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Vasotocin and oxytocin modulation of the endocrine and behavioral response to an aggressive challenge in male Siamese fighting fish

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ABSTRACT

Aggressive behavior is an adaptive trait present across all taxa. However, the neuroendocrine mechanisms regulating it, particularly in fish, are not well understood. Oxytocin (OXT) and arginine vasotocin (VT) are known modulators of aggression, but their actions remain controversial. This study tested the possible modulation of endocrine and behavioral responses to an aggression challenge by these nonapeptides in Siamese fighting fish, *Betta splendens*, a species known for its intrinsic aggressiveness. Male *B. splendens* were injected with different dosages of either Manning compound or L-368,899, VT and OXT receptor antagonists respectively, and were exposed to a mirror challenge for 30 min. While all fish displayed high levels of aggression toward their mirror image, no differences were observed between control-injected and treatment fish. However, blocking VT inhibited the post-fight increase in plasma levels of the androgen 11-ketotestosterone (KT). To further investigate this result, testis tissue from males was incubated with and without VT and Manning compound, and KT levels were measured after 180 min. Results showed a direct effect of VT on *in vitro* KT secretion, indicating the presence of VT receptors in the testes of this species. Overall, the study does not support a modulatory role of VT or OXT in aggressive behavior, although VT might be implicated in the regulation of peripheral androgen response to aggression in *B. splendens*.

1. Introduction

Aggression is an adaptive behavior used to establish dominance hierarchies, compete for resources, protect offspring, and as a form of defense (Koolhaas and Bohus, 2003). It can be observed between conspecifics (as defined by Aronson and Tinbergen, 1953; and Lorenz, 1967), but also as a form of heterospecific interactions when competing for resources, especially when there is an overlap in the ecological niche (Grether et al., 2013). Studies usually focus on the ethological significance of aggressive behavior, its phylogenetic and ontogenetic development, or, in the context of human and veterinary medicine studies, understanding and controlling pathological aggression. However, a clear picture of the mechanisms underlying variation in aggressive behavior, within and across species, is still lacking (reviewed by Way et al., 2015). This is particularly true for non-mammalian species, including fish, despite the diversity of aggressive behaviors that can be found across the >30,000 described fish species (Froese and Pauly, 2024).

Aggressive behavior can be modulated by neurotransmitters, such as dopamine, 5-HT, histamine, nitric oxide, and by hormones from the hypothalamic-neurohypophysial system (HNS), hypothalamic-pituitary-interrenal axis (HPI) and hypothalamic-pituitary-gonadal axis (HPG) (Filby et al., 2010). The nonapeptides oxytocin (OXT) and vasotocin (VT) have also been implicated in the modulation of aggression (Kelly and Wilson, 2020). They are highly conserved across taxa and their evolution is tightly linked, with their genes located in the same chromosome, with evidence supporting the hypothesis that they are adjacent paralogous genes resulting from a local duplication (Theofanopoulou et al., 2021). The fish homologues of the mammalian OXT and VT have been referred to in the literature as isotocin (IT) and arginine vasotocin (AVT), respectively (Wircer et al., 2016). However, because their sequence differs only in two (OXT/IT) and one (VT/AVT) amino acid, a universal nomenclature for the ligands and receptors of this family has been proposed (Theofanopoulou et al., 2021) and here the terms oxytocin (OXT) and vasotocin (VT) are used. The two nonapeptides also share similar functions such as the regulation of reproductive behavior,

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stress response, metabolism, circadian and seasonal rhythms, cardiovascular system, and osmoregulation (Balment et al., 2006; Jurek and Neumann, 2018). They are also involved in the modulation of aggression, but their specific role and mechanisms of actions are still to be determined, with contradictory results reported in the literature. Generally, in mammals, VT is linked to an increase in the expression of aggression (Albers, 2012). A similar effect has been reported for some fish species, (*e.g.*, non-territorial phase males of bluehead wrasse *Thalassoma bifasciatum,* Semsar et al., 2001; males of the beaugregory damselfish *Stegastes leucostictus*, Santangelo and Bass, 2006; zebrafish *Danio rerio*, Teles and Oliveira, 2016) although the opposite effect (*e.g.*, territorial phase males of bluehead wrasse, Semsar et al., 2001; males of the brown ghost knifefish *Apterootus leptorhynchus*, Bastian et al., 2001; males and females of zebrafish, Filby et al., 2010) has also been described. VT has also been associated with social status and territorial behavior, for example: in the peacock blenny *Salaria pavo,* the cell size is higher in the parvocellular nuclei in parasitic sneaker males relative to nest-holder males and females (Grober et al., 2002); in the rock-pool blenny *Parablennius parvicornis,* the cell number is higher in smaller non-nesting male than in larger nesting males, if corrected for body mass (Miranda et al., 2003); in the masu salmon *Oncorhynchus masou*, the intensity of hybridization signal of VT in male breeders is lower in the parvocellular population and higher in the gigantocellular population, while no differences were detected in the magnocellular population (Ota et al., 1999); and, in zebrafish the VT cell number in dominant males is lower in the parvocellular population, and higher in the magnocellular and gigantocellular population (Larson et al., 2006). Thus, differences in the size and number of VT cells across brain areas appear to be linked to specific reproductive or dominance phenotypes. Hence, VT expression can correlate with alternative phenotypes (Greenwood et al., 2008) and have a role in shaping dominant-subordinate relationships (da Silva et al., 2021).

Less information is available on the role of OXT on aggression. In mammals, OXT seems to be associated with maternal aggression while in males its role is still ambiguous (reviewed in Lee et al., 2009). In fish, contradictory evidence on the relationship between OXT and aggression has also been reported. For example, OXT administration to the preoptic area/anterior hypothalamus (POA-AH) induces fictive aggressive vocalization in parasitic sneaker males, but not in territorial males, of the plainfin midshipman *Porichthys notatus* (Goodson and Bass, 2000); while in other species OXT administration does not produce any effects in aggressive displays (*e.g.*, males of the beaugregory damselfish, Santangelo and Bass, 2006, and in males and females of the cichlid *Neolamprologus pulcher*, Reddon et al., 2012). Depending on the dominance and reproductive status, distinct OXT and VT expression patterns have been observed (*e.g.*, in the Mozambique tilapia *Oreochromis mossambicus*, Almeida et al., 2012; in the three-spined stickleback *Gasterosteus aculeatus*, Kleszczyńska et al., 2012; in the zebrafish, Teles et al., 2016; and in the round goby *Neogobius melanostomus*, Sokołowska et al., 2020).

