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The role of serotonin 5-HT$_{2C}$ receptors in anxiety and social status in the zebrafish *Danio rerio*

Nuno Félix Paiva Alves

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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
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Mestrado em Biodiversidade, Genética e Evolução

Departamento de Biologia

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Resumo

Ansiedade é um aspeto fulcral de senciência animal e que afeta em grande medida comportamento animal. Ao longo dos anos, o estudo de como stress e ansiedade podem influenciar animais de diferences espécies, especialmente os que vivem em condições laboratoriais, tem sido dado grande foco, visto que os animais são expostos a vários tipos de stress ao longo das suas vidas que podem causar mudanças comportamentais e fisiológicas. Um dos tipos mais prevalentes de stress é o stress social, que resulta das interações com outros indivíduos, especialmente conspecíficos, sendo os seus impactos maiores e mais relevantes em espécies que vivem em grupo. O estabelecimento de hierarquias e a manutenção de diferentes estatutos sociais são uma fonte constante de stress para todos os indivíduos que vivem em grupo, especialmente subordinados, já que estes são mais defensivos e tímidos do que indivíduos dominantes e exibem mais comportamentos tipo-ansiosos. Diferentes paradigmas têm sido usados para estudar stress social em várias espécies modelo diferentes, sendo o foco maior em roedores e primatas não-humanos, e muito menor em peixes teleósteos.

O peixe-zebra (*Danio rerio*) é uma espécie que vive em grupo, nativo do Sudeste asiático, e muito usado em várias áreas científicas, como é o caso das neurociências devido á alta homologia genética e fisiológica com modelos mamíferos. No entanto, o conhecimento obtido de estudos com esta espécie ainda é relativamente limitado, em comparação com mamíferos, nomeadamente em questões comportamentais, mas também neurofisiológicas.

A serotonina (5-HT) é um neurotransmissor com papéis centrais e periféricos que está envolvido na regulação do comportamento animal e envolvido em ansiedade e em comportamentos tipo-ansiosos em vertebrados e invertebrados. Como todos neurotransmissores, as suas ações são levadas a cabo através da ligação com seus recetores. De todas as classes, a classe de recetores 5-HT_2 é muito importante para o estudo de ansiedade, em particular o subtipo 5-HT_{2C}. Dado o conhecimento já adquirido acerca do papel dos recetores 5-HT_{2C} em ansiedade e a importância que o peixe-zebra tem ganho dentro das neurociências, são necessários trabalhos que estudem a modulação deste subtipo de recetores no comportamento e ansiedade social de peixes teleósteos, já que são poucos os estudos publicados. Como tal, eu fiz um estudo em peixes-zebra onde o papel dos recetores 5-HT_{2C} em ansiedade social foi analisado.

O objetivo principal deste estudo foi perceber se subordinação e manipulação do sistema serotoninérgico com um agonista direto e específico do recetor 5-HT_{2C} (MK-212) e um antagonista também direto e específico do mesmo recetor (RS-102221) poderiam influenciar o comportamento de peixes-zebra adultos num teste do tanque novo e como estatuto social e o sistema serotoninérgico interagiriam. Subordinação resultou num aumento do tempo passado no terço mais fundo do aquário, natação errática, e congelamento, comportamentos normalmente exibidos por indivíduos mais ansiosos. O tratamento com MK-212 também teve resultados ansiogénicos, com uma interação significativa com estatuto social no tempo passado no terço mais fundo do tanque. Por outro lado, o tratamento com RS-102221 diminuiu o tempo passado no teço mais fundo do tanque e natação errática, mas só em indivíduos controlo e dominantes, enquanto que em subordinados teve o efeito oposto, o que poderá indicar que a ação deste fármaco é dependente de estatuto social ou que em subordinados terá havido uma dessensibilização dos recetores 5-HT_{2C} a esta droga. O tratamento com RS-102221 não resultou em alterações significantes no congelamento. Não foram encontradas quaisquer mudanças significativas como resultado de subordinação ou modulação dos níveis de serotonina na velocidade de natação dos indivíduos estudados. Finalmente, não foram detetadas diferenças nos comportamentos entre machos e fêmeas.

Os resultados mostraram que subordinação resulta em aumento de ansiedade em peixes-zebra e que a modulação do sistema serotoninérgico através dos recetores 5-HT_{2C}, afeta a exibição de comportamentos tipo-ansiedade.

Palavras-chave: peixe-zebra (*Danio rerio*), serotonina, MK-212, RS-102221, estatuto social, ansiedade, teste do tanque novo

Abstract

Anxiety is a key aspect of animal sentience, one which greatly affects animal behaviour. Over the years, the biggest focus has been given to the study of how stress and anxiety can impact different species, especially those that live in laboratory conditions. Indeed, throughout their lives, animals are under different types of stress, which can cause behavioural and physiological changes. One of the most prevalent types of stress is social stress, caused due to constant interactions with other individuals, usually conspecifics, and, thus, its impacts are more notorious in group-living species. The establishment of hierarchies and the maintenance of the different social status are a constant source of stress for all individuals of the group, more so for subordinates, who have been shown to be more defensive and shier and exhibit more anxiety-like behaviours in comparison to dominants. Different paradigms have been used to study this type of stress in many different model species, particularly rodents and non-human primates and the focus given to teleosts much smaller.

The zebrafish (*Danio rerio*) is a group-living species, native to the South East Asia, that has become a very appealing and useful model species for many scientific areas, such as the neurosciences due to the high genetic and physiological homology with mammalian models. However, the knowledge obtained from studies using this species is still lacking, in comparison to mammals, not just regarding behaviour but also in regards to neurotransmitters and how they may affect it.

Serotonin (5-HT) is a neurotransmitter with central and peripheral roles that modify animal behaviour. It is known to be involved in anxiety and anxiety-like behaviours in both vertebrates and invertebrates. Like all neurotransmitters, its actions are carried from the binding with its receptors. Of the several classes, the 5-HT_2 receptor class is a very important one for the study of anxiety, in particular the 5-HT_{2C} receptor subtype. Given the knowledge already gained about the role of the 5-HT_{2C} receptors in anxiety and the importance that zebrafish has gained in neurosciences, works that study the modulation of this subtype of receptors in the behaviour and social anxiety of teleosts are of great need, since there few studies published regarding the topic. As such, I made a study in zebrafish where the role of 5-HT_{2C} receptors in social anxiety was analysed.

The main goal of this study was to know if subordination and manipulation of the serotonergic system with a 5-HT_{2C} receptor-specific direct agonist (MK-212) and a

5-HT_{2C} receptor-specific direct antagonist (RS-102221) could affect the behaviour of adult zebrafish in a novel tank test and how social status and the serotonergic system would interact. I report that subordination resulted in increased time spent in the bottom third of the tank, erratic swimming and freezing, which are known anxiety-like behaviours. The treatment with MK-212 also had anxiogenic properties, with a significant interaction with social status in the time spent in the bottom third. On the other hand, the treatment with RS-102221 had the expected anxiolytic results only in the first two variables and only in control and dominant individuals, while in subordinates it had the opposing effect, which could indicate that the action of this drug is dependent on the social status of the treated animal or that, in subordinates, occurred a desensitization from the 5-HT_{2C} receptors to this drug. It also resulted in no significant alterations in freezing behaviour. Swimming speed was also measured, but no significant changes were found as a result of subordination nor of serotonin modulation. Moreover, sex did not seem to factor in the results.

The results showed that zebrafish individuals under social stress as a result of subordination have increased anxiety and that modulation of the serotonergic system, specifically 5-HT_{2C} receptors, affect the exhibition of anxiety-like behaviours.

Keywords: zebrafish (*Danio rerio*), serotonin, 5-HT, MK-212, RS-102221, social status, anxiety, novel tank test

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List of abbreviations

5-HTP	5-HYDROXYTRYPTOPHAN
5-HT_2	SEROTONERGIC RECEPTOR FAMILY 2
$_C$AMP	CYCLIC ADENOSINE MONOPHOSPHATE
CAS	CONSPECIFIC ALARM SUBSTANCE
CNS	CENTRAL NERVOUS SYSTEM
CRH	CORTICOTROPIN-RELEASING HORMONE
CUS	CHRONIC UNPREDICTABLE STRESS
DAG	DIACYLGLYCEROL
DH	DORSAL HIPPOCAMPUS
DMSO	1% DIMETHYL SULFOXIDE
GI	GASTROINTESTINAL
IP_3	INOSITOL TRIPHOSPHATE
MAO	MONOAMINE OXIDASE
MK-212	6-CHLORO-2-(1-PIPERAZINYL)PYRAZINE
OCT	ORGANIC CATION TRANSPORTER
PMAT	PLASMA MEMBRANE MONOAMINE TRANSPORTER
RS-102221	8-[5-(2,4-DIMETHOXY-5-(4-TRIFLUOROMETHYLPHENYLSULPHONAMIDO) PHENYL-5-OXOPENTYL]-1,3,8-TRIAZASPIRO[4.5]DECANE-2,4-DIONE
SERT	SEROTONIN TRANSPORTER
SSRI	SELECTIVE SEROTONIN REUPTAKE INHIBITOR

TRP	TRYPTOPHAN
VH	VENTRAL HIPPOCAMPUS

Introduction

1. Anxiety and social stress

Anxiety is a very important aspect of animal sentience, defined as an individual's ability to perceive and process key emotions and states and, in this way, to respond accordingly through a set of innate or learned behaviours (Proctor, 2012; Proctor, Carder, & Cornish, 2013). It is an emotional state that produces behavioural, neurological and physiological changes in the individuals and it occurs as a response to a potential, but unknown threat or outcome of an event (Steimer, 2002). It is often associated with fear, given that they are both alert signals to threats and dangerous events and both elicit adaptative responses. However, the main distinguishable aspect between both states is that anxiety deals on uncertainty, while fear is a response to a clear threat or to a known event outcome (Steimer, 2002). Alongside with pain and fear, animal anxiety has mostly been used in studies relating to human psychiatry, given how severe and prevalent anxiety disorders are and how much they affect individual's lives. Several different model organisms, such as rodents and zebrafish, have been employed for the study of anxiety and its associated behaviours (Campos, Fogaça, Aguiar, & Guimarães, 2013). A major reason for the employment of other animals to study human anxiety-related disorders is the evolutionary preservation of expression of emotions through individuals' behaviour, initially observed by Charles Darwin (Darwin, 1872). Traditionally, in studies involving animal models to study human disorders, the goal is to establish an evolutionary comparison between them and humans and to advance human psychopharmacotherapy through *e.g.*, development of anxiolytics. However, due to advances in areas such as ecology, endocrinology and neurosciences, as well as bigger concerns and focus on animal ethics and welfare, scientists have also studied how emotions, including anxiety, affect not just these model species but others as well. The goal of this type of research is to also improve the welfare of the laboratory animals through, for example, reduction or even elimination of potential stressors and reduction of potential anxiogenic causes. Individuals, throughout their lives, are constantly under the effect of several different factors and conditions that may cause some sort of stress, whether it be physical, physiological or mental, which can result, among other things, anxiety and putative changes in behaviour.

