine and killed by decapitation. All male animals were sampled in prespawning stage. Females were not processed. Brains with the attached pituitary were fixed in Bouin's solution, dehydrated and embedded in Paraplast (Fisherbrand, Fisher, Wash). Samples were then sectioned coronally at 15-µm intervals throughout the POA and AVT-ir neurons were labeled by immunohistochemistry. Each AVT-ir soma was assigned to either the pPOA, mPOA or gPOA group based on neuroanatomical position, somata morphology and size. The size and number of AVT-ir somata, and the size of AVT-ir nucleus were measured on coronal sections without prior knowledge of the corresponding social status.

Cell number from each cell subgroup of the POA was calculated based on cell counts done in duplicate under 600× magnification through a light-microscope. Counts were done on a single brain hemisphere and the number of total counts was doubled to obtain "whole brain counts", assuming symmetry. As neuronal cell bodies are highly irregular in shape and almost always split during histological sectioning, in order to avoid double counting, the profile counts were converted to neuronal numbers using an assumption-based correction factor proposed by Abercrombie (1946). This correction factor overcomes the problem of overestimation and makes adjustments for double counting to obtain 'true' cell numbers. In this method nuclear profile counts (n) are multiplied by mean nuclear diameter (D) divided by mean nuclear diameter plus section thickness (T) to yield neuron numbers (N); $N = (n \times D)/(D + T)$. The assumptions were that the nuclei were spherical and that all fragments of a sectioned nucleus were visible, with no lost caps.

Cell and nucleus profile area were computed from digital images of neurons at 600× with the software Image Pro Plus (Media Cybernetics) which was previously calibrated with a stage micrometer. Digital images of sections were captured by digital camera (Coolpix 4500, Nikon) attached to a bright-field, light microscope (Nikon, microphot FX). Thirty randomly chosen AVT-ir cells with clear nucleus were measured from pPOA and mPOA cell populations, while 8 gPOA randomly chosen cells were analyzed, due to reduced gPOA cell numbers. AVT-ir neuron/nucleus size (μm^2) was measured as the cross-sectional area of an AVT-ir cell/nucleus by tracing the cell somata's/nuclei's profile with a digitizing pen.

2.4. Effects of exogenous AVT on pituitary β-FSH and β-LH release in vitro

To study the effect of AVT on pituitary LH and FSH secretion, 24 randomly selected males were anesthetized in a 0.1% benzocaine solution, and their whole pituitaries were carefully and mechanically dissected from the brain and quickly washed in physiological solution. Afterwards, they were transferred to multi-well culture plates containing 120 µl of culture media (L15 in a 80% v/v dilution, supplemented with 10% fetal bovine serum, 10 mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin). Pituitaries were individually maintained in culture for 3 days at 25 °C (a single whole pituitary per well, 4 pituitaries per plate). After the first 24 h, culture media were removed and discarded (Day 0) in order to eliminate the effect of brain-derived factors which could have remained in the neurohypophysial terminals. Pituitaries were then cultured for the next 24 h, after which the media were removed and frozen at −20 °C (Day 1). Randomly selected pituitaries were then incubated for 24 h with different concentrations of AVT (Sigma–Aldrich). The used doses were 0.1, 1 and 10 µM based on published studies on the effect of *in vitro* administrated hypothalamic factors on fish pituitary cultures [7,43]. Control pituitaries were cultured in clean media. After 24 h, culture media were removed and frozen (Day 2) (Fig. 2a). All media were frozen after the addition of 1 µl of protease inhibitor (Sigma–Aldrich).

2.4.1. Western blot analysis and semi-quantification

β-FSH and β-LH released in media were semi-quantified by immunoblots of the pituitary culture media using heterologous

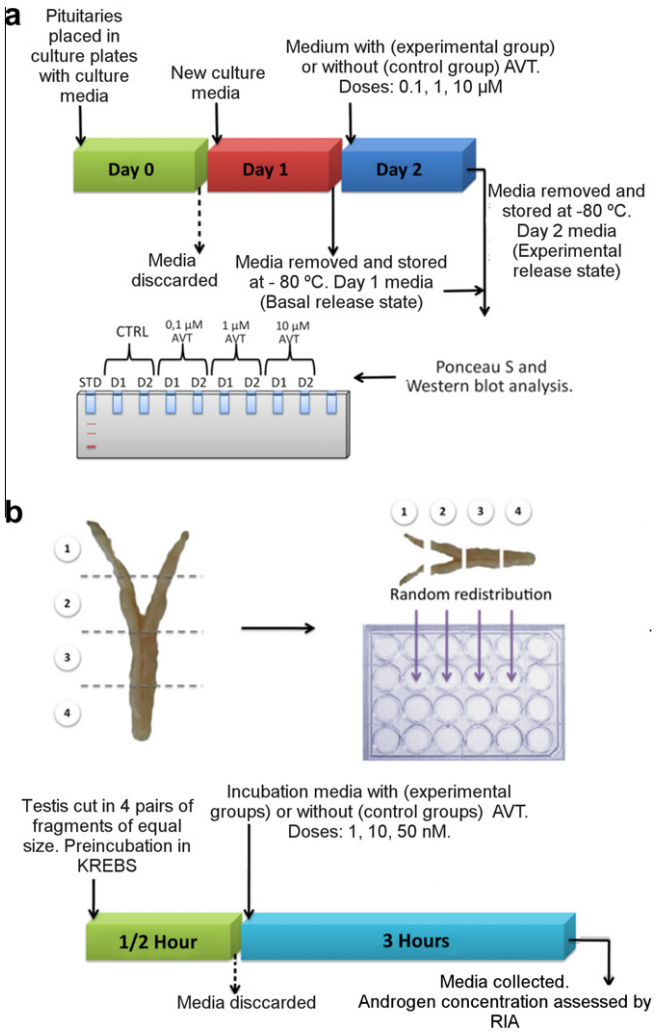

Fig. 2. Experimental design for (a) the single pituitary culture system and (b) the testicular fragments incubation. (b) General view of a single fish testis pair. Dash lines show cutting points into 4 pairs of fragments of equal size randomly assigned to each treatment. D1: Day 1; D2: Day 2; CTRL: control; STD: Molecular Weight Standard.