OXT and VT also have a reproductive role that is not only limited to the modulation of sexual and courtship behavior but can also act on steroidogenesis (VT) and in the regulation of the female reproductive cycle (OXT) (Gonçalves et al., 2024). Therefore, a connection between these two nonapeptides and sex steroids is plausible, especially because sex hormone receptors have been observed in different brain areas, including the preoptic area where OXT and VT neurons are located. Additionally, the presence of the VT receptor in Leydig cells in the testes, the primary site of androgen production, has been observed in two different fish species: the catfish *Heteropneustes fossilis* (Rawat et al., 2019) and bluehead wrasse (Lema et al., 2012). However, most studies aiming to understand the crosstalk between these two pathways have usually focused on how sex steroids modulate the nonapeptide systems (for a review see Mennigen et al., 2022) rather than exploring the reverse interaction.

The Siamese fighting fish *Betta splendens* has been emerging as a model species for the study of non-mammalian aggression because of its high levels of male aggression and stereotypical and conspicuous aggressive displays. Individuals of this species are solitary, with males establishing and vigorously defending a territory from other males and building a bubble nest from which they attract females for mating (Jaroensutasinee and Jaroensutansinee, 2001). The male-male aggression interactions in this species are well-defined, starting with threat displays that include the distension of fins, the opening of the opercula, and caudal swings toward the opponent, followed by attack behavior with bites and charges if the opponent is not deterred (Ramos et al., 2021; Simpson, 1968; Vu et al., 2020). The aggressiveness of this species, which is endemic to parts of Southeast Asia, has been recognized for centuries by locals, who began using males in staged fights as a national pastime (Smith, 1945). Winner lines are bred while loser lines are discarded, leading to the establishment of "fighter lines", morphologically different and more aggressive than wild-type fish (Ramos and Gonçalves, 2019; Verbeek et al., 2007). The selection for winners also seems to have modulated the response of the endocrine system during aggression, with fighters showing a post-fight increase in plasma KT levels but not in cortisol (F), while wild-type fish show an increase in both hormones (Ramos and Gonçalves, 2022). The modulation of aggression in this species by VT and OXT is not yet clear, with only one study showing a negative effect of OXT administration on aggression (Oliveira et al., 2022), while no studies have been published on VT. On the other hand, a robust post-fight androgen response after an aggressive challenge has been found in this species independent of the type of stimulus – mirror, live conspecifics, or video playback – with androgen levels showing no correlation with the duration nor the frequency of aggressive behavior (Alex et al., 2023; Ramos et al., 2021; Ramos and Gonçalves, 2022).

Here, we tested the potential modulation of VT and OXT on the aggressive and endocrine responses elicited by the presence of a mirror in male *B. splendens* through intraperitoneal injection of VT and OXT antagonists. The aim was to contribute to the still poorly understood role of these neuropeptides in the modulation of aggressive behavior and their possible interaction with the described androgen response to an aggression challenge in *B. splendens*.

2. Material and methods

2.1. Animals and Housing

All subjects used in the experiments were 26 months old *B. splendens* males from the F2 generation of a cross between a wild-type and a fighter strain (for details on this line see Ramos et al., 2021). As these strains differ in the expression of aggressive behavior and endocrine response to aggression (Ramos and Gonçalves, 2019, 2022) the rationale of using hybrids was to capture potential variation in the OXT and VT system across different strains of the species. Prior to the experiment, fish were isolated, without visual contact with other conspecifics, for at least 48 h in 9W × 9D × 20H cm aquaria, containing a small ceramic shelter. Fish were fed twice a day with dry food (Golden pearl diet and Atison's Betta Pro) in the morning and live food (adult artemia) in the afternoon, except on the day of the experiments, when no food was given. Conditions in the group and isolation tanks were similar, with the temperature kept at 28 ± 1 °C, the photoperiod set to 12L:12D, and tank water supplied by reverse osmosis (RO) water with salinity kept at 4 ± 1 g/L.

2.2. Receptor Antagonist Treatments

Fish were divided into seven treatments, consisting of intraperitoneal injections with one of the following compounds: a saline solution of 0.9 % NaCl in Milliq water (control group, C); the OXT antagonist L-368,899 hydrochloride (CAT#2641; CAS 1603112–62-9; Tocris Bioscience) at 1 μg/g body weight (L1-low dosage), 3 μg/g body weight (L3-medium dosage), 5 μg/g body weight (L5-high dosage); the VT antagonist

Manning compound (CAT#V2255; CAS 73168–24-8; Sigma) at 1 μg/g body weight (M1-low dosage), 3 μg/g body weight (M3-medium dosage), 5 μg/g body weight (M5-high dosage). Each fish was weighed before the start of the experiment so that the injection volume could be adjusted for the fish's body weight. The injections were performed with a 30G syringe. The choice of the antagonists and the concentrations resulted from a literature review. L-368,899 is a non-peptidergic antagonist that inhibits OXTR in mammals, resulting in a significant change in behavior (Boccia et al., 2007). Moreover, *in vitro* studies have shown that it has a higher affinity for the oxytocin receptor with respect to the vasopressin receptor (Landin et al., 2020; Manning et al., 2012). It is also important to emphasize that L-368,899 is an unbiased antagonist which means that it can block at the same time the OXTR signaling through both Gq and Gi protein subunit pathways (Williams et al., 2020). Initially, the same L-368,899 dosages used by Landin et al. (2020) in zebrafish were tested in *B. splendens*, specifically 50 and 100 μg per gram fish. However, in *B. splendens*, 50 and 100 μg induced the death of some individuals, and accordingly, it was considered an overdosage. The same dosages were tested with zebrafish in our laboratory without any obvious complications for the tested subjects, suggesting that this is a species-specific difference in tolerance to the compound. The Manning compound is the most commonly used V1a receptor antagonist in research, known to have almost no antidiuretic activity (Guillon et al., 2004), and to block the VT signaling in teleosts, with visible impacts in behavior (Semsar et al., 2001; Semsar and Godwin, 2004; Yaeger et al., 2014). In these studies, a dosage of 3.2 μg/g body weight was used. Since there were no articles in *B. splendens* with the use of these antagonists, the dosages of 1 μg/g body weight, 3 μg/g body weight and 5 μg/g body weight were chosen respectively as the lowest, the medium, and the highest dosages for both neuropeptides.

