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Interaction between vasotocin and gonadal hormones in the regulation of reproductive behavior in a cichlid fish

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Abstract

Vasotocin (VT) has been associated with the regulation of different aspects of social behavior (e.g., mating and aggression). Given the fact that androgens are also known to regulate reproductive behavior, we hypothesized that VT and androgens could be interacting, rather than acting independently, in the regulation of reproductive behavior. In the present study, we aimed to understand the effect of VT and its interaction with gonadal hormones (putatively androgens) on different aspects of reproductive behavior of a polygynous and territorial cichlid fish, the Mozambique tilapia (*Oreochromis mossambicus*). Using a within-subject design, we treated territorial males, that were previously castrated or sham-operated, with different dosages of VT as well as with a *V1A* receptor antagonist (Manning compound) and subsequently analyzed their behavior towards females and towards an intruder male. Our results showed that VT affected the behavior of territorial males towards females but not towards males. Specifically, VT-treated males interacted less with females (i.e., spent less time touching the transparent partition that allowed visual contact with females) and were less aggressive towards females than saline-treated males. Moreover, in sham-operated males, blocking *V1A* receptors increased the frequency of bites towards females in comparison to saline-treated males, but not in castrated males. This result suggests that VT down-regulates aggressiveness towards females through the action of *V1A* receptors in the gonads (putatively decreasing androgen secretion), and that androgens up-regulate this behavior. In summary, our results suggest that VT may modulate social behavior, through an interaction with gonadal hormones.

Keywords Reproductive behavior · Aggressive behavior · Vasotocin · Manning compound · Mozambique tilapia

Introduction

Both gonadal steroids and neuropeptides have been implicated in the regulation of a wide range of social behaviors (reviewed in Gonçalves et al. 2017, for teleost fish). The canonical explanation for this multiplicity of regulators of social behaviors has relied on the existence of a shared brain network for different social behaviors (aka social behavior network, Newman 1999; Goodson 2005; O'Connell and Hofmann 2011). In this network, each brain region constitutes a node, expressing receptors for steroid hormones and neuropeptides, which further modulate the state of the network (e.g., estrogen: Forlano et al. 2005; Hawkins et al. 2005; Muriach et al. 2008; androgen: Harbott et al. 2007; Munchrath and Hofmann 2010 vasotocin: Kline et al. 2011; Huffman et al. 2012 and isotocin: Huffman et al. 2012). Significantly, some of the effects of these modulators of social behavior can result from an interaction between these hormonal and peptidergic systems, such that their concurrent action mediates various aspects of social behavior.

Regarding the specific effect of VT on social behavior, several investigations manipulating the VT system in teleosts have obtained contrasting results (Godwin and Thompson 2012). Thus, a coherent pattern between species has not been found although Oldfield et al. (2015) have proposed an important evolutionary framework that tries to explain the relation between VT expression across species and consequently their aggressive behavior and mating system.

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Likewise, the effect of androgens on reproductive and aggressive behaviors is not straightforward. For instance, castration impairs courtship, spawning pit digging, and nuptial coloration in some species (e.g., Egyptian mouthbrooder, *Pseudocrenilabrus multicolor*, Reinboth and Rixner 1970; blackchin tilapia, *Sarotherodon melanotheron*, Levy and Aronson 1955; Burton's mouthbrooder, *Astatotilapia burtoni*, Francis et al. 1992; *O. mossambicus,* Almeida et al. 2014) but not in others (jewelfish, *Hemichromis bimaculatus*, Noble and Kumpf 1936; platinum acara, *Andinoacara latifrons*, Aronson et al. 1960, *S. melanotheron* and *Oreochromis upembae*, Heinrich 1967). While in the case of aggressive behavior, the exogenous administration of androgens increases aggression (*A. burtoni* and sheepshead minnow, *Cyprinodon variegatus*, Fernald 1976; Higby et al. 1991), however, androgen receptor antagonists or castration can either inhibit (*Amatitlania nigrofasciata*, Sessa et al. 2013; *A. burtoni*, Francis et al. 1992) or have no effect in aggression (*O. mossambicus* and *A. nigrofasciata*, van Breukelen 2013; Almeida et al. 2014).

Even though traditionally VT and androgens have been studied separately in the context of social behavior, some studies account for a crosstalk between these systems. In mammals, it has been shown that androgens modulate the vasotocin neural system, the mammalian homologue of VT (reviewed in Albers 2012). For example, castrated male rats present fewer vasotocin cell bodies and fiber density in several brain areas than control males; a difference which is restored with androgen replacement treatments (DeVries et al. 1985). Likewise, studies in lizards have reported the sexual dimorphism of the VT system, namely, that males have denser VT fibers in several limbic areas and that VT magnocellular cells of the paraventricular nucleus are larger than in females (e.g., tree lizard, *Urosaurus ornatus*, Kabelik et al. 2008). In addition, castration followed by testosterone replacement treatment increases the size of VT cells compared to castrated and saline treated males (desert-grasslands whiptail, *Cnemidophorus uniparens*, Hillsman et al. 2007; *U. ornatus*, Kabelik et al. 2008). Similar experiments conducted in birds and amphibians have shown the same pattern, i.e., that testosterone plays a key modulator role of the brain VT system also in these taxa (e.g., bullfrogs, *Rana catesbeiana*, Boyd 1994; birds: reviewed in Panzica et al. 2001). In fish, some studies account for morphological differences in the VT system between males and females. For instance, females of the Hawaiian sergeant damselfish, *Abudefduf abdominalis*, seem to have increased density fibers than males (Maruska 2009) while females of the halfspotted goby, *Asterropteryx semipunctata*, have more or larger VT cells than males (Maruska et al. 2007). Interestingly, these differences may change between reproductive seasons (see, for instance, Maruska et al. 2007).

