



Brain levels of nonapeptides in four labrid fish species with different levels of mutualistic behavior



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ABSTRACT

There is strong evidence that brain nonapeptides are implicated as modulators of a wide array of social and reproductive behaviors in fishes. However, the question remains, as to whether there is a link between the distribution of active nonapeptides across brain regions and fishes specific behavioral phenotypes. To explore this link we compared the nonapeptides' profile across the brains of fishes representing different degrees of mutualistic behavior (here: cleaning behavior). Herein we studied the quantitative distribution of both nonapeptides, arginine vasotocin (AVT) and isotocin (IT), in the brains of four species of fish belonging to the family *Labridae*: two are obligatory cleaners throughout their entire life (*Labroides dimidiatus* and *Labroides bicolor*), one species is a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous), and one is a non-cleaner species, corallivorous throughout its entire life (*Labrichthys unilineatus*). The biologically available AVT and IT concentrations were measured simultaneously in distinct brain macro-areas: forebrain, optic tectum, cerebellum and brain stem, using liquid chromatography–tandem mass spectrometry (LC–MS/MS). We showed that the levels of both AVT and IT varied significantly across species, as measured in the whole brain or in the specific macro-areas. Significantly higher AVT concentrations in the cerebellum which were found in the obligate cleaners seemed to be related to expression of mutualistic behavior. On the other hand, the higher levels of brain IT in the non-cleaner *L. unilineatus* suggested that these might be linked to the development of sexual dimorphism, which occurs only in this non-cleaner species.

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1. Introduction

One of the most important questions to ask when studying social behavior concerns physiological processes that underlie the species-specific behavioral variation. It is not surprising that the integrative approach to behavior and physiology has received considerable attention in fishes (Johnsson et al., 2006). Teleost fish are able to exhibit unparalleled diversity when it comes to social organization and behavioral output thus providing an excellent opportunity to search for patterns of variation in physiological proxies associated with social divergence. For instance, some species live a solitary life while others tend to aggregate in schools and develop the social skills that serve them well across their lifespan

(Bshary, 2001). Additionally, some fishes exhibit cognitive abilities considered to be complex in social settings, which make them relevant models to study social behavior and cognition (Bshary et al., 2014). There are several categories of physiological factors including neurotransmitters and neuromodulators in the brain that can be related to the behavioral plasticity of teleost fish (for review: Oliveira, 2006). Among those signaling molecules are the nonapeptides, arginine vasotocin (AVT) and isotocin (IT), evolutionary predecessors of well-known mammalian arginine vasopressin (AVP) and oxytocin (OT). They belong to ancient family of neurohormones (nonapeptides) with a well-conserved structure and core functions across vertebrate taxa (Acher and Chauvet, 1995; Goodson and Bass, 2001). There is strong evidence that brain nonapeptides are implicated in a wide array of social and reproductive behaviors in fishes (Godwin and Thompson, 2012), however, the question remains, whether there is a link between

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the distribution of active nonapeptides across brain regions and the specific behavioral phenotypes. To explore this link we compare the nonapeptides' profile across the brains of fishes representing different degrees of mutualistic behavior (here: cleaning behavior). Herein we study the quantitative distribution of both nonapeptides, AVT and IT, in the brains of four species of cleaner fish belonging to the family *Labridae*: two are obligatory cleaners throughout their entire life (*Labroides dimidiatus* and *Labroides bicolor*), one species is a facultative cleaner (*Labropsis australis*); juveniles are cleaners and adults are corallivorous), and one is a non-cleaner species, corallivorous throughout its entire life -*Labrichthys unilineatus* (Fulton and Bellwood, 2002; Cole et al., 2009; Cole, 2010).

Cleaner fishes are specialized organisms that inspect the body surface, gill chambers and mouth of cooperating larger fishes (known as clients) in search of ectoparasites, mucus and dead or diseased tissues (Limbaugh, 1961; Feder, 1966; Gorlick et al., 1987; Losey et al., 1999; Côté, 2000). Interestingly, the best known species of cleaner fish, the Indo-Pacific bluestreak cleaner wrasse *L. dimidiatus*, often feed on healthy tissues, mucus or scales instead of on clients' ectoparasites, dead or diseased tissues what causes conflict between cleaner and client referred to as cheating (Grutter and Bshary, 2003). In order to minimize conflict, these cleaners need to eat against preference, i.e. ectoparasites, dead or diseased tissues instead of mucus which they prefer (Grutter and Bshary, 2003). Moreover, individuals use a diverse behavioral repertoire that includes individual recognition of their clients, manipulation of client decisions, reconciliation, punishment, advertising of their cleaning services and tactical deception to survive (reviewed by Bshary and Côté, 2008; Bshary and Noë, 2003; Bshary, 2001). Other species of labrid cleaner fishes are less commonly used in behavioral studies. For example, Oates and colleagues revealed that the individuals *L. bicolor* have larger home ranges than *L. dimidiatus* but show uneven frequency of use of different areas within their home ranges (Oates et al., 2010). This reduces the chance of repeated interactions between the same partners and thus may influence the outcome of these fish cooperative decisions; as a result *L. bicolor* cheats more often than does *L. dimidiatus* (Oates et al., 2010, 2012). The facultative cleaner, *L. australis* engages in cleaning solely in the juvenile stage but as an adult shares similar eating habits with *L. unilineatus*, feeds on the mucus produced by the coral polyps (Cole et al., 2009; Cole, 2010). All four species are protogynous hermaphrodites, e.g. individuals are born female and at some point in their lifespan may change sex to male to become harem owners (Robertson, 1972), however, only *L. unilineatus* exhibits two sex-related color forms. All species inhabit similar environment, in coral reefs (Cole, 2010).

