



Forebrain neuropeptide regulation of pair association and behavior in cooperating cleaner fish



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HIGHLIGHTS

- Animals establish privileged relationships which contribute to behavioral variation.
- We asked if intra-pair association index is linked with brain AVT and IT changes.
- And whether these mechanisms relate to changes in interspecific service quality.
- Variation in pair relationship was found to influence male and female cleaner fish differently.
- Variation in brain neuropeptide levels is linked to conditional cooperative outcomes.

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ABSTRACT

Animals establish privileged relationships with specific partners, which are treated differently from other conspecifics, and contribute to behavioral variation. However, there is limited information on the underlying physiological mechanisms involved in the establishment of these privileged ties and their relationship to individual cooperation levels. The Indo-Pacific bluestreak cleaner wrasse *Labroides dimidiatus* often forages in mixed-sex pairs when cleaning fish clients. Intra-couple conflicts often arise during a joint client inspection, which may alter the overall quality of cleaning service provided. Here we tested two hypotheses: a) whether intra-pair association (i.e. association index), measured with joint interspecific cleaning and intraspecific behavior, is correlated with neuroendocrine mechanisms involving forebrain neuropeptides arginine vasotocin (AVT) and isotocin (IT) and b) whether these neuropeptide level shifts relate to an individual's interspecific service quality. We found that partner support (number of cleaning interactions and tactile stimulation) received by male cleaners increased with association index. When cleaners inspected clients alone, cleaners' cheating decreased with association index for females but not males. AVT levels did not differ according to sex or association level. Forebrain IT levels increased with association index for males, whereas no relationship was found for females. Finally, cleaner cheating varied between sex and forebrain IT levels. Findings indicate that variation in pairs' relationships influences male and female cleaner fish differently and contributes to the variation of brain neuropeptide levels, which is linked to distinct cooperative outcomes.

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1. Introduction

Behavioral variation between individuals is recognized as a fundamental force shaping social interactions and the evolution of complex social behavior that includes cooperation [1,2]. These behavioral differences prompt individuals to learn more about others (before and during

interactions), which may lead to distinct cooperative outcomes. For instance, they may influence individuals to invest more when dealing with partners that reciprocate or to abandon uncooperative partners for more cooperative ones [2]. One of the greatest contributors to individual behavioral variation is the existence of social ties or familiarity, which encourages the establishment of individuals' relationships with specific partners (e.g. pairbonding, alliances, and friendships [3]). However, there is limited information on the underlying physiological mechanisms that involve the establishment of social ties and their direct consequences to individual behavioral variation (see [4]).

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The formation and maintenance of stable male–female relationships are sustained by selective socio-sexual behavior between the pair, and by preference for one partner over other potential ones (e.g. pair bonds; [5,6]). But the maintenance of long lasting social attachments should also be sustained by specific neurohormonal frameworks, which should be linked to the behavioral responses of any paired individual, on two different levels: a) at the expression of a series of “bilateral” behaviors between pair partners and b) on “multilateral” interactions between the pair and the social environment [4]. Two neuropeptides are considered to be critical mediators of partner-preference formation and social attachment: oxytocin (OT) and arginine vasopressin (AVP) [7]. The neuropeptide OT is well known for its links to social bonds, which includes affectionate behavior between partners [8], displays of romantic love, and increases of prosociality within the context of cooperative behavior. For example, exogenous administration of OT seems to be responsible for an increase of investment in communal and cooperative activities in meerkats [9], the facilitation of partner-directed behavior in marmosets [10], and the promotion of humans’ trust and reciprocity [11,12]. The role of AVP in partner recognition and bonding, mostly derived from rodent studies, shows that both AVP and OT are involved but they have sex specific roles; for example, males and females are more sensitive to AVP and OT, respectively, which may be due to different brain receptor distributions [13,14].

The converse may also occur, with neuropeptide levels changing in response to behavioral variations in socio-sexual and affiliative behavior. For instance, partner support modulates the rise of OT plasma levels in both men and women [15] and physical contact (such as massaging, hugging and/or grooming) promotes the elevation of OT levels in humans and other primates and also in rodents [8,16,17]. In pair-bonded tamarins, OT levels relate to the amount of grooming and mutual contact in females and sexual behavior of males [18]. Measures of relationship distress correlate with OT levels in women and with AVP levels in men [19]. This suggests that shifts in levels of neuropeptide are directly linked with social environment and partnership quality. However, to be able to broadly understand these findings, we must look at vertebrate species other than primates and other mammals.

The Indo-Pacific bluestreak cleaner wrasse *Labroides dimidiatus* is often found in mixed-sex pairs. Male cleaner wrasses are harem holders and most frequently live and clean in pairs, usually with the largest female of his harem although the male also visits other females regularly [20]. Cleaners provide a service by removing ectoparasites, dead or damaged tissue of other visiting reef fish (hereafter referred as ‘clients’) [21]. However, cleaners prefer to feed on client mucus, which is detrimental to the client and constitutes cheating [22]. Instances of cheating contribute to a conflict of interests between clients and cleaners [22]. Clients need cleaners to eat against their preference in order to gain a good cleaning service which they achieve by: a) refraining from visiting a cleaner that provided a poor service in the past, b) avoiding cleaners they observe cheating other clients and/or c) by aggressively punishing cheating cleaners with chases [23].