Moreover, vasotocin seems to regulate gonadal steroidogenesis since in vitro studies in rodents report the existence of vasotocin receptors in the testis and that vasotocin influences the production of androgens by Leydig cells (Meidan and Hsueh 1985; Tahri-Joutei and Pointis 1989; Bathgate and Sernia 1994), even though the precise mechanism is not known. In the Leydig cells, it may act as a paracrine regulator of steroidogenesis or in an autocrine fashion since vasotocin mRNA has also been found here (Ivell et al. 1992). Interestingly, a study in rabbits and rats has shown that both oxytocin and vasotocin elicit tonic contractions in erectile and ejaculatory tissues, via vasotocin receptors (Gupta et al. 2008), suggesting that several gonadal functions could also be modulated by vasotocin. In teleosts, VT receptors have also been found in the testis (catfish, *Heteropneustes fossilis* and Amargosa pupfish, *Cyprinodon nevadensis amargosae*, Lema 2010; Lema et al. 2012) and, in the case of the catfish, these receptors were detected within the interstitial tissue, which contains Leydig cells. Moreover, in the Central American cichlid, *Cichlasoma dimerus*, it was found that this neuropeptide is expressed in the testis and that its administration stimulates the production of androgens on testis incubation cultures (Ramallo et al. 2012).

In the present study, we used a polygynous species, the Mozambique tilapia, *Oreochromis mossambicus*, which is a freshwater fish with a lek-breeding system (Fryer and Iles 1972). In this species, males form dense aggregations in territories, which they dig, defend, and where they attract females to mate (Oliveira and Almada 1998). There are two different male phenotypes, which can reversely change due to fluctuations in the social environment (Oliveira and Almada 1998). Males that establish territories and court females are typically larger and present a dark nuptial coloration. These territorial males are very aggressive to intruders, while, in contrast, subordinate males have a silver color pattern like females with whom they school. A previous study has shown that castration impairs reproductive but not aggressive behavior in this species (Almeida et al. 2014), suggesting that different neuroendocrine mechanisms regulate these kind of behaviors. To clarify this subject, we treated castrated and sham-operated territorial males with different dosages of VT and a potent VT receptor *V1A* antagonist, Manning compound (Manning et al. 2012), using a within-subject design, and subsequently analyzed their behavior towards females and males. With this study, we aimed to (1) characterize the effects of VT on reproductive and aggressive behavior and (2) to investigate a putative interaction of VT and gonadal hormones on the regulation of these behaviors. We predicted that VT would increase courting (Bastian et al. 2001) and reduce aggressive behavior (as in Huffman et al. 2015). Also, we expected that castrated males and sham males behaved differently when VT-treated but did not have a definite direction of the expected results, due to the lack of previous studies.

Materials and methods

Animals and housing

Fish used in this study came from a stock held at ISPA. Fish were maintained in stable social groups of 4 males and 5 females per group, in glass tanks (120 × 40 × 50 cm, 240 L) with a fine gravel substrate. Tanks were supplied with a double filtering system (sand and external biofilter; Eheim) and constant aeration. 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, a 12L:12D photoperiod and fed with commercial cichlid sticks. The social status of the males was monitored daily. Dominance status of the males was assessed based on the dark body coloration and the possession of a spawning pit on the substrate (Oliveira and Almada 1996).

Experimental procedure

Twenty-two territorial males (mean body mass ± SEM: 31.92 g ± 2.25 g; mean standard length ± SEM: 10.20 cm ± 0.27 cm) were isolated in test tanks (47 cm × 24 cm × 30 cm). On one side of the test tank, there was placed an adjacent demonstration tank (70 cm × 37 cm × 30 cm; demo tank 1) containing 4 females, while on the opposite side of the test tank, there was another demonstration tank (18 cm × 30 cm × 15 cm; demo tank 2) with an opaque partition between them (Fig. 1). Focal fish had visual access to the females of demo tank 1. Two days after isolation (day 2), focal males were either sham operated (SHAM group, $n = 11$) or castrated (CAST group, $n = 11$), then returned to test tank. Surgery was performed according to Almeida et al. 2014, to guarantee total excision of gonad tissue. On day 5, a demonstrator male, of similar size to the focal male, was placed in demo tank 2. On day 6, focal males received an intraperitoneal injection (ip) with one of the following compounds: vehicle solution, VT acetate salt (4 different dosages: 0.125, 0.25, 0.5, or 1 µg/g; Sigma V0130) or the specific VT receptor *V1A* antagonist, Manning compound, ([β-Mercapto-β,β-cyclopentamethyle nepropionyl[1], O-Me-Tyr[2], Arg[8]]-Vasopressin (Kruszynski et al. 1980); Sigma V2255). VT and Manning dosages were defined according to previous studies (Lema and Nevitt 2004; Filby et al. 2010). Chemicals were dissolved in saline vehicle solution (0.9% sodium chloride). After the injection, the behavior of the focal fish towards the females of the demo tank 1 was observed for 15 min. Then, an opaque partition was placed between the focal fish and the female's demo tank to avoid visual contact between them. Next, the opaque partition separating the focal male tank and the demo tank 2 was lifted. Thus, the focal fish was given visual access to the male in the demo tank 2 for 15 min and the behavior of the focal fish was noted. Every 2 days, the focal fish were ip injected with another treatment and observed in the behavioral assays with the same females and the same demonstrator fish. The order of exposure of each focal fish to the different treatments was randomized (VT, Manning or saline); however, the order of stimuli presentation was always the same, i.e., first to females than to males.

