



Nonapeptide levels in male cleaner fish' brains during interactions with unfamiliar intra and interspecific partners

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Abstract

In nature, cleaner fish face complex situations arising from a highly demanding socio-environment that includes conspecific and mutualistic relationships. The nonapeptide arginine vasotocin (AVT) is known to play a relevant role in the expression of mutualistic behaviour in the Indo-Pacific bluestreak cleaner wrasse (*Labroides dimidiatus*). However, the role of the second nonapeptide isotocin (IT) in mediating social behaviour has yet to be established. Here we investigated whether there is a link between the distribution of active nonapeptides across brain regions and cleaners' social behaviour. Male cleaner fish were exposed to different social contexts: (i) an unfamiliar male conspecific (conspecific context), (ii) an unfamiliar interspecific partner (blond naso tang, *Naso elegans*; mutualistic context) and (iii) a ball (non-social context). Furthermore, to distinguish between physicochemical and visual stimulations, cleaners were also exposed to an unfamiliar conspecific or an unfamiliar interspecific partner placed in a separate, smaller aquaria while inside the experimental tank. We then measured biologically available AVT and IT levels in distinct brain macro-areas, forebrain, optic tectum, cerebellum and brain stem, using high-performance liquid chromatography with fluorescence detection. We did not find any association between the levels of AVT and social contexts. On the other hand, we found lower levels of IT in the forebrain of cleaners in contact with a conspecific compared to those introduced to a client and a client inside another aquarium. Additionally, cleaners displayed aggression but only towards other conspecifics. These findings indicate that (i) IT in cleaners' forebrain is linked with mutualistic engagement between cleaners and clients, (ii) stimulation of IT pathways in the forebrain is probably linked with the visual recognition of potential clients and (iii) physicochemical signals are essential to trigger aggressive displays in male conspecifics.

Significance statement

The visual stimulation of IT pathways in the forebrain of male cleaner wrasses while introduced to sympatric clients is associated with the establishment of cleaner-client mutualistic relationship; physicochemical signals are important to trigger aggressive displays in male cleaners introduced to conspecifics.

Keywords Mutualisms · *Labroides dimidiatus* · Communication · Visual contact · Physicochemical interactions · Nonapeptides

Murilo S. Abreu and Ewa Kulczykowska shared first authorship.

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Introduction

In nature, cleaners face complex situations arising from a highly demanding socio-environment which include the need to engage in conspecific and mutualistic behaviours. These different relationships are expected to alter cleaners' behaviour and/or the levels of nonapeptides arginine vasotocin (AVT) and isotocin (IT) across specific brain regions. The nonapeptides hormones AVT and IT are well-known homologues of mammalian arginine vasopressin and oxytocin, respectively (Hoyle 1999), and have attracted much attention due to their role in the regulation of social and reproductive behaviours (for review, see Bass and Forlano 2008; Godwin 2010; Godwin and Thompson 2012). In regard to social behaviour, brain nonapeptides are involved in several aspects of fish social interactions, namely, in social status acquisition (Greenwood et al. 2008; Almeida et al. 2012; Almeida and Oliveira 2015), affiliative behaviour (Reddon et al. 2015) and mutualistic behaviour (Cardoso et al. 2015b; Soares et al. 2017). Previous studies examining the functions of the nonapeptides in the Indo-Pacific bluestreak cleaner wrasse, *Labroides dimidiatus*, demonstrated that intramuscular administration of AVT reduced their propensity to engage in interspecific cleaning activities with their clients (Soares et al. 2012) and a dimension of cooperativeness of individuals (Cardoso et al. 2015c). Additionally, the quantitative distribution of AVT and IT in the brains of four species of labrid fish, two obligatory cleaner species (engage in cleaning throughout their life, *L. dimidiatus* and *Labroides bicolor*), a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous) and a non-cleaner (the corallivorous *Labrichthys unilineatus*), strongly suggested that high AVT level in the cerebellum of the obligate cleaners is associated with expression of mutualistic behaviour (Kulczykowska et al. 2015a). Moreover, 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). Further examination of mixed sex pairs of cleaner wrasse *L. dimidiatus* demonstrated that in males, forebrain IT levels increased with the level of pair association (Cardoso et al. 2015b).

Here, we investigate whether cleaners' response to different social partners is associated with the levels of AVT and IT in the brain. Individual male cleaner wrasses (the Indo-Pacific *L. dimidiatus*) were introduced to one of these treatments: (i) an unfamiliar male conspecific, (ii) an unfamiliar interspecific partner (a sympatric client) or (iii) a ball (non-social condition) (see Fig. 1). Furthermore, to distinguish between physicochemical and visual stimulations, cleaners were also exposed to an unfamiliar conspecific or an unfamiliar interspecific partner placed in separate, smaller aquaria while inside the experimental tank (Fig. 1). In the natural habitat (the Indo-Pacific region), the naso tang (the Indo-Pacific *Naso elegans*; family Acanthuridae) is a

potentially regular client of cleaners *L. dimidiatus*. Indeed, behavioural interactions between these cleaners and clients *N. elegans* are well described in our recent paper (Soares et al. 2017). On the other hand, behavioural interactions between cleaners and conspecifics have received far less attention.