Because cleaners may inspect clients alone or simultaneously with a partner, the quality of cleaning service provided to the clients may also be a source of intra-couple conflict when cleaning together [24]. Intra-couple conflicts arise because the benefits of cheating can be gained by only one cleaner during a joint inspection (e.g. the first to cheat will induce the client to leave). However, the service provided to clients by paired inspections is of better quality mainly because females behave more cooperatively in joint inspections than during solitary ones [24]. This happens because the larger male cleaner punishes (i.e. aggressively chases) the females that cheat whereas females never chase the male [25]. Moreover, in laboratory conditions, male to female punishment seems to vary according to circumstances, with males punishing their female partners more severely when high value client models are at stake or when partners are similar in size [26]. Males tend to behave more aggressively with unfamiliar females, with such females responding by behaving more cooperatively [27]. However, compared

with laboratory environments involving pairs of cleaners confined to a limited aquarium space, and consequently continuously paired, in natural conditions the situation is not always as extreme. Instead, in naturally behaving pairs, pair association may decrease with increasing harem size, because male visitation rate to other females should also vary. This should result in pairs with a wide range of different relationships, varying in association strength.

Here, we examine possible correlates of variation in forebrain neuropeptide levels of arginine vasotocin (AVT) and isotocin (IT), fish homologs of mammalian AVP and OT respectively, by simultaneously measuring pair association (i.e. at the intraspecific level), and how these neuropeptides correlate with individual interspecific cooperative levels in naturally coupled pairs. The preoptic area (i.e. anterior hypothalamus), which is located in the forebrain, contains a high density of AVP/AVT-OT/IT elements and is a primary site of behavioral integration of vertebrates [28]. We tested two hypotheses: 1) whether the intra-pair relationship, measured by the rates of joint interspecific cleaning and intraspecific behavior, is correlated with brain levels of AVT and IT and 2) whether these mechanisms are also associated with individuals’ interspecific service quality. The method we used measures the concentration of free forebrain nonapeptides AVT and IT after their dissociation from non-covalent complexes. This provides information based solely on the biologically active fraction of peptides, which is engaged in conversion of environmental signals into specific reaction of individuals (e.g. behavioral expression [29,30]). Moreover, our study provides a novel approach to the neuroendocrine mechanisms of behavioral variation aiming at a tropical reef fish living in natural conditions.

2. Methods

2.1. Field methods

This study was conducted on two reefs around Lizard Island (Lizard Island Research Station, Australia, 14° 40’ S, 145° 289’ E) between September and October 2012. All observations and collections were made by two SCUBA divers, between 10:00 and 15:00 h. Twenty cleaner fish (10 naturally-coupled male–female pairs) were selected randomly from cleaning stations that varied in depth between 3 and 10 m. Males are always larger than and dominant to their female partner. Each cleaner (male and female) was then randomly assigned to one of the two divers in place. Both cleaners (male and female) were then observed and videotaped (during the same session) for the next 45 min using video cameras in waterproof cases (Sony HDR-XR155) from a distance of between 2 and 3 m. At the end of the observation, the pair was captured using hand and barrier nets. Total length (TL) and total weight (TW) of females ranged from 6.3 to 8.5 cm (mean \pm SD: 7.25 \pm 0.65 cm) and 2.5 to 6.2 g (3.82 \pm 1.14 g) and for males from 7.5 to 9 cm (8.35 \pm 0.45 cm) and 4.3 to 7.3 g (5.73 \pm 0.99 g), respectively. The sex of the individuals was confirmed by direct inspection of the gonads. Only a maximum of three couples were observed per day. After capture, fish were immediately brought to the field station where they were anesthetized (overdose of MS-222, Sigma) until muscular and opercular movements completely ceased, after which they were killed by decapitation. The forebrain, composed of olfactory bulbs, telencephalon and diencephalon, was extracted and placed in a cryo-Eppendorf tube, immediately frozen, and stored at -80°C in a liquid nitrogen container. The container was then taken by air to the mainland (resulting in samples being in liquid nitrogen for 10 to 15 days), and from there transported by air to Poland, in dry ice (an additional 2 days), for subsequent analysis.

2.2. Quantification of nonapeptides by high performance liquid chromatography with fluorescence detection (HPLC-FL)

Brain samples were weighed, for further calculation of nonapeptides’ levels (peptide content was expressed per milligram of brain tissue).

Then they 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 (12,000 g, 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 -80 °C prior to HPLC analysis.

Before quantitative analysis, the samples were re-dissolved in 40 μ L of 0.1% TFA (trifluoroacetic acid) in 30% acetonitrile and divided into two for replication. Pre-column derivatization of AVT and IT was performed according to the procedure by [31]. For derivatization reaction, 20 μ L of sample and 20 μ L of 0.2 M phosphate buffer (pH 9) were mixed, and then 3 μ L of NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg in 1 mL of acetonitrile) was added. The solution was heated at 60 °C for 3 min, cooled on ice, acidified with 4 μ L of 1 M HCl and eluted in a HPLC column. Derivatized samples were measured with Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA). Chromatographic separation was achieved on an Agilent ZORBAX Eclipse XDB-C18 column (150 mm \times 4.6 mm I.D., 5 μ m particle size). The gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in H₂O) and solvent B (0.1% TFA in acetonitrile: H₂O (3:1)). A linear gradient was 40–65% of eluent B in 20 min. Flow rate was set at 1 mL/min and the column temperature set to 20 °C. Injection volume was 47 μ L. Fluorescence detection was carried out at 530 nm with excitation at 470 nm.

