Physiology & Behavior 139 (2015) 314–320

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Arginine vasotocin reduces levels of cooperative behaviour in a cleaner fish

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HIGHLIGHTS

- In nature, cleaner fish need to invest in unrelated partners to yield current and future benefits.
- We confirm the importance of the AVT/AVP system as an agent affecting levels of cooperation.
- AVT offers a potential mechanistic pathway for the reported flexible service quality.

ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form 13 November 2014
Accepted 14 November 2014
Available online 20 November 2014

Keywords:
Cleaner fish
Cooperative levels
Labroides dimidiatus
Neuropeptides
Arginine vasotocin
Isotocin
Decision-making

ABSTRACT

Cooperation between unrelated individuals usually involves investments that often mean a decrease in immediate payoffs, but ensure future benefits. Here we investigated the potential role of the neuropeptides Arginine-vasotocin (AVT) and Isotocin (IT) as proximate agents affecting individuals' cooperative levels in the Indo-pacific bluestreak cleaner wrasse *Labroides dimidiatus*. Their 'client' reef fish partners only benefit from interacting if cleaners eat ectoparasites and refrain from gleaning preferred client mucus. Thus, cleaners must control their impulse to eat according to their preference, and eat less preferred items to maintain ongoing interactions and avoid clients' leaving or punishing. We found that solely the experimental transient higher dosage of AVT led to a decrease of cleaners' willingness to feed against their preference, while IT and AVT antagonists had no significant effects. The sole effect of AVT on cleaner's performance may imply a link between AVT's influence and a potential activation of a stress response. Our results confirm the importance of the AVT/AVP system as an agent affecting levels of cooperation, offering a potential mechanistic pathway for the reported flexible service quality that cleaners provide their clients.

1. Introduction

Cooperation between unrelated individuals often involves investments, which means a decrease in immediate payoffs (for the actor), in order to contribute to the enhancement of benefits in another individual [1]. Evolutionary models usually focus on questions related to potential strategies, which may ensure that investments yield future benefits and hence stabilise cooperation [1–3]. However, current models are agnostic about proximal mechanisms that need to be in place to enhance the individuals' ability to decide whether or not to invest.

Knowledge on how changes in an individual's physiological/neurological state affect cooperative and social behaviour is needed [4,5], in order to understand variation within and between individuals as well as between species. The neuropeptides arginine vasopressin (AVP) and oxytocin (OT) are well known modulators of a diverse range of vertebrate social processes and emotions, including that of humans [6–8]. For example, within humans, experimental setups aiming to increase OT levels have demonstrated that these mediate rises in prosociality, which include trust [9,10] generosity [11,12], empathy [12], and social memory [13], while behavioural manifestations of prosociality have now been linked to individual differences in rs53576 genotype of the OT receptor [14]. Partner support is also a good facilitator of increases in OT plasma levels in both men and women [15]. Regarding AVP, studies have now examined its effects (via intranasal administration) on human facial responses linked to social communication, revealing that AVP influences the response to ambiguous social stimuli [16] and that its effects are sex specific with respect to responses towards same-sex faces, i.e. agonistic in men and affiliative in women [17]. Finally, in a recent study, Rilling and colleagues [18], demonstrate that intranasal AVT and IT administration mediate biassed effects in human males and

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http://dx.doi.org/10.1016/j.physbeh.2014.11.052
0031-9384/

females interacting in a Prisioner's Dillemma task. Taken together, the above studies suggest that these systems offer a general mechanistic framework involved in the regulation of complex social processes.

The nonapeptides AVP and OT neural expression and gene regulation appear to be widely conserved across vertebrates and have peripheral (hormonal) as well as central (neuromodulator) actions [19]. Indeed, recent work in non-mammalian vertebrates indicates that the social function of OT may be ancient in terms of its evolutionary framework [20]. However, both systems are highly pleiotropic, affecting a wide range of behaviours across functional contexts (e.g. pair bonding, parental care, anxiety, memory, recognition, communication and aggression; for reviews please see [21,22]). For example, in teleost fish, studies have found a relation between IT and the increase of sociality in goldfish, *Carrasius auratus* [23], and with zebrafish, *Danio rerio* [24], while under the influence of AVT, Thompson and Walton [23] found that exogenous administration of AVT inhibited approach behavior. Regarding cooperative contexts, in meerkats (*Suricata suricata*), individuals treated with OT were observed to increase their investment in communal and cooperative activities [25], while in a cooperative breeding fish (*Neolamprogus pulcher*), IT increased the response to social information, namely in increasing individual sensitivity to differences in opponent size and aggressive feedback [26].