Recent studies have identified several neurohormones that may influence different aspects of cleaner fish behaviour (Soares et al., 2011, 2012, 2014; Cardoso et al., 2015a,b,c; Paula et al., 2015). The role of AVT has been found to play a key role in the modulation of cleaners' behavioural activities, namely that exogenous administration of AVT decreases cleaners' propensity to engage in cleaning interactions with their clients (Soares et al., 2012). More recently, new studies have demonstrated that exogenous administration of AVT decreases the cleaners' willingness to feed against preference (Cardoso et al., 2015b), that AVT affects the learning competence of cleaners (Cardoso et al., 2015c) and that endogenous variations of AVT and IT are linked the regulation of pair association and behaviour of cleaners (Cardoso et al 2015a). Furthermore, comparative neuroanatomical studies have revealed that obligate cleaners *L. dimidiatus* have smaller and less numerous AVT-ir neurons in the gigantocellular preoptic area (gPOA) compared to non-cleaners *L. unilineatus* (Mendonça et al., 2013). However, there is currently no information either on the availability of active AVT and IT at putative target regions in the brain of labrid fishes or on

the link between the distribution of active nonapeptides across brain regions and the specific behavioral phenotypes. Therefore, this study has been undertaken to characterize the quantitative distribution of AVT and IT in different brain macro-areas and to compare the quantitative levels of AVT and IT in the different brain macro-areas in labrid fishes that represent different levels of mutualistic behavior.

The AVT and IT concentrations were measured in the distinct brain macro-areas: forebrain, optic tectum, cerebellum and brain stem, using liquid chromatography–tandem mass spectrometry (LC–MS/MS). We determined the concentration of free nonapeptides after their dissociation from non-covalent complexes, which is the sole nonapeptide fraction allowing that specific receptors to act as neurotransmitters and/or neuromodulators in the brain. This analytical approach, which permits the measurement of bio-active nonapeptides AVT and IT at their site of action, has been used successfully in several other fish species (Gozdowska et al., 2006; Kleszczyńska et al., 2012; Almeida et al., 2012; Kleszczyńska and Kulczykowska, 2013; Sokołowska et al., 2013; Martos-Sitcha et al., 2013; Kulczykowska and Kleszczyńska, 2014; Kulczykowska et al., 2015; Reddon et al., 2015). Given the above information, differences are expected to appear selectively in AVT and IT quantitative levels for species and brain macro-areas implicated in the regulation of mutualistic behavior.

2. Methods

2.1. Fishes

The fishes used in this study were imported to Portugal by a local distributor (Tropical Marine Centre, Lisbon, Portugal). A total of 16 *L. dimidiatus* (standard length (SL), mean \pm SEM = 5.703 \pm 0.265; body weight (BW), mean \pm SEM = 2.767 \pm 0.391), 11 *L. bicolor* (SL = 6.470 \pm 0.269; BW = 4.216 \pm 0.716), 15 *L. australis* (SL = 6.564 \pm 0.284; BW = 4.517 \pm 0.636) and 12 *L. unilineatus* (SL = 8.725 \pm 0.422; BW = 6.465 \pm 1.795) were used in this study. All animals were adult females in order to avoid any potential influence of fish sex on the results. The sex of all individuals was confirmed by direct inspection of the gonads and when needed also with the help of an acetocarmine stain (Guerrero and Shelton, 1974). All animals were collected outside the breeding season.

2.2. Brain sampling

All fishes were sacrificed with an overdose of tricaine solution (MS222, Pharmaq; 500–1000 mg/L) and the spinal cord sectioned. The brain was dissected under a stereoscope (Zeiss; Stemi 2000) into four macro-areas: forebrain (olfactory bulbs + telencephalon + diencephalon), optic tectum, cerebellum and brain stem. Brain areas were then weighted in an analytical balance OHAUS.