antisera from *Fundulus heteroclitus*, the specificity of which has been previously tested in this species [42]. Briefly, 12.5 µl of each pituitary culture medium was separated by electrophoresis on 15% sodium dodecylsulfate–polyacrylamide gel (SDS–PAGE). After that, proteins and molecular markers (ColorBurst™ Electrophoresis Marker [M.W. 8000–220,000], Sigma–Aldrich) were transferred to a nitrocellulose membrane (Sigma–Aldrich) for 60 min at 75 V. Then, membranes were washed in Tris-buffer solution Tween (TBST; 100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20; pH 7.5), and blocked with TBST containing 3% nonfat dry milk and 3% BSA at 4 °C overnight. Later, they were incubated for 3 h at room temperature with either β-FSH or β-LH primary antisera (1:2000). After 4 washes in TBST, membranes were incubated with a biotinylated anti-rabbit IgG (Sigma–Aldrich) (1:2000), washed again and finally incubated with a streptavidin complex conjugated to alkaline phosphatase (1:2000) (Dako) for 60 min at room temperature. After washing, the reaction was visualized on nitrocellulose using an alkaline phosphatase developing kit (BCIP/NBT, Vector Blue, Dako). Finally, membranes were air-dried, digitalized and optical density semi-quantification was performed using Image Gauge version 3.12 (Fuji Photo Film) software. A pituitary culture assay with mammalian GnRH from 0.01 to 1 µM, followed by Western blotting for β-LH, served as a positive control as the stimulatory effect of GnRH on LH pituitary cells is well known. Results showed a clear effect on LH release as evidenced by Western blot (Supplementary Fig. 2) [7].

In order to avoid a possible loading error in the SDS–PAGE, pituitary hormone release was normalized to a 148-kDa protein that was only present in the culture media [7,41]. This protein was visualized by Ponceau S in the nitrocellulose membrane, digitalized and semi-quantified. Previous analysis by immunoblot of somatolactin, a piscine pituitary hormone, from serially diluted pituitaries culture media (5, 10, 15, 20 µl) showed that this method was sensitive enough to detect differences between treatments (Supplementary Fig. 3) [7]. Medium content of β-FSH and β-LH from each pituitary were evaluated as follows: culture media of each treatment from day 1 were loaded in SDS–PAGE besides the day 2 culture medium (Fig. 2a). Day 1 was considered as an indicator of the gland release state, hence day 2 medium was normalized to day 1 in order to compare values obtained from each pituitary surrounding medium due to un-controlled variables present in the study animals.

2.5. Effects of exogenous AVT on testicular androgen release in vitro

To investigate the effect of vasotocin on testicular androgen release, 5 adult territorial males were anesthetized in a 0.1% benzocaine solution and killed by decapitation. Their testis were dissected and washed in physiological solution. Each pair of testis was then cut in 4 pairs of fragments of equal size (Fig. 2b) and pre-incubated for 30 min, continuously shaken, at room temperature in 24-well culture plates containing 110 µl of incubation media (KREBS Ringer glc HEPES, pH 7.35–7.4 NaCl 0.9%, KCl 1.15%, $CaCl_2$ 1.22%, KH_2PO_4 2.11%, $AgSO_4$ $7H_2O$ 3.8%, glc 5.4%, HEPES 0.026 M). After the pre-incubation period, media were changed and fragments pairs were then incubated in 110 µl medium with or without different AVT concentrations (control, 1 nM AVT,

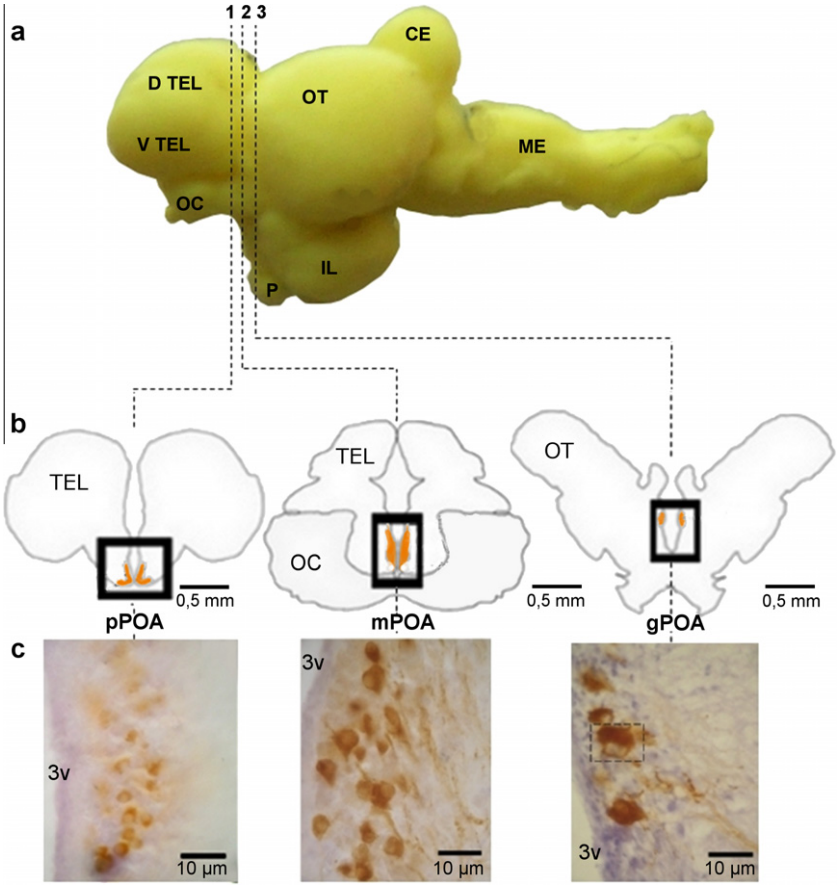

Fig. 3. (a) Lateral view of *C. dimerus* brain. Dashed lines show the position of transverse sections shown on b and c. The series 1, 2 and 3 correspond to the anterior, medium and posterior POA, respectively. (b) Camera lucida drawings from POA transverse sections highlighting the brain positions where AVT-ir cells were observed. (c) Microphotograph of transverse sections from pPOA (1), mPOA (2) and gPOA (3), where typical cell size and morphology of the three cell populations are shown. Enclosed within the dashed box lies a gPOA cell intercalated between mPOA cells. CE: cerebellum; D TEL: dorsal telencephalon; IL: inferior lobe; ME: medulla; OC: optic chiasm; OT: optic tectum; P: pituitary; TEL: telencephalon; V TEL: ventral telencephalon. Scale bar: in image.