Here we use one of the best studied cooperative models, the Indopacific bluestreak cleaner wrasse *Labroides dimidiatus*, to investigate how changes in individuals' neuropeptide levels (IT and AVT systems) may be implicated in the mechanisms underlying the adjustment of individuals to the existence of partner control mechanisms in cooperative interactions between unrelated individuals. The cleaners are visited by the other reef fish species (so called clients) for ectoparasite removal [27,28]. A conflict of interest occurs because cleaners prefer to eat client mucus, which constitutes cheating [29]. As clients respond to non-cooperative cleaners with attacking (punishing), leaving or avoidance [30–32], cleaners need to adjust their feeding behaviour to feeding on clients' ectoparasites (against their preference). The problem can easily be abstracted in laboratory experiments involving plates and two types of food, where cleaner wrasses but not closely related species can learn to eat against their preference if that allows them to continue to forage [33,34]. This experimental paradigm has been used successfully in the last few years, having resulted in over a dozen published studies focusing on cleaner wrasses [29,30,33–44] and captures the essence of cleaning interactions as demonstrations of key results have been reproduced in experiments with real cleaner–client interactions [32]. We made use of the experimental design to test how the AVT and IT systems influence the cleaners' ability to feed against preference in order to prolong their foraging interactions.

Nonapeptides seem to be good candidates to modulate cleaner wrasses' decision-making, related to cleaning behaviour. In a first study concerning this system, Soares and colleagues [45] found that AVT administration caused a decrease on interspecific cleaning interactions, while its V1a receptor antagonist (Manning compound) had opposite effects in mediating a rise in cleaners' dishonesty via central effects on the V1a-type receptors. More recently, further support for the involvement of AVT on cleaning behaviour was provided by a comparative neuroanatomical study, where an association between AVT gigantocellular preoptic area (gPOA) neurons and the expression of cleaning behaviour in cleaning wrasses was found [46]. However, given AVT's overall effects regarding our system [45], it was still unclear how it would directly influence cleaners' predisposition to eat against preference and hence how it may contribute to conditional cooperative outcomes. Thus, we expect to find differences in the extent of neuropeptide influence to affect cleaner wrasses foraging decisions, namely that the blocking of AVT effects (via the V1a receptor antagonist Manning compound) should promote a decrease in cooperative levels (more eating according to preference, as it was mentioned in [45]) while the opposite should be observed by the agonist (AVT injection). Regarding IT, we predict that by exogenously increasing its levels, we may observe an enhancement of cleaners' ability to identify and properly respond to social stimuli, which should have a direct influence on their levels of feeding against preference. Nevertheless, concerning IT, few relevant results have been found so far, during previous manipulations in the wild [45].

2. Methods

2.1. Experiments

Experiments were conducted at the fish housing facilities of the Oceanário de Lisboa (Lisbon, Portugal). We used 9 wild caught cleaner wrasses that originated in Maldives and were directly imported to Portugal by a local distributor. The fish were kept in individual aquaria ($100 \times 40 \times 40$ cm) combined in a flow through system that pumped water from a larger cleaning tank ($150 \times 50 \times 40$ cm) that served as a natural filter. Each tank contained an air supply and a commercial aquarium heater (125 W, Eheim, Jäger). Small PVC pipes (10–15 cm long; 2.5 cm diameter) served as shelter for the fish. Nitrite concentration was kept to a minimum (always below 0.3 mg/l). Fishes were fed daily with mashed prawn flesh or a mixture of mashed prawn flesh and fish flakes spread on plastic (Plexiglas) plates [47].