2.3. Brain AVT and IT assay: sample preparation

Brain samples were sonicated in 1 mL Milli-Q water (Microson™ XL, Misonix, USA), acidified with glacial acetic acid (3 μ L) and placed in a boiling water bath for 3.5 min. Then, homogenates were centrifuged (12,000g, 20 min, 4 °C) and supernatants decanted and loaded onto previously conditioned (3 mL methanol, 3 mL Milli-Q water) solid phase extraction (SPE) columns (100 mg/1 mL, C18 Bakerbond, J.T. Baker). To purify samples, columns were washed successively with 1 mL of 5% acetic acid, 1 mL Milli-Q water and 1 mL of 5% methanol. The peptides were eluted using 2 mL of ethanol: 6 M HCl (2000:1, v/v). The eluate was evaporated to dryness using a Turbo Vap LV Evaporator

(Caliper Life Sciences, USA). Samples were then frozen and stored at -70°C prior to LC–MS/MS analysis.

2.4. LC–MS/MS analysis

AVT and IT analysis was performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using Infinity 1290 System (Agilent Technologies, USA) coupled with triple quadrupole mass spectrometer Agilent 6460A equipped with a JetStream ESI source. Chromatographic separation was done using Agilent Poroshell 120 SB–C18 column ($3\text{ mm} \times 100\text{ mm}$, $1.8\ \mu\text{m}$ particle) preceded by online automatic SPE extraction with precolumn switching program to remove matrix. SPE was achieved on Eclipse XDB–C18 ($4.6\text{ mm} \times 15\text{ mm}$, $3.5\ \mu\text{m}$ particle) precolumn. The mobile phase consisted of solvent A: 0.1% acetic acid in H_2O (v/v) and solvent B: 0.1% acetic acid in acetonitrile: water (3:1) (v/v). A gradient elution was used starting with 5% B for 3 min, followed by linear gradient from 5% to 32.5% B in 11 min. Next, the column was washed at 95% for 5 min and then was equilibrated for 5 min at starting conditions after each analysis. The injection volume was $10\ \mu\text{L}$. Flow rate of the mobile phase was set at 0.6 mL min^{-1} and the column temperature at 20°C . To minimize contamination of the mass spectrometer the column flow was directed only from 7.0 to 15.0 min into the mass spectrometer using switching valve.

JetStream ESI source was operated in positive ionization mode using the following settings: capillary voltage 3500 V, nozzle voltage 500 V, drying gas temperature 300°C and flow 5 L min^{-1} , nebulizer gas pressure 45 psi, sheath gas temperature 250°C and flow rate 11 L min^{-1} . The analyses were monitored in the multiple reaction monitoring mode (MRM). The monitored mass transitions for AVT were set at $m/z\ 525.5 \rightarrow 517.2$ (dwell time 200 ms, fragmentor voltage 143 V, collision energy 73 V) and for IT: $m/z\ 483.7 \rightarrow 136.1$ (dwell time 200 ms, fragmentor voltage 74 V, collision energy 53 V).

Limit of detection (LOD, signal-to-noise ratio 3:1) and limit of quantification (LOQ, signal-to-noise ratio 10:1) were evaluated on a basis of six repetitions of analysis of blank sample and standard with concentration close to LLQ. LOD was $0.4\text{ fmol }\mu\text{L}^{-1}$ for AVT and $0.8\text{ fmol }\mu\text{L}^{-1}$ for IT. LOQ was $1.3\text{ fmol }\mu\text{L}^{-1}$ for AVT and $2.5\text{ fmol }\mu\text{L}^{-1}$ for IT. This is a slightly modified variant of the assay described by Gozdowska et al. (2013).

2.5. Statistical analyses

A total of 57 female labrids were used for nonapeptides' measurement. In case of IT, 16 *L. dimidiatus*, 11 *L. bicolor*, 15 *L. australis*

and 12 *L. unilineatus* were successfully analyzed. For AVT, some nonapeptides' levels were below the limit of detection and therefore the final sample sizes were as follows: 10 *L. dimidiatus*, 11 *L. bicolor*, 13 *L. australis* and 9 *L. unilineatus*. Variables were log transformed to conform to parametric parameters of homogeneity of variances (assessed by Levene's test). Brain levels of AVT and IT for the whole brain and each brain macro-areas (forebrain, optic tectum, cerebellum and brain stem) were compared by using one way independent measures ANOVAs, followed by Tukey pos-hoc HSD tests. All tests were 2 tailed and performed in the software package SPSS Statistics, version 22.

2.6. Ethics statement

The animal experimentation procedures used in this study followed the Association for the Study of Animal Behaviour and the Animal Behaviour Society guidelines for the treatment of animals in behavioral research and teaching.

