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Serotonergic mechanisms of dominance and aggression in the common waxbill

Beatriz do Couto Pereira Azevedo Saldanha

Dissertação de Mestrado apresentada à
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

Existe uma necessidade em compreender como a resposta comportamental está integrada com a fisiologia de um indivíduo, uma vez que esta é influenciada por diversos fatores. A resposta comportamental pode estar ligada a um panorama genético, mas a resposta fisiológica do indivíduo também contribui para lidar com o meio em que este vive e obter um melhor entendimento das interações sob certos contextos. Ao responder às quatro perguntas de Tinbergen (também conhecidas como níveis de análise) é possível ter uma abordagem lógica e compreensiva na explicação do comportamento social através de, por exemplo, uma abordagem proximal, onde os mecanismos ou os aspetos fisiológicos estão inseridos. Por exemplo, neurohormonas podem atuar centralmente (no cérebro) e perifericamente, entrando no sistema circulatório desse modo, afetando e coordenando variadas funções, incluindo aspetos sociais do comportamento, em vertebrados, mas também em invertebrados.

A serotonina (5-HT) é um dos principais neurotransmissores monoaminérgicos e um dos sistemas mais conservado e predominante entre os vertebrados, tendo um papel importante na regulação do comportamento animal. Contudo, a sua ação não é simples nem bem compreendida, particularmente em aves, para quais informação ainda é muito limitada. O recetor 5-HT1A, localizado tanto pré- como pós-sinapticamente, é, por exemplo, um dos principais reguladores da ação serotonérgica, sendo o mais abundante e identificado para várias espécies, incluindo aves.

Para este trabalho, foi testada a influência da serotonina na dominância e agressividade através da manipulação dos seus níveis recorrendo a injeções com 8-OH-DPAT (um agonista do recetor 5-HT1A), de Fluoxetina (um inibidor seletivo de recaptura de serotonina; SSRI) e de WAY 100.635 (um antagonista do recetor 5-HT1A), em bicos-de-lacre comuns (*Estrilda astrild*). Recorrendo a observações comportamentais num teste de competição por comida, reparou-se que o tratamento com o SSRI Fluoxetina provocou uma diminuição geral da atividade, alimentação e agressividade, o que poderá ser devido a um tipo de efeito anxiogénico explicado pelo aumento dos mecanismos de ansiedade/medo, que poderão estar a atuar através, por exemplo, dos recetores 5-HT2. Por outro lado, o agonista 8-OH-DPAT, que atua seletivamente no grupo de recetores 5-HT1A, teve o efeito oposto de aumento de atividade, enquanto que nenhuns resultados significativos foram encontrados para o antagonista WAY 100.635. Supostos níveis baixos de serotonina poderiam explicar a ausência de resultados para o WAY 100.635 ou os resultados observados serem dependentes da dose. Por conseguinte, estudos futuros devem-se focar nos possíveis

efeitos de diferentes doses e quadros de ação temporais distintos, adicionalmente o uso de um antagonista da Fluoxetina (PCPA, para-clorofenilalanina), que provavelmente revelaria resultados opostos à Fluoxetina, considerando que é um composto mais forte. Aqui, discuto sobre como os efeitos serotonérgicos observados neste estudo podem atuar como moduladores adaptativos no comportamento nos bicos-de-lacre comuns, uma vez que são altamente sociais.

Palavras-chave: serotonina, 8-OH-DPAT, Fluoxetina, WAY 100.635, comportamento social, bico-de-lacre comum (*Estrilda astrild*)

Abstract

There's a need to understand how behavioural response is integrated with the individual's physiology and anatomy since it's influenced by several factors. Behavioural response can be linked to genetic landscape but individual's physiological response also contributes to deal with the surroundings and to have a better understanding of the interactions under certain contexts. By answering the Tinbergen's four questions (also known as levels of analyses) is possible to have a comprehensive and logical approach to explain social behaviour being for example, the proximate approach, the mechanisms or physiological aspects of behaviour. For example, neurohormones may act centrally (at the brain) and peripherally, by entering the circulatory system thus, affecting, mediating and coordinating several functions, including aspects of social behaviour, in vertebrates but also in invertebrates.

Serotonin (5-HT) is a main monoaminergic neurotransmitter and one of the most prevalent and conserved system among vertebrates, with an important part in the modulation of animal behaviour. However, its action is not simple nor well understood, particularly in avian models, for which information is yet sparse. The 5-HT1A receptor subtype, located both pre- and postsynaptically is, for example, one of main mediators of serotonergic action, being the most abundant and identified for several species, including birds.

Here, I tested the influence of serotonin on dominance and aggression, through its manipulation, resorting to injections of 8-OH-DPAT (a 5-HT1A receptor agonist), of Fluoxetine (a selective serotonin reuptake inhibitor; SSRI) and of WAY 100.635 (a 5-HT1A receptor antagonist), on common waxbills (*Estrilda astrild*). Using behavioural observations in a test of competition for food, I found that treatment with the SSRI Fluoxetine caused an overall decrease of activity, feeding and aggressive behaviour, which is probably an anxiogenic-like effect explained by increasing anxiety/fear mechanisms, acting through, for instance, 5-HT2 receptors. On the other hand, the agonist 8-OH-DPAT, which acts selectively on the 5-HT1A receptor group, had the opposed effect of increasing activity, while I found no detectable behavioural effects for treatment with the antagonist WAY 100.635. Putative lower baselines could explain the absence of results for WAY 100.635 or dose dependent effects. Future studies should focus on potential effects of different dosages and distinct time action frames and also the addition of a Fluoxetine antagonist (for instance, PCPA, para-chlorophenylalanine), which would probably reveal opposite results to Fluoxetine. I discuss how the

serotonergic effects observed here may act as adaptive modulators of behaviour in waxbills, in the context of their highly gregarious social organization.

Keywords: serotonin, 8-OH-DPAT, Fluoxetine, WAY 100.635, social behaviour, common waxbills (*Estrilda astrild*)

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* p≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

Attachments

Figure 13- Scheme of the distribution of treatments per cage. Pair1 (A&B), in the first week, receive one of the treatments and Pair2 (C&D) a placebo. The next week, for example, the Pair2 will now receive one of the treatments and the Pair1 will then receive placebo. In the third week, for instance the Pair1 will again receive treatment but not the same one from the first week and Pair2 placebo. This will happen until every individual receives all serotonergic treatments (Fluoxetine, WAY 100.635 and 8-OH-DPAT) once.

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5-HT	serotonin, 5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
5-HIAA	5-hydroxyindole acetic acid
SSRI	Selective Serotonin Reuptake Inhibitor
MAO	Monoamine oxidase
Trp	Tryptophan
CNS	Central Nervous System
5-HT1A	Serotonergic receptor family 1A
GLMM	Generalized Linear Mixed Model
SBH	Social Brain Hypothesis
LA	Lateral amygdala nuclei
8-OH-DPAT	(±)-8-Hydroxy-2-(dipropylamino) tetralin hydrobromide
WAY 100.635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide

1. Introduction

1.1. Studying animal behaviour: Genetic and physiological perspective

Understanding the relation between individual differences in behaviour and population organization and functioning has long been central to many scientific fields such as behavioural ecology and evolutionary biology (Sueur and Mery, 2017). Behaviour responds to intrinsic and extrinsic factors, being these physiological, ecological or social. The study of behaviour needs an understanding of how an individual's physiology and anatomy are integrated with its response (Tenney, 2014).

Within a group, each individual becomes a part of a network of social interactions that can impact the ecology and evolution of individuals, populations and species, taking many forms and influencing fitness (Silk *et al.*, 2003; Formica *et al.*, 2012; Kurvers *et al.*, 2014; Sueur and Mery, 2017). There is evidence that differences in behaviour can be due to genes. From a genetic point of view, changes at single loci can influence behaviour while many behavioural differences are probably defined by many different loci with complex epistatic relationships (Barnard, 1983). One way to see it, is that genes code for hormones (the biggest influencers of behaviour), for example by mediating synthesis and release but also hormones can regulate gene expression (Harden and Klump, 2015). For instance, steroid hormones affect behaviour by binding to intracellular receptors that act as genetic transcription factors, thus regulating expression of steroid-sensitive genes shaping neurotransmission, therefore providing changes in behavioural responses (a more physiological perspective; Oliveira, 2009; Harden and Klump, 2015). Genes assimilate evolutionary responses of former populations to select behaviours that increase fitness, which in turn become more common in that species (Tenney, 2014). Indeed, behavioural response is crucially linked to animal's genetic landscape together with environment, modulating animal's survival and fitness. Normally, individuals with higher fitness have a better chance to pass their genes on to the next generation. On the other hand, individual's physiological response helps to deal with life processes (Barnard, 1983).

In 1963, Nikolaas Tinbergen, the first zoologist and ethologist to perform field experiments to test hypotheses of social behaviour, recognised that biologists working with behaviour focused on different types of problems (Bateson and Laland, 2013;Koenig and Dickinson, 2018). This way defining four major categories to explain and provide a comprehensive and logical approach when studying animal behaviour: i) mechanism, ii)

ontogeny, iii) adaptive value and iv) phylogeny, currently separated into “proximate” and “ultimate” causes (Figure 1). Proximate mechanisms involve a range from hereditary, developmental to physiological aspects of behaviour (Koenig and Dickinson, 2018). On the other hand, ultimate causes, comprise evolutionary origins and selective processes that have shaped their functions (Koenig and Dickinson, 2018).

Tinbergen's Four Questions		Two objects of explanation	
		Sequence (Diachronic)	**Single form** (Synchronic)
Two kinds of explanation	**Proximate**	**Ontogeny** How does the trait develop in individuals?	**Mechanism** What is the structure of the trait?
	Evolutionary	**Phylogeny** What is the trait's evolutionary history?	**Adaptive significance** How have trait variations influenced fitness?

Fig. 1 - The proximate and ultimate (evolutionary) causes that make up for the Tinbergen's four questions: mechanism, ontogeny, phylogeny and adaptive value. Providing possible explanations for distinct behaviours. Image from Nesse (2019).

Although proximate causes do not explain the evolutionary basis of a particular behaviour, it can provide insight into the functions in which organisms are adapted to (Koenig and Dickinson, 2018). Not only the nervous system but also hormones play an important role mediating behaviour, exerting proximate influences on it. Among neuroendocrine modulators, neurotransmitters are a particular type of chemical messenger that enables the communication between neurons through a synapse and are known to have important consequences on behaviour. Studying social behaviour is important to advance social, cognitive and affective neuroscience and through animal research is possible to provide strong evidence that neuromodulators play a crucial role in many social behaviours (Crockett and Fehr, 2014).

1.1.1. Hormones, neuromodulators and behaviour

a) *What are hormones?*

Hormones are chemical messengers produced by endocrine glands, that enter the circulatory system in response to many internal and external stimuli (Barnard, 1983). Neurohormones, are secreted by special neurons within the nervous system, where they are transmitted along axons or into the blood again (see definitions in Table 1). These substances are known to affect physiological and behavioural functions, controlling and coordinating processes throughout the body (Nelson, 2009). These often work locally (as neurotransmitters), also interacting with other neurotransmitters and cytokines to influence behaviour (Nelson, 2009). In the Attachments (Table 4), are listed the major hormones acting on social behaviour in vertebrates and primary biological actions.

Table 1 - Chemical/hormonal definitions. Table adapted from Nelson (2000), Norris (2007) and Soares et al. (2010).

Agents	Definition
Hormone	An organic chemical messenger released by endocrine cells that travels the bloodstream interacting with other cells and causing a bio-response.
Neurohormone	Substance secreted by neurons into de blood circuit that may be stored in neurohemal organ prior to release.
Neuropeptide	A peptide hormone synthetized by a neuron.
Neurosteroid	A steroid hormone synthetized by a neuron.
Neuromodulator	Substances that enhance the excitatory or inhibitory responses of postsynaptic receptors (not directly activate).
Neurotransmitter	Chemical messenger that operates through the synaptic space.
Chemical messenger	Substance produced by a cell that will influence the function of another cell.

b) *How hormones affect behaviour?*

Hormones influence three systems: i) input systems (sensory), ii) integrators (CNS) and iii) output systems/effectors (e.g. muscles), so that a specific stimulus induces certain responses in an appropriate behaviour or social context (Barnard, 1983; Nelson, 2009). By affecting sensory capabilities, it consequently alters the individual's perception of its environment and therefore the response to particular stimuli. Various hormones also affect the development of the different structures that animals resort to perform distinct behaviours and also on the development of young animals (Barnard, 1983). If the neural networks underlying behaviour demonstrate a possibility for neural plasticity, so that it can produce different outcomes regarding the same stimulus/input to the network, changes can happen in behaviour (also depending on the motivational state of the animal; Oliveira, 2009). Structural reorganization and biochemical switching of neural networks are the main neural mechanisms proposed to mediate those changes. Biochemical switching can be achieved by different neuroactive molecules interacting with the circuit and altering its functional properties, that way allowing for a variable response of the same neuronal network under similar stimuli (promoting either excitatory or inhibitory states; Oliveira, 2009). These "neuroactive molecules" are released by neurons and interact with receptors at multiple sites in the neural system.

Furthermore, hormones can act as "primers" facilitating the action of other hormones rather than directly influencing on the nervous system (Barnard, 1983). Hormones can also have pleiotropic functions, e.g. have different effects on behaviour depending on the region acting (Barnard, 1983).

Hormones and neuroendocrine compounds affect and are affected by behaviour. It was possible to observe with androgens, for example, that these act as behavioural facilitators since they only act as modulators of neural pathways of social behaviour by increasing its expression (Simon, 2002; Oliveira, 2004; Oliveira, 2009). Androgens not only affect behaviour but also respond, leading to the reciprocal model (Figure 2) proposed by Oliveira (2009), which refers the feedback of behaviour on the endocrine system as a way to formulate a response towards changes in social environment (Oliveira, 2009), therefore altering behaviour expression in subsequent interactions (Mazur, 1976; Leshner, 1979; Oliveira, 2009). Another example is the elevation of blood estradiol in females rats by chemosensory cues of males, thereby stimulating proceptive or male-seeking behaviours and, similarly, the decreasing of circulating testosterone in males, in a period of time afterwards losing an aggressive encounter (Nelson, 2009). In other words, hormonal levels are influenced by social interactions in which an individual

participates, or to which it is exposed, modulating neural mechanisms that will consequently affect behaviour in social interactions (Figure 2; Oliveira, 2009).

