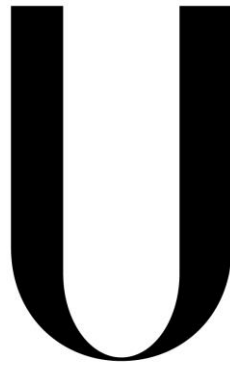


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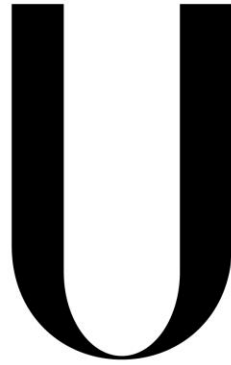
**The role of serotonin in neuromodulation of cleaner fish  
*Labroides dimidiatus* behaviour – a key species of coral reefs**

**José Ricardo Maceiras de Paula**

Dissertação  
Mestrado em Biologia da Conservação

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Orientadores:

Doutora Marta Soares

Professor Doutor Paulo Fonseca

**2013**

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**THIS WORK WAS PRESENTED IN THE FOLLOWING SCIENTIFIC MEETINGS:**

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Serotonin Neuromodulation of Cooperative Behaviour in a Cleaner Fish.  
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**Paula, J.\*;** Messias, J.; Grutter, A.; Oliveira, R.; Bshary, R.; Soares, M.  
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---

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## RESUMO

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Os ecossistemas de recife de coral têm vindo a sofrer um declínio massivo em abundância, diversidade e estruturação do habitat. Este declínio deve-se à sobre-exploração das pescas, poluição, doenças do coral e lixiviação. Entre 2050 e 2100, é expectável que a concentração de dióxido de carbono exceda as 500 partes por milhão e que as temperaturas globais aumentem pelo menos 2°C, excedendo significativamente os valores dos últimos 420,000 anos. O aquecimento global e a acidificação dos oceanos poderão comprometer a acreção de carbonato de cálcio, tornando os corais cada vez mais raros nos recifes. Prevê-se que esta sucessão de eventos resulte numa redução da diversidade e no desaparecimento de estruturas de carbonato, diminuindo a qualidade dos recifes, eventualmente, até ao seu colapso.

Nos recifes de coral, os organismos de limpeza, como o peixe limpador *Labroides dimidiatus*, Valenciennes, 1839, têm a função de remover ectoparasitas de outros organismos, denominados “clientes”. O comportamento de limpeza tem sido usado como um exemplo clássico de mutualismo, com benefício para os limpadores e para os clientes. Foi demonstrado que a remoção experimental do peixe limpador *L. dimidiatus* de áreas restritas de recifes “fragmentados”, durante 8 anos e meio, leva à diminuição de peixes clientes residentes nessas áreas. Estas alterações provocaram uma redução na taxa de crescimento e de sobrevivência dos peixes residentes. Com a remoção experimental verificou-se uma diminuição de 37% na abundância e 23% na riqueza específica comparativamente com os recifes controlo. A abundância de juvenis de peixes visitantes (peixes que normalmente se movem entre recifes) diminuiu em cerca de 65%. Estes dados sugerem que a presença de *L. dimidiatus* também afeta o recrutamento. Este estudo demonstrou que a presença do peixe limpador beneficia diretamente a comunidade de peixes de recife de coral, sob a forma de aumento de tamanho e taxa de sobrevivência. Adicionalmente, muitos dos grupos de peixes afetados podem também perturbar, indiretamente, outros organismos de recife, provocando assim um impacto mais geral na comunidade do recife de coral.

O peixe limpador do indo-pacífico *Labroides dimidiatus* conjuntamente com um restrito número de espécies das famílias Labridae e Gobiidae, apresenta o mais alto nível de especialização em comportamentos de limpeza, sendo classificado como limpador obrigatório, ou seja, a sua alimentação baseia-se exclusivamente no que consegue remover dos seus clientes, com os quais pode interagir mais de 2000 vezes

por dia. Distribui-se desde o Mar Vermelho até à Grande Barreira de Coral e o Mar de Coral, pode medir até 11,5 cm e habita recifes rochosos e de coral. De uma forma geral estes limpadores encontram-se em territórios específicos, designados por estações de limpeza, tanto em recife contínuo, como em recife “fragmentado”, tendo uma área vital muito limitada, restrita àquela estação. Estes vivem num sistema de harém, um macho com várias fêmeas, e apresentam mudança de sexo, sendo uma espécie protogínica. Poucos mutualismos foram tão estudados como o mutualismo de limpeza. Porém estamos ainda longe de compreender este mutualismo. Por exemplo, as razões causais (fisiológicas) que estarão na base do aumento da tolerância e motivação para interagir, seja com conspecíficos seja com indivíduos de outras espécies, estão ainda muito mal compreendidas.

O neurotransmissor serotonina é uma monoamina derivada do triptofano que pode ser encontrada no sistema nervoso central. Como em outros vertebrados, nos teleósteos a atividade serotoninérgica está inversamente associada com o status social. Para além disso, no bodião *Thalassoma bifasciatum*, Bloch, 1791, a administração de fluoxetina – um inibidor seletivo de recaptção de serotonina (SSRI) – diminui o comportamento agressivo em machos territoriais quando confrontados com intrusos, tanto em laboratório como na natureza. Consistentemente, o tratamento de ciclídeos boca de fogo (*Cichlasoma meeki* Brind, 1918) com p-clorofenilalanina, um inibidor de síntese da serotonina, resultou num aumento da agressividade quando se observavam ao espelho. Estes resultados estão em concordância com os dados similares encontrados em roedores que mostram que diferentes estratégias farmacológicas para aumentar o nível de serotonina resultaram numa diminuição do comportamento agressivo. Sabe-se também que as vias serotoninérgicas e os recetores de serotonina estão envolvidas nos processos de aprendizagem e memória. O uso de agonistas e antagonistas do recetor 5-HT<sub>1A</sub> em diversos estudos mostrou o efeito deste recetor tanto no bloqueio como na facilitação de processos de aprendizagem espacial e visual.

O objetivo deste trabalho é averiguar de que forma a serotonina atua na modulação do comportamento de *L. dimidiatus*. Para tal, foram aplicados in loco um SSRI, fluoxetina, um agonista dos receptores 5-HT<sub>1A</sub> e 5-HT<sub>7</sub>, denominado 8-OH-DPAT, um antagonista do receptor 5-HT<sub>1A</sub> o WAY 100.635 e um bloqueador de síntese de serotonina p-clorofenilalanina a peixes recolhidos na Lizard Island, Grande Barreira de Coral, Austrália (14°40'S; 145°28'E). Os meus resultados sugerem que o sistema serotoninérgico, principalmente através do recetor 5-HT<sub>1A</sub>, é um modulador importante dos processos cognitivos, motivacionais e percecionais. A ativação do sistema serotoninérgico esteve envolvida em processos motivacionais que levaram ao aumento

da predisposição para enverdar em interações de limpeza. Para além disso, a ativação através do recetor 5-HT<sub>1A</sub> levou a um aumento dos níveis de qualidade da interação, enquanto o bloqueio levou a uma diminuição dos níveis de cooperação interespecíficos e a um aumento das atividades conspécificas. Além das alterações no comportamento dos peixes limpadores através do sistema serotoninérgico, sabe-se que o contexto social e o comportamento dos clientes também contribui para a existência de alterações fisiológicas responsáveis pela regulação do comportamento cooperativo.

Foi também realizada uma experiência no Oceanário de Lisboa da qual o objetivo foi compreender como é que a serotonina influencia o processo de aprendizagem em *Labroides dimidiatus*, essencial para o estabelecimento e manutenção das interações mutualísticas entre este peixe e os seus clientes. Foram dadas injeções de fluoxetina, 8-OH-DPAT e WAY-100.635 para verificar a influência da serotonina na aprendizagem visual e espacial. Neste estudo, sobre a influência da serotonina na aprendizagem do peixe limpador, os meus dados sugerem que a serotonina tem um papel importante tanto na aprendizagem espacial como na visual. A aprendizagem foi influenciada através do recetor 5-HT<sub>1A</sub> e 5-HT<sub>7</sub>. O antagonista do recetor 5-HT<sub>1A</sub> WAY 100.635 atrasou a aprendizagem visual e o agonista do recetor 5-HT<sub>1A</sub> e 5-HT<sub>7</sub> atrasou a aprendizagem espacial. Apesar da aprendizagem espacial ser importante para este peixe ao nível de reconhecimento do território, esta não é relevante para a obtenção de alimento. No estudo do efeito da serotonina no comportamento, o antagonista WAY 100.635 teve um efeito “negativo” nalgumas medidas de qualidade de limpeza (e.g. duração de inspeção). Este antagonista levou a que os peixes limpadores interagissem menos (diminuição do interesse). Deste modo, o atraso causado pelo antagonista na aprendizagem visual pode estar relacionado com esta diminuição do interesse para interagir com clientes.

Estes dados mostram que a serotonina tem um papel essencial na regulação e manutenção das bases do comportamento cooperativo entre o peixe *Labroides dimidiatus* e os seus clientes.

Os recifes de coral estão a passar por alterações rápidas devido ao desaparecimento dos corais vivos, levando ao crescimento de outros organismos bentónicos nos corais mortos. Estes novos habitats são ideais para o desenvolvimento de ectoparasitas elevando a importância da manutenção das interações entre o peixe limpador *L. dimidiatus* e os seus clientes. Além disso, cada vez mais, são encontrados traços de drogas que modulam o sistema serotoninérgico em águas residuais. Os

tratamentos atualmente feitos nestas águas não são suficientemente eficientes para remover estes compostos.

O meu trabalho contribui para a compreensão dos mecanismos fisiológicos deste mutualismo de limpeza e para o conhecimento dos mecanismos causais para o estabelecimento de interações cooperativas entre este peixe limpador e os seus clientes.

### **Palavras chave:**

*Labroides dimidiatus*, serotonina, neuromodulação, aprendizagem, cooperação, mutualismo, recifes de coral, fluoxetina, 8-OH-DPAT, WAY 100-635 e p-clorofenilalanina.

# ABSTRACT

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The coral reef ecosystems have been experiencing massive declines in abundance, diversity and structure of habitat due to over-fishing, pollution, disease and coral bleaching. In coral reefs, cleaning species, such as cleaner fish *Labroides dimidiatus*, have the function of removing ectoparasites from client organisms, usually reef fish. The cleaning behaviour has been used as a classic example of mutualism, with benefit to cleaners and clients. It has been shown that total removal of *L. dimidiatus* from patch reefs leads to a drastic decrease in abundance and richness of fish client species. This leads to a need of understanding the physiological processes of mutual interactions between the cleaners and their clients. Serotonin is a neurotransmitter involved in the regulation of the social behaviour in all vertebrates. However, little is known about the role of serotonin in the modulation of cleaning behaviour and the physiological mechanisms involved in interspecific cooperative interactions.