3. Results

3.1. Brain AVT levels

Mean concentrations of AVT in the whole brain and brain macro-areas are given in Fig. 1a, Table 1 and in Fig. 2. Whole brain AVT levels differ significantly across species (one way ANOVA, $F_{3,38} = 29.964$, $p < 0.001$). The values in *L. bicolor* (LB) are significantly higher than those found in three other species (Tukey HSD tests, all $p < 0.001$, Fig. 1a). Both obligate cleaners have higher levels of brain AVT compared to those found in *L. australis* (LA) and *L. unilineatus* (LU) (Tukey HSD tests, all $p < 0.03$; Fig. 1a); in the last ones, AVT levels are similar to each other (Tukey HSD tests, LA vs LU: $p = 0.998$; Fig. 1a). Brain AVT levels also differ significantly at each brain region (one way ANOVAs, forebrain: $F_{3,38} = 8.792$, $p < 0.001$; optic tectum: $F_{3,38} = 14.577$, $p < 0.001$; cerebellum: $F_{3,38} = 11.396$, $p < 0.001$ and brain stem: $F_{3,38} = 9.977$, $p < 0.001$, see Table 1, Fig. 2). Highest brain AVT levels are found in most of brain macro-areas in *L. bicolor* (see Table 2, Fig. 2) except the cerebellum, where higher levels are solely presented in both obligate cleaners (Table 2, Fig. 2).

3.2. Brain IT levels

Mean concentrations of IT in the whole brain and brain macro-areas are given in Fig. 1b, Table 1 and in Fig. 3. Whole brain IT levels differ significantly across species (one-way ANOVA,

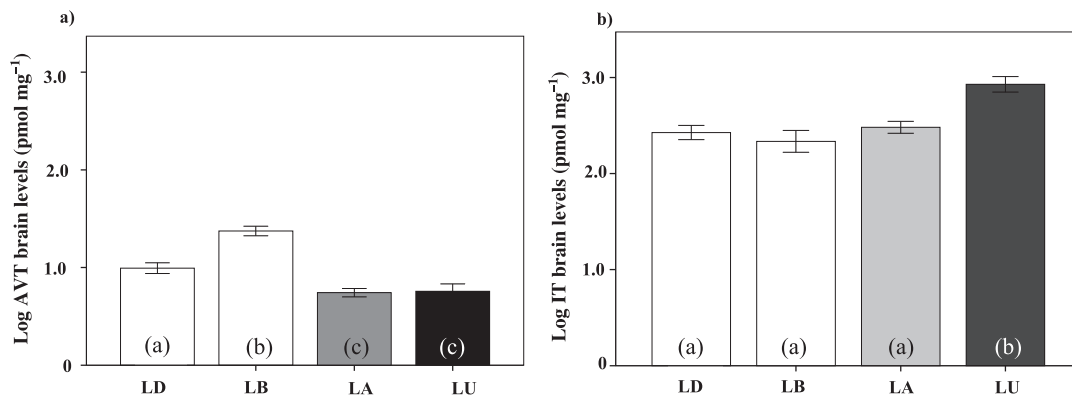


Fig. 1. (a) Whole brain levels of arginine vasotocin (AVT) (mean \pm SEM) and (b) whole brain levels of isotocin (IT) (mean \pm SEM) in four Labrid fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU – *Labrichthys unilineatus* expressed as $\log(\text{AVT (pmol mg}^{-1}\text{)})$ and $\log(\text{IT (pmol mg}^{-1}\text{)})$. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

Table 1
Arginine vasotocin (AVT) and isotocin (IT) levels (mean \pm SEM) in different brain macro-areas of four *Labrid* fish species (examined with One Way ANOVAs) expressed as $\log(\text{AVT (pmol mg}^{-1}\text{)})$ and $\log(\text{IT (pmol mg}^{-1}\text{)})$.

Brain region	Neuropeptides	Species				Statistics
		<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichthys unilineatus</i>	
Forebrain	AVT	0.49 \pm 0.06	0.82 \pm 0.07	0.47 \pm 0.05	0.45 \pm 0.04	$F_{(3,41)} = 8.79; p < 0.001$
	IT	1.98 \pm 0.10	1.80 \pm 0.14	1.99 \pm 0.07	2.38 \pm 0.10	$F_{(3,56)} = 5.15; p = 0.003$
Optic tectum	AVT	0.16 \pm 0.04	0.61 \pm 0.08	0.18 \pm 0.03	0.21 \pm 0.06	$F_{(3,41)} = 14.55; p < 0.001$
	IT	1.60 \pm 0.08	1.44 \pm 0.14	1.60 \pm 0.09	2.03 \pm 0.14	$F_{(3,56)} = 4.68; p = 0.006$
Cerebellum	AVT	0.59 \pm 0.07	0.74 \pm 0.10	0.25 \pm 0.04	0.30 \pm 0.07	$F_{(3,41)} = 11.40; p < 0.001$
	IT	1.28 \pm 0.10	1.69 \pm 0.10	1.30 \pm 0.11	1.96 \pm 0.07	$F_{(3,56)} = 10.41; p < 0.001$
Brain stem	AVT	0.27 \pm 0.06	0.81 \pm 0.13	0.32 \pm 0.03	0.26 \pm 0.07	$F_{(3,41)} = 9.98; p < 0.001$
	IT	1.80 \pm 0.15	1.67 \pm 0.18	1.75 \pm 0.19	2.55 \pm 0.08	$F_{(3,56)} = 6.08; p = 0.001$