2.3. Experimental setup

Fish were randomly assigned to the seven treatments (control $n = 10$, L1 $n = 9$, L3 n = 9, L5 n = 9, M1 n = 10, M3 n = 10, M5 n = 9) and did not differ in weight (W) or standard length (SL) between groups (One-way ANOVA, W: $F_{(6,59)} = 0.106$, $p = 0.995$; SL: $F_{(6,59)} = 0.38$, $p = 0.889$). Each subject was injected intraperitoneally without anesthesia, as the effects on behavior were to be assessed shortly after the injection. *Betta* fish are large in relation to the needle used and the procedure was fast, with fish resuming normal swimming behavior almost immediately. Also, the prompt response to the mirror challenge after the acclimation period (see below) suggests that the injection procedure had minimal to no impact in the behavioral response. After being injected, the subjects were transferred to the test tank, which had a dimension of 25W × 12.5D × 20H cm, and contained approximately 3.3 L of system water. The recording of the behavior of the fish started immediately, with one side and one top Raspberry Pi camera module (V2) at a resolution of 1640 × 922 px and 30 fps. Each camera was connected to an independent Raspberry Pi board 4B, with one Raspberry Pi also controlling the activation of a smart screen that blocked the mirror during acclimation *via* a relay switch (Fig. 1). Illumination was provided by a diffuse LED panel, and the entire setup was enclosed in a box with white walls, accessible through small doors located on the long side. Thus, the illumination inside the box was consistent for each fish analyzed. A total period of 60 min was given for acclimation, with the first 30 min considered the recovery period from the injection procedure and the second half providing baseline data for the acclimation period. At minute 60, the smart screen, located on one side of the tank, was automatically activated to show the mirror, allowing the start of the experiment (Fig. 1). After 30 min, the fish was immediately removed from the test tank, anesthetized with buffered MS222 (concentration 400 mg/L), measured, and blood sampled (approximately 10 μL volume) with a heparinized 27G syringe. Blood was collected by inserting the needle under the scales of the middle part of the tail, just below the lateral line, at an angle of 45°, to reach the base of the vertebral column. After recovering from anesthesia, the fish were returned to their isolation tanks. The blood samples were centrifuged for 15 min to collect the plasma which was then transferred to a new clean tube and stored at −20 °C for subsequent hormonal analysis.

2.4. Behavioral observations

For each fish, data from the two cameras were combined using in-house deep-learning software to extract variables related to the 3D position in the tank (*i.e.*, time spent within 5 cm of the stimuli and within 4

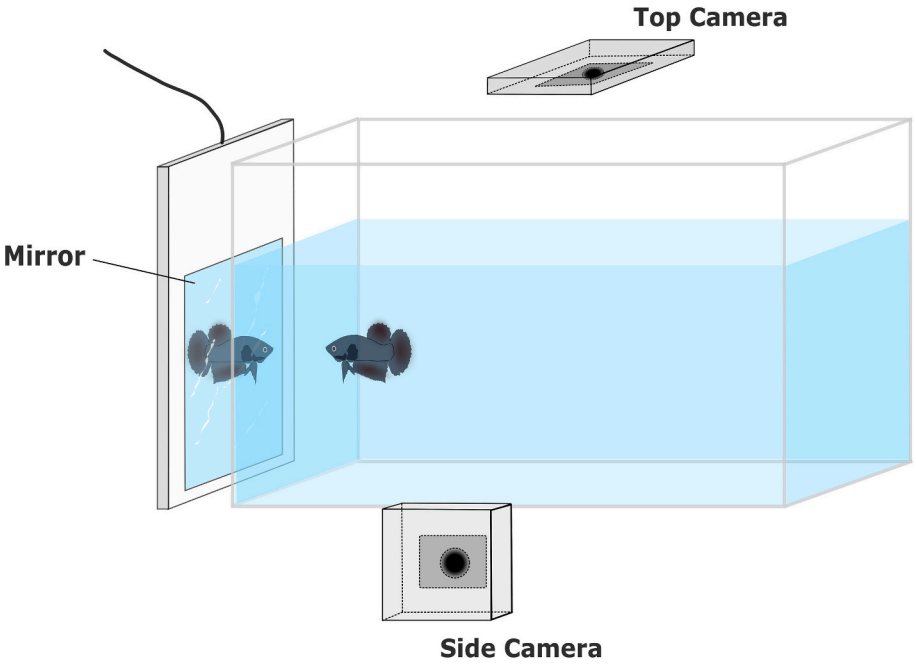

Fig. 1. Experimental set-up representation. The focal fish is exposed to a smart screen that, after an acclimation phase, is triggered to show a hidden mirror. The behavior was recorded with both a top and a side camera. Illumination was provided by a diffuse LED strip.

cm of the surface or the bottom, and head orientation), and the distance traveled, as well as variables associated with aggressive behavior such as the time with the opercula open and the number of charges. Moreover, since fish become darker when displaying aggression, a measure of color change was obtained by comparing the average grayscale value (from 0 - black to 255 - white) of a central area of the fish body during the acclimation and test periods, excluding the first and last 5 min of each phase (5 to 25 min and 35 to 55 min for the acclimation and test phases, respectively). For this, all frames where the fish was lateral to the side camera (*i.e.* ±20°, as determined from the top camera coordinates) were selected and the average grayscale value of a square with 16 × 16 pixels, centered on the fish blob, obtained. The delta of the difference in average body grayscale between the acclimation and test phases was used as the measure of color change and compared between treatments. Other aggressive behaviors such as the duration of unpaired fins distension, frequency of caudal swing, and frequency of attempted bites/mouth hits were manually scored with Boris v.7.13.6 software (Friard and Gamba, 2016). *B. splendens*, as a labyrinth fish, can breathe oxygen from air, and the frequency of surface air-breathing correlates with oxygen consumption during agonistic encounters, thus providing a measure of metabolic effort (Alton et al., 2013). Accordingly, the frequency of surface air-breathing was also recorded manually using the same software.

2.5. In vitro testicular androgen release

After euthanasia in buffered MS-222 (1 g/L), testis from nine individuals were collected, measured, and weighted. Each testis pair was separated into two single wells and treated as individual samples to reduce the number of fish needed for the procedure. Tissue was pre-incubated and continuously shaken in 150 mL of Ringer solution (7.2 pH, NaCl 116 mM, KCl 2.9 mM, $CaCl_2 \cdot 2H_2O$ 1.8 mM, HEPES 5 mM) until the collection was completed for all fish. Afterward, the samples were transferred into fresh Ringer solution and incubated as previously described for another 30 min. Samples were then randomly assigned to one of four treatments: control (Ringer); VT 100 nM; Manning 100 nM; or Manning 100 nM for 10 min, after which they were changed to VT 100 nM. After 180 min of incubation, the media were collected and stored at −20 °C for subsequent quantification of KT levels. The decision to analyze the levels of KT after 180 min of incubation was based on previous studies (Ramallo et al., 2012; Rodríguez and Specker, 1991), which performed similar experiments in two other fish species.