The aim of this project is to investigate how serotonin modulates the behaviour and the learning processes of *L. dimidiatus*. Serotonin agonists, antagonists, SSRIs and inhibitors of synthesis were injected in *L. dimidiatus* at Lizard Island to understand how serotonin neuromodulate cleaning behaviour. The activation of the serotonergic system has been involved in motivational processes that led to an increased predisposition to enrol in cleaning interactions. In addition the agonist of the 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptor led to an increase in the quality of the interaction. The antagonist decreased levels of interspecific cooperative activity and increased conspecific interactions. In addition to *in situ* experiments, I made a laboratory assay to understand how important learning processes of this fish are influenced by serotonin. In this study about serotonin influence in learning my data suggest that serotonin plays an important role in both spatial and visual learning. My study improve our knowledge on the physiological bases for this important mutualistic interactions in the coral reef ecosystem.

## Keywords:

*Labroides dimidiatus*, serotonin, neuromodulation, learning, cooperation, mutualism, coral reefs, fluoxetine, 8-OH-DPAT, WAY 100-635 and p-chlorophenylalanine.

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## ABBREVIATIONS

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5-HT	Serotonin
8-OH-DPAT	(±)-8-Hydroxy-2-(diethylamino) tetralin hydrobromide
AVP	Arginine vasopressin
AVT	Arginine vasotocin
DI	Dorsolateral telencephalon
Dm	Dorsomedial telencephalon
FLX	Fluoxetine
PCPA	p-chlorophenylalanine
SSRI	Selective serotonin reuptake inhibitor
WAY 100.635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide



# INTRODUCTION

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## **a) Coral reef ecosystem conservation**

Coral reefs are among the most biodiverse ecosystems and economically important in the world, providing ecosystem services that are vital to human societies (Moberg & Folke 1999). The coral reef ecosystems have been experiencing massive declines in abundance, diversity and structure of habitat due to over-fishing, pollution, disease and coral bleaching (Pandolfi et al. 2003). It is expected that the concentration of carbon dioxide will exceed 500 parts per million and that overall temperature will increase at least 2°C between 2050 and 2100, significantly exceeding the value of the past 420,000 years during which the majority of marine organisms evolved (Hoegh-Guldberg et al. 2007). Global warming and ocean acidification will likely compromise carbonate accretion of calcium, making corals becoming increasingly rare on reef systems. It is anticipated that this sequence of events will result in a reduction in diversity and loss of the carbonate reef structures, producing local stress, lowering the quality of the reefs, possibly until its collapse (Hoegh-Guldberg et al. 2007).

In coral reefs, cleaning species, such as cleaner fish *Labroides dimidiatus*, have the function of removing ectoparasites from client organisms, usually reef fish (Grutter 2002). Cleaning behaviour has been used as a classic example of mutualism, with benefit to the cleaners and their clients (Raihani et al. 2010; Waldie et al. 2011). It has been shown that experimental removal of cleaner fish *L. dimidiatus* for 8.5 years on restricted areas of patch reefs leads to a decreased abundance of resident clients as compared to control reefs (Waldie et al. 2011). This resulted in a reduction of the growth rate and of survival of resident fish. Indeed, a decrease of 37% in abundance and 23% in species richness was verified when compared with control reefs (Waldie et al. 2011). This study demonstrated that the presence of cleaner fish directly benefits the community of coral reef fish in the form of increased size and survival rate. Additionally, many of the fish groups affected can also disrupt, indirectly, other reef organisms, thus causing an impact on the wider community of the coral reef (Waldie et al. 2011).

## **b) Cleaner fish *Labroides dimidiatus* and cooperative behaviour**

Cooperation is generally defined as any interaction between individuals where the investment costs are outweighed by its benefits (Dugatkin 1997). When interactions take place between individuals of different species they are referred to as mutualisms

(Côté 2000). The mutualism between *Labroides dimidiatus* and its “client” fish involves removing tissue and ectoparasites from the “client” species (Bshary & Côté 2008). These mutualisms integrate two actors: a cleaner, small with a conspicuous coloration (Lettieri et al. 2009; Cheney et al. 2009) and a client, typically larger (Côté 2000). The complexity of the social context in which these individuals live ultimately facilitate the development of adaptations at behavioural and morphological levels (Bshary & Côté 2008).

The Indo-Pacific cleaner wrasse *Labroides dimidiatus* Valenciennes, 1839, presents the highest level of expertise in cleaning behaviours, being classified as an obligate cleaner (Côté 2000), *i.e.* their food intake is based solely on what they can remove from their clients, with whom they may interact more than 2000 times a day (Grutter 1997). The cleaner wrasse is a bony fish belonging to the family Labridae, and is distributed from the Red Sea to the Great Barrier Reef and Coral Sea; it inhabits rocky and coral reefs and can be up to 11.5 cm long (Kuitert 2000). In general these cleaners are in particular territories designated cleaning stations, both in continuous reef or in patch reefs, and their vital area is very limited, restricted to that station. They live in a harem system composed by one male with several females, and may change sex, being a protogynous species (Nakashima et al. 2000).

Trivers (1971) used the cleaning mutualism as an example to explain the concept of reciprocal altruism, worrying about the survival of the cleaners when inspecting piscivorous clients. However, reciprocal altruism assume that only one player (the predator) could be dishonest and that stability is achieved only by the occurrence of repeated interactions rather than specific monitoring mechanisms used by the potential victim (the cleaner). These asymmetries have been neglected for a long time until Axelrod & Hamilton (1981) formalized Trivers concept through the prisoner's dilemma (Iterated prisoner's dilemma framework) where both players can cooperate or not. In theory, in interactions between cleaners and predators, predators may be dishonest eating the cleaner, but cleaners and can also be dishonest eating mucus instead of ectoparasites (Bshary & Côté 2008). In fact, using choice experiments Grutter & Bshary (2003) verified that the cleaner wrasse *L. dimidiatus* prefers client mucus to isopod parasitic Gnathiidae – most abundant ectoparasites, a behaviour regarded as dishonest. So both cleaners and predators are in a situation where they can cooperate or be dishonest. However, if a predator finishes the game if the cleaner is dishonest, it will prevent the cleaner being dishonest again in the next encounter (Hammerstein & Hoekstra 1995). Consequently, cleaners are virtually "forced" to cooperate when facing predators (Bshary 2001; Soares et al. 2011) to avoid being eaten in case the predator has a dishonest response (Bshary & Bronstein 2004). Another pre-conflict strategy used

by the cleaners is the tactile stimulation (Bshary & Würth 2001; Grutter 2004; Soares et al. 2007) using the pelvic and pectoral fins to "massage" the client's body during the inspection. This behaviour not only exerts an anti-stress effect on clients but also increases their fitness (Soares et al. 2007), and reduces the probability of conflicts (Côté 2000; Bshary & Würth 2001).

Interactions between cleaners and non-predatory clients are asymmetric because eating the cleaner is not an option in these interactions. Thus, such clients cannot strengthen the cooperative behaviour through the threat of being dishonest in future interactions (Bshary & Côté 2008). Control mechanisms for cleaners' behaviour can depend on the existence of only one or more cleaning stations. Clients with access to a single cleaning station have aggressive responses to the cleaners when they are dishonest (Bshary & Grutter 2002a); other clients, however, can simply switch cleaning station for the next inspection (Bshary & Schäffer 2002). This illustrates the importance of the two alternative concepts for maintaining the cooperation between unrelated individuals: punishment (Clutton-Brock & Parker 1995) and partner choice (Bshary & Côté 2008). These concepts are a vital component of the Theory of Biological Markets (Nöe 1990, 2001; Nöe et al. 1991), originally developed to explain income disparities between partners. Bshary & Grutter (2002) first applied the theory of markets to biological mutualisms when studies of cleaning fish showed that the service quality, including immediate inspection, only depends on the ability of the client to visit another cleaning station.

Interactions between client and cleaner occur within a communication network (McGregor 1993, 2004). This allows clients to use prior information through analysing the behaviour of the cleaner when inspecting other clients. In the absence of apparent conflicts the newcomer invite the cleaner to clean by "posing" (Côté et al. 1998). However, aggression to or escape of the client allows the newcomer client to decide to avoid that cleaner (Bshary 2002c). This observation by potential clients keeps cleaners under pressure to well treat their clients, which is an example of audience effects (Bshary & Côté 2008).

*L. dimidiatus*, is one of the top 10 fish species exported to the United States (Wabnitz et al. 2003). Given the importance of this species to the ecosystem, and due to the fact that more than 180 species of its clients are at risk of local extinction (Comeros-Raynal et al. 2012), knowledge of the physiological processes that lead to the mutualistic interaction between these cleaners and their clients is of special importance because these interactions are essential to the coral reef ecosystem.

### **c) Physiological basis of the cleaning mutualism and aim of this study**

Although few mutualisms have been as much studied as cleaning behaviour (Bshary & Côté 2008). We are still far from understanding this mutualism. For example, the causal reasons (physiological) which form the basis of tolerance and increased motivation to interact either with conspecific or intraspecific individuals are still poorly understood. Up to now we know that clients with access to cleaning events appear to have lower stress levels (Bshary et al. 2007; Ros et al. 2011, 2012). It is also known that clients benefit to receive tactile stimulation by the cleaners (Soares et al. 2011). With respect to the cleaners, it is known that they do not react similarly to all kinds of clients, as they clean better certain ones, and that their behaviour modification in the presence of dangerous clients should probably be due to a temporary increase in stress (Soares et al. 2012b). The neuropeptide arginine vasotocin (AVT) is somehow connected with the cleaner's motivation to interact with its clients, but not necessarily with conspecifics (Soares et al. 2012a). Other hormonal systems are involved and, taking into account the fishes' learning speed and decision making, it is likely that neurotransmitters, such as dopamine or serotonin, contribute to these processes.

The neurotransmitter serotonin (5-HT) is a monoamine derived from tryptophan that can be found in the central nervous system (Hoyer et al. 1994). The serotonergic system is mainly known for controlling the aggressiveness, both in vertebrates and in invertebrates (Huber et al. 1997; Winberg et al. 2001), and its influence on the dynamics of agonistic interactions is known (Huber et al. 1997; Nelson & Chiavegatto 2001). As in other vertebrates (Nelson & Chiavegatto 2001), serotonergic activity in teleosts is inversely related to social status (Winberg et al. 1991, 1992, 1993, 1997b). In addition, in the wrasse *Thalassoma bifasciatum* Bloch, 1791, the administration of fluoxetine - a selective serotonin reuptake inhibitor (SSRI) - lowers aggressive behaviour in males when confronted with intruders (Perreault et al. 2003).

This study aims to study the role of serotonin in modulation of cooperative behaviour and learning on the cleaner fish *Labroides dimidiatus* in order to improve our knowledge on the physiological bases for this important mutualistic interactions in the coral reef ecosystem.