2.3. Behavioral analyses

For each video observation, we recorded the following measures: a) the number and duration (in s) of a cleaner's inspection of each client and b) the number of jolts (whole-body shudders, in response to cleaner fish mouth contacts that are a good correlate of cheating by cleaners, i.e. eating mucus [22]). Measures of intraspecific pair behavior involved: a) frequency of partner support received, defined as the number of cleaning interactions and number of physical contacts (tactile stimulation) received by an individual cleaner wrasse per observation and b) male to female punishment frequency (number of chases per observation). Interspecific cleaner fish service quality was measured using the frequency of jolts per 100 s of inspection).

2.4. Statistical analyses

As all cleaner wrasse pairs were randomly selected, they were independent measures. We calculated the association indices for pairs of cleaner wrasses using the "twice-weight index of association" [32]. To calculate an association index for each pair – male A and female A, we divided the number of cleaning interactions in which both male A and female A were engaging in cleaning together by the sum of that same number plus the number of interactions in which either male A or female A were observed to clean alone. We therefore generated association indices for each individual cleaner wrasse with its coupled partner. Events in which both cleaners (male and female) were observed to inspect the same client together are hereinafter referred to as "paired" while events in which cleaners were cleaning alone are defined as "unpaired". We then examined cleaner wrasses' behavior and how it may be linked to forebrain neuropeptide levels (with values controlled for forebrain weight) along this axis of association.

We first examined the importance of cleaner pairs' association level with: a) partner support received, which was log transformed to achieve normality and b) cleaner brain neuropeptide levels (IT and AVT). We used analysis of covariance (1-way-ANCOVA) with sex as a fixed factor (male, female) and cleaner association index as a continuous covariate.

We then examined the influence of cleaner pairs' association on cleaner wrasse service quality (client jolt rates), by conducting two separate analyses: one for cleaning in pairs and the other for when cleaning alone. This was due to an observation limitation: when cleaning in pairs, we could not identify which cleaner (male or female) was directly responsible for the client's jolt reaction. For paired cleaner wrasses we used a linear regression to examine the relationship between cleaner association and client size, however only larger clients (large clients ≥ 11 cm total length) were considered in the analysis (small fish had almost no jolts and so did not fit the assumptions of ANCOVA). For the unpaired cleaner wrasses, the initial model was also tested with client size, which was then dropped when found to be not significant. Non-significant interactions were dropped from the models (see results for P values). All single factors were retained in the final model, even if not significant (except for client size class). Relationships between behavioral measures and cleaner brain neuropeptide levels were examined by using the Pearson correlation coefficient. Residuals normality plots were examined and all showed a distribution of residuals that closely approximates a normal distribution (expected probability) [33]. All tests were 2 tailed and were done in SPSS Statistics, version 22.

3. Results

3.1. Intraspecific pair behavior

The amount of partner support received by cleaners varied positively with association index (ANCOVA $F_{(1,17)} = 15.79$, $P = 0.001$), and not between sexes ($F_{(1,17)} = 2.11$, $P = 0.164$, Fig. 1a, Table S1). Male chase (punishment) frequency directed at their paired female partners was not correlated with male association index (Pearson correlation test: $r = 0.07$, $N = 10$, $P = 0.83$, Fig. 1b).

3.2. Cleaner pair interspecific service quality (jolts)

For unpaired cleaner wrasses' jolt rate, client size class was not significant in any of the interactions or on its own (all $P \geq 0.313$), and so it was dropped from the model. There was a significant interaction between sex and association index indicating the slopes were different between sexes (ANCOVA; $F_{(1,16)} = 5.262$, $P = 0.036$, Fig. 2a, Table S2a). Visual examination of Fig. 2a indicates this is likely due to a negative relationship in females.

When inspected by paired cleaner wrasses, smaller clients rarely jolted (mean \pm SD = 0.289 ± 0.916 , Fig. 2b). For that reason, only larger clients were analyzed. We found a significant positive relationship between jolt rate and association index (linear regression; $F_{(1,8)} = 10.699$, $P = 0.01$, Fig. 2b, Table S2b).

3.3. Cleaner pair forebrain neuropeptide levels

IT levels varied according to an interaction between sex and association (ANCOVA: $F_{(1,16)} = 13.237$, $P = 0.002$, Table S3a). Visual examination of Fig. 3a indicates this is likely due to a positive relationship for males. AVT levels did not differ according to sex ($P = 0.597$, Table S3b) or with association level ($P = 0.113$, Table S3b). In females, there was a positive correlation between AVT and IT (Pearson correlation test: $r = 0.72$, $N = 10$, $P = 0.02$, Fig. 3c) in contrast to males, where there was no correlation between the two neuropeptides (Pearson correlation test: $r = -0.40$, $N = 10$, $P = 0.26$, Fig. 3c).