2.6. Hormonal analysis

KT levels in plasma and incubation media were measured using competitive enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical) following the manufacturer's instructions. For plasma, all standards and samples were measured in duplicate with a dilution in the EIA buffer of 1:150. In order to analyze all plasma samples, two assays had to be performed, and samples from the different treatments were equally distributed among the two plates. The intra-assay coefficient of variation from the two plates, calculated from the sample duplicates, was 4.9 % and 7.6 %. The analysis excluded two individuals because their levels were out of range (too low). For the testis incubation media, both samples and standards were measured in duplicate with a dilution in the EIA buffer of 1:300 in the same plate. The intra-assay coefficient of variation, calculated from the sample duplicates, was 5.1 %.

2.7. Data analysis

For all data, normality and homoscedasticity were tested using Shapiro-Wilk's and Levene's tests, respectively. If the parametric assumptions were not satisfied, the variables were logarithmically transformed and tested again for assumptions. If, after transformation, the variables did not meet the parametric assumptions, corresponding non-parametric tests were applied. First, we checked for any specific dosage effect of each compound administered on plasma hormone levels and the different behavioral variables recorded, including activity, position, and aggressive behaviors. These variables were tested with one-way ANOVA with dosage as a factor (low, medium, high), followed by Fisher's LSD test. Since no relevant differences between dosages were observed (see results), data from the different dosages of the same compound were pooled to compare saline (control), Manning, and L-368,899 treated individuals. A one-way ANOVA with treatment as a factor, followed by a post-hoc Dunnett's test for comparison with the control group, was performed to analyze the effects of the treatments on hormone levels, activity, aggression, and position in the tank. Differences in behavior between the acclimation and the mirror phases were analyzed using a paired *t*-test or a Wilcoxon signed-rank test, if data were not parametric. Additionally, two correlation matrices – one for each phase, acclimation and mirror test – were created to assess any possible correlations among activity, position in the tank, and aggressive behavior. Pearson correlation was used for continuous variables, and Kendall correlation was applied when Pearson's assumptions were not met. For the *in vitro* testis experiment, KT values corrected for testis weight were compared between treatments using a one-way ANOVA, followed by Fisher's LSD test. Eta squared estimates with confidence intervals were provided for ANOVAs and the Kruskal-Wallis test, while Cohen's *d* was calculated for pairwise comparisons. For the Wilcoxon signed-rank test, the effect size (r) was calculated as the z-score divided by the square root of the number of non-missing pairs. Statistical analyses were performed using R Statistical Software v. 4.3.0 (R Core Team, 2021).

2.8. Ethical note

All methods adhered to the ASAB/ABS "Guidelines for the Treatment of Animals in Behavioural Research and Teaching" (2012). This study followed the ethical guidelines enforced at the Institute of Science and Environment of the University of Saint Joseph and work with the species is approved by the Division of Animal Control and Inspection of the Civic and Municipal Affairs Bureau of Macau, license AL017/DICV/SIS/2016.

3. Results

3.1. Response to the mirror test

As described in previous studies, males readily attacked their mirror image. This behavior was quantified by comparing the fish's behavior during the acclimation and the mirror-exposed phases. All subjects were pooled together, independently of the treatments, to create two groups: acclimation and mirror. In response to the mirror, the fish increased the distance traveled (paired *t*-test $t(65) = -3.1$, $p = 0.003$, Cohen's $d = 0.382$), spent more time close to the surface (Time near surface: paired t-test $t(65) = -7.11$, $p < 0.0001$, Cohen's $d = 0.875$ / Time near bottom: paired t-test $t(65) = 8.22$, $p < 0.0001$, Cohen's $d = -1.011$) and near the mirror side (Wilcoxon signed-rank test $p < 0.0001$, $r = 0.511$). Additionally, the number of air-breathing events increased (paired t-test $t(65) = 6.14$, $p < 0.0001$, Cohen's $d = 0.628$), and the body color shifted toward a darker shade (Wilcoxon signed-rank test $p = 0.0006$, $r = -0.422$). These results confirm that the fish behaved as expected (Supplementary material Fig. 1). During the mirror fight, the frequency of air-breathing correlated with the frequency and duration of threat and attack behaviors, indicating a higher metabolic effort during the fight phase. Aggressive behaviors were positively correlated with one another, except for charges, which appeared to show a slight negative correlation with opercula opening. Body color was not correlated with any of the threat or attacks behaviors (Supplementary material Fig. 2B). During both the acclimation and mirror phases, the frequency of air-breathing was positively correlated with distance traveled, reflecting the metabolic effort required (Supplementary material Fig. 2).

3.2. Dosage effect

A comparison between the different dosages of the L-368,899 compound (1 μg/g body weight, 3 μg/g body weight, 5 μg/g body weight) did not reveal any relevant differences during either the acclimation or mirror phase (Table 1). Similarly, injections with different dosages of the Manning compound held differences for only a few of the analyzed variables (Table 1). For the L-368,899 compound, activity (distance traveled and air-breathing), position in the tank (close to the surface, close to the bottom, and close to the test area), body color, aggressive display (distended fins, open opercula, caudal swings, and charges), and hormone levels (plasma KT) did not show any significant differences. The same was observed for the three dosages of the Manning compound, except that during the acclimation phase, the distance traveled by individuals injected with the highest dosage was higher than that of those injected with the medium dosage ($p = 0.007$). During the mirror phase, individuals injected with the medium and highest dosages spent more time near the surface ($p = 0.03$, $p = 0.022$, respectively) and correspondingly less time spent near the bottom ($p = 0.03$, $p = 0.027$, respectively) compared to those injected with the lowest dosage. Additionally, fish injected with the highest dosage also performed more caudal swing displays than those injected with the medium ($p = 0.018$) and the lowest ($p = 0.05$) dosages (Supplementary material Fig. 3 and Fig. 4). However, as this was the only aggressive behavior that showed a significant difference between dosages, it was therefore not considered sufficient to justify selecting a specific dosage for further analyses.

Table 1
Dosage effect (L = L-368,899, M = Manning compound) of variables related to the activity (distance and air-breathing), position in the tank (time at the surface, time at the bottom, time near test), body morphology (color), aggressive behaviors (time with fins distended, time with open opercula, frequency of caudal swings, frequency of charges), during acclimation and mirror phases; and KT plasma level post-fight. F: Anova-test F-estimate; p: *p*-value; η^2: effect size estimate (eta squared), 95 % confidence interval. The *p*-values are reported as ≤0.05 (*).