In teleost fish, the AVT/IT neurosecretory system contains three main cell groups in the preoptic area (POA): gigantocellular, magnocellular and parvocellular that project fibres to multiple brain areas, such as the ventral telencephalon, the thalamus and the mesencephalon (Holmgvist and Ekström 1995; Saito et al. 2004). There is substantial evidence that variation in behaviour is associated not only with nonapeptide production/presence but also relates to variation in the distribution and abundance of nonapeptide receptors in various brain regions (Lema et al. 2015). The first insight into the quantitative levels of AVT and IT across different brain areas, of fish that exhibits different social behaviour has been provided for the African cichlid fish *Oreochromis mossambicus* (Almeida et al. 2012). We have also quantified the levels of both AVT and IT in different brain regions of cleaner fish (Cardoso et al. 2015b; Kulczykowska et al. 2015a). Drawing on our previous experience and on the available literature regarding the presence of AVT/IT and their receptors in various brain macro-areas, herein we investigate the quantitative distribution of AVT and IT in the forebrain, the optic tectum, the cerebellum and the brain stem.

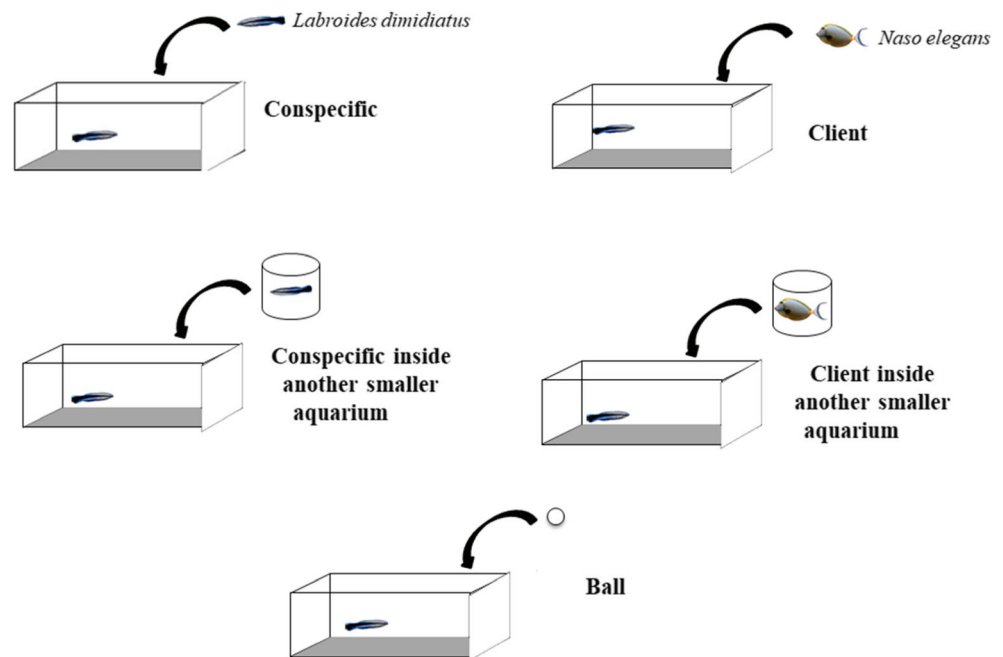
Following behavioural observations, we measured the concentrations of biologically available nonapeptides, AVT and IT, in the brains using high-performance liquid chromatography with fluorescence detection (HPLC-FL). We hypothesized that cleaners' behavioural response to different social partners (experimental treatments) would be associated with region-specific changes in brain AVT and IT levels.

Methods

Animals and housing

Experiments were conducted at the fish housing facilities of the Oceanário de Lisboa (Lisbon, Portugal). The specimens used in this study were the Indo-Pacific bluestreak cleaner wrasse, *L. dimidiatus*, and adult blond naso tang, *N. elegans* (family Acanthuridae, aka clients), all imported to Portugal by a local distributor (Tropical Marine Centre, Lisbon, Portugal). Total length (TL) and total weight (TW) of cleaner wrasses *L. dimidiatus* ranged from 5.2 to 7.2 cm (mean 6.4 ± standard deviation 0.6 cm) and 1.3 to 4.7 g (2.4 ± 0.7 g). Because dominance and sex-derived conflicts mostly occur when individuals live and clean as a couple, all male cleaner wrasses were kept and analyzed alone in 50 × 40 × 40 cm aquaria, to avoid further contextual variations. Cleaner wrasse sex was determined by measurement of gonad 17β-estradiol (see Soares et al. 2017 for detailed