3.4. Link between jolting rate and forebrain neuropeptide levels

When forebrain AVT levels were examined, client jolts rates did not differ between cleaner sexes (ANCOVA: $P = 0.787$, Table S4, Fig. 4a) or with AVT levels ($F_{(1,16)} = 1.752$, $P = 0.204$), though it should be noted that the interaction was nearly significant ($F_{(1,16)} = 4.47$, $P = 0.051$, Table S4, Fig. 4a). Regarding forebrain IT levels, client jolt rates differed

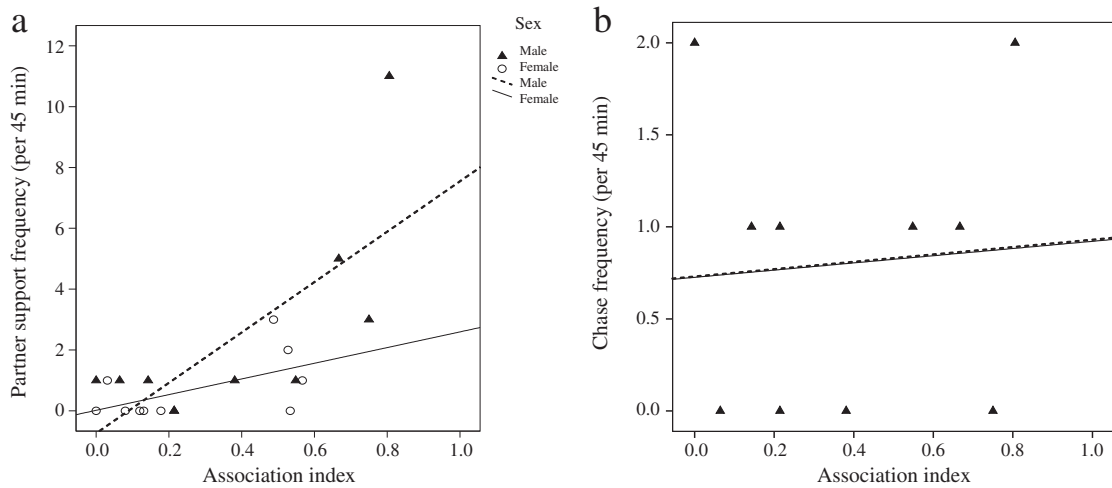


Fig. 1. The relationship between cleaner pairs' association index: a) frequency of partner support received according to sex (male and female) and b) male to female chase frequency.

between sexes ($P = 0.001$, Table S5, Fig. 4b) and also with IT levels ($F_{(1,17)} = 9.084$, $P = 0.008$, Table S5, Fig. 4b). Visual examination of Fig. 4b indicates that the average client jolt rate adjusted for cleaners' forebrain IT levels seems higher in female than male cleaners.

4. Discussion

Our data suggest that differences in pairs' affiliations influence male and female cleaner fish differently and contribute to the variation of brain neuropeptide levels, which is linked to distinct cooperative outcomes. These links will be further explored in the following sections of the discussion.

4.1. The influence of partner support to pair association levels and cleaner wrasse behavior

In natural conditions, male cleaner wrasses hold territories that encompass several breeding females, which they visit frequently [20, 34]. Usually, these males are found living and cleaning with the biggest female of their harem [20]. Partnership maintenance is characterized by partner-directed behaviors, which include: physical contact (either by

cleaning or providing tactile stimulation to each other, which we refer to as "partner support"), male to female punishment, and sexual displays. Our results indicate that pair association quality is related to how much partner support they receive from each other, for males in particular (but see Fig. 1a). Moreover, pair association is also related to how well cleaners engage in pair-joint interspecific cleaning events. Indeed, when cleaning in pairs, client size played an important role in cleaners' behavior, with more associated pairs producing a higher jolt rate in larger clients (better food sources). Although we were unable to ascertain the contribution of each sex to the overall jolt rate during these joint-cleaning events, we found that male and female cleaner wrasses behaved differently when cleaning unpaired. This appeared to be mostly driven by females, which tended to become more honest whenever more associated with their male partner. This should happen because males punish females that cheat and cause clients to leave [24, 25]. Consequently, females become more cooperative when cleaning in pairs [24]. Interestingly, these more associated females seem to maintain higher honesty levels when inspecting clients alone, which may mean that they continue to respond to male influence even when these do not directly participate in the cleaning event. Males living in more associated partnerships may be generally more present at

