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NEUROENDOCRINOLOGY OF COOPERATION:

The role of Neuropeptides on the modulation of Mutualistic Behaviour of the Indo-Pacific Cleaner Wrasse (*Labroides dimidiatus*)

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À minha querida Marta
Tudo começa e acaba em ti...

Abstract

Interspecific cleaning interactions are a classical textbook example of mutualistic cooperation. One of the most notorious cleaning mutualisms involve the Indo-Pacific Bluestreak cleaner wrasse *Labroides dimidiatus*, which are known to interact with an average of 2300 visitor reef fish per day. In contrast to the increasing knowledge on the functional aspects of cleaning mutualisms in the last decades, their underlying physiological mechanisms are still relatively rare. One major class of neuromodulators that is involved in the control of social behaviour and that seems to be co-opted for the regulation of cleaning behaviour is a group of nonapeptides of the arginine vasopressin /oxytocin family (AVP/OT). The general aim of this study is to attempt to link functional aspects of decision-making underlying the cleaning behaviour with their proximate mechanisms, namely to determine the contribution of the neuropeptides arginine vasotocin (AVT) and isotocin (IT) for the regulation of adjustments of individual cleaner wrasses' behavioural output.

This study yielded several findings. First, I tested the influence of AVT upon the cleaners' ability to solve two different problems that in principle differ in ecological relevance and are associated with two different memory circuits and found that AVT affected the learning competence of cleaners as individual performance showed distinct response selectivity to AVT dosage levels. However, only in the ecologically relevant task was their learning response improved by blocking AVT via treatment with the antagonist Manning compound. Next I examined if neuropeptides may be implicated in the mechanisms underlying the adjustment of individuals to the existence of partner control mechanisms in cooperative interactions between unrelated individuals and discovered 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. Then I asked if the establishment of privileged ties and the quality of association between cleaner wrasse pairs is correlated with neuroendocrine mechanisms involving forebrain neuropeptides and whether these neuropeptides level shifts relate to individual's interspecific service quality. Here I found that variation in pairs' relationship influence male and female cleaner fish differently and contribute to the variation of brain neuropeptide levels, which is linked to distinct cooperative outcomes. Finally, I explore the link between these neuroendocrine pathways and the expression of mutualistic behaviour

in fishes by comparing the brain quantitative distribution of AVT and IT across the overall and in selected areas of the brain; aiming at four closely related species of labrids that differ in the degree to which they depend on cleaning. The levels of both AVT and IT varied significantly across species, as measured in the whole brain or in specific macro-areas. More importantly, significantly higher AVT levels in cerebellum and in the whole brain were found in the obligate cleaner species, which seems to be related to expression of mutualistic behaviour.

Overall, my study suggests that the neuropeptidergic system but mostly AVT pathways play a pivotal role in the regulation of interspecific cooperative behaviour and conspecific social behaviour among stabilized pairs of cleaner wrasses.

Keywords: Interspecific cooperation; Cleaning mutualisms; *Labroides dimidiatus*; Neuroendocrinology; Neuropeptides; Arginine vasotocin; Isotocin

Resumo

As interações interespecíficas de limpeza são exemplos clássicos de cooperação mutualística. Um dos mais emblemáticos exemplos de mutualismo de limpeza envolve o bodião limpador do Indo-Pacífico *Labroides dimidiatus* que interage em média com cerca de 2300 espécies de peixes do recife de coral por dia. Estes bodiões removem ectoparasitas e tecido infectado ou morto da superfície dos peixes que os visitam (referidos como clientes) e dependem exclusivamente das interações mutualísticas de limpeza para obter o seu alimento. Os bodiões limpadores encontram-se em territórios específicos, designados por *estações de limpeza*, e vivem num sistema de harém (espécie poliginica e protogínica). As interações de limpeza são geralmente iniciada pelos clientes, que adoptam uma posição específica ficando imóveis e abrindo a boca, opérculos e barbatanas peitorais, que demonstram assim que desejam ser limpos. Por sua vez, os limpadores exibem um comportamento específico de dança, nadando para cima e para baixo, chamando assim a atenção dos seus clientes. Uma estratégia de pré-conflito utilizada pelos limpadores é a da estimulação táctil, durante a qual, usando as barbatanas pélvicas e peitorais, “massajam” o corpo do cliente durante a inspecção, e enquanto o faz não se alimenta. Apesar do crescente conhecimento sobre os aspectos funcionais do mutualismo de limpeza, pouco se sabe sobre os mecanismos fisiológicos que estão na base destas interações de cooperação. O grupo dos nonapeptidos da família da Arginina vasopressina/ Oxytocina (AVP/OT) é uma classe importante de neuromodeladores que está relacionada com a regulação do comportamento social e que por conseguinte poderá estar envolvida na modelação do comportamento cooperativo de limpeza. Um estudo recente realizado no campo demonstrou que a administração exógena de AVT no bodião limpador contribui para a diminuição as interações de limpeza, sem afectar da mesma forma as suas relações conspécificas destes limpadores. O principal objectivo deste estudo é relacionar os aspectos funcionais das tomadas de decisão que determinam o comportamento de limpeza com os seus mecanismos proximais, isto é, tentar perceber de que forma os neuropéptidos, a Arginina vasotocina (AVT) e a Isotocina (IT) (homólogos nos peixes da AVP e OT) contribuem para a regulação da flexibilidade das respostas comportamentais dos bodiões limpadores.

Em primeiro lugar, testei em laboratório a influência da AVT sobre a capacidade do bodião limpador resolver duas tarefas sociais que diferiam na sua relevância ecológica, e

que estão associadas a dois circuitos de memória diferentes. Verifiquei que a AVT influenciou a capacidade de aprendizagem do limpador e o seu comportamento revelou ser sensível a diferentes dosagens deste neuropéptido. Na tarefa de aprendizagem por “pista” - a tarefa ecologicamente relevante - o limpador melhorou a sua aprendizagem quando a AVT foi bloqueada através da administração de um composto antagonista – o composto Manning. Já na aprendizagem espacial, tarefa ecologicamente não relevante para o contexto de limpeza, apenas o tratamento com uma dose baixa de AVT diminui a capacidade de aprendizagem do bódio limpador.

Seguidamente, foi examinado, também em condições controladas, se os neuropeptídeos estariam implicados nos mecanismos que ajustam o controlo exercido pelos parceiros sociais envolvidos neste tipo de interação interspecífica. Os bódios limpadores preferem o muco (com alto valor energético) aos ectoparasitas mas a fim de assegurarem relações duradouras com os seus clientes precisam comer contra a sua preferência, controlando a impulsividade de ingerirem apenas o que preferem. Verificou-se que apenas uma dose elevada de AVT levou os bódios limpadores a comerem menos vezes contra a sua preferência. Os antagonistas da AVT e da IT não revelaram ter resultados significativos.

Depois foi estudado (em condições naturais) se o estabelecimento de ligações privilegiadas e a qualidade da associação dos casais de bódios limpadores estariam relacionados com os mecanismos neuro endócrinos que envolvem os neuropéptidos no *forebrain* (área cerebral associada ao comportamento social) e ainda se os níveis de neuropéptidos nesta área cerebral influenciariam a qualidade do serviço de limpeza prestado quando limpavam sozinhos. Ficou demonstrado que a variação do índice de associação dos casais de limpadores influencia machos e fêmeas de forma distinta e contribui para a variação dos níveis de neuropeptídeos no cérebro, que por sua vez leva a respostas comportamentais diferentes. Os machos *L. dimidiatus* que estabeleciam pares com maior índice de associação tinham também valores de IT e eram mais desonestos (comiam a favor da sua preferência) com maior frequência. De igual modo, as taxas de “batota” das fêmeas pareceram relacionar-se com os seus níveis de IT no forebrain, mas menos dependentes do índice de associação. Fêmeas com valores mais elevados de IT e AVT demonstraram ser mais desonestas, mas contrariamente aos machos, as fêmeas apresentaram mais “batoteiras” pertenciam a casais onde existiam maior instabilidade.

Por último analisei a ligação entre as vias neuroendócrinas e a expressão do comportamento mutualístico ao comparar os níveis de AVT e IT no cérebro (como um todo e nas diferentes áreas cerebrais que o compõem) em quatro espécies de labrídeos próximos filogeneticamente mas que diferem na expressão do comportamento de limpeza (*L. dimidiatus* e *Labroides bicolor* – limpadores obrigatórios, *Labropsis australis* limpador facultativo e um não limpador - *Labrichthys unilineatus*). Verificou-se que os níveis de AVT e IT variavam significativamente entre espécies quando medidos como um todo e em cada uma das macroáreas cerebrais (cerebelo, tecto óptico, tronco cerebral e *forebrain*). Os limpadores obrigatórios, *L. dimidiatus* e *Labroides bicolor*, apresentaram níveis de AVT significativamente maiores no cerebelo e no cérebro total, o que pode estar relacionado com a expressão do comportamento mutualístico. Os níveis de IT não parecem estar relacionados directamente com a expressão do comportamento mutualístico mas os valores elevados de IT no *L. unilineatus* sugerem que estes nonapéptidos poderão estar relacionados com o desenvolvimento do dimorfismo sexual, que apenas existe na espécie de não limpador (todas as outras espécies são monomórficas).

Em resumo, o meu estudo sugere que o sistema neuropeptidérgico, em especial as vias neuronais de AVT desempenham um papel crucial na regulação do comportamento cooperativo interespecífico e comportamento social conspecífico entre casais estáveis de budiões limpadores.

Palavras-chave: Cooperação interspecífica; Mutualismo de limpeza; *Labroides dimidiatus*; Neuroendocrinologia; Neuropeptidos; Arginina vasotocina; Isotocina.

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Author's statement

This thesis produced four scientific research papers:

Chapter 2: Cardoso SC, Bshary R, Mazzei R, Paitio JR, Oliveira RF, Soares MC (2015) Arginine vasotocin modulates learning in the mutualistic cleanerfish *Labroides dimidiatus*. Behavioral Ecology and Sociobiology (*accepted*).

Chapter 3: Cardoso SC, Paitio JR, Oliveira RF, Bshary R, Soares MC (2015) Arginine vasotocin reduces levels of cooperative behaviour in a cleaner fish. Physiology and Behavior 139, 314-320.

Chapter 4: Cardoso SC, Grutter AS, Paula JR, André GI, Messias JP, Gozdowska M, Kulczykowska E, Soares MC. Forebrain neuropeptide regulation of pair association and behaviour in cooperating cleaner fish. Physiology and Behavior (*in review*).

Chapter 5: Cardoso SC, Kulczykowska E, Gozdowska M, André GI, Paula JR, Ślebioda M, Oliveira RF, Soares MC. Brain levels of nonapeptides in labrid fish species with different levels of mutualistic behaviour. (*to be submitted*).

CHAPTER
1

General Introduction

1. Cooperation and mutualistic behaviour

Biologists have been fascinated by the topic of cooperation for more than one hundred years. Organisms are conceived in the sense of promoting their successful evolution, investing only in their own benefits in detriment of their competitors (Nowak, 2006). Cooperation is defined as any interaction between individuals, in which the costs of investment of partnership supersede the benefits in question (Sachs et al., 2004) with an increase in fitness for all of those involved (Buston and Balshine, 2007). The ‘problem’ of cooperation arises because natural selection favours individuals that maximize their benefits and minimize their costs. A crucial issue is therefore individual fitness, which is to be measured by the real value of one’s investment against the outcome of cooperative actions.

In recent years, functional research on the diversity, evolution and stability of cooperative behaviour has generated a considerable amount of knowledge (Sachs et al., 2004). Major advances have been achieved in the basic understanding concerning the evolution of social behaviour, solving the confusion between the basic condition and the mechanisms that promote cooperation (i.e. an increase of the helper’s inclusive fitness either through direct or indirect benefits), and the ecological settings (e.g. low migration, or high between group competition) that cause a positive selection on helping. In short, research has followed four evolutionary hypotheses of cooperation: Kin selection, mutualism, reciprocity or retribution, and manipulation (Krebs and Davies, 1993). For instance, the kin selection hypothesis explains the cooperation between related individuals, where the co-operant individual can obtain direct genetic benefits thanks to the reproductive success of his related conspecific (Hamilton, 1964). Mutualism hypothesis (different from interspecific cooperation described further on the text) is referred as cooperation where direct genetic benefits are involved for all the individuals participating in the social interaction; for instance when two individuals work together in order to defend a mating territory and, that way, increasing the likelihood of a successful reproduction of both individuals. As an alternative, the evolutionary hypothesis of reciprocity or retribution solely provides direct immediate benefits to the receiving individual of the cooperative act, and the co-operant may also benefit from this cooperation, though only in the future (Trivers, 1971). Lastly, the manipulation hypothesis states that individuals behave in a cooperative way because they are compelled or coerced to do so, mainly because the costs of not cooperating are too high. As an example we have

the individuals hatching eggs of others alongside with theirs, not because they may have some kind of benefit, but simply because the attempt of eliminating the other eggs may destruct their own eggs. However, in general, individuals may cooperate simply because this is a way of enhance their own survival probabilities or of reproductive success, therefore acquiring genetic benefits either in the present or in the future. They can even obtain indirect genetic benefits by cooperating with kin related individuals; therefore their offspring can also benefit (Griffin and West, 2003). Cooperation does not exclusively occur within the same species. In fact when occurring between individuals of different species is often referred to as Mutualism (Bshary & Bergmüller, 2008). Trivers (1971) proposed that both cooperative types, intraspecific and mutualistic might evolve and be maintained when the same individuals interact with each other repeatedly. His idea of "reciprocal altruism" was formalized about a decade later by Axelrod and Hamilton (1981), when they found, in a computer simulated tournament - involving a large variety of strategies -, that a strategy named "Tit-for-Tat", could emerge as a cooperative solution for the game. In the "Tit-for-Tat" game, the player starts the game cooperating and, in subsequent rounds, the player response according to his partner behaviour in previous rounds. Nonetheless, the proposed strategies set by theoreticians, as "Tit-for-Tat", were not adequate to understand cooperative interactions in nature, especially interspecific interactions. Three other theoretical concepts became promising frameworks to explain the evolutionary stability of cooperation and mutualism: By-product mutualism, Pseudoreciprocity and Biological Market Theory. By-product's mutualism is applied to intraspecific and interspecific cooperative interactions and it is settled in the following assumption: each individual acts selfishly while the benefit arises as a simple "by-product" of its behaviour (Brown, 1983). Oddly enough, as there is no investment, one cannot actually say it is an altruistic behaviour. Pseudoreciprocity differs from By-product mutualism in two ways: a) one or more partners invest mutually; (b) the investment makes to cooperate as the best option for the investing partner (Connor, 1986). The Biological Market Theory (Noë, 2001) proposes that mutualistic interactions can be seen as the exchange of goods and services between partners, although differing in their degree of control on the change of those same goods and services. Partners should be selected amongst a likely number of partners and the emphasis of the theory has been to understand how the level of supply/demand of the exchanged services and goods, in combination with the opportunity of choosing the partner, may set the exchange value (Noë, 2011; Hoeksema e Swartz, 2003). The concepts of by-product, mutualism, pseudo reciprocity

and the choosing partners (Biological Markets) have revealed to be more adequate in the understanding of persistent cooperative behaviour in the mutualistic interactions. Both cooperative interactions (intra and interspecific) can be maintained if the parties involved keep the involved benefits in the relationship (Komdeur, 2006). There are several examples of these mutualistic interactions in nature: coral reefs (polyps and zooxantellae), diverse pollinators mediating the reproduction of many species of plants, clown fish and its relation with anemones, ants its relation with fungi, and cleaning mutualisms.

1.1 Cleaning Mutualisms

Cleaning mutualism refers to the removal of ectoparasites and dead or infected tissue by species named *cleaners* from other species that actively visit them, know as *clients*. There are examples of fish, birds and crustaceans which acquire their food through cleaning other individuals, usually larger than them. Though these interactions exist in several environmental contexts, they also occur in marine environments where both the parties involved are usually fish (Losey et al., 1999; Côté, 2000). Obligate cleaner fish, which exhibit a large degree of specialization, belong to *Labridae* and *Gobidae* families and feed almost exclusively from what they remove from their *clients* (Losey et al., 1999; Bshary, 2001). These *cleaners* are mostly found in specific territories known as *cleaning stations*, places actively visited by their *clients* and where they adopt an incentive position (they lay still, or in an oblique position), showing their desire to be inspected (Côté, 2000; Bshary, 2001). The *cleaner*, on his turn, drives to the *client*, inspecting the body surface, gills and mouth, removing ectoparasites, mucus, and the dead or ill tissue (Losey et al., 1999; Côté, 2000). These cleaning interactions, apparently simple, involve complex behaviours and strategies, alongside presenting constant challenges for the individuals involved. Hormones help animals to react and solve complex and practical issues, acting as mediators of short and long-term behavioural and physiological responses (Adkins-Reagan, 2005).

2. Hormones, social behavioural modulation and social behaviour network (BSBN)

Hormones should not be seen as causal agents but instead as modulators, changing the probability of expression of a certain behaviour, given they act on the neural mechanisms underlying behavior (Oliveira, 2005). Of all hormones present in vertebrates, the groups that have received the most attention and emphasis are sex hormones (androgens, estrogens), stress hormones (cortisol) and neuropeptides (arginine-vasopressin (AVP)/oxytocin (OT)).

Newman (1999) proposed the existence of a brain social behaviour network (BSBN) in mammals, with bidirectional connections between pairs, and which is involved in the regulation of countless types of social behaviour; from aggression up to affiliation, bonding and parental behaviour. Afterwards, Goodson (2005) confirmed the presence of this network in other classes of vertebrates, even identifying homologous areas for each node in the different classes and/or taxonomy. We may find in the mammal's BSBN the following areas/nodes: the extended medial amygdala (medial amygdale, mAMY, and the medial bed nucleus of stria terminalis, BNSTm), the lateral septum, the preoptic area (POA), the anterior hypothalamus (AH) and the ventromedial hypothalamus (VMH), and, also, the periaqueductal grey (midbrain) in mammals and other vertebrates' homologous structures (Goodson, 2005). In teleost fish were identified six areas/nodes which are determinant for the regulation of their social behaviour: the supracommisural nucleus of the ventral telencephalon (Vs; amygdala homologue); POA; AH; ventral nucleus of the ventral telencephalon (Vv, septum homologue); anterior tuberal nucleus (at least part of this nucleus is homologous to the VMH) and midbrain (Goodson, 2005). Steroids' receptors and neuropeptides exist in different nodes of this network, suggesting that social behaviour is associated with the modulation of these hormones. Hormonal steroids are also known for regulating neuropeptides' synthesis and their receptors, thus acting as indirect factors in the neuromodulator system of the AVP/OT. This system acts on a peripheral level and at the central brain's level and it can even affect behaviour expression on mutual levels, as it occurs with dopaminergic reward system and with the serotonergic system (Skuse and Galager, 2009). Therefore, the endocrine modulation of social behaviour can occur in multiple forms and in an integrated manner: a) peripheral effects (sex hormones and stress hormones – such as cortisol, neuropeptides - AVT/OT); or centrally, by the direct effects of the neuropeptides, or by the neurotransmitters (dopaminergic and

serotonergic systems); and, lastly c) through the effect of peripheral hormones in the central neurotransmitters (Fig. 1). Neuropeptides of the AVP/OT's family have, thus, the capacity of altering this neural network and therefore the behavioural response (Goodson, 2005), becoming then optimal candidates for regulating the behaviours expressed during the mutualistic interactions.

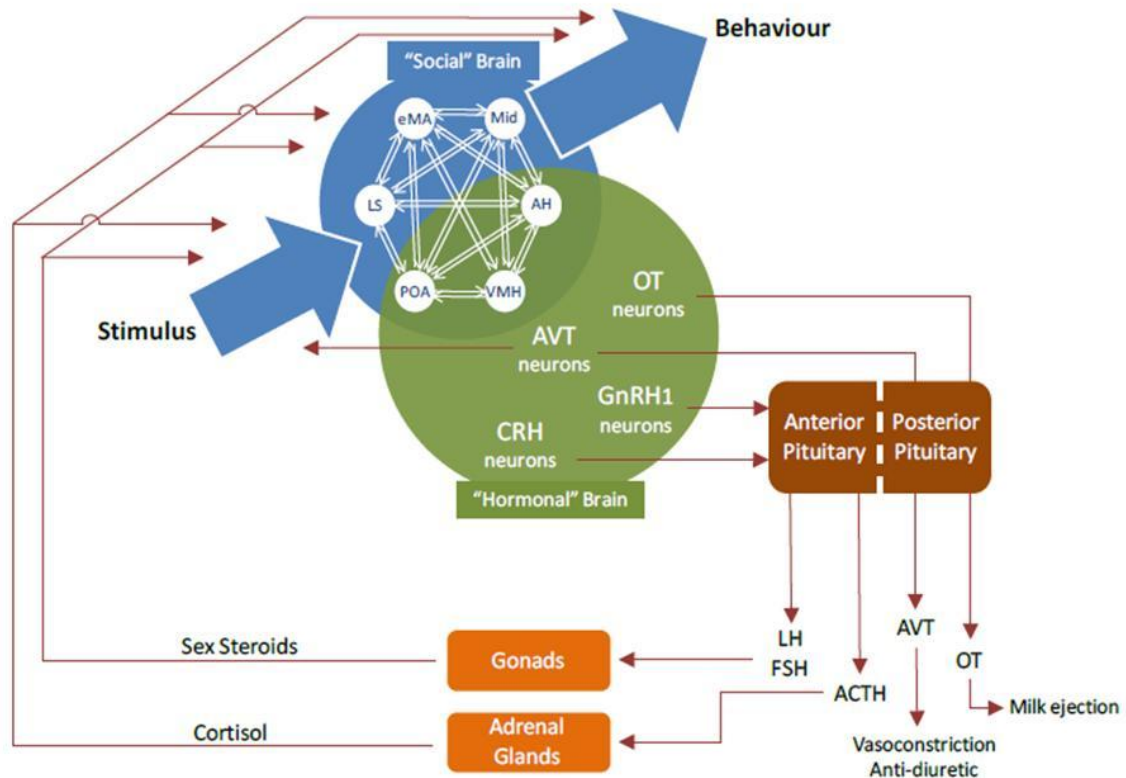


Figure 1. Hormones modulation on BSBN via central effects of neuropeptides and steroids produced by peripheral glands (from Soares et al., 2010).

3. Neuropeptides as modulators of social behaviour

The regulation mechanisms of neuropeptides on behaviour have been under discussion in numerous studies in the past few decades. The AVP and OT found in mammals and their homologous in non-mammals (arginine vasotocine – AVT), mesotocine (MT, in birds, reptiles and amphibians) and isotocine (IT in teleost fish) - Table 1, are known currently by playing a determinant role in the modulation of social behaviour (Godson and Bass, 2001; Lim and Young, 2006).