In group-living species, one of the most prevalent and important types of stress is socially derived, because it happens via interactions with other individuals such as predators or conspecifics. Many different paradigms have been used to study social stress and the frequency of the exposure to the stressor is also changeable, varying from acute to more chronic exposures. This variety of available model species, ranging from invertebrates to fish and mammals (Blanchard, McKittrick, & Blanchard, 2001; Curran & Chalasani, 2012; Steenbergen, Richardson, & Champagne, 2011), study paradigms (some are highlighted further below), and duration of stress exposure helps to bring a more complete image of how social interactions affect individual behaviour.

While most scientific paradigms usually focus in agonistic behaviour as a cause of social stress, other paradigms emphasize social isolation or social proximity – crowding (Blanchard et al., 2001). For instance, “crowding” can be defined as a large number of individuals living in and sharing a restricted space, therefore competing for resources (Beery & Kaufer, 2015). While many species may live in groups, when the number of individuals in these groups become larger for the available space, it also contributes as a source of stress (Love & Zelikowsky, 2020). However, in many cases, the information regarding the minimal space requirements or the optimal group size is scarce. In addition, when studying crowding-derived stress, one may or may not take in consideration affiliative and/or agonistic interactions between the individuals of the group (Blanchard et al., 2001). Other factors can also impact the effects of crowding stress on behavior; in zebrafish, males seem to be more susceptible to Microsporidia infections (Chow, Xue, & Kent, 2016), which can be greatly induced by crowding (Ramsay, Watral, Schreck, & Kent, 2010), Social isolation, on the other hand, is the opposite of crowding, and it happens when an animal is physically separated from others (Mumtaz, Khan, Zubair, & Dehpour, 2018). The extent of the impact social isolation has on an individual is also dependent on the existence of social cues (visual, olfactive and/or auditory), since they have been shown to mitigate stress in social species (Daniel & Bhat, 2022). Overcrowding and isolation are both sources of stress. However, depending on other variables, such as sex, the degree of their impact on the individuals’ behaviour may differ. For instance, male rats (*Rattus norvegicus)* are seemingly more stressed under social crowding, while social isolation appears to have a significantly larger impact in females of the same species (Brown & Grunberg, 1995; Haller & Halász, 1999). In *Macropodus opercularis* (Paradise fish), isolation resulted in increased aggressiveness in both sexes but males showed higher lateral display than females and the approach response was stronger reinforced via mirror image stimulation in males (Davis, Harris, & Shelby, 1974).

For example, one of the most used paradigms in the study of social stress are dyadic encounters, in which two individuals are paired up and confrontations ensue, leaving a winner and a loser. The winner usually exhibits more offensive behaviours, such as attacks and chases, whereas the loser usually tries to avoid the other individual (if possible), freezing, spending more time in a specific area or assuming a more submissive posture, such as, for example, laying on the back in the case of rodents (Blanchard et al., 2001; Blaser & Gerlai, 2006; Bozi et al., 2021). As a result of social defeat, loser individuals typically show more anxiety-like behaviours in tests designed to study anxiety, like the novel tank test in the case of fish (Bozi et al., 2021), and forced swimming and black/white box tests in rodents (Keeney & Hogg, 1999), whereas winners and dominant individuals tend to be less anxious and, at the same time, bolder and more affiliative (Dahlbom, Lagman, Lundstedt-Enkel, Sundström, & Winberg, 2011; Shively, 1998). Different behavioural endpoints can be observed and studied (*e.g.*, freezing, running/swimming away, elevated or reduced aggression, depending on the individual, etc.), which can be very good indicators of stress but other indices can also be used, such as physiological changes (*e.g.*, changes in cortisol levels). However, any measured indicator may be dependent on species, age, sex and other factors (Blanchard et al., 2001). In this paradigm, social hierarchies between conspecifics can also be observed and studied. The establishment of dominant/subordinate relationships is physically, psychologically, and physiologically demanding for both dominant and subordinate individuals. In mammals, offensive behaviours are increased in dominant individuals and defensive behaviours are increased in subordinates, with consequent changes in weight, cortisol levels, and monoamine levels, in both groups (Tamashiro, Nguyen, & Sakai, 2005). In fish, it has a similar effect, as subordinates show increased cortisol levels and reduced brain cell proliferation (Maruska, Carpenter, & Fernald, 2012; Sørensen, Nilsson, Summers, & Øverli, 2012; Tea, Alderman, & Gilmour, 2019), while dominant individuals also show increased cortisol levels immediately after winning fights (Øverli, Harris, & Winberg, 1999). Stress and the physiological changes tend to increase in species whose social hierarchies are not stable, whenever the dominant status of any given individual may be challenged (Dahlbom, Backström, Lundstedt-Enkel, & Winberg, 2012). However, in some species, sex may also be important to consider. While in species such as the zebrafish or cynomolgus macaque (*Macaca fascicularis*), hierarchies are formed in both male and female groups (despite possible differences In relation to the stability) (Dahlbom et al., 2012; Riddick et al., 2009; Shively, 1998), in other species, such as rodents, the formation of hierarchies are not as evident in females as they are in males (Tamashiro et al., 2005).

2. Zebrafish: the model species

2.1. Taxonomy and characterization

Danio rerio (Hamilton 1822) is a species member of the superorder Ostariophysi. The members of this superorder share a common physical characteristic, which is the presence of the Weberian apparatus, a set of small ossicles that connect the auditory system to the swim bladder, which serves as a sound amplifier (Diogo, 2009). Another important characteristic of this superorder, relevant for social behaviour, is the existence, in many species, of a conspecific alarm substance (CAS) that, when released as result from injury to the club cells located in the epidermis which produce the substance, triggers a fright reaction in other fish (Maximino et al., 2019). This CAS has been studied in different species, including zebrafish (Lima-Maximino et al., 2020; Maximino et al., 2018; Silva et al., 2021), making this superorder important to study in social contexts. The zebrafish is also a member of the Cyprinidae family, the largest fish family.

Zebrafish adults can be identified by its small length, fusiform body and, most notably, its horizontal blue stripes on the side of the body that extend to the limits of the caudal fin (Conradsen & McGuigan, 2015). In this species, there is some sexual dimorphism (Conradsen & McGuigan, 2015). The most noticeable phenotypical external difference between the two sexes lies in the colour of the stripes on the side of the body (Conradsen & McGuigan, 2015). Males exhibit gold stripes in between the blue ones, whereas females sport silver stripes In addition, females' belly is usually wider and whiter, while males have a more stream-line body type and bigger anal fins (Conradsen & McGuigan, 2015) (Fig.1).

Figure 1. Female (top) and male (bottom) zebrafish. Photography by Tohru Murakami (https://www.flickr.com/photos/8659392@N07/13896905021).

Endemic to the Southeast Asia, the zebrafish is a group-living species that inhabits fresh bodies of water such as ponds, canals, ditches and small rivers (Vishwanath, 2010). Regarding behaviour, it is a highly social species with an innate shoaling behaviour which forms highly organized schools, whose size is dependent on availability of resources, as well as environmental features (Shelton et al., 2020; Suriyampola et al., 2016). This innate shoaling behaviour offers a degree of protection against predators (Delcourt, Denoël, Ylieff, & Poncin, 2013; Engeszer, Da Barbiano, Ryan, & Parichy, 2007; Pitcher, 1983) and it is interesting since zebrafish adults exhibit no parental care (Engeszer et al., 2007). In captivity, it forms stable social hierarchies between both males and females, which are observed in a short amount of days with dyadic encounters (Bozi et al., 2021; Filby, Paull, Bartlett, Van Look, & Tyler, 2010; Paull et al., 2010). In these social hierarchies, several behaviours can be observed that are indicative of social rank. For example, dominants are more aggressive (Paull et al., 2010), more mobile, spend less time sheltering and interact more with novel objects (Dahlbom et al., 2011) while subordinate individuals exhibit more anxiety-like behaviours, such as freezing, erratic swimming and longer times spent in a particular area (Winberg & Nilsson, 1993a). These differences in social status may also be reflected in their reproductive success, as dominant males have more control over

spawning sites and higher reproductive success while dominant females have more offspring with the dominant male (Paull et al., 2010).

2.2. The importance of behavioural studies

For quite some time, zebrafish has been a very important animal model for a wide array of scientific areas such as genetics, toxicology, oncology, neurobiology and other areas (Kinth, Mahesh, & Panwar, 2013). There are several reasons for this species being so appealing in science, such as being easily kept in laboratory, short life cycle (~3 months), able to reproduce several times all year round in the laboratory, large number of transparent eggs spawned in each clutch, very rapid embryonic development, fully sequenced genome, and existence of many well-studied strains. (Fonseka, Wen, Foster, & Kennedy, 2016; Genario, de Abreu, Giacomini, Demin, & Kalueff, 2020; McCammon & Sive, 2015).

Zebrafish has also become increasingly more used to study stress and anxiety and to model psychological disorders (Bencan, Sledge, & Levin, 2009; Lillesaar, 2011; Steenbergen et al., 2011). This species can exhibit anxiety-like behaviours, such as swimming in the bottom or along the edges of the tank, freezing or alterations of swimming speed, which, in turn, are attenuated by anxiolytics (Bencan et al., 2009). Furthermore, a high genetic and physiological homology to mammalian models makes this species very useful for behavioural research (Lillesaar, 2011; Steenbergen et al., 2011). In addition, genetic tools have also been commonly employed in studies involving zebrafish, as well as pharmacological models, the latter exemplified by, for instance, serotonergic modulations (Kalueff, Stewart, & Gerlai, 2014). Indeed, many different tests have been presented and used to study zebrafish behaviour. A common behavioural paradigm used is the novel tank test (geotaxis test), in which the individuals are transported to a new environment and left to explore it, while their behaviour is monitored (Cachat et al., 2010). Typical behaviours observed in this species during this test are bottom-dwelling, erratic swimming and freezing (Cachat et al., 2010). There is some evidence that zebrafish behaviour in the novel tank test (NTT) is modulated by the serotonergic system. For instance, the use of buspirone (a 5-HT_{1A} receptor agonist) and use of SSRIs reduced bottom-dwelling (Bencan et al., 2009; Sackerman et al., 2010), while the use of GR 55562 (a 5-$HT_{1B/D}$ receptor antagonist) produced anxiolytic effects as well (Nowicki, Tran, Muraleetharan, Markovic, & Gerlai, 2014). Meanwhile, the treatment with 5-HT_{1A}, 5-HT_2 and 5-HT_3 antagonists resulted in increments of anxiety-like behaviours (Nowicki et al., 2014). Other commonly used tests include the light/dark preference test (scototaxis) and, much like the novel tank test, it is a sensitive test for

the use of drugs impacting anxiety (Maximino et al., 2012). However, in the literature, there is inconsistency between tests in regards to the manipulation of the serotonergic system and its respective effect on anxiety-like behaviours (Maximino et al., 2012; Maximino, Puty, et al., 2013). For example, the use of acute fluoxetine (2.5 mg/kg), a SSRI, was anxiolytic in the novel tank test but anxiogenic in the scototaxis test, and the use of a tryptophan hydroxylase inhibitor (-*para*-chlorophenylalanine) also had opposite effects between the tests (anxiogenic in the former, anxiolytic in the latter) (Maximino, Puty, et al., 2013). A possible explanation for this is that different tests measure different aspects of anxiety, since different stressors are involved (Maximino et al., 2012; Maximino, Puty, et al., 2013). Another hypothesis is that the tests have different sensitivities to the use of drugs targeting serotonin receptors (Maximino, Puty, et al., 2013).