# Chapter 1: Serotonin neuromodulation of cooperative behaviour in a cleaner fish

## INTRODUCTION

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In recent decades biologists have developed theories to explain the evolution of cooperative behaviour (Trivers 1971; Axelrod & Hamilton 1981; Côté 2000; Bshary & Côté 2008). Cooperation is generally defined as any interaction between individuals where costs, *e.g.* investment in the partnership, are outweighed by the benefits (Dugatkin 1997). Nevertheless, because natural selection favours individuals that maximize their benefits and minimize their costs, it should enhance those who are dishonest in cooperative interactions (Sachs et al. 2004). Furthermore, any organism promotes its evolutionary success by considering selfishly their own benefits in the detriment of its competitors (Nowak 2006) affecting their own individual fitness, which can be measured by the actual value of their investment against the result of cooperative actions (Trivers 1971). So, what conditions can we consider that may explain why unrelated individuals help others? While lots is now known about functional strategies and how these integrate on individuals benefits to cooperate, very little is known about the proximate mechanisms involved. Presently it is acknowledged that it is necessary to integrate function and mechanism to better understand behaviour (McNamara & Houston 2009)

Proximal cooperation mechanisms should be based on regulation of physiological and neural state of the individual (County 1987; Bartlett & DeSteno 2006; Rutte & Taborsky 2007). Current models of cooperation fail to integrate these hormonal factors that may lead to changes in cooperative and social behaviour (Barta et al. 2011). Serotonin is a major neurotransmitter that is responsible for the regulation of social behaviour in vertebrates including humans (Winberg et al. 1991, 1992, 1993, 1997b; Raleigh et al. 1991; Ferris & Jr 1997; Villalba et al. 1997; Summers et al. 1998; Larson & Summers 2001; Fox et al. 2009). For example, in humans serotonin is associated with vulnerability to mood disorders (Fox et al. 2009).

In spite of having undergone a genome duplication in their evolutionary history that led to the change in the number of proteins involved in the synthesis, transport and signalling in the serotonergic system, the pattern of expression of these proteins in teleost continued to be similar to other vertebrates (Maximino & Lima 2013). In animal

models (e.g., fish, reptiles, rats, and primates), studies have demonstrated that more aggressive males have lower serotonergic activity than less aggressive ones (Blanchard et al. 1991; Raleigh et al. 1991; Summers 1997). Similarly, in teleost fish, the serotonergic activity is inversely related to the social status of the individual (e.g., *Salvelinus alpinus* Linnaeus, 1758 (Winberg et al. 1991, 1992); *Oncorhynchus mykiss* Walbaum, 1792 (Winberg et al. 1993) and cichlids *Astatotilapia burtoni* Günther, 1893 (Winberg et al. 1997b)). In blue-headed wrasses *Thalassoma bifasciatum* Bloch, 1791, injections with fluoxetine, a selective serotonin reuptake inhibitor (SSRI), decrease aggression levels against intruders (Perreault et al. 2003). Moreover, in the rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) the prolonged diet (7 days) with additional L-tryptophan, a precursor of serotonin was also found to be responsible for a decrease in aggression levels against territorial intruders (Winberg et al. 2001). Contrarily, when firemouth cichlids (*Cichlasoma meeki* Brind, 1918) are treated with p-chlorophenylalanine and face their mirror reflection, aggressiveness increases (Adams & Liley 1996).

Changes in the serotonergic activity are able to produce alterations in behaviour of animals by neuronal signalling pathways such as via arginine vasopressin (AVP) (Ferris et al. 1997; Delville et al. 1998). Fibers of serotonin and AVP-producing neurons are located in the same areas in the brain and serotonin can influence the release of this peptides thus the interaction between the AVP and serotonin (and nonmammalian homolog arginine vasotocin - AVT) may be particularly important in social behaviour (Ferris & Jr 1997). When *T. bifasciatum* is injected with fluoxetine less expression of AVT is observed in all regions of the preoptic area (Semsar et al. 2004). In this fish, AVT is a modulator of the social status and reproductive and aggressive behaviour which reinforces the idea that serotonin may be producing changes in social behaviour through AVT pathway (Godwin et al. 2000; Semsar et al. 2004). In cleaner wrasse *Labroides dimidiatus* exogenous administration AVT led to a substantial decrease of most mutualistic interactions without causing changes in interactions with conspecifics, suggesting a differential effect of AVT in cleaning behaviour and not a general effect on social behaviour and showing that AVT pathways might have a role in regulation of cooperative behaviour (Soares et al. 2012b).

Serotonin activity is also linked to stress and to the response of the Hypothalamic–pituitary–adrenal axis (Winberg et al. 1997a; Höglund et al. 2002). Goldfish (*Carassius auratus*) injected with 8-OH-DPAT agonist of the 5-HT<sub>1A/7</sub> receptors increase their cortisol in comparison with control subjects injected with saline (Lim et al. 2013). In addition to the effect of 8-OH-DPAT effect is also observed in vitro with

cisapride agonist of 5-HT<sub>4</sub> receptor (Lim et al. 2013) suggesting that serotonin, in fish, can directly stimulate cortisol secretion via multiple 5-HT receptors showing a relationship between serotonin and stress.

In this study I aim to understand how exogenously administered agonist of serotonin's 5-HT<sub>1A</sub>/5-HT<sub>7</sub> receptors 8-hydroxy-2-(di-n-propyl amino)tetralin (8-OH-DPAT), the antagonist of 5-HT<sub>1A</sub> receptor WAY 100.635, selective tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA) (which is a rate-limiting enzyme in the biosynthesis of serotonin (Jéquier et al. 1967)) and the SSRI (selective serotonin reuptake inhibitor) fluoxetine, are able to modulate the cooperative behaviour of female cleaner wrasse *L. dimidiatus* (Valenciennes, 1839). Serotonin receptor 5-HT<sub>1A</sub> is normally linked with the regulation of aggressive behaviour, as numerous studies showed that: in one hand agonists, as 8-OH-DPAT, lead to a decrease in aggression (de Boer & Koolhaas 2005; Clotfelter & O'Hare 2007) and, in the other hand antagonists, as WAY-100,635, decrease aggressive behaviour (Lopez-Mendoza et al. 1998). This receptor is also linked with sociability and impulsivity (Winstanley et al. 2005; Thompson et al. 2007) making it a potential pathway of modulation of cooperative behaviour.

In the mutualism between the cleaner wrasses *L. dimidiatus* and their visiting reef fishes (usually refer as "clients"), cleaners inspect the body surface, gills and sometimes mouth of clients (Côté 2000). During an interaction with clients, cleaners can choose between cooperate, *i.e.* remove ectoparasites, or to cheat, by eating mucus from clients, which they prefer. Conflicts arise when cleaners chose to cheat – the reaction of clients to cleaners' bites is usually referred as jolting (cleaners sometimes take bites to which the clients respond with a short body jolt that usually is a behaviour associated with cheating by cleaner fish) (Bshary & Grutter 2002b; Soares et al. 2008). As a way to mitigate the conflict and invest in the interaction cleaners often provide tactile stimulation to their clients, which is characterized by a massage in the dorsal area of the clients with its pelvic and pectoral fins (Bshary & Würth 2001; Grutter 2004). Cleaners have demonstrated to have a diverse set of behaviours to convince their clients to visit, increase the duration of inspection and to promote future interactions (Bshary 2011). To best understand how the behaviour of the cleaner fish is modulated by serotonin, manipulations and observations were conducted in the field. I wanted to determine whether exogenous administration through peripheral injections affect the likelihood to engage in cleaning behaviour through the proportion of clients that are inspected and the proportion of cleaners that change from one client to the next and if it would directly affect the quality of inspection (by calculating the time cleaners spent inspecting clients, how often clients responded to the bites of cleaners and proportion of interactions in

which the cleaner applied tactile stimulation). I also aimed to determine if these effects were extended to conspecific behaviour. I hypothesize that in addition to a general effect of the serotonin, 5-HT<sub>1A</sub> receptor must have an important role in maintaining the cooperative behaviour and in the establishment of mutualistic relationships.

# MATERIAL AND METHODS

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## Field methods

This study was conducted on ten different reefs around Lizard Island (Lizard Island Research Station, Australia, 14° 40'S, 145° 289'E) between August and October 2012. We used 45 females of blue streak cleaner wrasse *L. dimidiatus* to test the effect of 5-HT neurotransmitter on neuromodulation of cleaning behaviour. All observations and manipulations were made by one SCUBA diver always accompanied by a diving buddy, between 9:00 and 15:00 hours. Cleaner fish were selected haphazardly across the reefs and cleaning stations varied in depth between 1 and 10 m. All individuals were captured using hand and barrier nets and measured to the nearest mm (TL-total length). TL of these individuals ranged from 5.5 cm to 8.0 cm. Fish body weight was measured from a length-weight regression (unpublished data). We then gave the focal female an intramuscular injection of one of five compounds: saline (0.9 NaCl); fluoxetine (Sigma F132); ( $\pm$ )-8-hydroxy-2-(dipropylamino) tetralin hydrobromide (8-OH-DPAT, Sigma H8520); WAY-100,635 (Sigma W108) or p-chlorophenylalanine (PCPA, Sigma C6506). Injection volumes were always 15  $\mu$ l per gram of body weight (gbw) and this process never exceeded 2 min. All dosages were based on previous studies that focused on other model species of teleost fish: a concentration of 5ul/gbw – gram of body weight (Perreault et al. 2003); 1ul/gbw of 8-OH-DPAT and 1.5ul/gbw of WAY-100,635 as in (Clotfelter & O'Hare 2007) and PCPA 5ul/gbw according to a previous study focusing on *Lythrypnus dalli* and *Betta splendens* (Lopez-Mendoza et al. 1998; Clotfelter & O'Hare 2007; Lorenzi et al. 2009).

The order of the treatments was randomized for each dive and all treatments used independent cleaner fish. Once an individual was released it was then observed and videotaped for the next 45 min using video cameras in waterproof cases Sony HDR-XR155.

## Behavioural analysis

During each video analysis, we recorded the following measures: a) species and TL of each client (estimated visually to the nearest cm) visiting the cleaning station; b) the number of clients that adopted the species-specific immobile pose, which signals the need to be cleaned (Côté et al. 1998) including those that ended up not being inspected

by the cleaning fish; c) the number of inspections and duration (in sec.) of a cleaner's inspection towards each client e) the number and duration of tactile stimulations provided (where a cleaner touches, with ventral body and fins, the body of the client and no feeding is involved (Bshary & Würth 2001); f) the number of jolts (cleaners sometimes take bites to which the clients respond with a short body jolt that usually is a behaviour associated with cheating by cleaner fish (Bshary & Grutter 2002b)) by clients and the client's reaction following each jolt and e) conspecific-directed behaviour such as: focal cleaner swimming closely with partner, whether it provided or received tactile stimulation, if inspected or otherwise cleaned by partner, and all agonistic interactions, including charges where one individual rapidly advanced towards the other partner like described in Soares et al. 2012.

