



## Parental care and neuropeptide dynamics in a cichlid fish *Neolamprologus caudopunctatus*

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### ABSTRACT

The hypothalamic neuropeptides arginine vasopressin (AVP) and oxytocin (OT) modulate social behavior across a wide variety of species. However, the role of arginine vasotocin (AVT) and isotocin (IT, the teleost homologs of AVP and OT) in regulating biparental care especially in the context of monogamy is not well studied. Here, using high-performance liquid chromatography (HPLC), we investigated how bioactive whole brain AVT and IT neuropeptide levels vary in relation to the phase of the breeding cycle and sex, in a monogamous biparental cichlid fish, *Neolamprologus caudopunctatus*. Since non-caring individuals of this species readily cannibalize eggs, but caring parents never do, we further investigated whether there might be changes in AVT/IT whole brain levels that correspond to the transition from a non-breeding, egg cannibal to an egg caring parent. We found that AVT levels were higher in females than in males and that AVT levels were highest when the need to defend the young was greatest. Breeding pairs that had a strong pair-bond and a higher frequency of nest care had the highest levels of AVT, whereas individuals that spent little time close to their breeding partner, displayed aggression towards their partner and neglected their nest duties (signs of a weak pair bond), had lower whole brain AVT levels. Isotocin (IT) levels did not differ between sexes and we did not detect any variation across the breeding cycle, with pair-bonding scores or with parental behavior. Our results show that whole brain AVT levels are linked to the breeding cycle, nest maintenance and pair-bonding score in this species. Furthermore, our study highlights species and sex-specific nonapeptides patterns in bi-parental caring fish and contributes to the increasing knowledge on neuroendocrinological mechanisms underlying parental care.

### 1. Introduction

There is substantial evidence that hypothalamic neuropeptides are implicated in a wide array of social and reproductive behaviors in vertebrates. For instance, female rats (*Rattus norvegicus*) guard, nurse and lick their pups after giving birth, while unmated virgin female rats strongly avoid or even attack pups (Feldman and Bakermans-Kranenburg, 2017). However a single injection of the hypothalamic neuropeptide oxytocin (OT) will induce maternal behavior in virgin females (Pedersen et al., 1982). Similarly, in domestic chickens (*Gallus domesticus*), maternal nest attendance and post-hatching care is mediated by the avian homolog of oxytocin - mesotocin (Chokhalomwong et al., 2013). Such results across a wide variety of taxa have led researchers to the assertion that the neuropeptide oxytocin (OT) is an important modulator of maternal care (Bales and Saltzman, 2016; Lee

et al., 2009), whereas arginine vasopressin (AVP), a closely related hypothalamic neuropeptide, is thought to influence male parental care (Bales and Saltzman, 2016). However, in a recent study on poison frogs (*Ranitomeya imitator*), a single injection of arginine vasotocin (AVT, a homolog of AVP) reduced parental care behavior and did so in both males and females (Schulte and Summers, 2017).

Whereas the mechanisms underlying parental care and the role of OT and AVP have been well studied in mammals, our understanding of the impact of non-mammalian homologs of these neuropeptides such as AVT (arginine vasotocin) and IT (Isotocin) on parental care in other taxa is less established. Additional information linking parental behavior with nonapeptides in other taxa would enhance our ability to determine which mechanisms modulating parental care are common across animals and which mechanisms are species specific and likely evolved via special selective challenges. Here we studied the role of

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AVT and IT in relation to parental behavior in a biparental cichlid fish from Lake Tanganyika.

In teleosts, apart from parental care, it has been well established that IT and AVT regulate a variety of other social behaviors, such as aggression (Larson et al., 2006; Santangelo and Bass, 2006; Greenwood et al., 2008; Filby et al., 2010; Santangelo and Bass, 2010; Oldfield and Hofmann, 2011; Godwin and Thompson, 2012; Kleszczyńska et al., 2012), submission (Reddon et al., 2012, 2014), proximity-seeking behavior (Thompson and Walton, 2004; Filby et al., 2010) and territoriality or space use (Oldfield et al., 2015). There are also a small but growing number of studies that suggest that AVT and IT influence parental behavior in fishes (Maruska et al., 2007; O'Connell et al., 2012; Kleszczyńska et al., 2012; DeAngelis et al., 2017, 2018). For example, an isotocin receptor antagonist blocked male parental care in convict cichlids, *Amatitlania nigrofasciata*, (O'Connell et al., 2012) and a similar result was found in the clownfish *Amphiprion ocellaris*, (DeAngelis et al., 2017). Moreover, IT receptor gene-expression was up-regulated in caring *Amphiprion ocellaris* males (DeAngelis et al., 2018), while an arginine vasotocin antagonist increased male parental care (*Amphiprion ocellaris*, DeAngelis et al., 2017).

In fishes, AVT and IT are mainly expressed in neuronal populations of the preoptic area (Godwin and Thompson, 2012), an important brain region for the regulation of sexual, aggressive and parental behaviors (O'Connell and Hofmann, 2011). There are three types of neuronal populations that secrete these neuropeptides; these differ in size (from smallest to largest) and in position in the preoptic area (caudal to rostral) called: 1) parvocellular, 2) magnocellular and 3) gigantocellular cell groups (Godwin and Thompson, 2012; Oldfield et al., 2015; Reddon et al., 2017). Centrally, the magnocellular and gigantocellular AVT producing cells are thought to play a role in regulating aggression and approach (Santangelo and Bass, 2010; Dewan and Tricas, 2011; Godwin and Thompson, 2012; Ramallo et al., 2012); while the parvocellular cells are linked with the regulation of submissive behavior and withdrawal (Godwin and Thompson, 2012; Reddon et al., 2017). Furthermore, the axon terminals of these neurons extend to the neurohypophysis, where the secretions of these neurosecretory cells are picked up by the circulatory system and transported to target organs exerting a wide range of peripheral effects (Godwin and Thompson, 2012).

Cichlids make excellent model systems for endocrinological investigations of the physiological basis of care, because they all perform parental behaviors, with different species employing different forms of parental behaviors (Balshine-Earn and Earn David, 1998; Goodwin et al., 1998). Cichlids provide care for young either by mouth brooding (protecting young inside their buccal cavity) or by substrate guarding, with bi- or uni-parental care (Goodwin et al., 1998; Reynolds et al., 2002; Sefc, 2011; Balshine, 2012). In contrast to the many studies examining the ultimate causes of parental care variation, studies investigating proximate mechanisms underlying parental behavior in fishes are more scarce (but see Desjardins et al 2008; Bender et al., 2008a, 2008b; Grone et al., 2012; Reddon et al., 2017) and studies focused on biparental caring fish species are the rarest of all (O'Connell et al., 2012).