Table 1. Biochemical evolution of nine-amino-acid neuropeptides (adapted from Insel and Young, 2000).

Isotocin	C Y I S N C P I G A	Fish	C Y I Q N C P R G A	Vasotocin
Mesotocin	C Y I Q N C P I G A	Amphibia	C Y I Q N C P R G A	Vasotocin
		Reptiles		
		Birds		
Oxytocin	C Y I Q N C P L G A	Mammals	C Y F Q N C P R G A	Vasopressin

3.1 The Arginine Vasopressin/Oxytocin's (AVP/OT) System

AVP is a neurohypophyseal hormone differing only of OT hormone in two positions in the sequence of the nine amino acids which compose it. The genes of these two peptides arose prematurely in the vertebrates' lineage, from a duplication event: these genes are in the same chromosome, separated by a small intergenic zone and there are transcribed in opposite directions (Burbach et al., 2001; Donaldson et al., 2008). AVP has peripheral functions, acting at the level of blood vessels through the AVPR1a receptor in the vasoconstriction's control and, moreover, through the AVPR2 receptor in the kidney, in the control of the hydrous' equilibrium (Barberis et al., 1992; Hiber et al., 1999). That is also the reason why AVP is also known between endocrinologists as the antidiuretic hormone (ADH). OT is, as AVP (and their non-mammal homologous), synthesized in the periventricular nucleus (PVN) and supraoptical (SON) of the hypothalamus. The cells of these region project axons into the posterior part of the pituitary gland, where they release OT into the bloodstream in the neurohypophysis (Burbach et al., 2006). The peripheral effect of the OT is essentially related with reproduction (For example: induction of uterine contractions during the process of giving birth, and milk production). Nevertheless, AVP and OT's action refers not only at a peripheral level (as stated above), but also in an extensive list of social behaviours, from aggression and reproductive behaviour to affiliate behaviour, such as parental behaviour, pair bonding and social recognition (Goodson and Bass, 2001; Lim and Young, 2006; Kaverne and Curly, 2004; Burbach et al., 2006; Young, 2008). Regarding OT, it is know only one receptor but AVP has three different receptors (V1a, V1b and V2). These receptors distribution is heterogeneous, the majority of V2 receptors can be found in the periphery, all of the receptors can be found in the brain, and

the two subtypes of V1 receptors regulate behaviour. In other words, in order to distinguish the effects and actions of AVT, we need specific antagonists and agonists for each receptor.

AVP and OT's role in the formation of social memories, namely in the learning process of distinguishing familiar and non familiar individuals, has been studied in lab rats (reviewed by Lim and Young, 2006). Dantzer and his colleagues (1987) were the first to show the OT's involvement in the formation of social memories, and this was further demonstrated with transgenic lab rats without OT genes (OT-knockout). These lab rats did not present any deficit in olfactory abilities or in any other kind of the learning process, but could not recognize familiar individuals, even after several interactions (Ferguson et al., 2000). And with a simply OT administration before an interaction, these rodents would recognize a given individual in a posterior interaction (Ferguson et al., 2002). In monogamous voles, OT has a very important role in partner recognition and in pair bonding (Young and Wang, 2004). Recently has been demonstrated that OT has a very important effect in social recognition in humans. The intranasal administration of OT improves the ability of recognizing familiar faces, but it had no effect in non-social stimuli memorization (Rimmele et al., 2009). As for the involvement of AVT in social recognition, Feifel and colleagues (2009) have shown it by discovering the Brattelboro rat, that fail to exhibit AVP, and which are completely unable of social recognition. Thus lays the demonstration that AVP influences the olfactory recognition in rats.

For animals, the valence (to be positive or negative) assessment of social stimuli is very important. The amygdala has a fundamental role in providing valence to a social stimulus, and it is considered the centre of danger detection in the brain, since it is activated when a potential danger appears (LeDoux, 2007). Responding to the contact with unfamiliar conspecifics or predators, the amygdala is activated and it enables an anxiety or flee response (Stein et al., 2002). Whalen and his colleagues (1998) have shown that when humans are presented with scared faces, there is activation of the amygdala while Meyer-Lindenberg and his colleagues (2005) have shown that diminishing the activation of the amygdala is associated to hyper sociability. OT and AVP seem to have opposite effects in the activation of the amygdala and, consequently, in the determination of a social stimuli valence. It has been observed that, in humans, the inhalation of OT reduces the activity of the amygdala in response to scared faces presentation (Kirsh et al, 2005). However, AVP

seems to enhance the subjective perception of threat, even in emotionally neutral stimuli (Thompson et al., 2004; 2006). The opposite effects of OT and AVP in the amygdala activation seem to regulate the expression of autonomous signals of fear, which may affect the motivation for cooperation, that is, they influence the trust in a partner in a social context. OT intranasal administration has enhanced trust behaviour and diminished the activation of the amygdala in a trust game (Baumgartner et al., 2008). Therefore, OT seems to enhance the trust by diminishing the amygdala's activation, and even diminishing the stress state associated to the probability of non-reciprocation. Social interactions may be compensatory, leading to the repetition of interactions with the same partner. It is the neuronal dopaminergic mesolimbic pathway that, through the circuit of reward learning, allows encoding the valence of the reward's signal in social interactions. The dopaminergic mesolimbic pathway seems to be involved in decision making, when we are referring to reciprocal interchange (Rilling et al., 2008). OT and AVP receptors interact with the dopaminergic receptors in voles reward centre (Young and Wang, 2004) and, potentially, in other mammals (revised by Fisher et al., 2006). In mammals, OT seems also to be involved in regulating prosocial behaviour in the context of cooperation (Madden and Clutton-Brock, 2011). These peptides' regulate behaviour however this is dependent accordingly to species, gender, phenotype and social context (Goodson and Bass, 2001). The neuroanatomical expression pattern and the physical regulation of OT/AVP genes are extraordinary similar in vertebrates. Venkatesh and collaborators (1997) have illustrated in their study the notable "conservation" of genetic expression of OT/AVP system in the molecular mechanisms of regulation.

3.2. Social Behaviour in Teleostean: Modulation of AVT and IT neuropeptides

Brief description of AVT and IT neuroanatomy

So far, in fish studies (amongst cartilaginous and osseous), the AVT and IT neurons are almost exclusively found in the preoptic area (POA), in the anterior hypothalamus area - even though there have also been identified cellular populations of AVT in the anterior tuberal hypothalamus in several species, such as: *Porichthys notatus* (Goodson et al., 2003), *Astatotilapia burtoni* (Greenwood et al., 2008) and in two butterflyfish species' - *Chaetodon multicoloratus* e *Chaetodon miliaris* (Dewan et al., 2008). In the POA, one can find three neural groups: The parvocellular (pPOA), the magnocellular (mPOA) and the gigantocellular (gPOA).

3.2.2 Modulation in social behaviour

The effect of AVT and IT neuropeptides in fish behaviour has motivated a great number of experimental studies, most of all in the scope of reproduction. For example, it was found a positive association between AVT and the behavioural changes of courtship (Carneiro et al., 2003; Lema and Nevitt, 2004), aggressive behaviour (Santagello Bass, 2006; 2010) and pair bonding in monogamous species of fish (Oldfield and Hofmann, 2011). Studies in other species have found a negative association between AVT and social behaviour (Goodson and Bass, 2001; Lema and Nevitt, Basitian et al., 2001). Thompson and Walton (2004) studied the effect of these neuropeptides regarding approach to conspecific stimuli and found that under the effect of AVT, individuals *Carassius auratus* failed to approach conspecifics while the opposite was observed when administering IT. Recently in a comparative study Dewan and colleagues (2011) found a relationship between the AVT system and social behaviour focussing on seven different species of butterflyfish species (*Chateodontidae*), which affected aggressive, affiliate and mating behaviour. Moreover, AVT and IT, promoted sociability if administered peripherally in zebrafish (Braidia et al., 2011). Reddon and his colleagues (2012) observed that intraperitoneal injection enhanced the sensitivity to social stimuli and the rate of submissive behaviour in social cichlids that cooperate (*Neolamprologus pulcher*). Studies in another monogamous cichlid, *Amatitlania nigrofasciata*, revealed an IT role in the promotion of parental care, but did not affect the pair bonding of previously established couples (O'Connell et al., 2012).

In short, behavioural neuroendocrinology studies have mostly focussed on conspecific relations and behaviours, often in the context of reproduction. However, not much is known regarding the physiological basis of cooperative behaviour. The relationship between the Indo-Pacific cleaner wrasses, *Labroides dimidiatus*, and their coral reef fish clientele is a classic example cooperation and mutualistic behaviour (Trivers, 1971). Thus, it is a prime candidate to study the action of neuropeptides in the context of interspecific cooperative behaviour.

4. *Labroides dimidiatus* study model and endocrinology of mutualistic cleaning behaviour

The obligate cleaner wrasse *L. dimidiatus* individuals are highly specialized cleaners, depending exclusively from what they obtain from clients (Côté, 2000; Summer et al., 2005). The cleaner wrasses inhabit small areas referred to as *cleaning stations*. They are harem protogynous hermaphrodites, a single male dominates several females, and these females may eventually change sex (Nakashima et al., 2000). Cleaner wrasses advertise their cleaning services through a ritualized dance, luring the attention of their clients (Côté 2000; Becker et al., 2005). This seems a perfect association, considering the clients are free from the harms of parasites; while the cleaning wrasses have easy access to a meal (Côté, 2000; Bshary and Côté, 2008). However, conflicts arise because the cleaning wrasses frequently feed on mucus and fish scales of its clients (Grutter and Bshary, 2003), which is referred to as “cheating”, and is harmful for the clients' health (Grutter, 1997; Grutter and Bshary, 2004). In order to prevent wrasses from *cheating*, clients developed several control mechanisms which assure the cooperation/honesty of cleaners (Bshary and Grutter, 2004; Mills and Côté, 2010): clients may switch between cleaning stations, or pursue the “dishonest” wrasse (punishment), and in the case of predators may even try to eat the cleaners (Bshary and Wurst, 2001; Bshary and Grutter, 2002, 2005; Mills and Côté, 2010). Male cleaner wrasses also use this last control mechanism (punishment) with female wrasses when they make the client leave the cleaning station due to dishonesty during the cleaning process (Raihani et al., 2010). Usually, punished females become more cooperative in later interactions (Raihani et al., 2012). During a cleaning inspection, cleaner wrasses provide tactile stimulation with their pectoral and pelvic fins, massaging their clients (Grutter, 2004; Soares et al., 2011). Tactile stimulation is used to: (a) manipulate clients to stay longer in cleaning stations (Bshary and Wurst, 2001); (b) lure the watching audience that the cleaner is honest (image scoring) to attract other clients with a higher nutritional value (Bshary, 2002, Bshary and Grutter, 2006; Soares et al., 2014); (c) And, finally, as a conflict management strategy - cleaners usually initiate the cleaning interactions with predator fishes by providing tactile stimulation (Grutter, 2004). Tactile stimulation provision is a costly investment for cleaners because these do not feed during the process (Bshary and Wurst, 2001). Tactile stimulation has a calming/relaxing effect for the clients, lowering their stress levels and enhancing their fitness (Ross et al., 2011; Soares et al., 2011).

In summary, there is an extensive knowledge on the behaviour and ecology of this species, while less is known on the physiological mechanisms underlying cleaner wrasses

cleaning behaviour. Recently Soares and colleagues (2012) have shown that AVT regulates cleaning behaviour. When injected intramuscularly with AVT, cleaner wrasses diminished their cleaning activities, as well as the quality of the service they give when compared with individuals injected with a saline solution (control solution). When injected with AVT's antagonist, the Manning compound (Manning et al., 2008), cleaners inspected more clientes, however the quality of the service was lower. Interestingly, the exogenous administration of AVT produced no effects in reducing conspecific interactions of cleaning wrasses as it happened with their clients. On the contrary, there was a predisposition enhancement to interact with conspecifics, accompanied by an increasing number of tactile/massage stimulation during those said interactions (Soares et al., 2012). AVT's involvement in the regulation of cleaning behaviour was tested once again in a comparative neuroanatomical study between the cleaning wrasses (*L. dimidiatus*) and another phylogenetically close species of wrasse that never cleans, the corallivorous *Labricthys unilineatus*. Mendonça and colleagues (2013) found smaller and less numerous AVT-producing neurons in the POA in the cleaner species. Recently, Soares and colleagues (2014) demonstrated that changes in cortisol levels are associated with behavioural shifts in the cleaning service provided by cleaners to their clients. Neurotransmitters such as serotonin and dopamine seem to also have a modulating and motivating role in the expression of cleaning behaviour (Paula et al, *in press*, Messias et al, *in review*).

5. Objectives and outline of the thesis

This thesis intends to contribute to the knowledge of physiological basis of interspecific cooperative behaviour, namely the influence of neuropeptides in the modulation of cleaning behaviour between the cleaning wrasse *L. dimidiatus* and clientes.

In chapter two, I asked if the learning performance of the cleaner wrasse is regulated by AVT. I tested the influence of AVT upon the cleaners' ability to solve two different problems that in principle differ in ecological relevance and are associated with two different memory systems. Namely, the first experiment (cue discrimination) is socio-ecologically more relevant while the second experiment (place discrimination) is not. Furthermore, the two different learning tasks presumably rely on different neural substrates.

In chapter three, I investigated the potential role of the neuropeptides AVT and IT as proximate agents affecting individuals' cooperative levels. As mentioned above, '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.

Taking into consideration the first field study by Soares and colleagues (2012) which demonstrated that AVT pathways play a pivotal role in the regulation of interspecific cooperative behaviour and conspecific social behaviour among stabilized pairs of cleaner fish, in chapter four I tried to find out more about the underlying physiological mechanisms involved in the establishment of pair privileged ties and its relationship to individual cooperation levels. In chapter four I tested two hypotheses: a) whether intra-pair association (i.e. association index), measured with joint interspecific cleaning and intraspecific behaviour, is correlated with neuroendocrine mechanisms involving forebrain AVT and IT and b) whether these neuropeptides level shifts relate to individual's interspecific service quality.

In chapter five, I explore the link between the neuropeptidergic pathways and the expression of cleaning behaviour in fishes by comparing the brain quantitative distribution of AVT and IT across the overall and in selected areas of the brain. For that I selected four closely related species of cleaner fish belonging to the family Labridae: two are obligatory cleaners throughout their entire life (*L. dimidiatus* and *Ls bicolor bicolor*), one species is a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous), and one is a non-cleaner species, corallivorous throughout its entire life (*L. unilineatus*).

Finally, in chapter six I synthesise all of my findings and comment on the importance of the neuropeptidergic system for the modulation of this classic example of cleaning mutualism: the cleaner wrasse system.

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CHAPTER
2

**Arginine vasotocin modulates learning in
the mutualistic cleanerfish *Labroides
dimidiatus***

ABSTRACT

Social environmental complexity induces structural and biochemical changes in animals' brains, which are linked to the improvement of animals' learning abilities. The neuropeptide arginine-vasopressin (AVP) has been shown to play a significant role in the regulation of social behaviour, particularly in the formation of social memories and individual recognition. Given that distinct neural systems, which may vary in the distribution of AVP receptor expression, are implicated in differences in individual learning processes, here we asked if the learning performance of the Indo-Pacific bluestreak cleaner wrasse (*Labroides dimidiatus*) is regulated by arginine-vasotocin (AVT). We tested the influence of AVT upon the cleaners' ability to solve two different problems that in principle differ in ecological relevance and are associated with two different memory systems. We found that AVT affected the learning competence of cleaners as individual performance showed distinct response selectivity to AVT dosage levels. However, only in the ecologically relevant task was their learning response improved by blocking AVT via treatment with the antagonist Manning compound. Our findings demonstrate that AVT pathways, which are implicated in the regulation of interspecific behavior (i.e. a cleaner's willingness to seek interactions with clients), are also linked to individual learning ability in the context of mutualistic behavior, and in tune with socio-ecological demands.

Keywords: Cleanerfish; Learning abilities; *Labroides dimidiatus*; Neuropeptides; Arginine vasotocin; Social-ecological demands

Introduction

In cooperative contexts, learning and memory are prerequisites that enable individuals to decide where, when and how to invest without being cheated by their counterparts (Brosnan et al. 2010). These decision rules are also imperative to the process of choosing amongst potential partners and to determine the course of action of each interaction. All of these behavioral modules require distinct discriminative abilities that rely on specific types of learning and memory and may be underlined by different anatomical and neurochemical circuits in the brain. Recent empirical studies show that the neural basis of learning and memory processes are identical between teleost fish and other vertebrates, namely mammals (Salas et al. 2006). Like tetrapods, teleost fish rely not only on egocentrically referenced mechanisms for orienting, based on stimulus-response simple associations but are also able to use allocentric map-like representation of the environment (Nadel 1991; Broglio et al. 2003; Salas et al. 2003) In mammals, the pallial amygdala is linked to emotional learning and memory, whereas the hippocampus is involved in spatial, contextual, or relational memory (Portavella et al. 2002). In teleost fish, evidence shows that some cognitive abilities might be related to the forebrain, specifically to the dorsolateral (Dl) and the dorsomedial (Dm) telencephalon, which are most likely homologues of the hippocampus and amygdala of mammals, respectively (Northcutt 1995; Salas et al. 1996a,b; López et al. 2000a,b; Wullimann and Mueller 2004). For example, training goldfish in a spatial context produces a significant and selective spatial learning-related increase of protein synthesis of Dl neurons (Vargas et al. 2000). Likewise, lesions studies show that Dl lesions cause impairments in place learning and memory in goldfish when trained in a plus-maze surrounded by distal visual cues (Rodriguez et al. 2002). The involvement of Dl in spatial cognition seems to be extremely selective, as damage to this area does not affect cue learning or other egocentric strategies (Salas et al. 1996a; López et al. 2000a).

Apart from knowing the neural mechanisms involved in learning, it is also important to study how these mechanisms are modulated by hormones and neuromodulators that contextualize them into an internal state of the organism. Given their acknowledged role in the regulation of social behaviour (Goodson and Bass 2001; Lim and Young 2006; Goodson and Thompson 2010), nonapeptides of the arginine-vasopressin (AVP) family (arginine vasotocin, AVT, in non-mammals) are major candidate modulators of socially relevant learning, which for instance occurs in mutualistic relationships. The

central behavioural actions of AVT/AVP are mainly mediated by its V1a receptor subtype in both mammals and non-mammals (Everts and Koolhaas 1999; Lema 2010; Lema et al. 2010). These central actions are vital for the formation of social memories and for individual recognition in mammals (Lim and Young 2006). In fish AVT has been implicated both in the regulation of reproductive and aggressive behaviours among conspecifics (e.g. promotion of territorial behaviour – Santangelo and Bass 2006a, b; changes in courtship behaviour – Carneiro et al. 2003; Lema and Nevitt 2004; pair formation- Oldfield and Hofmann 2011, and in the regulation of interspecific cooperative behaviour, cleaning mutualisms – Soares et al. 2012; Mendonça et al. 2013) The role of AVT in the regulation of interspecific interactions has also been recently addressed in the context of cleaner fish mutualisms. It was found that AVT administration caused a decrease on interspecific cleaning interactions, while its antagonist (Manning compound) had opposite effects in mediating a rise in cleaners' dishonesty (Soares et al. 2012). Nevertheless, the role of AVT on cognitive abilities underlying cooperative behaviour in fish has not been investigated yet.

In this paper, we asked if the same neuropeptide that was implicated in the regulation of interspecific behavior was also linked to individual learning ability in the context of their mutualistic behavior. For this purpose we used the cleaner wrasse *Labroides dimidiatus* to study AVT' effects on learning of two tasks, distinct in terms of socio- ecological relevance. These cleaner wrasses live on coral reefs throughout the tropical Indo-Pacific and feed exclusively on other visiting reef fish (usually referred as client fish) ectoparasites, mucus and scales (Randall 1958; Côté 2000; Bshary and Côté 2008). One of the unique characteristics of this system comes from a conflict of interests between partners since cleaners prefer to cheat by eating client mucus, which is detrimental to the client (Grutter and Bshary 2003). Cleaners manage this conflict of interest by adjusting service quality to client identity and situation. For example, they are able to distinguish between predator and non-predator clients as well as between familiar and unfamiliar individuals (Tebbich et al. 2002; Bshary et al. 2011), and to behave more cooperatively if bystander clients are present (Bshary and Grutter 2006; Pinto et al. 2011). A widespread ability of cleaners is to largely feed against their preference, so that clients do not terminate interactions prematurely or fail to return for another inspection (Bshary and Grutter 2003; Bshary and Grutter 2005). Thus, it appears that cleaners are able to calculate payoffs without overvaluing current benefits against both immanent and delayed future benefits (Grutter and Bshary 2003).

In laboratory conditions, the learning process can be investigated by using Plexiglas plates “clients” offering food as proxies for “real clients”. This experimental paradigm has been used extensively and successfully in the last few years, both on cleaner wrasses (Grutter and Bshary 2003; Bshary et al. 2011; Bshary and Grutter 2006; Bshary and Grutter 2003; Bshary and Grutter 2005; Wismer et al. 2014; Gingins et al. 2013; Raihani et al. 2012a,b,c; Raihani et al. 2010; Salwiczek and Bshary 2010; Danisman et al. 2010; Bshary et al. 2008) and on other model system of marine cleaning mutualism such as the Caribbean cleaning gobies *Elacatinus* spp and the sabre tooth blennies *Plagiotremus* spp (Soares et al. 2010; Bshary and Bshary 2010; Bshary and Bshary 2012). This experimental paradigm captures the essence of cleaning interactions as demonstrations of key results have been reproduced in experiments with real cleaner-client interactions (Pinto et al. 2011). Moreover, Wismer and colleagues (2014) found that cleaner wrasses that lived in a socially simple environment (i.e. small isolated reefs instead of continuous fringing reefs) failed to learn and perform in ways consistent with previous studies; thus, variation in social context and familiarity with clients may have major relevance to this system. In the current experimental design, we used different colour patterns, which allow for individual identification of plates. We mimicked a situation that occurs regularly under natural conditions, namely that two clients seek service simultaneously (Bshary and Côté 2008). In such situations, cleaners have to make a choice which client to inspect first, with the risk that the ignored client will leave (Bshary and Schäfer 2002). In these experiments, the ignored plate was always immediately removed by the experimenter. Client species differ with respect to their nutritional value, i.e. parasite load (Grutter 1994; Bansemmer et al. 2002; Soares et al. 2008), and we introduced this aspect by offering food on one plate only. Thus, cleaners had to learn to identify the food source.