### **Statistical analysis**

As mentioned before, in my cleaning behaviour observations following neuroendocrine manipulations, all cleaner fish were randomly selected, thus these were independent measures. Interspecific cleaner fish behaviour was measured along two different behavioural categories: a) measures of likelihood to interact with clients and b) measures of cleaning quality, a measure of degree of cooperativeness. Both of these measures in turn have some behavioural correlates. Therefore, I measured cleaners' likelihood to interact as: 1) proportion of inspected clients and 2) Proportion of cleaners switching from a current client to queuing client. Measures of cleaning service quality included: 1) duration of inspection by cleaners, 2) frequency of jolts per 100 sec of inspection, 3) proportion of interactions in which tactile stimulation was used by cleaners 4) duration of tactile stimulation provided by cleaners. All data were analysed using non-parametric tests because the assumptions for parametric testing were not met. We therefore tested for differences between treatments using a Kruskal-Wallis analysis of variance with neuropeptide groups as a fixed factor, followed by planned Mann-Whitney U test in order to compare each treatment with the control (saline) group.

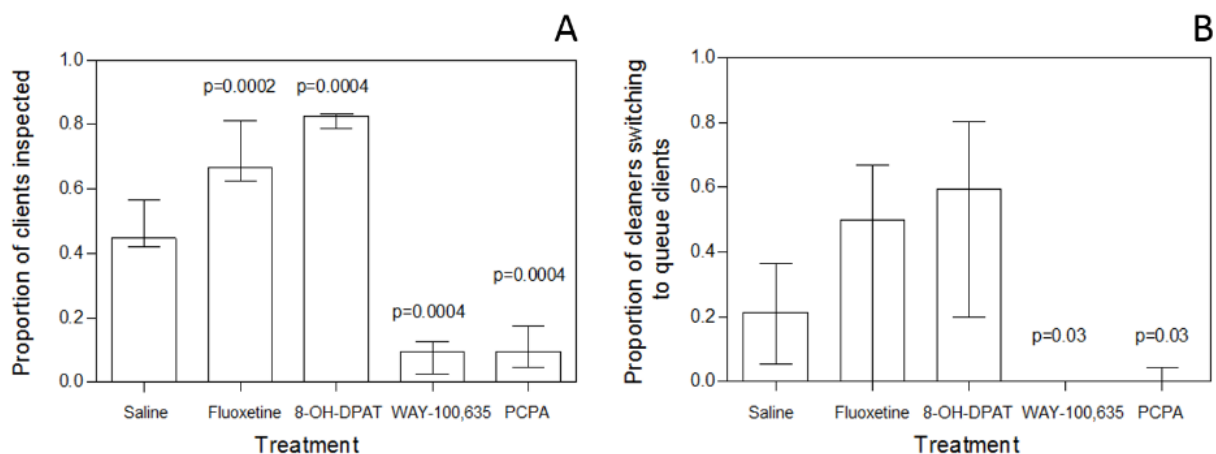
Observations of a cleaner fish behaviour directed at conspecifics were also independent measures. Intraspecific behaviour was measured by cleaner-cleaner cleaning interactions and pair close swimming events. Data were analysed using a one-way Kruskal-Wallis analysis of variance, followed by planned Mann-Whitney U tests to search for specific differences between each treatment and the control (saline) group.

# RESULTS

## a) Serotonin Effect on the Likelihood to Engage in Cleaning Behaviour

There were significant effects of treatment on both measures of a cleaner fish's likelihood to interact with clients: the proportion of clients that were inspected (Kruskal-Wallis test,  $\chi^2 = 36.10672$ ;  $df = 4$ ;  $p < 0.0001$ , Figure 1A) and the proportion of cleaners switching from a current client to queuing client (Kruskal-Wallis test,  $\chi^2 = 17.43083$ ;  $df = 4$ ;  $p = 0.0192$ , Figure 1B).

Regarding to proportion of clients inspected, both agonists fluoxetine and 8-OH-DPAT significantly increased the proportion of clients inspected while WAY 100-635 and PCPA significantly decreased this proportion (Mann-Whitney test: fluoxetine vs saline,  $U=3$   $n_1=9$ ,  $n_2=9$ ,  $p=0.0002$ ; 8-OH-DPAT vs. saline,  $U=0$   $n_1=9$ ,  $n_2=9$ ,  $p=0.00004$ ; WAY 100-635 vs. saline,  $U=0$   $n_1=9$ ,  $n_2=9$ ,  $p=0.00004$ ; PCPA vs. saline,  $U=0$   $n_1=9$ ,  $n_2=9$ ,  $p=0.00004$ , Figure 1A). WAY 100,635 and PCPA significantly decreased the cleaners' probability to switch to queuing clients when inspecting a client while cleaners treated with Fluoxetine and 8-OH-DPAT shown no significant effects (Mann-Whitney test: fluoxetine vs. saline,  $U=31$   $n_1=9$ ,  $n_2=9$ ,  $p=0.43628$ ; 8-OH-DPAT vs. saline,  $U=22.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.11349$ ; WAY 100-635 vs. saline,  $U=16.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.03146$ ; PCPA vs. saline,  $U=17$   $n_1=9$ ,  $n_2=9$ ,  $p=0.039984$ , Figure 1B).

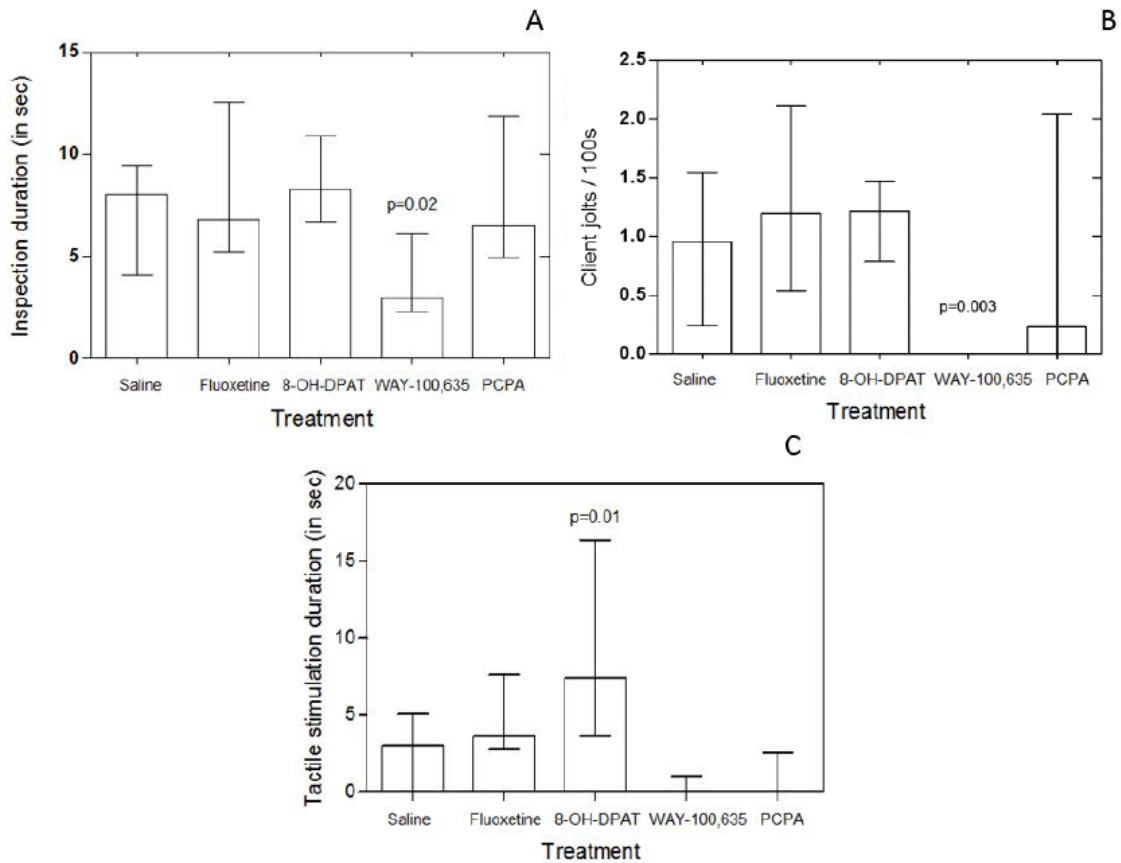


**Figure 1:** The effect of 8-OH-DPAT, fluoxetine (FLX), PCPA and WAY-100635 (WAY) on the cleaning behaviour of the cleaner fish *Labroides dimidiatus*, measured in the field and compared with a control (saline) on serotonin effect on the likelihood to engage in cleaning behaviour: A) the proportion of clients that was inspected (number of clients' cleaned/total number of visits) and B) proportion of cleaners switching from a current client to queuing client (number of times cleaner switched to a queue client/total queuing clients).

## **b) Serotonin Effect on Cleaning Quality Levels**

There were also significant effects of treatment on measures of cleaning service quality: the duration of inspection (Kruskal-Wallis test,  $\chi^2 = 5.869565$ ;  $df = 4$ ;  $p = 0.0359$ , Figure 2A ); the frequency of client jolts in response to cleaner fish bites (Kruskal-Wallis test,  $\chi^2 = 16.54150$ ;  $df = 4$ ;  $p = 0.0007$ , Figure 2B), the latter being a measure of cleaner fish cheating; and the duration of tactile stimulation provided in an inspection (Kruskal-Wallis test,  $\chi^2 = 18.0$ ;  $df = 4$ ;  $p = 0.0002$ , Figure 2C).

Only WAY 100,635 caused a significant decrease increase in cleaner fish's inspection duration (Mann-Whitney test: fluoxetine vs. saline,  $U=36$   $n_1=9$ ,  $n_2=9$ ,  $p=0.73044$ ; 8-OH-DPAT vs. saline,  $U=30$   $n_1=9$ ,  $n_2=9$ ,  $p=0.3865$ ; WAY 100-635 vs. saline,  $U=15$   $n_1=9$ ,  $n_2=9$ ,  $p=0.024434$ ; PCPA vs. saline,  $U=38$   $n_1=9$ ,  $n_2=9$ ,  $p=0.86330$ , Figure 2A). Also only WAY 100,635 decreased large clients' body jolts when compared with saline treatment (Mann-Whitney test: fluoxetine vs. saline,  $U=34$   $n_1=9$ ,  $n_2=9$ ,  $p=0.60481$ ; 8-OH-DPAT vs. saline,  $U=30$   $n_1=9$ ,  $n_2=9$ ,  $p=0.38650$ ; WAY 100-635 vs. saline,  $U=9$   $n_1=9$ ,  $n_2=9$ ,  $p=0.0039$ ; PCPA vs. saline,  $U=31$   $n_1=9$ ,  $n_2=9$ ,  $p=0.43628$ , Figure 2B). Concerning to duration of tactile stimulation 8-OH-DPAT significantly increased time that a cleaner spend providing it to clients (Mann-Whitney test: fluoxetine vs. saline,  $U=28.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.29732$ ; 8-OH-DPAT vs. saline,  $U=14.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.01875$ ; WAY 100-635 vs. saline,  $U=18$   $n_1=9$ ,  $n_2=9$ ,  $p=0.0503$ ; PCPA vs. saline,  $U=25.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.19025$ , Figure 2C).