In *N. caudopunctatus*, both parents actively participate in rearing the offspring and do so mainly by defending the breeding cavity that contains the eggs, larvae and free swimming young (Ochi and Yanagisawa, 1999), making this monogamous species (Schaedelin et al., 2015) an ideal model organism to investigate proximate causes of parental care for both sexes. Before providing care, *N. caudopunctatus* must find or attract a mate, form a pair-bond, construct a nest and reproduce. The parental phase concludes once the young become independent and breeders revert to the non-breeding state (Fig. 1A). Whereas breeding *N. caudopunctatus* are diligent parents, non-breeding individuals are voracious cannibals (Cunha-Saraiva et al., 2018). The propensity for egg cannibalism ends abruptly when spawning occurs, initiating the caring phase, which is maintained by the uninterrupted presence of

eggs and larvae (Cunha-Saraiva et al., 2018). In contrast to other biparental fish species (Schwanck, 1986; Lavery and Keenleyside, 1990), both females and males *N. caudopunctatus* perform egg cannibalism. Thus, *N. caudopunctatus* also offers an excellent opportunity to study the underlying endocrinological mechanisms of care and associations with the transition from egg cannibal to egg care giver.

The aims of our study were: 1) to characterize the whole brain neuro-endocrinological profile of bioactive AVT and IT across the breeding cycle of *Neolamprologus caudopunctatus*, 2) to investigate sex differences in whole brain levels of AVT and IT, and 3) to explore links between the levels of IT/AVT and pair bonding, brood care, brood defense and nest maintenance. To address these aims, we sampled males and females *N. caudopunctatus* across the breeding cycle, from the non-breeding phase to the post-spawning stage (i.e. 10 days after egg laying). We then measured and compared biologically active whole-brain AVT and IT in non-breeders, pair bonded pre-spawning pairs, pair bonded fish that had just spawned and post-spawning pairs with older young. We predicted that during the transition from non-caring egg cannibal to caring parent would be reflected in AVT and IT whole-brain levels, and that if involved in care these neuropeptides would be higher after young had hatched and care is more intensive. Additionally, we experimentally explored the relationship between the degree of brood care, brood defense, nest maintenance and strength of the pair-bond and the levels of these two biologically active nonapeptides. We predicted that isotocin (IT) would modulate brood care behavior and pair-bonding while arginine vasotocin (AVT) would modulate antagonistic behaviors, such as brood defense.

## 2. Material and methods

### 2.1. Study animals and housing conditions

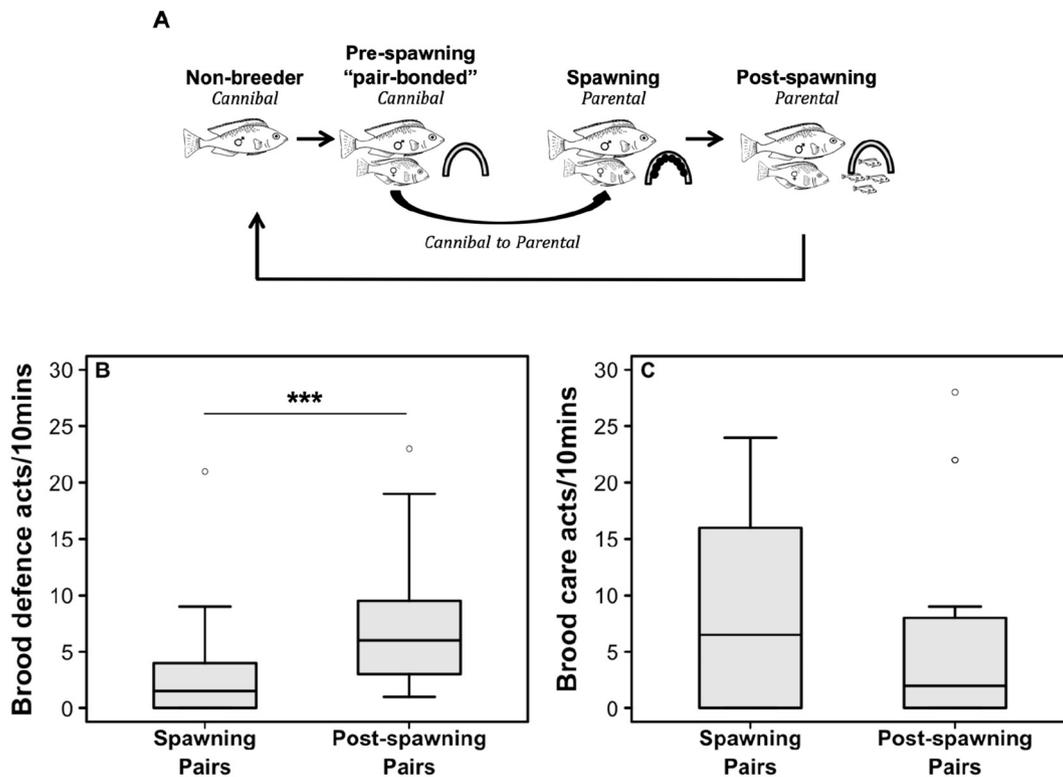
*Neolamprologus caudopunctatus* is a socially and genetically monogamous (Schaedelin et al., 2015) cichlid fish from Lake Tanganyika, Africa. Breeding pairs are sexually monomorphic and excavate a cavity together, in which they lay and fertilize eggs and guard the offspring. The breeding pair defends their free swimming young for up to 40 days mostly against conspecific non-breeders (both juveniles and adults) (Ochi and Yanagisawa, 1999), who are frequent and ravenous consumers of young (Cunha-Saraiva et al., 2018).