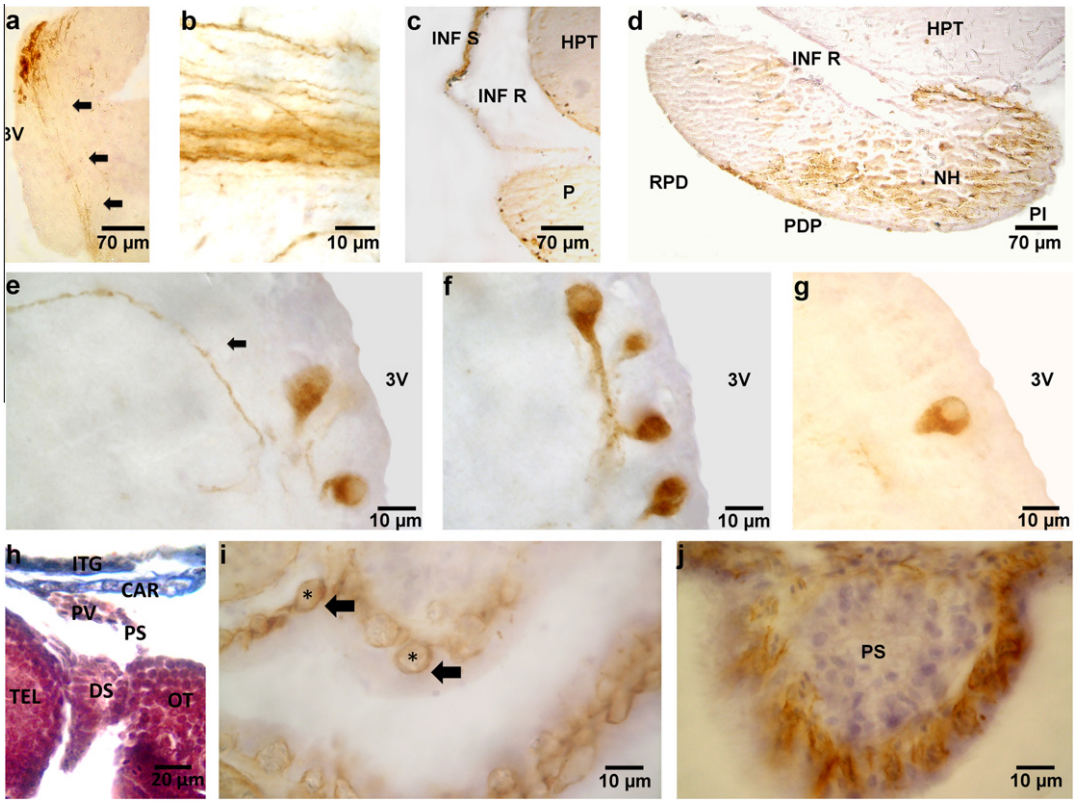

Fig. 4. Transverse sections, unless indicated (a) Fibres (arrows) from mPOA cells proyecting towards the infundibular stalk. (b) Detail from AVT-ir fibres. (c) Anterior infundibular stalk with AVT-ir fibres entering the pituitary (sagittal section). (d) AVT-ir fibres entering the pituitary (sagittal section). Immunolabeling was strongest in the proximal pars distalis (PDP) and pars intermedia (PI). Scarce AVT-ir fibres were observed associated to the rostral pars distalis (RPD). (e) Magnocellular neurons. The arrow indicates an AVT-ir fibre. (f) 1-cell thick layer of AVT-ir mPOA cells on the dorsal and posterior POA. (g) Isolated gPOA cell within the limiting most posterior region of the POA. (h) General view of parasagittal section of the pineal complex in this species. (i) Sagittal section of pineal complex's dorsal sac. AVT-ir fibres (arrows) are seen in close association with dorsal sac cells (∗). (j) Pineal stalk surrounded by AVT-ir fibres in the dorsal sac. 3 V: third ventricle CAR: cartilage; HPT: hypothalamus; INF R: infundibular recess; INF S: infundibular stalk; ITG: integument; DS: dorsal sac; OT: optic tectum; P: pituitary; PS: pineal stalk; PV: pineal vesicle; TEL: telencephalon. Scale bar: in image.

10 nM AVT and 50 nM AVT; Sigma–Aldrich, [Arg8]-vasotocin acetate salt), based on published works that investigated the effect of AVT on testosterone production by the rainbow trout testis in culture [47]. Each pair of testicular fragments was randomly assigned to one of the treatments, in such manner that the fragments coming from a single fish were exposed to all treatments. Fragments were then incubated for 3 h at room temperature with constant shaking. The incubation medium was later collected and frozen at −20 °C until assayed. Androgen release to the medium was quantified by RIA (DSL-4000 ACTIVETestosterone Coated-Tube Radioimmunoassay Kit) using a heterologous antibody against testosterone with a 45% cross-reactivity with 11-ketotestosterone (Fig. 2b). Thus in this work, the term "androgens" is used in reference to the collective measure of testosterone and 11-ketotestosterone released into the media, even though androgens include many other steroids such as androstenedione, dehydroepiandrosterone, dihydrotestosterone, etc., not measured in the study.

2.6. Distribution of C. dimerus AVT gene expression

2.6.1. Expression analysis by RT-PCR

To study AVT gene expression in a variety of organs, total RNA was isolated from the POA-hypothalamic brain region, head kidney, trunk kidney, intestine, muscle, testis and liver of a randomly selected adult male using Trizol (Invitrogen). To eliminate genomic DNA, each sample was treated with DNase (Amplification Grade DNase I, Sigma–Aldrich) at 37 °C for 30 min, followed by inactivation at 70 °C for 10 min. After phenol extraction and ethanol precipitation, first-strand cDNA was synthesized from 1 μg total RNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma–Aldrich) according to manufacturer's instructions for poly (dT) oligos. Using first strand cDNA from the POA brain region, RT-PCR was done with degenerate primers designed from consensus regions of AVT preprohormone cDNA sequences from the african cichlid, *Haplochromis Burtoni* (GenBank: AF517935.1), two species from the Labridae family (*Parajulis poecilepterus* [GenBank: DQ073094.1] and *Halichoeres tenuispinis* [GenBank: DQ073098.1]) and a pleuronectiform (*Platichthys flesus* [GenBank: AB036517.1]). The primary RT-PCR degenerate primer pair sequences were as follows: AVT-Forward (5′-GCTACATCCAGAA(T/C)TGCC-3′) and AVT-Reverse (5′-TCAGAGTCCACCATACAGC-3′). Reaction conditions for primary PCR were 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min. RT-PCR reactions were analyzed on 2% agarose gel. The expected band (289 bp, GenBank: JQ924782) was purified using a purification kit (AccuPrep™, Gel Purification Kit, Bioneer) according to manufacturer's instructions, sequenced using a 3130xl Genetic Analyzer (Applied Biosystems) and identity confirmed by using BLAST (NCBI). Specific primers were then designed based on the sequence obtained, in order to study AVT expression in *C. dimerus*' different organs. Primer pair sequences were: AVT-Forward-Specific (5′-ACGAGCGCTGCCTGAGACTG-3′) and AVT-Reverse-Specific (5′-TCGGAGTTGCAGCAGAGT-3′). Optimal annealing temperature was assessed by gradient PCR using first strand cDNA from the POA as substrate. Reaction conditions were 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 50 °C; 51.6, 53.6, 58, 60.9, 62.6, 64.1 or 65 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min. Finally, using first strand cDNA from the different organs, reaction conditions for PCR under optimized