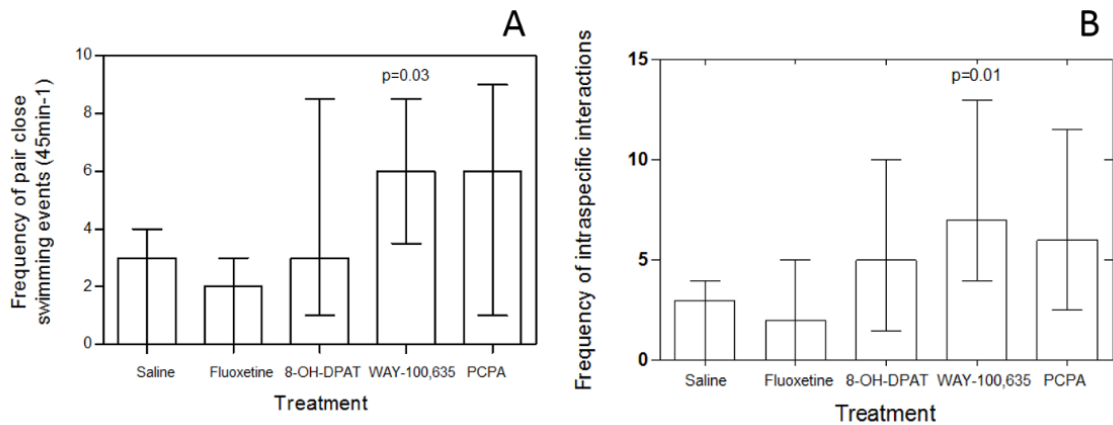


**Figure 2:** The effect of 8-OH-DPAT, fluoxetine (FLX), PCPA and WAY-100635 (WAY) on the cleaning behaviour of the cleaner fish *Labroides dimidiatus*, measured in the field and compared with a control (saline) on serotonin effect on the cleaning quality: A) client inspection duration at cleaning stations (in seconds) (total inspection duration/total number of inspections); B) number of client jolts per 100 s of inspection and C) tactile stimulation duration (total time of tactile stimulation/total number of inspections with tactile stimulation).

### c) Serotonin Effect on a Cleaner Fish's Likelihood to Interact with Conspecific Partners

Overall, there was a significant effect of treatment on the frequency in which focal cleaners were observed to swim closer together (paired) with their conspecific partners (Kruskal-Wallis test,  $\chi^2 = 7.648221$ ;  $df = 4$ ;  $p = 0.0404$ , Figure 3A) and on how frequently these interact with their conspecific partners (Kruskal-Wallis test,  $\chi^2 = 7.2$ ;  $df = 4$ ;  $p = 0.0384$ , Figure 3B). WAY 100,635 injected cleaners were less often seen swimming in close contact with their partners when compared to those injected with saline whereas no effects were found with fluoxetine, 8-OH-DPAT or with PCPA (Mann-Whitney test: fluoxetine vs. saline,  $U=35$   $n_1=9$ ,  $n_2=9$ ,  $p=0.66647$ ; 8-OH-DPAT vs. saline,  $U=34$   $n_1=9$ ,  $n_2=9$ ,  $p=0.60481$ ; WAY 100-635 vs. saline,  $U=16.5$   $n_1=9$ ,  $n_2=9$ ,  $p= 0.03147$ ; PCPA vs.

saline,  $U=22.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.11349$ , Figure 3A). Focal cleaner fish injected with WAY 100,635 also interacted more with their conspecific partners than those injected with saline and again no effects were found for fluoxetine, 8-OH-DPAT or for PCPA (Mann-Whitney test: fluoxetine vs. saline,  $U=38$   $n_1=9$ ,  $n_2=9$ ,  $p=0.86331$ ; 8-OH-DPAT vs. saline,  $U=26$   $n_1=9$ ,  $n_2=9$ ,  $p=0.22241$ ; WAY 100-635 vs. saline,  $U=12$   $n_1=9$ ,  $n_2=9$ ,  $p=0.01061$ ; PCPA vs. saline,  $U=20.5$   $n_1=9$ ,  $n_2=9$ ,  $p=0.07701$ , Figure 3B).



**Figure 3:** The effect of 8-OH-DPAT, fluoxetine (FLX), PCPA and WAY-100635 (WAY) on the cleaning behaviour of the cleaner fish *Labroides dimidiatus*, measured in the field and compared with a control (saline) on cleaner fish's likelihood to interact with conspecific partners: **A**) frequency of pair close swimming events (45 min<sup>-1</sup>) and **B**) frequency of intraspecific interactions (every type of interaction cleaner-cleaner).

# DISCUSSION

My results show a causal association between the effects of serotonin availability shifts and changes of cleaner fish' behavioural response. When compared with saline control, cleaners treated with fluoxetine and the agonist 8-OH-DPAT were more willing to interact with clients while those treated with the antagonist WAY 100.635 and PCPA interacted less and switched less between clients. In terms of measures of service quality (*i.e.* degree of cooperation), the antagonist WAY 100,635 caused a decrease in motivation for cleaner to cheat but it also caused a decrease in time spent in interacting with clients, which indicated that they were less cooperative. On the contrary, 8-OH-DPAT increased the duration providing tactile stimulation. Interestingly, the contrary pattern was found when it came to conspecific-directed behaviour: cleaners injected with WAY 100.635 swam more in close contact with conspecific and interacted more with them (Tab. 1). My results are key information as to reveal the importance of the serotonergic system in modulating the cooperative behaviour.

**Table 1:** Summarized table with the effect of 8-OH-DPAT, fluoxetine, PCPA and WAY-100635 on cleaner fish *Labroides dimidiatus* behaviour.

Behavioural response	Subjects analysed	Fluoxetine	8-OH-DPAT	WAY 100.635	PCPA
<b>Interspecific</b>					
1) Likelihood to engage in cleaning behaviour					
a) Proportion of clients inspected (Figure 1A)	Cleaner	↑	↑	↓	↓
b) Proportion of cleaner switching from a current client to a queing client (Figure 1B)	Cleaner/Client	↔	↔	↓	↓
2) Cleaning service quality					
a) Inspection duration (Figure 2A)	Cleaner/Client	↔	↔	↓	↔
b) Frequency of client jolts in response to cleaner bites/100s (Figure 2B)	Client	↔	↔	↓	↔
c) Duration of tactile stimulation provided to clients (Figure 2C)	Cleaner	↔	↑	↔	↔
<b>Intraspecific</b>					
a) Frequency of paired close-swimming events (Figure 3A)	Cleaner	↔	↔	↑	↔
b) Frequency of intraspecific interactions (Figure 3B)	Cleaner	↔	↔	↑	↔

Legend: Arrows indicate the effect, relative to saline, on the behaviours of interest: '↑' denotes an increase in display, '↓' a decrease, and '↔' indicates no effect detected.

### **a) Mechanisms of serotonin action**

Serotonin action occurs via activation of 5-HT receptors that can be grouped into seven subfamilies (5-HT<sub>1-7</sub>) based on sequence homology and signalling mechanisms (Kroeze et al. 2002). All serotonergic receptors, with the exception of 5-HT<sub>3</sub> receptor which is a ligand-gated ion channel, are heterotrimeric guanine nucleotide binding protein (G-protein)-coupled receptors (Wang et al. 2013). All receptors are located in post-synapse, and some of these modulate the ion flux to cause depolarization (5-HT<sub>2A,2C,3,4</sub> receptors) or hyperpolarization (5-HT<sub>1A</sub> receptor), furthermore some subtypes (such as 5-HT<sub>1A,1B</sub> receptor) are located on serotonergic neurons themselves and can act as serotonin autoreceptors at a somatodendritic and terminal level in the nerve (Barnes & Sharp 1999). The 5-HT<sub>1A</sub> receptor functions as an inhibitory receptor involved in autoregulation of the serotonin neuronal function and is found at postsynaptic sites in the brain (King et al. 2008). The distribution of this receptor and the availability of highly selective agonists and antagonists makes it a potential target to study the action of serotonin on behaviour (Meneses 1999; de Boer & Koolhaas 2005; King et al. 2008).

We now know that both in vertebrates and in invertebrates the activation of specific 5-HT receptors may be connected with the modulation of specific behaviours (Barnes & Sharp 1999). The 5-HT<sub>1A</sub>, 5-HT<sub>2A/C</sub> receptors highlight the wide variety of studies reporting behavioural and physiological responses to agonists of these receptors can cause (Barnes & Sharp 1999; Thompson et al. 2007) but there is still much to discover about the effect of these receptors activation or inhibition on cooperative behaviour.

### **b) Serotonin modulation of interspecific cleaning behaviour**

My results demonstrate that the administration of fluoxetine and 8-OH-DPAT led to a general increase in the likelihood to engage cleaning interactions by substantially increasing the predisposition of the cleaner wrasse to approach and inspect clients (see Fig. 1) while that WAY 100.635 and PCPA have the opposite effect. This suggests that 5-HT<sub>1A</sub> receptor might play a fundamental role in modulation cleaners motivation to interact but it could not be the single receptor involved as both the fluoxetine and PCPA produced similar effects as 8-OH-DPAT and WAY 100.635. For example, results on image mirror stimulation in *Betta splendens* treated with 8-OH-DPAT showed decreased aggressive behaviour, but did not reveal any such effect with the antagonist WAY 100.635 thus making it impossible to find which receptor mediated the effect (Clotfelter & O'Hare 2007), as 8-OH-DPAT stimulates both 5-HT<sub>1A</sub> and 5-HT<sub>7</sub>. As I observed a general effect of serotonin on the likelihood to engage in cleaning interactions

and not just an isolated effect of 5-HT<sub>1A</sub> receptor (via the antagonist WAY 100.635), the generalized effect observed may be related to other receptors such as 5-HT<sub>2A</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub> involved in mood and social behaviour (Barnes & Sharp 1999; King et al. 2008).

Serotonin can also lead to the release of AVT through the interaction between the serotonergic fibers and neurons producing AVT (see introduction). According to Soares et al. (2012) exogenous administration of AVT leads to a decrease in the likelihood to engage cleaning interactions without affecting the expression of behaviour with conspecifics, suggesting that the AVT has a differential effect on cleaning behaviour and no effect general social behaviour. My results suggest that serotonin may be causing an effect on the likelihood to engage cleaning interactions through: at least the expression of AVT mRNA can be affected by fluoxetine (Semsar et al. 2004) and is reasonable to hypothesize that other serotonin related compound could have an effect on AVT in addition to the normal effect through serotonergic receptors, which suggests that the motivation to interact with clients is modulated by an the link between the serotonergic system and AVT system.