In this study, we used a total of 80 wild-caught *N. caudopunctatus* (40 males and 40 females) from the most southern tip of Lake Tanganyika, Zambia, Africa. All fish were sexually mature. The fish were sexed and measured for standard length (SL in mm: from the tip of the longest jaw to the end of the caudal peduncle), total length (TL in mm), and body mass (M, in grams, see Table 1 for all the body morphological measures). Fish were kept in a mixed sex stock aquaria (160 L, 96 cm × 45 cm × 38 cm) at a constant water temperature of 26 ± 1 °C under a 12/12 h light/dark cycle with access to breeding shelters on a 3 cm layer of sand. Fish were fed once daily with either frozen food (a mixture of artemia, cyclops, daphnia and red mosquito larvae) or with tropical fish flakes.

### 2.2. General procedures and experimental protocol

Breeding pairs were allowed to form freely in a large mixed-sex stock aquaria (1:1 sex ratio); free mate choice results in faster and more successful breeding compared to artificially composed or forced pairings in *N. caudopunctatus* (Cunha-Saraiva et al., 2018). Each formed pair was removed and transferred to a 45 L experimental tank, equipped with 2 cm of sand as substrate, half a flower pot as a breeding shelter, a heater and a sponge filter. We checked each breeding pair daily for signs of nest construction, egg laying, social stability and general health.

To investigate possible changes of neuropeptide levels (IT and AVT) across the breeding cycle, we sampled the behavior of 10 pairs before



**Fig. 1.** (A) The diagram illustrates the different reproductive stages of *Neolamprologus caudopunctatus*'s breeding cycle. Non-breeding individuals and pre-spawning breeding pairs are egg cannibals, while spawning and post-spawning pairs are non-cannibals. A graphical representation of (B) brood defence and (C) brood care across two reproductive phases (spawning and post-spawning); and (D) Nest maintenance across three reproductive phases (pre-spawning, spawning and post-spawning). Ten breeding pairs were observed and sampled at each reproductive stage. Grey boxes represent females and males pooled together. Box plots show the interquartile range (IQR) of each group analyzed with whiskers extending to 1.5 × the IQR. Horizontal lines represent medians. Outliers were included in the analysis and are depicted on the graphs as white circles. Significant differences between reproductive stages are depicted on the graph as bold stars \*\*\*P < 0.001, \*\*P < 0.01, \*P < 0.05.

egg laying (pre-spawning pairs), 10 pairs within the first 24 h of egg laying (spawning pairs) and another 10 pairs at ten days post spawning (post spawning pairs), a time when the pair actively defends its free

swimming young (Fig. 1A). Additionally, we sampled 10 non-breeding males and females collected from our same-sex stocks aquaria, where the individuals had not yet paired or reproduced. In addition to

**Table 1**

Measured morphological characteristics (SL = standard length, M = mass, BM = brain mass and GSI = gonadal somatic index) of the *Neolamprologus caudopunctatus* individuals used in the current study. A total of 10 males and 10 females were used in all the morphological measures and behavioral assays of the reproductive stages, however, HPLC values of whole brain AVT and IT could not be obtained from all individuals. This led to different final sample sizes across treatments. All values presented as mean ± standard error of the mean (SEM).

Reproductive stage	Sex	Data type	N	M(g)	SL(mm)	BM(mg)	GSI
Non-breeder	Male	HPLC: IT	8	3.9 ± 0.2	52.7 ± 1.2	27.5 ± 1.3	0.22 ± 0.04
		HPLC: AVT	10	4.0 ± 0.2	53.1 ± 0.9	27.4 ± 1.1	0.22 ± 0.04
Pre-spawning	Female	HPLC: IT	9	3.1 ± 0.2	48.2 ± 0.9	21.9 ± 1.4	1.86 ± 0.39
		HPLC: AVT	8	3.0 ± 0.2	47.8 ± 1.0	22.0 ± 1.6	1.93 ± 0.44
	Male	Behavior	10	3.9 ± 0.2	53.4 ± 1.0	27.5 ± 1.2	0.24 ± 0.03
		HPLC: IT	9	3.9 ± 0.2	53.2 ± 1.1	27.4 ± 1.3	0.24 ± 0.03
Spawning	Female	HPLC: AVT	9	3.8 ± 0.2	53.2 ± 1.1	27.0 ± 1.2	0.25 ± 0.04
		Behavior	10	2.6 ± 0.1	46.5 ± 0.8	20.8 ± 1.0	3.69 ± 0.64
		HPLC: IT	10	2.6 ± 0.1	46.5 ± 0.8	20.8 ± 1.0	3.69 ± 0.64
	Male	HPLC: AVT	10	2.6 ± 0.1	46.5 ± 0.8	20.8 ± 1.0	3.69 ± 0.64
		Behavior	10	3.8 ± 0.2	53.3 ± 1.1	27.8 ± 1.6	0.23 ± 0.04
		HPLC: IT	8	3.8 ± 0.2	53.6 ± 1.0	26.5 ± 1.7	0.19 ± 0.04
Post-spawning	Female	HPLC: AVT	10	3.8 ± 0.2	53.3 ± 1.1	27.8 ± 1.6	0.23 ± 0.04
		Behavior	10	2.8 ± 0.1	47.7 ± 0.8	23.1 ± 0.9	0.96 ± 0.06
		HPLC: IT	9	2.8 ± 0.1	47.7 ± 0.9	23.0 ± 1.0	0.92 ± 0.04
	Male	HPLC: AVT	10	2.8 ± 0.1	47.7 ± 0.8	23.1 ± 0.9	0.96 ± 0.06
		Behavior	10	4.0 ± 0.3	53.5 ± 1.6	27.1 ± 1.8	0.27 ± 0.04
		HPLC: IT	8	4.1 ± 0.3	54.6 ± 1.8	28.1 ± 2.2	0.25 ± 0.04
Post-spawning	Female	HPLC: AVT	9	4.0 ± 0.3	53.6 ± 1.8	27.4 ± 2.1	0.27 ± 0.04
		Behavior	10	2.8 ± 0.2	46.7 ± 1.0	22.9 ± 0.8	0.93 ± 0.08
	Male	HPLC: IT	8	2.7 ± 0.2	46.6 ± 1.3	23.4 ± 0.8	0.96 ± 0.09
		HPLC: AVT	9	2.7 ± 0.2	46.5 ± 1.1	23.0 ± 0.8	0.94 ± 0.09

collecting behaviors we used high performance liquid chromatography techniques to quantify whole brain IT/AVT levels.