Variables	Acclimation - L	Mirror - L	Acclimation - M	Mirror - M
#Activity				
Distance traveled	$F_{2,25} = 1.72$ $p = 0.2$ $\eta^2 = 0.12$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 1.45$ $p = 0.254$ $\eta^2 = 0.10$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 4.58$ $p = 0.02$ * $\eta^2 = 0.27$ 95 % CI = [0.03, 1.00]	$F_{2,25} = 0.16$ $p = 0.853$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]
Air-breathing	$F_{2,25} = 1.25$ $p = 0.304$ $\eta^2 = 0.09$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 2.69$ $p = 0.087$ $\eta^2 = 0.18$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 2.45$ $p = 0.106$ $\eta^2 = 0.16$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 2.26$ $p = 0.126$ $\eta^2 = 0.15$ 95 % CI = [0.00, 1.00]
#Position in the tank				
Time near Surface	$F_{2,25} = 0.82$ $p = 0.452$ $\eta^2 = 0.06$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 2.85$ $p = 0.076$ $\eta^2 = 0.19$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.70$ $p = 0.507$ $\eta^2 = 0.05$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 3.72$ $p = 0.038$ * $\eta^2 = 0.23$ 95 % CI = [0.01, 1.00]
Time near Bottom	$F_{2,25} = 0.77$ $p = 0.475$ $\eta^2 = 0.06$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 1.77$ $p = 0.191$ $\eta^2 = 0.12$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 1.01$ $p = 0.378$ $\eta^2 = 0.07$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 3.59$ $p = 0.043$* $\eta^2 = 0.22$ 95 % CI = [0.00, 1.00]
Time near Test Area	$F_{2,25} = 0.48$ $p = 0.624$ $\eta^2 = 0.06$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.70$ $p = 0.508$ $\eta^2 = 0.05$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.56$ $p = 0.578$ $\eta^2 = 0.04$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 1.84$ $p = 0.18$ $\eta^2 = 0.13$ 95 % CI = [0.00, 1.00]
#Body morphology				
Color	$F_{2,25} = 0.1$ $p = 0.902$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.39$ $p = 0.679$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.21$ $p = 0.816$ $\eta^2 = 0.02$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 1.60$ $p = 0.221$ $\eta^2 = 0.11$ 95 % CI = [0.00, 1.00]
#Aggressive Behaviors				
Distended fins		$F_{2,25} = 1.39$ $p = 0.268$ $\eta^2 = 0.10$ 95 % CI = [0.00, 1.00]		$F_{2,25} = 0.24$ $p = 0.786$ $\eta^2 = 0.02$ 95 % CI = [0.00, 1.00]
Open Opercula		$F_{2,25} = 0.31$ $p = 0.733$ $\eta^2 = 0.02$ 95 % CI = [0.00, 1.00]		$F_{2,25} = 1.58$ $p = 0.227$ $\eta^2 = 0.11$ 95 % CI = [0.00, 1.00]
Caudal swing		$F_{2,25} = 0.12$ $p = 0.887$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]		$F_{2,25} = 3.58$ p = 0.043 * $\eta^2 = 0.22$ 95 % CI = [0.00, 1.00]
Charges		$F_{2,25} = 0.45$ $p = 0.641$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]		$F_{2,25} = 0.65$ $p = 0.53$ $\eta^2 = 0.05$ 95 % CI = [0.00, 1.00]

	Post-fight - L	Post-fight - M
#Hormonal level		
KT	$F_{2,23} = 0.38$ $p = 0.686$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]	$F_{2,25} = 0.38$ $p = 0.691$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]

3.3. Treatment effect

Due to the general absence of behavioral or endocrine differences between different dosages of either antagonist, data from all dosages for the same compound were pooled together for further analysis. For both the acclimation (Supplementary material Fig. 5) and mirror-fight phases (Fig. 2), the position in the tank, distance traveled and frequency of air-breathing did not differ between the control (C), Manning (M) and L-368,899 (L) groups (Fig. 2A, B; Table 2). Regarding aggressive behaviors, no significant differences between treatments were detected in the time spent with the opercula open, fins distended, frequency of caudal swings, charges, or body color (Fig. 2C, D; Table 2).

In contrast to the behavioral results, there were differences between groups in post-fight plasma KT levels, with Dunnett's test as post-hoc analysis revealing that the group injected with the Manning compound showed lower levels of KT compared to the control ($p < 0.001$) group (Fig. 2E; Table 2).

3.4. In vitro testicular androgen release

The different treatments had a significant effect on the release of KT by the testis (one-way ANOVA $F_{(3,32)} = 6.75$, $p = 0.001$, $\eta^2 = 0.39$, 95 % CI = [0.14, 1.00]). Specifically, the treatment with 100 nM of VT increased the KT release compared to the control ($p = 0.0001$) and to the other two treatments [Manning (100 nM) $p = 0.0132$; Manning (100 nM) + VT (100 nM) $p = 0.0151$] (Fig. 3).

4. Discussion

The purpose of the present study was to investigate the effect of the nonapeptides OXT and VT modulation on aggressive behavior and post-fight androgen secretion in *B. splendens*. Overall, both OXT and VT antagonists, administered at three different dosages, failed to induce significant changes in the duration and frequency of aggressive behaviors. Furthermore, no impact of the compounds on general swimming patterns and frequency of air-breathing during the acclimation phase were found. These results contrast with the majority of the studies on VT modulation, where other fish species treated with the Manning compound showed different behaviors in potentially agonist contexts. Blocking VT signaling usually induces a decrease in the expression of aggressive behavior; for example, in the clownfish *Amphirion ocellaris*, during a 10 min dyadic fight, treatment with the Manning compound reduced the frequency of bites and charges compared to the saline-treated fish, with no effect on submissive behaviors (quivers and flees) (Yaeger et al., 2014). In the monogamous convict cichlid *Amatitlania nigrofasciata*, VT/OXT antagonist decreased agonist behavior on the first day of the treatment (Oldfield and Hofmann, 2011). In the beaugregory damselfish, the Manning compound decreased the number of bites, while VT increased them (Santangelo and Bass, 2006). In the bluehead wrasse, the Manning treatment affected territorial aggression differently, decreasing the frequency and duration of chasing by T-TP (territorial terminal phase) males toward IP (female-like initial phase) individuals, while showing no significant effect toward TP (terminal phase) males (Semsar et al., 2001). In a field experiment on the same species, high-ranking individuals treated with the Manning compound remained more on the reef and did not display a dominant phenotype over the spawning site (Semsar and Godwin, 2004). Finally, in male goldfish *Carassius auratus*, injection with the Manning compound stimulated the approach response to other conspecifics (Thompson and Walton, 2004). Research focused on the assessment of the OXT system on aggression using antagonists is still lacking. Only one study, conducted in clownfish, showed that the administration of the OXT antagonist (desGly-NH2-d(CH2)5[D-Tyr2,Thr4]OVT) did not influence aggression, which aligns with our result (DeAngelis et al., 2017).