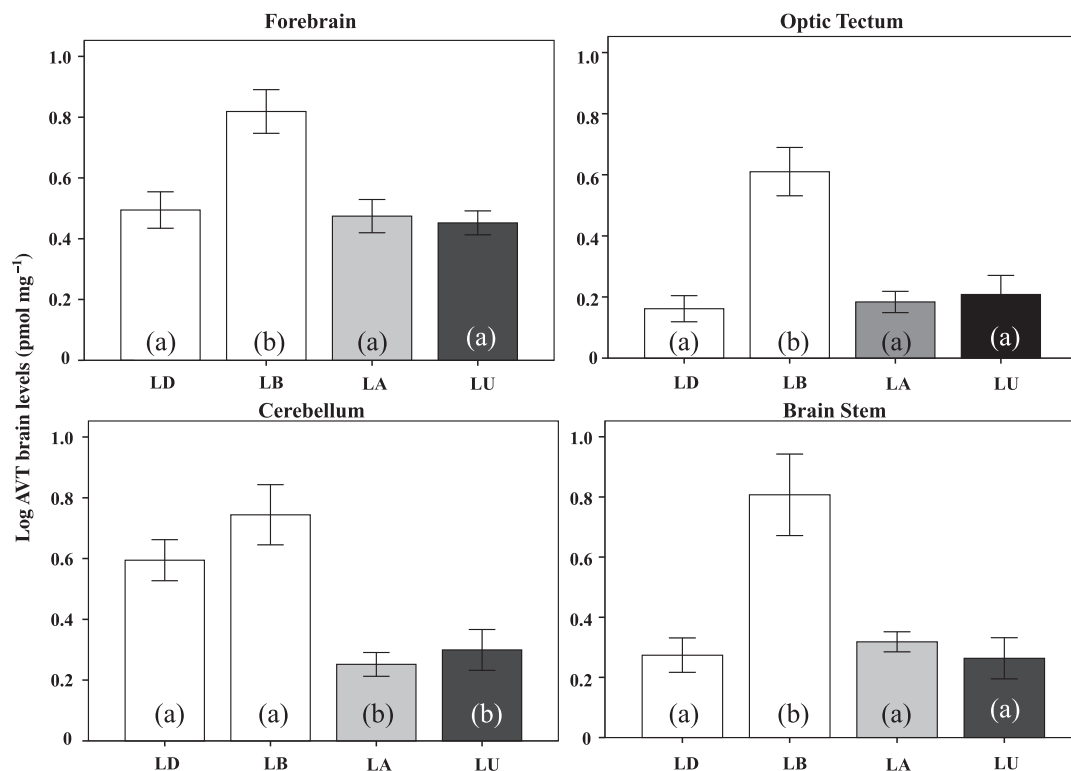


Fig. 2. Levels of arginine vasotocin (AVT) in different brain macro-areas (mean \pm SEM): Forebrain, Optic Tectum, Cerebellum and Brain Stem in four *Labrid* fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU – *Labrichthys unilineatus* expressed as $\log(\text{AVT (pmol mg}^{-1}\text{)})$. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

$F_{3,53} = 9.714, p < 0.001$, Fig. 1b). The values in *L. unilineatus* are significantly higher than those found in three other species (Tukey HSD tests, all $p < 0.001$, Fig. 1b). Similarly, brain IT levels also differ significantly at each brain region (one way ANOVAs, forebrain: $F_{3,53} = 5.152, p = 0.003$; optic tectum: $F_{3,53} = 4.675, p = 0.006$; cerebellum: $F_{3,53} = 10.405, p < 0.001$ and brain stem: $F_{3,53} = 6.068, p < 0.001$, see Table 1). The highest brain IT levels are found in most of brain macro-areas in *L. unilineatus* (see Table 3, Fig. 3) except the cerebellum, where higher levels are solely observed in both *L. unilineatus* and *L. bicolor* (see Table 3, Fig. 3).

4. Discussion

This study provides new data on the constitutive profiles of nonapeptides AVT and IT across the female brains of species that differ in the expression of mutualistic behavior. We showed that the levels of both AVT and IT varied significantly at the level of

the whole brain and in specific brain macro-areas, such as the forebrain, optic tectum, cerebellum and brain stem.