In addition to the rise in the motivation to interact, I observed that the compounds directly linked to the 5-HT<sub>1A</sub> receptor also affected the quality of cleaning as: cleaner fish treated with 8-OH-DPAT spent more time providing tactile stimulation during inspections. Indeed, the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT has been successfully demonstrated to reduce aggression in fish (Clotfelter & O'Hare 2007) in birds (Sperry et al. 2003) and rodents (Haug et al. 1990; de Boer et al. 1999; Ferris et al. 1999). Injections with 8-OH-DPAT can lead to an increase in cortisol in goldfish when compared with the control (Lim et al. 2013) thus it might suggest that 8-OH-DPAT is increasing levels of stress in cleaners through the activation of the hypothalamic–pituitary–interrenal axis resulting in the production of cortisol (Winberg et al. 1997a; Höglund et al. 2002). In this context, the rise of cortisol levels made them to respond by investing further and cooperating more as a reinforcement of a positive interactive outcome (a.k.a. reducing the scope for aggressive client responses) which may explain either the increase on cleaning quality or the reduction in aggressive behaviour (DiBattista et al. 2005). The effects of short term rises of cortisol levels has been reported on a previous study by Bshary et al. 2011, which caused cleaners to increase their levels of cooperation.

However, no further effect of 8-OH-DPAT was to be found in the inspection duration and on the amount of client-jolts, which might indicate that the agonist of the 5-HT<sub>1A</sub> receptor does not have a generalized effect on the quality of cleaning. Cleaner fish

treated with the antagonist WAY 100.635 spent less time interacting and consequently also cheated less frequently (translated by jolts of clients). As these compounds (8-OH-DPAT and WAY 100.635) are respectively agonist 5-HT<sub>1A/7</sub> and antagonist of the 5-HT<sub>1A</sub> and we only found effect with those two this might mean that 5-HT<sub>1A</sub> receptor and maybe 5-HT<sub>7</sub> have an important role in the regulation of quality of service by itself and on the degree of cooperativeness.

### **c) Serotonin regulation of cleaner fish likelihood to interact with conspecific partners**

The cleaner wrasse is part of a complex harem social system, in which intraspecific interactions and establishment of social status are relatively frequent (Robertson 1974; Bshary & Côté 2008). Contrarily to the effect observed previously with interspecific interactions, WAY 100.635 did not have the same effect of reducing conspecific interactions. Under the effect of WAY 100.635, cleaners increase their willingness to interact with conspecifics. Many studies in vertebrates show that the more aggressive males (which are usually dominant males) have lower serotonergic activity than males less aggressive (Blanchard et al. 1991; Raleigh et al. 1991; Winberg et al. 1993; Matter et al. 1998),. In this study I've focused exclusively on female cleaner wrasses, which occupy a more subordinate role in comparison to their male partners. Female cleaner wrasses are often punished by their male counterparts, especially if they are closer in size to males (this is associated with a potential increase in competition levels via a change in social rank; see (Raihani et al. 2012)). It is therefore possible that the effect of the antagonist WAY 100.635 may stimulate potential changes in social status towards the male and other females in the proximity, thus increasing the scope for conspecific interactions.

### **Concluding remarks**

Most of the studies that attempt to explain the effect of serotonin on social behaviour are focused on aggressive behaviour directed at other conspecific (Blanchard et al. 1991; Raleigh et al. 1991; Winberg et al. 1993; Huber et al. 1997; Matter et al. 1998; Perreault et al. 2003). This study is the first to link the role of serotonin to the modulation of interspecific cooperative behaviour. I hypothesized that the serotonergic system, mainly through the 5-HT<sub>1A</sub> receptor, and as a main modulator cognitive, motivational and perceptual mechanism, would directly influence cleaner wrasse

interspecific and conspecific behaviour. My prediction was confirmed: the activation of the serotonergic system via receptor 5-HT<sub>1A</sub> was involved in motivational processes and in the increase of interactive quality levels while the blocking of its pathways would be responsible of a decrease of interspecific cooperative levels and a rise of conspecific motivational activities. However, other receptors pathways could also be involved on cleaner fish motivational levels to interact with its clientele, such as 5-HT<sub>7</sub> (as 8-OH-DPAT is also an agonist of this receptor) and 5-HT<sub>4</sub> (as seen in in vitro experiments, see introduction). While changes of available serotonin levels was observed to change cleaner wrasses behaviour, it is also known that the influence of social context and behaviour of conspecifics and clients is also a contributor for the existence of physiological changes in different areas of the brain that should be responsible for the regulation of cooperative behaviour (Goodson 2005; Soares et al. 2010). I propose that during the evolution of cleaning mutualisms both the serotonergic system, mainly through the 5-HT<sub>1A</sub> receptor, and AVT had an essential role and were probably recruited to regulate intraspecific and interspecific interactions. Further studies should illustrate the role of mechanisms other than the serotonin and AVT, such as the dopaminergic system, and also should dwell on the specific influence of serotonin levels and how these activate different brain areas according to distinct social contexts.

## Chapter 2: The role of serotonin for cleaner fish learning competence

### INTRODUCTION

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Monoaminergic systems are known to be involved in the regulation of behaviour both in vertebrates and in invertebrates (Huber et al. 1997; Winberg et al. 2001). Serotonin plays a primary role in the expression of aggressiveness and it is known that influences the dynamic agonistic interactions (Huber et al. 1997; Nelson & Chiavegatto 2001). Both dominant and subordinate individuals experience an increased metabolism of serotonin after territorial disputes, but only subordinates maintain this turnover (Summers et al. 1998; Overli et al. 1999).

Serotonergic fibers are projected to different areas of the brain (Lillesaar 2011). Serotonergic receptors (their agonists and antagonists) are involved in learning and memory processes (King et al. 2008). In mammals, serotonin receptors 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> show a higher distribution areas involved in learning and memory as the hippocampus, amygdala and cortex (Meneses & Hong 1997; Barnes & Sharp 1999; Meneses 1999). The serotonin receptor 5-HT<sub>1A</sub> functions as an somatodentritic inhibitory receptor, is also found at postsynaptic sites in the brain and is involved in autoregulation of serotonergic neuronal function (King et al. 2008). The 5-HT<sub>1A</sub> receptor distribution and the availability of many highly selective antagonists and partial agonists, makes this receptor an ideal target for study of serotonin role in this processes (King et al. 2008). This receptor has also a role as modulator of glutamatergic and cholinergic neurons, which are linked to memory and learning processes (King et al. 2008). The use of the full agonist of 5-HT<sub>1A</sub> receptor, the compound 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) has shown to be influent to a high range of responses which include improvement in cognitive tasks to impairment (Carli et al. 1995; Kant et al. 1996; Misane et al. 1998) . For example, in rats, low doses of 8-OH-DPAT can enhance cognitive performance while higher doses can impair performance in spatial learning and active passive avoidance tests (Misane et al. 1998). On the other had, studies with WAY 100.635, the antagonist of the 5-HT<sub>1A</sub> receptor have reported a high diversity of results, from a complete absence of effect to impairment and behavioural improvement, which can related with the type of the cognitive task and the specificity of this compound (King et al. 2008). Another mechanisms in which serotonin seem to be related to learning and

memory, is through the effects of the full 5-HT reuptake / transporter, which is responsible for the re-uptake of serotonin released into the synaptic cleft (Meltzer, M.D et al. 1998). The serotonin reuptake inhibit or otherwise facilitate the absorption of serotonin in neurons located in areas involved in cognitive processes (Jacobs & Azmitia 1992). Indeed, the selective serotonin reuptake inhibitors (SSRIs) facilitate the consolidation of learning : for example, the SSRI fluoxetine is able to reverse the effect of impaired learning consolidation which was previously induced by serotonergic agonists such as 8-OH-DPAT (Meneses & Hong 1997).

The serotonergic system is highly conserved in different groups of vertebrates (Winberg et al. 1991, 1992, 1993, 1997b; Raleigh et al. 1991; Ferris & Jr 1997; Villalba et al. 1997; Summers et al. 1998; Larson & Summers 2001) and in spite of teleost fish have genomic duplication which altered the number of genes encoding proteins related to the synthesis, transport and signaling of serotonergic system the expression pattern of these proteins is equal to the other groups of vertebrates (Maximino & Lima 2013). Recent studies have shown that the neural basis of learning and memory are identical between the teleost and other vertebrates, including mammals (Salas et al. 2006). In teleost fish mechanisms for orientation and association of simple response to a stimulus are not solely based on egocentrically referenced processes but also use allocentric map-like representations of the environment (Broglio et al. 2003). The lateral telencephalic pallium (LP) and the medial telencephalic pallium (MP) of fish are probably the homologous of the hippocampus and amygdala of mammals, respectively (Nieuwenhuys & Meek 1990; Butler 2002). In a study with goldfish in which these were trained to a spatial task, the authors reported a significant induction of protein synthesis associated with spatial learning selective neurons in the LP (Vargas et al. 2000). In a test with visual cues, lesions in the LP produced impairment in place learning and memory (Salas et al. 1996). The role of LP in space cognition appears to be extremely selective since when this area is affected, only changes in spatial learning but not in the cue directed learning are reported (Salas et al. 1996; López et al. 2000). In teleost telencephalic areas contain a considerable number of serotonergic innervation, but the density of serotonergic fibers varies between areas (Kah & Chambolle 1983; Frankenhuis-van den Heuvel & Nieuwenhuys 1984; Ekström & Van Veen 1984; Meek & Joosten 1989; Corio et al. 1991; Pierre et al. 1992; Batten et al. 1993; Khan & Thomas 1996; Adrio et al. 1999; Rodríguez-Gómez et al. 2000; Kaslin & Panula 2001). In mammals, the hippocampus, involved in memory and spatial learning, is supplied by a rich serotonergic innervation mainly from median raphe populations and the pallial amygdala is relationated with emotional learning and memory and receives extensive

serotonergic innervation from the dorsal raphe nuclei (Imai et al. 1986; Morin & Meyer-Bernstein 1999). The raphe serotonergic innervation of the zebrafish dorsal telencephalon, in particular of the lateral zone (DI), is very dense and fewer serotonergic fibers are observed in the medial zone (Dm) of dorsal telencephalon originating from the raphe fibers (Lillesaar 2011).