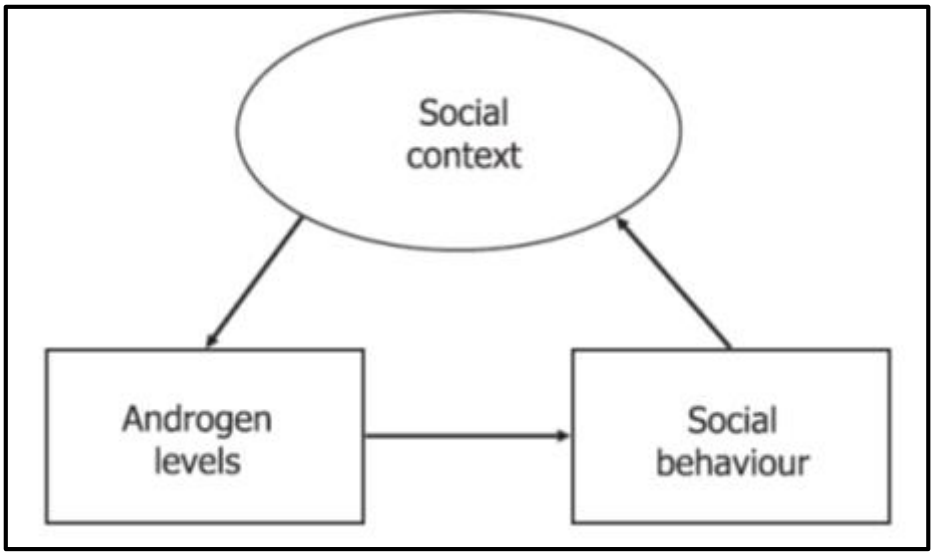

Fig. 2 - The Reciprocal model proposed in Oliveira (2009), with androgens as example of hormone that influences and it is influenced by social behaviour and context.

c) *Chemical neuromodulation: the importance of monoamines*

Catecholamines and neuropeptides are the major classes of neuromodulators, their action on behaviour being extensively documented (Oliveira, 2009). Monoamines neurotransmitters are important bioactive substances in the central nervous system (CNS). These have a variety of essential physiological roles in the modulation of behaviour such as motor control, cognition, emotion and endocrine modulation (Libersat and Pflueger, 2004). They also affect the regulation of motivated behaviours where the serotonergic system takes part on the control of aggressive motivation, demonstrated both in vertebrates as in invertebrates (Kravitz, 2000; Huber; 2005; Oliveira, 2009; Swallow *et al.*, 2016). Many neurologic and neuropsychiatric disorders, like schizophrenia are due to dysfunctions in monoamine neurotransmission, particularly of dopamine and serotonin (Libersat and Pflueger, 2004). Thus, these chemical messengers (i.e. monoamines, sex steroids) may act as neuromodulators of behaviour adapted to a given context (Oliveira, 2009). Several neuroendocrine mechanisms and respective molecules (i.e. serotonin, dopamine, noradrenaline, vasopressin, estrogen and testosterone, etc.) have been identified to regulate vertebrate behavioural responses.

To obtain an integrated holistic response, all these mechanisms are expected to be coordinated by a neuroendocrine control centre (Oliveira, 2009). Steroid hormones, for instance, seem to play an important role in the manipulation of the neural networks of social behaviour (also known as conserved nodes in the brain of vertebrates) when facing changes in social environment, causing the behavioural plasticity (Figure 3; Oliveira, 2009).

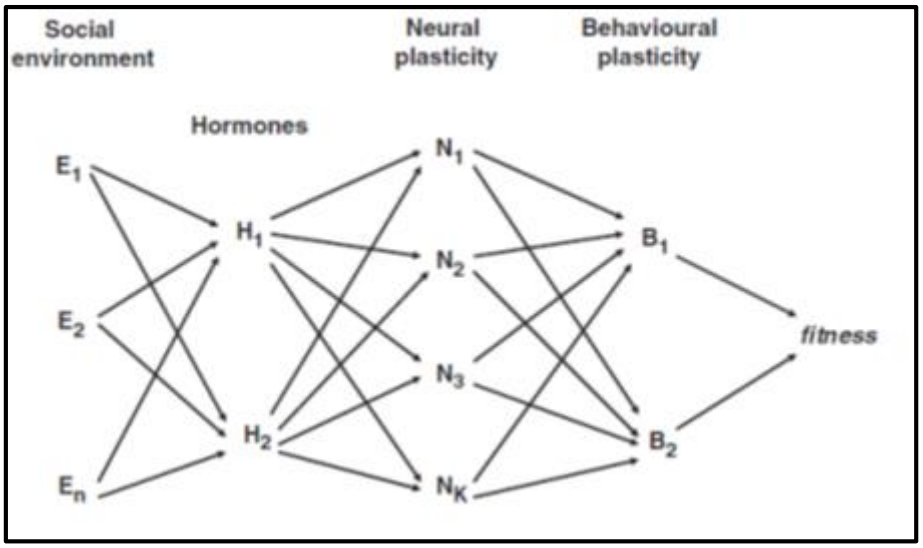

Fig. 3 - Diagram suggested by Oliveira (2009), proposing relationship between social environment, hormones, neural circuits (plasticity), social behaviour and fitness. How variations in social environment/context may induce consequent changes in hormone levels thereby affecting fitness.

d) *The social brain*

The brain is considered one of the most “expensive” organs that has the ability to control body’s functions (Dunbar, 1998; Dunbar and Shultz, 2007; Frith, 2007). The social brain is understood as the set of brain regions that are dedicated to social cognition allowing individuals to interact with others (Frith, 2007). A broad interpretation of the social brain hypothesis (SBH) is that “*individuals living in stable social groups face cognitive demands that individuals living alone (or in unstable aggregations) do not*”, as mentioned in the study by Dunbar and Shultz (2007). In groups, these have to maintain cohesion by meeting their own requirements as well as coordinate behaviour with conspecifics. Research has supported that many key brain regions and neurotransmitters, such as serotonin, play major roles in decision-making (Lee and Goto, 2018). Decision-making involves many cognitive mechanisms in selecting possible actions and is known to be influenced by social environments, for instance if an individual is under conflict/stress. Therefore, species that live in groups exhibit synchronized and coordinated actions, which normally propagate within the group (Lee and Goto, 2018).

Although more studied in mammals, these biological preconditions are not ubiquitous across vertebrates. In a study of Emery (2006), it was suggested “intelligence” in birds since these possessed traits such as use and manufacture of tools, episodic-like memory, predicted the behaviour of conspecifics and possibly understood their mental states. These are supposedly related with brain size but in avian models that relationship between sociality and brain size is complex and no clear connection was found (Emery *et al.*, 2007).

Serotonin (5-HT), in particular, has been shown to be linked to aggression in a wide and diverse range of species (Kravitz, 2000; Sperry *et al*., 2003; Dennis *et al*., 2008; Lorenzi *et al.*, 2009; Theodoridi *et al*., 2017). However, the nature of the connection is not simple and the role of this amine is still to be well understood. This study will focus on effects of serotonin on social behaviour of the common waxbill, namely aggressive behaviour, through the experimental manipulation of serotonin availability.

1.2. Serotonin

1.2.1. What is serotonin?

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter (i.e., a compound mediating communication between nerve cells), which also acts as a neurogenic/immune modulator. 5-HT plays a major role in dendrite growth, altering the expression of its receptors (Dennis *et al.*, 2013) and also regulating cell proliferation, neuronal differentiation and synaptogenesis (Lillesaar, 2011). Moreover, it interacts directly with catecholamines (other neurotransmitters) during development and adult neural processes, or indirectly through alterations in monoamine oxidases (MAOs; Lorenzi *et al.*, 2009; Dennis *et al.*, 2013; Ziomkiewicz, 2016). Monoamines are a group of chemical modulators, that when released at the synapses, exert their actions by activating specific receptors, serving as physiological modulators of animal behaviour (Aksoy, 2017). Some main examples of monoamines neurotransmitters include serotonin, dopamine and norepinephrine.

1.2.2. Where are these found?

Neurons carrying 5-HT have been found in all major metazoan clades and identified in the central nervous system (CNS) of many vertebrates suggesting an early appearance during animal evolution (Figure 4; Lillesaar, 2011; Celada *et al.*, 2013). The serotonergic system is phylogenetically, among the most prevalent and most conserved neurotransmitter systems, exhibiting a number of evolutionary differences in neuroanatomical organization and receptor pharmacology among different species (Turleijski, 1996; Walker *et al*., 1996; Higley and Linnoila, 1997; Insel and Winslow, 1998; Celada *et al.*, 2013). For instance, in avian models, more specifically the pigeon's brain (*Columba livia*), it was possible to conclude that it conforms to the same organizational principles as the mammalian brain as most of the modules (that is the definition given to the distinct subsets of the brain network regions) were functionally and/or anatomically comparable (homologous) to the ones that are revealed when carrying out analysis in human brain (Shanahan *et al.*, 2013). Therefore, despite the differences existing due to evolutionary processes, in this respect the avian brain conforms to the same organizational principles as the mammalian brain (Jarvis *et al.*, 2005; Shanahan *et al.*, 2013).

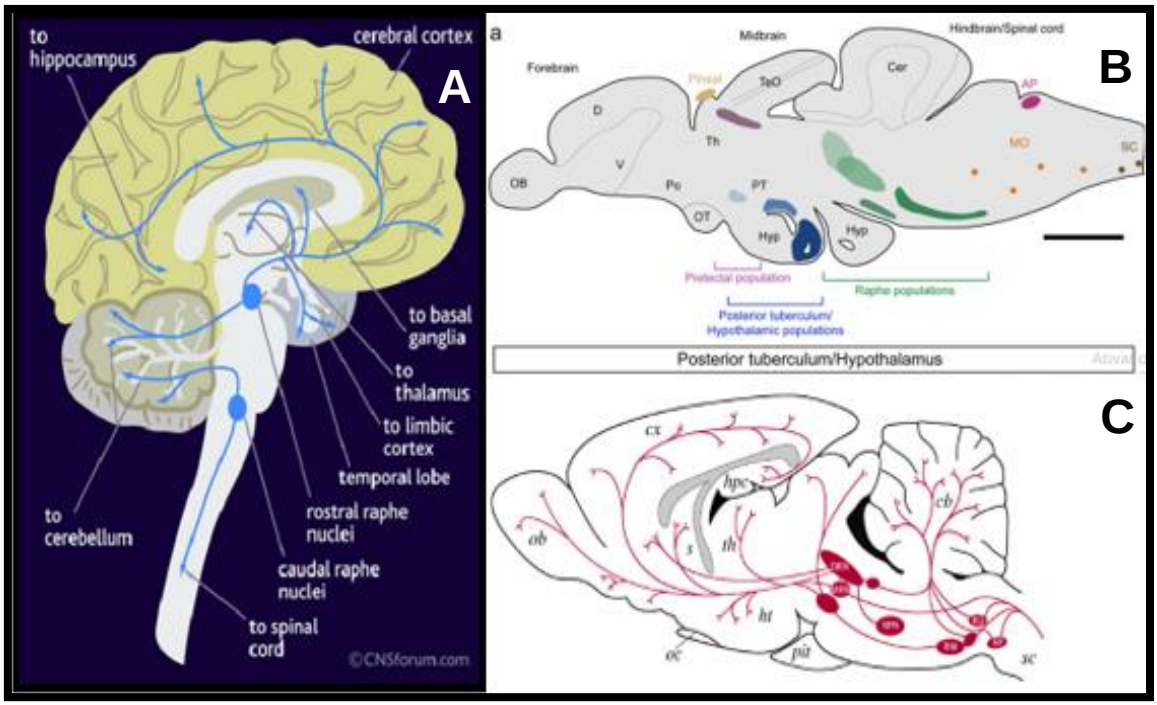

Fig. 4 - The serotonergic pathways in the **(A)** human brain, **(B)** fish brain and **(C)** rat brain. Images from cnsforum.com, Lillesaar (2011) and Ögren et al. (2008), respectively.

The serotonergic system includes central and peripheral serotonin, as seen in Figure 5, in a chicken brain (*Gallus gallus domesticus*; de Haas and van der Eijk, (2018)). The central 5-HT has primary sources of production in the raphe nuclei of the brainstem (CNS), whose projections extend to various brain regions such as hippocampus, cerebral cortex, amygdala, hypothalamus and pituitary (Clotfelter *et al.*, 2007; Ziomkiewicz, 2016; de Haas and van der Eijk, 2018). However, 90% of serotonin (peripheral 5-HT) is synthesized exteriorly to the CNS, being found in blood platelets and several organs, but more specifically in the enterochromaffin cells of the gastrointestinal tracts (Ziomkiewicz, 2016; de Haas and van der Eijk, 2018). Beyond that, with few exceptions, it can also be found, in the pineal gland as a precursor of melatonin (diencephalon/forebrain), hindbrain and/or spinal cord, myenteric cells in the digestive system, beta-cells in the pancreas, parafollicular cells in the thyroid, ovaria cumulus cells, dorsal root ganglia and taste buds (Trowbridge *et al.*, 2010; Lillesaar, 2011). In birds, it can be in the retina (Rios *et al.*, 1997) but again, majorly including the posterior tuberculum and hypothalamus (Lillesaar, 2011).