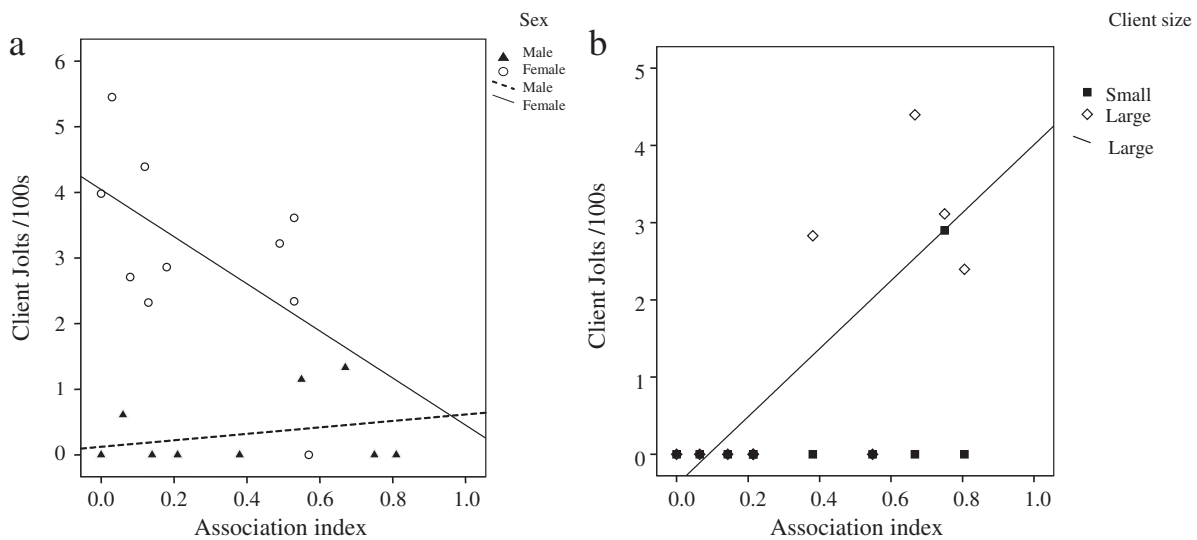


Fig. 2. The relationship between cleaner pairs' association index and: a) unpaired cleaner pair interspecific service quality (jolts) according to sex (male and female) and b) paired cleaner pair interspecific service quality (jolts) according to client size – small clients (total length < 11 cm) and large clients (total length ≥ 11 cm) – analysis was solely performed for larger clients.

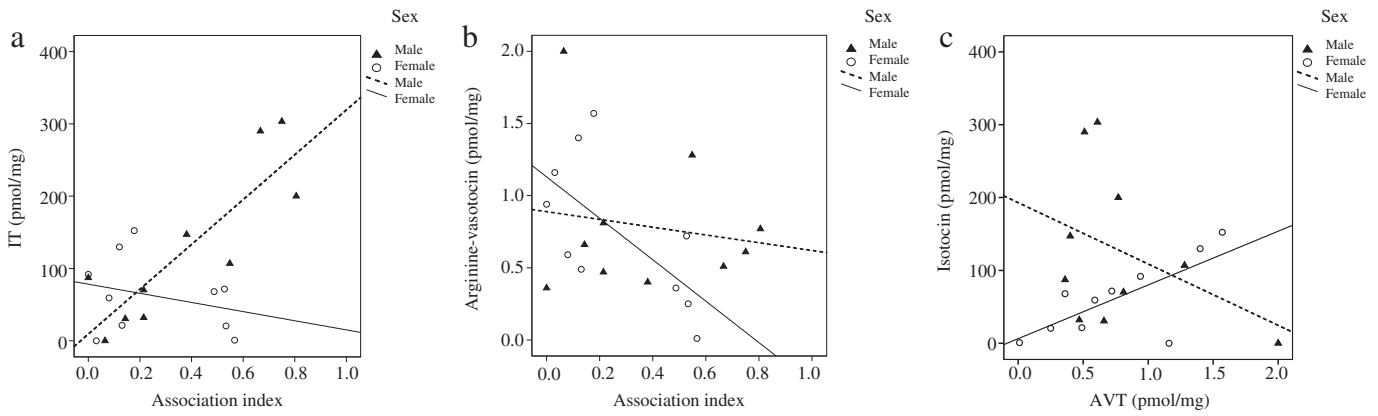


Fig. 3. The relationship between cleaner pairs' association level and: a) forebrain isotocin levels (pmol/mg), b) forebrain arginine-vasotocin levels (pmol/mg), and c) the relationship between forebrain isotocin and arginine-vasotocin levels (pmol/mg), according to sex (male and female).

cleaning stations (even when not engaging in joint cleaning interactions) and spend less time cleaning alone or visiting other (less preferred) females. The increase of the overall jolt rates during inspection of larger clients should most likely be due to male and not to female cheating behavior. The increase of female honesty works as a counterbalancing factor against male cheating level rises which allows for pairs to provide better cleaning service overall and become a better choice for these larger clients [24].

Contrary to previous studies done in controlled conditions [26,27], we did not observe a link between the increase of honesty in females and a rise of male to female punishment levels. However, we did find that more associated females provided more partner support (which includes tactile stimulation) to their male partners, a form of behavior typically used to prolong interactions and to reconcile with clients after cheating [35]. This could potentially contribute to a decrease in intra-couple tension and to a decrease in the need to punish. Nevertheless, these low frequencies of male to female punishment levels seem to suffice to promote a change in female behavior when cleaning in pairs.

4.2. IT, pair association and male cleaner wrasse stress levels

We found that more associated males received greater amounts of partner support provided by female cleaner wrasses, and exhibited

higher levels of forebrain IT. OT, the mammalian equivalent to IT, is released by positive physical contact between partners (which results in higher levels of plasma OT [15], and can regulate anxiety levels and stress coping [36–39]). In fish, Soares and colleagues [29] showed that physical contact alone is enough to reduce stress in a coral reef fish, however the link to IT brain levels is yet to be disclosed. Nevertheless, we can speculate that in male cleaner wrasses, the relatively high levels of forebrain IT may be related to the amount of support received, which could potentially underlie reductions of stress levels (as observed in other model systems, see [38]). Male higher brain IT levels may also correspond to a rise in partner tolerance, which would be expressed in an increase in time spent in joint-cleaning events without an increase in male to female punishment.