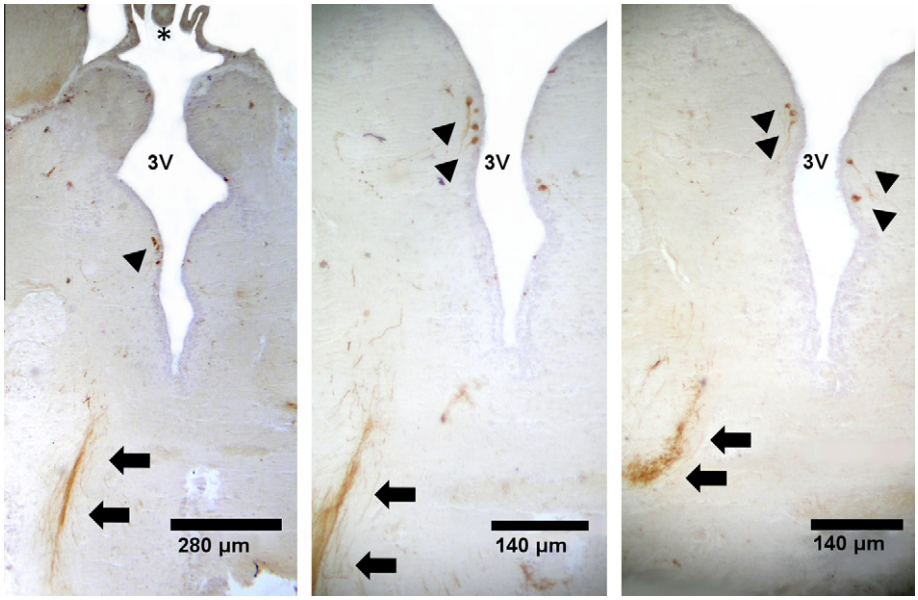

Fig. 5. Transverse sections of posterior POA. Arrows show the position of the preoptic-hypophyseal tract where most AVT-ir mPOA and gPOA cells converged. Arrow heads show gPOA cells with ventrally projecting axons. The asterisk points the position of the pineal complex. Scale bar: in image.

temperature conditions were 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min.

2.6.2. Immunohistochemical localization of AVT in testis cryostat sections

Testis were fixed in formaldehyde 4% overnight at room temperature, cryoprotected in a 20% sucrose phosphate-buffered solution (pH 7.4) at 4 °C and embedded in cryoplast (Biopack). Samples were cut transversally on a cryostat at 15 μm intervals and mounted onto gelatin coated slides. The following procedures were carried out at room temperature. Sections were washed in phosphate-buffered saline, 0.15% Triton X-100 (pH 7.4), treated for 30 min with PBS, 0.15% Triton X-100 containing 5% nonfat dry milk, incubated with primary rabbit antiserum (1:500) for 16 h at 4 °C, washed in PBS, 0.15% Triton X-100, and incubated for 45 min in biotinilated anti-rabbit IgG (Sigma–Aldrich) (1:800). Sections were finally incubated with STRP–HRP (Dako) (1:600) and visualized with DAB in TRIS buffer (pH 7.6) and 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, and mounted. To avoid false positives caused by the immunohistochemistry itself, replacement of primary antisera with PBS was performed.

2.7. Statistical analysis

Statistical analysis of data related to AVT-ir cells' morphometry associated to social status was performed using nested design analyses of variance (ANOVA). Statistical significance was established at the $p < 0.05$ level. The effect of AVT on pituitary gonadotropin release *in vitro* was analyzed by a randomized block design ANOVA, followed by Fisher's Least Significance Difference (LSD) test. Statistical significance was corrected using Bonferroni correction to $p < 0.025$. The effect of AVT on testicular androgen release *in vitro* was analyzed by a randomized block design ANOVA, followed by Fisher's LSD test. Data were presented as means ± SEM.

3. Results

3.1. Localization of AVT-ir cell bodies and their axonal projections

Cell bodies: In adult males of *C. dimerus*, AVT-ir neurons were localized exclusively within the POA. Vasotocinergic cell populations extended from behind the anterior commissure to the posterior POA, above the optic chiasm, forming an arch-like structure. Immunoreactivity was observed in all 3 cell populations forming the POA: pPOA, mPOA and gPOA, lining the border of the third ventricle. The most anterior and ventral AVT-ir cells were those of the pPOA, immediately posterior to the anterior commissure. These cells were round or oval in shape, of small diameter (6.5 ± 0.5 μm) and densely packed against the border of the third ventricle (Fig. 3, series 1). The mPOA cells extended dorsal and posteriorly from the pPOA, and were the most numerous. Cells from the mPOA were round or pyriform in shape, with eccentric spherical nucleus, and larger soma diameter (9.2 ± 0.5 μm) than pPOA cells (Fig. 3, series 2). On transverse sections, the number of mPOA cells lining the border of the third ventricle decreased dorsally, to the extent that only a 1-cell thick layer of AVT-ir cells was observed on the most dorsal and posterior positions. Located between these cells, there were isolated gPOA cells. The gPOA cell population was the most caudal with the largest somata within the POA (11.7 ± 1.1 μm). The gPOA cells were generally irregular in shape, with spherical and eccentric nuclei (Fig. 3, series 3). Quite commonly, isolated gPOA cells were found at the limiting most posterior region of the POA, where the other 2 cells populations (pPOA and gPOA) were absent (Fig. 4g).

Axonal projections: The greatest density of AVT-ir axons observed occurred within the POA (Fig. 4a and b), where fibres formed a dense preoptic-hypophyseal (PO-H) tract (Fig. 5) that entered the pituitary through the anterior and posterior infundibular stalks (Fig. 4c and d). At the pituitary level AVT-ir was observed at the proximal pars distalis and mainly at the pars intermedia, while the rostral pars distalis showed scarce immunolabeling (Fig. 4d).