2.3. Behavioural sex differences in zebrafish

Few studies have been published analysing behavioural differences between male and female zebrafish and so understanding about behavioural differences in males and females is limited. For instance, males are usually thought to be more mobile and explorative (Ariyomo, Carter, & Watt, 2013; Dahlbom et al., 2011; Reolon, de Melo, da Rosa, Barcellos, & Bonan, 2018) but this is also affected by age (Philpott, Donack, Cousin, & Pierret, 2012). However, very often there is disagreement between results. For example, it has been reported that males were more aggressive than females, especially in some specific contexts (*e.g.*, spawning) (Love & Zelikowsky, 2020; Paull et al., 2010), but other studies have reported conflicting results (Ariyomo et al., 2013; Dahlbom et al., 2012; Moretz, Martins, & Robison, 2007; Rambo et al., 2017).

Regarding the stability of the social hierarchies in this species, differences between sexes have also been reported. Females exhibit more stable social hierarchies, whereas in male-only social groups, subordinates tend to challenge the dominant individual's position for a longer period (Dahlbom et al., 2012; Paull et al., 2010). This may induce, in males, higher stress, and, as a result, higher brain serotonergic activity is seen in this sex (Dahlbom et al., 2012). However, social status itself does not seem to incur sex differences in the expression of anxiety-like behaviours (Bozi et al., 2021), despite some studies indicating increased cortisol levels in male but not female subordinates (Tea et al., 2019) and others reporting higher anxiety-like behaviours in non-stressed female zebrafish (Fontana, Cleal, & Parker, 2020). This latter work did not assess the effects of social stress on anxiety-like behaviour, however. These sex differences in anxiety-like behaviours have also been

seen in studies manipulating the levels of neurotransmitters, including serotonin. In Winberg & Thörnqvist, (2016), male and female zebrafish were subjected to fluoxetine by immersion and the effects on behaviour in an open field test was assessed. It was observed that females, regardless of treatment, significantly moved less distance, spent more time immobile and transitioned less between zones than males. However, said differences are not seen in studies involving other neurotransmitters. In López Patiño, Yu, Yamamoto, & Zhdanova, (2008), cocaine withdrawal was used to study anxiety-like behaviours in male and female zebrafish. It was reported that it did not result in sex differences in the expression on anxiety-like behaviours, despite differences in their development. No differences were also seen in the levels of brain dopamine.

3. Serotonin: a key neurobiological system

3.1. Classification

Serotonin (5-HT) belongs to the monoamine group. Monoamines are neuromodulators with an amine group linked to an aromatic ring. Besides 5-HT, this group includes dopamine, epinephrine, norepinephrine, among other compounds, and are some of the most well-studied neurotransmitters, with their roles in behaviour, emotion, memory and neurological disorders being extensively reviewed (Azizi, 2020; Kurian, Gissen, Smith, Heales, & Clayton, 2011; McEntee & Crook, 1991).

3.2. Distribution and evolution

In animals, 5-HT neurons are found in the CNS as well as peripherally (Gaspar & Lillesaar, 2012). 5-HT produced outside of the CNS is primarily found in the enterochromaffin cells of the gastrointestinal (GI) tract and accounts for ~90% of the total concentration (Berger, Gray, & Roth, 2009; Trowbridge, Narboux-Nême, & Gaspar, 2011). Less than 10% can be found in blood platelets (which are important storage sites for this neurotransmitter) and the remainder is found in the CNS and in other cells, such as myenteric cells (digestive system), β-cells (pancreas), parafollicular cells (thyroid), cumulus cells (oocytes), dorsal root ganglia, taste buds, and in the retina (Charnay & Leger, 2010; Masson, 2019; Mohammad-Zadeh, Moses, & Gwaltney-Brant, 2008; Trowbridge et al., 2011).

5-HT is an evolutionarily old compound, being present in most metazoans and also in some protozoans. Interestingly, despite expected differences as a result of evolution, its roles are widely preserved across every phylum from protozoans to the most recent species (Turlejski, 1996; Walker, Brooks, & Holden-Dye, 1996). For instance, activation of specific 5-HT receptors (families 5-HT_1 and 5-HT_{4-7}) regulates adenylate cyclase activity in vertebrates and this action is also seen in the protozoan genus *Tetrahymena* (Turlejski, 1996). Furthermore, the known actions of 5-HT in behaviour and several aspects of cognition found in vertebrates can also be seen in insects (Huser et al., 2012). Additionally, the 5-HT system of the bird brain observes the same functional and/or anatomical principles as the human brain (Jarvis et al., 2005; Shanahan, Bingman, Shimizu, Wild, & Güntürkün, 2013). Interestingly, 5-HT is also found in plants, with different functions from those seen in other groups (Pelagio-Flores, Ortíz-Castro, Méndez-Bravo, MacÍas-Rodríguez, & López-Bucio, 2011; Ramakrishna,

Giridhar, & Ravishankar, 2011). This brings interesting questions about the evolution of this compound and new avenues of neurotransmitters research.

3.2.1. Teleost fish vs mammals

Over the years, studies on teleost fish, in particular zebrafish, have provided knowledge about the properties of many different neurotransmitters and modulators and this knowledge has enabled scientists to establish comparisons between fish and mammals (Bacqué-Cazenave et al., 2020; Gaspar & Lillesaar, 2012; Winberg & Nilsson, 1993a). However, despite zebrafish's increasing importance in neurosciences, there is still a degree of overfocus in mammals when it comes to studying physiology.

Regarding the 5-HT system, teleosts and mammals reveal some similarities. In both taxa, 5-HT is involved in mostly the same roles, whether it be in the CNS or in the periphery, and, as such, many behavioural observations in studies with 5-HT done in mammals are also present in studies in teleosts and vice-versa (some examples are given in the "Overall function" section below) (Bacqué-Cazenave et al., 2020; Winberg & Nilsson, 1993a).

In mammals, brain (central) 5-HT is mainly produced in the raphe nuclei, an area of the brainstem containing 5-HT neurons with several projections to other regions of the brain (Charnay & Leger, 2010) (Fig.2, A). Based on their location and their projections to other brain areas, the raphe are divided, in mammals, in two groups: the rostral group and the caudal group, each composed of multiple nuclei (Hornung, 2010), with each group being involved in behaviour (Deakin & Graeff, 1991; Dos Santos, De Andrade, & Zangrossi, 2005; Pobbe & Zangrossi, 2005; Sena et al., 2003; Takase, Barone, & Nogueira, 2000; Vicente, Zangrossi, dos Santos, de Macedo, & Andrade, 2008), cognition (Li et al., 2016; Nakamura, 2013) and movement (Flaive, Fougère, van der Zouwen, & Ryczko, 2020; Veasey, Fornal, Metzler, & Jacobs, 1995). In teleosts, the raphe nuclei also possess 5-HT neurons and project to other areas in similar fashion and may, indeed, have similar roles (Adrio, Anadón, & Rodríguez-Moldes, 1999; Ekström & Van Veen, 1984; Kaslin & Panula, 2001; Timothy & Forlano, 2020). Also, similarly to mammals, the teleost raphe nuclei display an organization that correlates to their function, based on their ontogeny and connectivity (Lillesaar, 2011). In zebrafish, four different populations of raphe 5-HT populations have been identified, also divided in two groups: the superior and the inferior raphe nuclei (Fig.2, B). The superior raphe nuclei, constituted by the three most rostrally situated nuclei, project to the midbrain and the forebrain, while the inferior raphe nuclei projects to the hindbrain and spinal cord.

This suggests that the raphe nuclei and their 5-HT populations have been conserved throughout the course of evolution in vertebrates (Lillesaar, 2011).

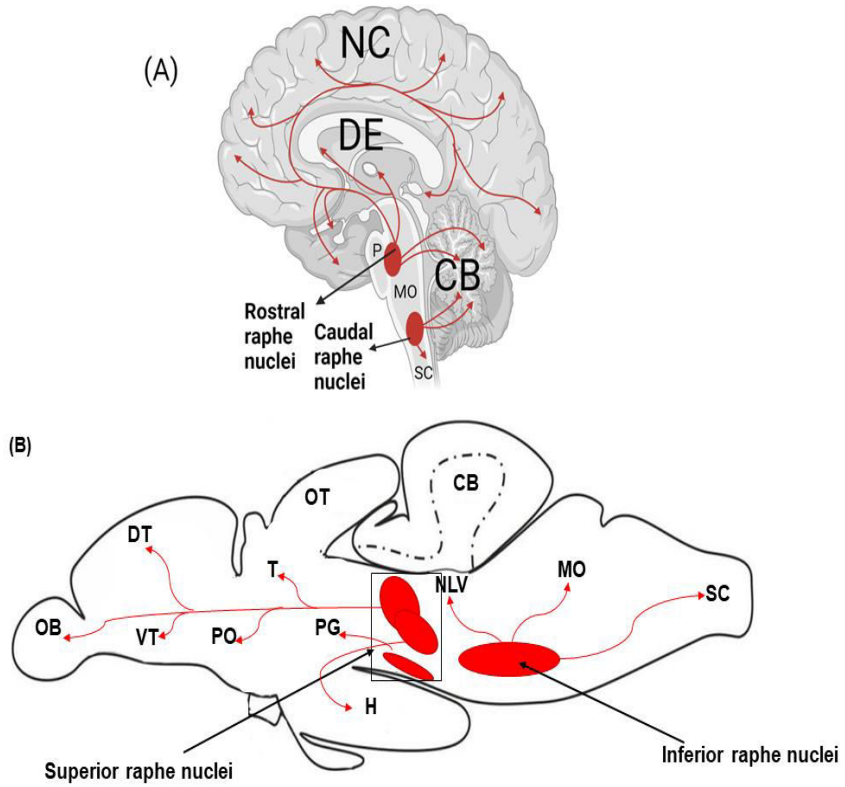

Figure 2. 5-HT projections from the raphe nuclei in human and zebrafish brains. (A) Sagittal cut of a human brain. Adapted from "Anatomy of the Brain" by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates. (B) Dorsal view of a zebrafish brain. Adapted from (Zhang, Ge, & Yuan, 2015), (Lillesaar, 2011) and (Gaspar & Lillesaar, 2012). Abbreviations: (A) CB – Cerebellum; DE – Diencephalon; MO – Medulla Oblongata; NC – Neocortex; P – Pons; SC – Spinal cord; (B) CB – Cerebellum; DT – Dorsal telencephalon; H – Hypothalamus; MO – Medulla Oblongata; NLV – Nucleus lateralis valvulae; OB – Olfactory bulb; OT – Optic Tetum; PG – Preglomerular complex; PO – Preoptic area; SC – Spinal cord; T – Thalamus; VT – Ventral telencephalon.