4.1. AVT and mutualistic behavior

Both obligate cleaners *L. dimidiatus* and *L. bicolor* had higher levels of whole brain AVT compared to non-cleaners *L. australis* and *L. unilineatus*. This pattern was repeated in the cerebellum. The neurophysiological mechanisms and neural circuitry of the cerebellum is considered quite conservative across vertebrate species (Rodríguez et al., 2005). Cerebellum is known as a motor control center however in teleost fishes it is also implicated in several cognitive and emotional functions, particularly in associative learning and memory processes (Rodríguez et al., 2005). Individuals of obligatory cleaner fish species have to interact with a large range of client species repeatedly, which they need to recognize and memorize for future interactions (Bshary and Côté, 2008). Because associative learning and memorizing is the core

Table 2

Tukey pos-hoc HSD tests for arginine vasotocin (AVT) levels in different brain macro-areas of four *Labrid* fish species. Significant correlations are marked with * for $p < 0.05$ and ** for $p < 0.01$.

Brain regions	Species				
		<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichthys unilineatus</i>
Forebrain	<i>Labroides dimidiatus</i>	–	$p = 0.002^*$	$p = 0.995$	$p = 0.963$
	<i>Labroides bicolor</i>	$p = 0.002^*$	–	$p = 0.001^*$	$p = 0.001^*$
	<i>Labropsis australis</i>	$p = 0.995$	$p = 0.001^*$	–	$p = 0.994$
	<i>Labrichthys unilineatus</i>	$p = 0.963$	$p = 0.001^*$	$p = 0.994$	–
Optic tectum	<i>Labroides dimidiatus</i>	–	$p < 0.001^{**}$	$p = 0.992$	$p = 0.946$
	<i>Labroides bicolor</i>	$p < 0.001^{**}$	–	$p < 0.001^{**}$	$p < 0.001^{**}$
	<i>Labropsis australis</i>	$p = 0.992$	$p < 0.001^{**}$	–	$p = 0.99$
	<i>Labrichthys unilineatus</i>	$p = 0.946$	$p < 0.001^{**}$	$p = 0.99$	–
Cerebellum	<i>Labroides dimidiatus</i>	–	$p = 0.457$	$p = 0.007^*$	$p = 0.039^*$
	<i>Labroides bicolor</i>	$p = 0.457$	–	$p < 0.001^{**}$	$p = 0.001^*$
	<i>Labropsis australis</i>	$p = 0.007^*$	$p < 0.001^{**}$	–	$p = 0.966$
	<i>Labrichthys unilineatus</i>	$p = 0.039^*$	$p = 0.001^*$	$p = 0.996$	–
Brain stem	<i>Labroides dimidiatus</i>	–	$p < 0.001^{**}$	$p = 0.981$	$p = 1.000$
	<i>Labroides bicolor</i>	$p < 0.001^{**}$	–	$p = 0.001^*$	$p < 0.001^{**}$
	<i>Labropsis australis</i>	$p = 0.981$	$p = 0.001^*$	–	$p = 0.967$
	<i>Labrichthys unilineatus</i>	$p = 1.000$	$p < 0.001^{**}$	$p = 0.967$	–