2.2. Learning against preference task

We followed Bshary and Gruter [33] protocol, with some minor modifications. Cleaners learned to feed from the plates within 1–3 days of exposure. The plates had a variety of patterns (Fig. 1) and each cleaner was exposed to all different protocol steps (plaque pattern) as to become accustomed to the presentation of unfamiliar stimuli (to avoid potentially neophobic cleaners). The experiments began after the fish had been in captivity for at least 15 days. The "learning against preference task" consisted of three phases, namely: (a) an initial preference test; (b) learning phase; and (c) foraging test without any hormonal treatment. The plates used in the experiment were attached to a 40 cm long lever that allowed the experimenter to simulate the behaviour of the client fishes (fleeing, or just calmly leaving after the cleaner finished foraging).

In the initial preference test we offered the cleaners an unfamiliar plate with three prawn items and three flake items (Fig. 1). The sequence of the 6 items (prawn or flake) placed in the grid cells was determined by using tables of random sequences of 0 and 1, where 0 represented prawn and 1 represented flake. The cleaners could eat all items but plates were removed once a cleaner stopped feeding with items still remaining. After three trials that allowed cleaners to become familiar with the plates, we conducted the initial preference test. We then offered the plate three times to each cleaner and scored the first three items eaten. This meant that we could possibly find a 100% preference for either prawn or flakes.

In the learning phase each cleaner was subjected to six learning trials. Cleaners were trained such that eating the less preferred food items (fish flakes) had no consequences, while eating a preferred item led to the immediate removal of the plate ('fleeing'). In each trial, the plate was offered to the fish again after 60 s until the cleaner ate a second preferred food item. There were two parts in this phase: the first where we used a plate with 12 flakes and 2 prawns; and a second where we used a plate with 3 flakes and 3 prawns (equal number of the 2 different items).

In the foraging experiment each cleaner was allowed to interact once with the plate that did not respond to the cleaner's foraging behaviour. In other words, eating a preferred food item had no negative consequences. We scored the first 3 items eaten, allowing the possibility of a 100% bias for either food.

2.3. Neuropeptide treatment

This part consisted of the final foraging experiment but this time each cleaner was sequentially and haphazardly treated (intramuscularly) with

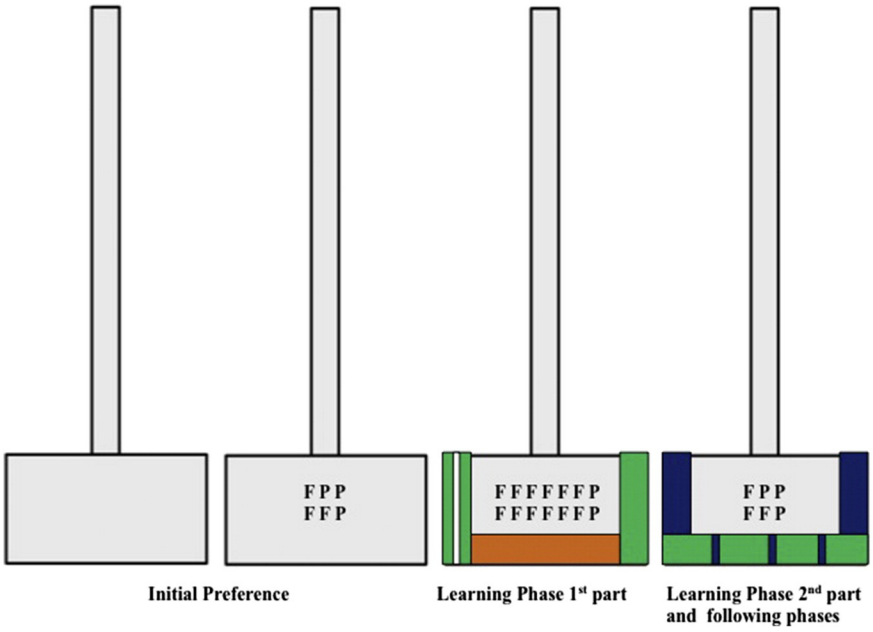

Fig. 1. Plate patterns during the different phases of the experiment. Each item is represented by one letter: F — fish flake and P — prawn.