Despite similarities between raphe nuclei in both taxa, there is a notable difference in regards to the production of 5-HT in the CNS. Whereas, in mammals, central 5-HT is produced only in the raphe nuclei, in teleosts, as well as other phylogenetic groups, 5-HT is also produced in other brain areas, such as the hypothalamus, pretectum, medulla oblongata, and spinal cord (Lillesaar, 2011; Lillesaar, Stigloher, Tannhäuser, Wullimann, & Bally-Cuif, 2009; Maximino, Gomes, et al., 2013; Rosner, Chagnaud, & Wullimann, 2020; Timothy & Forlano, 2020). It suggests that, during the course of evolution, the functions of 5-HT neurons outside the raphe nuclei in vertebrates other than mammals were shifted to being executed in the raphe nuclei in eutherians (Lillesaar, 2011).

Another difference comes in the number of enzyme isoforms present in mammals and in teleosts. For some of the enzymes related to the 5-HT system, the number of isoforms in teleosts is usually higher than the number of isoforms seen in mammals, which, in some cases, it is attributable to a teleost-specific genome duplication event that occurred (Herculano & Maximino, 2014; Maximino, Gomes, et al., 2013). However, this does not happen with all enzymes (Bellipanni, Rink, & Bally-Cuif, 2002; Shih, Chen, & Ridd, 1999; Teraoka et al., 2004). In the case of the monoamine oxidase (MAO) enzyme, the situation is even reversed since is present in two different isoforms in mammals but only one in zebrafish, which, given the results of genetic studies of the human sequences, it is likely due to a duplication event of an ancestral gene that occurred in mammals (Shih et al., 1999).

3.3. Biosynthesis and metabolism

Monoamines are derived from certain amino acids. In the case of 5-HT, it is formed by the hydroxylation of L-tryptophan (Trp) to 5-hydroxytryptophan (5-HTP, an intermediate), followed by decarboxylation to 5-HT (Höglund, Øverli, & Winberg, 2019) (Fig.3). This is a two-step process, with the first one (hydroxylation from Trp to 5-HTP by tryptophan hydroxylase) being the rate-limiting one, due to the rapid decarboxylation of 5-HTP (Boadle-Biber, 1993; Höglund et al., 2019). After the action of 5-HT is carried, the monoamine is reabsorbed to the presynaptic neuron. The uptake of 5-HT occurs via two systems. The first system, adequately named serotonin transporter (SERT), has a high-affinity to 5-HT and it is dependent on Na^+, K^+ and Cl^- ions (Herculano & Maximino, 2014; Rudnick, 2006). The second system is, in opposition to SERT, independent on Na^+ and it is composed by three different organic cation transporter (OCT) isoforms (OCT1-3) and by plasma membrane monoamine transporter (PMAT). After 5-HT is reabsorbed, it is mainly metabolized in the liver in a two-step reaction as well, first to 5-hydroxyindole acetaldehyde (5-HIAL) and then to 5-hydroxindole acetic acid (5-HIAA), which is then excreted in the urine (Fig. 3).

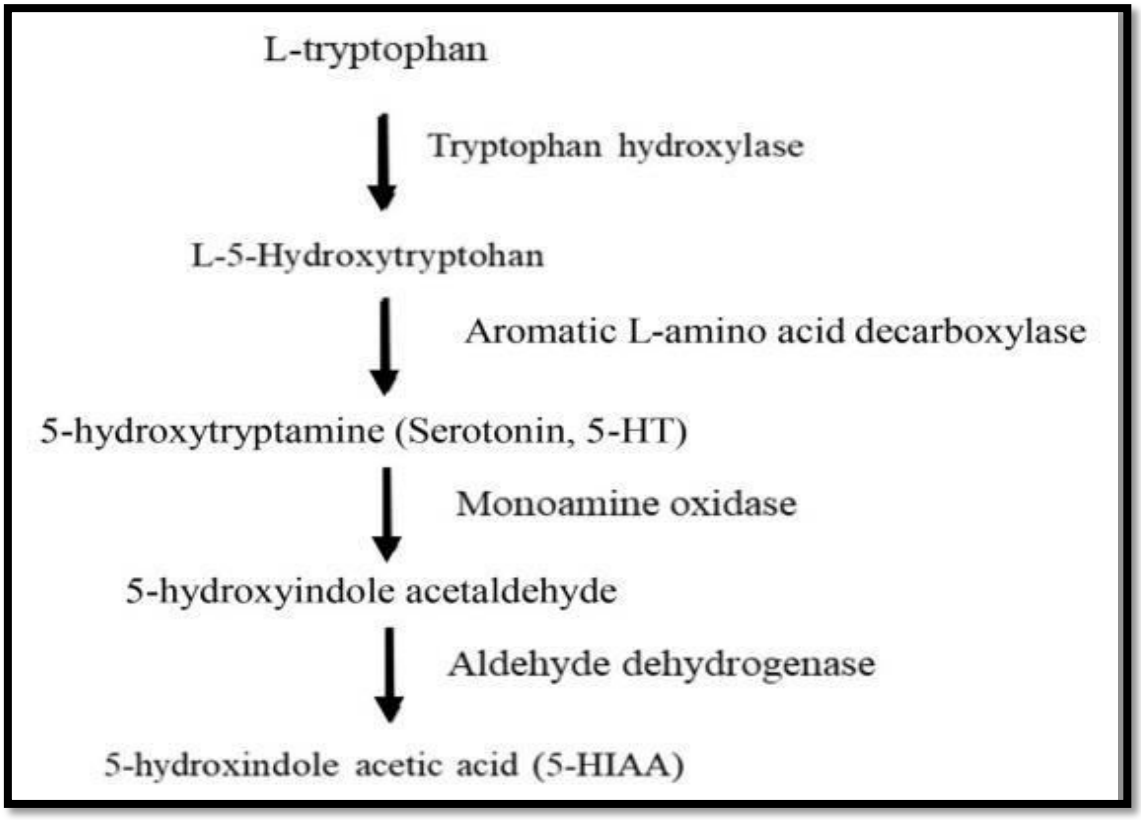

Figure 3. Biosynthesis and metabolism of 5-HT. Image taken from (Höglund et al., 2019).

Lastly, 5-HT is also a precursor to melatonin, a hormone greatly involved in the control of the circadian rhythm and skin colour changes (Sprenger, Hardeland, Fuhrberg, & Han, 1999; Zimova et al., 2018).

3.4. 5-HT receptors

5-HT actions are carried through the binding with its receptors. In vertebrates, they are currently divided in 7 subfamilies (with some being further divided in different subtypes) and are found in the CNS, as well as in other peripheral cells (muscle, stomach, blood vessels and platelets) (Hoyer & Martin, 1995; Katzung, 2018). Interestingly, these receptors share little homology with each other, suggesting that the divergence occurred very early across evolution (Peroutka & Howell, 1994; Walker et al., 1996). Most 5-HT receptors are G-protein coupled receptors essential for signal transduction via regulation of second messengers cyclic adenosine monophosphate (cAMP) or inositol triphosphate (IP_3) and diacylglycerol (DAG) (von Zastrow, 2018). The only exception is the 5-HT_3 receptor subtype which is a ligand-gated Na^+/K^+ ion channel, responsible for depolarization of the plasma membrane (Derkach, Surprenant, & North, 1989). Of all the extant receptor subfamilies and subtypes, one that is

important in the context of anxiety but, at the same time, only starting to receive more attention from the mid-2000s onward is the 5-HT_{2C} receptor subtype (Alves, Pinheiro, Motta, Landeira-Fernandez, & Cruz, 2004; Heisler, Zhou, Bajwa, Hsu, & Tecott, 2007; Kimura et al., 2009). Despite its discovery in the 1980s (Pazos, Hoyer, & Palacios, 1984), more studies have been published focusing on other 5-HT receptor subtypes, such as the 5-HT_{1A}. Additionally, a lack of selective ligands, in comparison to other subtypes, has also made difficult understanding the actions of this receptor (Pytliak, Vargová, Mechírová, & Felšci, 2011). As such, its roles in anxiety have started to be better understood only more recently.

3.4.1. 5-HT_{2C} receptors

The **5-HT_{2C}** receptor subtype was originally named 5-HT_{1C} but was renamed after being found to have higher homology to the 5-HT_2 class, as well as similar pharmacological and functional features as members of this class (Berg, Maayani, Goldfarb, & Clarke, 1998; Hoyer, 2007; Hoyer & Martin, 1995). This subtype has been found to be present in several brain areas but, unlike other subtypes, not outside of the CNS (Giorgetti & Tecott, 2004; Hoyer, 2019). One key behavioural aspect it affects is anxiety. For instance, 5-HT_{2C} receptor knockout mice showed reduced anxiety-like behaviours in four different assays and a reduced activation of corticotropin-releasing hormone (CRH) neurons (Heisler et al., 2007), while transgenic mice expressing higher levels of the receptor in the forebrain exhibited increased anxiety-like behaviour (Kimura et al., 2009). Direct injections of 5-HT_{2C} receptor agonists in the mice ventral hippocampus resulted in anxiogenic effects. However, these effects were not exhibited when injected in the dorsal hippocampus, suggesting different roles of this receptor in different brain regions (Alves et al., 2004). It is also a target in antidepressant and antipsychotic therapies, with several studies being conducted with different compounds. For example, agomelatine and mirtazapine are used as antidepressants by increasing dopamine and norepinephrine levels via antagonism with 5-HT_{2C} receptors (Kasper & Hamon, 2009; Millan et al., 2000), while fluoxetine and other SSRIs act on 5-HT_{2C} receptors by impeding the reuptake of 5-HT therefore stimulating the receptors' activity (Ni & Miledi, 1997; Pytliak et al., 2011). Not many studies on this receptor subtype have been conducted in zebrafish, but it has been shown in this species that 5-HT_{2C} receptor agonists and antagonists have effects on fear and anxiety (Silva et al., 2021; Yang et al., 2021). There have also been reported notable differences between mammals and fish, in particular zebrafish. For instance, in zebrafish, two copies of this receptor are found (Schneider et al., 2012; Sourbron et al., 2016), as opposed to one in mammals.

Moreover, the 5-HT_{2C} receptor-binding pocket is conserved in both isoforms in zebrafish (Silva et al., 2021). Additionally, in mammals, the receptor undergoes RNA editing (Burns et al., 1997), creating different polymorphisms that vary not just between species, but also between tissues (Hoyer, 2019) and show different levels of constitutive ability, *i.e.* a receptor's ability to propel a signal transduction and to lead to the production of a second messenger without the need of a ligand (Teitler, Herrick-Davis, & Purohit, 2005). However, RNA editing has not been yet detected in zebrafish (Schneider et al., 2012). Lastly, in larvae, this receptor is expressed in similar brain areas as in mammals (*e.g.,* thalamus, hypothalamus, olfactory system) (Schneider et al., 2012).