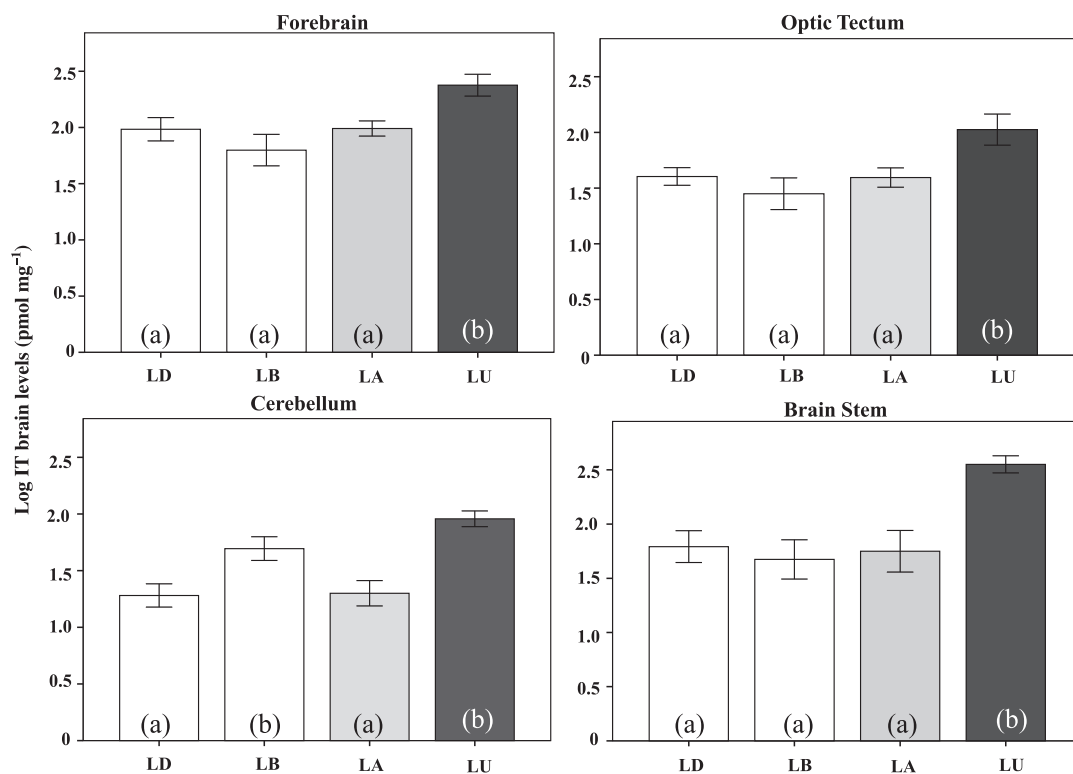


Fig. 3. Levels of isotocin (IT) in different brain macro-areas (mean \pm SEM): Forebrain, Optic Tectum, Cerebellum and Brain Stem in four *Labrid* fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU – *Labrichthys unilineatus* expressed as $\log(\text{IT} (\text{pmol mg}^{-1}))$. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

of the most of the behavioral tactics shown by obligate cleaners, an involvement of the cerebellum can be expected. Thus the higher levels of AVT found solely in the cerebellum of both cleaner species may suggest the participation of AVT in processes of associative learning and memorizing connected with cleaning behavior.

However, there are also noticeable behavioral differences between individuals of obligate cleaners *L. dimidiatus* and *L. bicolor*. For instance, individuals of *L. bicolor* have larger home ranges and display higher tendency to be aggressive with their clients (e.g. they cheat more) than *L. dimidiatus* (Oates et al., 2010, 2012; Mills and Côté, 2010). Thus the higher AVT levels in the whole

brain and in the forebrain, optic tectum and brain stem in *L. bicolor* may be related to behavioral characteristics mentioned above. In fishes, aggressiveness and territoriality are usually linked with AVT in the brain, as well as with the enhanced activity of neurons producing AVT indicated by the size of AVT-producing neurons and AVT mRNA expression. For instance, in the three-spined stickleback (*Gasterosteus aculeatus*), aggressiveness and territoriality corresponded with high concentrations of whole brain AVT (Kleszczyńska et al., 2012). Furthermore studies have found that aggressive behavior in the territorial butterflyfish species was coupled with larger AVT-ir neurons in the gigantocellular POA and also

Table 3
Tukey pos-hoc HSD tests for isotocin (IT) levels in different brain macro-areas of four *Labrid* fish species. Significant correlations are marked with * for $p < 0.05$ and ** for $p < 0.01$.

Brain regions	Species				
		<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichthys unilineatus</i>
Forebrain	<i>Labroides dimidiatus</i>	–	$p = 0.587$	$p = 1.000$	$p = 0.039^*$
	<i>Labroides bicolor</i>	$p = 0.587$	–	$p = 0.557$	$p = 0.002^*$
	<i>Labropsis australis</i>	$p = 1.000$	$p = 0.557$	–	$p = 0.044^*$
	<i>Labrichthys unilineatus</i>	$p = 0.039^*$	$p = 0.022^*$	$p = 0.044^*$	–
Optic tectum	<i>Labroides dimidiatus</i>	–	$p = 0.759$	$p = 1.000$	$p = 0.041^*$
	<i>Labroides bicolor</i>	$p = 0.759$	–	$p = 0.793$	$p = 0.005^*$
	<i>Labropsis australis</i>	$p = 1.000$	$p = 0.793$	–	$p = 0.035^*$
	<i>Labrichthys unilineatus</i>	$p = 0.041^*$	$p = 0.005^*$	$p = 0.035^*$	–
Cerebellum	<i>Labroides dimidiatus</i>	–	$p = 0.032^*$	$p = 0.999$	$p < 0.001^{**}$
	<i>Labroides bicolor</i>	$p = 0.032^*$	–	$p = 0.044^*$	$p = 0.321$
	<i>Labropsis australis</i>	$p = 0.999$	$p = 0.044^*$	–	$p < 0.001^{**}$
	<i>Labrichthys unilineatus</i>	$p < 0.001^{**}$	$p = 0.321$	$p < 0.001^{**}$	–
Brain stem	<i>Labroides dimidiatus</i>	–	$p = 0.956$	$p = 0.997$	$p = 0.007^*$
	<i>Labroides bicolor</i>	$p = 0.956$	–	$p = 0.988$	$p = 0.003^*$
	<i>Labropsis australis</i>	$p = 0.997$	$p = 0.988$	–	$p = 0.004^*$
	<i>Labrichthys unilineatus</i>	$p = 0.007^*$	$p = 0.003^*$	$p = 0.004^*$	–