The time to assess behavioral effects after injections was defined based on pilot studies and a study published by Mens et al. (1983). Accordingly, subcutaneous injections of vasotocin in rats resulted in an increase of peptide concentration in the cerebrospinal fluid 2 min after injection, reached a maximal level 5 min after injection, and were undetectable 1 h after administration. Moreover, Soares et al. (2012) also found that both VT and Manning compound pharmacological manipulations promote differences, within 60-min post-injection, in the cleaning behavior of the wrasse *Labroides dimidiatus*. Surgeries were performed with fish anaesthetized with MS-222 (tricaine methanesulfonate, 1000 mg/g, dilution 300 mg/L, Pharmaq).

Behavioral observations

Behavior of the focal male, either towards the females or interacting with the demonstrator male, was analysed in real-time using a computerized multi-event recorder software (Observer, Noldus technology, version 5). The analysis was based on the ethogram repertoire provided by Baerends and Baerends-Van Roon (1950). The frequency and duration of relevant behavioral patterns were quantified during female (i.e., touching the transparent partition, courtship, digging a spawning pit, bites at the transparent partition) and male (i.e., bites at the transparent partition, displays, attacks) interactions, over the 15 min observation period. Since only four focal fish (from the sham-treated group) courted females in a total of six trials, this variable was excluded from further analyses.

Data analysis

Behavioral variables were logarithmically transformed [log10 $(x + 1)$] to meet parametric assumptions. However, two variables, the frequency of bites towards females and the frequency of digging, did not follow the assumptions of normality. Outlier observations were identified and replaced by missing values using Dixon test, used for small sample sizes (Dixon 1963). For non-parametric variables, the latter test is not possible to apply. Thus, in these cases, extreme values were identified using the SPSS software (SPSS identify

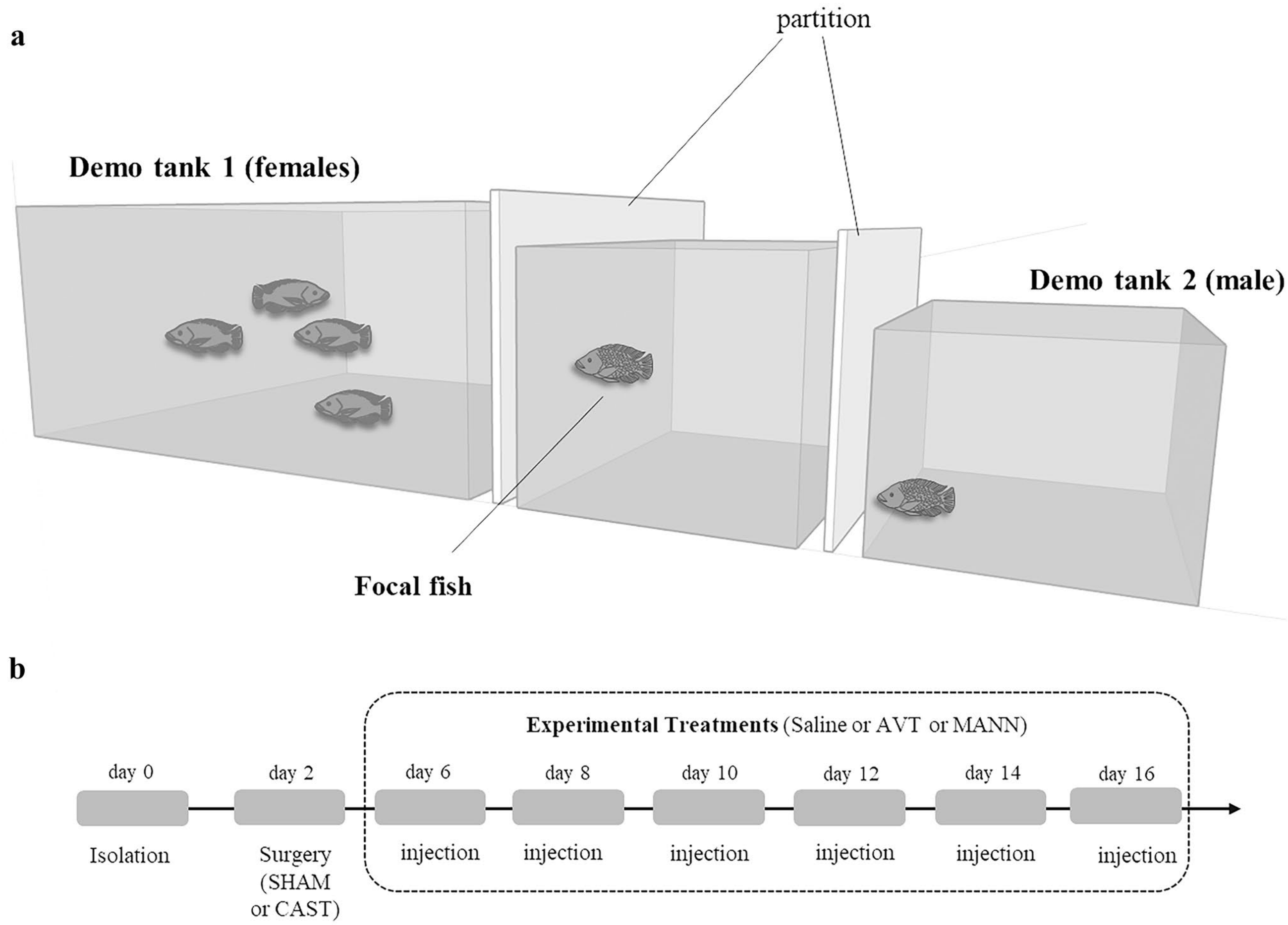


Fig. 1 Experimental design. **a** 3D diagram of the experimental setup. Males were isolated in test tanks. On each side, there were demonstration tanks (demo tank 1 with females and demo tank 2 with a demonstrator male). **b** Timeline of the experiment (within-subject design). In the first day of the experiment, focal males were isolated and on day 2 were submitted to surgery, either a sham operation (SHAM) or castration (CAST). From day 6 until day 16, focal fish received an intraperitoneal injection with one of the following compounds: vehicle solution, VT acetate salt (4 different dosages: 0.125, 0.25, 0.5, or 1 μg/g) or the specific VT receptor *V1A* antagonist, Manning compound. After each injection, the behavior of the focal fish towards the females of the demo tank 1 was observed for 15 min. Then, an opaque partition was placed between the focal fish and the female's demo tank and the focal fish was given visual access to the male in the demo tank 2 during 15 min. The order of exposure of each focal fish to drug treatments (VT dosages and antagonist) was randomized

values more than 3 box lengths/interquartile range from either hinge) and removed from further analyses.