Much like other neurotransmitters, most studies involving 5-HT and its receptors comes from mammals and, in a lot of cases, the 5-HT system is targeted via administration of SSRIs, which does not specifically target 5-HT receptor subtypes (even though it can bind to some (Ni & Miledi, 1997; Peng, Gu, Li, & Hertz, 2014)) but rather SERT (Maximino, Puty, et al., 2013; Pourhamzeh et al., 2021). As such, more in depth studies that specifically target the 5-HT_{2C} receptor subtype in fish are very much needed, since it would help establish evolutionary similarities and differences with other animal groups such as mammals. Moreover, given the growing importance of fish model species like zebrafish, it would help understand the 5-HT system better and provide more and better tools for the improvement of neurosciences and the development of newer and better pharmaceuticals (De Deurwaerdère, Chagraoui, & Cunningham, 2020).

3.5. Agonists and antagonists

Agonists are compounds that through several different mechanisms increase the release or action of a neurotransmitter, and those mechanisms include, reuptake inhibition, transporter blockade, increase of production, binding to the receptors and acting in the same way as the respective neurotransmitter, among others. This latter example are called direct agonists (Pleuvry, 2004). As such, 5-HT direct agonists activate 5-HT receptors in the same way as 5-HT itself. Meanwhile, antagonists are compounds that reduce the production of a neurotransmitter or prevent its action by, for example, blocking its receptor – direct antagonists. For instance, 5-HT direct antagonists block 5-HT receptors and inhibit their action. Both can be either selective or non-selective. Non-selective agonists and antagonists can bind to any receptor, but they may have a bigger affinity for a given receptor. For example, 5-carboxamidotryptamine (5-CT) is a non-selective agonist but has a bigger affinity for 5-HT_1 receptors (Yamada,

Sugimoto, Noma, & Yoshikawa, 1998). On the other hand, selective agonists and antagonists only bind to a specific receptor (Table 1).

MK-212 is a selective 5-HT_{2C} agonist and has been shown to affect anxiety-like behaviours (Clineschmidt, 1979). Administration of various doses of MK-212 to non-anxious humans led to increases in cortisol and prolactin levels, as well as increased anxiety (Bastani, Nash, & Meltzer, 1990; Lowy & Meltzer, 1988). Additionally, microinjections of this drug produced anxiogenic effects in rats under elevated plus-maze and light/dark tests (Alves et al., 2004; Cruz et al., 2005; Kshama, Hrishikeshavan, Shanbhogue, & Munonyedi, 1990). However, it has been hypothesized that low doses of the drug may have anxiolytic properties, after a study in mice showed that the individuals systemically injected with 0.1 or 0.2 mg/Kg of MK-212 spent less time in the dark compartment in a light/dark test, which may be attributable to differences between the species (Kuznetsova, Amstislavskaya, Bulygina, Tibeikina, & Popova, 2006).

RS-102221 is a selective antagonist at 5-HT_{2C} receptors (Bonhaus et al., 1997) and has shown anxiolytic (Bell, Duke, Gilmore, Page, & Bègue, 2014; Kuznetsova, Amstislavskaya, Shefer, & Popova, 2006; Scarpelli, Alves, Landeira-Fernandez, & Cruz, 2008; Warhaftig et al., 2021) and anti-depressive effects (Wankhar, Syiem, Pakyntein, Thabah, & Sunn, 2020) in rats but other studies have shown no effect of this drug on anxiety (Nic Dhonnchadha, Bourin, & Hascoët, 2003).

Table 1. 5-HT receptor agonists and antagonists.

Name	Selectivity	Receptor(s)	Reference
Agonists:			
5-CT	Non-selective	5-HT_{1A-D}/5-ht_5/5-HT_7	(Yamada et al., 1998)
8-OH-DPAT	Non-selective	5-HT_{1A}/5-HT_7	(Hedlund et al., 2004)
LSD	Non-selective	All classes except 5-HT_3 and 5-HT_4	(Aghajanian & Marek, 1999)
Zolmitriptan	Selective	5-HT_{1B}/5-HT_{1D}	(Martin et al., 1997)

MK-212	Selective	5-HT_{2C}	(Clineschmidt, 1979)
Cisapride	Selective	5-HT_4	(Kim et al., 2016)
Antagonists:			
Ketanserin	Non-selective	5-HT_{1D}/5-HT_2/5-HT_7	(Pytliak et al., 2011)
Mirtazapine	Non-selective	5-HT_1/5-HT_2/5-HT_3	(Millan et al., 2000)
Ritanserin	Non-selective	5-HT_{1D}/5-HT_2/5-ht_5/5-HT_7	(Pytliak et al., 2011)
WAY 100.635	Selective	5-HT_{1A}	(Fletcher et al., 1995)
RS-102221	Selective	5-HT_{2C}	(Bonhaus et al., 1997)
Alosetron	Selective	5-HT_3	(Clayton et al., 1999)

3.6. Overall role in behaviour and brain functions

5-HT is involved in a wide array of functions, both in the CNS and outside of it. In non-nervous tissue, it is involved in vascular tone, cardiovascular function and development, bone formation, regulation of the GI tract, among others (Berger et al., 2009; Mohammad-Zadeh et al., 2008), all depending on the receptor to which it binds and the location. In the CNS, it is involved in many aspects of cognition and behaviour, such as memory and learning, feeding and other reward-associated behaviours, mood and stress coping, nociception, social behaviour and status, aggressiveness and anxiety, in many vertebrate and invertebrate species, highlighting the evolutionary preservation of this system (Bacqué-Cazenave et al., 2020). Moreover, it has also been associated with many neuropsychiatric disorders, like depression, schizophrenia and Alzheimer's disease, with several drugs developed for the treatment of these conditions being on the market (Geldenhuys & Van der Schyf, 2011; Pourhamzeh et al., 2021). As such, the study of 5-HT is very important for the study of cognition, particularly in teleosts, considering their wide distribution of 5-HT cells in the brain. In more recent times, it has become possible to distinguish projections originating from the raphe nuclei

and projections coming from other locations, thus, not only enabling comparisons to mammals regarding raphe nuclei 5-HT innervations but also providing more reasons to the use of teleosts model species, like the zebrafish, as alternatives to mammals (Lillesaar et al., 2009; Timothy & Forlano, 2020).

3.6.1. Sociality and aggression

5-HT has been shown to modulate sociality in a plethora of species. In general, lower levels of 5-HT are associated with decreased sociality and higher isolation (Kiser, Steemers, Branchi, & Homberg, 2012) and upregulation leads to higher frequency of pro-social behaviours (Raleigh, Brammer, McGuire, & Yuwiler, 1985). These social behaviours and interactions can be represented in different ways. For example, an important aspect of sociality is cooperation. The cleaning interactions of bluestreak cleaner wrasses (*Labroides dimidiatus*) are a good example of the relation between fish cognition and sentience. These wrasses remove ectoparasites and dead tissue from their clients' skin, gills and mouth, meaning that the clients get cleaned while the cleaners get a source of food (Soares, 2017). In this species, increasing 5-HT levels or activating 5-HT_{1A} receptors resulted in higher motivation for cleaning behaviours, while blocking this receptor or downregulating 5-HT synthesis resulted in opposite effects (Paula, Messias, Grutter, Bshary, & Soares, 2015).

Another example of cooperation comes from the guppies (*Poecilia reticulata*) and their conditional approach strategy when inspecting predators. In this species, some individuals separate from the shoal and approach the predator and only get if accompanied by another conspecific that behaves in the same manner, thus reducing the risk of getting injured (Croft et al., 2006; Dugatkin, 1988; Pimentel et al., 2019). 5-HT was shown to play a role in this cooperative-like behaviour when phasic increases of 5-HT levels promoted this conditional approach, while blocking 5-HT receptors with the non-selective antagonist metergoline decreased it (Pimentel et al., 2019). The link between cooperation and 5-HT is also present in humans. For instance, the use of SSRIs promotes cooperation (Knutson et al., 1998; Tse & Bond, 2002) while tryptophan depletion decreases it (Wood, Rilling, Sanfey, Bhagwagar, & Rogers, 2006).

As mentioned before, another representation of social interactions are agonistic encounters (fights between conspecifics), which are also impacted by 5-HT, thus, meaning it to be also involved in aggression. For instance, stimulation of the 5-HT system in fish usually results in decreased aggression (Clotfelter, O'Hare, Mcnitt, Carpenter, & Summers, 2007; Perreault, Semsar, & Godwin, 2003; Theodoridi,

Tsalafouta, & Pavlidis, 2017) but it is not always the case. In toadfish (*Opsanus beta*), for example, intraperitoneal injections of fluoxetine, a commonly used SSRI, resulted in increased aggression (McDonald, Gonzalez, & Sloman, 2011). There has also been established connections between aggression and 5-HT levels in other taxa (aan het Rot, Moskowitz, Pinard, & Young, 2006; Audero et al., 2013; Dennis, Fahey, & Cheng, 2013; Higley et al., 1996; Lee & Goto, 2018). However, one must be cautious when reporting associations between 5-HT functions in the brain and aggressiveness, as these functions are dependent on which brain areas are studied. For example, less aggressive rainbow trout (*Oncorhynchus mykiss*) individuals possess higher 5-HT concentration and turnover in the brain stem and optic tectum than more aggressive ones, but the relationship is reversed in the hypothalamus (Øverli, Pottinger, Carrick, Øverli, & Winberg, 2001).

Both cooperation and aggressiveness may help establish social hierarchies. Since 5-HT is associated with both types of behaviours, this suggests that 5-HT is also linked to social status and the establishment of hierarchies. However, it is dependent on social contexts and may be even dependent on the species as well (Kiser et al., 2012; Knutson et al., 1998). For example, in crayfish (*Astacus astacus*), losers in agonistic encounters do not fight other individuals in the short time after the original encounter, which can be reversed by 5-HT injections (Huber, Smith, Delago, Isaksson, & Kravitz, 1997). Also in this species, changes in social status result in changes in the 5-HT system (Edwards & Kravitz, 1997). This change in social status as a result of modifications of the system is also seen in other vertebrates (Larson & Summers, 2001). In zebrafish, while manipulations of the system, namely increases of serotonin levels via treatment with fluoxetine, did not result in changes in social status, it did result in decreased aggression of dominants and, in subordinates, reduced freezing and increased aggression in some individuals (Theodoridi et al., 2017). Higher 5-HT levels have also been associated to lower social status (Dahlbom et al., 2012; Loveland, Uy, Maruska, Carpenter, & Fernald, 2014; Sørensen et al., 2012; Winberg & Nilsson, 1993b). However, this may be dependent on brain areas. For instance, dominant individuals have higher 5-HT activity in the telencephalon (Teles, Dahlbom, Winberg, & Oliveira, 2013). Interestingly, there also seems to be differences between sexes, with males exhibiting higher brain 5-HT activity in zebrafish (Dahlbom et al., 2012) and in the bluebanded goby (*Lythrypnus dalli*) (Lorenzi, Carpenter, Summers, Earley, & Grober, 2009). This association between domination/subordination and 5-HT is also present in mammals, including humans. Studies where the 5-HT system were manipulated whether by tryptophan supplementation or by treatment with drugs showed a reduction

in dominant behaviours (aan het Rot et al., 2006; Knutson et al., 1998), although higher social status can also be achieved via cooperation which is linked to high 5-HT levels (Raleigh, McGuire, Brammer, Pollack, & Yuwiler, 1991).