Immunoreactive fibres were also observed associated to the pineal complex (Fig. 4h). Cells from the dorsal sac (Fig. 4i and j) were in close association with surrounding AVT-ir fibres.

3.2. Morphometry of AVT-ir cell bodies in territorial vs non-territorial males

After immediate hierarchy establishment, social status had no effect on the number or nuclear size of AVT-ir neurons in the preoptic area ($p > 0.05$; Fig. 6a and b). Differences were observed however, in soma size (Fig. 6c). Territorial males had pPOA neurons 23% smaller

than those present in NT males (soma size: T vs NT: 55 ± 3.9 μm^2 vs 71 ± 8.6 μm^2, $p = 0.027$). No differences were observed however in mPOA and gPOA cell's size between T and NT males.

3.3. Effects of exogenous AVT on pituitary β-FSH and β-LH release in vitro

Vasotocin affected the release of LH and FSH *in vitro*, though response profiles were clearly specific. LH exhibited a triphasic response to AVT, where the lowest (0.1 μM) and highest (10 μM) doses of AVT elicited a maximal increase in pituitary secretion, reaching the basal release state, whereas the intermediate (1 μM) concentration did not show any effect over LH release (Fig. 7a). On the other hand, the highest AVT concentration in the medium (10 μM) induced a maximal increase in pituitary FSH release, above the gland release state, the intermediate (1 μM) concentration showed an intermediate response, while the lowest (0.1 μM) AVT concentration had no effect over FSH secretion (Fig. 7b).

3.4. Effects of exogenous AVT on testicular androgen release in vitro

Testicular androgen release was stimulated *in vitro* by AVT. Exposure of testicular fragments to 1 nM AVT, had no effect over androgen synthesis, while 50 nM AVT increased it in more than 150% ($p < 0.01$). In turn, intermediate AVT values (10 nM) elicited an intermediate response (Fig. 8).

3.5. Distribution of C. dimerus AVT gene expression

Specific PCR products of AVT preprohormone were only amplified from cDNAs obtained from testis and POA brain region of adult male *C. dimerus* (Fig. 9). AVT mRNAs were not observed in the absence of reverse transcriptase in the RT-PCR mix, showing there was no genomic DNA contamination in the samples (Fig. 9a, RT-). The partial nucleotide sequence obtained from *C. dimerus* brain mRNA consisted of a 236 bp fragment. There was a high degree of sequence identity between *C. dimerus* and other cichlids' AVT preprohormone (96% *Herichthys mincklei* and *H. cyanoguttatus* and 91% *A. burtoni*) and other Perciform species (87% *H. tenuispinis* and *P. poecilepterus*). Also, there was a certain degree of sequence identity between *C. dimerus* and other cichlids and Perciforms' isotocin precursor (86% *H. tenuispinis* and *P. poecilepterus*), an AVT paralogue.

Immunohistochemical localization of AVT in testis revealed the presence of small immunoreactive cells in the interstitial compartment. AVT-ir cells were scarce, tapered shaped with thin cytoplasmatic extensions and dispersed within the interstitial compartment (Fig. 9b–e).

4. Discussion

This study provides an initial characterization of the vasotocinergic system, and its potential association with reproductive physiology and social behavior in males of *C. dimerus*. Differences in AVT-ir cells' soma size associated to social status show a direct link between AVT and the early establishment of social hierarchy. Whether it is non-territorial behavior that modifies cell morphology, or larger pPOA cells that prompts submissive behavior, remains to be seen. Indirect evidence from the observation of axonal projections, pituitary and testis culture, suggest a possible role of AVT either at a central level acting as a neuromodulator, and/or at a peripheral level, acting as a neurohormone with target sites as close as the pituitary, or as distant as the testis. In *C. dimerus*, AVT promotes pituitary gonadotropin release and testicular androgen synthesis *in vitro*. AVT mRNA and peptide expression were found in the testes, with IHC identifying small cells from the interstitial compartment of the testis. Though many studies have noted AVT expression in the testes of various vertebrates [13,38,52], to our knowledge this is the first report of AVT peptide detection at a cellular level in a teleost testis.