the following compounds: saline (0.9 NaCl; reference treatment); AVT (V0130 — Sigma) at 0.5 μg/gbw (low dosage) and 2.5 μg/gbw (high dosage); IT (H-2520 — Bachem) at 0.5 μg/gbw (low dosage) and 2.5 μg/gbw (high dosage); Manning compound (V2255 — Sigma- [b-Mercapto-b,b cyclopentamethylenepropionyl1,O-me-Tyr2, Arg8]-Vasopressin) at 3.0 μg/gbw; .and Atosiban [Bachem: H-6722.0050 (50 mg) (Deamino-Cys1, D-Tyr(Et)2,Thr4,Orn8)-Oxytocin(RWJ 22164)]) at 3.0 μg/gbw. Injections were always given in the morning and on alternate days. Manning compound is a commonly used antagonist of the AVP type 1a receptors (V1a), which in teleost fish include both subtypes V1a1 and V1a2, that also have some affinity with the OT receptors in mammals [48]. Atosiban is an antagonist of the OT/IT receptors, but has also been referred to have some affinity for AVP/AVT receptors [48]. The use of these two antagonists is a tentative to disentangle the route of action of any effects of the nonapeptides. Each cleaner was weighed before the onset of the experiment so that the injection volume could be adjusted to body weight. Each cleaner was injected with each compound in the adequate volumes according to their weight (0.5 μl/gbw). After each injection, each cleaner was allowed to interact once with the plate that did not respond to the cleaner's foraging behaviour (similarly to part c of the "learning against preference task"). We also scored the first 3 items eaten, allowing the possibility of a 100% bias for either food item.

2.4. Ethical note

No fish suffered any detectable injury or mortality as a result of the injections or behavioural testing. The methods for animal housing, handling and experimental protocols were assessed and approved by the Portuguese Veterinary Office (Direcção Geral de Veterinária, licence # 0420/000/000/2009) and adhere to the ASAB/ABS Guidelines.

2.5. Statistical analysis

We used a counterbalance sequence (between and within subjects) in which each cleaner was used for all treatment compounds. Data were analysed using non-parametric tests because the assumptions for parametric testing were not met. Wilcoxon matched pair tests were used to evaluate whether each cleaner learned to eat against their preference, comparing with the initial preference and also comparing with the saline treatment. We also used Wilcoxon matched pair tests (uncorrected) to compare each hormonal treatment with the reference (saline) treatment. All tests were 2 tailed and were done in SPSS Statistics, version 22.

3. Results

3.1. Initial preference test and learning against preference task

All 9 cleaners ate more prawn items than flakes in the initial phase preference test (80%, Fig. 2). Cleaners that had been exposed to the plaques being removed after they had eaten prawn, ate significantly less prawn items after the learning against preference phase than during the initial preference tests (Wilcoxon-test, $n = 9, Z = -2.67, p =$ 0,008, Fig. 2). The effect was still significantly observed when cleaners were injected with saline ($n = 9, Z = -2.31, p = 0.02$, Fig. 2). There were no significant differences between the prawn eaten by the cleaners after they learned to eat against their preference and when they were treated with saline ($n = 9, Z = -1.82, p = 0.069$, Fig. 2).

3.2. Final foraging experiments with neuropeptide treatments

We found that only individuals treated with the high dosage of AVT increased their preference for prawn compared with the saline group, after they had successfully learned to eat against preference ($n = 9$, $Z = -2.02$, $p = 0.04$, Fig. 3). None of the remaining treatments produced significant foraging differences (AVT 0.5 vs saline: $n = 9, Z = -0.06, p = 0.95$; IT 0.5 vs saline: $n = 9, Z = -1.61, p = 0.11$; IT 2.5 vs saline: $n = 9, Z = -1.02, p = 0.31$; Manning compound vs saline: $n = 9$, $Z = -1{,}12$, $p = 0.26$ and Atosiban vs saline: $n = 9, Z = -0.71, p = 0.47$, Fig. 3).