### 2.3. Behavioral assays and scoring

Before and after egg laying, we conducted two focal behavioral observation trials on the pairs; one consisted of a 10-minute brood care assay and was followed by a 2-minute brood defense assay. We collected all within-pair behaviors during both the brood care and defense assays (including all performed and received aggression and submission). We recorded all offspring care and nest maintenance behaviors only during the brood care assay. The behavioral assays were always conducted between 10 h and 13 h to avoid possible diurnal fluctuations in whole brain IT/AVT levels (Gozdowska et al., 2006; Lema et al., 2010). Each observation period began with a 2-minute habituation period. We divided parental behaviors into two categories: i) nest care, which included any acts of digging and or sand transport away from the nest and ii) egg care, which included nest cavity visits, egg inspection, cleaning and fanning. We also recorded any aggressive and submissive behaviors performed towards partners. A full description of all the behaviors recorded for *N. caudopunctatus* can be found in Cunha-Saraiva et al., 2018. After the brood care assay, we conducted the brood defense assay by placing a perforated transparent plexiglass cylinder containing three juvenile *N. caudopunctatus* (between 30 and 45 mm, standard length SL) into the aquaria which allowed both visual and chemical communication between the pairs and the intruders. We used juvenile *N. caudopunctatus* as intruders because as mentioned above they are known to be voracious egg predators (Cunha-Saraiva et al., 2018) and pose a serious potential threat to young against which pairs will readily defend. The 2-minute brood defense assay would begin as soon as one of the breeding pair performed a single aggressive display or act towards the juveniles in the cylinder. We also recorded latency to attack, which differed across the breeding cycle: for pre-spawning pairs, attacks began on average after  $84 \pm 27.1$  s; for spawning pairs attacks began after a mean of  $135 \pm 44.5$  s; while for post-spawning pairs (with hatched free-swimming young) the average time until attack was on average only  $18 \pm 12.8$  s. All aggressive behaviors towards the intruders (the potential brood cannibals, the *N. caudopunctatus* juveniles) and between the breeding pair were recorded.

### 2.4. Brain isotocin and arginine vasotocin assays

To collect the brains, we captured the experimental pair immediately after the brood defense assay and euthanized both individuals with an overdose of anesthetic (MS222, Sigma, 1000 mg/l) followed by spinal cord severance. We then extracted the whole brain from each fish. Capture and dissection occurred within 2 min of approach to the tank. Each brain was weighed (Table 1) and frozen immediately in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . After brain tissue collection, we dissected and measured the gonads and liver for each fish and confirmed the sex of each fish anatomically.

We determined the AVT and IT content in the brains using high performance liquid chromatography with fluorescence and UV detection preceded by solid-phase extraction (SPE). The frozen brains were thawed before being sonicated in 1 ml Milli-Q water (MicrosonXL, Misonix, Farmingdale, NY). We added glacial acetic acid (2.5  $\mu\text{l}$ ) to the homogenates, and then placed the samples in a boiling water bath for 3.5 mins. The extracts were cooled on ice, and then centrifuged at 8000g for 20 mins at  $4^\circ\text{C}$ . We loaded the supernatants onto preconditioned (1 ml MeOH, 1 ml distilled water) SPE columns (30 mg  $\text{ml}^{-1}$ , STRATA-X, Phenomenex, Torrance, CA). We passed water (600  $\mu\text{l}$ ) and then 0.1% trifluoroacetic acid (TFA) in 5% acetonitrile (600  $\mu\text{l}$ ) through the columns to wash away impurities, and then eluted the peptides by  $2 \times 600 \mu\text{l}$  of 80% acetonitrile. The eluates were evaporated using Turbo Vap LV Evaporator (Caliper Life Science, Hopkinton, MA) and frozen immediately. Then the samples were stored at  $-80^\circ\text{C}$  prior to

HPLC analysis.

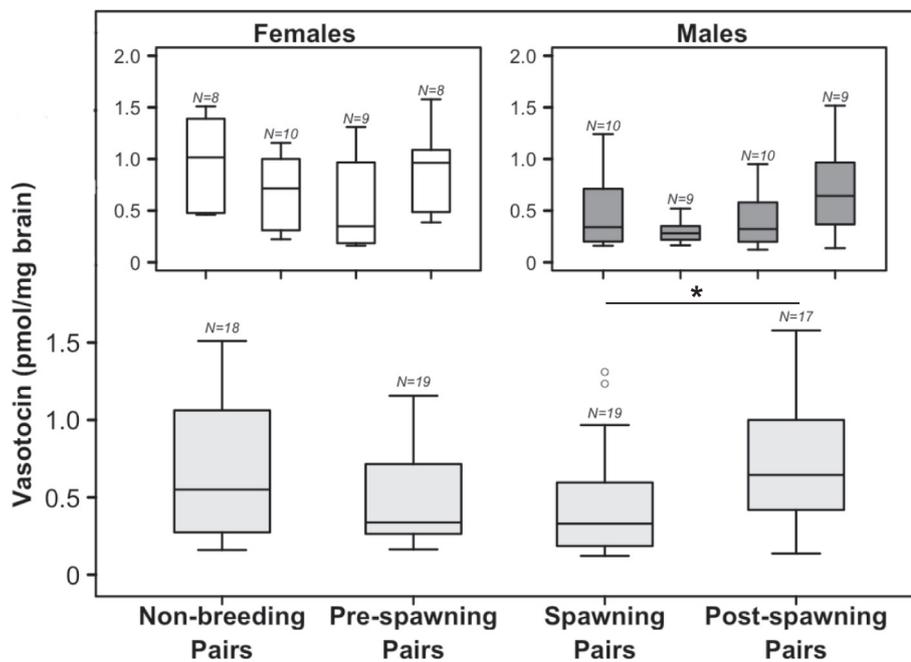
Before quantitative analysis, we re-dissolved the samples in 50  $\mu\text{l}$  of 0.1% TFA in 30% acetonitrile and divided them into two aliquots. The derivatization of AVT and IT in each of the 25  $\mu\text{l}$  samples was performed using 3  $\mu\text{l}$  of 4-fluoro-7-nitro-2,1,2-benzoxadiazole (NBD-F) solution (30 mg NBD-F in 1 ml of acetonitrile) in 25  $\mu\text{l}$  borate buffer (0.05 M, pH 8.0). We heated the solution at  $40^\circ\text{C}$  for 10 min in a dry heating block and then cooled it down on ice before adding 5  $\mu\text{l}$  of 1 M HCL. We measured the derivatized samples with Agilent 1200 series Quaternary HPLC system (Agilent Technologies, Santa Clara, CA). Chromatographic separation was achieved on a ZORBAX Eclipse XDB-C18 column (Agilent Technologies,  $150 \times 4.6$  mm I.D., 5  $\mu\text{m}$  particle size). A gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in  $\text{H}_2\text{O}$ ) and solvent B (0.1% TFA in acetonitrile:  $\text{H}_2\text{O}$  [3:1]). A linear gradient was 40–65% of eluent B in 20 min. We set the flow rate at  $1 \text{ ml min}^{-1}$  and the column temperature at  $20^\circ\text{C}$ . The injection volume was 58  $\mu\text{l}$ . The fluorescence detection was carried out at 530 nm with excitation at 470 nm and UV at 340 nm. Our analytical procedure permits the measurement of biologically active AVT and IT, and this technique had been used successfully in several fish species including closely related cichlids (Gozdowska et al., 2006; Almeida et al., 2012; Kleszczyńska et al., 2012; Martos-Sitcha et al., 2013; Kleszczyńska and Kulczykowska, 2013; Sokołowska et al., 2013; Reddon et al., 2015). AVT and IT were analyzed simultaneously in every sample and data expressed as pmol of peptide per brain weight (mg).