Behavioral variables were analyzed using Linear Mixed Models (LMM) with castration (sham-operated or castrated) and VT treatment (saline, VT 0.125 μg/g, VT 0.25 μg/g, VT 0.5 μg/g, VT 1 μg/g, Manning) as fixed effects and focal fish as a random effect. Homoscedasticity was confirmed with Levene's test. Plots of residuals, fitted values and estimated random effects were used to confirm assumptions of LMM. Planned comparisons were set a priori and used to test for specific differences between the saline and the other treatments and between SHAM and CAST group within each treatment. P-values were adjusted for multiple testing using the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995).

Regarding the frequency of bites towards females and the frequency of digging, despite the lack of normality and homoscedasticity of these variables we still used a LMM analysis due to the lack of an equivalent nonparametric test and to avoid loss of data due to missing values (e.g., fish that froze during observations).

Effect sizes were computed for LMM tests (omega-squared, ω^2) and for planned comparisons (Cohen's d). Statistical analysis was performed using IBM SPSS® statistics v.21, and R (Team 2015) with the following packages: nlme (LMM), multcomp (planned comparisons), sjstats (effect sizes) and outliers (Dixon test). Degrees of freedom may vary between the analyses due to missing values.

Results

Behavior towards females

The time spent by the focal fish interacting with females (i.e., the time spent touching the transparent partition that allowed visual contact with females) changed significantly with VT treatment ($F_{(5,91)} = 17.92$, $p < 0.001$, $\omega^2 = 0.47$) but did not differ significantly between sham and castrated males ($F_{(1,20)} = 0.02$, $p = 0.90$, $\omega^2 = -0.05$). The interaction between VT treatment and castration was also not significant ($F_{(5,91)} = 0.99$, $p = 0.430$, $\omega^2 = 0.00$). After VT injection, independently of dosage and castration, males significantly decreased the time spent interacting with females in comparison with the saline injected treatment (Table 1, Fig. 2a). Castrated fish injected with Manning decreased the time of interaction with females compared with saline-injected castrated fish (Table 1, Fig. 2a).

Table 1 – Effect of castration and chemical treatment on the behavior of the focal male towards females: effect sizes and planned comparisons

Planned comparisons	SHAM			CAST			SHAM vs CAST		
	z	*p*	*d*	*z*	*p*	*d*	*z*	*p*	*d*
Time spent in interaction									
VT 0.125 µg/g vs saline	**−4.54**	**<0.001**	**2.40**	**−5.65**	**<0.001**	**0.86**			
VT 0.25 µg/g vs saline	**−4.58**	**<0.001**	**1.46**	**−5.08**	**<0.001**	**1.17**			
VT 0.5 µg/g vs saline	**−3.62**	**<0.001**	**0.97**	**−3.93**	**<0.001**	**1.24**			
VT 1 µg/g vs saline	**−5.81**	**<0.001**	**1.66**	**−4.80**	**<0.001**	**1.31**			
Manning vs saline	−0.16	0.93	0.33	**−2.22**	**0.05**	**3.90**			
Saline							0.48	0.84	3.34
VT 0.125 µg/g							−0.32	0.92	0.88
VT 0.25 µg/g							0.08	0.94	0.04
VT 0.5 µg/g							0.23	0.93	0.08
VT 1 µg/g							1.03	0.48	0.43
Manning							−0.92	0.52	1.08
Frequency of bites									
VT 0.125 µg/g vs saline	**−2.59**	**0.017**	**0.80**	**−3.19**	**0.003**	**nd**			
VT 0.25 µg/g vs saline	**−3.54**	**0.002**	**1.51**	**−3.19**	**0.003**	**nd**			
VT 0.5 µg/g vs saline	−2.03	0.067	0.97	**−2.91**	**0.007**	**0.92**			
VT 1 µg/g vs saline	**−3.61**	**0.002**	**1.39**	**−3.19**	**0.003**	**nd**			
Manning vs saline	**3.50**	**0.002**	**1.01**	−0.47	0.73	0.12			
Saline							−0.75	0.56	0.21
VT 0.125 µg/g							−1.29	0.26	nd
VT 0.25 µg/g							−0.34	0.74	nd
VT 0.5 µg/g							−1.54	0.18	0.07
VT 1 µg/g							−0.38	0.74	nd
Manning							**−4.14**	**<0.001**	**0.76**
Frequency of digging spawning pit									
VT 0.125 µg/g vs saline	−1.08	0.39	nd	**−4.03**	**<0.001**	**nd**			
VT 0.25 µg/g vs saline	−1.05	0.39	nd	**−3.98**	**<0.001**	**nd**			
VT 0.5 µg/g vs saline	−1.10	0.39	nd	**−3.84**	**<0.001**	0.85			
VT 1 µg/g vs saline	−1.10	0.39	nd	**−4.03**	**<0.001**	**nd**			
Manning vs saline	**2.59**	**0.03**	**0.02**	1.61	0.25	0.73			
Saline							**2.73**	**0.02**	**0.97**
VT 0.125 µg/g							0.01	1	nd
VT 0.25 µg/g							−0.08	1	nd
VT 0.5 µg/g							0.002	1	nd
VT 1 µg/g							0	1	nd
Manning							1.15	0.29	0.00