4. Discussion

In this study we asked if AVT or IT were implicated in the regulation of cleaner wrasse's decisions to feed against their preference, as they have to do under natural conditions, in order to cooperatively eat ectoparasites instead of preferred mucus. In the first part of our study, we could replicate earlier studies which had shown that removal of a food source in response to cleaners eating preferred food items leads to

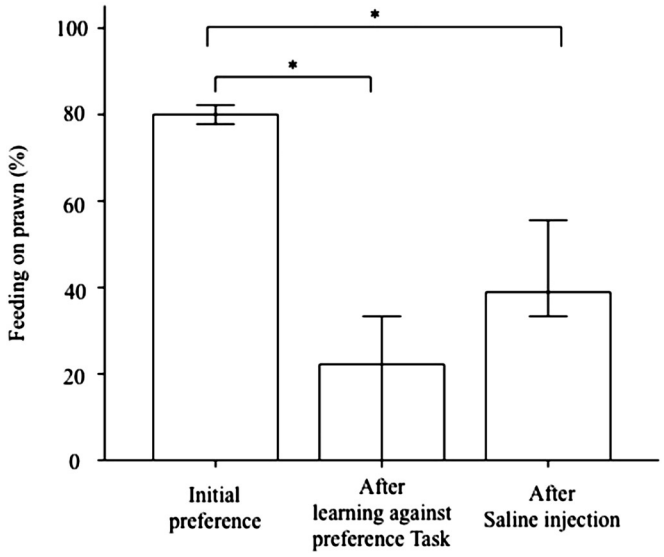

Fig. 2. The percentage of prawn items eaten in an initial preference test, after the individuals learned to eat against their preference and when treated with saline solution. Shown are the medians and the interquartiles. Symbols above bars represent P values which refer to Wilcoxon matched pair tests (*, $p < 0.05$). Sample sizes (number of individual cleaner fish) are of n = 9 for all experimental phase groups.

cleaners selectively feeding against their preference [33]. All nine subjects behaved accordingly. Then our main experiment tested how these neuropeptides affect their willingness to eat unpreferred food first. Against our predictions, the administration of AVT rather than the blocking of its V1a-type receptors by the antagonist Manning compound, caused a decrease of cleaners' levels of feeding against preference. IT and its antagonist (Atosiban) had no measurable effects. It is possible that none of the dosages used (for agonist and antagonist) was appropriate to result in significant effects. As for the lack of effects observed in the treatment with the V1a-type antagonist Manning compound, these might also be underlined by its affinity for OT/IT-type receptors [48]. The sole effect of AVT highest dosage on cleaners performance may imply a link between AVT's influence and a potential activation of a stress response. We also explore the potential links between AVT and IT effects in social memory and recognition and/or impulsive choice control (absence or presence of self control) in the following sections of the discussion.

4.1. Neuropeptide modulation of cleaners' stress levels

The effects of AVT on cleaner wrasses' behaviour may be produced via the activation of a stress response, since AVT influences adrenocorticotropin (ACTH) production and thus cortisol secretion [49,50]. For instance, Huffman and colleagues [50] have recently shown that exogenous administration of AVT is able to produce significant rises in the circulating cortisol levels in both dominant and subordinate individuals. When occurring naturally, changes in cortisol concentration (allostatic load) may preclude a shift in animal's energetic demands, which are sometimes associated with predictable or unpredictable adaptations, resulting in more access to mates, food sources or for instance shifts in social status [51]. For cleaners, recent experiments demonstrate that elevations in cortisol levels are responsible for the decrease of cooperative levels amongst those cleaners that cheat more often (e.g. "biting" cleaners [52]). Thus, in the cleaner fish system, the increase of circulating cortisol levels does not lead to a reduction in activity levels (as shown by Huffman and colleagues [50] for cichlids) but is otherwise associated with significant behavioural strategic changes [52]. Interestingly,