### 2.5. Statistical analysis

We used R statistical software 3.5.1 to perform the analyses. Prior to any statistical test, all dependent variables were categorized according to their distribution and transformed when necessary to reach normal distribution. We opted to remove one outlier in IT whole brain levels identified by the Grubbs test in the outliers package ( $G = 3.25$ ,  $U = 0.84$ ,  $P = 0.02$ , we were blind to the reproductive stage and sex of individuals while conducting this test). No outlier was identified in whole brain AVT levels ( $G = 2.39$ ,  $U = 0.91$ ,  $P = 0.54$ ). Isotocin and arginine vasotocin whole brain levels were both log transformed to meet normality. To determine how IT and AVT whole brain levels change across the breeding cycle, we performed an ANOVA (aov function, from the stats package) for each neuropeptide with body mass (M) and standard length (SL) as covariates, and reproductive stage (non-breeding, pre-spawning, spawning and post-spawning), sex (male and female) and their interactions as factors. Effect sizes estimates were computed as follows: Eta-square for analysis of variance (etaSquared function, from the lsr package, Navarro, 2015) and Cohen's d for pairwise comparisons (cohen.d function, from the effsize package, Torchiano, 2018). Additionally, we performed linear regression analysis between levels of biologically active whole brain neuropeptides (AVT and IT) and GSI (gonadal somatic index) for both females and males, separately. GSI values were computed as follows:  $\text{GSI} = (\text{gonad mass} / (\text{body mass} - \text{gonad mass})) \times 100$ .

Non-significant factors were removed from the final models (**Mass (M)** IT:  $\eta^2 = 0.01$ ,  $F\text{-test}_{1,59} = 0.88$ ,  $P = 0.35$ , AVT:  $\eta^2 = 0.007$ ,  $F\text{-test}_{1,63} = 0.68$ ,  $P = 0.41$ ; **Standard length (SL)** IT:  $\eta^2 = 0.01$ ,  $F\text{-test}_{1,59} = 1.09$ ,  $P = 0.29$ , AVT:  $\eta^2 = 0.004$ ,  $F\text{-test}_{1,63} = 0.41$ ,  $P = 0.52$ ; **interaction** IT:  $\eta^2 = 0.05$ ,  $F_{3,61} = 1.25$ ,  $P = 0.29$ , AVT:  $\eta^2 = 0.02$ ,  $F_{3,65} = 0.87$ ,  $P = 0.45$ ). To test for differences between the different stages of the breeding cycle, we used glht as a general linear hypothesis testing function in R (Hothorn et al., 2008) by using user-defined contrasts (Non-breeding vs Spawning; Pre-spawning vs Spawning; Spawning vs Post-spawning; Non-breeding vs Post-spawning; Pre-spawning vs Post-spawning). This is a single-step method test, which adjusts the P-values to the number of comparisons using Tukey contrasts.

In order to test if the amount of parental care behavior varied across



**Fig. 2.** A graphical representation of *Neolamprologus caudopunctatus* AVT whole brain levels for both females and males per reproductive phase. White boxes represent females and dark grey boxes represent males, whereas light grey represent females and males pooled together. Box plots are as in Fig. 1. Significant differences between reproductive stages are depicted on the graph as bold stars (Tukey Contrasts: Non-breeding vs Pre-spawning,  $t = 1.65$ ,  $P = 0.32$ , Cohen's  $D = 0.52$ ; Pre-spawning vs Spawning,  $t = -0.45$ ,  $P = 0.96$ , Cohen's  $D = 0.03$ ; Post-spawning vs Non-breeding,  $t = -0.76$ ,  $P = 0.85$ , Cohen's  $D = 0.18$ ; Pre-spawning vs Post-spawning,  $t = -2.4$ ,  $P = 0.07$ , Cohen's  $D = 0.77$ ), \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

the breeding cycle, we calculated a brood defense score from the defense assay (the sum of all observed aggressive behaviors towards the nest intruders), a brood care score (the sum of all observed care behaviors including egg care and cavity visits) and a nest maintenance score (digging out substrate) from the brood care assay for each individual in our study. All behavioral variables were SQRT transformed and then used as the response variable in a generalized linear model, while reproductive stage and sex were used as predictor variables. Each model was validated by assessing the distribution of its residuals and AIC (Johnson and Omland, 2004).

Finally, to explore the link between the frequency of specific behaviors and the levels of biologically active whole brain nonapeptides, we performed spearman correlations (all correlations were corrected for multiple comparisons using "holm" P-value adjustment method (Aickin and Gensler, 1996)). We explored how whole brain AVT and IT levels correlated with: 1) brood defense, 2) brood care 3) nest maintenance and 4) pair-bonding scores. Pair-bonding scores were computed by using the proximity scores (percentage of time the pair spent within one body length) and the within-pair aggression frequency (the sum of aggressive acts between the male and female); all of these measures were recorded during the brood care assay. We conducted a linear regression analysis of the proximity scores and within-pair aggression frequency (LM:  $R^2 = 0.06$ ,  $F_{1,58} = 5.24$ ,  $P = 0.02$ ) and then use the residuals as a pair-bonding score. A positive residual score referred to a highly social and unaggressive individual (high proximity score and low within-pair aggression), while a negative score indicated a highly aggressive and asocial individual (low proximity score and high within-pair aggression). We linked these pair-bonding scores to whole brain bioactive neuropeptide levels. Additionally, we perform spearman correlations between pair-bonding score and brood defense behavior for both males and females.