We conducted two experiments that differed with respect to the relevant cues available to identify the correct choice. In experiment 1, cleaners had to identify the plate that consistently provided food, while in experiment 2; they had to identify a location where food was consistently provided. As cleaners exclusively feed on the surface of clients that are themselves mobile, the first experiment (cue discrimination) is socio-ecologically more relevant while the second experiment (place discrimination) is not. Furthermore, the two different learning tasks presumably rely on different neural substrates, with cue learning being Dm dependent whereas place learning is Dl dependent. AVT seems to be a good candidate to affect both types of learning since V1a receptors are widely distributed throughout the forebrain in other teleost species (Huffman et al. 2012).

However, variance in the expression of nonapeptide receptors on areas of relevance for learning and memory, such as the Dl and Dm may allow for differential effects of AVT on Dl and Dm dependent learning. Here we predict that, given the general effect of AVT on social behaviour and its already established effect on cleaner fish cooperative behaviour in particular, AVT should preferentially alter (i.e. inhibit) the putatively Dm-dependent and ecologically relevant task of cue learning.

Methods

Experiments

Experiments were conducted at the fish housing facilities of the Oceanário de Lisboa (Lisbon, Portugal). We used 12 wild caught *L. dimidiatus* that originated in Maldives and were directly imported to Portugal by a local distributor. The fish were kept in individual aquaria (100x40x40 cm) combined in a flow through system that pumped water from a larger cleaning tank (150x50x40 cm) that served as a natural filter. Nitrite concentration was kept to a minimum (always below 0.3 mg/l). Each tank contained an air supply and a commercial aquarium heater (125W, Eheim, Jäger). Small PVC pipes (10–15 cm long; 2.5 cm diameter) served as shelter for the fish. Each aquarium was divided into two compartments separated by an opaque partition. The partition could be closed or opened by pulling up the partition. Experiments were carried out between February and November 2012 in the individual tanks of each fish. Before the start of the experiments, fish were trained to feed from a Plexiglas plate as a substitute for the client fish that serve as food source in nature. Cleaners took approximately three days to learn to eat from plates.

Experiment 1: Cue discrimination task

For each treatment we had two plates (acting as clients) with two different color patterns: one was deemed to be the one from which cleaner needed to start eating first (correct pattern) and the second that could wait but had no food/reward available (incorrect pattern). All plates were initially introduced to cleaners, the day before the start of experiments, with a small piece of prawn in the back. Cleaners were submitted to one session, in alternate days. Each session was composed of 10 trials, until the individual had learned (three successive sessions in which the individual chose the correct pattern plate in at least seven out of ten trials, two successive sessions in which the cleaner chose the correct pattern plate at least eight out of the 10 trials or just one session with at least nine

out of 10 trials). The first trial began 10 minutes after the compound treatment and consisted of successive presentation (10 minutes inter-trial interval) of the two pattern color plates until the completion of 10 trials. The “correct” pattern plate had available prawn while the “incorrect” pattern plate had inaccessible prawn (covered with transparent tape which prevented cleaners to smell the item) at the back, so out of sight for cleaners during the choice process. Experimental individuals would then have complete access to both plates; a correct choice would enable both plates to stay inside the aquarium, while an incorrect choice would result in immediate removal of the correct plate, preventing cleaners from eating the food item (Fig. 1).

Experiment 2: Place discrimination task

In this task, the cleaner was challenged with learning a specific side of the plate on which to start eating (spatial learning), instead of a correct color pattern plate (cue learning). Plates were initially introduced to cleaners with a small piece of prawn in the back, one plate at the time, and the day before the beginning of experiments. Cleaners were submitted to one session, on alternate days. Each was, composed of 10 trials, until the individual reached our learning criterion as described for the first experiment. The correct side was chosen haphazardly and was maintained between trials and sessions but it changed between compound treatments. The first trial began 10 min after the injection of the compound treatment and consisted on successive presentation of the two pattern color plates until the outcome of 10 trials. The “correct” side plate had available prawn while the “incorrect” side plate had inaccessible prawn (covered with transparent tape). Experimental individuals would then have complete access to both plates; a correct choice would enable both plates to stay inside the aquarium, while an incorrect choice would consequently have the correct plate (and the only one with accessible food) immediately removed, preventing cleaners from eating the food item (Fig. 1).

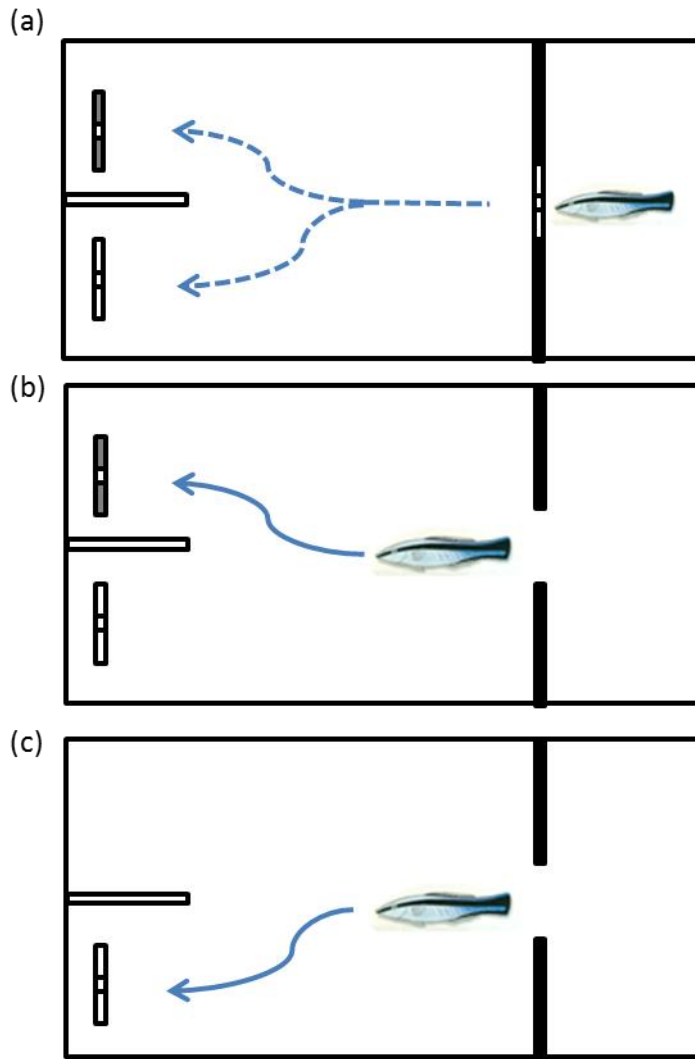


Figure 1 - The experimental aquarium (view from above) was divided into a back compartment (right) and the test compartment (left) with help of an opaque PVC partition. a) Initial phase b) The two pexiglas plates are placed and if cleaner choose the correct pattern/side, both plates will stay in the aquarium and eat one piece of shrimp. c) The two pexiglas plates are placed and if cleaner choose the incorrect pattern/side only the incorrect plate remains, with no shrimp available. The two Plexiglas plates were visually separated from each other with help of a dividing transparent partition wall in-between the plates. The tested fish could move into the test compartment when an opaque partition at the side of the back compartment was pulled up.

Neuropeptide treatment

Cleaners were weighed before the onset of the experiment so that injection volume could be adjusted to body weight. Each cleaner was sequentially and haphazardly injected (intramuscularly) with all the compounds: saline (0.9 NaCl); AVT (V0130– Sigma) at a low (0.5 $\mu\text{g}/\text{gbw}$) and a high (2.5 $\mu\text{g}/\text{gbw}$) dose; Manning compound [3.0 $\mu\text{g}/\text{gbw}$, V2255– Sigma- [b-Mercapto-b,b cyclopentamethylenepropiony]1,O-me-Tyr2, Arg8]-Vasopressin]

and Atosiban [3.0 µg/gbw, Bachem: H-6722.0050 (50mg) (Deamino-Cys1,D-Tyr(Et)2,Thr4,Orn8)-Oxytocin(RWJ 22164)]. Injections were always given in the morning, so each individual could in total be injected a maximum of 8 times with each compound. However, the real number of injections was usually smaller but dependent on individual learning speed under the effect of each compound. Manning compound is a commonly used antagonist of the AVP type 1a receptors (V1a), which in teleost fish include both subtypes V1a1 and V1a2, and which has also shown relative affinity for the OT receptor in mammals (Manning et al., 2008). Atosiban is an antagonist of the OT/IT receptors but has also been referred to have some affinity for AVP/AVT receptors (Manning et al., 2008). If indeed, putative AVT effects are being mediated by AVT1a receptors in competition with IT receptors, then the use of both Manning and Atosiban will help us to demonstrate their effects more clearly.

Statistical Analysis

In both tasks, the same cleaners were used in all treatment groups. Sample size on the place discrimination task is smaller because two of the cleaners were not able to learn the task in the control treatment (saline injection). Data were analysed using one-way Repeated Measures (RM) ANOVA to test for main effects of neuropeptide treatment and *a posteriori* planned comparisons of least squares means in order to contrast the effect of each neuropeptide treatment with the reference (saline) group. All statistical tests shown in this study were two tailed. Although it would be interesting to compare both tasks, these are not comparable because the second task involves reversal learning.

Results

Experiment 1: Cue Discrimination task

We found a significant main effect of treatment on cleaners' ability to solve the cue discrimination problem (RM ANOVA, $F_{4,44} = 7.05$, $p = 0.0018$, Fig. 2a). Cleaners injected with Manning compound needed significantly fewer sessions to complete the task than the saline group, whereas those treated with Atosiban needed significantly more learning sessions than the saline group to solve the problem (planned comparisons: Manning compound vs saline, $F_{1,11} = 12.57$ $p = 0.004$; Atosiban vs saline, $F_{1,11} = 5.18$, $p = 0.04$; Fig. 2a).

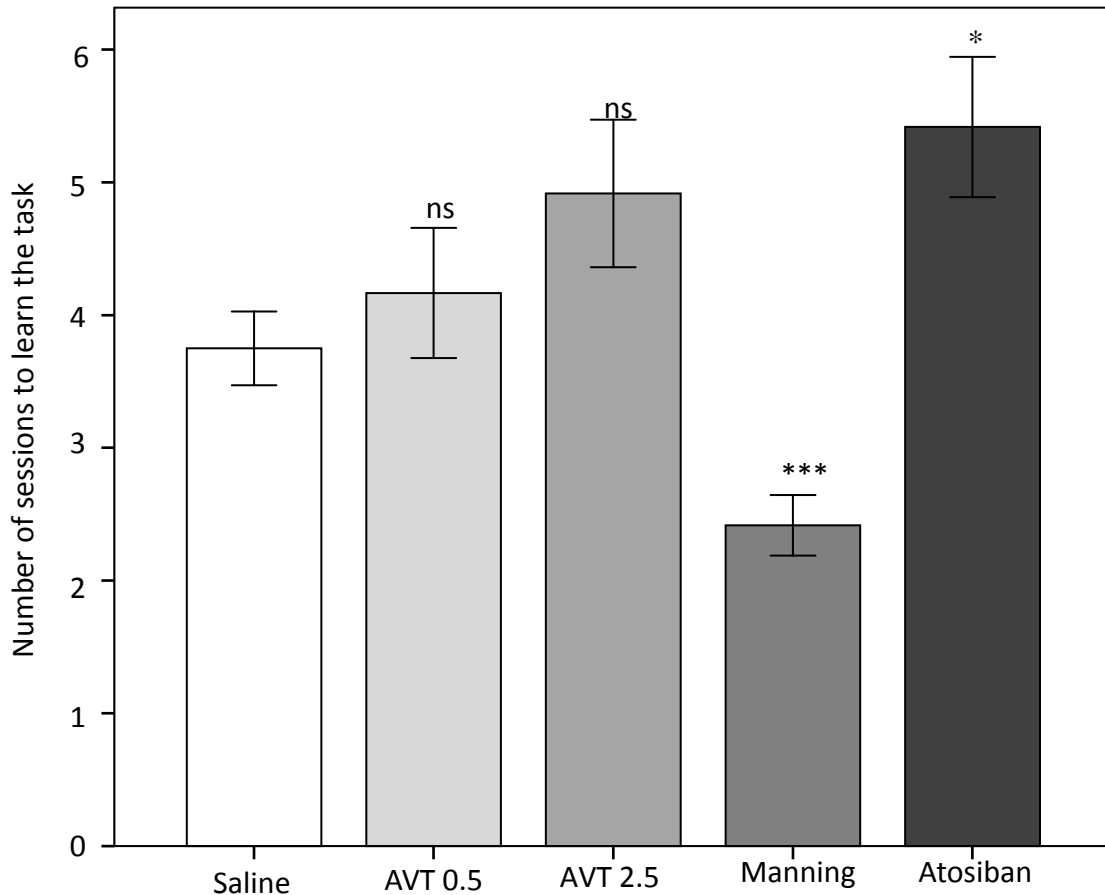


Figure 2. a) The effect of the neuropeptides arginine-vasotocin (AVT), manning compound (Manning) and Atosiban on learning behaviour of the cleaner fish *L. dimidiatus* in the ecologically relevant cue learning task, compared with a control (saline). Symbols above bars represent P values which refer to planned comparisons of least squares means effect of each neuropeptide treatment group against the reference (saline) group (*, <0.05; ***, <0.01; ns, >0.05).

Neither of the AVT dosages had an effect on learning (planned comparisons: AVT 0.5 vs saline, $F_{1,11} = 0.41$, $p > 0.05$; AVT 2.5 vs saline, $F_{1,11} = 3.11$, $p > 0.05$, Fig 2a). A similar pattern was observed in the learning curves for each treatment, where it is clear that the Manning compound learning curve is distinct from the curves of the other treatments (Fig. 2b).

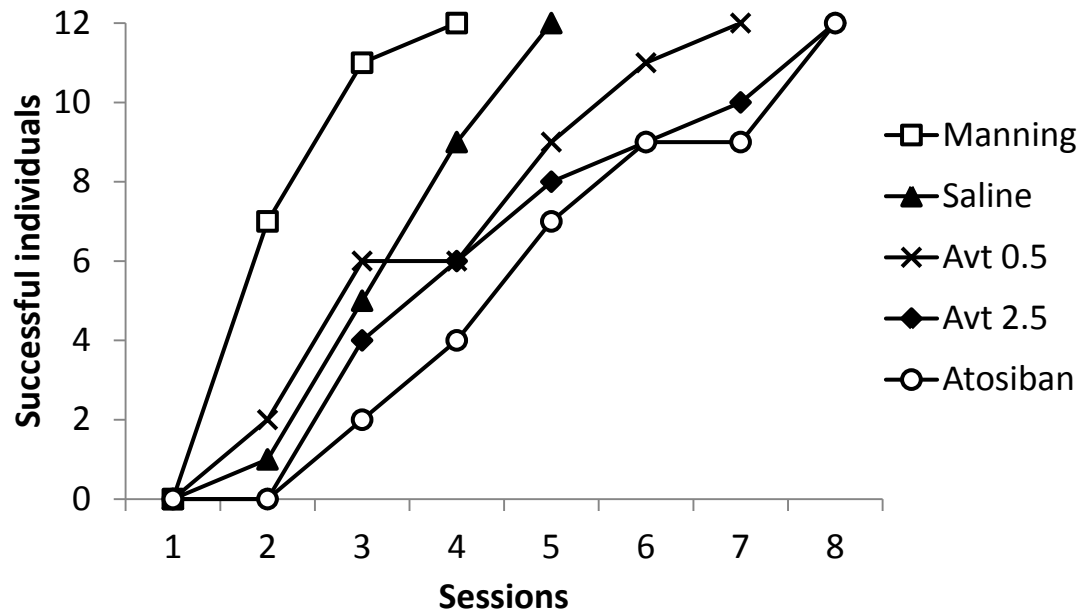


Figure 2b. Learning curves generated by the different treatments (number of cleaner wrasses that successfully learned the task in each session) Cleaners had a maximum of 8 sessions to learn the task.

Experiment 2: Place discrimination task

We found a marginally non-significant trend for the main effect of treatment on cleaners' ability to learn the place discrimination task (RM ANOVA, $F_{4, 36} = 2.38$, $p = 0.069$; Figure 3a). Planned comparisons revealed that cleaners treated with the lower dosage of AVT needed a higher number of sessions to complete the task than fish from the saline treatment (planned comparisons: AVT 0.5 vs saline, $F_{1,9} = 10.51$, $p = 0.01$; AVT 2.5 vs saline, $F_{1,9} = 0.08$, $p > 0.05$; Manning compound vs saline, $F_{1,9} = 0$, $p = 1$; Atosiban vs saline, $F_{1,9} = 0.07$, $p > 0.05$; Fig. 3a). The learning curves were similar among the different treatments, but the learning curve of the lower dosage of AVT was the most distinct from the curve generated by the control treatment (saline) (Fig. 3b).

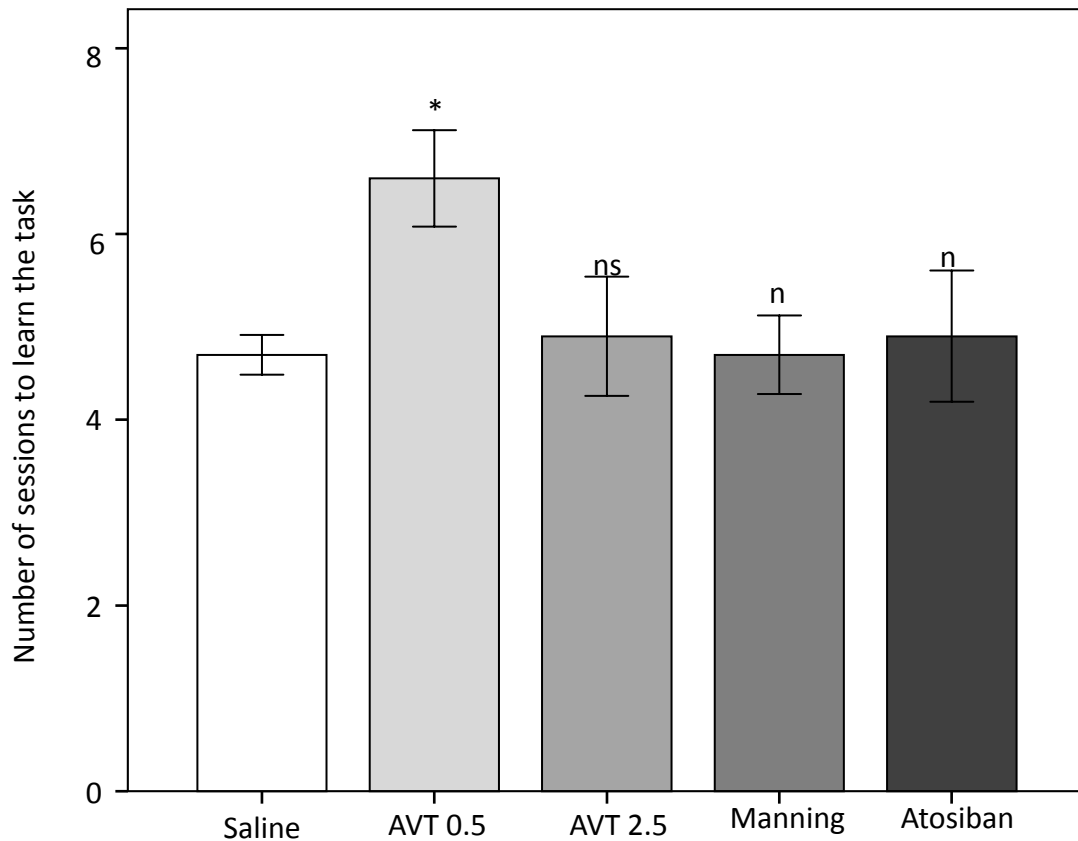
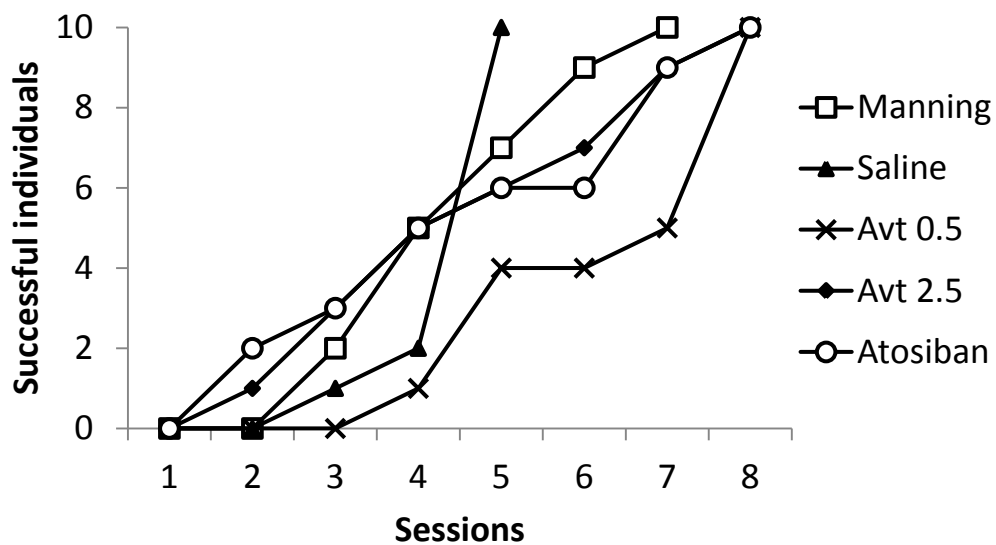


Figure 3a. The effect of the neuropeptides arginine-vasotocin (AVT), manning compound (Manning) and Atosiban on learning behaviour of the cleaner fish *L. dimidiatus* in the ecologically non-relevant place task, compared with a control (saline). Symbols above bars represent P values which refer to planned comparisons of least squares means effect of each neuropeptide treatment group against the reference (saline) group (*, <0.05; ns, >0.05). **b)** Learning curves generated by the different treatments (number of cleaner wrasses that successfully learned the task in each session) Cleaners had a maximum of 8 sessions to learn the task.



Discussion

Our results show that AVT affects the learning competence of cleaners in both learning tasks, which seem to be related to two parallel memory systems and vary in ecological relevance. In the cue learning task there was a non-significant trend for increasing dosages of AVT to delay learning while the AVT antagonist (Manning compound) significantly increased learning speed in relation to the reference treatment (saline injection). In the place learning, only the small AVT dosage significantly delayed learning speed in comparison to saline treated fish. Thus, contrary to our prediction, our results showed that the negative effects of AVT on learning seem to be independent of the learning task. Moreover, these effects are associated to the specificity of dosage and to the sensitivity of the tasks regarding AVT.