Groups: *SHAM* sham fish, *CAST* castrated fish, *z* *z*-test estimate, *d* effect size estimate (Cohen's *d*), *p* *p*-value after multiple comparison adjustment; statistically significant values are in bold

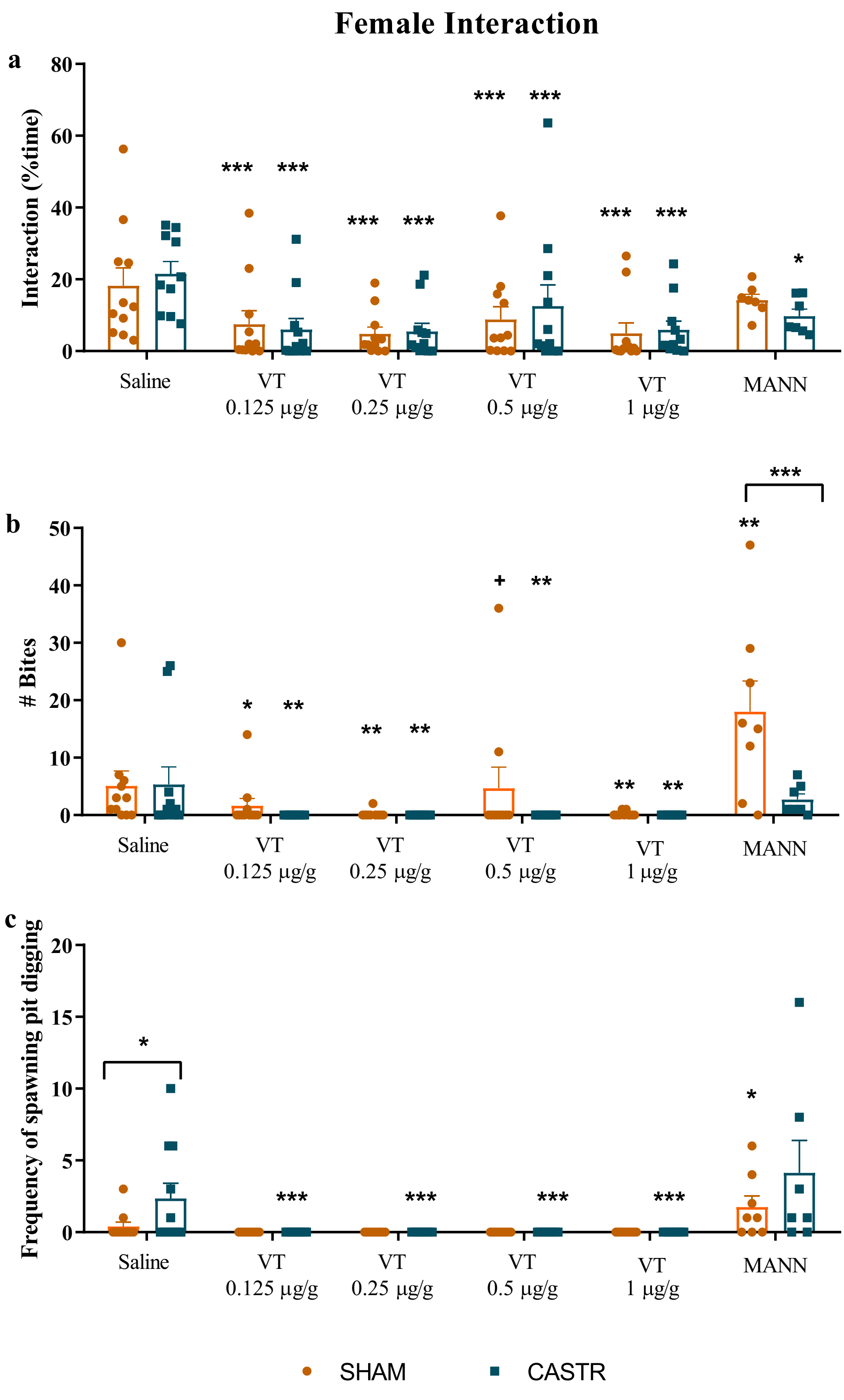


Fig. 2 Behavioral measurements of the focal fish during females' interaction after each experimental treatment **a** time spent interacting with females; **b** frequency of bites; **c** frequency of spawning pit digging. Groups: SHAM, sham fish; CAST, castrated fish. MANN: Manning compound. *significant difference for $p < 0.05$; **significant difference for $p < 0.01$; ***significant difference for $p < 0.001$

The frequency of bites towards females decreased significantly with VT treatment ($F_{(5,91)} = 14.64$, $p < 0.001$, $\omega^2 = 0.41$) and with castration ($F_{(1,20)} = 5.37$, $p = 0.03$, $\omega^2 = 0.17$). The interaction between AVT treatment and castration was also significant ($F_{(5,91)} = 2.60$, $p = 0.03$, $\omega^2 = 0.08$). There were no differences in the control treatment (i.e. saline injected fish) in terms of frequency of bites towards females between the sham-operated and castrated males. Both sham-operated and castrated males injected with VT significantly decreased their bites towards females in comparison with the saline injected treatments (Table 1, Fig. 2b). After the Manning injection, sham-operated fish significantly increased the frequency of bites in comparison with the saline treatment (Table 1, Fig. 2b), and there was a significant difference between the sham-operated and castrated fish in the Manning treatment (Table 1, Fig. 2b).

The frequency of spawning pit digging in the presence of females changed significantly with VT treatment ($F_{(5,86)} = 13.20$, $p < 0.001$, $\omega^2 = 0.40$) but there was no effect of castration ($F_{(1,20)} = 1.84$, $p = 0.19$, $\omega^2 = 0.04$). The interaction between VT treatment and castration was not significant ($F_{(5,86)} = 1.48$, $p = 0.21$, $\omega^2 = 0.03$). After VT injection, castrated males significantly decreased digging frequency in comparison with saline injected males (Table 1, Fig. 2c). In sham-operated males, there were no differences between the saline and VT injected treatments (Table 1, Fig. 1c). After Manning injection, sham-operated males significantly increased digging in comparison with the saline treatment (Table 1, Fig. 2c).