## 2.6. Ethical note

The procedures in this study were approved by the University of Veterinary Medicine Vienna institutional ethics and animal welfare committee and conform to the Austrian national authority's procedures and follow paragraph 26 of the Animal Experiments Act, Tierversuchsgesetz 2012-TVG 2012 (permits Austria: GZ 68.205/0145-WF/V/3b/2016; 68.205/0064-WF/V/3b/2017; 68.205/0093-WF/V/

3b/2018).

## 3. Results

### 3.1. Brood care, brood defense and nest maintenance

The frequency of brood defense increased over the course of a reproductive phase from spawning (first 24 h after egg laying) to the post-spawning stage (10-days after egg laying) ( $\eta^2 = 0.22$ ,  $F_{1,37} = 12.82$ ,  $P < 0.001$ , Fig. 1B). In contrast, brood care (the sum of nest visits and egg care in the nest) decreased in frequency from spawning to post-spawning stage, but this decrease did not quite reach statistical significance ( $\eta^2 = 0.02$ ,  $F_{1,37} = 3.27$ ,  $P = 0.07$ , Fig. 1C). We observed an increase in nest maintenance across the reproductive cycle ( $\eta^2 = 0.12$ ,  $F_{2,56} = 4.58$ ,  $P = 0.01$ ; Tukey Contrasts: Pre-spawning vs Post-spawning,  $t = 3.01$ ,  $P = 0.01$ , Cohen's  $D = 0.78$ , but the other stages of the cycle did not differ from each other: Pre-spawning vs Spawning,  $t = 1.31$ ,  $P = 0.36$ , Cohen's  $D = 0.39$ ; Spawning vs Post-spawning,  $t = 1.70$ ,  $P = 0.21$ , Cohen's  $D = 0.41$ ). These changes in behavior were observed in both males and females, but females were overall more active than males in both brood care and nest maintenance (**brood care**:  $\eta^2 = 0.76$ ,  $F_{1,37} = 130.2$ ,  $P < 0.001$ ; **nest maintenance**:  $\eta^2 = 0.14$ ,  $F_{1,56} = 10.98$ ,  $P = 0.001$ ), while males performed more brood defense than females (**brood defense**:  $\eta^2 = 0.10$ ,  $F_{1,37} = 6.11$ ,  $P = 0.01$ ).

### 3.2. Neuro-endocrinological profiles of AVT and IT

Whole brain AVT levels were lowest during the spawning phase (first 24 h after egg laying) and increased significantly by the post-spawning defense stage when parents are actively defending young (10-days after egg laying) (Reproductive stage:  $\eta^2 = 0.12$ ,  $F_{3,65} = 3.60$ ,  $P = 0.01$ ; Spawning vs Post-spawning,  $t = -2.84$ ,  $P = 0.02$ , Cohen's  $D = 0.75$ ; for no other stage of the reproductive cycle were AVT levels significantly different, Fig. 2). Females had higher levels of whole brain AVT than did males (Sex:  $\eta^2 = 0.13$ ,  $F_{1,65} = 12.20$ ,  $P < 0.001$ , Fig. 2). However, the whole brain AVT levels were not linked with investment in gonadal tissues (gonadosomatic index, **Females**: LM  $R^2 = -0.007$ ,  $F_{1,33} = 0.75$ ,  $P = 0.39$ ; **Males**: LM  $R^2 = -0.02$ ,  $F_{1,36} = 0.17$ ,  $P = 0.68$ ). Levels of IT in the whole brain did not vary across the

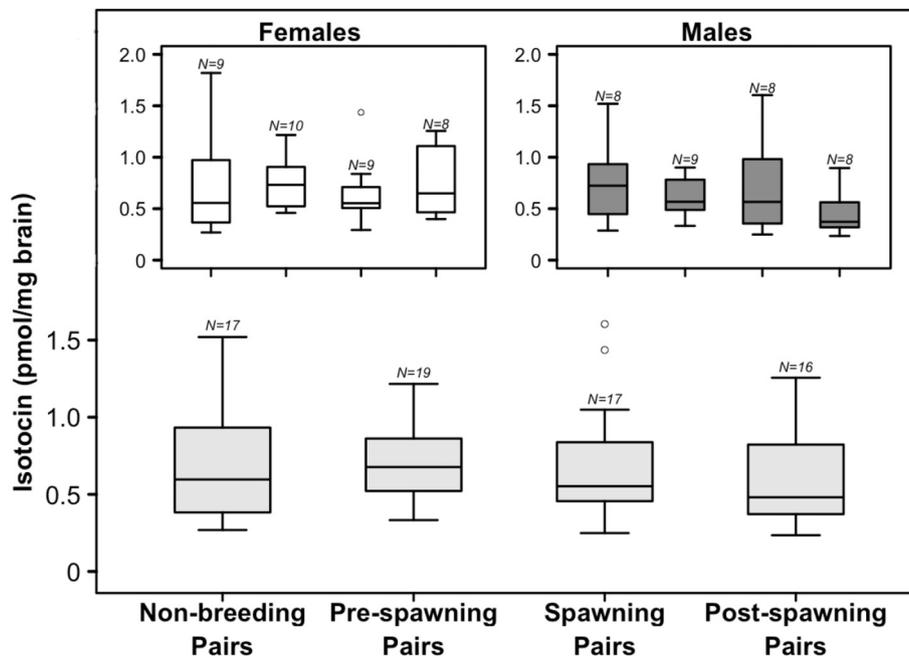


Fig. 3. A graphical representation of *Neolamprologus caudopunctatus* IT whole brain levels for both females and males per reproductive phase. Box plot details are as in Figs. 1 and 2, white boxes represent females and dark grey boxes represent males, whereas light grey represent females and males pooled together.

reproductive cycle, nor did males and females differ in their whole brain IT levels (Reproductive stage:  $\eta^2 = 0.02$ ,  $F_{3,61} = 0.61$ ,  $P = 0.60$ ; Sex:  $\eta^2 = 0.02$ ,  $F_{1,61} = 1.89$ ,  $P = 0.17$ , Fig. 3). Similar to our results on whole brain AVT levels, we found no link between GSI and whole brain IT levels (**Females**: LM  $R^2 = -0.02$ ,  $F_{1,34} = 0.18$ ,  $P = 0.66$ ; **Males**: LM  $R^2 = 0.04$ ,  $F_{1,31} = 2.64$ ,  $P = 0.11$ ).