4.3. AVT, pair association and female cleaner wrasse behavior

Neuropeptides' modulating role in partner recognition and bonding have revealed that males and females respond differently. For instance, female rodents are more influenced by changes in exogenous OT and males by AVP changes [40–42]. However, few studies have solely focused on the effects of the AVP/AVT system as a mediator of the female component affiliative behavior, compared to those involving male models [43]. It is known that for female cleaner wrasses, the

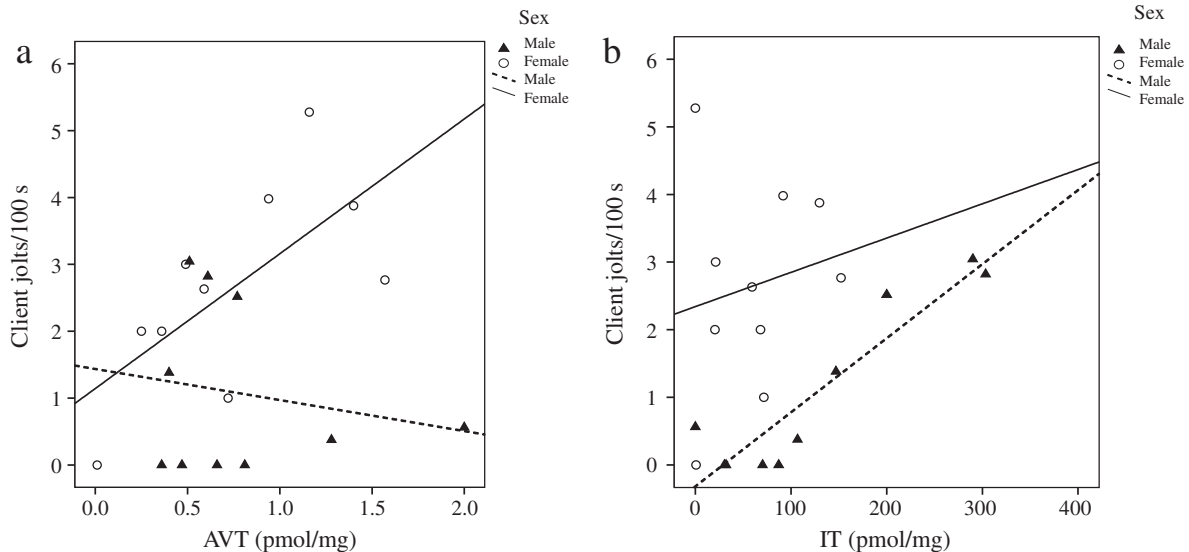


Fig. 4. The relationship between cleaner pair interspecific service quality (jolts) and a) forebrain isotocin levels (pmol/mg) and b) forebrain arginine-vasotocin levels (pmol/mg), according to sex (male and female).

exogenous elevation in the levels of AVT (via injection of receptor agonist) causes a decrease in their propensity to engage in interspecific cleaning activities, while the same is not observed for conspecific directed behavior [44]. This implies that high levels of AVT contribute to a reduction of cleaner wrasse propensity to cooperate with its clients, however, at a conspecific level AVT's influence on female behavior is still unclear. In the current study, female endogenous levels of forebrain AVT seem to be correlated to their cheating levels (see Fig. 4a), while the same was not observed in male cleaner wrasses. Also, higher client jolts rates were mostly observed whenever clients were inspected by sole females, which belonged to lower quality partnerships. The AVP/AVT system is highly implicated in the neuroendocrine stress response processes in vertebrates [45]: for example, in response to stress, gene expression of AVP/AVT is upregulated in parvocellular neurons in mammals and in teleost fish [46,47]. Female cleaner wrasses living in lower quality partnerships may suffer a rise in anxiety, promoted by a context of higher social instability, which could then be related to an increase in endogenous brain AVT levels. However, no direct significant link was found between female association index and their brain AVT levels.

4.4. Neuropeptide role in the modulation of cheating by pairs of cleaner wrasses

In the cleaner wrasse system, dominance is usually expressed in the amount of male to female punishment or in intraspecific female-to-female competition. In contrast, the amount of partner support (provided or received) should contribute to a decrease in intra-couple tension. All these variables influence intra-pair stability, which in turn will affect their opportunity to gain access to better food sources (clients). Individuals respond to social instability with a series of endocrine and neural adaptations that will not only modulate expression of brain neuropeptide levels but also of other endocrine compounds that work in an integrative mode, such as androgens and stress steroids [4]. Conversely, stability should also have an effect on individual physiological response. We found that male cleaner wrasses living in stronger/stable pair associations had higher levels of IT and also cheated more frequently. Female cleaner wrasses' cheating rates were also linked to their forebrain IT levels, but these were less dependent on pair association. Moreover, females with higher levels of forebrain AVT (which also corresponded with higher levels of IT) appeared to cheat more often. In contrast to males, females' higher cheating frequencies seem to relate to partnership instability. These females may clean more frequently alone and may thus be less controlled by their male partners.