The fact that the pharmacological manipulation of the AVT system had an effect on both cue and place learning, which is putatively dependent on Dm and Dl, respectively, suggests that AVT receptors and fiber varicosities might be present in both circuits in cleaner wrasses (as found in other species – Dewan et al. 2011). To date, three AVT receptors subtypes (i.e. V1a1, V1a2, and V2) have been described in teleost fish (Lema 2010; Lema et al. 2012). Moreover, AVT may also bind to the receptors of isotocin (the fish homologue of oxytocin) with significant affinity. This is consistent with our results (concerning atosiban' effects), as recent mapping of nonapeptide receptors in the brain of teleost species have shown that V1a and isotocin receptors are widely distributed throughout the forebrain, being present both on Dm and Dl areas (Kline et al. 2011; Huffman et al. 2012) . The dose dependent effects described above can be explained by variable expression of the different receptor subtypes at each memory circuit. For example, in the place learning task only the lower AVT dosage was effective in slowing down learning, which could mean that at higher AVT concentrations, the binding to other AVT and isotocin receptors (e.g. V1a2), would compromise the specific effects of binding to a specific AVT receptor (e.g. V1a1). In the cue learning task, the learning enhancing effects found for the V1a antagonist (Manning compound) and the observed non-significant trend for a dose-related detrimental effect of AVT suggests a major role of endogenous AVT and V1a-sensitive circuitry in the mediation of learning in the ecologically relevant task, as no role for endogenous AVT was demonstrated in the non-ecologically relevant task. Moreover, the inhibitory effect of AVT in the place-learning task may also suggest that spatial representations might also have an influence on cleaning

efforts, for instance in the identification of more appropriate or favorite sites to interact with clients or with conspecific partners (within the territorial boundaries).

The differential response of the two learning tasks to the pharmacological manipulations of the AVT system used in this study may also depend on the binding of AVT to V1a-type or to non-AVT receptors, which may be differently expressed across the two brain memory circuits. The V1a-type receptors (both subtypes V1a1 and V1a2) are generally the most predominant AVT receptors found in the teleost brain (Kline et al. 2011; Huffman et al. 2012), but these subtypes may not be equally expressed or functionally similar. For example, in the grouper (*Epinephelus adscensionis*), the V1a2 subtype is more expressed in the brain than the V1a1 subtype (Kline et al. 2011), and the expression of the V1a2 subtype is closely linked with sex, reproduction, and behavior, whereas V1a1 receptors in the brain are associated with stress response (Lema et al. 2012). Higher activity of AVT/V1a2 circuitry in the brain has been associated with space use and social dominance, whereas pair bonding seemed to be mediated by a different, less active AVT/V1a2 circuitry (Oldfield et al. 2013). Cleaner wrasses, interspecific cooperative behaviour is regulated by the V1a-type brain receptors, which was inferred via pharmacological treatment with the V1a antagonist Manning compound (Soares et al. 2012), however differences between the effects of both V1a subtypes could not be specified. Nevertheless, the regulation of cooperative behaviour should probably rely on shifts at the AVT/V1a2 circuits, a hypothesis that is amenable for further testing. Moreover, it should be noted that the negative effects of AVT on learning in cleaner wrasses contrast with memory enhancing effects of AVP on hippocampus dependent learning in rodents (Alescio-Lautier and Soumireu-Mourat 1998; Alescio-Lautier et al. 2000). Future work focussing on how AVT regulates the molecular signalling pathways (e.g. LTP, bdnf), related to learning and memory in cleaner wrasses is needed to further explore these contrasting results.

Another potential explanation for the effects of AVT upon cleaner wrasses' behaviour is that it could also occur via the activation of a stress response, because AVT influences adrenocorticotropin (ACTH) production and cortisol secretion (Balment et al. 2006), which could in turn prompt an inhibition of learning. Indeed, recent work has shown that exogenous injections of AVT cause a stress response (increased circulation levels of cortisol), which lead to a reduction in behavioural activity (Huffman et al. 2014). However, in cleaner wrasses, the exogenous administration of AVT produced a substantial decrease of most interspecific cleaning activities, but it did not affect the expression of

behavior directed towards conspecifics (Soares et al. 2012). Moreover, the effects of increasing cortisol levels in cleaner wrasses seem to differ considerably from other systems. A recent study demonstrated that elevations in cortisol levels are responsible for transient shifts towards a cheating strategy in cleaner fish (Soares et al. 2014). Thus, increasing cortisol levels does not lead to a reduction in activity levels but it rather seems to be associated with specific behavioural changes in the cleaner wrasse system. It is thus difficult to directly associate the effects of AVT's learning inhibition reported here to the potential rise of circulating cortisol.

Previous evidence on the cleaner wrasse system has demonstrated that cleaners can recognize individual clients (Tebbich et al. 2002) and are able adjust to past experience with them (Bshary and Grutter 2003; Bshary and Grutter 2005). This means that cleaners cooperative live comes with specialized cognitive skills that include social recognition and learning, which might be presumably mediated by AVT and IT (Soares et al. 2012). In our study we tested AVT's relevance for cleaners' ability to solve two social learning tasks that are putatively associated with different neural circuits. Our results indicate that both learning tasks are differently affected according to task and have dose specific effects. These can be explained by the differential sensitivity of the two neural circuits involved in the two learning types to the different AVT receptors. However, it may also reflect a significant variance in terms of relevance of these circuits on their daily operation: one being extensively used when interacting with clients and the other providing some sort of spatial discrimination and territoriality. Overall, our findings demonstrate that AVT/V1a pathways, which are implicated in the regulation of interspecific behavior, i.e. a cleaner's willingness to seek interactions with clients, are also linked to individual learning ability, in the context of their mutualistic behavior, and in tune with their social-ecological demands.

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CHAPTER
3

Arginine vasotocin reduces levels of cooperative behaviour in a cleaner fish

ABSTRACT

Cooperation between unrelated individuals usually involves investments that often mean a decrease in immediate payoffs, but ensure future benefits. Here I 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 cleanerwrasse *L. 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. I 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. My 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.

Keywords: Cleaner fish; Cooperative levels; *L. dimidiatus*; Neuropeptides; Arginine vasotocin; Isotocin; Decision-making.

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 (Bshary and Bergmüller, 2008). Evolutionary models usually focus on questions related to potential strategies, which may ensure that investments yield future benefits and hence stabilise cooperation (Bshary and Bergmüller, 2008; Lehmann and Keller, 2006; West et al., 2007). 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 (Barta et al., 2011; Soares et al., 2010), 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 (Goodson, 2008; Ross and Young, 2009; Goodson and Thompson, 2010). For example, within humans, experimental setups aiming to increase OT levels have demonstrated that these mediate rises in prosociality, which include trust (Kosfeld et al., 2005; Baumgartner et al., 2008), generosity (Zak et al., 2010; Barraza and Zak, 2009), empathy (Barraza and Zak, 2009), and social memory (Rimmele et al., 2009), while behavioural manifestations of prosociality have now been linked to individual differences in rs53576 genotype of the OT receptor (Kogan et al., 2012). Partner support is also a good facilitator of increases in OT plasma levels in both men and women (Grewen et al., 2005). 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 (Thompson et al., 2004) and that its effects are sex specific with respect to responses towards same-sex faces, i.e. agonistic in men and affiliative in women (Thompson et al., 2006). Finally, in a recent study, Rilling and colleagues (2014), demonstrate that intranasal AVT and IT administration mediate biased effects in human males and females interacting in a Prisoner's Dilemma 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 (Giligan et al., 2003). Indeed, recent work in non-mammalian vertebrates indicates that the social function of OT may be ancient in terms of its evolutionary framework (Goodson et al., 2009). 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 MacDonald and MacDonald, 2010; Albers, 2012). For example, in teleost fish, studies have found a relation between IT and the increase of sociality in goldfish, *Carrasius auratus* (Thompson and Walton, 2004), and with zebrafish, *Danio rerio* (Braidà et al., 2012), while under the influence of AVT, Thompson and Walton (2004) 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 (Madden and Clutton-Brock 2011), 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 (Reddon et al., 2012).

Here we use one of the best studied cooperative models, the Indopacific bluestreak cleaner wrasse *L. 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 (Côté, 200; Bshary and Côté, 2008). A conflict of interest occurs because cleaners prefer to eat client mucus, which constitutes cheating (Grutter and Bshary, 2003). As clients respond to non cooperative cleaners with attacking (punishing), leaving or avoidance (Bshary and Grutter, 2002; Bshary and Schäffer, 2002; Pinto et al., 2011), 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 (Bshary and Grutter, 2005; Gingins et al., 2013). This experimental paradigm has been used successfully in the last few years, having resulted in over a dozen published studies focusing on cleaner wrasses (Grutter and Bshary, 2003; Bshary and Grutter, 2002; Bshary and Grutter, 2005; Gingins et al., 2013; Wismer et al., 2014; Raihani et al., 2010; 2012a,b,c; Salwiczeck et al., 2011; Bshary et al., 2011;

Danisman et al., 2010; Bshary et al., 2008; Bshary and Grutter, 2006) and captures the essence of cleaning interactions as demonstrations of key results have been reproduced in experiments with real cleaner–client interactions (Pinto et al., 2011). 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 (2012) 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 (Mendonça et al., 2013). However, given AVT's overall effects regarding our system (Soares et al., 2012), 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 Soares et al., 2012) 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 (Soares et al., 2012).

Methods

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 × 40 × 40 cm) combined in a flow through system that pumped water from a larger cleaning tank (150 × 50 × 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 (Grutter and Bshary, 2004).

Learning against preference task

We followed Bshary and Gruter (2005) 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).

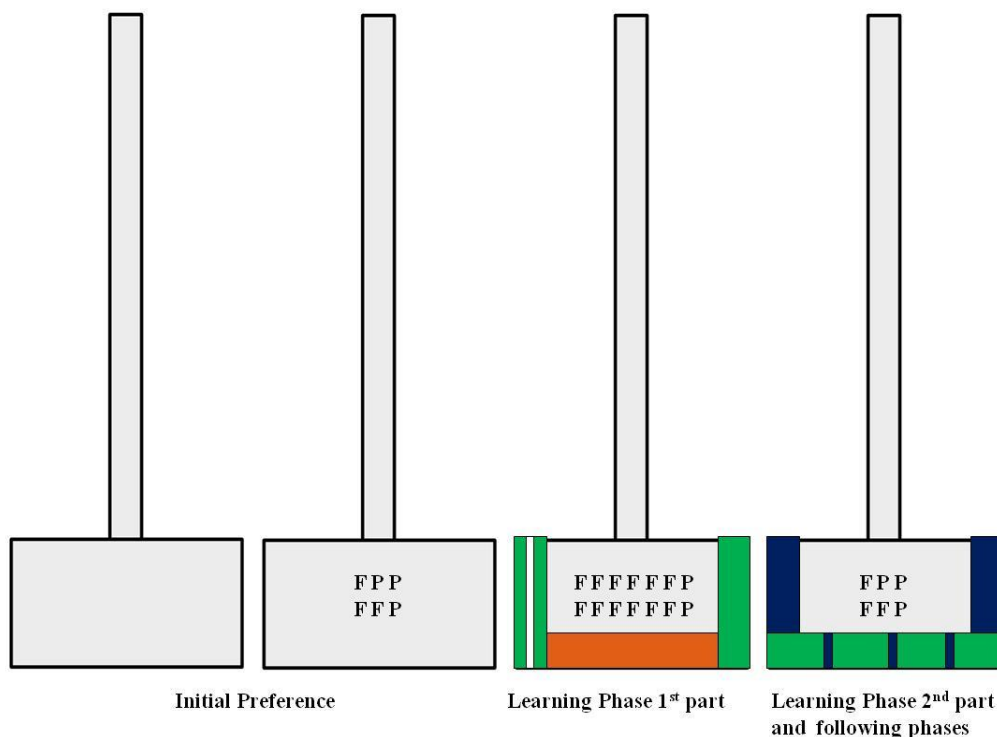


Figure 1. Plate patterns during the different phases of the experiment. Each item is represented by one letter: F — fish flake and P — prawn.

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.

Neuropeptide treatment

This part consisted of the final foraging experiment but this time each cleaner was sequentially and haphazardly treated (intramuscularly) with 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 (Manning et al., 2008). Atosiban is an antagonist of the OT/IT receptors, but has also been referred to have some affinity for AVP/AVT receptors (Manning et al., 2008). 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 (0.5µl/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.

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.

Results

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).

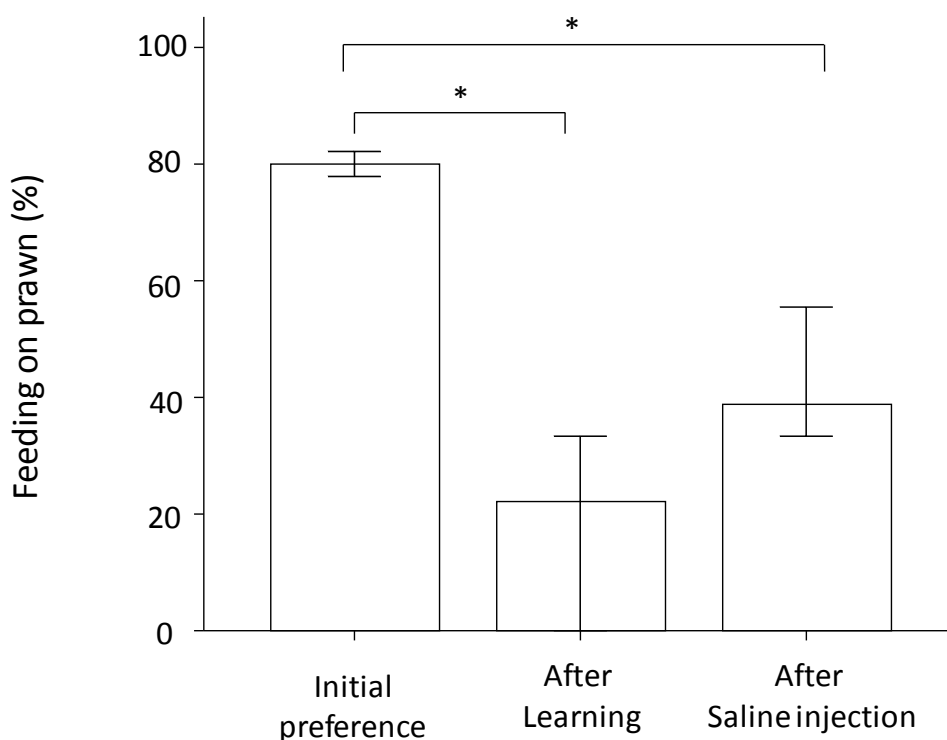


Figure 2. The percentage of prawn items eaten in an initial preference test, after the individuals learned to eat against their preference and when treated with saline solution. Shown are the medians and the interquartiles. Symbols above bars represent P values which refer to Wilcoxon matched pair tests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

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. 8). 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).

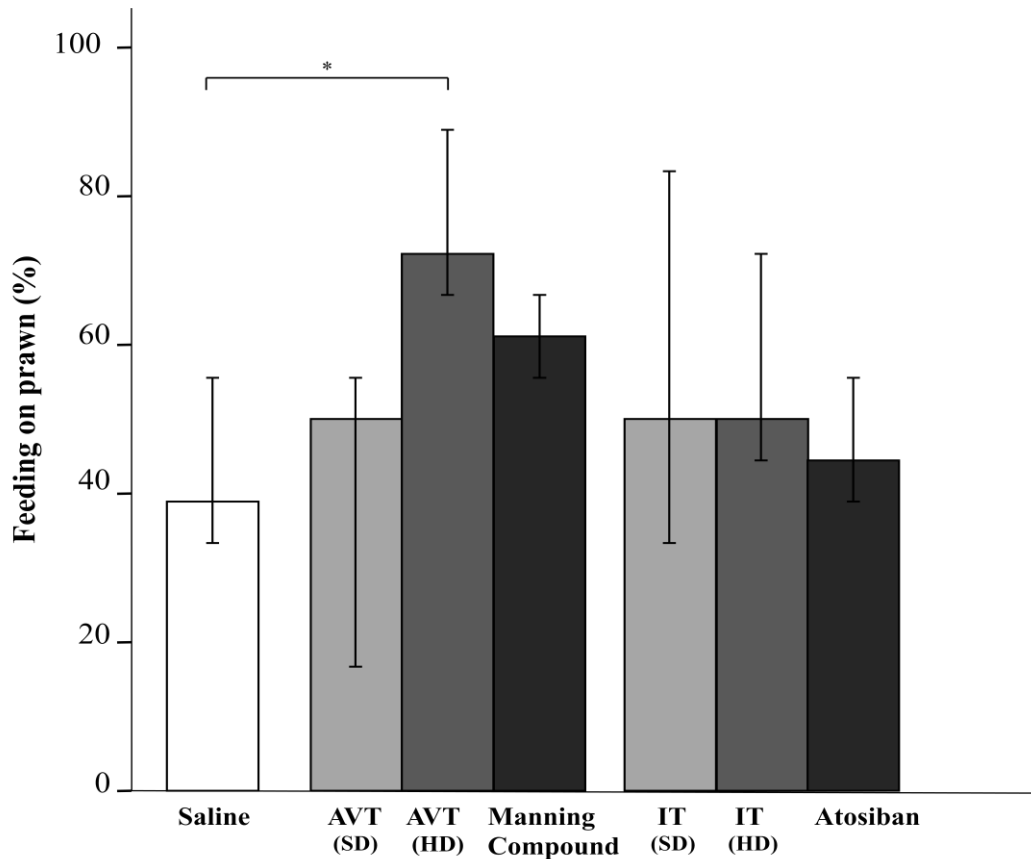


Figure 3. The percentage of prawn items eaten in all treatment groups: saline, AVT (smaller dosage—SD), AVT (higher dosage—HD), Manning compound, IT (SD), IT (HD) and Atosiban. Light grey bars represent a smaller dosage and dark grey bars represent a higher dosage of AVT and IT, while black bars represent 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.

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 cleaners selectively feeding

against their preference (Bshary and Grutter 2005). 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 V1atype 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 (Manning et al., 2008). 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.

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 (Balment et al., 2006; Huffman et al., 2014). For instance, Huffman and colleagues (2014) 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 (Goymann and Wingfield, 2004). 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 Soares et al., 2014). 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 2014, for cichlids) but is otherwise associated with significant behavioural strategic changes Soares et al., 2014. Interestingly, 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 (Soares et al., 2014). 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 (Creel et al 2013) and facilitates a behavioural shift responsible for a decrease of cleaners' levels of feeding against preference.

Neuropeptide modulation of cleaner fish social memory and recognition

There is experimental evidence that cleaners can recognise individual clients (Tebbich et al., 2002) and that cleaners adjust to past experience with them (Bshary and Grutter, 2002; 2005). 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 – Winslow and Insel, 2004). In rodents, AVP is crucial for the enhancement of social recognition and social memory (Young and Wang, 2004; Bielsky et al., 2005; Feifel et al., 2009) while in humans it seems to affect social communication, demonstrating biased effects between genders (Thompson et al., 2004, 2006; Rilling et al., 2014). Moreover, sex steroids and glucocorticoids (as mentioned above) may interact with AVP and OT in various ways (Goodson and Bass, 2001; De Vries and Simerly, 2002; Choleris et al., 2009). For example, both peripheral injections and chronic central infusions of AVP into castrate male rats reduce their social recognition skills towards juvenile conspecifics (Bluthé and Dantzer, 1992). Castration seems to reduce AVP expression in various limbic brain areas (Zhou et al., 1994), which are known to directly influence reward learning of social interactions (Choleris et al., 2009). For cleaner wrasses, elevation in the levels of AVT decrease cleaners' propensity to engage in interspecific cleaning activities (Soares et al., 2012). As cleaners are protogynous 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 (Bielsky and Young, 2004). The development of the OT knockout mice further established the role of OT in social recognition (Ferguson et al., 2000; Choleris et al., 2003; Crawley et al., 2007). While in

rodent models, AVT facilitates social information consolidation, the OT's major role is at the level of acquisition (Winslow and Insel, 2004). 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 (Soares et al., 2012). 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.

Neuropeptide modulation of impulsive choice control

Lack of impulse control has been associated with reduced activity of the serotonergic system (Soubrie, 1986). For example, in humans impulsive violent behaviour is indicative of low serotonin turnover rate (Linnoila et al., 1983; Virkkunen et al., 1995), while lower levels of serotonin have been demonstrated to result in stimuli response inhibition, leading to a rise in impulsivity and aggression (Young, 2013). Research mostly done in rodents has confirmed that serotonin has a key role in altering the secretion and release of AVP and OT (Jørgensen et al., 2003), 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 – Ferris et al., 1997). 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) (Ferris, 1996; Perry and Grober 2003; Semsar et al., 2004) . On the contrary, a hyporeactive serotonin system may result in enhanced AVP activity and aggression in both animals and humans (Ferris, 2000), 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 (Crockett et al., 2009). 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).

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 (Grutter and Bshary, 2003). 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.

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CHAPTER
4

**Forebrain neuropeptide regulation of pair
association and behaviour in cooperating
cleaner fish**

ABSTRACT

Animals establish privileged relationships with specific partners, which are treated differently from other conspecifics, and contribute to behavioural 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 behaviour, is correlated with neuroendocrine mechanisms involving forebrain neuropeptides arginine vasotocin (AVT) and isotocin (IT) and b) whether these neuropeptides level shifts relate to 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, cleaner's 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' relationship influence male and female cleaner fish differently and contribute to the variation of brain neuropeptide levels, which is linked to distinct cooperative outcomes.

Keywords: Behavioural Variation; Cleaner fish; Arginine-vasotocin; Isotocin; Forebrain; Male-female partnerships; Cooperative behaviour

INTRODUCTION

Behavioral variation between individuals is recognized as a fundamental force shaping social interactions and the evolution of complex social behavior that includes cooperation (Sherratt and Roberts, 2001; McNamara and Leimar, 2010). These behavioural 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 (McNamara and Leimar, 2010). One of the greatest contributors to individual behavioural variation is the existence of social ties or familiarity, which encourages the establishment of individual's relationships with specific partners (e.g. pairbonding, alliances, friendships; Silk, 2003). However, there is limited information on the underlying physiological mechanisms that involve the establishment of social ties and their direct consequences to individual behavioural variation (see Soares et al., 2010).