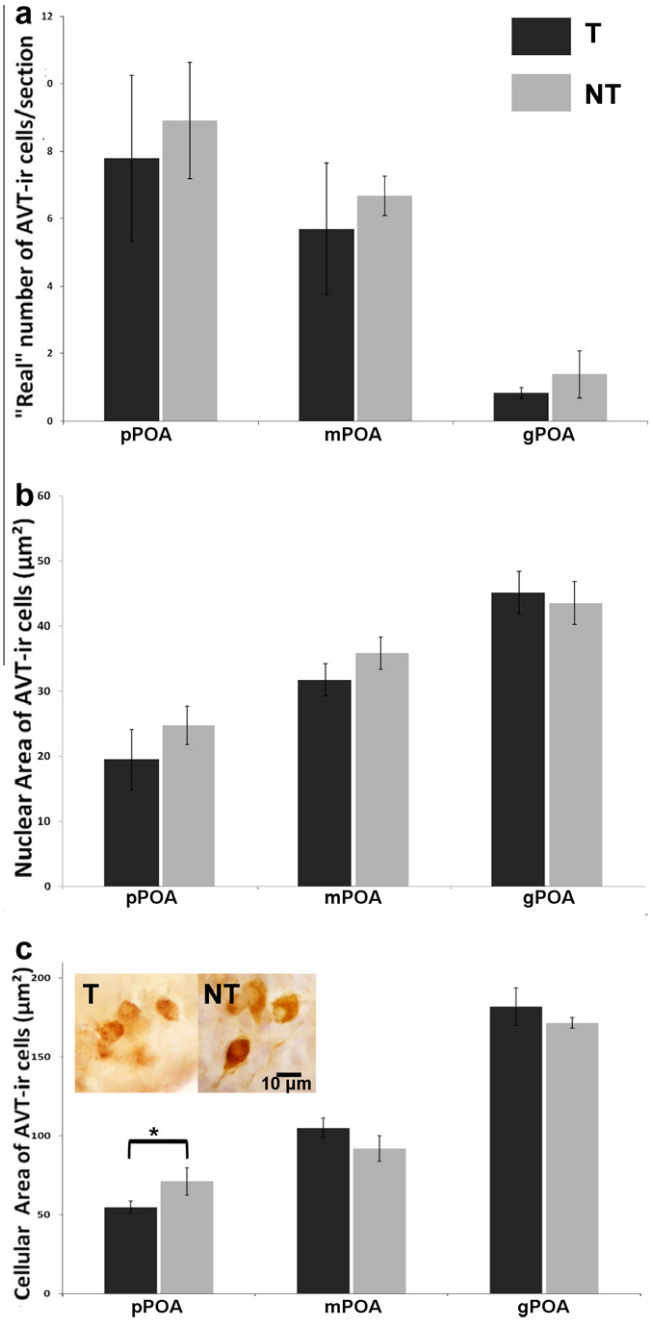

Fig. 6. Morphometry of AVT-ir cell bodies in territorial vs not-territorial males. (a) "Real" number of AVT-ir cells per 15 μm section from pPOA, mPOA and gPOA cells populations. No significant differences were observed between territorial ($n = 4$) and non-territorial males ($n = 4$) for any of the cell populations ($p > 0.05$). (b) Mean nuclear area (μm^2) of AVT-ir cells associated with social status. No significant differences were observed between territorial ($n = 4$) and non-territorial ($n = 4$) males for any of the cell populations ($p > 0.05$). (c) Mean soma size (μm^2) of AVT-ir cells from each of the three cells populations. pPOA cells associated to non-territorial status (n = 4) were 23% larger compared to those associated to territorial males (n = 4) (nested design ANOVA: p = 0.027; Fisher's LSD). No significant differences were observed between mPOA and gPOA cells populations ($p > 0.05$). Inset shows a microphotograph of a smaller pPOA cell from a territorial male (left) compared to a larger from a non-territorial individual (right). Scale bar: in image. In all cases data was presented as means ± SEM and data error bars indicate SEM for cell population. Asterisk indicates significant differences.

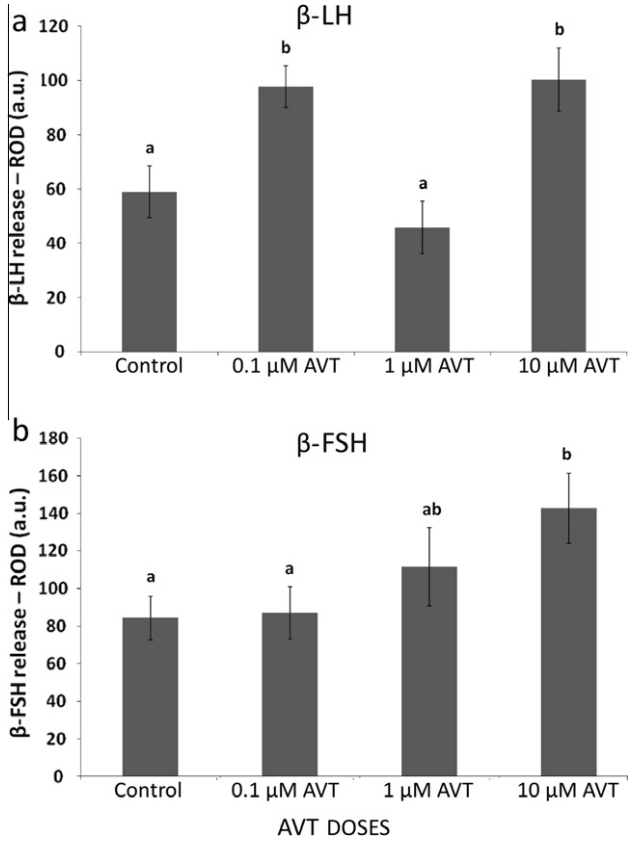

Fig. 7. Effects of exogenous AVT on pituitary β-FSH, and β-LH release. Semiquantitative analysis of pituitary hormone release from single-cultured pituitaries of *C. dimerus* after treatment with increasing doses of AVT (0.1, 1 and 10 μM; $n = 6$ per treatment). Values are expressed in arbitrary units (a.u.) as means ± SEM of the pituitary hormone released optical density of day 2 relativized to day 1; ROD = relative optical density. (a) Triphasic response in pituitary β-LH release (randomized block design ANOVA: $p = 0.007$; Fisher's LSD). (b) Dose-dependent increase in pituitary β-FSH release (randomized block design ANOVA: $p = 0.007$; Fisher's LSD).

In *C. dimerus* AVT-ir neurons were located exclusively within the POA, distributed among the parvo-, magno-, and gigantocellular subpopulations. This pattern of distribution is consistent with all vertebrate species studied to date, including agnathans and gnathostomes, and these cells project both to a variety of brain regions and to the pituitary [37]. In teleost species as diverse as the reef butterflyfish (family Chaetodontidae, [10]), medaka (*Oryzias latipes*) [39], goldfish (*Carassius auratus*) [44] and the Death Valley killifish (*Cyprinodon nevadensis*) [29], AVT-ir neurons are located almost exclusively in the POA and anterior hypothalamus, distributed among a population of densely packed, small cells and two other population of larger cells surrounding the recess of the third ventricle, pPOA, mPOA and gPOA respectively. In two species of the reef butterflyfish (*Chaetodon multicinctus* & *C. miliairs*), Dewan et al., 2008 [10], described a group of additional AVT-ir neurons present in the ventral tubular hypothalamus, posterior to the POH tract, of even smaller soma diameter than the pPOA cells. This population was not detected in *C. dimerus*. In turn, in both species of butterfly fish, the most numerous cell subpopulation was the pPOA, in contrast to that observed in *C. dimerus*, where the mPOA neurons were most abundant.

Similar to the conserved pattern observed for AVT-ir somata, vasotocinergic fibres also show a high degree of neuroanatomical conservation. Vasotocin projections are found throughout the teleost brain, especially in forebrain and hindbrain regions [10,14,17,25,34,48]. Surprisingly, in *C. dimerus*, no immunolabeling was observed in extra-hypothalamic regions, except for the pineal complex, where AVT is believed to play a role in the coordination between photoperiodic cues and reproductive events [27]. Noteworthy is the fact that the above mentioned studies where AVT fibres were broadly observed across the brain of different fish species, used frozen IHC protocols in contrast to the paraffin based protocol used here. Thus is quite possible that failure to detect fibres outside the POA-Hypothalamic region results from methodological reasons.

In *C. dimerus* the association observed between AVT-ir nerve terminals with the adenohypophysis, suggests a possible role of AVT as a moderating factor for the release of pituitary hormones.