4.5. Concluding remarks

Understanding how individuals decide and how their social environment influences these decisions may provide valuable insights on trade-offs and possible constraints that contribute to the maintenance and evolution of cooperative interactions. In our study, we show that different cleaner wrasse partnerships affect individual behavior in relation with two relevant neuropeptide systems (AVT and IT). Moreover, we identify a link between forebrain IT levels and cleaner wrasse behavior, which seems to depend on the level of pair association. Further testing is necessary to establish a causal relationship between forebrain IT level and pair association levels in accordance to sex, and how it may influence individual cleaner wrasse cooperative behavior.

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Author contributions

MCS designed the study. JRP and JPM collected the samples in the field. GIA and MG prepared and run HPLCs. JRP analyzed behavioral videos. SCC, ASG, EK and MSC analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.physbeh.2015.03.024>.

References

- [1] T.N. Sherratt, G. Roberts, The role of phenotypic defectors in stabilizing reciprocal altruism, *Behav. Ecol.* 12 (2001) 313–317.
- [2] J.M. McNamara, O. Leimar, Variation and response to variation as a basis for successful cooperation, *Philos. Trans. R. Soc. B* 365 (2010) 2627–2633.
- [3] J.B. Silk, Cooperation without counting: the puzzle of friendship, in: P. Hammerstein (Ed.), *Genetic and Cultural Evolution of Cooperation*, MIT Press, Cambridge, Massachusetts, 2003, pp. 37–54.
- [4] M.C. Soares, R. Bshary, L. Fusani, W. Goymann, M. Hau, K. Hirschenhauser, R.F. Oliveira, Hormonal mechanisms of cooperative behaviour, *Philos. Trans. R. Soc. B* 365 (2010) 2737–2750.
- [5] D. Kleiman, Monogamy in mammals, *Q. Rev. Biol.* 52 (1997) 39–69.
- [6] A. Fuentes, Re-evaluating primate monogamy, *Am. Anthropol.* 100 (4) (1999) 890–907.
- [7] L.J. Young, Z. Wang, The neurobiology of pair bonding, *Nat. Neurosci.* 7 (2004) 1048–1054.
- [8] K.C. Light, K.M. Grewen, J.A. Amico, More frequent partner hugs and higher oxytocin levels are linked to lower blood pressure and heart rate in premenopausal women, *Biol. Psychol.* 69 (1) (2005) 5–21.
- [9] J.R. Madden, T.H. Clutton-Brock, Experimental peripheral administration of oxytocin elevates a suite of cooperative behaviours in a wild social mammal, *Proc. R. Soc. B* 278 (2011) 1189–1194.
- [10] A.S. Smith, A. Ágmo, A.K. Birnie, J.A. French, Manipulation of the oxytocin system alters social behavior and attraction in pair-bonding primates, *Callithrix penicillata*, *Horm. Behav.* 57 (2010) 255–262.
- [11] M. Kosfeld, M. Heinrichs, P.J. Zak, U. Fischbacher, E. Fehr, Oxytocin increases trust in humans, *Nature* 435 (2005) 673–676.
- [12] T. Baumgartner, M. Heinrichs, A. Vonlanthen, U. Fischbacher, E. Fehr, Oxytocin shapes the neural circuitry of trust and trust adaptation in humans, *Neuron* 58 (2008) 639–650.
- [13] H.P. Nair, L.J. Young, Vasopressin and pair-bonding formation: genes to brain to behaviour, *Physiology* 21 (2005) 146–152.
- [14] K.A. Young, Y. Liu, Z. Wang, The neurobiology of social attachment: a comparative approach to behavioral, neuroanatomical, and neurochemical studies, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 148 (4) (2008) 401–410.
- [15] K.M. Grewen, S.S. Girdler, J. Amico, K.C. Light, Effects of partner support on resting oxytocin, cortisol, norepinephrine and blood pressure before and after warm partner contact, *Psychosom. Med.* 67 (2005) 531–538.
- [16] K. Uvnäs-Moberg, Oxytocin may mediate the benefits of positive social interaction and emotions, *Psychoneuroendocrinology* 23 (8) (1998) 819–835.
- [17] C. Crookford, R.M. Wittig, K. Langergraber, T.E. Ziegler, K. Zuberbühler, T. Deschner, Urinary oxytocin and social bonding in related and unrelated wild chimpanzees, *Proc. R. Soc. B* 280 (2013) 20122765.
- [18] C.T. Snowdon, B.A. Pieper, C.Y. Boe, K.A. Cronin, A.V. Kurian, T.E. Ziegler, Variation in oxytocin is related to variation in affiliative behavior in monogamous, pairbonded tamarins, *Horm. Behav.* 58 (2010) 614–618.