The formation and maintenance of stable male-female relationships are sustained by selective socio-sexual behaviour between the pair, and by preference for one partner over other potential ones (e.g. pair bonds - Kleiman, 1997; Fuentes, 1999). But the maintenance of long lasting social attachments should also be sustained by specific neurohormonal frameworks, which should be linked to the behavioural responses of any paired individual, on two different levels: a) at the expression of a series of "bilateral" behaviours between pair partners and b) on "multilateral" interactions between the pair and the social environment (Soares et al., 2010). Two neuropeptides are considered as critical mediators of partner-preference formation and social attachment: oxytocin (OT) and arginine vasopressin (AVP) (Young and Wang, 2004). The neuropeptide OT is well known for its links to social bonds, which includes affectionate behaviour between partners (Light et al., 2005), displays of romantic love, and increases of prosociality within the context of cooperative behaviour. For example, exogenous administration of OT seems to be responsible for an increase of investment in communal and cooperative activities in meerkats (Madden and Clutton-Brock, 2011), the facilitation of partner-directed behaviour in marmosets (Smith et al., 2010), and the promotion of humans' trust and reciprocity (Kosfeld et al., 2005; Baumgartner et al., 2008). The role of AVP in partner recognition and bonding, mostly derived from rodent studies, shows that both AVP and OT are involved but that 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 (Nair

and Young, 2005; Young et al., 2008).

The converse may also occur, with neuropeptide levels changing in response to behavioural variations in socio-sexual and affiliative behaviour. For instance, partner support modulates the rise of OT plasma levels in both men and women (Grewen et al., 2005) and tactile stimulation (such as massaging, hugging and/or grooming) promotes the elevation of OT levels in humans and other primates and also in rodents [Light et al., 2005; Uvnäs-Moberg, 1998; Crockford et al., 2013]. In pair-bonded tamarins, OT levels relate to the amount of grooming and mutual contact in females and sexual behaviour of males (Snowdon et al., 2010). Measures of relationship distress correlate with OT levels in women and with AVP levels in men (Taylor et al., 2010). 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 however the male also visits the other females regularly (Robertson, 1972). Cleaners provide a service by removing ectoparasites, dead or damaged tissue of other visiting reef fish (hereafter referred as 'clients') (Côté, 2000). However, cleaners prefer to feed on client mucus, which is detrimental to the client and constitutes cheating (Bshary and Grutter, 2002). Instances of cheating contribute to a conflict of interests between clients and cleaners (Bshary and Grutter, 2002). 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 (Bshary and Côté, 2008).

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 (Bshary et al., 2008). 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 (Bshary et al., 2008). This happens because the larger male cleaner punishes (i.e. aggressively chases) the females that cheat whereas females never chase the male (Raihani et al., 2010). 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 (Raihani et al., 2012a) . Males tend to behave more aggressively with unfamiliar females, with such females responding by behaving more cooperatively (Raihani et al., 2012b). 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 behavioural integration of vertebrates (Goodson and Bass, 2001). We tested two hypotheses: 1) whether intra-pair relationship, measured by the rates of joint interspecific cleaning and intraspecific behaviour, is correlated with brain levels of AVT and IT and 2) whether these mechanisms are also associated with individual's 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. behavioural expression, Kleszczyńska et al., 2013; Gozdowska et al., 2013). Moreover, our study provides a novel approach to the neuroendocrine mechanisms of behavioural variation aiming at a tropical reef fish living in natural conditions.

METHODS

Field methods

This study was conducted on two reefs around Lizard Island (Lizard Island Research Station, Australia, 14° 40'S, 145° 28'9"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 minutes 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 ± 0.65 cm) and 2.5 to 6.2 g (3.82 ± 1.14 g) and for males from 7.5 to 9 cm (8.35 ± 0.45) and 4.3 to 7.3 g (5.73 ± 0.99), 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-ependorf tube, immediately frozen, and stored at -80C in a liquid nitrogen container. The container was then taken by air to 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.

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: 6M HCl (2000:1, v/v). The eluate was evaporated to dryness using a Turbo Vap LV Evaporator (Caliper Life Science, USA).

Samples were then frozen and stored at -80 °C prior to HPLC analysis.

Before quantitative analysis, the samples were re-dissolved in 40 µL 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 derivatisation reaction, 20 µL of sample and 20 µL of 0.2 M phosphate buffer (pH 9) were mixed, and then 3 µL of NBDF (30 mg in 1mL of acetonitrile) was added. The solution was heated at 60°C for 3 min, cooled on ice, acidified with 4 µL 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 x 4.6 mm I.D., 5 µm 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 µL. Fluorescence detection was carried out at 530 nm with excitation at 470 nm.

Behavioural analyses

For each video observation, we recorded the following measures: a) the number and duration (in sec) 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 (Côté et al. 1998). Measures of intraspecific pair behaviour 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 sec of inspection).

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” (Cairns and Schwager, 1987). 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’ behaviour 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 analysis: one for cleaning in pairs and 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 behavioural 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) (Zuur et al., 2010). All tests were 2 tailed and were done in SPSS Statistics, version 22.

RESULTS

Intraspecific pair behaviour

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 1). 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. 9b).

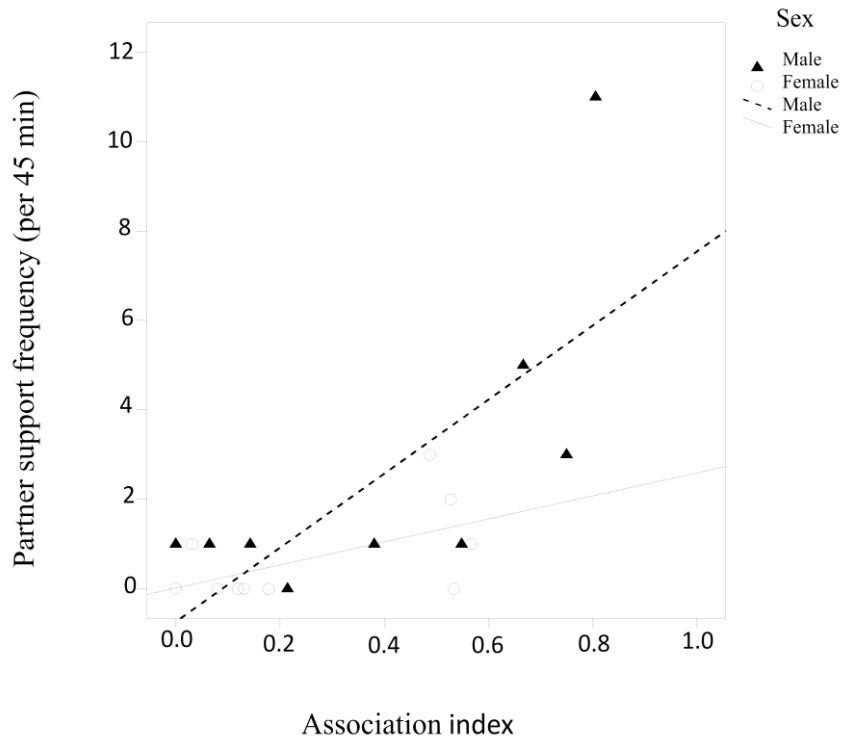


Figure 1a. The relationship between cleaner pairs' association index: frequency of partner support received according to sex (male and female).

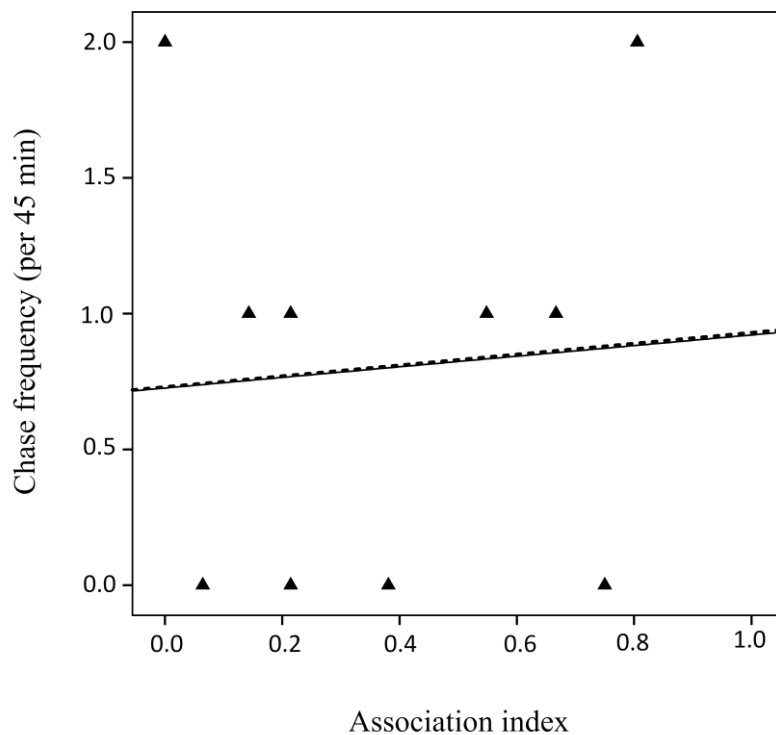


Figure 1b. The relationship between cleaner pairs' association index: male to female chase frequency.

Table 1. Statistical results of a one-way analysis of covariance. Sex is fixed and the association index is a covariate. Sex = male or female. Partner support is the dependent variable. $P < 0.05$ values are *; significant term mentioned in the main text are in bold.

Source	Numerator DF	Denominator DF	Sum of Squares	<i>F</i>	<i>P</i>
Sex	1	17	0.097	2.113	0.164
Association	1	17	0.724	15.792	0.001*

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 (1-way ANCOVA; $F_{(1,16)} = 5.262$, $P = 0.036$, Fig. 2a, Table 2a). Visual examination of Fig. 10a 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 analysed. 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 2b).

Table 2. Statistical results of a one-way analysis of covariance for unpaired cleaner wrasses and a linear regression for paired cleaner wrasses. Sex was fixed and association index is a covariate. Events in which both cleaners (male and female) were observed to inspect the same client together are defined as "paired" while events in which cleaners were cleaning alone are defined as "unpaired". a) unpaired, and b) paired cleaner wrasses.

Jolt rate as the dependent variable. For paired cleaner wrasses, only larger clients were considered. See Table S1 for definitions of bold and * values.

a) Unpaired cleaner wrasses

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Sex	1	16	30.501	< 0.0001*
Association	1	16	3.020	0.101
Sex*Association	1	16	5.262	0.036*

b) Paired cleaner wrasses

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Association	1	14.660	11.991	0.004*

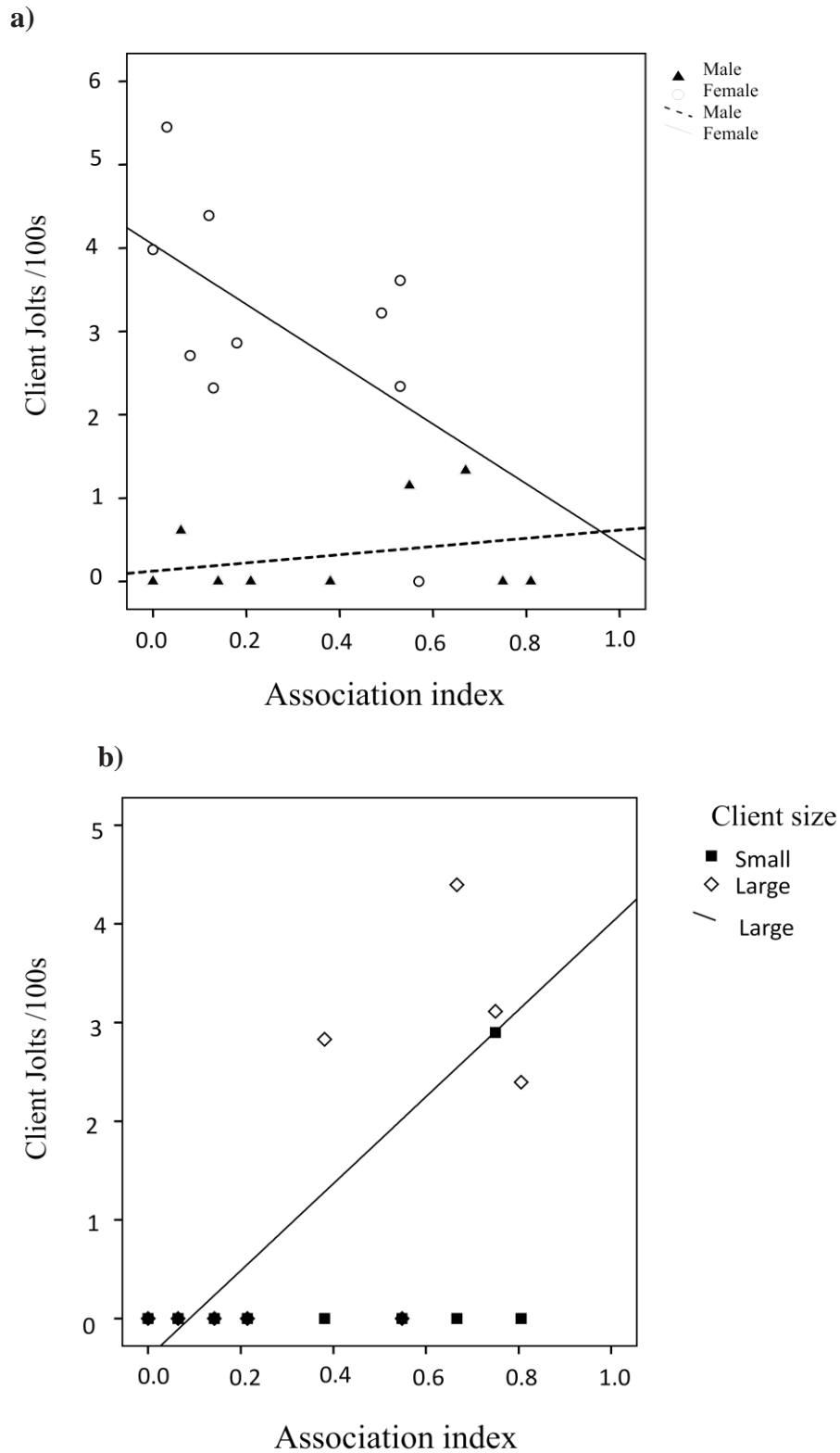


Figure 2. The relationship between cleaner pairs' association index and: a) unpaired cleaner pairs interspecific service quality (jolts) according to sex (male and female) and b) Paired cleaner pairs interspecific service quality according to client size - small clients (total length <11 cm) and large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

Cleaner pairs forebrain neuropeptide levels

IT levels varied according to an interaction between sex and association (ANCOVA: $F_{(1,16)} = 13.237$, $P = 0.002$, Table 3a). Visual examination of Fig. 3a indicates this is likely due to a positive relationship in males. AVT levels did not differ according to sex (ANCOVA: $P = 0.597$, Table 3b) or with association level ($P = 0.113$, Table 3b). In females, there was a positive correlation between AVT and IT (Person 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).

Table 3. Statistical results of one-way analysis of covariance. Sex is fixed and association index is a covariate. See Table 2 for definitions of bold and * values.

a) Forebrain isotocin as the dependent variable

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Sex	1	16	2.854	0.111
Association	1	16	5.789	0.029*
Sex*Association	1	16	13.237	0.002*

b) Forebrain arginine-vasotocin as the dependent variable

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Sex	1	17	0.290	0.597
Association	1	17	2.800	0.113

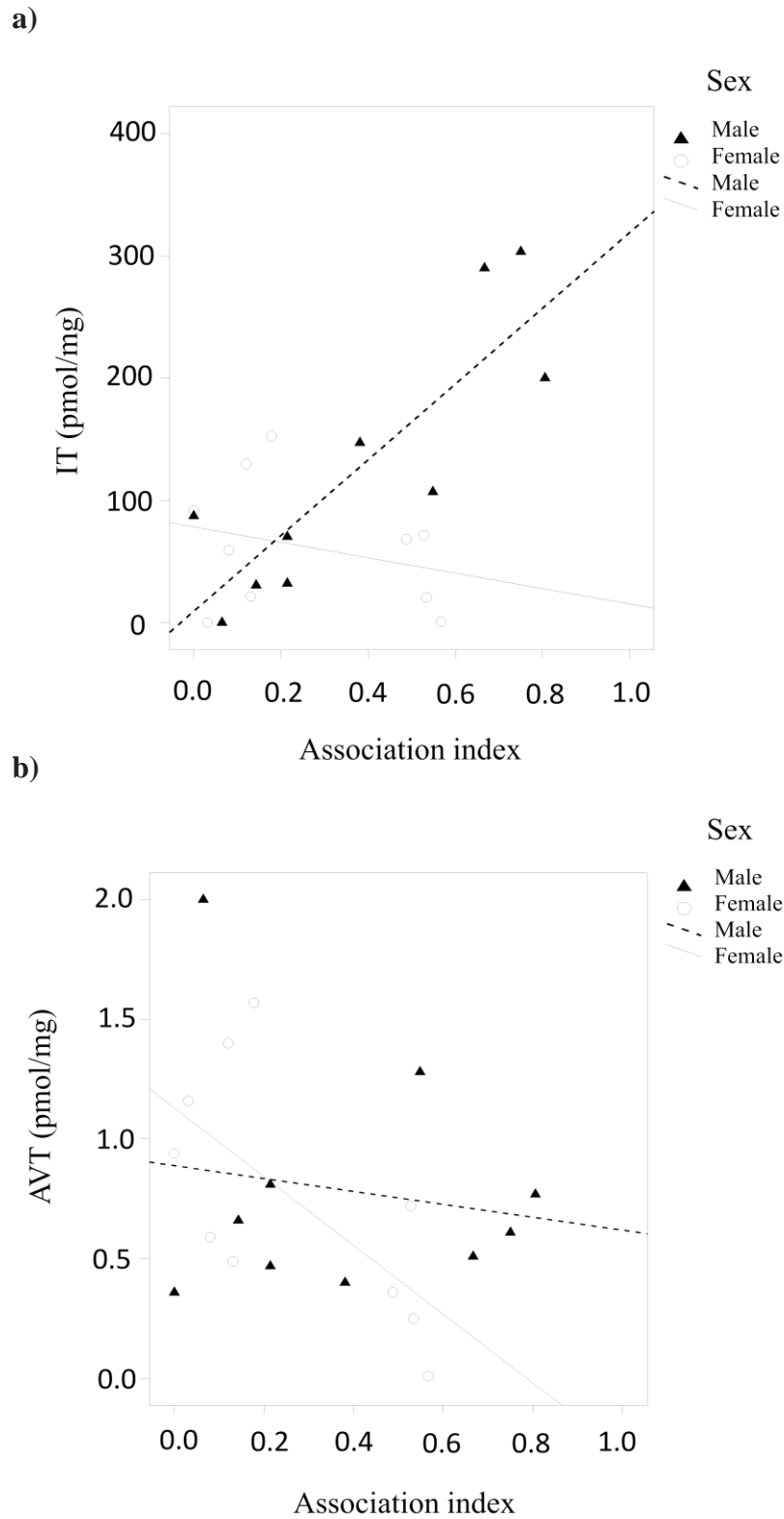


Figure 3. The relationship between cleaner pairs' association level: a) forebrain isotocin levels (pmol/mg) and: b) forebrain arginine-vasotocin levels (pmol/mg).

c)

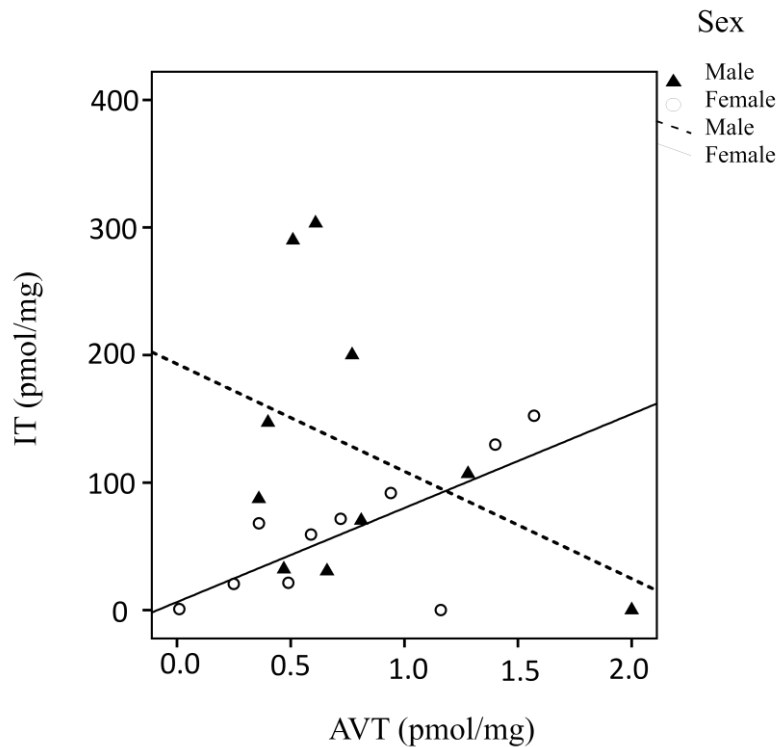


Figure 3. The relationship between cleaner pairs' association level and: c) the relationship between forebrain isotocin and arginine-vasotocin levels (pmol/mg), according to sex (male and female).

Link between jolting rate and forebrain neuropeptide levels

When forebrain AVT levels were concerned, client jolts rates did not differ between cleaner sexes (ANCOVA: $P = 0.787$, Table 4, 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 4, Fig.4a). Regarding forebrain IT levels, client jolt rates differed between sexes ($P = 0.001$, Table 5, Fig. 4) and also with IT levels (ANCOVA $F_{(1,17)} = 9.084$, $P = 0.008$, Table 5, 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.

Table 4. Statistical results of one-way analysis of covariance. Sex is fixed, forebrain arginine-vasotocin (AVT) is a covariate and jolt rate is the dependent variable. See Table S1 for definitions of bold and * values.