with a higher AVT-fiber density in the telencephalon (Dewan et al., 2008; Dewan and Tricas, 2011). In African cichlid fish (*Astatotilapia burtoni*), territorial, aggressive males exhibited higher level of AVT mRNA expression in posterior POA than non-territorial and non-aggressive ones (Greenwood et al., 2008). However, all quoted studies were executed in males, whereas our study was performed in females. Unfortunately there are no studies linking AVT to aggressiveness in females to refer. Therefore we should be more circumspect about drawing conclusions regarding the relationship between nonapeptides and aggressiveness of females.

As mentioned previously, individuals of *L. bicolor* patrol larger territories than do *L. dimidiatus* (Mills and Côté, 2010), *L. australis* and *L. unilineatus* (M.C. Soares, personal observations). Thus the highest AVT levels in the brain stem of *L. bicolor* may also be related to the maintenance of high level of motor-related activity. For example, Thompson et al. (2008) found that at the brain area, the modulation of AVT is associated with motor control. The higher levels of AVT found within the optic tectum of *L. bicolor* may be coupled with the remarkable roaming abilities of this species, because vision plays a pivotal role in roaming behavior. Adult females of two non-cleaner species – *L. australis* and *L. unilineatus* (as a reminder: only juvenile *L. australis* are cleaners but these were not included in our study), have significantly lower AVT levels in the cerebellum and the whole brain. Therefore we hypothesize that higher AVT levels just in the cerebellum (which resulted also in higher AVT levels in the whole brain) can be related to the expression of mutualistic behavior. Specific behavioral features of *L. bicolor* as distinct aggressiveness, territoriality and roaming ability can be linked to higher AVT levels in other brain regions as the forebrain, optic tectum and brain stem (which resulted also in higher AVT levels in the whole brain).

4.2. IT and mutualistic behavior

Over the past few years our knowledge about the brain IT and its association with social behavior has significantly increased. For instance, Thompson and Walton (2004) found that IT intracerebroventricular infusions stimulate social approach in goldfish. Also, both AVT and IT promote sociability when given peripherally to zebrafish – *Danio rerio* (Braidia et al., 2011), while in the cooperative breeder *Neolamprologus pulcher*, intraperitoneal injections of IT enhance sensitivity to social stimuli and increase submission rates in social groups (Reddon et al., 2012). In the current study, only the non-cleaners *L. unilineatus* have significantly higher IT brain levels (overall and at each brain macro-area) compared to

all other species. Interestingly, females of facultative cleaners *L. australis* which are non-cleaners as adults have similar levels of IT (measured in their whole brain and at each brain macro-area) as obligate cleaners *L. dimidiatus* and *L. bicolor*. Therefore, at a first look, higher brain IT seem to be related to adamant whole-life non-cleaning behavior expressed by *L. unilineatus*.

There is also one more circumstance that cannot be overlooked. Namely, while all of the species are protogynous hermaphrodites, three are monomorphic (*L. dimidiatus*, *L. bicolor* and *L. australis*) and only the non-cleaner *L. unilineatus* displays phenotypic sexual dimorphism (males and females are different according to size and specific coloration). The evident sexual dimorphism in brain AVT and IT concentrations was presented in the black molly (*Poecilia sphenops*) and the three-spined stickleback which demonstrate clear phenotypic sexual dimorphism (Kulczykowska et al., 2015; Kleszczyńska and Kulczykowska, 2013). Therefore we can speculate that higher levels of IT in the brains of *L. unilineatus* may be linked to the development of sexual dimorphism, which only occurs in this non-cleaner species.

4.3. Concluding remarks

AVT is known to be involved in the expression of mutualistic behavior in cleaner fishes (Soares et al., 2012; Mendonça et al., 2013; Cardoso et al., 2015a,b,c). Here we have provided, for the first time, data on AVT and IT levels in different brain macro-areas in cleaner and non-cleaner labrid fishes. Higher AVT levels in the cerebellum and the whole brain seem to be related to expression of mutualistic behavior. Our data further highlights the potential relevance of AVT to modulation of aggressiveness toward clients, and roaming behavior of the obligate cleaner *L. bicolor*. In contrast, brain IT does not seem to be related to expression of mutualistic behavior. However, higher levels of IT in *L. unilineatus* suggest that these might be linked to the development of sexual dimorphism, which occurs only in this non-cleaner species. Future integrative and comparative work, considering behavior and a larger number of species, is necessary to unravel the neural circuits underlying cleaning behavior and how these are modulated by nonapeptides.

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