The establishment of different social phenotypes in males of *C. dimerus*, was accompanied by differences in brain vasotocinergic neural populations. Non-territorial individuals had pPOA neurons 23% larger compared with T males. There were no differences between the two social phenotypes with respect to number of cells per section and nuclear area for any of the 3 subpopulations analyzed. Average BW and total body length, which could possibly account for allometric based differences, did not differ between T and NT males (BW, T vs. NT: 45.7 ± 9.6 g vs. 45.1 ± 8.6 g, $p = 0.30$. Total body length, T vs. NT: 12.6 ± 0.9 cm vs. 12.5 ± 0.8 cm, $p = 0.98$).

While larger AVT-ir cell bodies may reflect changes in the synthesis, accumulation or peptide release that would be necessary for the maintenance or modulation of social behaviors, additional studies are needed to test this hypothesis in *C. dimerus*. Quantitative PCR (Q-PCR) analysis of AVT mRNA associated to social status should help dim some light into the subject. On the one hand, studies in other species of teleost have shown that the relationship between soma size and gene expression of a given peptide, do not always correlate. For example, in the protogynous Bluehead wrasse (*Thalassoma bifasciatum*) different aspects of neural phenotype, as soma size and peptide synthesis appear to be regulated independently [51]. On the other hand, in *A. burtoni* for example, there is a close positive relationship between GnRH-ir neurons soma size and gonad size [15], a feature regulated, among others, by gonadotropins through the HPG axis. This socially induced mechanism in cell size change provides potential for fast and adaptive change in the neuroendocrine system without neural death or addition.

It is possible that the pPOA nucleus, which mainly projects to the pituitary [24], may be involved in the modulation of behaviors

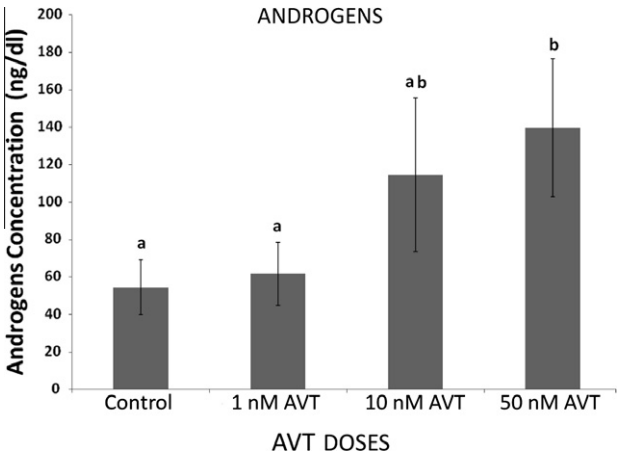

Fig. 8. Effects of exogenous AVT on testicular androgen release *in vitro*. Dose-dependent increase in androgen synthesis (ng/dl relativized to the estimated weight of each pair of fragments) by testicular fragments of equal size exposed to different AVT concentrations (1, 10 and 50 nM $n = 5$ per treatment) (randomized block design ANOVA: $p = 0.0054$, Fisher's LSD; $n = 5$). Heterologous antiserum raised against testosterone had a 45% crossreactivity with 11-ketotestosterone.

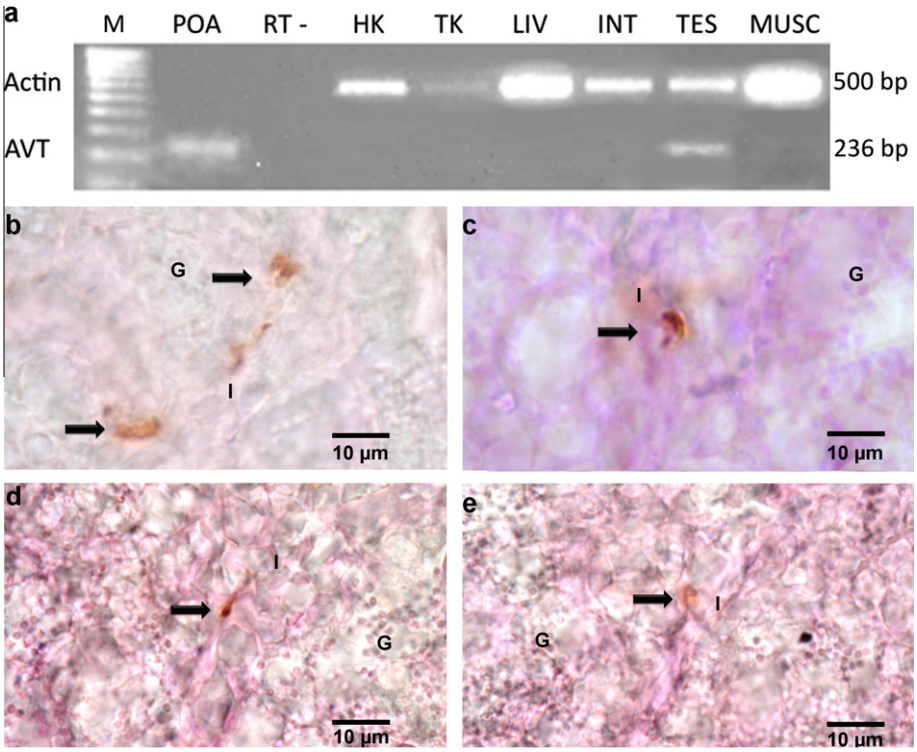

Fig. 9. (a) Distribution of *C. dimerus* AVT gene expression. RT-PCR detection followed by PCR amplification of AVT preprohormone in different organs. Actin was used as an internal control of RNA extraction and cDNA synthesis. M: DNA marker; POA: preoptic area (positive control); RT-: negative control, HK: head kidney; TK: trunk kidney; LIV: liver; INT: intestine; TES: testis; MUSC: muscle. (b–e) Testicular immunohistochemical localization of AVT-ir cells in cryostyat sections. Small and scarce AVT-ir cells were distributed in the testis' interstitial compartment. Most cells were tapered shaped (b), some with thin cytoplasmatic extensions (d). Arrows point the position of AVT-ir cells. G: example area of germinal compartment; I: example area of interstitial compartment.