### 3.3. Brood care, brood defense, nest maintenance and the degree of pair-bonding

Levels of whole brain AVT and IT did not correlate with the frequency of brood care (Table 2) nor with brood defense (Table 2). However, individuals that invested considerable time and energy in constructing and maintaining their nest (high nest maintenance scores) had higher whole brain AVT levels (Fig. 4A), but no such pattern was observed with whole brain IT levels (Table 2). Additionally, pair-bonding scores were marginally positively correlated with whole brain AVT levels (Fig. 4B, Table 2). Individuals that were more aggressive towards their breeding partner and spent little time close to their breeding partner tended to have low whole brain AVT levels, whereas individuals who remained close to their breeding partner and were not aggressive towards their mate tended to have higher whole brain AVT brain levels. Whole brain IT levels showed no relationship with pair-bonding score (Table 2).

Brood defense was not correlated with within-pair aggression in

Table 2

Sample sizes, Spearman rank correlation coefficients and P-values of behavioral responses and whole brain nonapeptide concentration. Significant P-values are highlighted in bold font whereas P-values below 0.10 are shown as italic numbers.

Measure	AVT			IT		
	n	rho	P	n	rho	P
Brood care	36	0.16	0.68	33	0.15	0.41
Brood defense	35	0.02	0.92	32	0.001	0.99
Nest maintenance	55	0.25	<b>0.04</b>	52	-0.04	0.78
Pair-bonding score	55	0.28	<i>0.06</i>	52	-0.14	0.33

males (Spearman correlation:  $\rho = -0.07$ ,  $P = 0.70$ ), but a negatively correlation was observed in females (Spearman correlation:  $\rho = -0.36$ ,  $P = 0.04$ ). In other words, females that performed high levels of brood defense performed few aggressive acts towards their partners compared to females that had a low frequency of brood defense. These results suggest that individuals that were aggressive in a parental context were not necessarily also aggressive in a mating or pairing context.

### 4. Discussion

In *N. caudopunctatus*, on average, whole brain arginine vasotocin (AVT) levels were higher in females than in males and levels were lowest during spawning, increasing significantly by the post-spawning stage, a period when both parents must vigorously defend their free swimming young. In contrast, whole brain isotocin (IT) levels did not vary between the sexes or across the reproductive cycle. Brood care (the sum of nest visits and egg care), nest maintenance and brood defense were highly sex-specific with a continuous increase in both sexes from pre- to post-spawning; males defended more than females, while females tended the eggs and maintained the nest more than males. Also, neither whole brain AVT nor IT levels seemed to directly link with brood care and brood defense frequencies. However, more aggressive and asocial fish that mostly neglected their nest duties had low AVT levels, while caring and highly affiliative fish with a high frequency of nest maintenance had high AVT whole brain levels. The link between neuropeptides and behavior is a bidirectional one, AVT levels may have influenced behavior but it is also equally possible that performance of these caring and defense behaviors increased the neuropeptides levels (Becker et al., 2002; Choleris et al., 2013).

We found no clear link between whole brain AVT levels and the transition from egg cannibal to egg caring as there was no obvious change in AVT levels between pre-spawning (egg cannibal stage) and spawning phases (when egg consumption ceases) of the reproductive cycle. Whole brain IT levels also did not vary in relation to this switch. Our inability to find a neuroendocrinological link for the transition from egg cannibal to egg caring might be due to our methodological approach (Oldfield et al., 2015) as whole brain measures can easily mask small but important local changes of nonapeptide release. It is

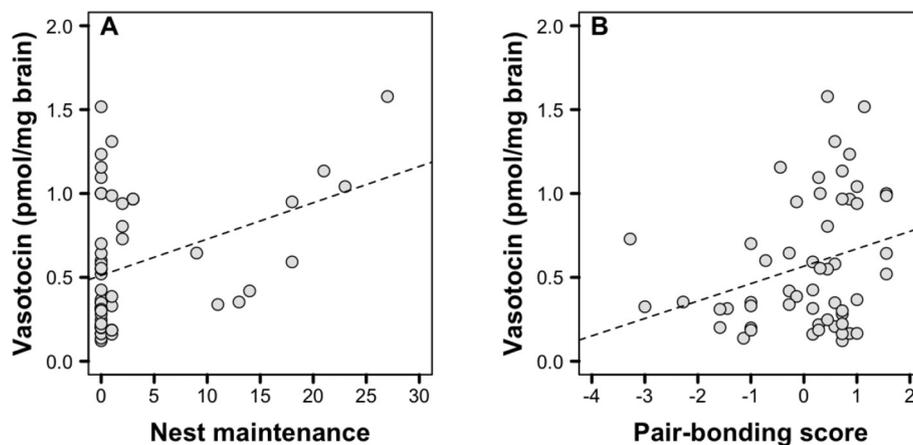


Fig. 4. *Neolamprologus caudopunctatus* AVT whole brain levels in relation to A) nest maintenance frequency and B) pair-bonding score. The light grey points represent females and males pooled together whereas linear regression lines are depicted as dashed lines.

possible that what is modulating this important behavioral switch point are specific neural circuits in particular brain regions, such as the hypothalamus or preoptic area (POA). Moreover, these effects in particular regions are mediated by specific receptor-binding (Balment et al., 2006). Therefore, future studies are now needed to disentangle specific effects of nonapeptide expression in socially relevant brain regions, such as, POA and hypothalamus. Uncovering the mechanism underlying cannibalism would significantly enhance our understanding of this complex behavior.