Fig. 1 Experimental setup. Male *Labroides dimidiatus* was exposed to a conspecific (*L. dimidiatus*), a client (*Naso elegans*), a conspecific inside another smaller aquarium, a client inside another smaller aquarium and a ball



methodology). Tangs were kept in stock aquaria of $100 \times 40 \times 40$ cm in groups of five to ten individuals. All aquaria were combined in a flow through system that pumped water from a larger sump ($150 \times 50 \times 40$ cm) that served as a mechanical and biological filter. Nitrite concentrations were kept below 0.3 mg/L. Each tank contained an air supply and a commercial aquarium heater (125 W, Eheim, Jäger). PVC pipes (15–20 cm long; 20 cm diameter) served as shelter for the fish. Experiments were carried out in the individual smaller tanks ($50 \times 40 \times 40$ cm).

Experimental design and sampling

On each experimental day, one of the following treatments was randomly exposed to each subject cleaner wrasse while kept in aquarium of $50 \times 40 \times 40$ cm: (a) a conspecific ($n = 8$) (*L. dimidiatus*), (b) a client ($n = 9$) (*N. elegans*), (c) a conspecific inside another smaller aquarium ($20 \times 10 \times 40$ cm) ($n = 7$), (d) a client inside another smaller aquarium ($20 \times 10 \times 40$ cm) ($n = 9$) and (e) a ball ($n = 8$) (non-social context control) (≈ 50 g, white ball (solid rubber core and a cover made of thermoplastic ionomer), approximately 5 cm in diameter, which stayed at the bottom, completely sessile) (see Fig. 1). Experimental aquaria were divided by opaque partitions that prevented any visual contact between fish during experiment. After a 2–5-min stabilization period, the recording started. Behaviour was videotaped for the next 60 min while the experimenter left the room (see section behavioural analyses below). At the end of experiments, each cleaner wrasse was rapidly captured and sacrificed with an overdose of tricaine solution (MS222, Pharmaq; 1000 mg/L) and the spinal cord sectioned. The brain was immediately dissected under a stereoscope (Zeiss; Stemi 2000) into four macro-areas,

forebrain (olfactory bulbs + telencephalon + diencephalon), optic tectum, cerebellum and brain stem, and stored at -80 °C prior to the analysis of AVT and IT levels.

Quantification of nonapeptides by high-performance liquid chromatography with fluorescence detection

Brain samples were weighed for later calculation of nonapeptides' levels (peptide contents are expressed per milligram of brain sample) and sonicated in 1 mL Milli-Q water (Microson™XL, Misonix, USA), acidified with glacial acetic acid (2.5 μ L, final concentration in homogenate solution was 0.25%) and placed in a boiling water bath for 3.5 min. The homogenates were then centrifuged (12,000 \times g, 20 min, 4 °C), and supernatants were decanted and loaded onto previously conditioned (1 mL methanol, 1 mL Milli-Q water) solid-phase extraction (SPE) columns (30 mg/1 mL, Strata-X, Phenomenex). To purify samples, columns were washed successively with 0.6 mL Milli-Q water and 0.6 mL of 0.1% TFA (trifluoroacetic acid) in 5% acetonitrile. The peptides were eluted using 1.2 mL of 80% acetonitrile. The eluate was evaporated to dryness using a Turbo Vap LV (Caliper Life Science, USA), and the residue was frozen and stored at -80 °C prior to HPLC analysis. Before quantitative analysis, the residue was re-dissolved in 40 μ L of 0.1% TFA in 30% acetonitrile. Pre-column derivatization of AVT and IT was performed according to the procedure by Gozdowska and colleagues (Gozdowska et al. 2013). For derivatization, 20 μ L of sample and 20 μ L of 0.2 M phosphate buffer (pH 9) were mixed, and 3 μ L of NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg in 1 mL of acetonitrile) was then added. The solution was heated at 60 °C for 3 min, cooled on ice,