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Sex	1	16	0.076	0.787
AVT FB	1	16	1.752	0.204
Sex*AVT FB	1	16	4.472	0.051

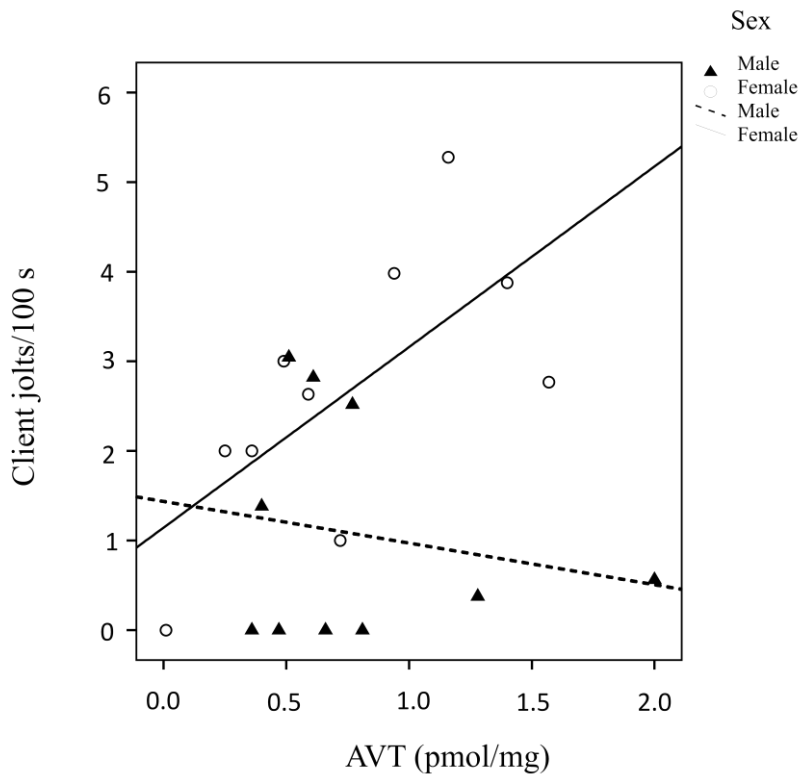


Figure 4a. The relationship between cleaner pairs interspecific service quality (jolts) and forebrain isotocin levels (pmol/mg)

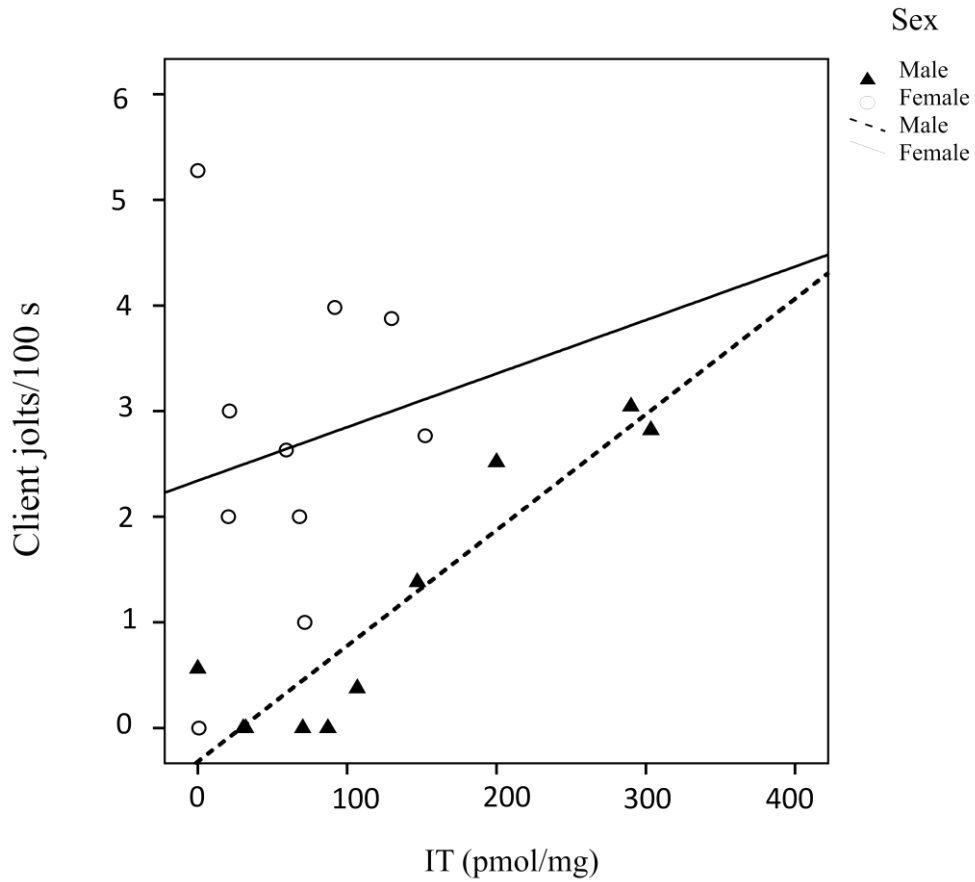


Figure 4b. The relationship between cleaner pairs interspecific service quality (jolts) and forebrain arginine-vasotocin levels (pmol/mg), according to sex (male and female).

Table 5. Statistical results of one-way analysis of covariance. Sex is fixed, forebrain isotocin (IT) is a covariate and jolt rates is the dependent variable. See Table S1 for definitions of bold value. See Table S1 for definitions of bold and * values.

Source	Numerator DF	Denominator DF	<i>F</i>	<i>P</i>
Sex	1	17	15.615	0.001*
IT FB	1	17	9.084	0.008*

DISCUSSION

Our data suggest that changes in a pair's relationship influence male and female cleaner fish differently and contribute to the variation of brain neuropeptide levels, which are linked to distinct cooperative outcomes. These links will be further explored in the following sections of the discussion.

The influence of partner support to pair association levels and cleaner wrasse behaviour

In natural conditions, male cleaner wrasses hold territories that encompass several breeding females, which they visit frequently (Robertson, 1972; Nakashima et al., 2000). Usually, these males are found living and cleaning with the biggest female in their harem (Robertson, 1972). Partnership maintenance is characterized by partner-directed behaviours, 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 (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' behaviour, 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 alone. 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 (Bshary et al., 2008; Raihani et al., 2010). Consequently, females become more cooperative when cleaning in pairs (Bshary et al., 2008). Hence, the increase of the overall jolt rates during inspection of larger clients should most likely be due to male and not to female cheating behaviour. The increase of female honesty works as a counterbalancing factor against male cheating level rises which allows for pairs to overall provide better cleaning service and become a better choice for these larger clients (Bshary et al., 2008).

Contrary to previous studies done in controlled conditions (Raihani et al., 2012a,b), 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 behaviour typically used to prolong interactions and to reconcile with clients after cheating (Grutter, 2004). 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 behaviour when cleaning in pairs.

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 – Windle et al., 1997; Uvnäs-Moberg, 1997; Neumann, 2008; Taylor et al., 2006). In fish, Soares and colleagues (2011) 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 wrasse, the relatively high levels of forebrain IT may be related to the amount support received which could potentially underlie reductions of stress levels (as seen in other model systems, see Neumann, 2008). Male higher brain IT levels may also relate to a rise in partner tolerance, which would be expressed in a increase in time spent in joint-cleaning events without an increase in male to female punishment.

AVT, pair association and female cleaner wrasse behaviour

The neuropeptide modulating roles 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 (Insel and Shapiro, 1992; Carter, 1998; Lim and Young, 2006). However, few studies have solely focused on the effects of the AVP/AVT system as a mediator of female component affiliative behaviour, compared to those involving male models (Nephew, 2010). It is known that for female cleaner wrasses, the 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 behaviour (Soares et al., 2010). Although this implies that high levels of endogenous AVT contribute to a reduction of cleaner wrasse propensity to cooperate with its clients, however, at a conspecific level AVT's influence on female behaviour 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 - Backström et al., 2011): for example, in response to stress, gene expression of AVP/AVT is upregulated in parvocellular neurons in mammals and in teleost fish (Aubry et al., 1999; Gilchrist et al., 2000). 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.

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. Contrarily 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.

Concluding remarks

Understanding how individuals decide and how their social environment influence 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 behaviour in relation with two relevant neuropeptide systems (AVT and IT). Moreover, we identify a link between forebrain IT levels and cleaner wrasse behaviour, 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 behaviour.

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CHAPTER
5

**Brain levels of nonapeptides in labrid fish
species with different levels of mutualistic
behaviour**

ABSTRACT

Evolutionary and ecological comparative approaches provide unique knowledge into how social organization variation influences brain and behaviour. Marine cleaning mutualisms offer a promising setting system to study the physiological mechanisms and evolution of cooperative behaviour as closely related species may differ markedly in the degree to which they depend on these interactions for their diet. Here we aimed at finding out if differences in cognitive abilities related to mutualistic behaviour are reflected in the brain levels of two neuropeptides: arginine vasotocin (AVT) and isotocin (IT), across four close-related species of Labrid fish: two are obligatory cleaners throughout their entire life (*Labroides dimidiatus* and *L. bicolor*), one other species that is a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous), and one “non-cleaner” species (*Labrichthys unilineatus*). We showed that the levels of both AVT and IT varied significantly across species, as measured in the whole brain or in the specific macro-area. Significantly higher AVT levels in cerebellum and in the whole brain were found in the obligate cleaner species, which seems to be related to expression of mutualistic behaviour. On the other hand, brain IT is not related to expression of mutualistic behaviour, but higher levels of IT in *L. unilineatus* suggest that these might be linked to the development of sexual dimorphism, which occurs only in this non-cleaner species.

Keywords: Arginine vasotocin; isotocin; mutualistic behaviour; labrid fish

Introduction

Evolutionary and ecological comparative approaches provide unique knowledge into the consequences of social organization variation in brain and behaviour (Phelps et al., 2010). Indeed, one of the major questions related to the study of social behaviour concerns the physiological processes that underlie the species-specific behavioural variation and how evolutionary changes in the structure, localization and interactions between brain areas influence and are influenced by neuromodulatory agents (Taborsky and Oliveira, 2012; Zupanc and Lamprecht, 2000). There are several categories of neuromodulators in the brain that may be related to the behavioural plasticity in teleost fish, among them are the nonapeptides arginine vasotocin (AVT) and isotocin (IT), evolutionary predecessors of mammalian arginine vasopressin (AVP) and oxytocin (OT). Both peptides belong to evolutionary ancient family of neurohormones (nonapeptides), quite conserved across phyla, similar in structure and differing by only two amino acid residues (reviewed by Caldwell et al., 2008). Their remarkable plasticity and sensitivity to environmental factors make them suitable candidates to explore the regulation of complex social behaviour in vertebrates ranging from humans to fishes (Insel and Young, 2000).

Much of the current evidence of the contribution of these neurohormones to social behaviour come from studies on mammals, namely close related species from the genus *Microtus* (voles). Research on the highly social prairie voles have been strengthened by the extensive comparative work done on monogamous and non-monogamous species, and demonstrated that the AVP system mediates behavioural patterns associated with pair-bonding, social recognition, spatial memory and reward in males (Freeman and Young, 2013). In females, pair-bonding seems to involve the OT pathways, which mediate social recognition and reward (Dore et al., 2013). There is also strong evidence that the nonapeptides are key proximate regulators of social behaviour in many other mammals as the deer mice (Bester-Meredith et al., 1999), the African naked mole rats (Kalamatianos et al., 2010), the marmosets (Smith et al., 2010), the titi monkeys (Jarcho et al., 2011), the tamarins (Snowdon et al., 2010) and the wild chimpanzees (Crockford et al., 2013). However, our understanding of the link between environmental and cognitive demands, and nonapeptides' presence at different brain areas is still limited. Teleost fish have unparalleled diversity among vertebrates in terms of social organization and should thus become the relevant model systems to explore the link. For instance, some species are found in solitary conditions most of their lives while others aggregate with several others

(schools) and engage socially during their entire life span (Bshary, 2001). Moreover, some exhibit cognitive abilities considered to be complex in social settings, which makes them relevant models to study social behaviour and cognition (Oliveira, 2013; Bshary et al., 2014). This allows to search for patterns of variation in the nonapeptide system which can be associated with social variation. However, very few studies have addressed this question. In this respect, the most relevant study so far, examined AVT neuronal phenotypes across seven different species of butterflyfishes (Chaetodontidae) living in similar environmental conditions, i.e. tropical coral reefs, but exhibiting divergent social systems. It was found that increased aggressive behaviour in the territorial species was associated with larger gigantocellular AVT cells in the preoptic area (POA) and higher fiber density in the telencephalon (Dewan et al., 2008, 2011; Dewan and Tricas, 2011). Conversely, it was found in two Labrid species, varying on the expression of mutualistic behavior that the cooperative one had smaller and less numerous gigantocellular AVT cells in the POA (Mendonça et al., 2013). However, these studies, which were focused on AVT producing neurons in the POA, did not provide any information on the availability of the peptide at different putative target regions in the brain. Therefore, there is a need for comparative quantitative studies of nonapeptide distribution in brain regions of interest for the different behaviours under study. In this study we aim to fill this gap.

Marine cleaning mutualisms and specifically cleanerfish species, offer a promising setting system to study the physiological mechanisms and evolution of cooperative behaviour as closely related species may differ markedly in the degree to which they depend on interactions with other species for their diet (Feder, 1966). Cleaners are fishes that inspect the body surface, gill chambers and mouth of cooperating larger fishes (known as clients) in search of ectoparasites, mucus and dead or diseased tissue (Limbaugh, 1961; Feder, 1966; Gorlick et al., 1987; Losey et al., 1999; Côté, 2000). In the best known species of cleanerfish, the Indo-Pacific bluestreak cleaner wrasse *Labroides dimidiatus*, conflicts over the quality of each interaction are frequent because these often feed on healthy tissue, mucus or scales instead on clients' ectoparasites (referred to as cheating, Grutter and Bshary, 2003). So, to minimize conflicts these cleaner have to eat against preference (i.e. ectoparasites instead of mucus, which they prefer, Grutter and Bshary, 2003). Individuals belonging to this species are referred to make use of a highly diverse behavioural repertoire that includes individual recognition of their clients, manipulation of client decisions (on different levels of action), reconciliation, punishment, advertising of their cleaning services, tactical deception and indirect reciprocity based on image scoring

(reviewed by Bshary and Côté, 2008; Bshary and Noe, 2003; Bshary, 2001). Moreover, these cleaners may also clean in stable male-female pairs which will contribute to a variation in the outcome service quality towards clients, depending on whether they clean alone or in pairs (Bshary et al., 2008). Studies focussing on other species of closely related cleanerfish are not as frequent as those aimed at *L. dimidiatus*. However, recent work on *Labroides bicolor* revealed its' specific features useful in this study. Individuals of this species have larger home ranges that are occupied in at an uneven rate (e.g, they spend more time in some areas of their territories). Thus, the likelihood that repeated interactions will occur with the same partner changes drastically. This fact will influence the outcome of their cooperative decisions and also on the response of their partners (Oates et al., 2010). Moreover, comparison of diets and observations of cleaning behaviour between the two cleaner species demonstrated that *L. bicolor* fed more on client mucus (e.g. cheated) and interacted with fewer species (Oates et al., 2012). Contrarily to *L. dimidiatus*, *L. bicolor* individuals mostly seek preferred clients actively to initiate interactions with them and seem to exhibit less control to eat against their preference (Oates et al., 2010). These studies came to demonstrate that not all cleaners are the same and that we should expect behavioural and physiological variation between closely related species.

The previous studies in cleanersfish have shown that AVT plays a key role as a physiological modulator of cooperative behaviour, as high levels of this neuropeptide decrease cleaners' likelihood to engage in cleaning interactions with their clients (Soares et al., 2012). Moreover, recent work has found that the experimental transient elevation of AVT levels leads to a decrease of cleaners' willingness to feed against preference (Chapter 3). The role of IT remains less clear. Cardoso and colleagues (in review) has found a link between forebrain IT levels and cleaner wrasse's less cooperative behaviour but such is dependent on variation in intra-pair relationship. In the present study, we aim to find if both nonapeptides' levels measured in different brain regions, i.e. forebrain, optic tectum, cerebellum and brain stem are related to variation in cooperative behaviour across four close-related species of Labrid fish belonging to the tribe Labrichthyini: two are obligatory cleaners throughout their entire life (*L. dimidiatus* and *L. bicolor*), one other species that is a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous), and one "non-cleaner" species (*Labrichthys unilineatus*) (Westneat and Alfaro, 2005; Cowman et al., 2009). Our selected species vary in their mutualistic behaviour (cleaning) and inhabit similar environments (Cole, 2010).

Methods

Study species

We studied two Labrid fish (Fig 1; tribe Labrichthynes) that are obligatory cleaners throughout their entire life (*L. dimidiatus* and *L. bicolor*), one other species that is a facultative cleaner (*Labropsis australis*; juveniles are cleaners and adults are corallivorous), and one “non-cleaner” species [*L. unilineatus*; corallivorous throughout its entire life, Fulton and Bellwood, 2002; Cole et al., 2009, Cole, 2010). We used a total of 16 *L. dimidiatus* (standard length, mean \pm SEM=5.703 \pm 0.265; body mass, mean \pm SEM=2.767 \pm 0.391), 11 *L. bicolor* (SL= 6.470 \pm 0.269; BW=4.216 \pm 0.716), 15 *L. australis* (SL=6.564 \pm 0.284; BW=4.517 \pm 0.636) and 12 *L. unilineatus* (SL=8.725 \pm 0.422; BW=6.465 \pm 1.795). All fish were purchased from a local distributor (Tropical Marine Centre, Lisbon, Portugal) and only adult females were used in order to avoid spurious effects of possible sex differences. The sex of all individuals was first confirmed by direct inspection of the gonads and when needed also with the help of an acetocarmine stain (Guerrero and Shelton, 1974).

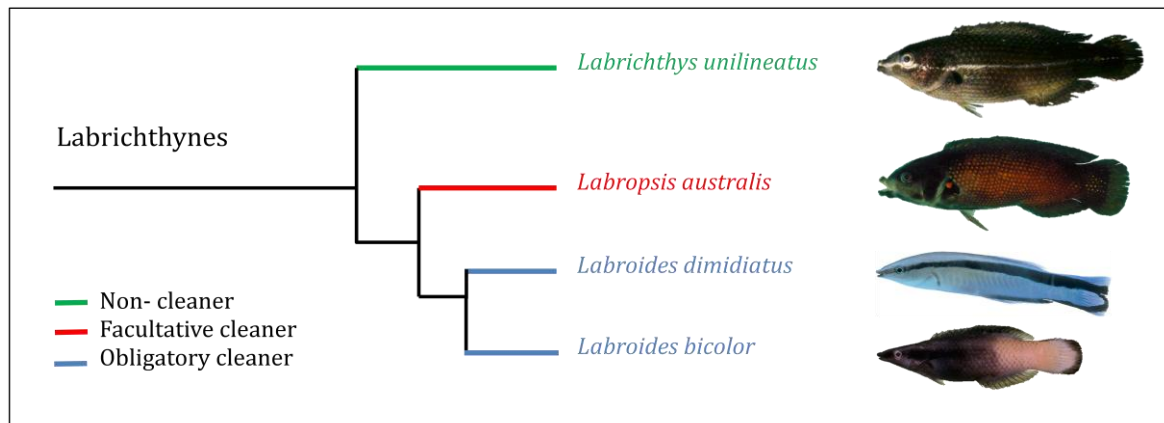


Figure 1. Cladogram with the Labrichthynes lineage, which included studied species (adapted from Cowman et al., 2009)

Brain sampling

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

Brain AVT and IT assay: sample preparation

Brain samples were sonicated in 1 mL Milli-Q water (Microson™XL, Misonix, USA), acidified with glacial acetic acid (3 µL) 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: 6M HCl (2000:1, v/v). The eluate was evaporated to dryness using a Turbo Vap LV Evaporator (Caliper Life Science, USA). Samples were then frozen and stored at -70 °C prior to LC-MS/MS analysis.

LC-MS/MS analysis

AVT and IT analysis was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using Infinity 1290 System (Agilent Technologies, USA) coupled with triple quadrupole mass spectrometer Agilent 6460A equipped with a JetStream ESI source. Chromatographic separation was done using Agilent Poroshell 120 SB-C18 column (3 mm x 100 mm, 1.8 µm particle) preceded by online automatic SPE extraction with precolumn switching program to remove matrix. SPE was achieved on Eclipse XDB-C18 (4.6 mm x 15 mm, 3.5 µm particle) precolumn. The mobile phase consisted of solvent A: 0.1% acetic acid in H₂O (v/v) and solvent B: 0.1% acetic acid in acetonitrile: water (3:1) (v/v). A gradient elution was used starting with 5% B for 3 min, followed by linear gradient from 5% to 32.5% B in 11 min. Next, the column was washed at 95% for 5 min and then was equilibrated for 5 min at starting conditions after each analysis. The injection volume was 10 µL. Flow rate of the mobile phase was set at 0.6 mL min⁻¹ and the column temperature at 20 °C. To minimize contamination of the mass spectrometer the column flow was directed only from 7.0 to 15.0 min into the mass spectrometer using switching valve.

JetStream ESI source was operated in positive ionization mode using the following settings: capillary voltage 3500 V, nozzle voltage 500 V, drying gas temperature 300 °C and flow 5 L min⁻¹, nebulizer gas pressure 45 psi, sheath gas temperature 250 °C and flow rate 11 L min⁻¹. The analyses were monitored in the multiple reaction monitoring mode (MRM). The monitored mass transitions for AVT were set at m/z 525.5 → 517.2 (dwell

time 200 ms, fragmentor voltage 143 V, collision energy 73 V) and for IT: m/z 483.7 → 136.1 (dwell time 200 ms, fragmentor voltage 74 V, collision energy 53 V).

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

Statistical Analysis

Variables were log transformed to conform to parametric parameters of homogeneity of variances (assessed by Levene's test). We then compared AVT and IT brain levels for the whole brain and for each brain area (forebrain, optic tectum, cerebellum and brain stem) by using 1-way ANOVAs, followed by Tukey pos-hoc tests HSD tests. We sampled a total of 57 female wrasses for IT measurements (16 *L. dimidiatus*, 11 *L. bicolor*, 15 *L. australis* and 12 *L. unilineatus*). For AVT some individuals failed to produce a reliable peak, and were therefore removed from the analysis (final sample sizes: 10 *L. dimidiatus*, 11 *L. bicolor*, 13 *L. australis* and 9 *L. unilineatus*). All tests were 2 tailed and were performed in the software package SPSS Statistics, version 22.

Results

Whole brain mean concentrations of AVT and IT are given in Fig 2. Mean concentrations of AVT and IT in studied brain macroareas are also given in Table 1.

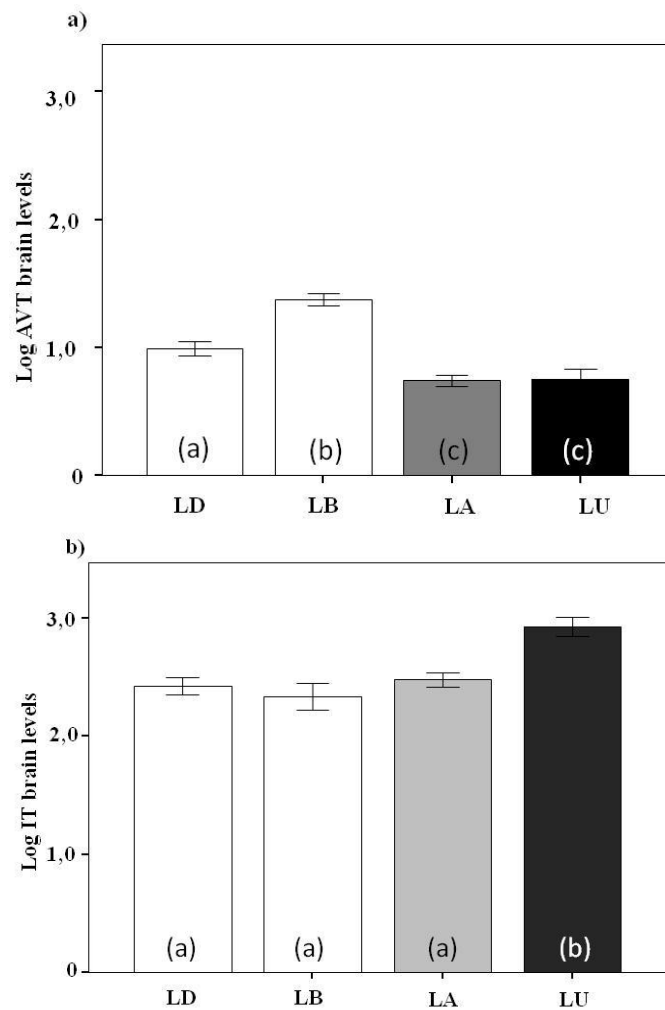


Figure 2. Whole brain levels of (a) arginine vasotocin (AVT) and b) isotocin (IT) across four close related Labrid fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU - *Labrichthys unilineatus*. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

Tabela 1 - Arginine vasotocin (AVT) and isotocin (IT) concentrations (mean \pm SEM) in different brain areas of different species (differences examined with One-way ANOVAs).