Behavior towards an intruder male

There were no effects of either VT treatment ($F_{(5,93)} = 2.05$, $p = 0.08$, $\omega^2 = 0.05$) or castration ($F_{(1,20)} = 1.\ 72$, $p = 0.20$, $\omega^2 = 0.03$) in the frequency of bites towards the intruder male (Fig. 3a). The interaction between VT treatment and castration was also not significant ($F_{(5,93)} = 0.52$, $p = 0.\ 76$, $\omega^2 = -0.03$).

There were no effects of either the VT treatment ($F_{(5,94)} = 2.15$, $p = 0.07$, $\omega^2 = 0.05$) or castration ($F_{(1,20)} = 0.72$, $p = 0.41$, $\omega^2 = -0.01$) in the frequency of displays towards the intruder male (Fig. 3b). The interaction between VT treatment and castration was also not significant ($F_{(5,94)} = 0.51$, $p = 0.77$, $\omega^2 = -0.03$).

There was a significant effect of VT treatment ($F_{(5,93)} = 3.53$, $p = 0.006$, $\omega^2 = 0.11$), but not of castration ($F_{(1,20)} = 1.19$, $p = 0.29$, $\omega^2 = 0.01$), in the time the focal fish spent displaying towards the intruder male (Fig. 3c). The interaction between VT treatment and castration was not significant ($F_{(5,93)} = 0.65$, $p = 0.67$, $\omega^2 = -0.02$). Visual inspection of Fig. 3c suggests the occurrence of an effect for castrated fish injected with VT (dose 1 µg/g). However, after correcting p-values for multiple comparisons, there were no significant differences between treatments (Table 2).

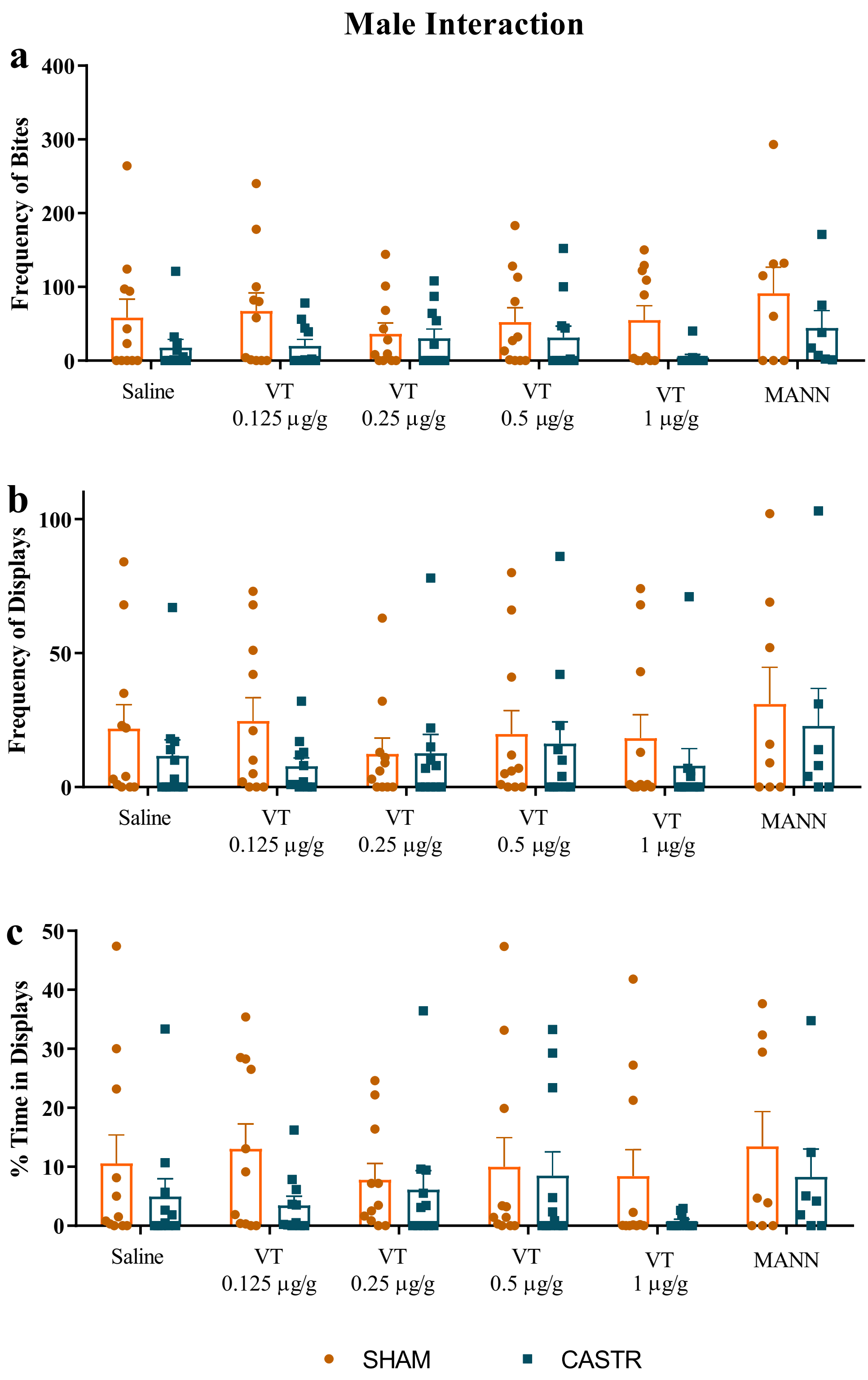
Discussion

In this paper, we have investigated the putative effects of gonadal hormones, through castration, VT, and the interaction between gonadal hormones and VT on the reproductive behavior of the cichlid fish *O. mossambicus*. Castration had no effect on the aggressive behavior of the focal male towards the intruder male but affected the behavior of breeding males towards females increasing the digging behavior involved in the construction of a spawning pit. Also, the present study showed that pharmacological VT manipulations affected the behavior of focal males towards females but not towards males.