There are two possible explanations for the differences between our study and previous work. The first hypothesis is that the dosages used may not have been high enough to induce changes. Since no studies were available on the injection of the Manning and L-368,899 compounds in fighting fish, the chosen dosages were based on previous experiments in fish (Mahlmann et al., 1994; Semsar et al., 2001; Semsar and Godwin, 2004; Yaeger et al., 2014). However, the clear effect on KT secretion by the Manning compound indicates that the antagonist did induce a significant physiological effect. Also, dosages for L-368,899 of 50–100 μg/g body weight used by Landin et al. (2020) in zebrafish induced mortality in male *B. splendens*, suggesting that a dosage of 5 μg/g body weight should have been enough to induce behavioral impacts, if these were dependent on OXT modulation. In the only other study in *B. splendens* where researchers tested the impact of OXT on aggression, low dosage (2.5 μg/g body weight dissolved in water) enhanced "combat" (sum of charges and bites) behaviors but not "display behaviors" (Oliveira et al., 2022). However, the high dosage of OXT (7.5 μg/g body weight dissolved in water) had an opposite effect, inhibiting both display and combat behaviors. Therefore, in addition to variation across species in sensitivity to pharmacological manipulations, as evident from the different response of *B. splendens* and zebrafish to the same concentration of L-368,899, differences across studies in tested dosages and modes of administration may contribute to the observed variability in the response to OXT and VT manipulation. Further studies with higher dosages of Manning and L-368,899 may be conducted to further assess the possible role of these compounds in *B. splendens* aggression. Also, genetic manipulation in *B. splendens* is now facilitated by the publishing of the species' genome (Fan et al., 2018; Zhang et al., 2022) and using transgenic knockout lines for OXT and VT could help avoid possible pharmacological issues (*e.g.*, dosages, administration routes).

The second hypothesis is that VT and OXT do not significantly modulate aggression in *B. splendens*. Indeed, other studies have failed to find an effect on aggression using nonapeptide receptors' antagonists. For instance, in the previously cited study on clownfish, OXT antagonist showed no effect on aggression (DeAngelis et al., 2017). Similarly, for VT injection with the Manning compound did not induce any changes in aggressive behavior in zebrafish (Filby et al., 2010), nor did it in the Amargosa River pupfish *Cyprinodon nevadensis amargosae* (Lema and Nevitt, 2004), or in juvenile rainbow trout *Oncorhynchus mykiss* (Backström and Winberg, 2009). However, it is noteworthy that in these previous studies, when fish were injected with VT, a reduction in aggressive behavior was observed. This suggests that the different effects of agonists and antagonists are likely related to their specificity and affinity for the VT receptors, especially considering that *B. splendens*, like zebrafish, appears to possess two orthologous for the V1a receptor, which are supposedly targeted by the Manning compound. It should also be investigated whether nonapeptides regulate aggressive behavior, not in male-male interactions but rather in male-female interactions, as demonstrated by Almeida et al. (2023).

Considering VT, its action appears to be relevant in dominant-subordinate relationships and specific reproductive phenotypes, as observed in various species (*e.g.*, in the peacock blenny, Grober et al., 2002; in the rock-pool blenny, Miranda et al., 2003; in the masu salmon, Ota et al., 1999; in zebrafish, Larson et al., 2006; and in cichlid, Culbert et al., 2024). During the mirror test, assessing dominance and subordination is not possible because there is no resolution of the fight. Therefore, in *B. splendens*, the potential link or influence of VT and OXT on dominant-subordinate relationships remains to be investigated. This raises relevant questions for future studies, as the scope of this research was to examine the effects of the nonapeptides on aggressive displays, rather than on the resolution of fights.

It should be highlighted that the number of fish tested with the two compounds was relatively high ($N = 27$ for L-368,899 and $N = 29$ for Manning), and variance between fish was minimized by using same-age siblings. Additionally, possible biases in the quantification of behaviors were mitigated by using automated scripts. Thus, the data strongly suggest that, for the tested dosages, Manning and L-368,899 did to significantly impact aggressive behavior in *B. splendens*.