Neuropeptides	Species				Statistics
	<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichthys unilineatus</i>	
AVT	0,49 \pm 0,06	0,82 \pm 0,07	0,47 \pm 0,05	0,45 \pm 0,04	F _(3,41) =8,79; p<0,001
IT	1,98 \pm 0,10	1,80 \pm 0,14	1,99 \pm 0,07	2,38 \pm 0,10	F _(3,56) =5,15; p=0,003
AVT	0,16 \pm 0,04	0,61 \pm 0,08	0,18 \pm 0,03	0,21 \pm 0,06	F _(3,41) =14,55; p<0,001
IT	1,60 \pm 0,08	1,44 \pm 0,14	1,60 \pm 0,09	2,03 \pm 0,14	F _(3,56) =4,68; p=0,006
AVT	0,59 \pm 0,07	0,74 \pm 0,10	0,25 \pm 0,04	0,30 \pm 0,07	F _(3,41) =11,40; p<0,001
IT	1,28 \pm 0,10	1,69 \pm 0,10	1,30 \pm 0,11	1,96 \pm 0,07	F _(3,56) =10,41; p<0,001
AVT	0,27 \pm 0,06	0,81 \pm 0,13	0,32 \pm 0,03	0,26 \pm 0,07	F _(3,41) =9,98; p<0,001
IT	1,80 \pm 0,15	1,67 \pm 0,18	1,75 \pm 0,19	2,55 \pm 0,08	F _(3,56) =6,08; p=0,001

AVT brain levels

Overall, whole brain AVT levels differ significantly across species (1-way ANOVA, $F_{3,38}=29.964$ $p < 0.001$) with *L. bicolor* (LB) scoring higher values compared to the three other species (Tukey HSD tests, all $p < 0.001$, Fig 2a) and with both obligate cleanerfish species having higher levels of brain AVT compared to *L. australis* (LA) and *L. unilineatus* (LU) (Tukey HSD tests, all $p < 0.03$; Fig 2b), while these last species did not differ between each other (Tukey HSD tests, LA vs LU: $p=0.998$; Fig 2a). Brain AVT levels also differ significantly at each individual brain region (1-way ANOVAs, forebrain: $F_{3,38}=8.792$ $p < 0.001$; optic tectum: $F_{3,38}=14.577$ $p < 0.001$; cerebellum: $F_{3,38}=11.396$ $p < 0.001$ and brain stem: $F_{3,38}=9.977$ $p < 0.001$, see Table 2, Fig 3). Again, *L. bicolor* had significantly higher AVT brain levels in most brain regions compared to the other species (see Table 3, Fig. 4) except for the cerebellum, where both obligate cleanerfish species had higher AVT levels than *L. australis* and *L. unilineatus* (Table 3, Fig 4).

IT brain levels

Overall, whole brain IT levels differed significantly across species (1-way ANOVA, $F_{3,53}=29.964$ $p < 0.001$, Fig 1c) with the non cleanerfish species *L. unilineatus* showing significantly higher levels than all other species (Tukey HSD tests, all $p < 0.001$, Fig 2b). Similarly, brain IT levels also differed significantly at each individual brain region (1-way ANOVAs, forebrain: $F_{3,53}=5.152$ $p = 0.003$; optic tectum: $F_{3,53}=4.675$ $p = 0.006$; cerebellum: $F_{3,53}=10.405$ $p < 0.001$ and brain stem: $F_{3,53}=6.068$ $p < 0.001$, see Table 1). Specifically, *L. unilineatus* had higher levels of IT in the forebrain, optic tectum and brain stem compared with all other species. In the cerebellum both *L. unilineatus* and the obligate cleanerfish *L. bicolor* had significantly higher values when compared to *L. dimidiatus* and *L. australis* (see Table 3, Fig. 3).

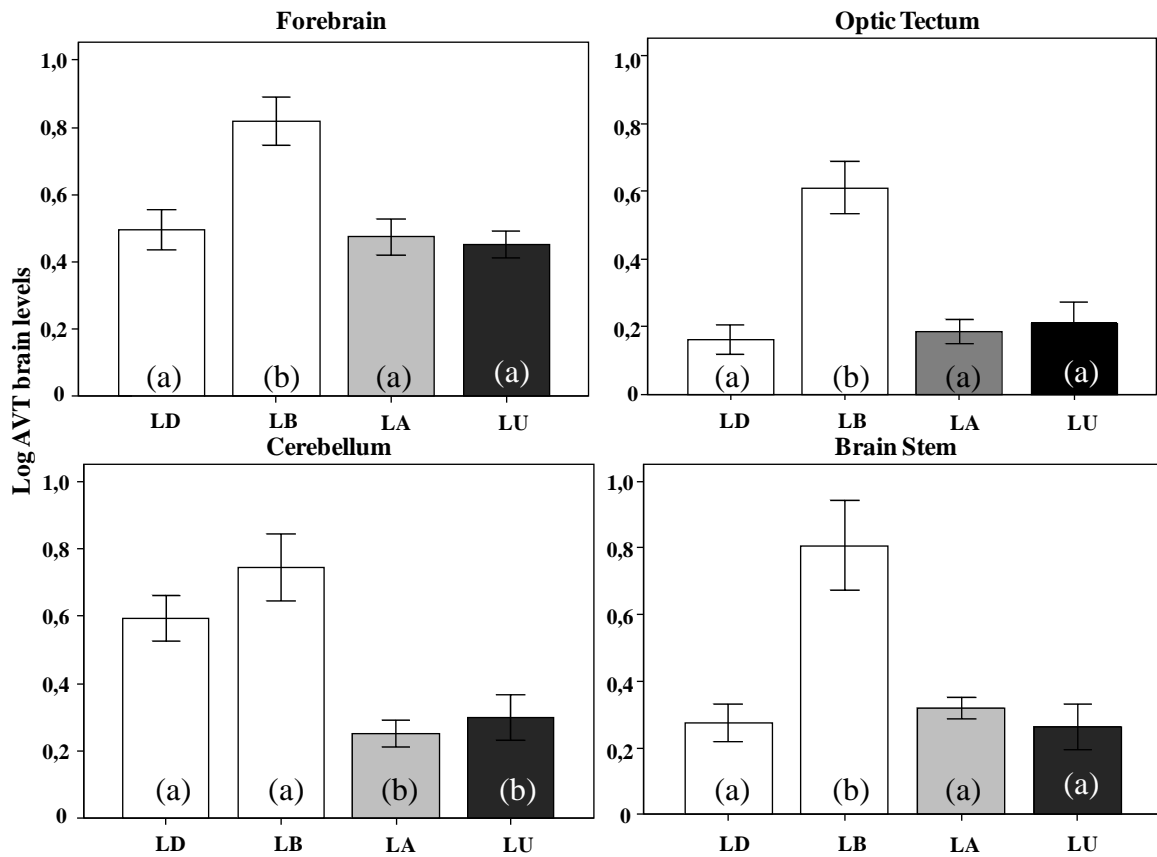


Figure 3. Levels of arginine vasotocin (AVT) in different brain areas (Mean \pm SE): forebrain, optic tectum, cerebellum and brain stem across four close related Labrid fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU - *Labrichthys unilineatus*. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

Table 2. Tukey pos-hoc HSD tests for arginine vasotocin levels between different brain areas and across different species. Significant correlations are marked with * for $p < 0.05$ and ** for $p < 0.01$

Brain regions	Species				
		<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichtys unilineatus</i>
Forebrain	<i>Labroides dimidiatus</i>	-----	$p=0,002^*$	$p=0,995$	$p=0,963$
	<i>Labroides bicolor</i>	$p=0,002^*$	-----	$p=0,001^*$	$p=0,001^*$
	<i>Labropsis australis</i>	$p=0,995$	$p=0,001^*$	-----	$p=0,994$
	<i>Labrichtys unilineatus</i>	$p=0,963$	$p=0,001^*$	$p=0,994$	-----
Optic Tectum	<i>Labroides Dimidiatus</i>	-----	$p<0,001^{**}$	$p=0,992$	$p=0,946$
	<i>Labroides Bicolor</i>	$p<0,001^{**}$	-----	$p<0,001^{**}$	$p<0,001^{**}$
	<i>Labropsis australis</i>	$p=0,992$	$p<0,001^{**}$	-----	$p=0,99$
	<i>Labrichtys unilineatus</i>	$p=0,946$	$p<0,001^{**}$	$p=0,99$	-----
Cerebellum	<i>Labroides Dimidiatus</i>	-----	$p=0,457$	$p=0,007^*$	$p=0,039^*$
	<i>Labroides Bicolor</i>	$p=0,457$	-----	$p<0,001^{**}$	$p=0,001^*$
	<i>Labropsis Australis</i>	$p=0,007^*$	$p<0,001^{**}$	-----	$p=0,966$
	<i>Labrichtys unilineatus</i>	$p=0,039^*$	$p=0,001^*$	$P=0,996$	-----
Brain Stem	<i>Labroides Dimidiatus</i>	-----	$p<0,001^{**}$	$p=0,981$	$p=1,000$
	<i>Labroides Bicolor</i>	$p<0,001^{**}$	-----	$p=0,001^*$	$p<0,001^{**}$
	<i>Labropsis australis</i>	$p=0,981$	$p=0,001^*$	-----	$p=0,967$
	<i>Labrichtys unilineatus</i>	$p=1,000$	$p<0,001^{**}$	$p=0,967$	-----

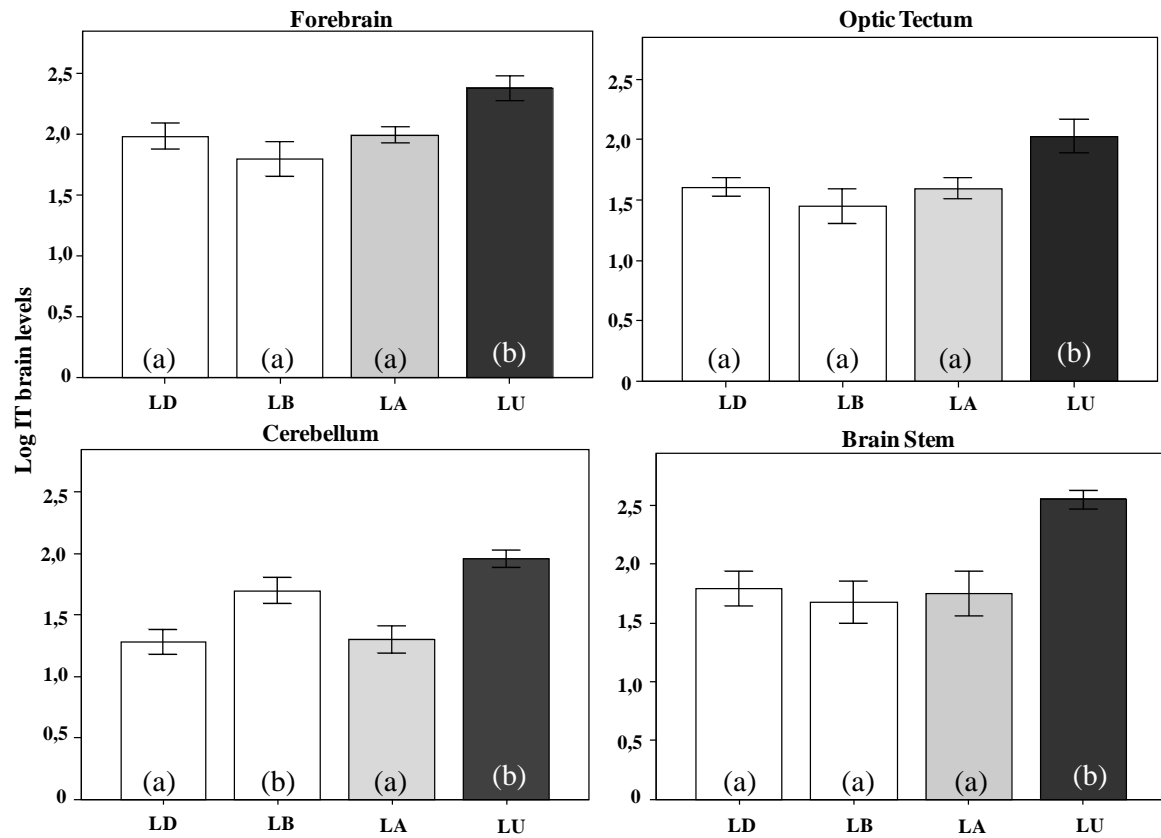


Figure 4. Levels of isotocin (IT) in different brain areas (Mean \pm SE): forebrain, optic tectum, cerebellum and brain stem across four close related Labrid fish species: LD – *Labroides dimidiatus*, LB – *Labroides bicolor*, LA – *Labropsis australis* and LU - *Labrichthys unilineatus*. Means with different subscripts were significantly different from each other in Tukey pos-hoc HSD tests.

Table 3. Tukey pos-hoc HSD tests for isotocin levels between different brain areas and across different species. Significant correlations are marked with * for $p < 0.05$ and ** for $p < 0.01$.

Brain regions	Species				
		<i>Labroides dimidiatus</i>	<i>Labroides bicolor</i>	<i>Labropsis australis</i>	<i>Labrichtys unilineatus</i>
Forebrain	<i>Labroides dimidiatus</i>	-----	$p=0,587$	$p=1,000$	$p=0,039^*$
	<i>Labroides bicolor</i>	$p=0,587$	-----	$p=0,557$	$p=0,002^*$
	<i>Labropsis australis</i>	$p=1,000$	$p=0,557$	-----	$p=0,044^*$
	<i>Labrichtys unilineatus</i>	$p=0,039^*$	$p=0,022^*$	$p=0,044^*$	-----
Optic Tectum	<i>Labroides Dimidiatus</i>	-----	$p=0,759$	$p=1,000$	$p=0,041^*$
	<i>Labroides Bicolor</i>	$p=0,759$	-----	$p=0,793$	$p=0,005^*$
	<i>Labropsis australis</i>	$p=1,000$	$p=0,793$	-----	$p=0,035^*$
	<i>Labrichtys unilineatus</i>	$p=0,041^*$	$p=0,005^*$	$p=0,035^*$	-----
Cerebellum	<i>Labroides Dimidiatus</i>	-----	$p=0,032^*$	$p=0,999$	$p<0,001^{**}$
	<i>Labroides Bicolor</i>	$p=0,032^*$	-----	$p=0,044^*$	$p=0,321$
	<i>Labropsis Australis</i>	$p=0,999$	$p=0,044^*$	-----	$p<0,001^{**}$
	<i>Labrichtys unilineatus</i>	$p<0,001^{**}$	$p=0,321$	$p<0,001^{**}$	-----
Brain Stem	<i>Labroides Dimidiatus</i>	-----	$p=0,956$	$p=0,997$	$p=0,007^*$
	<i>Labroides Bicolor</i>	$p=0,956$	-----	$p=0,988$	$p=0,003^*$
	<i>Labropsis australis</i>	$p=0,997$	$p=0,988$	-----	$p=0,004^*$
	<i>Labrichtys unilineatus</i>	$p=0,007^*$	$p=0,003^*$	$p=0,004^*$	-----

Discussion

In this study, we showed that the levels of both AVT and IT in the brain varied significantly across our studied Labrid species that express different degrees of mutualistic behaviour. These differences occurred both at the level of the whole brain and at the specific brain macro-areas, i.e. forebrain, optic tectum, cerebellum and brain stem.

Both obligate cleanerfish species *L. dimidiatus* and *L. bicolor* had higher levels of whole brain AVT compared to non-cleaners. This pattern is repeated in cerebellum. The neurophysiological mechanisms and neural circuitry of the cerebellum is considered quite conservative across vertebrate species (Rodriguez et al., 2005). In teleost fishes, cerebellum is more than a selective area of motor control and it is implicated in several cognitive and emotional functions, particularly in associative learning and memory processes (Rodriguez et al., 2005). Individuals of obligatory cleaner species have to interact with a large range of client species which they need to recognize and memorize for future interactions (Bshary and Côté, 2008). This profusion of interactions may become easily predictable due to the constant amount of behavioural feedback received. Because associative learning and memorizing is the core of the most of the behavioural tactics shown by species of obligate cleanerfish, the involvement of the cerebellum is not a surprise. The higher levels of AVT solely in the cerebellum of both cleaner species may suggest the participation of AVT in processes of associative learning and memorizing connected with cleaning behaviour.

The full time cleaner, *L. bicolor*, which exhibits wider home ranges and a higher tendency to be more aggressive with their clients (e.g. they cheat more, Oates et al., 2010, 2012; Mills and Cote, 2010) than *L. dimidiatus*, had higher AVT levels in whole brain and at each specific brain macro-area, i.e. forebrain, optic tectum, cerebellum and brain stem. Thus the higher AVT levels in *L. bicolor* seem to be related to aggressiveness and territoriality of this species. Indeed, much evidence regarding the action of AVT is associated with dominance, aggressive behaviour and territoriality. For instance, Dewan and colleagues (Dewan et al., 2008, 2011) found that aggressive behavior in the territorial butterflyfish species was linked with larger AVT-ir neurons in the gigantocellular POA and also with a higher AVT-fiber density in the telencephalon. In the three-spined stickleback (*Gasterosteus aculeatus*), aggressiveness and territoriality correspond with high concentrations of whole brain AVT: the highest brain concentrations have been observed in the most aggressive males while these take care of the eggs (Kleszczyńska et al., 2012).

Relationship between AVT and aggression during paternal care has been also observed in the plainfin midshipman where the aggressive males that guard eggs have larger AVT-immunoreactive (AVT-ir) cells in POA compared to males without nests (Foran, and Bass, 1998). Furthermore, in African cichlid fish (*Astatotilapia burtoni*), territorial, aggressive males exhibit higher level of AVT mRNA expression in posterior POA than non-territorial and non-aggressive ones [Greenwood et al., 2008]. However, we have to keep in mind that all quoted studies were executed in males, whereas our study was performed in females. Unfortunately there are no studies of the link between AVT and aggressiveness in females to refer to, but it appears that AVT and IT levels in the brain and their response to different social situations are sex-dependent; for instance, female three-spined sticklebacks kept alone have higher AVT and lower IT concentrations in their brains relative to females kept in groups, while males kept alone have higher AVT levels and show no differences in IT concentrations relative to males kept in groups (Kleszczyńska and Kulczykowska, 2013). Therefore we should be more circumspect about drawing conclusions on the relationship between neuropeptides and aggressiveness of females on the ground of data gained from studies of male response. At the brain stem, the modulation of AVT has been associated with motor control (Thompson et al., 2008) which in the case of *L. bicolor* potentially enables the maintenance of high motor-related activity levels, necessary to patrol their larger territories (Mills and Cote, 2010). Moreover, the higher levels of AVT also found within the optic tectum may be coupled with strong roaming abilities of this species, which are mostly dependent on vision. Adult females of our other two species - *L. australis* and *L. unilineatus* which are non-cleaners (only juvenile *L. australis* are cleaners but these were not included in our sample), both revealed to have significantly lower AVT levels in cerebellum and in the whole brain than two cleaners. Thus higher AVT levels in cerebellum and in the whole brain seems to be related to expression of mutualistic behaviour and can be influenced by aggressiveness, territoriality and roaming behaviour, as we observe in case of *L. bicolor*.

Over the past few years our knowledge about the brain IT and its association with specific fish behaviour has significantly increased. An intracerebroventricular infusion of IT stimulated social approach in goldfish (Thompson and Walton, 2004) and both AVT and IT promoted sociability when given peripherally to zebrafish (*Danio rerio*; (Braidă et al., 2011). Reddon and colleagues (2012) found that intraperitoneal injections of IT resulted in enhanced sensitivity to social stimuli and increased submission rates in social groups of the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. Isotocin is also

involved in courtship of the three-spined stickleback (Kleszczyńska et al., 2012). Moreover, in-depth studies in the monogamous cichlid, *Amatitlania nigrofasciata* indicate that IT plays a significant role in promoting paternal care likely via peptide release from the parvocellular POA neurons but it does not appear to play a significant role in affiliative behaviour towards the mate in an established pair bond (O'Connell et al., 2012). A previous study in monogamous cichlid fish showed that nonapeptides mediate the formation of a pair bond but not its maintenance (Oldfield and Hofmann, 2011). However, much is still to be discovered regarding the role of IT as modulator of cleaning behaviour. For instance, it is known that the OT/IT system is involved in the cooperation between bonded individuals (Madden and Clutton-Brock, 2011). However, the role of IT in cleaning behaviour has been harder to disclose. Recent data, has pointed out for a link between brain IT levels and cleaner wrasse's less cooperative behaviour. An influence of IT was mostly seen in males and it was dependent on variation in intra-pair relationship (Chapter 4).

In this study, only the non-cleaner species *L. unilineatus* showed significantly higher IT brain levels (overall and at each brain macro-area) compared to all other species (either obligatory or facultative cleaner species). Interestingly, adult females of facultative cleaner *L. australis* which are in fact non-cleaners, had similar levels of IT (measured in their whole brains and brains macro-areas) than those of obligate cleaners. Therefore brain IT does not seem to be related to the expression of mutualistic behaviour. On the other hand, *L. australis* is a cleaner as *L. dimidiatus* and *L. bicolor* during its juvenile stage. Thus the flexibility and facultative cleaning episodes in life history may be reflected in lower brain IT than that of adamant non-cleaner *L. unilineatus*. Interestingly, while all species exhibit female to male sex change (protogynous hermaphrodites), only the non-cleaner *L. unilineatus* displays phenotypic sexual dimorphism (males and females are different according to size, colour and behaviour) while all three Labroides species are monomorphic. There is evident sexual dimorphism in brain AVT and IT concentrations in species demonstrating clear phenotypic sexual dimorphism as black molly (*Poecilia sphenops*) and three-spined stickleback (Kulczykowska et al. in press, Kleszczyńska and Kulczykowska, 2013). Therefore we can speculate that higher levels of IT in the brains of *L. unilineatus* are linked to the development of sexual dimorphism, which only occurs in this non-cleaner species.