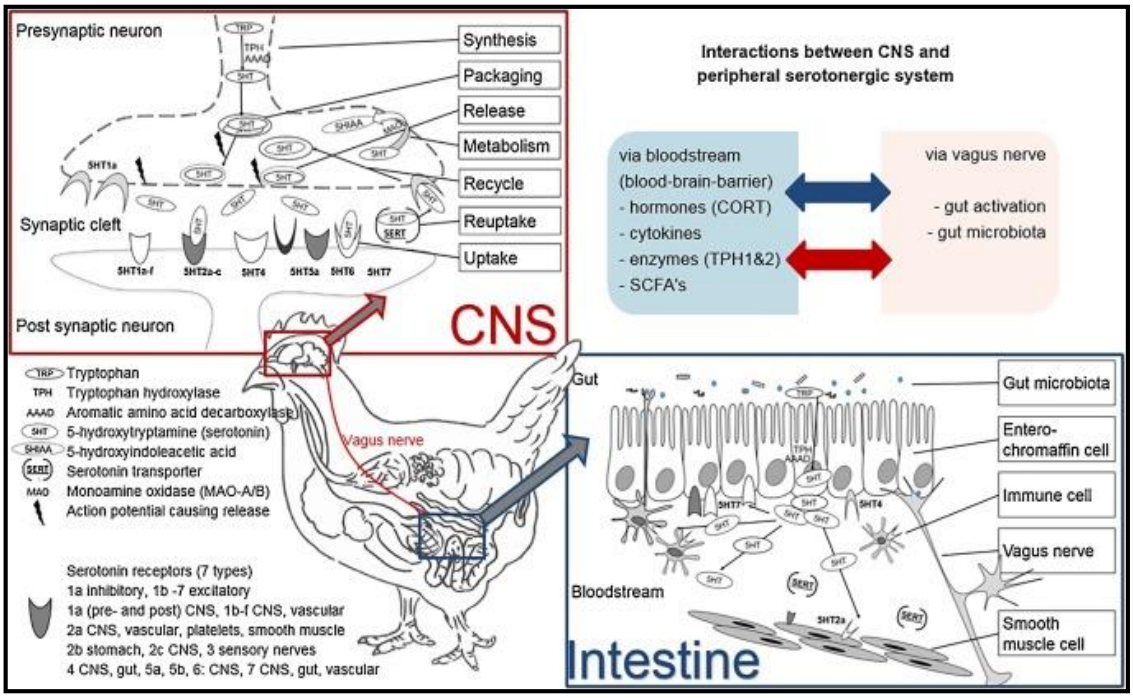

Fig. 5 - Central and peripheral serotonin (5-HT) in an avian model (chicken; *Gallus gallus domesticus*). Image obtained from de Haas and van der Eijk (2018). Central serotonin is synthesized in the CNS, stored in presynaptic vesicles being then released into the synaptic cleft/synapse, where it can bind to multiple receptors. The peripheral 5-HT is synthesized in the intestine (enterochromaffin cells). These two can interact with each other, through bloodstream or vagus nerve.

1.2.3. How is serotonin synthesized?

Both central and peripheral serotonin synthesis are limited by the levels of tryptophan (Trp) in the diet (Young and Leyton, 2002; de Haas and van der Eijk, 2018). A positive connexion between Trp availability and brain 5-HT production is well conserved within the vertebrate lineage (Höglund *et al*., 2019). Accordingly, serotonin is synthesized in a two-step process from the dietary amino acid L-tryptophan, which is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Upadhyay, 2003; Clotfelter *et al.*, 2007; Ziomkiewicz, 2016; Höglund *et al.*, 2019; Pimentel *et al.*, 2019), being widely distributed throughout the body.

In the brain, a transporter that also carries other amino-acids actively takes Trp (Figure 6; Upadhyay, 2003). The metabolic pathway is initiated by the hydroxylation of the Trp to its intermediate, 5-hydroxytryptophan (5-HTP), being subsequently decarboxylated becoming 5-HT (see Figure 6). The tryptophan hydroxylase, that performs the conversion to 5-HT is widely distributed and has a broad substrate specificity being the rate limiting enzyme in the pathway (Upadhyay, 2003). It's believed that the rate of serotonin synthesis is reflected in its release, often quantified as the concentration of the catabolite 5-hydroxyindole acetic acid (5-HIAA; Höglund *et al.*,

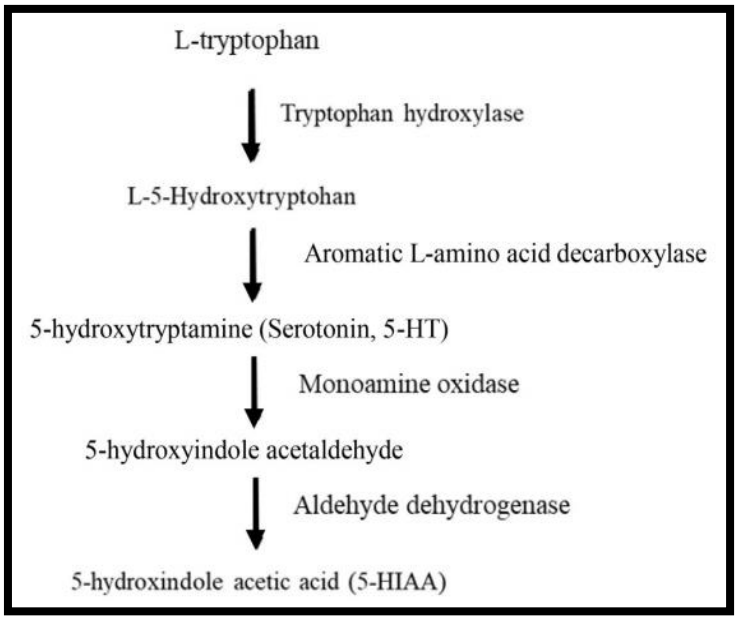

Fig. 6 - Biosynthetic/Metabolic pathway of serotonin (5-HT). Image from Hoglund et al. (2019).

2019). The principal route of metabolism of 5-HT involves a two-step process: i) first, where a monoamine oxidase (MAO) creates 5-HIAA; ii) secondly, where a Na+ dependent carrier mediated uptake process terminates the action of 5-HT (Upadhyay, 2003).

Posteriorly, platelets to acquire 5-HT, have a transporter localized in the exterior membrane to capture the serotonin that comes from the respective transporter located in the outer membrane of serotonergic axon terminals, since they do not have the enzymes required for its synthesis (Upadhyay, 2003).

Like any classical neurotransmitter, once 5-HT is released into the synapses during neuronal activity, it binds to a diverse class of receptors on the postsynaptic membrane (Hoyer *et al*., 2002; Clotfelter *et al.*, 2007; Lillesaar, 2011). Through a stimulation of the postsynaptic neuron, 5-HT is reabsorbed from synapses through serotonin transporter located on the presynaptic neuron, where some is metabolized into 5-HIAA (Clotfelter *et al.*, 2007; Ziomkiewicz, 2016). Levels of synaptic serotonin might be regulated by selective serotonin reuptake inhibitors (SSRIs; Ziomkiewicz, 2016).

1.2.4. How does serotonin work? The serotonergic brain function

The complex actions of 5-HT are mediated by a large family of receptors placed in different organs (Upadhyay, 2003; Meeter *et al.*, 2006; Ögren *et al.*, 2008). Within the CNS, the serotonergic system mediates a large number of functions due to its widespread innervation of the whole neuraxis (Celada *et al.*, 2013). Serotonergic neurons are originated in the dorsal and median raphe nucleus of the brainstem; whose ascending projections are throughout the brain. Following the release from the serotonergic nerve terminal, serotonin can act on several types of receptors that can be located pre or postsynaptically (Ögren *et al.*, 2008). Therefore, due to this neuronal organization, neuromodulators when released can have different effects in different brain regions according to the type of receptor activated (Crockett and Fehr, 2014). This has led to an intensive research to acquire more knowledge and identify the contributions of serotonin receptors subtypes in cognitive functions.

At least 16 different subtypes of 5-HT receptors have been isolated in mammals and classified into 7 major families according to pharmacological, transductional and structural criteria (Polter and Li, 2010; Hoyer *et al.*, 1994; Ögren *et al.*, 2008; Celada *et al.*, 2013). Being the 5-HT1 (A, B, D, E, F), 5-HT2 (A, B, C), 5-HT3 (A, B, C), 5-HT4, 5-HT5 (A, B), 5-HT6 and 5-HT7. It is known that at least 5-HT1 is distributed in birds since studies carried out with these involve manipulations and analyses of 5-HT1A/B/D receptors (Hartig *et al*., 1992; Sperry *et al*., 2003; Dennis *et al*., 2008; de Haas and van der Eijk 2018; dos Santos *et al.,* 2015).

Among the many existing receptors, the 5-HT1 family has received major attention because of the high density expression in the limbic and motor brain areas of vertebrates (5-HT1A and 5-HT1B, respectively; Celada *et al*., 2013). Even though being located postsynaptically to 5-HT axons, both these receptors are also autoreceptors in 5-HT neurons and therefore control the overall as well as the local activity of the system (Celada *et al.*, 2013). The differences in receptors' protein structure and consequent affinities for different serotonergic compounds, allows to identify selective ligands, either agonist or antagonist, for each receptor variant that can directly stimulate or block the receptors, being highly or less selective (Hoyer *et al.*, 1994; Crockett and Fehr, 2014).

There is evidence that the 5-HT system remains the primary mediator of aggressiveness, especially in birds with the 5-HT1 family as main subject of interest (Dennis *et al.,* 2008). Further studies support the role of 5-HT1 receptor family (mainly 5-HT1A receptor subtype) regarding modulation of behaviour (i.e. aggressiveness,

memory tasks, water and food intake, among many others; Saadoun and Cabrera, 2002; Sperry *et al.*, 2003; Dennis *et al.*, 2008; Dennis *et al.*, 2013).

a) *5-HT1A receptor*

In mammals, like rodents and humans, 5-HT1A receptors are highly expressed by different neuronal types in prefrontal cortex, suggesting an important role in the control of mood and emotions as well as cognitive processes (Celada *et al*., 2013; Paula et al., 2015). Another study mentions that these type of receptors, 5-HT1A, in birds, are expressed in prosencephalic areas thus being involved in visual and cognitive functions (dos Santos *et al.*, 2015). An autoradiographic study of pigeons indicated moderate to high expression in forebrain regions (including, for example, the hippocampus), and results indicate dense concentration in the brainstem and in the periventricular preoptichypothalamic areas (Figure 7; dos Santos *et al.*, 2015).

These receptors are divided into two distinct groups based on their location: i) autoreceptors, located on the soma and dendrites of serotonergic neuron of raphe nucleus (Polter and Li, 2010; dos Santos *et al.*, 2015) and ii) heteroreceptors, located on non-serotonergic neurons, primarily of the limbic areas, such as dendrites and soma of glutamatergic pyramidal neurons, for instance (Polter and Li, 2010; dos Santos *et al.*, 2015). Such mediation remains unclear, especially in birds (Hoyer *et al.*, 1994; Dennis *et al*., 2008).

In a study by Dennis *et al.* (2013), with chicken chicks as subject, when injecting low doses of a 5-HT agonist, 5-HT1A expression was greatest in the hypothalamus and raphe nucleus although results could be related to the 5-HT1A receptor group that it was binding to (autoreceptor or heteroreceptor), thus supporting the existence of those two distinct groups also in avian models.

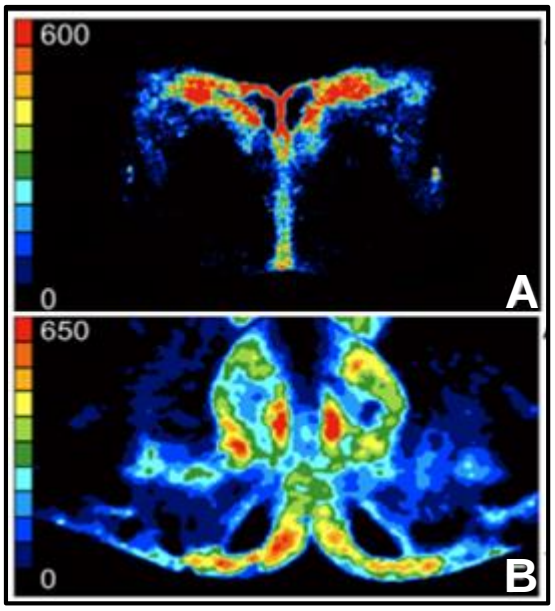

Fig. 7 - Colour-coded autoradiographs of 8-OH-DPAT binding sites in the pigeon (*Columba livia*): **(A)** brainstem and **(B)** hypothalamus, respectively. The colour-coding indicates the density of binding sites. Image adapted from dos Santos et al. (2015).

Activation of the autoreceptors suppresses firing of serotonergic neurons, this way reducing 5-HT activity and having an anti-serotonergic effect (Sprouse and Aghajanian, 1987). On the other hand, activation of heteroreceptors usually promotes serotonergic effects (Carey *et al.*, 2004; Polter and Li, 2010). Exogenous administration of 5-HT and 5-HT1A agonists may lead to an overall reduction in the probability of action potential firing (Celada *et al.*, 2013).

5-HT1A receptors are linked to anxiety-related and mood-related behaviours since these participate in some pathways that affect kinases that, posteriorly, may alter behaviours such as those mentioned above: anxiety, depression, fear and stress (Griebel *et al.*, 1994; Sánchez and Meier, 1997; Polter and Li, 2010; Maximino *et al.*, 2013; Garcia-Garcia *et al.*, 2014). There is evidence suggesting that normal expression of this receptor is required in the first weeks of life for the emergence of normal anxiety, otherwise individual's, in time, may develop pathological levels of anxiety (Polter and Li, 2010; Garcia-Garcia *et al.*, 2014). Anxiety-like behaviours are controlled in the brain by the neurotransmitter serotonin as confirmed in some studies (Fossat *et al.*, 2014). The exact role of the 5-HT1A receptors in the development and/or in the mediation of emotional behaviours has been complicated to elucidate taking into account that this receptor exists as two distinguished populations.

b) *Agonists, Antagonists and Selective Serotonin Reuptake Inhibitors*

Agonists (Figure 8B), bind to the receptor and mimic the actions of the neuromodulator (Crockett and Fehr, 2014). On the other hand, antagonists (Figure 8B), bind to the receptor and block the actions of the respective neuromodulator (Crockett and Fehr, 2014). When agonists bind to postsynaptic receptors, their effect is to increase the neuromodulator function, but agonists and antagonists can also influence the neuromodulator function if binding to autoreceptors (Crockett and Fehr, 2014). Autoreceptors are located on the neurons that produce and release neurotransmitters and when activated these inhibit those functions. The main mechanism for selective serotonin reuptake inhibitor (Figure 8A; SSRI), is the inhibition of neurotransmitter presynaptic reuptake, increasing extraneuronal monoamine concentrations both in the dorsal raphe nucleus as well as in the forebrain (Sperry *et al.*, 2003; Carlini *et al.*, 2012; Crockett and Fehr, 2014).