- [19] S.E. Taylor, S. Saphire-Bernstein, T.E. Seeman, Are plasma oxytocin in women and plasma vasopressin in men biomarkers of distressed pair-bond relationships? *Psychol. Sci.* 21 (1) (2010) 3–7.
- [20] D.R. Robertson, Social control of sex reversal in coral-reef fish, *Science*, 177, Science Publishers, Enfield (NH), 1972. 563–592.
- [21] I.M. Côté, Evolution and ecology of cleaning symbioses in the sea, *Oceanogr. Mar. Biol.* 38 (2000) 311–355.
- [22] R. Bshary, A.S. Grutter, Asymmetric cheating opportunities and partner control in a cleaner fish mutualism, *Anim. Behav.* 63 (2002) 547–555.
- [23] R. Bshary, I.M. Côté, New perspectives on marine cleaning mutualism, in: C. Magnhagen, V.A. Braithwaite, E. Forsgren, B.G. Kappor (Eds.), *Fish, Behaviour*, 2008, pp. 563–592.
- [24] R. Bshary, A.S. Grutter, A.S.T. Willener, O. Leimar, Pairs of cooperating cleaner fish provide better service quality than singletons, *Nature* 455 (2008) 964–967.
- [25] N.J. Raihani, A.S. Grutter, R. Bshary, Punishers benefit from third-party punishment in fish, *Science* 327 (2010) 171.
- [26] N.J. Raihani, A.I. Pinto, A.S. Grutter, S. Wismer, R. Bshary, Male cleaner wrasses adjust punishment of female partners according to the stakes, *Proc. R. Soc. B* 279 (2012) 365–370.
- [27] N.J. Raihani, A.S. Grutter, R. Bshary, Female cleaner fish cooperate more with unfamiliar males, *Proc. R. Soc. B* 279 (2012) 2479–2486.
- [28] J.L. Goodson, A.H. Bass, Forebrain peptides modulate sexually polymorphic vocal circuitry, *Nature* 403 (2001) 769–772.
- [29] M.C. Soares, R. Oliveira, A.F.H. Ros, A. Grutter, R. Bshary, Tactile stimulation lower stress in fish, *Nat. Commun.* 2 (2011) 534.
- [30] A. Kleszczyńska, E. Kulczykowska, Stocking density influences brain arginine vasotocin (AVT) and isotocin (IT) levels in males and females of three-spined stickleback (*Gasterosteus aculeatus*), *Gen. Comp. Endocrinol.* 183 (2013) 14–16.
- [31] M. Gozdowska, M. Ślebioda, E. Kulczykowska, Neuropeptides isotocin and arginine vasotocin in urophysis of three fish species, *Fish Physiol. Biochem.* 39 (4) (2013) 863–869.
- [32] S.J. Cairns, S.J.A. Schwager, A comparison of association indices, *Anim. Behav.* 34 (1987) 1454–1469.
- [33] A.F. Zuur, E.N. Ieno, C.S. Elphick, A protocol for data exploration to avoid common statistical problems, *Methods Ecol. Evol.* 1 (2010) 3–14.
- [34] Y. Nakashima, Y. Sakai, K. Karino, Female–female spawning and sex change in a harem coral-reef fish, *Labroides dimidiatus*, *Zool. Sci.* 17 (2000) 967–970.
- [35] A.S. Grutter, Cleaner fish use tactile dancing behaviour as a preconflict management strategy, *Curr. Biol.* 14 (2004) 1080–1083.
- [36] R.J. Windle, N. Shanks, S.L. Lightman, C.D. Ingram, Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats, *Endocrinology* 138 (1997) 2829–2834.
- [37] K. Uvnäs-Moberg, Oxytocin linked antistress effects—the relaxation and growth response, *Acta Physiol. Scand.* 161 (1997) 38–42.
- [38] I.D. Neumann, Brain oxytocin: a key regulator of emotional and social behaviours in both females and males, *J. Neuroendocrinol.* 20 (2008) 858–865.
- [39] S.E. Taylor, G.C. Gonzaga, L.C. Klein, P. Hu, G.A. Greendale, T.E. Seeman, Relation of oxytocin to psychological stress responses and hypothalamic–pituitary–adrenocortical axis activity in older women, *Psychosom. Med.* 68 (2) (2006) 238–245.
- [40] C.S. Carter, Neuroendocrine perspectives on social attachment and love, *Psychoneuroendocrinology* 23 (1998) 779–818.
- [41] T.R. Insel, L.E. Shapiro, Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles, *PNAS* 89 (1992) 5981–5985.
- [42] M.M. Lim, L.J. Young, Neuropeptidic regulation of affiliative behavior and social bonding in animals, *Horm. Behav.* 50 (4) (2006) 506–517.
- [43] B.C. Nephew, Behavioral roles of oxytocin and vasopressin, *Neuroendocrinology and Behaviour*, InTech, Rijeka, 2010.
- [44] M.C. Soares, R. Bshary, R. Mendonça, A.S. Grutter, R.F. Oliveira, Neuropeptide modulation of cooperative behaviour: arginine vasotocin decreases prosocial behaviour in cleanerfish, *PLoS ONE* 7 (2012) e39583.
- [45] T. Backström, J. Schjolden, Ø. Øverli, P. Thörnqvist, S. Winberg, Stress effects on AVT and CRF systems in two strains of rainbow trout (*Oncorhynchus mykiss*) divergent in stress responsiveness, *Horm. Behav.* 59 (2011) 180–186.
- [46] J.M. Aubry, V. Bartanusz, D. Jezova, X. Belin, Y. Kiss, Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity, *J. Neuroendocrinol.* 11 (5) (1999) 377–384.
- [47] B.J. Gilchrist, D.R. Tipping, L. Hake, A. Levy, B.I. Baker, The effects of acute and chronic stresses on vasotocin gene transcripts in the brain of the rainbow trout (*Oncorhynchus mykiss*), *J. Neuroendocrinol.* 12 (8) (2000) 795–801.