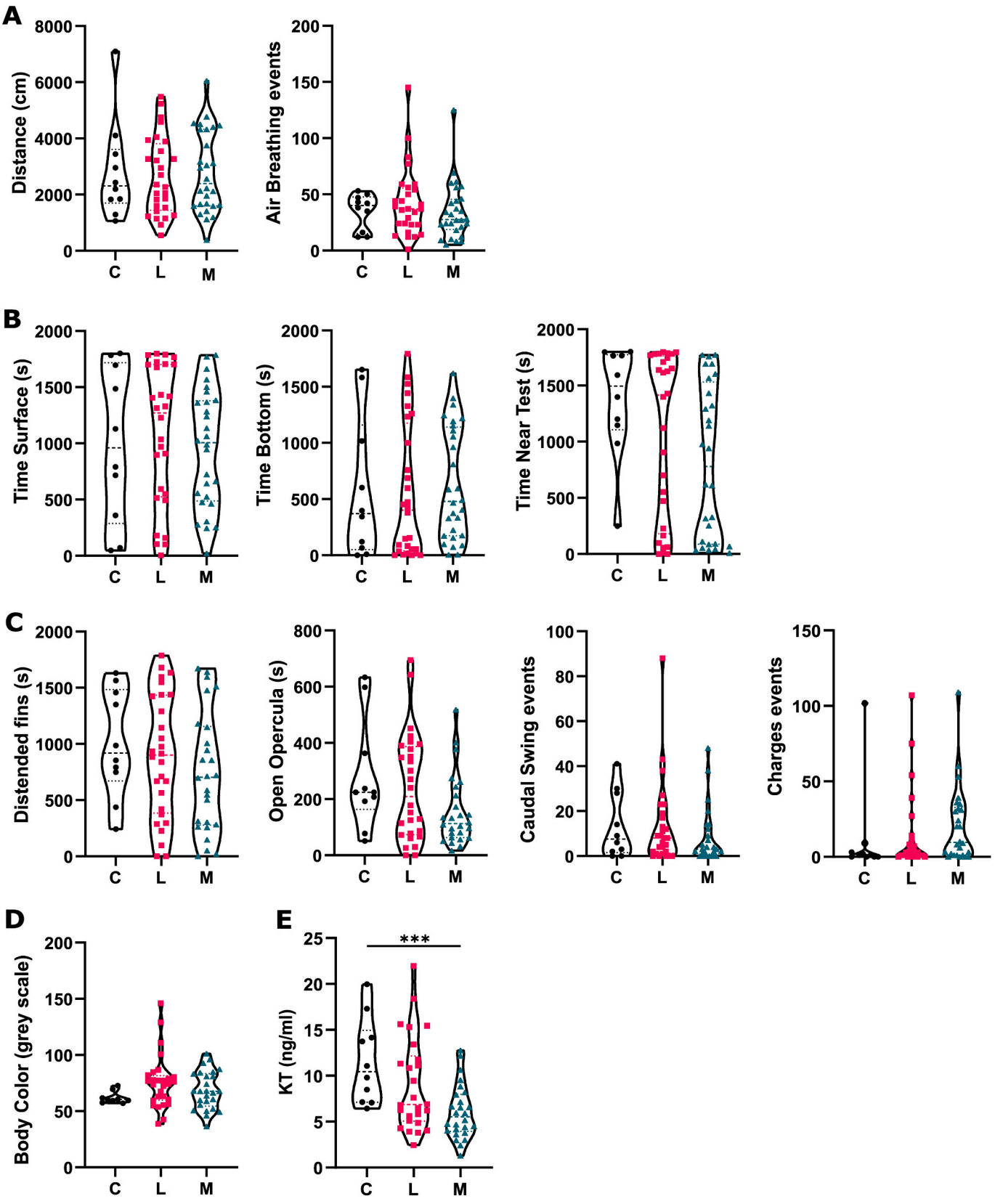

Fig. 2. Treatments (C = control, L = L-368,899, M = Manning compound) effect on activity (distance traveled, frequency of air-breathing) (A); position in the tank (time at the surface, time at the bottom, time near test) (B); aggressive behaviors (time with fins distended, time with open opercula, frequency of caudal swings, frequency of charges) (C); and body color (D) during the fight, and KT plasma level post-fight (E). The *p*-values are reported as ≤0.001 (***).

Table 2
Main effects of variables, at the treatments level, related to the activity (distance and air-breathing), position in the tank (time at the surface, time at the bottom, time near test), body morphology (color), aggressive behaviors (time with fins distended, time with open opercula, frequency of caudal swings, frequency of charges), during acclimation and mirror phases; and KT plasma level post-fight. F: Anova-test F-estimate; p: *p*-value; η^2: effect size estimate (eta squared), 95 % confidence interval. The *p*-values are reported as ≤0.001 (***).

Variables	Acclimation	Mirror
#Activity		
Distance traveled	$F_{2,63} = 0.58$ $p = 0.565$ $\eta^2 = 0.02$ 95 % CI = [0.00, 1.00]	$F_{2,63} = 0.07$ $p = 0.935$ $\eta^2 = 0.002$ 95 % CI = [0.00, 1.00]
Air-breathing	$F_{2,63} = 0.39$ p = 0.679 $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]	$F_{2,63} = 0.37$ $p = 0.695$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]
#Position in the tank		
Time near Surface	$F_{2,63} = 0.97$ $p = 0.385$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]	$F_{2,63} = 0.34$ $p = 0.711$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]
Time near Bottom	Kruskal Wallis chi-squared = 1.55 df = 2 $p = 0.461$ $\eta^2 = 0.007$ 95 % CI = [0.00, 1.00]	$F_{2,63} = 1.34$ $p = 0.269$ $\eta^2 = 0.04$ 95 % CI = [0.00, 1.00]
Time near Test Area	$F_{2,63} = 0.68$ $p = 0.511$ $\eta^2 = 0.02$ 95 % CI = [0.00, 1.00]	$F_{2,63} = 1.66$ $p = 0.198$ $\eta^2 = 0.05$ 95 % CI = [0.00, 1.00]
#Body morphology		
Color		$F_{2,63} = 0.14$ $p = 0.869$ $\eta^2 = 0.004$ 95 % CI = [0.00, 1.00]
#Aggressive Behaviors		
Distended fins		$F_{2,63} = 1.17$ $p = 0.317$ $\eta^2 = 0.04$ 95 % CI = [0.00, 1.00]
Open Opercula		$F_{2,63} = 0.91$ $p = 0.408$ $\eta^2 = 0.03$ 95 % CI = [0.00, 1.00]
Caudal swing		$F_{2,63} = 0.46$ $p = 0.635$ $\eta^2 = 0.01$ 95 % CI = [0.00, 1.00]
Charges		$F_{2,63} = 2.96$ $p = 0.06$ $\eta^2 = 0.09$ 95 % CI = [0.00, 1.00]

	Post-fight
#Hormonal level	
KT	$F_{2,61} = 7.73$ $p = 0.001$ *** $\eta^2 = 0.2$ 95 % CI = [0.06, 1.00]

We also examined whether the antagonists could modulate the post-fight plasma KT response. In males of this species, like in other cichlid fish species (*e.g.*, *Astatotilapia burtoni*, Alcazar et al., 2016, Desjardins and Fernald, 2010, Maruska and Fernald, 2010; *Pundamilia* spec., Dijkstra et al., 2012; and *N. pulcher*, Taves et al., 2009), a post-fight increase in plasma androgen levels, including after a mirror fight, has been consistently observed and shown to be robust (Alex et al., 2023; Ramos et al., 2021; Ramos and Gonçalves, 2022). No impact of OXT manipulation with L-368,899 was detected in KT levels. A similar result has been observed in zebrafish (Altmieme et al., 2019), though in a different social context, where the administration of L-368,899 prior to courtship behavior did not influence KT levels. However, blocking VT receptors with Manning significantly suppressed the post-fight KT response, potentially due to either central or peripheral effects of Manning. As reviewed by Mennigen et al. (2022), the nonapeptide system can have hypophysiotropic effects, influencing gonadotropin production directly in the pituitary, or play endocrine and paracrine roles targeting the testis. To further test the results obtained *in vivo*, the effects of VT and Manning in KT secretion by testicular tissue was investigated *in vitro*. After 180 min of incubation, there was a clear effect of VT in KT release, with higher levels recorded in the VT-administered wells, in comparison with all the other treatments. KT levels were detectable in the control wells, showing basal KT release, as described in previous studies (Leal et al., 2009; Tovo-Neto et al., 2020). The Manning compound did not block KT release, confirming that basal secretion of KT by testicular tissue is not dependent on VT. These results suggest the presence of VT