8-OH-DPAT is the first selective developed compound to be used as an agonist for the 5-HT1A receptor and it is known to reduce brain 5-HT synthesis and release by activating raphe 5-HT somatodendritic autoreceptors which serve to regulate serotonin neuronal activity (Montgomery *et al.*, 1991; Arborelius *et al.*, 1993; Ögren *et al.*, 2008; Celada *et al.,* 2013). Its main action is to facilitate serotonin activity via 1A receptor action (i.e. Fletcher *et al.,* 1993; Celada *et al*., 2013; Crockett and Fehr, 2014). Although its action can depend upon the balance of activation of the two differentially located populations (pre- and postsynaptically) of 5-HT1A receptors (Fletcher *et al.*, 1993; Ögren *et al.*, 2008). For many years, 8-OH-DPAT was thought to be the more selective 5-HT1A receptor agonist and was a frequently used drug (Meneses and Perez-Garcia, 2007; Ögren *et al.*, 2008) but more recently it was demonstrated that it also displays an affinity for 5-HT7 receptors known to influence learning and memory, in mammals (Meneses and Perez-Garcia, 2007; Ögren *et al.*, 2008; Perez-Garcia and Meneses, 2009). 5-HT1A receptor also plays a part in memory, in avian models (Dennis *et al.*, 2008; Dennis *et al.*, 2013) and also in mammals (Fletcher *et al.*, 1996; Perez-Garcia and Meneses, 2009).

WAY 100.635 is a selective antagonist with low intrinsic activity, that can penetrate easily into the brain, blocking 5-HT activity and it's being much used to study the functional role of brain 5-HT1A receptors (Fletcher *et al.*, 1996; Ögren *et al.*, 2008). With greater potency and selectivity for 5-HT1A receptors, it has been demonstrated to be an antagonist of 8-OH-DPAT (Fletcher *et al.*, 1996; Ögren *et al.*, 2008).

Finally, Fluoxetine is as selective serotonin reuptake inhibitor (SSRIs) and as any other SSRI, it blocks the neuronal uptake for 5-HT, increasing extracellular serotonin concentrations in the dorsal raphe nucleus as well as in the forebrain (Sperry *et al.*, 2003). By inhibiting its reuptake, it facilitates its activity. Its best known as a treatment of depression and anxiety in humans (Lesch and Mössner, 1998; Paula *et al.*, 2015).

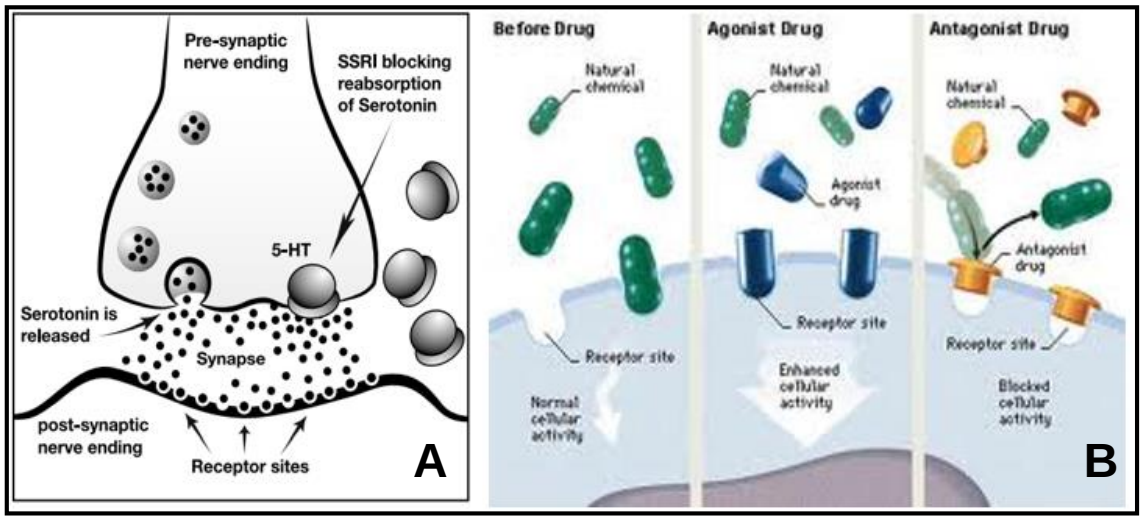

Fig. 8 - Actions of **(A)** SSRI, **(B)** agonist and antagonist. Images from Lattimore et al. (2005) and Receptor interactions-Agonists and Antagonists (RxCAM ApotheCare-Wordpress.com).

1.3. The influence of serotonin in social behaviour

Although recently discovered (about 50 years ago), the study of serotonergic functions is still an expanding field (Meneses, 1999; Meneses and Perez-Garcia, 2007). The function of central serotonergic system is yet not fully understood but growing evidence indicates that it is involved and plays a significant role in many actions from mood, stress response, depression, social interactions, locomotor activity, aggression, learning, memory, feeding, reward-related behaviours; all the way to the appraisal of pain, in a wide variety of species, vertebrates and invertebrates (Insel and Winslow, 1998; Meneses, 1999; Daw *et al.*, 2002; Beulig and Fowler, 2008; Schweighofer *et al.*, 2008; Polter and Li, 2010; Crockett *et al.*, 2010; Lillesaar, 2011; Dennis *et al.*, 2013; Theodoridi *et al.*, 2017; de Haas and van der Eijk, 2018). Therefore, serotonin is a great candidate monoamine for regulating social behaviour, since it is implicated in so many central functions (Soares *et al.*, 2016).

Serotonin neurons project from the brainstem, innervating diffusely all areas of the brain, modulating many aspects of brain function and prosocial behaviours (Young, 2013). As mentioned before, the reciprocal model is also applied to the serotonergic

system. Serotonin modulates behavioural responses to environmental stimuli (Kiser *et al.*, 2011). 5-HT plays a key role in shaping social responses and the system itself is highly responsive to social influences (Kiser *et al.*, 2011). Kiser *et al.* (2011), by reviewing serotonin effects in human, monkey and rodent social behaviour, involving parental attachment and caregiving, social play, aggressiveness, cooperation and sexual behaviour, concluded that 5-HT is positively sensitive to social factors.

Social behaviours encompass a variety of interactions at different levels, for example from feeding aggregations until multigenerational family groups with cooperative brood care (Koenig and Dickinson, 2018) and 5-HT is a neuromodulatory driver that can influence these different social behavioural interactions (Paula *et al.*, 2015; Ziomkiewicz, 2016). When living in groups, individuals must resort to specific tactics that include a mixture of affiliative (e.g. food sharing, allopreening), cooperative, aggressive, coordinated behaviours that will depend on the species, social organization and ecological niche (Ziomkiewicz, 2016).

1.3.1. Investment and Sociality

Possible explanations of how sociality evolved come from studies with different taxonomic groups, such as birds (Koenig and Dickinson, 2004; Ebensperger and Hayes, 2016). It involves a long-term spatial and temporal proximity of conspecifics resulting in the formation of social groups with attributes such as group size, stability and kin structure (Ebensperger and Hayes, 2016). Subsequently, many forms of cooperation may evolve, like strategies of reproduction and offspring care are shared within social groups (Silk, 2007; Ebensperger and Hayes, 2016). The social behaviour is better understood by considering the costs and benefits of sociality since interacting with other individuals can be inherently dangerous. Individuals in groups may benefit by cooperating to gain access to food and other resources besides the increased vigilance and group defence (Koenig and Dickinson, 2018). These often choose for sophisticated forms of communication and cooperation to enhance group's overall success (Koenig and Dickinson, 2018).

Serotonin may be one of the neuromodulators that directly influences sociality, for instance, through the quality of cooperative behaviours (Paula *et al.*, 2015; Pimentel *et al.*, 2019). A possible mechanism may occur via the interaction with other systems, like dopaminergic neuronal system with studies supporting the influence of 8-OH-DPAT administration affecting dopamine activity in the limbic system (critical to social stimuli;

Montgomery *et al.*, 1991; Dalley and Roiser, 2012; Paula *et al.*, 2015). Additionally, in humans, it was shown that since serotonin fibres overlap with oxytocin-containing cells in the paraventricular and supraoptic nuclei of the hypothalamus, it can establish a relationship between them (Emiliano *et al.*, 2007). It was also observed that the enhancement of serotonin levels results in fewer quarrelsome behaviours, increased dominant behaviour, cooperative communication and play during mixed-motive games, in adult humans (Knutson *et al*., 1998; Tse and Bond, 2002; Paula *et al.,* 2015). In locusts, an increase in serotonin levels seems to transform previous solitary individuals to gregarious ones (Anstey *et al.*, 2009; Paula *et al.*, 2015).

1.3.2. Impulsivity, Movement and Motivation

Impulsivity is described as poor self-control, characterized by quick decision making, without any forethought or consideration of any possible consequences (Dalley and Roiser, 2012). Serotonin has been implicated in impulsive aggression via emotion regulation mechanisms (Crockett *et al.*, 2008). Many studies have shown that reduced levels of 5-HT in the CNS promotes impulsive behaviours (Bizot *et al.*, 1999; Mobini *et al.*, 2000; Crockett *et al.*, 2008; Dalley and Roiser, 2012). The role of serotonin in impulsivity has been studied primarily using forebrain 5-HT depletion and resorting to pharmacological agents such as SSRIs, which increase extracellular serotonin concentrations. Additionally, systemic treatment with 8-OH-DPAT seems to suppress 5-HT neuronal firing producing impulsive choice (Winstanley *et al.,* 2005; Miyazaki *et al.*, 2012).

The term “Motivation” defines an internal state that influences the relationship or intervenes between stimulus and response (Barnard, 1983). Stimulus can evoke two types of response within the brain: i) directly related to the stimulus, visual stimuli that evokes responses in the visual centres of the brain, affecting auditory centres and so on; and ii) less specific, due to incoming pathways that connect with non-specifics to other brain centres, becoming later aroused. This means that a stimulus does not only raise a direct response but may also increase the individual’s responsiveness to other, unrelated stimuli (Barnard, 1983). In a study by Paula *et al.* (2015) with fish as subject, it was possible to observe a general increase in the motivation to engage in interactions following administration of Fluoxetine and the serotonin 5-HT1A receptor agonist 8-OH-DPAT. Whilst WAY 100.635 produced the opposite effect.

5-HT is also involved in gain control through its projections to the spinal cord enhancing activity with increasing motor output, as seen in humans, cats and rats (Elliott *et al.*, 1990; Jacobs *et al.*, 2002; Wei *et al.*, 2014). Locomotor activity is influenced by the activation of 5-HT1A receptors, decreasing or increasing depending on the doses, in rats (Ögren *et al.*, 2008). In a study with sparrows (*Melospiza melodia morphna*; Sperry *et al.*, 2003), no negative effects where observed in motor behaviour, when testing 8-OH-DPAT or Fluoxetine but results could be due to the low doses administered.

Manipulating serotonin levels can also cause a depressive/apathetic state. Regarding some studies, the hypofunction of serotonin (5-HT) has emerged has a leading candidate for depression (Tse and Bond, 2002; Meeter *et al.*, 2006). Depression can have serious effects on social functioning and also affecting the group/family and society (Tse and Bond, 2002).

1.3.3. Aggression, Social status and Dominance

a) *Adaptive value*

Aggressive behaviours may be largely instinctive and may also be adaptive as they can help the survival, access to resources and potential mates (Koenig and Dickinson, 2018). Aggressive and defensive behaviours are common and widely observed especially in species living in groups (Drews, 1993), and are the basis of social dominance hierarchies (Chase and Lindquist, 2009; Paull *et al.*, 2010; Theodoridi *et al.*, 2017). The existence of a social hierarchy is essential in the organization of many groups that undertake collective and coordinated activities with the dominant members being often privileged with those benefits (Ziomkiewicz, 2016). An individual's position usually determines its priority of access to resources thus social status can influence health, aging and fitness measures (Holekamp and Strauss, 2016). Even though aggression may be normally useful for the hierarchy formation, once established, a stable hierarchy can largely suppress further aggression and unwanted fights among group members (Holekamp and Strauss, 2016).

Although, normally, social hierarchies are stable, it has been shown that social status can be reversed due to environmental or husbandry conditions (Theodoridi *et al.*, 2017). Usually, "subordinates" have limited access to resources, allowing them, as weaker or less skilful members, to minimize consequences of aggressive encounters with conspecifics (Ziomkiewicz, 2016). When social structure is ignored or misinterpreted, it can lead to aggression from conspecifics, exclusion from the social group or even death (Watanabe and Yamamoto, 2015; Ziomkiewicz, 2016).

For gregarious animals that live in organized groups, where cooperative skills are required to attain dominant positions, general aggressiveness and physical strength are often insufficient to define a position in the social hierarchy (Ziomkiewicz, 2016). In the most common cases of gregarious birds, dominance is determined by intrinsic factors such as body size, fighting ability, personality traits or other attributes that can directly affect the interactions. The formation of social hierarchies is linked with the activation of specific brain regions, with, the serotonergic system being identified as one of the critical parts of the neural circuitry influencing expression of dominance behaviour (Holekamp and Strauss, 2016).

b) *5-HT modulation of aggressive behaviour*

Although scientific evidence suggests that serotonergic system is connected to the establishment and maintenance of social rank, it is still unclear whether this status itself is the cause or consequence of the differences observed at the physiology and behaviour between dominant and subordinate individuals (Theodoridi *et al*., 2017). Aggression is not an inflexible response triggered by some stimuli, in fact, an individual's tendency to attack relies on the activity of key structures and pathways in the nervous system and by the levels of particular circulating hormones in the blood (Huntingford, 2019).

Serotonin has been indicated to regulate aggressive behaviour both in vertebrate as in invertebrate species (Kravitz, 2000; Huntingford, 2019) similarly, pharmacological manipulations with agonists or antagonists further support that statement (Tse and Bond, 2002; Sperry *et al.*, 2003; Dennis *et al.*, 2008; Lorenzi *et al.*, 2009; Lillesaar, 2011; Maximino *et al.*, 2013; Aksoy, 2017).