3.6.2. Stress and anxiety

Much like aggression and sociality, stress is also associated with the 5-HT system. In general, stress results in higher 5-HT activity in the brain (Backström & Winberg, 2017; Blanchard et al., 2001; Winberg & Nilsson, 1993a). Different animal models and protocols have been used to study various stressors and their link with 5-HT. For example, the chronic unpredictable stress (CUS) model, which consists of exposing individuals to several random stressors over the course of several weeks, is used in rodents as a model of anxiety and depression (Monteiro et al., 2015). Recently, the involvement of 5-HT receptors (more specifically 5-HT_{2C}) was studied using an agonist and an antagonist and was found that the former had anxiolytic properties while the latter had anti-depressive activity (Wankhar et al., 2020). However, in another model widely used to test anxiety-like behaviours – the elevated plus-maze - 5-HT_{2C} agonists were tested via direct injections into the ventral (VH) and dorsal hippocampus (DH) and was reported anxiogenic effects in the former (Alves et al., 2004; Scarpelli et al., 2008) but not in the latter (Alves et al., 2004). This suggests different roles for different areas when it comes to anxiety (Alves et al., 2004; Ohmura et al., 2020). In zebrafish, there is some evidence for a participation of this receptor in anxiety- and fear-like behaviours as well: (Silva et al., 2021) showed that treatment with MK-212 and WAY-161503, another 5-HT_{2C} receptor agonist, blocked alarm reactions in zebrafish, while RS-102221 blocked part of this response as well as anxiety-like behaviour in the novel tank test after exposure to an alarm substance.

As aforementioned, there is a great number of studies published linking social status and 5-HT levels and activity. The interaction with conspecifics within a hierarchy is in itself a stress factor – social stress. Subordinate individuals, given they are the ones that suffer most in a hierarchy, whether being due to attacks/chases, restricted access to resources, are the ones more subjected to chronic social stress (Backström & Winberg, 2017) and 5-HT also is connected to this. Indeed, in teleosts, subordinate individuals typically have higher activation of the 5-HT system (Dahlbom et al., 2012; Loveland et al., 2014; Sørensen et al., 2012; Winberg & Nilsson, 1993b) and exhibit several anxiety-like behaviours (*e.g.*, reduced locomotor activity, freezing, bottom-dwelling, reduced appetite and food intake …) which are thought to be associated with the 5-HT system (Winberg & Nilsson, 1993a). However, the formation of a hierarchy

through agonistic encounters is also a stress factor in winners, as they have a rapid activation of the 5-HT system and of plasma cortisol levels (Øverli et al., 1999). The changes in 5-HT in the brain of individuals under stress do not occur in equal manner throughout the various areas, as some areas may be more impacted by others (Emerson, Kappenman, Ronan, Renner, & Summers, 2000; Øverli et al., 2001; Teles et al., 2013). Changes that occur in the 5-HT profile of the individuals and that are created by social stress are also mirrored by other stress factors, such as restraint, as most areas impacted by one (hippocampus, nucleus accumbens, locus ceruleus) are also impacted by the other (Emerson et al., 2000).

5-HT functions in instances of stress are also dependent on stress coping styles. Typically, two different coping styles are described: proactive and reactive (Koolhaas, De Boer, Buwalda, & Van Reenen, 2007). Proactive individuals tend to be more aggressive, bolder and more plastic, whereas reactive individuals are characterized by behavioural inhibition (Backström & Winberg, 2017; Koolhaas et al., 2007). In general, proactive individuals are thought to have lower brain 5-HT levels and reactive individuals to possess higher (Butler, Whitlow, Roberts, & Maruska, 2018), but the association between stress coping style and 5-HT concentrations is also dependent on brain areas. For example, in two strains of rainbow trout selectively bred for high (HR, reactive) and low (LR, proactive) stress responsiveness, differences in 5-HT functions occur. HR individuals have higher 5-HT concentrations and turnover in the brain stem and in the optic tectum than LR fish, but less so in the hypothalamus (Øverli et al., 2001). A similar situation, when it comes to different 5-HT levels in relation to stress coping styles, occurs also in the Atlantic salmon (Thörnqvist, Höglund, & Winberg, 2015).

3.6.3. Social plasticity and aversive brain system

One of the most interesting abilities animals possess is the regulation of their behaviour in regards to different social contexts from previous experiences in order to optimize their relationships with another individual or group, an ability called social plasticity (Oliveira, 2012). Studies in several fish species showed correlations between social plasticity and the 5-HT system and also differences between brain regions. For instance, treatment with SSRIs, which inhibit serotonin reuptake and, therefore, increase the extracellular levels of 5-HT, produces alterations in aggressive behaviours of both dominant and subordinate individuals in fish (Theodoridi et al., 2017), as well as other animal groups, such as lizards (Larson & Summers, 2001) and mammals (Knutson et al., 1998). These changes can even be responsible for altering social

status (Edwards & Kravitz, 1997; Larson & Summers, 2001). Additionally, in green anole (*Anolis carolinensis*), previous social interactions can not only have great impact on the individuals' social status but more so than their stress coping style (Korzan & Summers, 2007).

These changes in behaviour as a result of social interactions are usually accompanied by neural plasticity, that is, the ability of an individual's CNS to adapt itself in response to different stimuli. This is observed in several different fish species, such as the zebrafish and cichlids, with differences in cell proliferation (Maruska et al., 2012), neural activation (Miller et al., 2017), gene expression (Carpenter, Maruska, Becker, & Fernald, 2014; Maruska, Zhang, Neboori, & Fernald, 2013; Renn, Aubin-Horth, & Hofmann, 2008; Teles, Cardoso, & Oliveira, 2016), and changes in the functional connectivity along the social decision-making network (SDMN), an evolutionarily conserved network in vertebrates which encompasses the social behaviour network and the mesolimbic reward system in the forebrain and midbrain and whose pattern of activation underlies the expression of a specific behaviour (Teles, Almeida, Lopes, & Oliveira, 2015). While still on the topic of social decision-making, this concept is very much important for social plasticity as an individual, in different social contexts, is forced to choose between different behaviours in order to achieve a certain goal. 5-HT is involved in these choices. For example, alteration of 5-HT levels in different taxa has been associated with changes in aggression (aan het Rot et al., 2006; Audero et al., 2013; Clotfelter et al., 2007; Dennis et al., 2013; Higley et al., 1996; Lee & Goto, 2018; Perreault et al., 2003; Theodoridi et al., 2017) while, in humans, individuals with reduced 5-HT levels showed more negative reactions to unfairness without compromises in other emotional aspects (Crockett, Clark, Tabibnia, Lieberman, & Robbins, 2008) and, in another study, decreased cooperation (Wood et al., 2006).

As mentioned, the reward system can be associated with the roles of 5-HT, but 5-HT is also associated with aversion, making understanding the how exactly 5-HT affects reward processing difficult (Cohen, Amoroso, & Uchida, 2015; Fischer & Ullsperger, 2017; Hu, 2016). This association between 5-HT and aversion is often seen as behavioural inhibition in aversive situations, which associates stimuli and punishments, regardless of responses (Cohen et al., 2015; Crockett, Clark, Apergis-Schoute, Morein-Zamir, & Robbins, 2012; Crockett & Cools, 2015; Fischer & Ullsperger, 2017). Even though most evidence comes from studies in mammals, this link has been observed in teleosts, despite poor understanding of 5-HT innervations of

the brain regions associated with the reward system in this taxon. For example, inhibition of 5-HT transmission from the ventral habenula to the median raphe in zebrafish blunted adaptive avoidance learning while stimulation of ventral habenula neurons and subsequent triggering of the aversive expectation signal resulted in place avoidance behaviour (Amo et al., 2014). Moreover, both 5-HT_1 (namely 5-HT_{1A}) and 5-HT_2 receptors have been associated with regulation of aversive behaviours in this species (Maximino, Puty, et al., 2013; Nowicki et al., 2014).

4. Objectives and predictions

As shown above, 5-HT and social status alike play a role in modulating behaviour. However, there is still much to learn from fish models in behavioural sciences since a large portion of the knowledge regarding anxiety and 5-HT comes from studies in rodents and primates (Bacqué-Cazenave et al., 2020). Additionally, the link between 5-HT and social status needs to be better understood as most studies only offer a more general view (Dahlbom et al., 2012; McDonald et al., 2011; Øverli et al., 1999; Winberg & Nilsson, 1993a). Here I aim to provide a more in depth look specifically to the 5-HT receptor subtype (5-HT_{2C}) pathways, which has been associated in the past with anxiety (Alves et al., 2004; Heisler et al., 2007; Kimura et al., 2009), but has been understudied in fish. The goal of this study was to establish how 5-HT modulation and subordination could impact the anxiety-like behaviour of adult zebrafish using the novel tank test. Furthermore, sex was also taken as a factor in our study due to its importance in many aspects of biology overall and behaviour in particular, despite disparity found in the published literature.

I predicted that the exogenous administration of a selective 5-HT_{2C} receptor agonist MK-212 would increase anxiety-like behaviours, while the administration of the 5-HT_{2C} receptor antagonist RS-102221 would have anxiolytic properties, given the consensus surrounding these drugs found in the available literature. Furthermore, I expected that the stress caused by subordination would cause an increase in anxiety-like behaviours in comparison to control and dominant individuals. Additionally, it was also expected that the effects caused by the administration of both agonist and antagonist would be more substantial in subordinates than in dominants. Regarding sex, it is difficult to predict on whether or not it would affect the behaviour of the individuals since previous studies do not report sex differences in anxiety-like behaviours (Bozi et al., 2021), while others report subordinate males to be more stressed than subordinate females (Tea et al., 2019) or report that non-stressed female individuals are more anxious than males (Fontana et al., 2020). Nevertheless, I predict that there would not be sex differences in the behaviours, based on work reported in Bozi et al., (2021).

5. Materials and Methods

5.1. Animal handling

In total, 148 adult zebrafish were used for this experiment. Animals were obtained from a commercial breeder licensed for aquaculture and kept in laboratory for 4 weeks for acclimatization before the beginning of the experiments. During this acclimatization, the individuals were kept in mixed-sex 40 L tanks, with a maximum density of 25 fish per tank, and in a 14h light/10h dark photoperiod. The water present in the tanks was kept at room temperature (28 ºC), with a pH of 7.0-8.0, and water parameters were routinely monitored and kept at recommended levels (Lawrence, 2007). All experiments were carried out with consideration for fish welfare and approved by UEPA's IACUC under protocol 06/18.