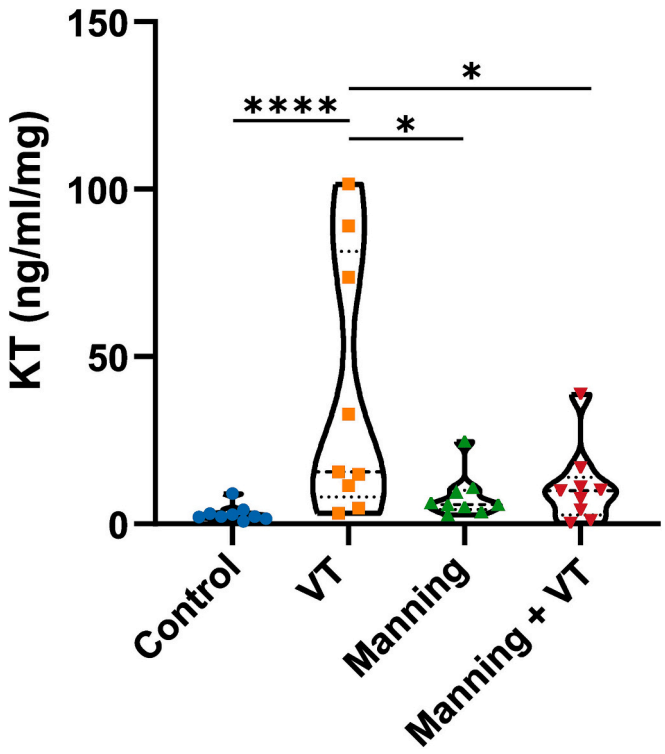

Fig. 3. *In vitro* KT secretion by testicular tissue under four different treatments: control (Ringer solution), VT (100 nM), Manning compound (100 nM), VT (100 nM) + Manning compound (100 nM). The *p*-values are reported as ≤0.05 (*), and ≤ 0.0001 (****).

receptors in *B. splendens* testes, similar to what has been described in other fish species (Lema et al., 2012; Rawat et al., 2019). Indeed, the impact of VT in KT release was not detected if the tissue was first incubated in Manning, suggesting that the mechanism of action of VT in KT release in the testis is through the activation of its receptors. Considering that the Manning compound is usually used to block the VTR1A receptor, we can suggest the presence of this receptor subtype in *B. splendens* testis, while acknowledging the potential presence of other orthologues. In zebrafish testis, for example, the avpr1aa transcript (orthologous to human VTR1A) has been found to be the most abundant with respect to the others receptor subtypes (Zanardini et al., 2024). Finally, previous *in vitro* studies using fish testicular tissue have already confirmed a stimulatory action of VT on androgen release (Ramallo et al., 2012; Rodríguez and Specker, 1991; Zanardini et al., 2024).

For the injected compounds to reach the brain, they must cross the blood-brain barrier (BBB), a semi-permeable membrane that protects the central nervous system (CNS) by preventing access to toxins and pathogens in the bloodstream (Wu et al., 2023). No study has directly assessed the ability of the chosen OXT or VT antagonists to cross the BBB in fish. However, numerous studies demonstrate that intraperitoneal injections of these antagonists can modulate various behaviors (*e.g.*, Landin et al., 2020; Semsar et al., 2001; Semsar and Godwin, 2004; Yaeger et al., 2014), suggesting a central role for these compounds. In other taxa, this area of research is also underexplored. A study in monkeys showed that intravascular administration of the oxytocin antagonist L-368,899 successfully entered the cerebrospinal fluid (CSF) and accumulated in the hypothalamus, septum, orbitofrontal cortex, amygdala, and hippocampus (Boccia et al., 2007). Considering that, in rodents (Manaenko et al., 2011; Wang et al., 2018), intraperitoneal injections have been shown to sufficiently deliver drugs to the brain compared to intravenous injections, it is reasonable to assume that L-368,899 can also access the brain *via* intraperitoneal injection. Furthermore, it is worth noting that fish, particularly zebrafish, possess a more ancestral endothelial BBB compared to mammals along with a less complex neurovascular unit, which includes all cell types in close proximity to neurons and CNS vasculature (O'Brown et al., 2018). This suggests that if the antagonists can cross the mammalian BBB, they are likely able to cross the fish BBB as well (Dunton et al., 2021; O'Brown et al., 2019). For the Manning compound, this type of research is not yet available. However, as previously noted, studies have demonstrated that its administration can influence behavior. Whether these effects result from central or peripheral actions remains uncertain. Considering our results, a peripheral effect targeting the gonads seems likely.

Taken together, the *in vivo* and *in vitro* results suggest that the post-fight androgen response is most likely enhanced by the action of VT in the testes. Interestingly, despite this effect of VT modulation in peripheral levels of androgens, no impact was observed in aggressive behavior, suggesting a decoupling between aggression and androgens. Indeed, this has already been suggested in other studies where castration (in *B. splendens*, Weiss and Coughlin, 1979; in Mozambique tilapia, Almeida et al., 2014; and in weakly electric fish, *Gymnotus omarorum*, Jalabert et al., 2015) or androgen administration (in bluehead wrasse, Semsar and Godwin, 2003; in *B. splendens*, Forsatkar et al., 2013) failed to increase aggressive behavior. At the same time, studies show that androgens can modulate aggressive behavior in different species: implants of KT induced increased aggressive behaviors in bluegill sunfish *Lepomis macrochirus* (Cunha et al., 2019; Rodgers et al., 2013); and testosterone injection increased frequency and duration of aggressive behavior, and also winning probability in the sheepshead minnow *Cyprinodon variegatus* (Higby et al., 1991). In addition, considering that aggression itself can modulate androgen levels (Desjardins et al., 2006), maybe an androgenic role as modulators, rather than mediators, of aggressive behavior is more likely, as suggested by Almeida et al. (2023).

5. Conclusions

This study is the first to investigate the possible role of OXT and VT antagonists in aggressive behavior in males of the Siamese fighting fish *B. splendens*. It is also among the most comprehensive studies in fish concerning the number of tested animals and dosages. While manipulation of OXT and VT did not significantly impact aggressive behavior, the peripheral secretion of KT during fights was inhibited by the administration of the VT, but not of the OXT, receptor antagonist. These results suggest that VT positively modulates KT release, likely *via* the activation of its receptors at the testicular level. The different effects of VT on androgen release and aggressive behavior further suggest a decoupling between aggression and peripheral androgen levels in this species.

CRediT authorship contribution statement

Bianca Fusani: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andreia Ramos:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Sara D. Cardoso:** Writing – review & editing, Software, Methodology, Investigation, Conceptualization. **David Gonçalves:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Author's contribution

D.G. and B.F. designed the experiment. B.F., S.D.C. and A.R. ran the behavioral trials and hormonal assays. B.F. performed the *in vitro* experiment. B.F. analyzed the data and prepared the figures and tables. All authors contributed to the writing and the revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2025.105728.

Data availability

Data will be made available on request.

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