Experimentally, decreasing levels of serotonin tends to increase aggression, with individuals becoming more bold and/or acquiring a dominant position while increasing levels has the opposite effect, being associated with decreased aggression, apprehensive and/or subordinate behaviour (Sperry *et al.*, 2003; Lorenzi *et al.*, 2009; Lillesaar, 2011; Tran *et al.*, 2013; Ziomkiewicz, 2016). This relationship between aggression and 5-HT seems to be consistent across vertebrates (Sperry *et al.*, 2003). However, in birds, the information is still limited (Sperry *et al.*, 2003; Dennis *et al.,* 2008; Dennis *et al*., 2013).

Regarding avian models, exogenous manipulations of the 5-HT system, using 8-OH-DPAT and Fluoxetine to measure its effects on aggressive behaviour found that both compounds influence a reduction of aggressive behaviour (Sperry *et al.*, 2003). Previous studies in non-passerine birds, also demonstrated that 5-HT modulates aggressive behaviour. In young domestic chickens, *Gallus domesticus*, when treated with tryptophan hydroxylase inhibitor, the frequency of aggressive encounters was negatively correlated with 5-HT levels (Buchanan *et al*., 1994). In food restricted pigeons, *Columba livia*, aggressive behaviour decreased after administration of the 5-HT precursor (Fachinelli *et al.*, 1996).

8-OH-DPAT, generally, appears to decrease aggressive behaviour in reptiles as well in mammals *(Olivier et al., 1995; Adams et al., 1996; Deckel et al., 1996; Sperry et al.*, 2003). These properties are probably mediated through presynaptic 5-HT1A autoreceptors rather than the postsynaptic heteroreceptors (Sperry *et al*., 2003; Carey

et al., 2004). On the other hand, Fluoxetine seems to have the same effect as 8-OH-DPAT, decreasing aggressive behaviour but also activity. Drugs that suppress serotonin action, antagonists like WAY 100.635, have been shown to increase aggression or counteract the effects of agonists in a variety of vertebrates (Clotfelter *et al.*, 2007).

1.4. Study's species: The common waxbill (*Estrilda astrild*)

The common waxbill (*Estrilda astrild*; Figure 9A) is a small finch native to Africa, originally endemic to some areas south of the Sahara and some islands (Figure 9B). It occurs mostly in open habitats such as savannas as well as areas with notable tall grasses, in proximity of water and feeding on herbaceous seeds (Goodwin, 1982; Clement *et al.,* 1993; Fry 2004). Although waxbills are resident year-round in most part of their range it is also common that they make vagrant, nomadic movements (Fry, 2004). Experiments in captivity (Steinbacher and Wolters, 1965; Nicolai and Steinbacher, 2007) show that common waxbills are very sensible to low temperatures (below 15ºC) and very negatively influenced, particularly in cold and wet weather conditions. Waxbills are found in flocks, they roost communally and form socially monogamous pairs that can nest in proximity of each other (Clement *et al.*, 1993; Payne, 2010). They are very gregarious, almost never found alone. Waxbills are mutually ornamented, with both sexes having red plumage and a vividly red bill, granting the species its common name. Ornamentation in waxbills does not have solely the purpose of mate attraction and choice, it also affects same-sex social interactions (Cardoso *et al.,* 2014).

Fig. 9 - **(A)** The common waxbill (*Estrilda astrild*; Photograph by Bruno Maia; https://www.flickr.com/photos/brunomiguelmaia/2542530220); **(B)** Endemic areas of distribution of the common waxbill by Stiels et al. (2011) and current distribution in Iberian Peninsula based on IUCN Red List, IUCN Red List (2018).

Due to pet trade they were introduced and also invaded many places worldwide (Figure 9B; Stiels *et al.*, 2011) and further invasions may still happen, since there's other places that can also offer environmentally suitable conditions for the species (Stiels *et al.*, 2011), and waxbills can use ecological niches quite different from native passerines (Batalha *et al*., 2013). In Europe, the largest invasion by waxbills begun in the 1960s, in areas near to the Portuguese coast, initially, slowly progressing but then expanding to most of Portugal and some regions of the southwest and northwest of Spain (Cardoso and Reino, 2018). In Portugal, they were introduced around 1964-1968, where they are currently widespread covering a vast range of ecological abiotic conditions, as well as locals that were colonized long ago, with higher population density and locals that were colonized recently, with lower population density (Sullivan *et al.*, 2012; Cardoso and Reino, 2018).

Being easy to maintain in captivity and highly gregarious, this species becomes ideal to study social behaviour. Previous studies (Funghi *et al.*, 2015, 2018) documented consistent differences in aggressiveness among waxbills when competing for food. The sexes did not differ in aggressiveness nor in position within the dominance hierarchies, although females were more aggressive towards other females than males, whereas males were equally aggressive to females and males (Funghi *et al.,* 2015). Individuals that were more aggressive scored higher in dominance hierarchies within groups, but aggression of subordinates towards more dominant individuals also happened. As such, common waxbills show weak social hierarchies, as might be expected for this granivorous and very gregarious species, which uses locally abundant resources that are difficult to monopolize (Funghi *et al.*, 2015, 2018). One factor that was related to

waxbill social dominance was body size, perhaps because larger body size confers fighting and resource-holding abilities (Funghi *et al.*, 2015).

1.5. Objectives

As mentioned, serotonin (5-HT) is one of the major neurotransmitters in the CNS, which influences innumerable behaviours and functions (Lillesaar, 2011). The main goal of this study is to observe how aggressive and dominant behaviours are influenced by serotonin in the common waxbill, resorting to pharmacological manipulations in captivity conditions. More specifically, to obtain better insights of the serotonergic system on social behaviour, in a social avian model. At the same time, I try to relate with the existing information on the ecology and social behaviour of the study species. This study will thus provide valuable information regarding the effects of serotonin on avian behaviour, since limited information is yet available for avian model systems.

To determine whether the behaviour of the waxbill is modulated by serotonin activity, manipulations and observations were conducted in captive conditions, with waxbills housed in mixed sex group. I measured the effect of serotonin by testing if exogenous administration of 2 serotonin activity facilitators (8- OH-DPAT and Fluoxetine) and 1 serotonin activity blocker (WAY 100.635; Table 2) differed from a control treatment in either: i) aggressiveness; ii) allopreening; iii) feeding and latency to the feeder or iv) motor activity.

Table 2 - Compounds used and respective effect and activity. Adapted from Paula et al. (2015).

Compound	***Effect***	***Serotonin Activity***
Fluoxetine	Selective serotonin reuptake inhibitor	Facilitates serotonin activity
8-OH-DPAT	5-HT1A receptor agonist	Facilitates serotonin activity
WAY 100.635	5-HT1A receptor antagonist	Blocks serotonin activity

I predicted that, by facilitating serotonin activity and increasing its levels, waxbills would decrease their social aggressiveness and, become more interested in interacting with other conspecifics (more allopreening). On the other hand, I foresaw that treatment with the antagonist WAY 100.635 would lead to an increase in aggressiveness and a decrease in positive social behaviours. These predictions are based on the consensus of past studies on other species (see above, section 1.3.3b), but it is possible that results with common waxbills differ, since serotonin effects on behaviour are dependent on many factors, such as the type of 5-HT1A receptor group being activated, the interaction with other neuroendocrine systems, stress, fear and anxiety-like behaviours (Cousins and Seiden, 2000; Bagdy *et al.*, 2001; Young and Leyton, 2002; Burghardt *et al.*, 2004; Ögren *et al.*, 2008; Maximino *et al.*, 2013; Tran *et al.*, 2013; Young, 2013; Garcia-Garcia *et al*., 2014; Theodoridi *et al*., 2017). To note that serotonin is a complex system yet to be fully understood, particularly in avian models where there is yet little information.

2. Materials and Methods

2.1. Housing of waxbills

Twelve male and 12 female common waxbills (*Estrilda astrild*) were obtained from certified breeders in September 2019, approximately 2 weeks before the start of the experiments (30th September) and housed as described below, to allow them to acclimatize to the aviary conditions. Birds were housed in six metal cages (88.5 x 30 x 40 cm) with 4 perches each and a gridded front (Figure 10). Each cage housed 2 males and 2 females. All waxbills were ringed for individual identification (with number and colour).

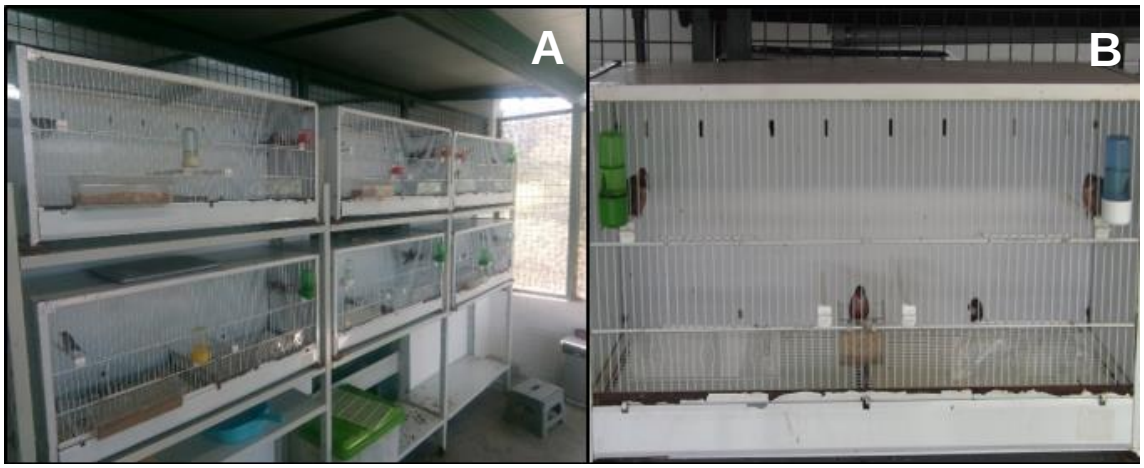

Fig. 10- Photographs from the **(A)** aviary and **(B)** from an individual cage during test.

Cages were in a sheltered outdoor aviary protected from wind and direct sunlight (Figure 10). Some of the aviary walls were made of grids to provide ventilation and temperature identical to the outside conditions. Natural light was complemented with artificial light in a cycle adjusted to the natural daily cycle. Birds were provided with *ad libitum* food in 2 long feeders (a commercial mix of seeds fitted for exotic birds, *Tropical Finches Prestige*, Versele-Laga) and water in 2 drinkers. Furthermore, fine sand with smashed oyster shells was placed at the bottom of the cages, to provide a calcium supplement. Bathtubs were made available twice a week.

The birds were regularly monitored to check their health welfare.

2.2. Experimental manipulation of the Serotonergic System

Experiments took place between the 30th September and the 13th November 2019. These occurred from Mondays until Wednesdays, in the morning period, between 9:30am and 12:45pm, approximately, as seen in Figure 11, to minimize the possible influence of daily cycle variations in stress or serotonergic levels.

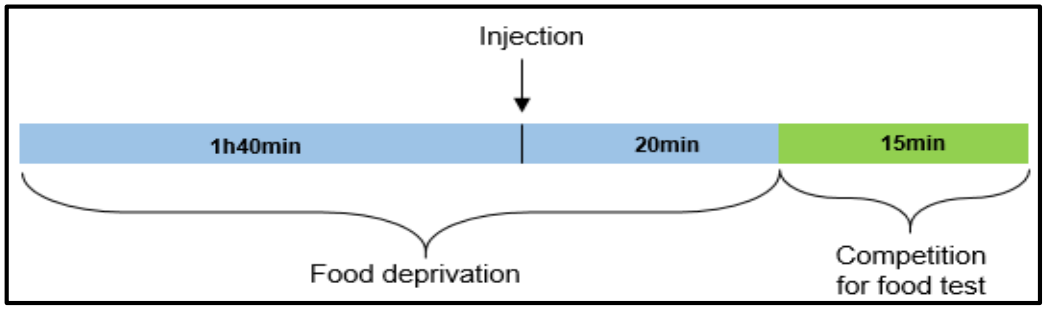

Fig. 11- Temporal scheme that was applied for each bird and respective treatment.

Before behavioural tests, individuals were subjected to one of four treatments, all resorting to intramuscular injection on the right side of the chest area, in the pectoral muscle. Each individual received all four treatments, in a balanced way, with a 7 days' interval between treatments. Individual A (male) and B (female) from the same cage received the same treatment, and the other pair of the same cage received control/placebo. After a week the individual A and B received one of three remaining treatments (see Attachments-Figure 13 for a scheme of the balanced order of treatments). This scheme was used for each cage. I tested two cages per day.

Each test started by putting a bird cage in food deprivation for 2 hours: after 1h40min of food deprivation, I removed each bird to inject the treatment accordingly to planning (Attachments-Figure 13), 2 with treatment and the other 2 with control. Following that, individuals were returned to their cage, still without food and waited another 20 minutes (making up the 2 hours) before starting the behavioural test of competition for food (see below).

The whole process of taking the birds from the cage, injection and placing them again in the cage was fast (approximately 10 minutes per cage) and the birds would return to their normal behaviour quickly (personal observation).

The following four treatments were applied: (±)-8-Hydroxy-2-(dipropylamino) tetralin hydrobromide (8-OH-DPAT, H8520 Sigma-Aldrich, Darmstadt, Germany), which is an agonist for 5-HT1A receptor; N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclo-hexanecarboxamide (WAY 100.635, W108 Sigma-Aldrich), which is an

antagonist of the 5-HT1A receptor; Fluoxetine (F132 Sigma-Aldrich), which is a selective serotonin reuptake inhibitor (SSRI) and a control treatment (saline solution, PBS).

Dosages for administering these treatments were based on the mean body weight of common waxbills. For this, the individuals were weighted and I used their mean body weight to calibrate dosages. The average body weight across all individuals was 8.66g, so approximately 9g. The volume of the injections was of 20µl for all treatments, administered with insulin syringes of 0.5ml, with the following concentrations.