5.2. Dyadic encounters and establishment of hierarchy

On day 0, immediately prior to dusk, 20 individuals were randomly moved in pairs to 10 experimental tanks (20 cm × 14.5 cm × 12.5 cm) and left overnight to acclimatize with the tank, recover from stress and return to normal behaviour. They were kept in these tanks for the next 5 straight days and allowed to establish dominance hierarchies. Throughout the 5 days, the individuals were observed for any putative physical injuries resultant of the interactions or other signs of poor health, in which case, the pair would be taken of the experiments and be substituted, so that the injured individual could be treated. However, no substitutions were needed to be done during the experiments. This time period has been shown in previous experiments to be sufficient for the establishment of hierarchies in this species (Bozi et al., 2021; Filby et al., 2010; Paull et al., 2010). On the morning of the 5th day, all experimental tanks were videorecorded for 5 minutes and X-Plo-Rat (Tejada, Chaim, & Morato, 2017) was used to assess different behaviours, following the work described in Pavlidis, Sundvik, Chen, & Panula, (2011) to determine dominant and subordinate status. The assessed behaviours were: 1) Chasing - the number of times one of the individuals swims directly toward the other individual and pursues it during the recorded 5 minutes (Paull et al., 2010); 2) Bottom-dwelling defined as the time the fish dwelled on the bottom third of the experimental tank (Bozi et al., 2021); 3) Freezing, defined as the time spent without moving in any direction (Blaser & Gerlai, 2006); and finally 4) Attacks – the number of agonistic events, with or without association to biting (Paull et al., 2010) (Fig.4). The control fish (n=48) were subjected to the treatments but were not subjected to the

dyadic encounters, having been transferred directly to the novel tank for the next phase of the experiments. After the video analysis, the individual that exhibited the highest frequency of chasing events and attacks and the lowest time spent freezing and/or bottom-duelling was considered the dominant one from the pair, and the individual that showed the inverse frequencies was considered to be the subordinate. This procedure was then repeated for another 4 rounds, signifying a total of 100 individuals used in the experiment.

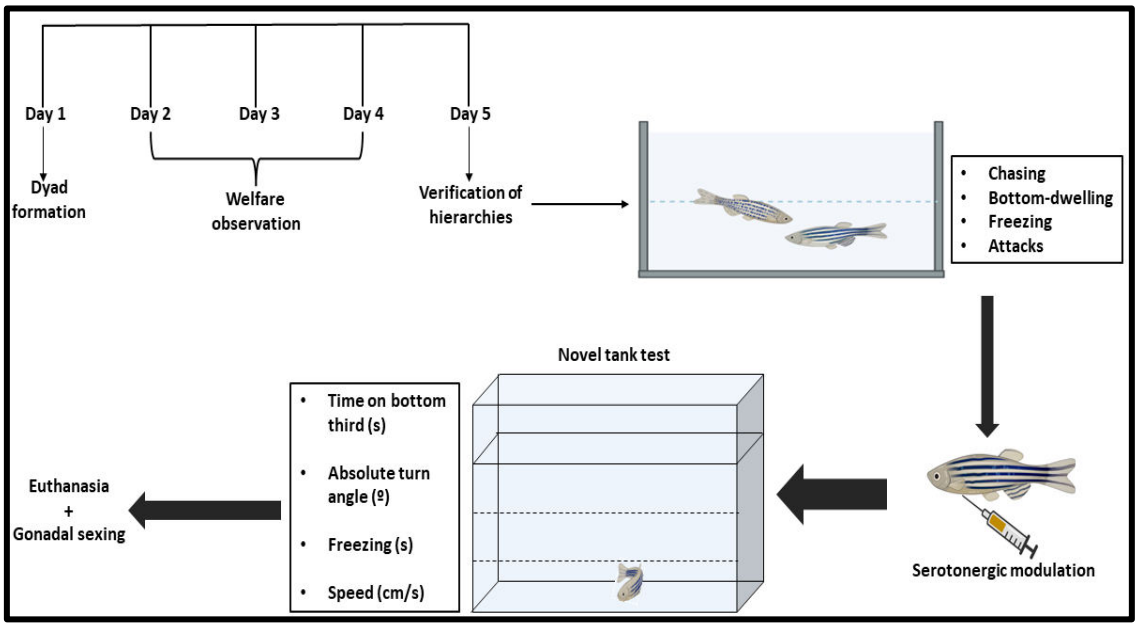

Figure 4. Representation of the experimental design. Dyads were allowed to be established on the experimental tanks on Day 1 and verification of hierarchy formation was done on Day 5 (the welfare of the individuals were monitored in between those days). The individuals were then moved into a novel tank test and observed for anxiety-like behaviour. Following the test, gonadal sexing took place. See "Material and Methods" for further information. Image adapted from (Bozi et al., 2021).

5.3. 5-HT modulation

The individuals underwent one of the following treatments: Cortland's salt solution, DMSO, MK-212 (2 mg/kg) and RS-102221 (2 mg/kg). The 5-HT_{2C} receptor agonist MK-212 was bought from Sigma-Aldrich (St Louis, USA) and dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin) (Wolf, 1963). The 5-HT_{2C} receptor antagonist RS-102221 was bought from Sigma-Aldrich (St Louis, USA) and dissolved in DMSO. Cortland's solution was used as a control for MK-212 and DMSO as a control for RS-102221, in accordance to their solubility. The individuals were anesthetized with colder water and the treatments were administrated intraperitoneally, as described in Kinkel, Eames, Philipson, & Prince, (2010). The treatments were randomly distributed in order to assure that, in each round

of dyadic encounters, there were always individuals that received each one of the 4 treatments. Animals were injected with one of the drugs or either of the vehicles immediately after recording of the last contest, and left to rest for 30 min. for the onset of the drug effect before being subjected to the novel tank test (Fig.4).

5.4. Novel Tank Test (NTT)

After ascribing dominant or subordinate status to the experimented individuals and injections, all animals (including the ones used as control) were carried out to the following phase of the procedures, the Novel Tank Test, used to assess anxiety-like behaviour. Each animal was then individually transported to a new aquarium (5 cm×24 cm×20 cm) and allowed to freely explore the space for 6 minutes. Their behaviour during this interval was recorded using a video camera positioned directly in front of the tank. The video recordings were filed and analysed using TheRealFishTracker, an automated video tracking software (available from http://www.dgp.toronto.edu/~mccrae/projects/FishTracker/). The assessed behavioural variables were the time spent on the bottom third of the tank (measured in seconds), the erratic swimming (absolute turn angle, measured in degrees(Bozi et al., 2021; Tran et al., 2017)), total amount of time spent freezing (measured in seconds), which was considered to happen whenever the individual would swim at a speed lower than 0.5 cm/s, and the average speed (measured in cm/s) (Fig.4). The time spent on the bottom third of the tank was the primary outcome, while the remaining variables were secondary. Speed was used as an outcome to assess any potential effects of the treatments on the animals' locomotion.

5.5. Euthanasia and gonadal sexing

Following the experimental design, the sex of the individuals was confirmed via observation of the individuals' gonadal tissue under a light microscope (Leica CTR4000). The animals were sacrificed by immersing them in ice-cold water, followed by spinal transection (Matthews & Varga, 2012). The individuals were then fixed in Bouin's fixative for 24h. To be better fixed, an incision was made on the ventral side of the individual. The fixed tissues were included in meth-acrylate glycol and sections were cut at 5 µm slices, which were collected onto glass slides. Following these steps, the slices were stained with hematoxylin-eosin to be observed under a microscope (Bozi et al., 2021). The oocytes were classified following the guidelines and descriptions presented in Selman, Wallace, Sarka, & Qi, (1993).

5.6. Statistical Analysis

The information regarding sex, treatment, status and measurements of the dependent variables (time spent in bottom third of the tank, absolute turn angle, freezing and speed) were compiled onto a table. The table was then imported to SPSS v.27 (IBM Corp, 2020). The data were first plotted to observe any qualitative differences between males and females in the different groups. The rationale for visual inspection is that there is evidence suggesting that differences seen between male and female brains may be insufficient and misleading for establishing relationships between sex and brain (Joel & Fausto-Sterling, 2016). Furthermore, these differences (both in humans and animals used in scientific research) may arise from one heterogeneous group rather than two separate ones. As such, it is suggested to observe for any possible interactions between sex and treatment and, if no interactions are suggested, the sex category should be omitted from the results' analysis (Joel & Fausto-Sterling, 2016). The preliminary plots showed no major differences between sexes in any group (Figs. 5-8) and, as such, the variable "Sex" was not included in the subsequent analyses.

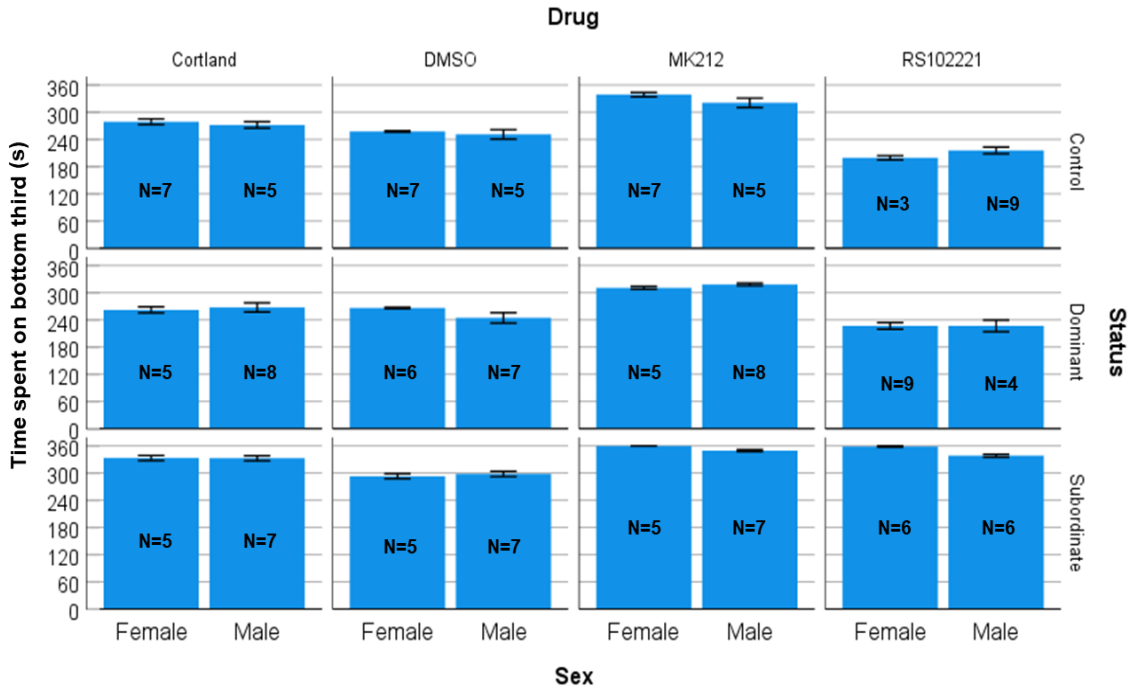

Figure 5. Preliminary plot of Time spent in bottom third by sex, status and drug treatment. Bars represent the means while the error bars represent the standard errors of the mean. N = number of individuals. Graph obtained in SPSS v.27.