In mutualism between the cleaner wrasse *Labroides dimidiatus* and its "clients" cleaners inspect the surface, gills and sometimes the mouth of his clients, eating ectoparasites, mucus, scales and dead or damaged tissue (Côté 2000; Bshary & Côté 2008). The cleaner species *Labroides dimidiatus* is able to distinguish between predators, not predators, families and unfamiliar clients and thus adjust their behavior to each type of client (Tebbich et al. 2002; Bshary et al. 2011). These cleaners are able to adjust their behavior depending on the presence or absence of third parties, behaving more cooperative when a bystander is present (Bshary & Grutter 2006). In this chapter I tried to understand whether serotonin is linked with the individual's ability to learn. Under laboratory conditions, I investigate the rules of decisions involving the learning process using plexiglas plates with different staining patterns simulating a variety of wild colors exhibited by clients visiting cleaner territories on coral reef, under the effect of serotonin 5-HT<sub>1A</sub> receptor agonists and antagonists and an SSRI. In the natural environment, it is common to see two or more clients visiting the territory of cleaner due to the abundance of these clients and the repetitive nature of these interactions (Bshary & Côté 2008). When two or more clients arrive at a cleaning station (cleaner fish territory), the cleaner has to choose which client you will be served first, that as clients vary in parasite load, is usually the client with greater food value (e.g. the more parasitized client) (Grutter 1995; Bansemmer et al. 2002; Soares et al. 2008). In captive conditions, I used the paradigm of Plexiglas plates to simulate the visit of two clients by introducing two different tasks: a first in which the cleaners have to learn to prioritize one pattern over another - cue discrimination task - which simulates what happens in nature with different types of clients coming to the cleaning station and in the second the cleaners had to learn to prioritize one side of the tank instead of another - spatial discrimination task. I expect to find relevant interactions between distinct serotonergic pathways and the ability of the cleaner wrasse to solve learning problems.

## MATERIAL AND METHODS

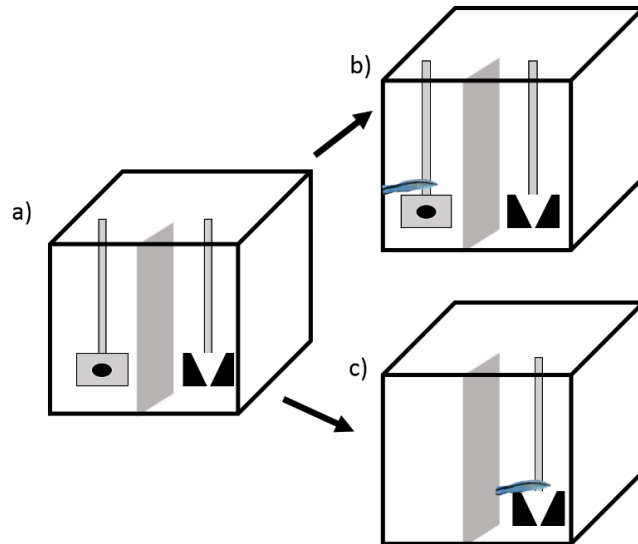
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Experiments were conducted at fish housing facilities of the Oceanário de Lisboa (Lisbon, Portugal). I used 8 *Labroides dimidiatus* that were wild caught in Maldives and directly imported to Portugal by a local distributor. All fish were kept in individual 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 tubes (10-15 cm long; 2.5 cm diameter) served as a shelter for the fish. Experiments were carried out between March and July 2013 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 natural conditions. Cleaners took approximately three days to learn to eat from plates.

### **Cue discrimination task**

In each treatment I used two Plexiglas plates with different colour patterns that simulated clients: one of these would be the sign by which the cleaner would have to start eating first (correct pattern) and with the other plate cleaner could only interact after interact with the first (incorrect pattern). All plates were initially shown to cleaners one day before the start of experiments, and all cleaners have been taught to eat from blank plates before the start of the experiments. Cleaners underwent a session on alternate days. Each session contained 10 trials, and the cleaners were subjected to different sessions until the individual has 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) until with a maximum of 8 sessions. The first trial began 10 minutes after the compound treatment and consisted on successive presentation with 10 minutes intervals of the two pattern color plates until the outcome of the 10 trials. The “correct” pattern plate had available prawn while the “incorrect” pattern plate had inaccessible prawn (covered with transparent tape). Experimental individuals would then have complete access to both plate but while a correct choice would enable both plates to stay inside the aquarium, an incorrect choice

would consequently have the correct plate (the only one with accessible food) be immediately removed, preventing cleaners to eat the food item (Fig. 1).



**Figure 1:** The experimental tank as seen from above. **a)** Early stage when the two boards are placed; **b)** after placing two Plexiglas plates if the cleaner choose the right pattern / side both plates stay in the tank allowing the cleaner to inspect both and receive the reward; **c)** after placing two Plexiglas plates if the cleaner chooses the wrong pattern / side plate only the wrong plate stays in the tank, and as it doesn't have available prawn the cleaner does not receive the reward. Both boards / sides were separated by a transparent partition.

### Side discrimination task

This task consisted of learning that it was a correct side to start eating unlike the previous task in which there was a right colour pattern to start. The plates were introduced with prawn to cleaners day before the start of the experiments. Cleaners were subjected to a session on alternate days, each session consisting of 10 trials, until the individual has learned (three successive sessions in which the individual chose the correct side plate in at least seven out of ten trials, two successive sessions in which the cleaner chose the correct side plate at least eight out of the 10 trials or just one session with at least nine out of 10 trials) until with a maximum of 8 sessions. The correct side was chosen randomly and was maintained between trials and session but it changed between compound treatments. The first trial began 10 minutes after the injection of the compound treatment and consisted on successive presentation of the two pattern color

plates with 10 minutes intervals until the outcome of 10 trials. The “correct” side plate had available prawn while the “incorrect” side plate had inaccessible prawn (covered with transparent tap). Experimental individuals would then have complete access to both plates but while a correct choice would enable both plates to stay inside the aquarium, an incorrect choice would consequently have the correct plate (and the only one with accessible food) be immediately removed, preventing cleaners to eat the food item (Fig. 1).

## **Hormonal treatment**

Cleaners were weighed before the onset of the experiment so that injection volume could be adjusted to body weight. Each cleaner was injected with all the compounds. I used the following compounds: saline (0.9 NaCl); Fluoxetine (Sigma F132); 8-OH-DPAT ((±)-8-hydroxy-2-(dipropylamino) tetralin hydrobromide, Sigma H8520) and WAY-100,635 (Sigma W108). All dosages were based on previous studies that focused, when possible, on other model species of teleost fish: a concentration of 5ul/gbw of fluoxetine, a selective serotonin reuptake inhibitor, was used as for *Thalassoma bifasciatum* (Perreault et al. 2003) were they showed that fluoxetine treatment decreases territorial aggression in a coral reef fish; 1ul/gbw of 8-OH-DPAT an agonist of 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors and 1.5ul/gbw of WAY-100,635 an antagonist of 5-HT<sub>1A</sub> receptor as in (Clotfelter & O’Hare 2007) *Betta splendens* treated with 8-OH-DPAT decreased aggressive behaviour and as in (Lopez-Mendoza et al. 1998) mice treated with WAY-100,635 increased attack rate.

## **Statistical analysis**

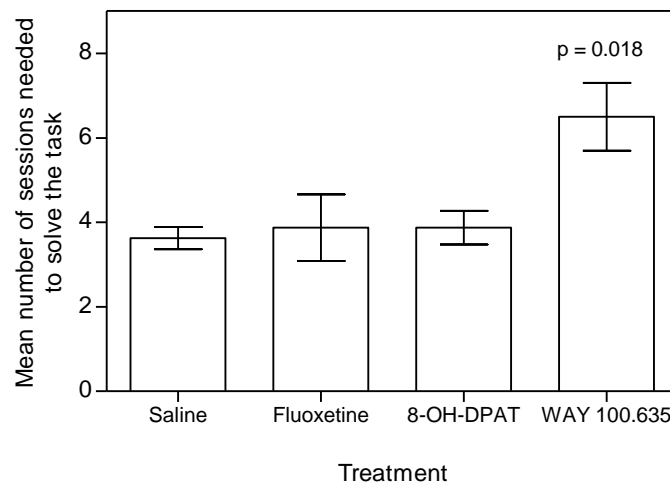
In both tasks, the same cleaners were used in all treatment groups. Data were analysed using one way repeated measures ANOVA, followed by planned comparisons of least squares means in order to compare each treatment with control (saline) group. All statistical test in this study were two tailed. Although it would be interesting to compare both tasks, these are not comparable because the second task (side discrimination) involves reversal learning, while the first (cue discrimination) did not

## RESULTS

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### Cue discrimination task

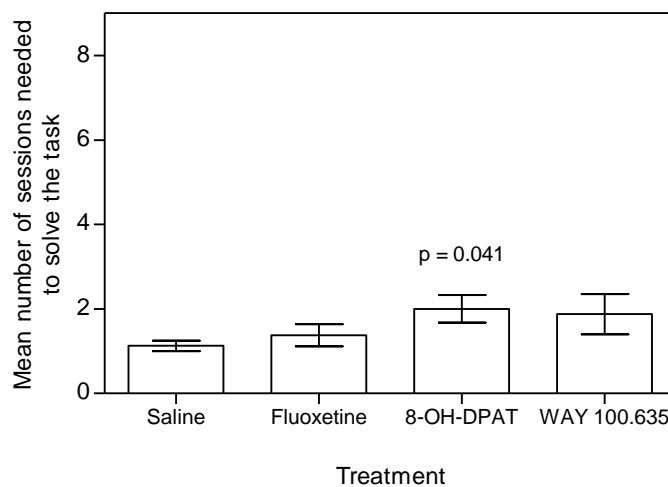
I found a significant main effect of treatment on cleaners' ability to solve the cue discrimination problem (RM ANOVA,  $F_{1,11} = 5.096$ ,  $p = 0.008$ , Fig. 2). Cleaners injected with WAY 100,635 needed more sessions to complete the task, contrarily to those treated with other treatments which needed less learning sessions to solve the task (planned comparisons: Fluoxetine vs Saline:  $F_{1,11} = 0.127$ ,  $p = 0.732$ ; 8-OH-DPAT vs Saline:  $F_{1,11} = 0.259$ ,  $p = 0.626$ ; WAY-100,635 vs Saline:  $F_{1,11} = 9.471$ ,  $p = 0.018$ ).



**Figure 2:** The effect of 5-HT<sub>1A</sub> receptor agonist (8-OH-DPAT), 5-HT<sub>1A</sub> receptor antagonist (WAY 100.635) and SSRI (fluoxetine) on the ability of cleaner wrasse to learn how to solve a cue task compared to the control (saline).

## Side discrimination task

Although we did not find a significant trend for the main effect of treatment on cleaners' capacity to learn the place discrimination task (RM ANOVA,  $F_{1,11} = 1.623$ ,  $p = 0.214$ , Fig. 3) when I examined the effect of each individual compound compared to control I found that the cleaners treated with 8-OH-DPAT needed a larger number of sessions to learn the side discrimination task (planned comparisons: Fluoxetine vs Saline:  $F_{1,11} = 0.636$ ,  $p = 0.451$ ; 8-OH-DPAT vs Saline:  $F_{1,11} = 6.236$ ,  $p = 0.041$ ; WAY-100,635 vs Saline:  $F_{1,11} = 2.333$ ,  $p = 0.170$ ).