related to submission and/or activation of the stress axis. Evidence from other teleost species shows that AVT synthesized by pPOA cells is involved in regulating the release of cortisol through the action of AVT on the hypothalamus–pituitary–adrenal axis [3,46]. Almeida et al. [1] quantified AVT levels in the pituitaries of males of the cichlid fish, *Oreochromis mossambicus*, of different social status. Subordinate animals had significantly higher levels of AVT than dominant males in the pituitary gland, which could point out to a possible activation of the stress axes through the synergistic action of AVT with corticotrophin releasing hormone (CRH) as an adrenocorticotropin (ACTH) secretagogue [3]. In spite of higher pituitary AVT levels in subordinate males, Almeida et al. [1], did not find a correlation between AVT pituitary levels and circulating cortisol concentration, allegedly due to uncoupled cortisol levels from social behavior as consequence of prolonged hierarchy stability. A recent study on *C. dimerus* demonstrated that non-territorial individuals have significantly higher cortisol levels than those of territorial males [2]. In the African cichlid, *A. burtoni*, AVT mRNA expression within the pPOA was negatively correlated with the production of territorial behaviors in both T and NT males and showed a strong positive correlation with the tendency of NT males to flee from T males [19]. More recently, Dewan and Tricas [11], found a negative relationship between the number and size of AVT-ir pPOA cells and offensive behavior in the multiband butterflyfish, *C. multicinctus*, under a simplified test paradigm that attempted to control for social context. Furthermore, Larson et al. [28] only observed AVT immunostaining in the pPOA of subordinates zebrafish, while it was absent in dominant males. Despite methodological and behavioral paradigms differences, these works along with the current study in *C. dimerus* points for a putative role of pPOA AVT in the activation/modulation of submissive neural circuits or inhibition of aggressive/dominance neural networks and elevated cortisol levels trough the action of AVT on the hypo thalamus–pituitary–adrenal axis, that could act jointly at a central level inducing typical non-territorial behavior.

The fact that mPOA and gPOA subpopulations did not differ between T and NT males, does not rule out by any means that neither group of cells is involved in the modulation of social behaviors. A more sensitive analysis by Q-PCR of RNA from the different subpopulations could reveal possible differences in AVT expression that do not correlate with general cell structure in the time course of this experiments. Also, allowing the established hierarchy to remain stable for longer periods of time (only 48 h in this case) could translate in detectable differences in cell number or soma/nuclear size.

Even though the effect of AVT on the stress axis is well characterized for some species, as stated above, there appears to be only few studies which have addressed the effect of AVT on the synthesis and gonadotropin release in teleosts. Groves and Batten [22], made pituitary cultures (*Poecilia latipinna*) exposed to different AVT doses in the medium. The study of the ultrastructure of gonadotrophs revealed that AVT apparently stimulated the synthesis of gonadotropins and probably their release. The size of the gonadotrophs, in general, increased slightly. There was an increase in the development of the rough endoplasmic reticulum, and cytoplasmic granulations increased very slightly [22], all indicative of increased peptide synthesis. In another study conducted by Batten [5], ultrastructural analysis of the pituitary revealed that some AVT-ir fibres were juxtaposed to the basal lamina and occasional discontinuities in it, allowing the intrusion of the fibres to the ADH and direct contact between AVT-ir fibres and gonadotrophs, although less so than with other cell types.

In *C. dimerus*, AVT affects the release of both gonadotropins quantified by Western blot and subsequent densitometric analysis. β-LH showed a triphasic response to increasing AVT doses, which

may imply the existence of at least two populations of pituitary AVT receptors with different affinities. In the Amargosa River pupfish (*C. nevadensis amargosae*), three putative AVT receptor cDNAs were identified. Two of these receptor cDNAs appeared to be paralogs that showed greatest homology to mammalian V1a-type receptors, while the third cDNA showed closest homology to mammalian V2-type receptors [31]. Pupfish male pituitary glands presented V1a1 and V2 transcripts. On the other hand, the effect of AVT on β-FSH release was dose-dependent and directly proportional. The presence of AVT-ir fibres in the proximal pars distalis, where *C. dimerus*' gonadotrophs are found, suggests a putative functional relationship between AVT and gonadotropin release *in vivo*. Recently, the distribution of the V1a2 receptor subtype was studied in the perciform, *Epinephelus adscensionis* [26], revealing its presence in the PPD of the pituitary.

Current results postulate a possible indirect effect (through gonadotropins) and a direct effect of AVT on testicular androgen release *in vitro*. AVT significantly increased androgen synthesis in a manner that is directly proportional. In testicular fragments incubated with the highest AVT dose, androgen synthesis increased more than twice compared to control. The androgen 11-KT is the most potent androgen in male teleosts [23] involved in the stimulation of secondary sexual characteristics, reproductive behavior and spermatogenesis [6]. In several species it has been linked with aggressive behavior as a result of the establishment of social hierarchy, where it may mediate long-term effects associated with the territorial status [45,53]. Thus AVT may not only be associated with the establishment of territorial aggressive displays at a central level, acting as neuromodulator, but acting as neurohormone and putative pituitary hormone-releasing factor, it may also stimulate androgen synthesis at the testis. Nonetheless due to the antibody 45% crossreactivity, it's not possible to distinguish whether the effect is over testosterone, 11-KT or both. Though the expression of the AVT receptor was not analyzed in *C. dimerus*, studies in other fish species have shown its expression in the testes [26]. In mammals, a receptor for AVP has been described in rodent's Leydig cells [33,35], where the ligand-receptor union affects cell steroidogenic activity.

PCR analysis revealed AVT preprohormone expression in the brain and testis of *C. dimerus*. On cryostat sections from testis, AVT-ir was observed in small isolated cells, tapered or oval shaped, within the interstitial compartment. The precise identity of these cells remains unknown; double IHC utilizing cellular markers of different cell types present in the interstitial compartment such as Leydig cells (i.e. 3-βHSD), myoid cells (i.e. α-smooth muscle actin), endothelial cells (i.e. VE-Cadherin) and fibroblasts (i.e. fibroblast growth factor surface proteins) will permit such identity recognition. AVT expression pattern in *C. dimerus* coincides with that observed for other vertebrate groups. In the air-breathing catfish (*Heteropneustes fossilis*) AVT expression was detected by a HPLC assay in the PO-H tract, ovary and testis. The latter showed the lowest expression levels and IHC on paraffin embedded sections of the testis, revealed no positive staining [52]. In mammals, there are many studies on AVP- and oxytocin-like peptides expressed in rat and human testicles [38]. Cultured Leydig and Sertoli cells revealed the presence of AVT immunostaining in Sertoli cells only [13]. AVT presence in the testis suggests a new action site of this nonapeptide. Its low expression hints for a restricted local effect, such that acting in a paracrine fashion, AVT synthesized in the testis, may regulate steroidogenesis and/or other processes involved in spermatogenesis.

5. Conclusion

Thus, in *C. dimerus*, the AVT system is highly complex with multiple sites of action along the HPG axis. AVT expression levels in pPOA cells are differentially related to distinct social classes in which increased AVT expression may be associated with behavioral and physiological changes typical of NT males.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012.08.015.

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