Table 1 Correlations (Spearman rank correlation coefficients) between different behavioural measures and brain AVT and IT levels in different brain macro-areas: forebrain, optic tectum, cerebellum and brain stem for two experimental treatments: conspecific context and client context. None of the significant correlations remained significant after analysis for the false discovery rate test

| Behaviour | Brain macro-areas | | | |
|---|--------------------------------|--------------------------------|-------------------------------|-------------------------------|
| | Forebrain | Optic tectum | Cerebellum | Brain stem |
| Arginine vasotocin (AVT) | | | | |
| Conspecific <i>N</i> = 8 | | | | |
| Cleaning bites | – | $r = 0.1000$ $p = 0.9500$ | $r = 0.8$ $p = 0.3333$ | $r = -0.8$ $p = 0.3333$ |
| Incidence of chases | – | $r = -0.8$ $p = 0.1333$ | $r = -0.4$ $p = 0.75$ | $r = -0.4$ $p = 0.75$ |
| Client <i>N</i> = 9 | | | | |
| Number of interactions | $r = 0.6667$ $p = 0.1611$ | $r = -0.4728$ $p = 0.2683$ | $r = -0.6669$ $p = 0.1667$ | $r = 0.3189$ $p = 0.5444$ |
| Average interaction duration | $r = 0.02857$ $p > 0.9999$ | $r = 0.07143$ $p = 0.9063$ | $r = -0.3000$ $p = 0.6833$ | $r = -0.3143$ $p = 0.5639$ |
| Proportion of interactions with tactile stimulation | $r = 0.9411$ $p = 0.0167$ | $r = -0.2224$ $p = 0.5905$ | $r = -0.6669$ $p = 0.1667$ | $r = 0.8407$ $p = 0.0444$ |
| Proportion of time providing tactile stimulation | $r = 0.7590$ $p = 0.1167$ | $r = -0.1853$ $p = 0.6429$ | $r = -0.6669$ $p = 0.1667$ | $r = 0.7247$ $p = 0.1222$ |
| Isotocin (IT) | | | | |
| Conspecific <i>N</i> = 8 | | | | |
| Cleaning bites | $r = -0.2571$ $p = 0.6583$ | $r = 0.2571$ $p = 0.6583$ | $r = 0.2$ $p = 0.7139$ | $r = -0.2$ $p = 0.7833$ |
| Incidence of chases | $r = 0.2571$ $p = 0.6583$ | $r = 0.7714$ $p = 0.1028$ | $r = 0.1429$ $p = 0.8028$ | $r = -0.7$ $p = 0.2333$ |
| Client <i>N</i> = 9 | | | | |
| Number of interactions | $r = -0.02899$ $p = 0.9333$ | $r = 0.1471$ $p = 0.7778$ | $r = 0.09009$ $p = 0.8595$ | $r = 0.05798$ $p = 0.9333$ |
| Average interaction duration | $r = -0.4857$ $p = 0.3556$ | $r = -0.3714$ $p = 0.4972$ | $r = -0.75$ $p = 0.0663$ | $r = -0.6$ $p = 0.2417$ |
| Proportion of interactions with tactile stimulation | $r = 0.7537$ $p = 0.1056$ | $r = -0.03036$ $p = 0.8833$ | $r = 0.6847$ $p = 0.1016$ | $r = 0.1518$ $p = 0.8333$ |
| Proportion of time providing tactile stimulation | $r = 0.4638$ $p = 0.3722$ | $r = -0.03036$ $p = 0.8833$ | $r = 0.5045$ $p = 0.2548$ | $r = 0.09108$ $p = 0.8833$ |

acidified with 4 μL of 1 M HCl and a 47 μL aliquot was injected into the HPLC system (1200 Series Quaternary HPLC System; Agilent Technologies, USA). Chromatographic separation was achieved on a ZORBAX Eclipse XDB-C18 column (150 mm \times 4.6 mm I.D., 5 μm particle size; Agilent, USA). A gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in acetonitrile: H_2O (3:1)). A linear gradient was 40–65% of eluent B in 20 min. The column temperature was 20 $^\circ\text{C}$ and flow rate 1 mL/min. The fluorescence of derivatized AVT and IT was measured at 530 nm with excitation at 470 nm.

Behavioural analyses

During each video analysis, we recorded the following: (1) the number and duration (in seconds) of cleaning inspections toward each client or conspecific; (2) the frequency

and duration of tactile stimulation provided (where a cleaner touches, with ventral body and fins, the body of the client and no feeding is involved (Bshary and Würth 2001)); (3) the number of jolts by clients (cleaners sometimes bite clients and they respond with a short body jolt which usually is a behaviour associated with cheating by cleaner fish (Bshary and Grutter 2002; Soares et al. 2008)); (4) the number and duration (in seconds) of chases where a cleaner (focal individual) rapidly advanced toward the other conspecific; and (5) the number of bites provided by the client. When clients chase cleaners, it is generally because cleaners were not solicited to clean (e.g. cleaners attempt to clean against the client) or after a cleaner's bite (measured by clients' body jolts). In the conspecific context, the incidences of chases may be due to size differences (if intruder is larger than the resident).