Based in previous studies with fish and other passerine/birds (Sperry *et al*., 2003; Paula *et al.*, 2015) I used: 1mg kg^{-1} body mass of 8-OH-DPAT, 1.5mg kg^{-1} body mass of WAY 100.635 and 10mg kg^{-1} body mass of Fluoxetine. Since the average body weight of the waxbills was approximately 9gr, the dosages were: 0.009mg for the 8-OH-DPAT, 0.0135mg for the WAY 100.635 and 0.09mg for Fluoxetine, diluted in 20µl of saline solution.

2.3. The Behavioural Test: Competition for food

A test of competition for food was applied. Many different tests can be used to study aggressive behaviour, of which competing for food is one of the simplest and most used (i.e. Schleuter and Eckmann, 2006; Ha, 2010). Also, protocols for assessing social aggressiveness in common waxbills were already successfully used before (Funghi *et al*. 2015, 2018).

Following Funghi *et al.* (2015, 2018) I made food competition tests by placing a feeder attached to the front grid of the cage, centrally, equidistant between the middle perches and video recorded the behaviour of the birds for 15 minutes. With these video recordings, I later used The Observer XT 11 (Noldus Information Techonology b.v., Wageningen, the Netherlands) to quantify the five following behavioural variables:

1) *Total duration feeder*: the total time, in seconds, that each individual spent on all its visits to the feeder;

2) *Latency to feeder*: calculated as the amount of time, in seconds, that the individual took to go to the feeder for the first time;

3) *Movements/Locomotor activity*: the total number of changes that the individual performs between different positions in the cage. These positions were: each of the four different perches, feeder and the ground. Every movement performed to

the adjacent perch or another more distant, feeder or ground was accounted as 1 movement. Movements within the same area (near the perch that already was on) were not quantified;

4) *Allopreening*: calculated as the amount of time, in seconds, that an individual preens or grooms another, being this a social behaviour;

5) *Aggressive Displays/Attacks*: the total number of times that an individual threatened another by opening the beak and stretching the neck, spreading the wings, tail upwards, basically considering the posture of the individual or whenever an individual caused the displacement of another, attacking physically, pecking, chasing/flying towards another individual (sometimes consecutively).

Behavioural quantification of the videos was always performed by the same observer, blindly to the experimental treatment (for this, the identification of the videos was previously coded).

2.4. Statistical Analysis

Inspection of histograms showed positively skewed distributions for *Allopreening*, *Latency to feeder* and *Aggressive Displays/Attacks*. I transformed each of them to approach normality by using a log(x+1) transformation, since there where zero values in the data. The *Total duration feeder* and *Movements/Locomotor activity* variables showed an approximately normal distribution.

To test if the control treatment differed from any of the serotonergic treatments, I performed Generalized Linear Mixed Models (GLMMs) for each one of the behavioural variables (*Total duration feeder*, *Latency to feeder*, *Movements/Locomotor activity*, *Allopreening* and *Aggressive Displays/Attacks*) with the *lmer()* function in the R package "lme4" (v.1.1-23; https://cran.r-project.org/web/packages/lme4/lme4.pdf).

In each GLMM, a behavioural trait was the dependent variable, treatment (Control, 8-OH-DPAT, Fluoxetine and WAY 100.635) was a fixed factor and individual identity was a random factor, to test for differences associated to each treatment on behaviour within individuals (repeated measures). I report the GLMM contrasts (i.e. the simple coefficients, without having run an ANOVA on the GLMM), which test for differences between one level of the treatment (the control treatment) and each of the remaining levels (8-OH-DPAT, Fluoxetine or WAY 100.635). The sex didn't have a significant interaction with treatment neither were there really significant differences between sexes thus deemed to be analysed together.

I examined residual plots and predicted distributions of residuals and response from these models resorting to *check_distribution()* and *check_model()* functions in the R package (version 4.0.0; http://www.R-project.org/), and in every case residuals were approximately normally distributed.

3. Results

a) Reaction to the feeder

When treated with the selective serotonin reuptake inhibitor (SSRI), Fluoxetine, individuals spent significantly less time at the feeder (*Total duration feeder*: t= -2.540; p= 0.0122, Table 3, Figure 12A) and took longer to go to the feeder for the first time (*Latency to feeder*: t= 3.188; p= 0.00177, Table 3, Figure 12B), comparing to the control treatment.

No other significant results were found with the remaining treatments (see Table 3).

b) Activity

The selective agonist 8-OH-DPAT was found to have a positive significant effect on activity, increasing activity inside the cage relatively to control (*Movements/Activity*: t= 2.568; p= 0.0113, Table 3, Figure 12C). Contrarily, the Fluoxetine treatment decreased their locomotor activity (*Movements/Activity*: t= -4.550; p= $1.15e^{-05}$, Table 3, Figure 12C).

c) Aggressive behaviour

Compared to control, Fluoxetine significantly decreased waxbills aggressive behaviour compared to the control (*Aggressiveness*: t= -2.918; p= 0.0041, Table 3, Figure 12D). Aggressive behaviour also showed a difference between sexes (t= 2.029; p= 0.0547, Table 3, Figure 12D).

d) Allopreening

Lastly, individuals performed less allopreening when treated with 8-OH-DPAT in comparison to the control (*Allopreening*: t= -2.070; p= 0.0403), as seen in Table 3 and Figure 12E.

Table 3 - GLMM for the different behaviours analysed in the Competition for food test. Positive t-values means an increase relatively to control; negative values indicate a decrease. Significant values in bold. (n=168)

GLMMs Results				
	β	**STD. ERROR**	***t-value***	***p***
Total duration feeder				
Intercept	142.53	21.45	6.645	$4.67e^{-07}$
8-OH-DPAT	23.48	21.05	1.115	0.2666
Fluoxetine	-53.47	21.05	-2.540	**0.0122**
WAY 100.635	-23.88	21.05	-1.135	0.2584
sex	22.78	29.05	0.784	0.4414
Latency to feeder				
Intercept	3.8252	0.4021	9.512	$8.31e^{-10}$
8-OH-DPAT	-0.1338	0.3490	-0.383	0.70199
Fluoxetine	1.1125	0.3490	3.188	**0.00177**
WAY 100.635	0.6400	0.3490	1.834	0.06878
sex	-0.9667	0.5500	-1.757	0.09274
Movements/Activity				
Intercept	47.612	7.386	6.446	$8.19e^{-07}$
8-OH-DPAT	17.948	6.990	2.568	**0.0113**
Fluoxetine	-31.802	6.990	-4.550	**$1.15e^{-05}$**
WAY 100.635	-6.594	6.990	-0.943	0.3471
sex	9.798	10.037	0.976	0.3396
Aggressiveness				
Intercept	1.4615	0.2447	5.972	$2.32e^{-06}$
8-OH-DPAT	0.4496	0.2592	1.735	0.0849
Fluoxetine	-0.7562	0.2592	-2.918	**0.0041**
WAY 100.635	-0.1870	0.2592	-0.722	0.4718
sex	0.6678	0.3291	2.029	0.0547
Allopreening				
Intercept	3.03102	0.39448	7.684	$2.81e^{-08}$
8-OH-DPAT	-0.88324	0.42672	-2.070	**0.0403**
Fluoxetine	0.05294	0.42672	0.124	0.9014
WAY 100.635	-0.64039	0.42672	-1.501	0.1357
sex	0.02590	0.52917	0.049	0.9614

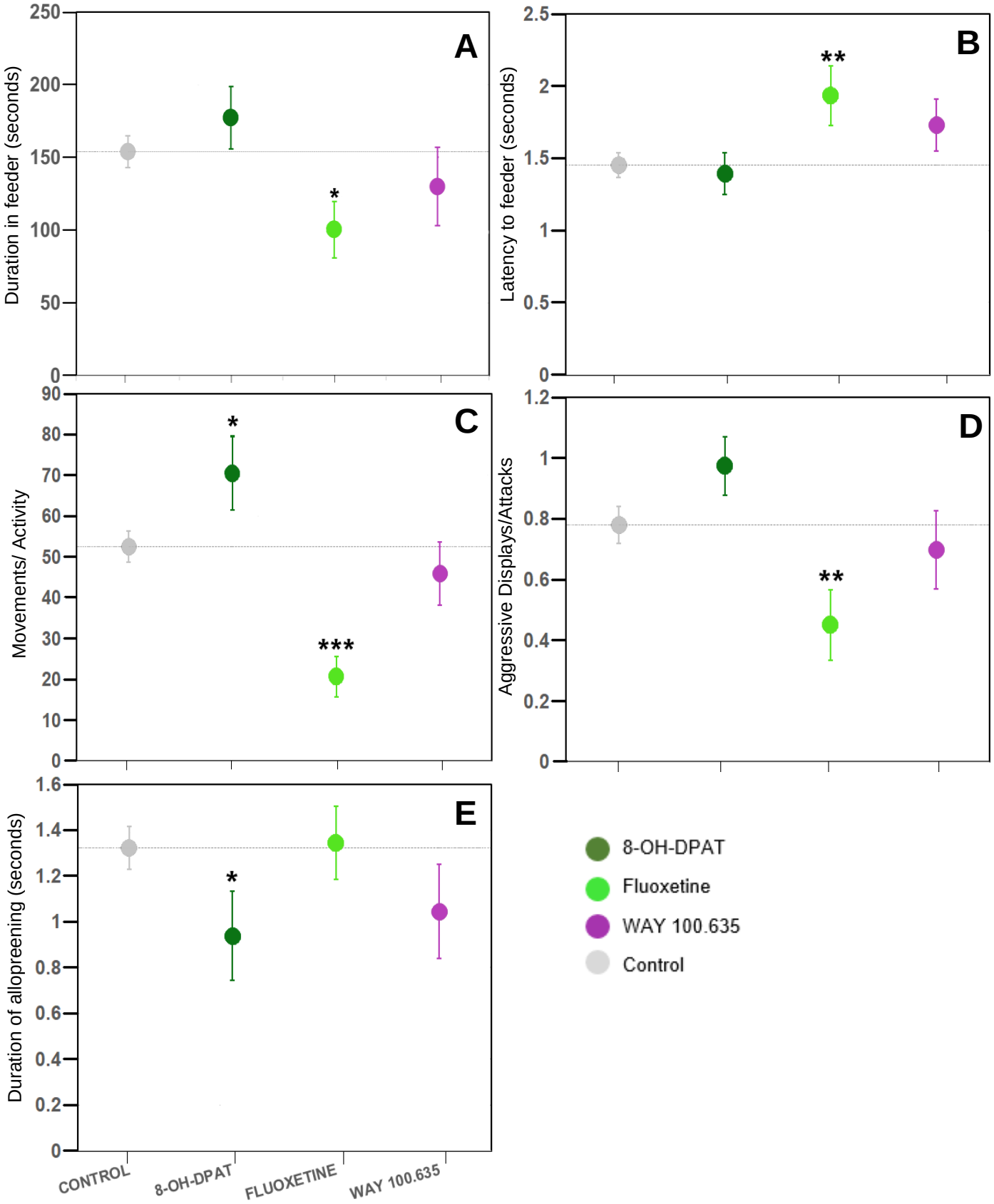

Fig. 12 - Effects of the compounds tested comparatively to control. **(A)** Duration spent at the feeder (in seconds); **(B)** Latency to feeder (in seconds); **(C)** Movements/Activity; **(D)** Aggressive Displays/Attacks; **(E)** Duration of allopreening (in seconds). Mean standard error is represented in each. Significance of contrast with the control treatment:

* p≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

4. Discussion

In the present study, the neuromodulatory role of serotonin was tested on waxbill behaviour by using an assay of competition for food. To understand the effects of serotonin system mediation in a non-sexual social context, I observed if acute shifts in 5-HT influenced feeding, activity levels and aggression in mixed-sex groups, using a highly gregarious bird with mild social hierarchies, the common waxbill (*Estrilda astrild*). I found that acute Fluoxetine (a selective serotonin reuptake inhibitor, SSRI) resulted in an overall decrease of waxbill activity levels, food response and time spent feeding and aggressive behaviour. On the other hand, the 1A agonist 8-OH-DPAT was shown to increase waxbills' activity levels while decreasing allopreening and treatment with the antagonist WAY 100.635 did not have discernible effects on behaviour.

Below I will discuss the results in accordance with the main behavioural variables analysed.

4.1. Serotonin modulation of feeding

Waxbills treated with Fluoxetine (SSRI) took longer to respond to food and spend less time at the feeder. No other treatment (8-OH-DPAT and WAY 100.635) was significantly observed to change individual's behaviour. However, visual analysis of the duration (total) spent feeding, shows the opposite action of the 8-OH-DPAT (increase in feeding time) compared to both Fluoxetine and WAY (both seemingly in the same direction). Thus, while not changing the response to the feeder, animals treated with 1A receptor agonist showed a non-significant tendency to feed more, compared with control and most interestingly, in respect to the SSRI inhibition of feeding.

Previous studies regarding the effects of 8-OH-DPAT on feeding are quite variable, depending on model species and contexts. For instance, in a study by Saadoun and Cabrera (2002), injection of 5-HT1A agonist, 8-OH-DPAT, 15 min just before refeeding resulted in food intake inhibition in overnight food-deprived chickens however, when injected 60 min after the beginning of refeeding it enhanced significantly the food intake. Those results also confirmed previous studies (Ebenezer *et al.,* 1999; De Vry and Schreiber, 2000) in which 5-HT agonists inhibited food intake in food-deprived individuals, but increased feeding in fed individuals. Where in starved pigs, the agonist injected intravenously 15 min before de feeding period, blocked the effects of food intake,

only in the first 5 min (Ebenezer *et al*., 1999). In addition, intracerebroventricular administration of the agonist 8-OH-DPAT may induce more water intake but no food intake, in pigeons starved for 24h prior to the experiment (Steffens *et al.*, 1997; Saadoun and Cabrera, 2002).