A previous study in the Mozambique tilapia has shown that gonadectomy impairs the expression of reproductive behavior, which can be rescued by androgen administration to castrated males, but has no effect on aggressiveness (Almeida et al. 2014). Contrary to what was expected, we did not report significant behavioral differences on the reproductive behavior between sham and castrated fish, except for the spawning pit digging (where we had an unexpected increase in the castrated fish in comparison with the sham-operated fish that we cannot explain), possibly due to distinct methodological and sampling conditions. For instance, Almeida et al. (2014) sampled male–female behavior without any intervention during eight days after castration while in the present study, we analysed focal fish behavior only 15 min after each manipulation and injection. Thus, we could not quantify courtship behavior, as mentioned earlier, since most fish did not court females, but decided to quantify the time focal fish spent touching the transparent partition that allowed visual contact with females. Therefore, we believe that the differences between the two studies may be explained due to the additional stressful conditions and differences in temporal behavioral observations.

Still, treatment with VT either in gonad-intact males or in gonadectomized males reduced their aggressiveness towards females. Given that all VT-injected males (either castrated or sham-operated) interacted less with females (i.e., spent less time touching the transparent partition that allowed visual contact with females), the observed reduction in aggressiveness could be interpreted as a consequence of a reduced interest in females in these males. In this and other's cichlid species, male's aggressiveness towards females is common and is part of their reproductive behavior. For instance, in the *Hemichromis bimaculatus*, when a female enters a male´s territory, the male usually displays aggressively and even tail beats and butts her (Baerends and Baerends-Van Roon 1950). Then, the male´s subsequent behavior is dependent on the female´s behavior. If

Fig. 3 Behavioral measurements of the focal fish during male interaction after each experimental treatment **a** frequency of bites; **b** frequency of displays; **c** time spent in displays. Groups: SHAM, sham fish; CAST, castrated fish. MANN: Manning compound



the female is not sexually receptive, she flees and the male chases and bites her (Baerends and Baerends-Van Roon 1950). However, when sexually receptive, the female stands against his attacks, assumes a subordinate attitude signalling herself as a potential partner and courtship behavior occurs (Baerends and Baerends-Van Roon 1950).

Moreover, there is a specific effect of the treatment with Manning compound in the frequency of bites in sham-operated

Table 2 – Effect of castration and chemical treatment on the behavior of the focal male towards the demonstrator male: effect sizes and planned comparisons

Planned comparisons	SHAM			CAST			SHAM vs CAST		
	z	*p*	*d*	*z*	*p*	*d*	*z*	*p*	*d*
Frequency of bites									
VT 0.125 µg/g vs saline	0.71	0.80	0.16	0.18	0.92	0.05			
VT 0.25 µg/g vs saline	−0.18	0.92	0.04	0.67	0.80	0.17			
VT 0.5 µg/g vs saline	0.51	0.82	0.12	0.30	0.92	0.08			
VT 1 µg/g vs saline	0.10	0.92	0.02	−1.25	0.64	0.61			
Manning vs saline	1.80	0.56	0.26	1.62	0.56	0.54			
Saline							−1.00	0.64	0.42
VT 0.125 µg/g							−1.30	0.64	0.55
VT 0.25 µg/g							−0.51	0.82	0.22
VT 0.5 µg/g							−1.11	0.64	0.47
VT 1 µg/g							−1.77	0.56	1.01
Manning							−1.04	0.64	0.25
Frequency of displays									
VT 0.125 µg/g vs saline	0.57	0.95	0.12	−0.07	0.95	0.017			
VT 0.25 µg/g vs saline	−0.93	0.81	0.21	0.10	0.95	0.023			
VT 0.5 µg/g vs saline	−0.21	0.95	0.05	0.38	0.95	0.086			
VT 1 µg/g vs saline	−1.26	0.81	0.27	−1.61	0.81	0.40			
Manning vs saline	1.21	0.81	0.12	0.37	0.95	0.19			
Saline							−0.74	0.92	0.31
VT 0.125 µg/g							−1.08	0.81	0.49
VT 0.25 µg/g							−0.19	0.95	0.09
VT 0.5 µg/g							−0.43	0.95	0.18
VT 1 µg/g							−0.92	0.81	0.40
Manning							−1.17	0.81	0.22
Time spent in displays									
VT 0.125 µg/g vs saline	1.21	0.59	0.24	0.04	0.97	0.01			
VT 0.25 µg/g vs saline	0.45	0.80	0.10	0.75	0.61	0.18			
VT 0.5 µg/g vs saline	−0.27	0.84	0.06	0.93	0.59	0.21			
VT 1 µg/g vs saline	−1.38	0.59	0.27	−1.94	0.59	0.61			
Manning vs saline	1.14	0.59	0.16	0.90	0.59	0.44			
Saline							−0.91	0.59	0.34
VT 0.125 µg/g							−1.54	0.59	0.66
VT 0.25 µg/g							−0.76	0.61	0.35
VT 0.5 µg/g							−0.27	0.84	0.11
VT 1 µg/g							−1.21	0.59	0.59
Manning							−0.95	0.59	0.14