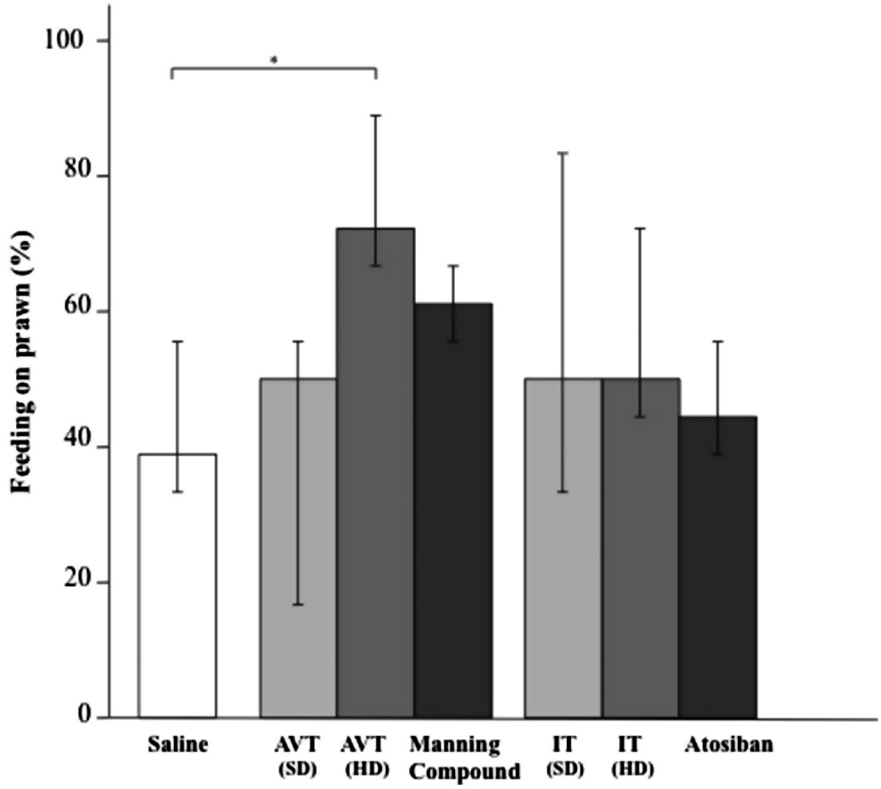

Fig. 3. The percentage of prawn items eaten in all treatment groups: saline, AVT (smaller dosage — SD), AVT (higher dosage — HD), Manning compound, IT (SD), IT (HD) and Atosiban. Light grey bars represent a smaller dosage and dark grey bars represent a higher dosage of AVT and IT, while black bars represent its antagonists (Manning compound and Atosiban, respectively). Shown are the medians and the interquartiles. Symbols above bars represent P values which refer Wilcoxon to matched pair tests for each neuropeptide treatment against the reference (saline) group (*, $p < 0.05$). Sample sizes (number of individual cleaner fish) are of n = 9 for all groups of treatments.

similarly to what is observed in the current study regarding the effect of antagonists Manning compound and Atosiban, the treatment with glucocorticoid antagonist also did not produce significant changes on cleaners' cooperative levels [52]. It is thus quite possible that the sole effects observed by AVT treatment (in its higher dosage) are being produced via the activation of the hypothalamic–pituitary–interrenal (HPI) axis, which causes an increase of available circulating cortisol [53] and facilitates a behavioural shift responsible for a decrease of cleaners' levels of feeding against preference.

4.2. Neuropeptide modulation of cleaner fish social memory and recognition

There is experimental evidence that cleaners can recognise individual clients [54] and that cleaners adjust to past experience with them [30,33]. Thus, memory and social recognition play a role on cleaners' competence to interact with clientele. There is ample evidence that in mammals, OT and AVP affect an individual's ability to remember individuals (usually conspecifics) [55]. In rodents, AVP is crucial for the enhancement of social recognition and social memory [56–58] while in humans it seems to affect social communication, demonstrating biassed effects between genders [16–18]. Moreover, sex steroids and glucocorticoids (as mentioned above) may interact with AVP and OT in various ways [59–61]. For example, both peripheral injections and chronic central infusions of AVP into castrate male rats reduce their social recognition skills towards juvenile conspecifics [62]. Castration seems to reduce AVP expression in various limbic brain areas [63], which are known to directly influence reward learning of social interactions [61]. For cleaner wrasses, elevation in the levels of AVT decrease cleaners' propensity to engage in interspecific cleaning activities [45]. As cleaners are protogyneous hermaphrodites and our subjects are most likely females, it is possible that AVT's influence in our system may work similarly to how AVP affects castrated rats: due to a potential short-term social recognition disruption, cleaners would then fail to identify and anticipate the response of the clients, which leads to behaving freely according to their preferences.