The high levels of whole brain AVT observed at the post-spawning stage might be a result of the increased motivation for offspring-care, given that this phase corresponds to the highest intensity of defense and need of care (Ochi and Yanagisawa, 1999). However, we did not find a direct link between whole brain AVT levels and brood care and defense. Instead we found that a high frequency of nest construction and maintenance appeared to be linked with high whole brain AVT levels. A strong and well-built nest is of extreme importance to *N. caudopunctatus* as pairs invest more in nest building when predators are present (Demus, 2010). In this species, females and males maintain the nest together, and spend a great amount of energy and time constructing a “sand barrier” to the nest opening that protects the offspring from potential egg and larvae predators (Ochi and Yanagisawa, 1998).

In *N. caudopunctatus*, within-pair aggression and brood defense were not positively correlated, indicating that these two types of antagonistic behaviors have different regulatory mechanisms. AVT is known to modulate aggressive behavior across taxa (Oldfield et al., 2015). In teleosts, aggressive males have larger gigantocellular AVT-ir cell bodies (Greenwood et al., 2008; Santangelo and Bass, 2010; Dewan and Tricas, 2011) and higher whole brain AVT levels (Greenwood et al., 2008; Filby et al., 2010) than non-aggressive males, which can facilitate territoriality (Oldfield et al., 2015). However, we could not find a direct link between brood defense and whole brain AVT levels, our results are similar to Almeida et al., 2012. In *N. caudopunctatus*, brood defense is a mutual resource (Tecot et al., 2016; Hinsch and Komdeur, 2017), since both breeding partners defend the breeding shelter. If biparental care is paramount for the successful completion of the breeding event, (i.e. offspring survival is low without both parents providing care), then conflict over care between male and female is likely to be zero or very low (Houston et al., 2005; Lessells, 2012). Thus, a high pair-bonding score between the two partners, indicates reduced intra-pair conflict, more mutual resource defense and would translate into fitness advantages for both partners (Nowicki et al., 2018; Sowersby et al., 2018).

Monogamous behavior is thought to be enabled by the neurobiological capacity to form and maintain selective social attachments, or pair-bonds, with a single mating partner (Young et al., 2019). In grey seals (*Halichoerus grypus*) and meerkats (*Suricata suricatta*) exogenous

increases in oxytocin lead to increases in proximity-seeking behavior (Madden and Clutton-Brock, 2011; Robinson et al., 2017). Also, mesotocin, an avian homolog of oxytocin, mediates pair-bonding behavior in monogamous zebra finches (*Taeniopygia guttata*) (Klatt and Goodson, 2013). In *N. caudopunctatus* females, but not in males, high levels of brood defense resulted in low levels of within-pair aggression, which feeds into the maintenance of a high pair-bonding score with a breeding partner. In a previous study on *N. caudopunctatus* within-pair aggression (a measure of pair-stability), did not predict filial cannibalism, thus the termination of care is unlikely caused by conflict between the sexes in this species. Maintenance of the pair-bond saves both individuals time and energy that would otherwise be needed regularly when forming a new pair-bond. In our study AVT did not appear to have modulatory function on brood defense behavior but was linked with pair-bonding. These results suggest that physical distance and interactions between breeding partners is regulated by AVT. We hypothesized that the increase in AVT brain levels from spawning to post-spawning might act as a trigger for the necessary increase of both nest maintenance (as detailed above) and pair-bonding score, which is essential for the successful bi-parental defense of the offspring over many weeks of care.

Contrary to our predictions, which were based on previous studies (O’Connell et al., 2012; Feldman and Bakermans-Kranenburg, 2017), whole brain IT levels did not appear to regulate or modulate the expression of parental care or the transition from egg cannibal to caring parent in *N. caudopunctatus*, our socially monogamous study species. Similar to our results, it has been shown in a biparental poison frog (*R. imitator*) that an exogenous increase of mesotocin, the amphibian and bird homolog of oxytocin, does not increase maternal and paternal behavior towards the eggs, but instead decreased clutch success (Schulte and Summers, 2017). Additionally, in male sticklebacks (*Gasterosteus aculeatus*) high levels of IT correspond to the courtship phase (Kleszczyńska et al., 2012), while egg laying females had lower IT brain levels compared to pre-spawning females (Kulczykowska and Kleszczyńska, 2014). Our study used whole brains and consequently the lack of neuroanatomical precision means we cannot conclusively exclude the possibility that in our study species parental care might still be modulated by IT expression in specific brain regions or via the activation of particular neural circuits that exert an effect at the gonadal level (Maruska and Fernald, 2011). Alternatively, as some researchers have argued, the effects of OT/IT may serve to more generally increase in the responsiveness to social environmental stimuli (i.e. an individual’s social sensitivity, known as the social salience hypothesis, Shamay-Tsoory and Abu-Akel, 2016). In teleosts, and in mammals, it appears that IT/OT, can have both positive and negative effects on social behavior. Exogenous IT reduced social-approach behavior in goldfish (*Carassius auratus*) (Thompson and Walton, 2004) and

increased submission while decreasing affiliative behavior in *Neolamprologus pulcher* (Reddon et al., 2012, 2015). Hence a fine resolution of brain anatomy is paramount to identify discrete IT circuits and characterizing their particular contributions to different aspects of parental care behavior.

#### 4.1. Conclusion

The post-spawning stage of care, when parents must defend free swimming young, appears to be the most critical phase of *N. caudopunctatus*' breeding cycle, with AVT, but not IT, acting as a neuromodulator for the successful completion of a breeding event. While the amount and quality of brood defense did not correlate with brain levels of either AVT or IT, high levels of AVT appeared to promote both a high investment in nest construction and strong pair-bonds which in turn might promote a strong brood defense. Effective and vigorous defense of young is essential for the successful completion of breeding, whereas weak brood-defense usually results with young being consumed by a hetero- or conspecific predators. *N. caudopunctatus* is particularly vulnerable to conspecific nest-intruders due to their spatial clustering (they live in dense aggregations) and the near constant competition for limited breeding shelters (Schaedelin et al., 2015). Breeding nest-owners can tackle this constant threat by allocating all their resources to brood- and nest-defense and by building up a sand barrier around the nest entrance which prevents other individuals from entering the nest (Cunha-Saraiva et al., 2018). The results of our study highlight the species and sex specificity of AVT and IT nonapeptides and emphasize the necessity to increase and extend our knowledge of the underlying mechanisms of parental care to amphibians, reptiles and fishes.

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#### Declaration of competing interest

We have no competing interests.

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