Groups: *SHAM* sham fish, *CAST* castrated fish, *z* *z*-test estimate, *d* effect size estimate (Cohen's *d*), *p* *p*-value after multiple comparison adjustment; statistically significant values are in bold

but not in castrated males treated that goes in the opposite direction (i.e., an increase in frequency of bites towards females). This specific result suggests the involvement of *V1A* receptors located in the gonads in a complex regulatory mechanism. These receptors could be regulating gonadal hormones (putatively androgens) production or release and consequently inhibiting aggression. Also, suggesting the interaction between VT and the gonads is the reported decrease of the spawning pit building behavior only in castrated males after VT injection, a behavior that is rescued when injected with the Manning compound. Several VT receptors have been described in teleost fish, namely, *V1A*, *V2A*, *V2B*, and *V2C*, but *V1A* receptors are the most distributed receptors in the brain of vertebrates (Lagman et al. 2013; Albers 2015). In addition, the *V1A* receptor has been detected in fish testis (Lema 2010; Lema et al. 2012) and a study in the rainbow trout, *O. mykiss*, reported that VT induced the production of androgens in immature cultured testes but not in mature testes (Rodríguez and Specker 1991). However, in the Central American cichlid, *C. dimerus*, VT stimulates the production of gonadotropins on pituitary extracts in vitro and androgens on testis culture of dominant fish (Ramallo et al. 2012). It was also detected VT mRNA and peptide within the interstitial tissue of the testis thus showing the presence and influence of VT in the HPG axis at a peripheral level, probably acting in a paracrine/autocrine fashion as a way to modulate steroidogenesis and/or spermatogenesis.

Interestingly, the present study showed that pharmacological VT manipulations affected the behavior of focal males towards females but not towards males. Since we did not randomize the order of the presentation of the fish stimulus, one may argue that drugs' effect was already absent when focal males interacted with males. However, since another study in a fish species showed an effect of VT and Manning within 60-min post-injection (Soares et al. 2012), we think that this is not the case, because we observed focal's behavior within 30-min post-injection.

In teleosts, VT is mainly expressed in neurons located in the POA in the anterior hypothalamus, that project to the neurohypophysis, where it is released to the bloodstream to act peripherally (reviewed in Godwin and Thompson 2012). These neurons also project to the ventral telencephalon, ventral thalamus, and mesencephalon (Saito et al. 2004; Huffman et al. 2012). There are different populations (parvo-, magno-, and giganto- cellular) of VT neurons that have been proposed to have different modulatory roles in social behavior (Greenwood et al. 2008). The absence of effect on aggressive behavior in male-male interactions supports the existence of a complex regulatory mechanism dependent on the concerted action of different subsystems composed of distinct VT populations (Greenwood et al. 2008; Loveland and Fernald 2017), probably because the peripheral administration of VT fails to stimulate these contrasting circuits in an independent manner. For instance, in the midshipman fish *P. notatus*, a well-studied fish model in the scope of vocal communication (see Bass 2008; Forlano et al. 2015 for comprehensive reviews), territorial males defend nests and attract females by using acoustic signals, agonistic ('grunts') and courtship sounds (long 'hums'), respectively. Interestingly, the VT delivery either in the forebrain or in the midbrain modulates different vocal circuits as shown by inducing distinct effects. VT treatment on the preoptic area–anterior hypothalamus decreases burst duration, whereas, at the midbrain level (specifically in the paralemniscal midbrain tegmentum), VT hampers call initiation by decreasing the number of vocal bursts and increasing response latency (Goodson and Bass 2000a, b).

Also, contrary to expectations, VT and the antagonist did not produce opposing effects in the time spent interacting with females. We cannot explain these results, but peripheral VT manipulations target multiple circuits/peripheral systems simultaneously, which may not occur during social interactions, and may have led to the complex patterns of effects we detected. Additionally, even though the Manning compound is a strong AVT receptor V1A antagonist, highly selective for *V1A* in comparison with *V2* receptors, it also acts as an OT antagonist (Manning et al. 2012), which may explain some of the results.

The lack of effect of castration on male-male aggressiveness corroborates the results obtained in our previous study (Almeida et al. 2014) supporting evidence for a moderator instead of a mediator role of androgens on aggressive behavior. Even though it is known that androgens favor aggression (e.g., Hirschenhauser and Oliveira 2006), it seems that they are not necessary for the expression of aggressive behavior, at least in this species.

Finally, VT neurons can also be modulated by gonadal steroids. Castration of Syrian hamsters reduces dramatically the expression of *V1A* receptors and ligand binding in the preoptic nucleus showing that androgens modulate sensitivity to vasotocin by affecting the number of *V1A* receptors (Young et al. 2000). Our study suggests that androgens favor aggressiveness towards females while VT has an inhibitory action on this behavior via *V1A* receptors. Unfortunately, we did not measure androgens in the current study, so these hypotheses need to be further examined, for instance, with hormonal assays or treating castrated fish with androgens.

Contrary to the literature, in the Mozambique tilapia, VT did not increase courting or affect aggressive behavior towards males but inhibited interaction and aggressiveness towards females, confirming that the action of this nanopeptide in behavior is species-specific. Moreover, we highlight the need to target specific populations of VT neurons, in order to clarify the role of VT in the modulation of social behavior through different putative regulatory circuits and also due to the structural similarity between vasotocin and oxytocin and their receptors (Donaldson and Young 2008; Albers 2015) which may lead to relevant crosstalk (reviewed in Stoop 2012; Kelly and Goodson 2014).

Author contribution OA and RO designed the experiments. OA performed behavioral experiments. AF analyzed the data. AF and RO wrote the paper, which was based on the Ph.D. thesis (chapter 3) of AF.

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Declarations

Ethics approval Animal experimentation procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986(86/609/EEC) and were approved by the Portuguese Veterinary Authority (Direcção Geral de Alimentação e Veterinária, Portugal; permit # 0421/000/000/2013).

Competing interests The authors declare no competing interests.

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