Moreover, we did not observe any link between brain IT levels and aggression in labrid fish species. Similarly, administration of IT seems to have no effect on the

aggressiveness of *N. pulcher* (Reddon et al. 2012). On the other hand, IT is significantly higher in aggressive dominant males of the three-spined stickleback that defend their territory (Kleszczyńska et al. 2012). The link between brain IT and aggressive behaviour in fish is not clear and again we cannot reach the conclusion about the relationship between nonapeptides and aggressiveness in females on basis of studies in males. It is also possible that the higher levels of IT observed in *L. bicolor* and *L. unilineatus* could be related to processing of specific social information or to learning of particular socio-sexual cues, which still need to be disclosed.

In summary, AVT is known to be involved in the expression of mutualistic behaviour in cleanerfish (Soares et al 2012, Mendonça et al 2013). Here, we provided for the first time comparative data on peptide levels in different brain regions of both cleaner and non-cleaner species which confirm that higher AVT levels in cerebellum and in the whole brain seem to be related to expression of mutualistic behaviour. Our study highlights the relevance of AVT in modulation of territory size (roaming behaviour) and interspecific aggressiveness of the obligate cleanerfish *L. bicolor*. On the other hand, brain IT is not related to expression of mutualistic behaviour but higher levels of IT in *L. unilineatus* suggest that these might be linked to the development of sexual dimorphism, which occurs only in this non-cleaner species. Further integrative and comparative work is clearly necessary to unravel the neural circuits underlying cleaning behaviour and how these are modulated by nonapeptides.

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CHAPTER
6

General Discussion

1. Modulation of cooperative behaviour - Overview

Considering the vast diversity of examples for cooperation, there likely exists an equal diversity of mechanisms that regulate cooperation. However, despite this wide variation in both the extent and complexity of interactions, cooperation is thought to have evolved from a combination of more “simple” social behaviours”, that gradually changed to constitute more specialized/”building-block” behaviours (Soares et al. 2010). For instance, specialized behaviours that will make an individual: (a) to tolerate the presence of conspecifics; (b) to coordinate its actions to produce behaviour that reduces immediate pay-offs; (c) to recognize partners and assess their behaviour and (d) to choose the appropriate responses from the available behavioural repertoire (Soares et al., 2010). Many of these problems may be solved with genetically determined rules (Brosnan et al. 2010). However, in many vertebrate animals, the brain mechanisms described above allow individuals to more flexibly respond to environmental stimuli.

2. Cognitive modules underlying cooperative behaviour

Cognitive abilities may be important for two different aspects of cooperation: First, cognition may help make coordination between partners more efficient and secondly, cognition may be important to make strategic decisions concerning the best behavioural option in a given situation (Brosnan et al., 2010). My focussed cleaner wrasse (*L. dimidiatus*) gathers characteristics and shows specialized cognitive abilities related to its mandatory cleaner demands, which will be explained further along in this chapter.

a) Evaluation of the social environment and learning

When individuals get involved in a social interaction it becomes necessary to evaluate its partner's behaviour in order to respond properly. Is the partner cooperating or investing less than it could, and will the benefits be enough to initiate and maintain a cooperative relationship? Neuromodulators will influence the perception of the social partner's effort, and will contribute to appraise the critical limit of each interaction (Soares et al., 2010). In some cases, the individuals react directly, in response to the partners' behaviour, as cleaner wrasses' clients set apart or pursue the cleaner if it feeds on mucus instead of ectoparasites (Bshary and Grutter, 2002). Another way of evaluating occurs through the observation of

the cooperative behaviour of a third party. In this way, those watching the interactions have access to information about potential partners in an indirect way, and may behave in a more adequate way in future interactions. On the other hand, those who are involved in the interactions also adjust their behaviour accordingly to the existence or nonexistence of an audience (audience effects – Early, 2010). Studies in humans reveal they are more cooperative when being watched by a third party than when they remain anonymous (Milinski et al., 2002). Pinto and his colleagues (2011) showed that cleaner wrasses increased their cooperative levels when observed by other clients, showing, for the first time, in non-humans, that the reputation before an audience increases cooperative behaviour.

b) Memory and social recognition

Social recognition is a necessary ability to distinguish cooperative and cheating partners between other more valuable partners and those who have less value (in other words, if they have more or less parasites). Cleaner wrasses showed abilities of individual recognition, when they spent more time next to familiar clients than to non-familiar clients (Tebbich et al., 2002). There are also clear evidences that cleaner wrasses are able to distinguish their resident clients (species which have restricted access to a certain cleaning stations) or non territorial clients, which hold more than a cleaning station in the area where they move (Bshary et al., 2002, Soares et al 2014). This way, and respecting the Theory of Biological Markets (Noë et al., 1991), the clients with more options in choosing a cleaning station have priority in accessing the cleaning service versus the clients with no options, when choosing cleaning stations (Bshary et al., 2002). This happens because clients with choosing options go to another cleaning station if they are not attended first (Bshary and Schäffer, 2002).

c) Social bonding

Social bonding mechanisms are necessary in the sense of enabling tolerance and maintaining associations amongst social partners (Chapter 4). In cooperative interactions winnings can be lost in time, and the connection may help overcoming the problem of non-immediate benefits (Soares et al., 2010). This means social bonding modulates the perception of benefits' exchange and, that way, may promote the maintenance of cooperative interactions. For instance, when cleaning in pairs, the quality of service

provided to clients should be modulated by the association strength of the partnership (chapter 4).

d) Tendency to approach partners

To engage in any cooperative interaction, animals need to have a predisposition to approach potential partners. The motivation to interact will need to surpass tendency of fleeing. Again, cleaner wrasses are good examples of standard high motivation to interact. In chapter 5, I also studied another species that exhibits high motivation to interact, another labrid species, *L. bicolor*. This tendency to approach other animals (in the case of the cleaners, other heterospecifics or clients) is even more surprising when it comes to interact to dangerous clients (piscivorous). Indeed, obligate cleanerfish are known to interact with dangerous clients while some species of cleaning gobies attend these clients preferentially (Soares et al., 2007). Cleaner wrasses also behave differently when interacting with predators: always start by providing tactile reward and refraining from cheating (Grutter 2004). These behavioural changes demonstrated by cleaning gobies and cleaner wrasses seem to be modulated by shifts in the regulation of stress and also by the dopaminergic system. Interestingly, facultative cleaners (species that clean only during juvenile stages) show high tendency to approach potential clients but solely if these are harmless (e.g. herbivorous, Côté, 2000).

e) Impulsivity and temporal discounting

In a cooperative context, individuals may sometimes choose to obtain a smaller reward with the purpose of maintaining future beneficial relationships. For instance, cleaner wrasses often eat against preference in order to gain longer access to clients (Grutter and Bshary, 2003). Thus, instead of removing clients' mucus and obtain an immediate reward (acts according to preference), the cleaner wrasse obtains a longest relationship but with less immediate effects, by controlling its impulsivity of eating what it prefers.

f) Partner choice

In order to choose the most appropriate partner, one has to develop what is known as “cooperative behavioural competence” (Soares et al., 2010). In order for that to happen, it

is necessary to: 1. analyse the partner and evaluate the signals of honesty/dishonesty; 2. recognize, memorize and categorize the previous interactions with the purpose of establishing lasting relationships; 3. apply different strategies (investment degree) regarding the context.

In the cleaner wrasse system, there is a conflict of interests between the social partners (cleaner and client) and, to resolve this conflict regarding the benefits achieved, both sides negotiate and compromise in two ways: using threat (reciprocity or abandonment of the cleaning station) or bargaining (when the cleaner uses tactile stimulation (Cant, 2014)). Cleaner wrasses show preferences for partners and alter their behaviour in accordance. Indeed, they manifest two distinct cleaning strategies (Bshary, 2002), which are not standard (Bshary and D'Souza, 2005): most "normal" cleaners show little interest in small clients and are rarely dishonest with bigger clients. However, cleaners (females) exhibit an alternative strategy: the so-called "biting cleaners", seek for small clients, to whom they provide tactile stimulation (Bshary, 2002). This behaviour brings about other larger clients that by observing these interactions (Pinto et al., 2011), seek to be cleaned by these "biting cleaners", which then become dishonest and remove mucus instead of ectoparasites. Recent findings show that changes in cortisol levels and in cortisol signaling (via GC receptor antagonism) are associated with these significant behavioural changes in the cleaning service provided by cleaners to their clients (Soares et al., 2014).

In short, the perception, evaluation and animals' behavioural response is adaptable to specific situations in a changing and complex world (Soares et al., 2010). My findings show that neuropeptides influence and are influenced by the components of cooperative behaviour.

3. Neuropeptide regulation of interspecific cooperative behaviour (e.g. cleaning)

This study yielded several findings. In chapter 2, I tested the influence of AVT upon the cleaners' ability to solve two different problems that in principle differ in ecological relevance and are associated with two different memory systems and found that AVT

affected the learning competence of cleaners as individual performance showed distinct response selectivity to AVT dosage levels. However, only in the ecologically relevant task was their learning response improved by blocking AVT via treatment with the antagonist Manning compound. Next, in chapter 3, I examined if neuropeptides may be implicated in the mechanisms underlying the adjustment of individuals to the existence of partner control mechanisms in cooperative interactions between unrelated individuals and discovered 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. Then, in Chapter 4, I asked if the establishment of privileged ties and the quality of association between cleaner wrasse pairs is correlated with neuroendocrine mechanisms involving forebrain neuropeptides and whether these neuropeptides level shifts relate to individual's interspecific service quality. Here I found that variation in pairs' relationship influence male and female cleaner fish differently and contribute to the variation of brain neuropeptide levels, which is linked to distinct cooperative outcomes. Finally, in Chapter 5, I explored the link between these neuroendocrine pathways and the expression of mutualistic behaviour in fishes by comparing the brain quantitative distribution of AVT and IT across the overall and in selected areas of the brain; aiming at four closely related species of labrids that differ in the degree to which they depend on cleaning. The levels of both AVT and IT varied significantly across species, as measured in the whole brain or in specific macro-areas. More importantly, significantly higher AVT levels in cerebellum and in the whole brain were found in the obligate cleaner species, which seems to be related to expression of mutualistic behaviour

Bellow, I will discuss in further detail the influence of AVT and IT in the behavioural response and decision making of the focussed study model the cleaner wrasse *L. dimidiatis* in each cognitive module described above.

a) Evaluation of the social environment and learning

In mammals, the amygdala is related with emotional learning and memorizing, and the hippocampus is related with spatial memory, either in context or by association (Portavella et al. 2002). In fish, evidence shows these cognitive abilities may be related to the forebrain, specifically to the dorsolateral (Dl) and the dorsomedial (Dm) telencephalon, which are most likely homologues of the hippocampus and amygdala of mammals,

respectively (Northcutt 1995; Salas et al. 1996a,b; López et al. 2000; Wullimann and Mueller 2004). Pharmacological manipulations of AVT had effect on both learning types (spatial and cue learning) in a cleaning context (Chapter 2). This points out for existence of AVT receptors in both circuits, as described in other fish species (Dewan et al., 2011). Indeed, the recent mapping of the neuropeptides receptors in fish brains species showed the receptors of V1a and IT are widely spread in the forebrain, being present in the D1 and Dm (Kline et al., 2011; Huffman et al., 2012). Moreover, the AVT effects seem to be dosage dependent, which might be related with the variation of expression of the different receptor in each memory circuit: one related with interspecific interactions and the other linked to the promotion of spatial discrimination or territoriality (Chapter 2).

b) Memory and social recognition

First evidence retrieved in natural conditions reported that elevation in the levels of AVT decrease cleaners' propensity to engage in interspecific cleaning activities (Soares et al., 2012). In chapter 3, and contrary to my initial predictions administration of AVT rather than blocking its V1a-type receptors by the antagonist Manning compound caused a decrease of cleaners' levels of feeding against preference, which may imply that there might exist a link between AVT effects and social memory and recognition.

For pairs of cleaners, variation of couple association and hence, levels of intra-couple recognition, seem to be linked to forebrain IT levels, particularly with male cleaner wrasses (Chapter 4). These males, which scored higher brain IT levels, were seen cleaning more often in pairs and receiving greatest amounts of conspecific support. In turn, the levels of chases or aggressive events were lower. In females, AVT seems to be related to couple dissociation, which could preclude a decrease of social recognition and a rise in impulsivity (these females were observed to cheat more often).

c) Social bonding

The establishment of privileged relationships between animals and the maintenance of coordinate behaviour between partners is a fundamental topic for cooperation and it is the aim of my Chapter 4. I found that the neuropeptide system is indeed involved in these mechanisms. As such, in Chapter 4 I show that the variation in pairs' relationship influence male and female cleaner fish differently, contributing to the variation of brain neuropeptide

levels and consequently to pairs' behavioural output. I specifically identify a link between forebrain IT levels and cleaner wrasse behaviour. In females, high levels of AVT in the forebrain seem to be related to their cheating levels, though the same has not been observed with its partners. It was also observed that females belonging to lower association rate couples were more dishonest with their clients when cleaning alone. The AVP/AVT system is highly implicated in the neuroendocrine stress response processes in vertebrates - Backström et al., 2011): in response to stress, gene expression of AVP/AVT is upregulated in parvocellular neurons in mammals and in teleost fish (Aubry et al., 1999; Gilchrist et al., 2000). 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 (Chapter 4).

d) Tendency to approach clients

As mentioned above, cleaning behaviour establishes a social relationship from where benefits can arise to both parties, thus becoming an incentive to investment and social approximation. The neuropeptide system is known to mediate social approach (Braida et al., 2012). A first field study concerning AVT mediation of cleaning activities revealed that AVT's elevation diminished interspecific cleaning behaviour (Soares et al., 2012). But on the contrary, AVT increased cleaner wrasses predisposition to interact with conspecific partners (i.e. paired close swimming) that was reciprocated by a rise in the levels of tactile stimulation received (Soares et al 2012). It was then hypothesized by Soares and colleagues (2012), that shift in endogenous AVT levels should modulate perceptive, motivational and cognitive mechanisms that, in turn will affect cleaner wrasse behaviour, both in conspecific social relationships and interspecific cooperative interactions. It remained to be discovered how would endogenous AVT and IT levels be recruited amongst brain areas and would those changed in comparison with other close-related species that do not engage in cleaning. If anything, AVT pathways would need to be regulated in a way to make individuals more capable to approach heterospecifics, using the same physiological mechanisms already in place that modulate conspecific behaviour. A comparative neuroanatomical study performed between cleaner wrasses *L. dimidiatus* and the non-cleaner corallivorous *L. unilineatus* revealed smaller and less numerous AVT-producing neurones in the POA in the cooperative species (Mendonça et al., 2013). Thus, if anything, one could expect cleaner organisms to have lower availability of AVT at

different putative target regions in the brain and overall. In Chapter 5 my findings show that the two obligatory cleaner species (*L. dimidiatus* and *L. bicolor*) have higher whole brain AVT levels compared to non-cleaners *L. australis* and *L. unilineatus*. Interestingly, this pattern is repeated in the cerebellum. This result is not entirely surprising considering that the cerebellum is linked to several cognitive and emotional functions, particularly to associative learning and memory processes (Rodriguez et al., 2005). Obligatory cleaners have to interact with a large range of client species repeatedly, which they need to recognize and memorize for future interactions (Bshary and Côté, 2008). The higher levels of AVT solely in the cerebellum of both cleaner species may suggest the participation of AVT in processes of associative learning and memorizing connected with cleaning behaviour in these species. Interestingly, the detected AVT values in all four wrasse species considered in Chapter 5, are far lower than those found in species which do not form interspecific relationships (Almeida et al., 2012; Kleszczyńska and Kulczykowska, 2013). In a nutshell, non-cleaner species have shown lesser levels of AVT both in the whole brains as in the cerebellum, which tells us higher AVT levels may be connected to the expression of mutualistic behaviour, and can be influenced by aggressiveness, territoriality and roaming behaviour, as revealed for *L. bicolor* (Chapter 5).

The role of IT in the context of interspecific cooperation is harder to decipher. In other fish species, IT seems to relate to social approach, in goldfish *Carassius auratus* (Thompson and Walton, 2004), to the promotion of sociability in zebra fish (Braidă et al., 2012), to the increase of sensitivity to social stimuli and the social submission of cichlids who cooperate in the reproduction (Reddon et al., 2012) and also in courtship of three-spined stickleback (*Gasterosteus aculeatus*) (Kleszczyńska et al., 2012). In the cleaner wrasse system, forebrain IT levels seemed to relate to male cleaner wrasses living in stronger/stable pair associations (Chapter 4). These males were found to clean more often with their preferential females, had access to larger clientes and cheated more frequently. Female cleaner wrasses' cheating rates seemed also linked to their forebrain IT levels, but these were less dependent on pair relationship (Chapter 4). Indeed, in the cleaner wrasse system, brain IT levels seems to vary conditionally to pair status or association. In Chapter 5, differences found amongst species and brain areas seemed again to relate less to mutualistic expression degree and more to do with adamant/organizational of a whole-life non-cleaning condition and the development of sexual dimorphism (*L. unilineatus*). The IT

levels registered in the cerebellum of *L. bicolor* and *L. unilineatus* may be related to the processing of relevant social information or learning of certain social/sex cues.

e) Impulsivity and temporal discounting

During cleaning events, cleaners have to make choices regarding investments and outcome benefits: for instance, either chose reward with a temporal mismatch or otherwise immediate benefits, taking some risks. That is, they need to control the tendency to win immediate benefits (eating accordingly to their preference - clients' mucus) in order to achieve cooperative stability with their clients, thus assuring future interactions (Grutter and Bshary, 2003). The absence of impulse control has been associated with reduced activity of the serotonergic system (Soubrie, 1986; Linoila et al., 1995). Serotonin seems to have an influential role in the altering of the secretion and release of AVP. A hyporeactive serotonin system may result in enhanced AVP activity and aggression which contributes to rise in impulsive or reactive behaviour towards stimuli. Cleaners' tendency to eat more preferred items (a response that contradicts what they had previously learned) following AVT treatment could be sustained on a rise of impulsive behaviour (Chapter 3). AVT levels rise could also be linked to an interruption of immediate social recognition, which made cleaners, fail to identify and anticipate clients' response, leading to free behaviour accordingly to their preferences (Chapter 3).

f) Partner selection

Neuroendocrine influence regarding partner selection in the cleaner wrasse system has been only partially disclosed. Soares and colleagues (2012) found that the V1a receptor antagonist (by using Manning compound) mediated a rise in cleaners' dishonesty via central effects on the V1a-type receptors. Indeed, by blocking the V1a receptors cleaners seemed to clean more and care less about maintaining good service quality. In Chapter 2 I show that the blocking of the V1a receptors significantly increased learning speed in relation to the reference treatment (saline injection) but solely on the cue learning experiment (considered to be contextually more relevant). Taking into consideration that cleaners need to tune their behaviour according to each specific situation presented and that being receptive to learn, associate and sometimes select the most beneficial situation then, it does seem that the AVT pathways are involved.

The effects of the AVT system on cleaner wrasse behaviour and partner selection might be working in integration with the hypothalamic- pituitary-interrenal tissue (HPI) stress axis (Balment et al 2006, Huffman et al 2014). Indeed, it is now known that changes in cortisol levels are related to how cleaners choose to approach clients and how these behave with them and even for the development of cleaning alternative tactics (Soares et al 2014). Future work is needed to investigate the link between AVT regulation and cortisol shifts and how this influences cleaner wrasses cooperative levels output.

4. The regulation of cleaning by neurotransmitter systems: is there a role for neuropeptides?

Parallel research on the cleaner wrasse system has recently pointed out for an interesting role of the serotonergic and dopaminergic systems. Paula et al (2015) found that enhancing serotonin made cleaner wrasses more motivated to engage in cleaning behaviour, and more likely to provide physical contact to clients (tactile stimulation) without spending more time cleaning or cheating more often. Blocking serotonin-mediated response resulted in an apparent decrease in cleaners' cheating levels and in an increase in cleaners' aggressiveness towards smaller conspecifics (Paula et al 2015). Serotonergic activity is linked to the AVT and OT systems. For instance, research, mostly done in rats, has confirmed that serotonin (and its agonists and selective serotonin reuptake inhibitors) play a key role in the altering the secretion and release of AVP and OT (Jørgensen et al 2003). Moreover, studies on hamsters are consistent with those done on teleost fish (Semsar et al. 2004), with the use of fluoxetine being responsible for a decrease in brain AVP levels (in hamsters) and lowering mRNA AVT levels in fish (Ferris, 1996, Semsar et al. 2004). If it is true that the rise of serotonin activity levels mediates a decrease in brain AVT production and release, then cleaner wrasses' behavioural response (which increased in their motivation to engage in cleaning) is in line with Soares and colleagues (2014) and now with my results in Chapter 2 and 3, which identified the involvement of the AVT pathways in cleaner wrasse behaviour and learning.

The Dopaminergic system has also been found related to cleaner wrasses behaviour modulation. Messias and colleagues (in review) show that by reducing the dopamine activity levels in cleaner wrasses it influences their effort to seek interactions and increase tactile stimulation provision, a behaviour that these individuals use to negotiate the

occurrence and duration of interactions with partners, but not necessarily influence the quality of their given service.

The understanding of the exact nature of the potential interactions between the neuropeptides AVT and IT with these evolutionary old and also highly conserved systems like the Serotonin and Dopamine will further the knowledge of these systems, how they work in integration and most importantly how they modulate interspecific cooperative behaviour.

5. Neuropeptide key influence in the building of cooperative behavioural competence - Final Considerations

AVT and IT neuropeptides are known as important modulators of teleost fish social behaviour and they have shown to be important regulators of mutualistic behaviour amongst fish (Soares et al., 2012). Here I show that AVT and to some extent IT pathways are indeed involved in the mediation of cleaner wrasse *L. dimidiatus*' behavioural output. Indeed, I found links of neuropeptidergic modulation in all the cognitive building blocks that presumably are the core ingredients in the making-of a cooperative species (see above). The occurrence of cooperative behaviour (cleaning), which varies in accordance to context, may be achieved by different combinations of neuromodulators that include AVT and IT but also cortisol, serotonin, dopamine and other different compounds. The “secret physiological recipe” that confers to an individual specific skills and cognitive abilities, enabling it to cooperate in a formal way seems to depend on the balance of the two studied neuropeptides (AVT and IT), interacting with other neuroendocrine/neuromodulator systems, and being regulated by the social context.

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