The effects of the OT/IT system are also highly associated to the functions of social recognition [64]. The development of the OT knockout mice further established the role of OT in social recognition [65–67]. While in rodent models, AVT facilitates social information consolidation, the OT's major role is at the level of acquisition [68]. If IT in cleaner wrasse was shown to similarly function at the level of acquisition, this would explain why we did not find significant effects of IT and its antagonist in the current study or during previous manipulations in the wild [45]. Another possibility is that significant effects would only be produced in more appropriate contexts, for instance if we had male–female cleaner couples in our laboratorial conditions. Indeed, recent data (Cardoso et al., unpublished data) has found a link between forebrain IT levels and cleaner wrasse's less cooperative behaviour however, it is dependent on variation in intra-pair relationship. This would contribute to refute the importance of the chosen-dosage hypothesis (above mentioned). Future testing is definitely needed so as to further demonstrate the role of IT in this system.

4.3. Neuropeptide modulation of impulsive choice control

Lack of impulse control has been associated with reduced activity of the serotonergic system [69]. For example, in humans impulsive violent behaviour is indicative of low serotonin turnover rate [70,71], while lower levels of serotonin have been demonstrated to result in stimuli response inhibition, leading to a rise in impulsivity and aggression [72]. Research mostly done in rodents has confirmed that serotonin has a key role in altering the secretion and release of AVP and OT [73], which were supported by previous evidence that demonstrated direct interactions between serotonin and the AVT/AVP systems (serotonin diminishes aggression by altering the activity of the AVP system [74]). For instance, the treatment with fluoxetine, a selective serotonin reuptake inhibitor, seems to be responsible for a decrease of AVP brain levels (in hamsters) and lowering of AVT mRNA abundances (in teleost fish) [75–77]. On the contrary, a hyporeactive serotonin system may result in enhanced AVP activity and aggression in both animals and humans [78], which contributes to a rise in impulsive or reactive behaviour towards stimuli. Indeed, in humans, the temporary acute lowering of serotonin seems to lead to a reduction of punishment induced inhibition without affecting motor response, while the magnitude of this inhibition is dose dependent [79]. These findings seem to correspond to our observed cleaner wrasse behavioural response: only the higher dosage of AVT increased cleaners' tendency to eat more preferred items (a response that contradicts what they had previously learned) and which was probably sustained on an expectation of aversive outcome (plate being removed from the aquarium).

4.4. Concluding remarks

Our results further confirmed the importance of the AVT/AVP pathways for the regulation of interspecific cooperative behaviour. The trade-off between payoffs at different points in time (inter-temporal discount) is experienced by cleaner wrasses in the wild, which need to overcome the tendency to gain immediate benefits to achieve cooperative stability, as to secure the possibility of future interactions [29]. Considering the potential role of AVT pathways mediating stress response and, in the regulation of impulse control or even in social memory and recognition, we could expect that these effects may also contribute in the assessment of clients' valence and also in the facilitation of the behavioural choice to apply in a following encounter. Future studies employing controlled pharmacological experiments with other compounds (such as serotonin or dopamine), brain region-specific analyses and specifically, testing the value of future rewards, will be necessary to better understand molecular pathways involved in cooperative interactions.

Funding statement

This study was financed by Fundação para a Ciência e Tecnologia (FCT, grant PTDC/MAR/105276/2008) to MCS. During this study SCC was supported by a FCT Ph.D. Fellowship. MCS is supported by the Project "Genomics and Evolutionary Biology", co-financed by North Portugal Regional Operational Programme 2007/2013 (ON.2 — O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF). RB is supported by the Swiss National Foundation. RFO is supported by FCT strategic plan PEst-OE/MAR/UI0331/2011.

Author contributions

MCS and RB designed the study. JRP and SCC collected the data. MCS and SCC analysed the data. SCC, MSC, RFO and RB wrote the paper. All authors discussed the results and commented on the manuscript.

Acknowledgments

We thank the principal curator of Oceanário de Lisboa (Núria Baylina) and staff for logistical support. The animal procedures used in this study have been approved by the Portuguese Veterinary Office (Direcção Geral de Veterinária, licence # 0420/000/000/2009).

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