In the present study, waxbills were food deprived 2 hours prior to the behavioural tests, with 1A agonist treatment resulting in a non-significant tendency to enhance feeding motivation compared to a significant decrease of the SSRI. Regarding Fluoxetine, results of previous studies are more coherent and in agreement with my present findings, with feeding rates decreasing, in multiple fish species (Gaworecki and Klaine, 2008; Mennigen *et al.,* 2010; Dzieweczynski *et al.*, 2016). Of course, these are not without exception, with the case of the cleanerfish *L. dimidiatus*, which increased feeding (cleaning) behaviour following treatment with both Fluoxetine and 8-OH-DPAT and seemed less motivated to feed under effect of the antagonist WAY 100.635 (Paula *et al.,* 2015). But for instance, a study with pigeons showed that WAY 100.635, when injected alone, had no effect on feeding behaviour (Santos *et al.*, 2009). The underlying reasons for such disparities (from previous studies and my current evidence) may be due to: i) different sensitivity of the 5-HT1 receptor in relation to physiological condition: hunger or satiety (it is also possible an interaction with other peptides ; Ebenezer, 1992; Saadoun and Cabrera, 2002); ii) site of injection which in this case was in the periphery, and condition the sizes of action by the agonist (may reach the receptors with different anatomical localizations, this way producing different responses) or iii) 8-OH-DPAT was developed for mammals, thus the differences between mammals and birds might underline possible differences in receptor mechanisms (receptor subtypes may work differently than the ones found in mammals; Saadoun and Cabrera, 2002).

4.2. Serotonin modulation of waxbill's activity levels

Waxbills activity levels were also inhibited following the Fluoxetine treatment. Similar effects have been demonstrated in fish, in which short-term exposure to Fluoxetine suppressed activity (Beulig and Fowler, 2008; Barry, 2013; Kohlert *et al.,* 2012; Dzieweczynski *et al.*, 2016). In zebrafish, individuals exposed to higher doses were less active and moved less than those exposed to a lower dose (Dagh, 2013; Dzieweczynski *et al.*, 2016). With waxbills, the decrease in activity could be due to the specificity of dosage or even a more complex mechanism of anxiety/fear response (Sánchez and Meier, 1997; Bagdy *et al.*, 2001; Maximino *et al.*, 2013; Theodoridi *et al.*, 2017).

However, contrarily to Fluoxetine's apparent anxiogenic effects, the exogenous transmission increase via 1A receptors revealed to have opposite and potentially anxiolytic effects, with waxbills raising their activity levels following treatment with 8-OH-DPAT, relatively to the control. Again, no results were observed for the WAY 100.635 treatment. Previous research with sparrows, revealed an absence of effects on motor activity via acute doses of 8-OH-DPAT and Fluoxetine. But for instance, Hillegaart and Hjorth (1989) and Harrison and Markou (2001), observed that 8-OH-DPAT injections into the median raphe nucleus, stimulated locomotor behaviour, implicating the site of injection and the type of 1A receptors activated (Evenden and Ängeby-Möller, 1990). But because activity may also relate to mood and motivation, and to behaviours related to depression, studies also show that sub-chronic administration of 5-HT1A receptor agonist, 8-OH-DPAT, may also reduce depressive behaviours while chronic administration inhibits depressive behaviour in novelty-suppressed feeding test (Santarelli *et al*., 2003; Polter and Li, 2010). Evenden and Ängeby-Möller (1990) demonstrates that this agonist, can affect locomotor activity in active individuals (mice and rats) immediately after injection but also long term, with animals changing from being hypoactive to hyperactive (elevated locomotor activity). The result seen right after injection was presumably mediated by the postsynaptic 1A receptor whilst the increased activity observed at lower doses might be mediated by presynaptic receptors.

4.3. Serotonin modulation of waxbills' aggressive behaviour

The serotonin system is considered a main mediator of aggressiveness in animals, including in birds (Sperry *et al.*, 2003; Dennis *et al.*, 2008). In waxbills, aggressive behaviour was also significantly reduced following treatment with Fluoxetine, which could be due to the overall anxiogenic effects that also reduced feeding and activity levels. Indeed, Fluoxetine treatment has revealed to reduce aggressive-like behaviour in many species (Sperry *et al.*, 2003; Hu, 2017), exhibiting a biphasic effect on agonistic behaviour influenced by dosage, type of aggression and test context (Hu, 2017). Fluoxetine acts as an inhibitor of neuronal serotonin reuptake thus enhancing 5-HT extracellular levels (at the synapse) additionally affecting both receptors identified to mediate aggression in birds, 5-HT1A and 5-HT1B (Sperry *et al.*, 2003; Dennis *et al.*, 2008; Hu, 2017). 5-HT1A in pigeons, appears to have similar binding affinity with the mammalian receptor (Challet *et al.*, 1996; Sperry *et al.*, 2003) while the 1B subtype has a binding profile similar to human rather than rats and mice (Waeber *et al.,* 1989; Sperry *et al.,* 2003). On the other hand, on chickens, treatment with para-chlorophenylalanine (PCPA), the SSRI antagonist, has shown that aggressive pecking increased over time (3-day test period), effects that seemingly diminished as brain 5-HT levels recovered over a 1-week period (see Buchanan *et al.,* 1994). Here I did not measure the timely effects of the SSRI injection over waxbill response, nor the influence of PCPA. But I could speculate that the effects on aggression levels of waxbills would be less pronounced according to time and that PCPA may also contribute to increase aggression levels. Future studies should focus on the putative opposite effects of SSRIs and PCPA on waxbill behavioural response.

The effects of 8-OH-DPAT on waxbill aggressive displays and attacks fell short on statistical significance. Visual inspection of Figure 12D, however, shows a trend (0.0849, see Table 3) for increased aggression following treatment with the 1A receptor agonist compared to control and contrarily to the decrease of effects via SSRI and WAY 100.635. Previous studies on other avian model systems revealed some variability. For instance, in chickens, the increased aggression observed coincide with the rise in 5-HT through 5-HT1A receptor pathways, contrarily to the inhibitory effect that would be expected with high concentrations in aggressive behaviour thus suggesting the existence of additional mechanisms underlying those changes (Dennis *et al*., 2008). On the other hand, de Boer and Koolhaas (2005) found that 5-HT1A receptor agonist inhibited aggressiveness by reducing 5-HT neurotransmission, defying the hypothesis were enhanced 5-HT levels decreases aggressive behaviour. In another study (Sperry *et al.*, 2003), 8-OH-DPAT with its high specificity for the 5-HT1A binding sites, reduced

aggressive behaviour in general, but some birds demonstrated a low agonistic response (with low-level of aggressiveness in non-aggressive individuals), suggesting that as a result of the potential anxiolytic effects of the agonist. These anxiolytic effects have also been shown in pigeons (Barrett *et al.,* 1994) as well as in rats (File *et al.,* 1996). These results could be dependent upon the activation of the type of 5-HT1A receptor, demonstrating to be the primary mediators of aggressive behaviour, therefore explaining waxbills response, even though not being significant.

The effects of the antagonist WAY 100.635, were again non-significant in respect to waxbill's aggressive behaviour, but were contrary to the effects of the 8-OH-DPAT (see Figure 12). In the study by Paula *et al.* (2015), WAY 100.635 caused treated individuals to be more aggressive towards conspecifics although in other studies this antagonist had no significant effect on aggression (Clotfelter *et al.*, 2007). Several rodent studies report that WAY 100.635 only affects aggressive behaviour when administered together with a serotonin agonist or a reuptake inhibitor (Bell *et al.,* 1999; Lopez-Mendoza *et al*., 1998; Sanchez, 1997; Clotfelter *et al.*, 2007). Despite that, Clotfelter *et al.* (2007), did not see any changes when WAY 100.635 was injected followed by 8-OH-DPAT which could be due to dosage.

4.4. Serotonin modulation of allopreening

Allopreening was significantly inhibited in waxbills treated by 8-OH-DPAT. The increase of their activity levels with increment in transmission via 1A receptor activation, together with a tendency (non-significant) to increase feeding (see Figure 12) and aggressive behaviour (see Figure 12D), may also lead to a decrease in social interest. To perform allopreening, individuals must not be moving and, since 8-OH-DPAT has the effect of increasing activity, as a collateral effect it may have reduced the allopreening behaviour. In other model animals, such as sparrows, 8-OH-DPAT decreased aggressive behaviour and led to a marked reduction in social interest and activity (Sperry *et al.*, 2003). This may be due to dosage, receptors being activated and/or even serotonin influence on other systems.

Interestingly, Fluoxetine did not decrease allopreening in waxbills. For instance, previous studies in rats (Bagdy *et al.*, 2001), have shown that, the acute administration of Fluoxetine caused a significant decrease of social interactions but otherwise contributed to a rise in time of self-grooming. Contrarily, in vervet monkeys, males treated

with Fluoxetine achieved dominance by increasing affiliative interactions (including grooming) with females in the group thus data being consistent with the hypothesis that increasing serotonergic activity facilitates affiliative interactions in males (Raleigh *et al.*, 1983).

4.5. The overall anxiogenic effects of Fluoxetine on waxbill behavioural responses

When serotonin levels were pharmacologically manipulated by blocking reuptake, waxbills behavioural response revealed to be overall anxiogenic (i.e. decreasing the overall activity such as feeding and response to the feeder, movements inside cage and aggressive displays/attacks only, continuing with allopreening similarly to control).

SSRIs are commonly known for treating a wide spectrum of anxiety disorders such as panic, social phobia, post-traumatic stress disorder as well as depression (e.g., Sánchez and Meier, 1997; Grillon *et al.*, 2007; Dzieweczynski and Hebert, 2012; Bocchio *et al.*, 2016; Dzieweczynski *et al.*, 2016) and Fluoxetine is one of the most widely used (and known). The known action of SSRIs involve blocking serotonin reuptake by inhibiting the serotonin transporter, causing an increase in serotonin availability (Burghardt and Bauer, 2013). Similarly, to the observed waxbill results, many other animal focused studies, using a variety of anxiety tests have reported anxiogenic-like effect by these SSRIs afterward acute treatment (Griebel *et al.*, 1994; Sánchez and Meier, 1997; Maximino *et al.*, 2013). However, there is a discrepancy between studies in which 5-HT reuptake inhibitors show anxiogenic-like behaviours, which is probably due to the different animal models representing qualitatively different types of “anxiety” or “fear”, where only some are potentiated by these compounds (Griebel *et al.*, 1994; Sánchez and Meier, 1997; Grillon *et al.*, 2007). In animals, acute serotonin can either increase (Griebel *et al.*, 1994; Bagdy *et al.*, 2001) or decrease (Inoue *et al.*, 1996, 2004; Sanchez and Meier, 1997) anxiety-like responses (Grillon *et al.*, 2007). These observations would be consistent with the hypothesis that serotonin affects multiple brain structures that mediate anxiety via multiple pathways and receptors (Graeff *et al.*, 1997; Grillon *et al.*, 2007).

Because SSRIs facilitate the acquisition of conditional fear responses (Burghardt *et al.*, 2004, 2007), evidence refers to the involvement of the amygdala, a brain region,

that is implicated in the acquisition and expression of fear conditioning thus in anxiety disorders (Burghardt *et al.*, 2007). The amygdala (LA) serves a major input of information from the cortex and thalamus (Ravinder *et al.*, 2013; Bocchio *et al.*, 2016) being posteriorly processed within the LA and basal nucleus finally relayed to surrounding/output structures (Ravinder *et al.*, 2013). A single systemic SSRI injection leads to an increase in amygdala extracellular serotonin (Bosker *et al.,* 2001; Ravinder *et al.*, 2013) which, complemented with the studies by Burghardt *et al.* (2004, 2007) indicate that this region may be an important site of action for the anxiogenic effects of acute SSRI treatment.

On the contrary, when administered chronically (long term), antidepressants drugs like Fluoxetine may acquire anxiolytic properties, which occurred with food-deprived rats involving novelty-supressed feeding (Griebel *et al.*, 1994; Santarelli *et al.*, 2003; Polter and Li, 2010).

Another potential reason for the generalized decrease of behavioural activity in waxbills may be related to the acute activation of inhibitory 5-HT1A autoreceptors in the raphe nuclei, which may lead to a reduction in the firing rate of the serotonergic raphe neurons (Piñeyro and Blier, 1999; Grillon *et al.*, 2007; Homberg, 2012). Again, after repeated exposure to SSRIs (i.e. when taken chronically), these type of receptors desensitize, leading to a disinhibition of the firing of raphe neurons (Homberg, 2012); although there's also the idea that the delayed therapeutic responses of these compounds are due to neuroplastic changes (Krishnan and Nestler, 2008; Homberg, 2012). The action of 5-HT1A autoreceptors in the anxiogenic-like effects arising from acute increased availability of serotonin is also confirmed by Grillon *et al*.'s study (2007). This activation leads to a reduced firing of ascending serotonin raphe neurons (Grillon *et al*., 2007). Moreover, results from Bagdy *et al.* (2001) suggest that anxiogenic-like responses of the SSRIs could possibly be attributable to the activation of 5-HT2C receptors (since Fluoxetine shows a modest affinity for these; Bagdy *et al.*, 2001), in the amygdala and adaptive changes of the 5-HT receptors (Westenberg and den Boer 1988; (Griebel *et al.*, 1994; Grillon *et al*., 2007) where the enhancement of 5-HT availability, after a single injection of SSRIs, would lead to a stimulation of the central 5-HT system, increasing anxiety levels.

While waxbills may be facing increased levels of anxiety, and an overall decrease in behavioural response, in the treatment with the SSRI Fluoxetine, they were not experiencing total "freezing behaviour". Freezing behaviour is a state of complete immobility, termed as conditioned fear stress(CFS)-induced freezing behaviour, that can

be used as model of anxiety (Hashimoto *et al*., 1996). In a review by Crawford and Masterson (1982), freezing behaviour is thought to be a species-specific defensive reaction in rats. In the present study, although waxbills decreased their motor activity, they didn't demonstrate this freezing-like behaviour since they would still perform allopreening at a similar level to the control. Homberg (2012) also found that freezing behaviour did not seem to change compared to control groups after acute SSRIs.

4.6. The seemingly opposite effects of serotonin facilitators in waxbill's behavioural output

In waxbills, the serotonergic system is mediating plasticity as a possible adaptive response to tolerable (mild) stress contexts. For instance, waxbills demonstrated to have an increase in aggressiveness and a significant decrease, with 8-OH-DPAT and Fluoxetine, respectively, the two enhancers of 5-HT availability, with 8-OH appearing to have an anxiolytic effect. The results could indicate the possible type of 5-HT1A receptor being activated (pre or postsynaptic). In rats (File *et al*., 1996), 8-OH-DPAT can either act as an anxiolytic (acting on presynaptic) or an axiogenic factor (acting on postsynaptic) thus results being ligand-specific, generally. 5-HT1A autoreceptors (presynaptic) have an inhibitory action of the firing rate of serotonergic neurons (Homberg, 2012; Celada *et al*., 2013) whereas 5-HT1A heteroreceptors have an enhancing action.