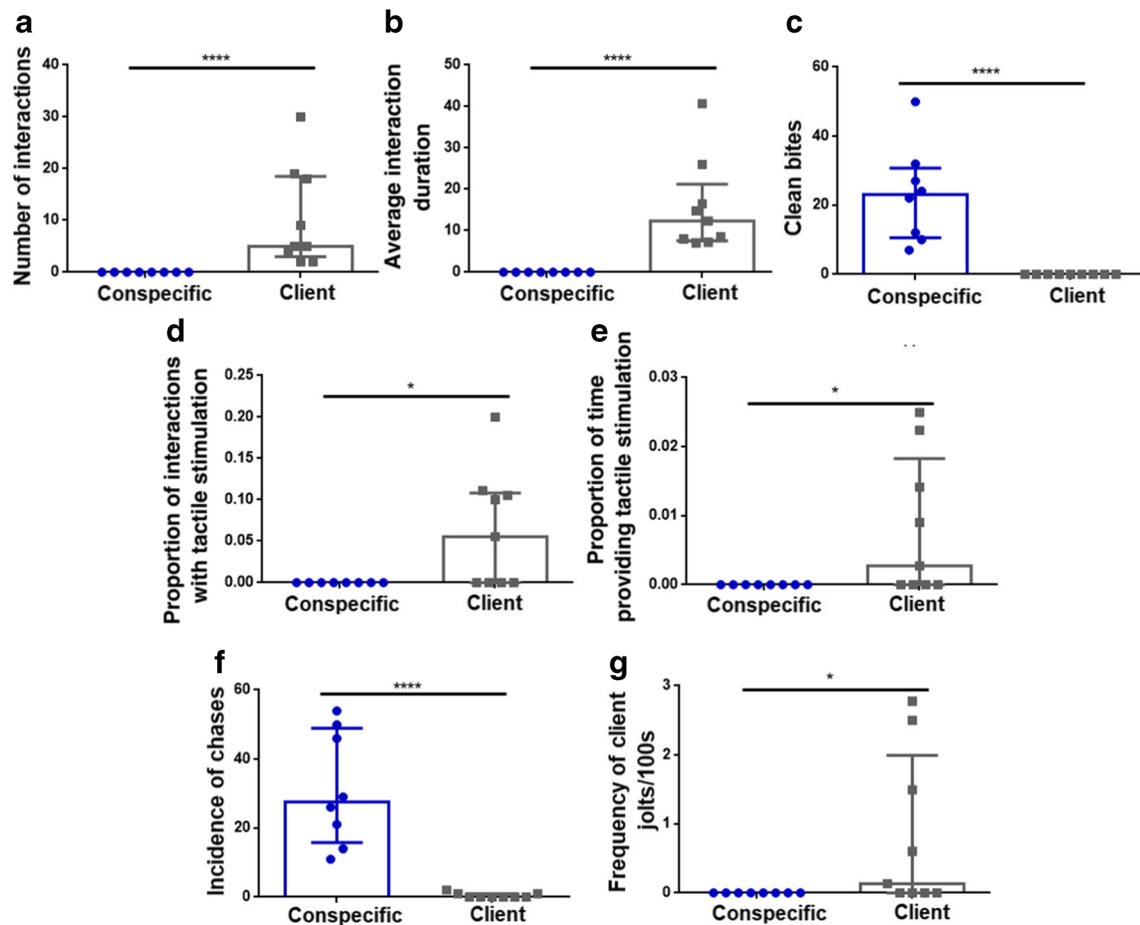


Fig. 2 Behavioural results. Analysis of interactions between a cleaner and a conspecific and a cleaner and a client. **a** Number of interactions. **b** Average interaction duration. **c** Cleaning bites. **d** Proportion of interactions with tactile stimulation. **e** Proportion of time providing

tactile stimulation. **f** Incidence of chases. **g** Frequency of client jolts/100 s. Medians and interquartile ranges are shown. Dots, in each group, represent each individual response. Significant values are shown above bars and refer to Mann-Whitney U tests (* $p < 0.05$; **** $p < 0.0001$)

Statistical analyses

A total of 41 male cleaners were used for measurement brain AVT and IT (mean \pm SD 29.4 \pm 35.7 pmol/mg). In four individuals, AVT levels were below the limit of detection (all brain areas) and in two individuals, IT levels were below the limit of detection (all brain areas). Data were analysed using non-parametric tests because the assumptions for parametric testing were not met. Mann-Whitney U tests were used to analyse behavioural differences between treatments. Kruskal-Wallis tests were performed to detect differences between groups (five treatments: conspecific, client, conspecific inside another smaller aquarium, client inside another smaller aquarium and ball) for each brain area and to search for differences amongst brain areas (four areas: forebrain, optic tectum, cerebellum and brain stem) followed by Dunn's post-hoc tests, which already include a Bonferroni adjustment to account for multiple comparisons to compare each treatment between each group. Finally, relationships within and between behavioural measures and clients' brain nonapeptide levels were examined using Spearman

correlation coefficients. We corrected the alpha values by applying the Benjamin-Hochberg false discovery rate correction reporting only those correlations that were significant (Hochberg 1988). Significance was accepted at $p < 0.05$. In the Table 1, all p values are reported.