**Figure 3:** The effect of 5-HT<sub>1A</sub> receptor agonist (8-OH-DPAT), 5-HT<sub>1A</sub> receptor antagonist (WAY 100.635) and SSRI (fluoxetine) on the ability of cleaner wrasse to learn how to solve a side discrimination task compared to the control (saline).

## DISCUSSION

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Serotonin plays an important role in the modulation of cooperative behaviour between the cleaner wrasse *Labroides dimidiatus* and their so-called "clients" mainly through 5-HT<sub>1A</sub> receptor (Paula et al, Chapter 1). Indeed, results show that only compounds linked to the 5-HT<sub>1A</sub> receptor affected these learning tasks. When faced with a cue learning task, involving visual memory, individuals injected with the antagonist of the 5-HT<sub>1A</sub> receptor WAY 100.635 showed delayed learning. Contrarily, when faced with a spatial learning task, involving recognition of space during a foraging related task, cleaners treated with WAY 100.635 didn't reveal any significant delay in learning, however when injected with the agonist of the 5-HT<sub>1A</sub> receptor 8-OH-DPAT these individuals took longer time to learn this task. Fluoxetine lack of effect on learning of both tasks suggests that the effect is mostly via actions on the 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptor and not a general serotonin effect.

My results show that the antagonism WAY 100.635 had a marked effect on the delay of learning, when solving a cue related problem. The use of the antagonist WAY 100.635 in studies of learning cognitive tasks has produced a variety of results from improvement to impairment, which should be linked to the type of cognitive problem, the ecology of each of the tested species and to the specificity of the compound, given that it has different effects at pre and post-synaptic 5-HT<sub>1A</sub> receptors (Forster et al. 1995). As the cleaner wrasse *L. dimidiatus* depends exclusively on the food they can get through interactions with their "clients", they must prioritize amongst clients because different clients or different orders of importance given to clients can result in fewer interactions and thus lower food gain (Grutter 1995; Bansemmer et al. 2002; Soares et al. 2008). So, for my model species, it is important to recognize visual cues in order to identify which client is a better and safer food source (Grutter 1995). Although studies have demonstrated that WAY 100.635 has an effect to block the impairment induced by agonists such as 8-OH-DPAT in learning of different tasks (Helsley et al. 1998), in the cue discrimination task associative memory should be essential. For example, in Rats subjected to associative learning tasks when treated with 8-OH-DPAT see their memory consolidation facilitated. However, if treated with the WAY 100.635 subsequently, their memory consolidation was completely blocked, suggesting that WAY 100.635 has an effect of reversing the process of associative memory (Manuel-Apolinar & Meneses 2004). Furthermore, systemic administration of WAY 100,635 decreases predator avoidance and risk assessment measures (Griebel et al. 1999, 2000), which could cause

cleaners to being unable to assess the risk involved in making the wrong choice and losing access to the plaque with available food. Indeed, WAY 100635 is described as a potent selective antagonist of the 5-HT<sub>1A</sub> receptor, which may reverse the effects of agonists of this receptor (Helsley et al. 1998). Because in many designs and behavioral tests remain inactive is therefore considered a "silent" antagonist (Fletcher et al. 1995). In fish, the area most related with emotional learning (Dm), have a low amount of serotonergic fibers which is perhaps why I observed such pronounced delaying effects of a single dosage of WAY 100.635 in the solving of the cue learning task. Although the agonist 5-HT<sub>1A</sub> receptor 8-OH-DPAT normally cause impairment in learning (Meneses & Terrón 2001) no effect was to be found on the cue discrimination task, which in turn confirms previous studies which point out that 8-OH-DPAT impairs spatial learning without affecting visual learning (Carli et al. 1995).

As stated above there is an increasing evidence suggesting a role for 5-HT<sub>1A</sub> receptor in learning and memory (Lüttgen et al. 2005). The administration of 5-HT<sub>1A</sub> receptor agonists, such as 8-OH-DPAT have been demonstrated to cause impairment of learning and memory in rodents introduced to various tasks, such as active and passive avoidance and Morris water maze (spatial learning) (Carli et al. 1995; Misane et al. 1998). The impairments caused by 8-OH-DPAT in different tests were attributed to stimulation of 5-HT<sub>1A</sub> postsynaptic receptors, 8-OH-DPAT, which may interfere with mechanisms related to the acquisition and storage of memory of recent events (Stiedl et al. 2000). This also suggests that 8-OH-DPAT may affect rats' performance in a passive avoidance task (the passive avoidance task is a fear-aggravated test used to evaluate learning and memory, in this test subjects learn to avoid an environment in which an aversive stimulus (such as a foot-shock) was previously delivered) by a mechanism which could be separated from effects on pain perception, general activity or emotional behaviour (Carli et al. 1995; Stiedl et al. 2000). Here, I show that cleaners also suffer a delay in learning, in the context of spatial task when treated with the agonist 8-OH-DPAT. These results are in line with what was already described in the literature concerning the effects of this compound, and directly shows that at least 5-HT<sub>1A</sub> receptor is involved in the regulation of spatial learning in a species that inhabits an environment where the input of visual information has a huge value.

Not effect was found of fluoxetine on both learning tasks. Fluoxetine is dose-dependent which with low doses no effect is observed and most of its effects occur only at high doses (Meneses & Hong 1995). Probably I did not observed any because the dose was too low to be observed effects. However, it is mostly likely possible that the effects of dosage are also dependent on behavioural context – for example I observed a

huge effect the same dosage of fluoxetine on cleanerfish motivational levels (chapter 1). Moreover, no compound led to any learning improvement. This could also be dependent on the dosage applied but also to the specific context of these learning problems. It is also possible that other specific but untested serotonin receptors are more related to an improvement of cleanerfish learning performance or perhaps other neuroendocrine systems, such as the dopamine- reward system.

This may be due to the fact that, first 8-OH-DPAT is related to learning impairment in several types of learning tests, second WAY 100635 is related to impairment, improvement, even lack of effect depending on the type of behavioral test (Carli & Samanin 1992; Egashira et al. 2006; King et al. 2008).

In addition to the direct involvement of the serotonin receptors on cleanerfish learning competence, it is also possible that the delayed seen in both tasks are somewhat linked to the system of arginine vasotocin (AVT). It is known that the serotonergic activity can produce changes in behaviour through AVT's pathway system (Ferris et al. 1997; Delville et al. 1998). For exemple, in hamsters serotonin diminishes aggression by suppressing the activity of the AVP system (Ferris 1996). Serotonin fibers and AVP-producing neurons (AVP is the mammalian homoloug of AVT) are located in the same areas in the brain and serotonin can influence the release of AVT peptides (Ferris & Jr 1997).

My results suggests that serotonin plays a role in learning both visual and spatial tasks in *L. dimidiatus*. In the cue learning task, linked to visual learning, and relevant to the mutualistic interactions between cleaner fish and their clients, the antagonist of the 5-HT<sub>1A</sub> receptor led to a delay of learning, which demonstrated that the 5-HT<sub>1A</sub> receptor might be involved in visual recognition and memory. However, in the spatial recognition task, the agonist of the 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> led to delay of spatial learning, suggesting that, similarly to mammals this receptor is connected to the recognition of space.

Although spatial learning is important for the cleaner wrasse in terms of the territorial recognition, this information is not relevant when it comes to foraging. In Chapter 1, the antagonist WAY 100.635 had an "negative" effect in certain cleaning quality measures (e.g. inspection duration) but the agonist 8-OH-DPAT had a leverage effect on tactile stimulation. WAY 100.635 caused cleaner to interacted less and less time - a diminuation of interest. The delay in cue task learning could related to this diminuation of interest to interact.

This study shows that serotonin plays an important role in the learning how to identify important cues and to recognize the territory which are essencial for

establishment and of cooperative behaviour between *Labroides dimidiatus* and its clients.

## CONCLUDING REMARKS

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Coral reefs undergo rapid changes with the disappearance of live corals leading to increased dead corals assemblages where other benthic organisms prosper (Artim & Sikkel 2012). These new habitats are ideal for the development of ectoparasites (Artim & Sikkel 2012) making increasing the importance of cleaning interactions between *Labroides dimidiatus* and their clients. Recently, traces of drugs that modulate the serotonergic system have been found in waste waters entering the sea, while it is known that sewage treatments inefficiently remove these compounds (Styrishave et al. 2011). It is thus imperative increase the knowledge on the implications of these compounds to potential behavioural changes of species, particularly those that directly influence the health and well being of many other inhabitants of coral reefs (aka, client species), such as the cleanerfish *L. dimidiatus*.

The present study tried to establish a link between the effect of serotonin and the behavioural and learning modifications in the cleaner wrasse, as a way to assess the physiological basis of these mutualistic interactions, which have a key role in coral reef ecosystem (Waldie et al. 2011).

On the study on serotonin modulation of the cleaner wrasse cooperative behaviour I suggest that the serotonergic system, mainly through the 5-HT<sub>1A</sub> receptor, a main modulator of cognitive, motivational and perceptual mechanisms, directly influences cleaner wrasse interspecific and conspecific behaviour. The activation of the serotonergic system via receptor 5-HT<sub>1A</sub> seems to be involved in motivational processes and in the increase of interactive quality levels. The blocking of its pathways is responsible of a decrease of interspecific cooperative levels and a rise of conspecific motivational activities. While shifts of available serotonin levels were observed to change cleaner wrasses behaviour, the influence of the social context and of the conspecifics' and clients behaviour can contribute to physiological changes in brain areas responsible for regulation of cooperative behaviour (Goodson 2005; Soares et al. 2010). I propose that, during the evolution of cleaning mutualisms, both the serotonergic (mainly through the 5-HT<sub>1A</sub> receptor) and AVT systems had an important role on regulating both intraspecific and interspecific interactions.

In the study of serotonin influence on cleaner fish learning competence I suggest that serotonin plays a role in learning of visual and spatial tasks. The present study shows that serotonin influences learning through the 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors. The serotonin antagonist WAY 100.635 delayed the learning of the visual task (cue learning) while the

agonist 8-OH-DPAT led to a delay of the spatial learning. Although spatial learning is important for cleaner wrasse recognition of space and territory on the coral reef, it is not usually used as a foraging ability. Visual cue learning has an increased ecological relevance for these fish, as they need to recognize their clients to adjust their behavior during each interaction to obtain the greatest benefit. This study shows that serotonin plays an important role in learning to identify important cues and to recognize the territory.

My work improves our information on the physiological mechanisms underlying cleaning mutualisms, and contributes to reveal causal mechanisms for the establishment of unrelated cooperative interactions.

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