Despite that, heteroreceptors also appear to play an important role in the regulation of anxiety and fear (Gross *et al.,* 2002), where in mice, restring 5-HT1A receptor function to the forebrain of the subjects, rescues anxiety-like behaviours (Polter and Li, 2010). Fluoxetine has a more general action of decreasing activity levels which, in turn, can influence not specifically the aggressive behaviour, since Fluoxetine can act on other receptors besides 5-HT1A receptor, complementing its action. For the role of Fluoxetine in waxbills, the present results seem in agreement with several studies and could indicate the different pathways through which these act but action/mechanism could be better interpreted if resorting also to a Fluoxetine antagonist, the PCPA. The chronic exposure to SSRIs can desensitize 5-HT1A autoreceptors thus these having an anxiogenic effect but these anxiogenic-like effects of Fluoxetine could be due to the activation of 5-HT2C receptors pathways (Bagdy *et al*., 2001), changes of the serotonergic receptors, as mentioned above.

Anxiety and stress are inducers of 5-HT release (Bland *et al*., 2003; Fujino *et al*., 2002, Rex *et al*., 2005) most essentially appearing when survival is threatened (Carhart-Harris and Nutt, 2017). The ability to tolerate stress has been proposed to be mediated by the enhanced postsynaptic 5-HT1A receptor signalling (Carhart-Harris and Nutt, 2017). This mediation could possibly be an adaptive response to mild levels of adversity since 5-HT may mediate passive coping/moderation of stress under adverse conditions which could be advantageous but also counterproductive (Puig *et. al*., 2005; Carhart-Harris and Nutt, 2017). Despite that, the plasticity and capacity for change, mediated by the 5-HT2A receptors (especially when the adversity reaches a critical point, such as life-threatening experiences), may confer significant evolutionary advantages as seen in humans (Stini, 1975; Strassman, 2000; Amargós-Bosch *et al.,* 2004; Anton *et al.*, 2014; Carhart-Harris and Nutt, 2017).

4.7. Serotonin putative influences on other neuroendocrine systems

One possible explanation suggested for the altered serotonin activity in allopreening, observed in waxbills, might occur: i) via the interaction with the dopamine system and/or ii) via regulation of the other serotonin receptor subtypes such as 2A, 6 and 7 (Paula *et al.*, 2015). Serotonin and other monoamines can influence behaviour by interacting with various neuropeptide neurotransmitters (Insel and Winslow, 1998). Evidence supports the existence of the interaction between the serotonergic and dopaminergic neuronal systems in the central nervous system (Sasaki-Adams and Kelley, 2001; Daw *et al*., 2002; de Haas and van der Eijk, 2018). 8-OH-DPAT has demonstrated to affect the dopamine neuronal activity, facilitating dopamine transmission and promote motivational arousal, which could be through the 5-HT1A receptor pathways located mainly in the limbic areas (Montgomery *et al*., 1991). These two systems are motivational opponents, also having different preparatory behavioural effects. They are thought to play an important role in the regulation of emotion and mood, for example, stimulating food intake by facilitating dopamine transmission or even having an antidepressant action (Montgomery *et al*., 1991; Sasaki-Adams and Kelley, 2001). Comparing with serotonin, dopamine is anatomically more widespread and behaviourally more diverse (Daw *et al.*, 2002). Although several early studies demonstrated an inhibitory role of serotonin upon the dopaminergic activity, more recent evidence suggests the contrary, but that seems to depend on doses and the serotonin receptors that are activated (Sasaki-Adams and Kelley, 2001). For example, in Arborelius *et al*.

(1993), low doses of 5-HT1A agonists seem to enhance dopamine transmission within the limbic system.

Despite that, the serotonergic system also plays an important role in the mediation of cortisol levels (Winberg *et al*., 1997; Höglund *et al.,* 2002) however, the response may depend on the state of the individual and the dosage used (Paula *et al*., 2015). 8-OH-DPAT, has also proved to influence cortisol levels (Winberg *et al*., 1997; Höglund *et al*., 2002; Paula *et al*., 2015), where increasing cortisol levels has effects on motivation to engage interactions between individuals (Soares *et al*., 2014; Paula *et al*., 2015) still results may depend on dosage (Soares *et al.,* 2016). In waxbills, the serotonergic system could be interacting with these two systems, acting the primary hypothesis where serotonergic and dopaminergic systems have opposite behavioural effects or mediating cortisol levels (Soares *et al*., 2017) but without further studies no affirmations can be done.

Serotonin is also one of the known monoamines with actions in vasopressin and oxytocin secretion from the neurohypophysis and that is also involved in the regulation of their expressions in the hypothalamus (Vacher *et al.*, 2002; Jørgensen *et al.*, 2003). Serotonin regulates vasopressin and oxytocin release due to its nerve fibres and receptors located in the supraoptic nucleus and the paraventricular nucleus (Jørgensen *et al.*, 2003). It may influence the vasopressin system, for example in hamsters, by suppressing its activity through the administration of Fluoxetine, SSRI, it can diminish aggression, leaving social interactions like grooming intact (Ferris, 1996; Jørgensen *et al.*, 2003). In the case of waxbills, the actions of SSRI acute treatment may have primed the vasotocin (homolog of vasopressin in birds) system, contributing to a rapid increase of stress/anxiety. But at this point, it is impossible to confirm without further studies.

5. Concluding Remarks

In general, the Fluoxetine treatment had a consistent significant effect of decreasing activity, including reducing movement, aggression and feeding. Thus, Fluoxetine may not have had a specific effect on aggression, but rather a general effect in decreasing all the activity. 8-OH-DPAT, the agonist, appears to have an opposite action to the SSRI, Fluoxetine, causing waxbills to be more active and (tendentially) more aggressive, but not more social (allopreening). WAY 100.635, the antagonist, was not found here to significantly affect behaviour.

The serotonergic mechanisms may act as an adaptive regulator of behaviour, namely of anxiety-related behaviour, which is an adaptive natural emotion beneficial for group-living organisms. Among the wide range of anxiety-like behaviours, the expression of fear can inform members of possible dangers or resources through ways of communications created by the group (Koenig and Dickinson, 2018) thus coping with the environment and be considered useful to possible survival strategies (Gutiérrez-García and Contreras, 2013). This coping with a stressful situation may cause an individual to simultaneously emit vocalizations, movements to escape, freeze or release “alarm pheromones” (Gutiérrez-García and Contreras, 2013).

Fluoxetine has been demonstrated to impact foraging (a behaviour present and necessary for this species) and activity also having an important role in the mediation of “defensive approach” (scototaxis-risk assessment, being anxiogenic for low doses) versus “defensive avoidance” (geotaxis, being anxiolytic; Maximino *et al.*, 2013) thus influencing fitness (Dzieweczynski *et al*., 2016). These changes in response and/or relationship across contexts may cause individual and population consequences if the behaviours that are being affected are crucial to survival (Dzieweczynski *et al.*, 2016). For instance, by affecting boldness, individuals fail to forage/explore, avoid predators or attract mates thus decreasing fitness (Arnold *et al*., 2014; Dzieweczynski *et al.*, 2016).

Overall, the significant results for Fluoxetine appear to suggest more of an anxiety/fear-like mechanism where the 5-HT2 receptors may have a prominent role whereas the action of 8-OH-DPAT seem to relate to 5-HT1A receptor group being activated. Fluoxetine and 8-OH-DPAT noticeable differences suggest that these act on waxbill behaviour through different pathways. Considering that waxbills are highly social, normally not aggressive and with less pronounced dominance statutory differences between conspecifics, these may have lower expression levels of serotonin, comparing for instance with other more aggressive avian species. These putative lower baseline

levels of serotonin could explain the absence of results by the antagonist WAY 100.635. Moreover, the results seen for the antagonist could also be dose dependent. In this study and in contrast to previous research (for example, Maximino *et al.* (2013), that used lower doses of WAY 100.635), our high dosage could be a potential reason for the absence of significant effects. In this case, because WAY 100.635 has a lower specificity/intrinsic activity regarding the 1A receptor, a lower dosage could amount to a clearer response. Thus, future studies should aim on potential effects of different dosages and distinct time action frames. Moreover, it is important to consider the possible influence of other receptors subtypes or groups/families. Although 5-HT1A receptor subtype is one of the most studied, most frequent and widely distributed, having crucial influence on behaviour, it is known that the 5-HT1B receptor subtype (for example) may also share a role in aggressive behaviours in avian models. Another interesting group, the 5-HT2 receptor, could also be relevant, but remains mostly understudied. Testing an antagonist of Fluoxetine, additionally, such as the para-chlorophenylalanine (PCPA), a tryptophan hydroxylase inhibitor, would most probably reveal opposite results to those of Fluoxetine as it did for other studies such as Buchanan *et al*. (1994), Lorenzi *et al.* (2009) or Maximino *et al.* (2013). The measurement of waxbills stress levels, invasively (blood) or non-invasively (faecal matter) would be crucial to confirm serotonin influence on waxbill anxiety response.

6. References

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7. Attachments

Table 4 - Some of the major hormones acting on social behaviour and respective source as well as primary biological action, in vertebrates. Adapted from Soares et al. (2010) and Nelson (2009).

Hormone	**Source**	**Major biological action**
Testosterone	Testis	Development of male reproductive tissues as well as the promotion of male secondary characters; Spermatogenesis.
Oestradiol	Ovaries	Regulation of oestrous and menstrual cycle; Uterine and other female tissue development.
Progesterone	Ovaries	Uterine and mammary gland development, pregnancy sustenance.
Cortisol	Adrenal glans	Increases carbohydrate metabolism; anti-stress hormone.
Corticosterone	Inter-renal glands	Increases carbohydrate metabolism; anti-stress hormone.
Arginine-vasopressin (AVP)	Hypothalamus	Increases water reabsorption in kidney. Raises arterial blood pressure.
Arginine-vasotocin (AVT)	Hypothalamus and pineal gland	Regulates reproductive organs.
Oxytocin(OT), mesotocin, isotocin (IT)	Hypothalamus	Stimulates milk letdown and uterine contractions during birth
Serotonin (5-HT)	CNS	Stimulates release of GH, TSH, ACTH, inhibits release of and LH

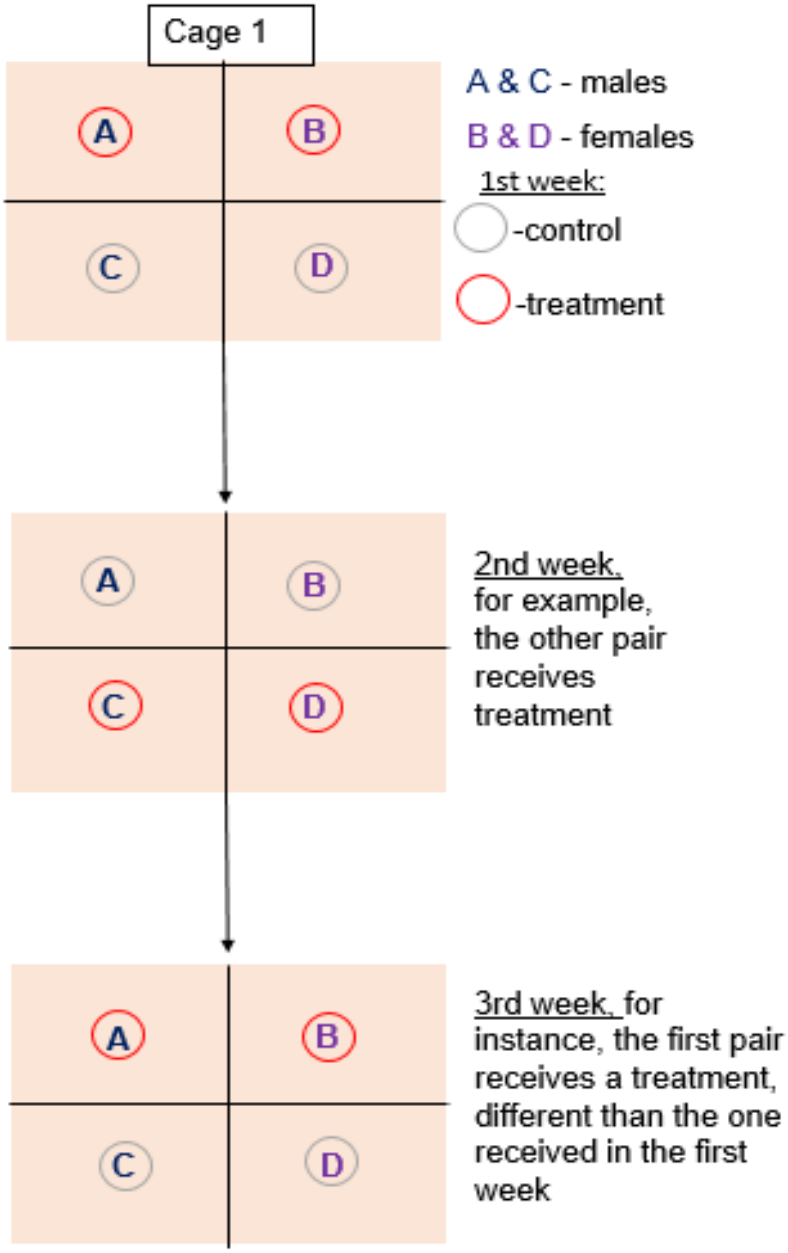

Fig. 13 - Scheme of the distribution of treatments per cage. Pair1 (A&B), in the first week, receive one of the treatments and Pair2 (C&D) a placebo. The next week, for example, the Pair2 will now receive one of the treatments and the Pair1 will then receive placebo. In the third week, for instance the Pair1 will again receive treatment but not the same one from the first week and Pair2 placebo. This will happen until every individual receives all serotonergic treatments (Fluoxetine, WAY 100.635 and 8-OH-DPAT) once.