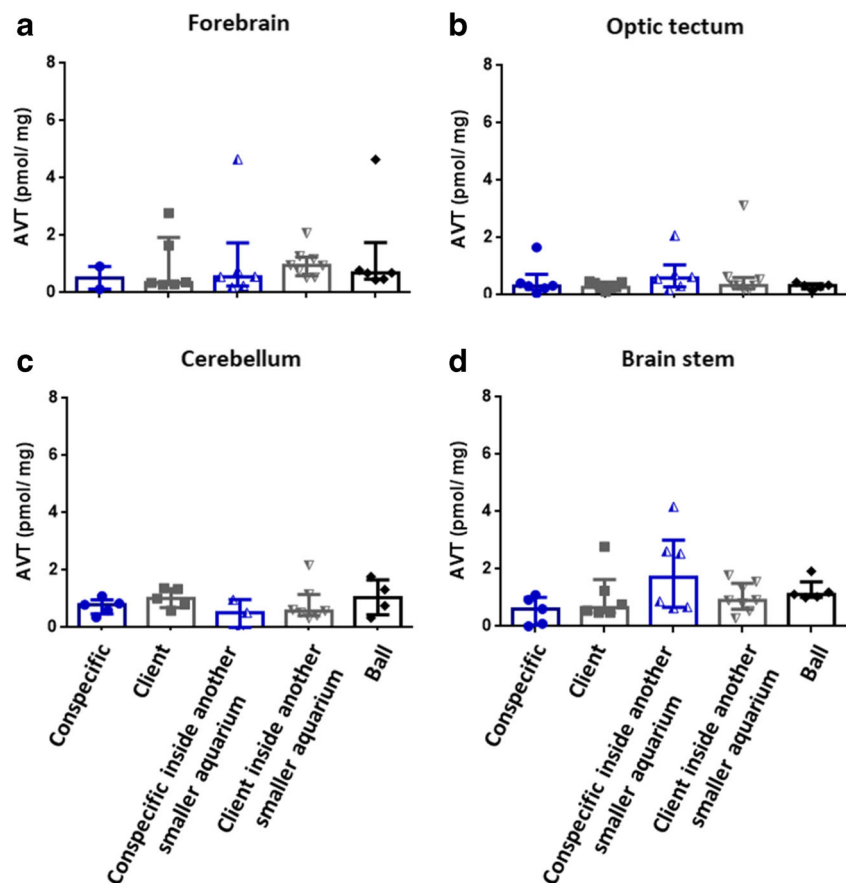
Data availability statements Data from the current study are available from the corresponding author upon reasonable request.

Results

Cleaner's behaviour

We demonstrate that the frequency of cleaning interactions was higher when cleaners were interacting with a client compared to those interacting with conspecific fish (Mann-Whitney U test, $U = 0$, $n_1 = 8$, $n_2 = 9$, $p < 0.0001$; Fig. 2a), with higher mean interaction time ($U = 0$, $n_1 = 8$, $n_2 = 9$, $p <$

Fig. 3 AVT levels in different brain macro-areas: forebrain, optic tectum, cerebellum and brain stem, exposed to different treatments. **a** AVT levels in the forebrain area. **b** AVT levels in the optic tectum area. **c** AVT levels in the cerebellum area. **d** AVT levels in the brain stem area. Medians and interquartile ranges are shown. Dots, in each group, represent each individual response



0.0001; Fig. 2b), higher proportion of cleaning interactions with tactile stimulation ($U = 16$, $n_1 = 8$, $n_2 = 9$, $p = 0.0294$; Fig. 2d), higher proportion of time providing tactile stimulation ($U = 16$, $n_1 = 8$, $n_2 = 9$, $p = 0.0294$; Fig. 2e) and also higher frequency of client jolts ($U = 16$, $n_1 = 8$, $n_2 = 9$, $p = 0.0294$; Fig. 2g). The frequency of cleaning bites ($U = 0$, $n_1 = 8$, $n_2 = 9$, $p < 0.0001$; Fig. 2c) and incidence of chases ($U = 0$, $n = 8$, $n_2 = 9$, $p < 0.0001$; Fig. 2f) increased in the conspecific context (Fig. 2). Finally, cleaners were not observed to approach or touch the ball (hereafter referred as control).

Cleaner's brain nonapeptides

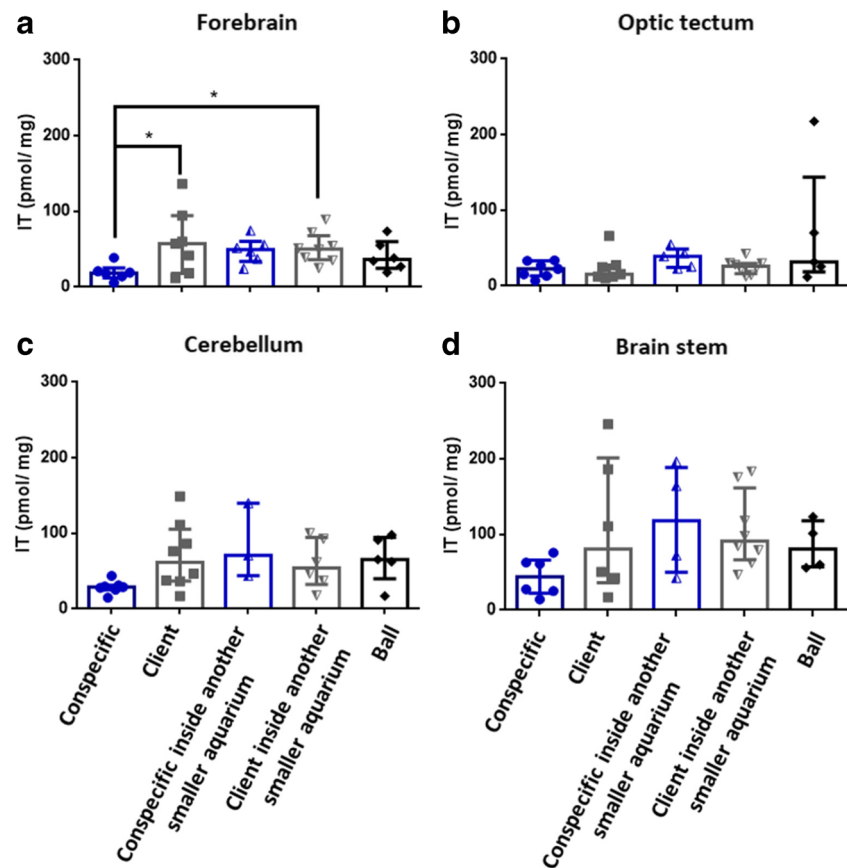
AVT brain levels did not vary significantly across brain areas, for all treatment groups (forebrain area (Kruskal-Wallis, $K = 3.438$, $p = 0.4873$), optic tectum area ($K = 3.646$, $p = 0.456$), cerebellum area ($K = 3.687$, $p = 0.452$) and brain stem area ($K = 5.839$, $p = 0.2115$)) (Fig. 3). Regarding the IT brain levels, significant differences were solely found in cleaners in contact with a conspecific, which had lower levels of IT in the forebrain compared to those introduced to a client and a client inside another aquarium ($K = 9.7$, $p = 0.0458$; Fig. 4a). There were no significant differences between the five groups in the remaining brain areas (optic tectum area ($K = 5.236$; $p = 0.2639$), cerebellum area ($K = 8.709$; $p = 0.0688$) and

brain stem area ($K = 5.489$; $p = 0.2407$)) (Fig. 4). Regarding the relationship between cleaner behaviour and brain nonapeptides, none of the correlations remained significant after calculation of Hochberg-adjusted alpha values (Table 1).

Discussion

In nature, cleaners divide their time between interacting with multiple interspecific clients and their conspecific partners. But because these cleaners live and clean in pairs, conspecific conflicts may frequently arise within pairs (Raihani et al. 2010) in addition to affiliative interactions, such as tactile stimulation (physical contact, touching partners with their pelvic fins (Bshary and Bronstein 2011) and cleaning (Cardoso et al. 2015b)); however, studies have mostly focused on the role of nonapeptides on mutualistic related mechanisms and scarcely on their conspecific domain. In this study, we examine whether the different social relationships (between unfamiliar individuals: cleaner and conspecific or cleaner and client) are expressed in cleaners' behaviour and/or AVT and IT levels in distinct brain macro-areas: forebrain, optic tectum, cerebellum and brain stem. We found that in experimental pairs, cleaner-conspecific or cleaner-client (Fig. 1) individuals were freely and

Fig. 4 IT levels in different brain macro-areas: forebrain, optic tectum, cerebellum and brain stem, exposed to different treatments. **a** IT levels in the forebrain area. **b** IT levels in the optic tectum area. **c** IT levels in the cerebellum area. **d** IT levels in the brain stem area. Medians and interquartile ranges are shown. Dots, in each group, represent each individual response. Significant values are shown above bars and refer to Dunn's post-hoc tests ($*p < 0.05$)



frequently interacting. Indeed, cleaners were mostly engaging in cleaning with the interspecific partners (*N. elegans*), chasing and biting their conspecifics and not responding to the ball (control non-social treatment). However, there were no correlations between cleaners' behaviour and their brain nonapeptides, AVT and IT. On the other hand, we found the lower levels of IT in the forebrain of cleaners in contact with conspecific compared to those introduced to a client and a client inside another aquarium. Additionally, cleaners displayed aggression toward unfamiliar conspecifics. Thus, the physicochemical signals are essential to trigger aggressive displays in male cleaners introduced to conspecifics.

In contrast to previous studies indicating the modulatory role of AVT in mutualistic behaviour of cleaners (Soares et al. 2012; Cardoso et al. 2015a, b, c), no differences were found in levels of AVT in distinct brain macro-areas amongst experimental groups. This could be due to the relative poverty of housing conditions which strongly differ from the natural habitats, including the absence of a relevant cue stimulus as a presence of more conspecifics and other client species, habitat diversity, etc. Overall, environmental enrichment which includes physical structures added to the captive environment (presence of sand, rocks, coral, plants etc. in the aquarium) has positive effects on fish behaviour, physiology and performance (for review: (Martins et al. 2012). It is also well established that housing conditions have significant

effects on the neural plasticity and cognitive ability of fish (Salvanes et al. 2013).

Our earlier studies of *L. dimidiatus* demonstrated the positive relationship between forebrain IT levels in male cleaners and their interspecific service quality (measured by the clients' jolting rate) indicating an important role of IT pathways and, in particular the forebrain IT, in mutualistic relationships (Cardoso et al. 2015b). In the present case, the lower levels of IT were shown in the forebrain of cleaners in contact with conspecific, compared to those in contact with clients (directly or while these were solely in visual contact - separated in small aquarium). It indicates that the visual stimulus is enough for the stimulation of cleaners' IT response. Additionally, it suggests that the stimulation of IT pathways in the forebrain of cleaner is linked with the visual recognition of potential clients.

The stimulation of IT pathways by visual recognition is not surprising because vision plays a pivotal role in the behaviour of many fish species, like in the reproductive behaviour in the Poeciliidae (Kodric-Brown and Nicoletto 2001; Tobler et al. 2006) and the sticklebacks (*Gasterosteus aculeatus*) (FitzGerald 1993; Braithwaite and Barber 2000; Kleszczyńska et al. 2012; Kulczykowska and Kleszczyńska 2014). Moreover, the visual stimulation is the signal triggering aggressive behaviour in male cichlid fish (*Oreochromis mossambicus*) (Oliveira et al. 2005) and in the three-spined

stickleback (*Gasterosteus aculeatus*) (Kleszczyńska et al. 2012), to the masculinization of the black molly (*Poecilia sphenops*) (Kulczykowska et al. 2015b) and to courtship in the painted goby (*Pomatoschistus pictus*) (Amorim et al. 2013). Interestingly, higher level of IT has been detected in the brain of the aggressive male three-spined sticklebacks attacking their mirror image (Kleszczyńska et al. 2012), confirming the role of visual signal in stimulation of IT pathways.

Although, there are no significant correlations between cleaner behaviour and brain nonapeptides in the current study, we have demonstrated that physicochemical interactions between conspecifics are essential for the production of aggressive displays in male cleaners *L. dimidiatus*. The role of physicochemical stimulation in building a relationship between conspecifics has been shown in our previous studies with mixed sex pairs of cleaner wrasses (Cardoso et al. 2015b, c). Furthermore, male cleaner wrasses demonstrate higher forebrain IT levels when receiving more cleaning and tactile stimulation from their bonded female partners (Cardoso et al. 2015b).

Additionally, physicochemical stimulation is essential to establish dominance hierarchies. For example, in males of the Mozambique tilapia (*O. mossambicus*), urine that is actively released during aggressive interactions between males probably contains male pheromones which may act as a “dominance” pheromone (Barata et al. 2007). On the other hand, in a experiment with mirrors carried out in the same species showed that chemical signals are not required to evoke aggression (Oliveira et al. 2005). Also in male three-spined sticklebacks, visual but not physiochemical signals are crucial for induction of aggressive behaviour as evidenced by experiments with mirrors (Kleszczyńska et al. 2012).

Overall, we demonstrated the importance of forebrain IT in mutualistic relationships between cleaner wrasses and sympatric clients. The stimulation of IT pathways in the forebrain of cleaners is probably linked with the visual recognition of the potential interspecific partners. Furthermore, physicochemical signals are essential to trigger aggressive displays in male cleaners in respect to other conspecifics.

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Authors' contribution MCS designed the study. SCC and GIA ran experimental procedures. GIA prepared the samples for HPLC. MG ran HPLC analysis. MM analysed behavioural videos. MSA analysed the data. MSA, EK and MCS wrote the paper. All authors discussed results and commented on the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Animal procedures used in this study were approved by the Portuguese Veterinary Office (Direcção Geral de Veterinária, license # 0420/000/000/2009) and were carried out in accordance with the approved guidelines.

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