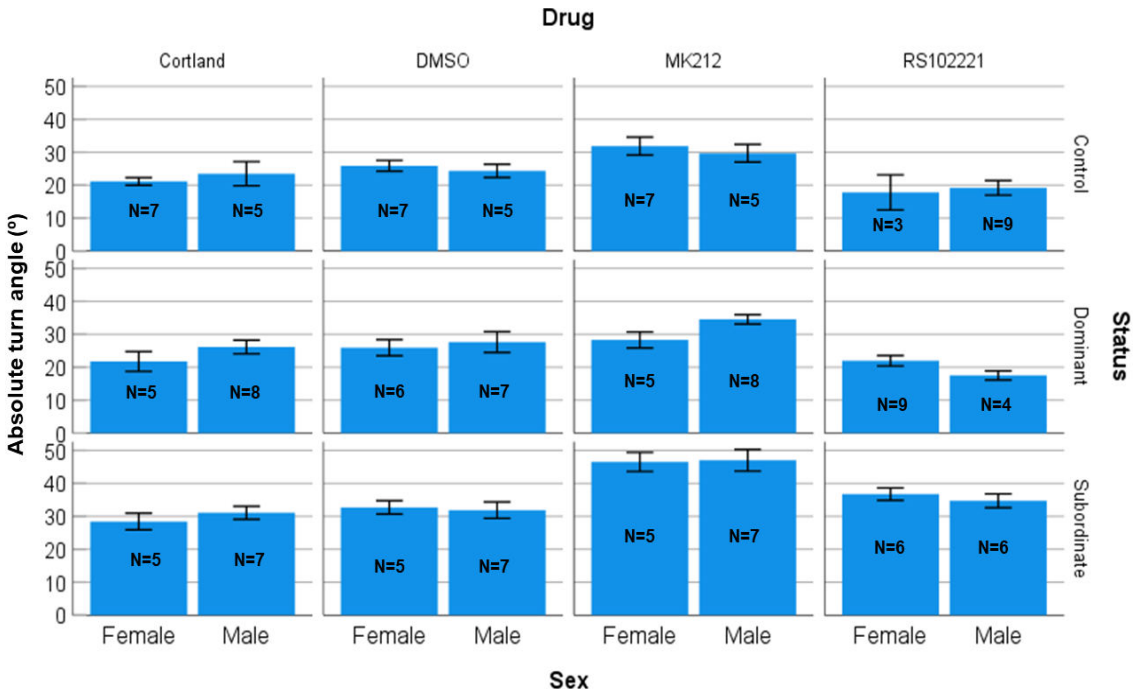

Figure 6. Preliminary plot of Absolute turn angle by sex, status and drug treatment. Bars represent the means while the error bars represent the standard errors of the mean. N = number of individuals. Graph obtained in SPSS v.27.

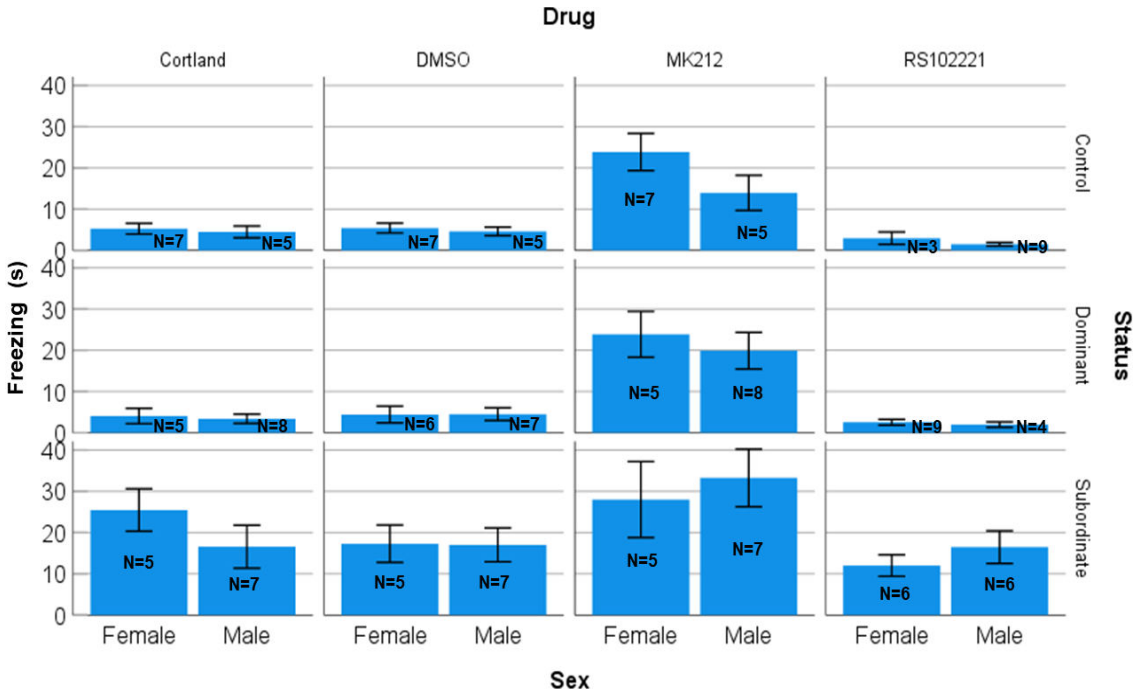

Figure 7. Preliminary plot of Freezing by sex, status and drug treatment. Bars represent the means while the error bars represent the standard errors of the mean. N = number of individuals. Graph obtained in SPSS v.27.

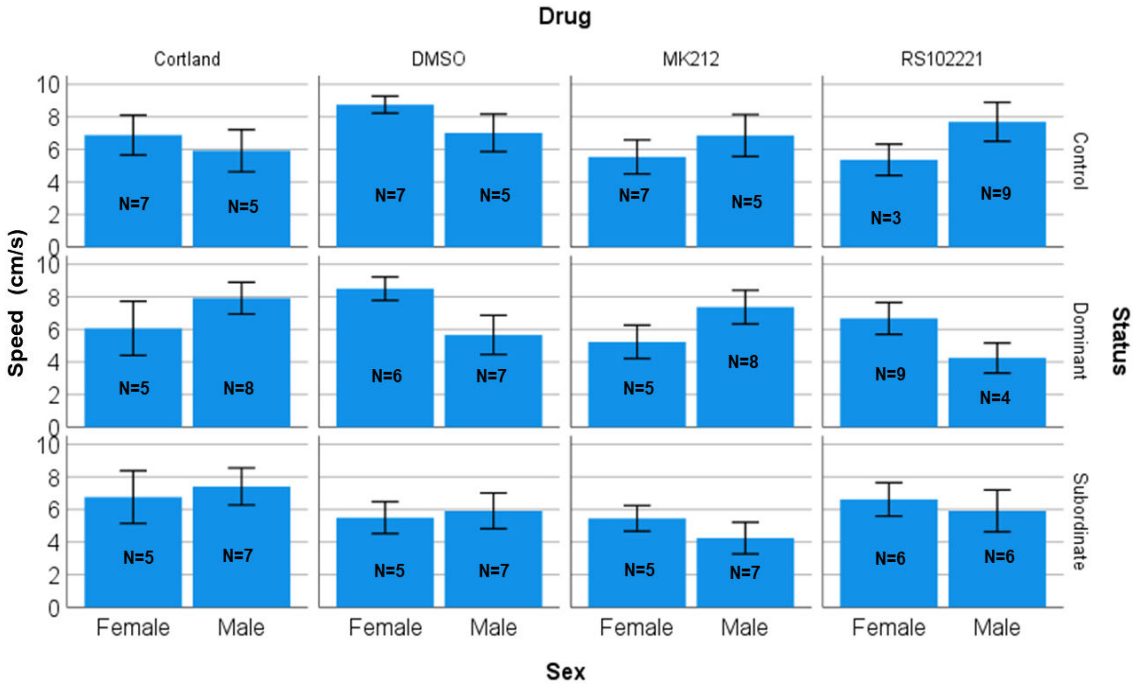

Figure 8. Preliminary plot of Speed by sex, status and drug treatment. Bars represent the means while the error bars represent the standard errors of the mean. N = number of individuals. Graph obtained in SPSS v.27.

Levene's tests were performed to check the homogeneity of variances on R v4.1.3 (R Core Team, 2022). For the variables "Time spent on the bottom third "and "Freezing", the Levene's tests showed that the variances were significantly different between the groups ($F(11, 136) = 1.94$, $p = 0.039$ and $F(11, 136) = 9.97$, $p = 3.937e^{-13}$, respectively). For the "Absolute Turn Angle" and "Speed" variables, the variances were homogenous ($F(11, 136) = 0.92$, $p = 0.618$ and $F(11, 136) = 0.34$, $p = 0.974$, respectively). Since the Levene's test for two of the variables indicated that the variances were significantly different (Pr (>F) <0.05) and remained that way despite attempts at transforming the data, the homogeneity of variances could not be assumed. Therefore, pairwise robust tests were chosen to be performed as post-hoc tests (Field, Miles, & Field, 2012). These tests possess one advantage in these scenarios and that is they do not make any sort of assumptions (*e.g.* homogeneity of variances, normality, etc). A robust two-way analysis of variance (ANOVA) on the variables "Drug" and "Status" was performed for every dependent variable, using the function *t2way*(), as well as pairwise post-hoc tests, using the *mcp2atm()* function. Both functions are present in the *WRS2* package. (Mair & Wilcox, 2020).

6. Results

6.1. ANOVAs results

Both social status and manipulation of 5-HT levels had a significant on the "Time spent in bottom third" variable, as well as for the "Absolute turn angle" and "Freezing" variables (Table 2). However, on the variable "Speed", neither "Status" nor "Drug" had a significant effect (Table 2). Additionally, there was only a significant interaction effect for the time spent in the bottom third of the tank (Table 2).

Table 2. Two-way ANOVA results (α=0.05).

Variable	Effect	Q value	p-value
Time spent in bottom third	Status	467.6353	0.001
	Drug	354.1668	0.001
	Interaction	162.3543	0.001
Absolute turn angle	Status	73.1301	0.001
	Drug	58.5263	0.001
	Interaction	13.3102	0.086
Freezing	Status	27.1897	0.001
	Drug	34.7118	0.001
	Interaction	4.3143	0.689
Speed	Status	3.1111	0.225
	Drug	5.3040	0.177
	Interaction	8.1637	0.303

6.2. Bottom-dwelling

As shown in Table 2, significant effects of status, drug and a status X drug interaction were found. The post-hoc tests showed that control and dominant individuals behave similarly when comparing Cortland's solution vs DMSO (p=0.176) and the same was found when comparing control and subordinate individuals (p=0.648). However, the difference in the time spent at the bottom significantly differed between dominant individuals and subordinates (p=0.025). Visual analysis of Fig.9., regardless of individual social status, the mean of the individuals treated with the saline control is higher than the mean of the individuals treated with DMSO. The pairwise tests revealed significant differences between control treatments, Cortland's solution and MK-212 (agonist), DMSO and RS-102221 (antagonist) and MK-212 and RS-102221 ($p<0.001$ in all comparisons) (Fig.9).

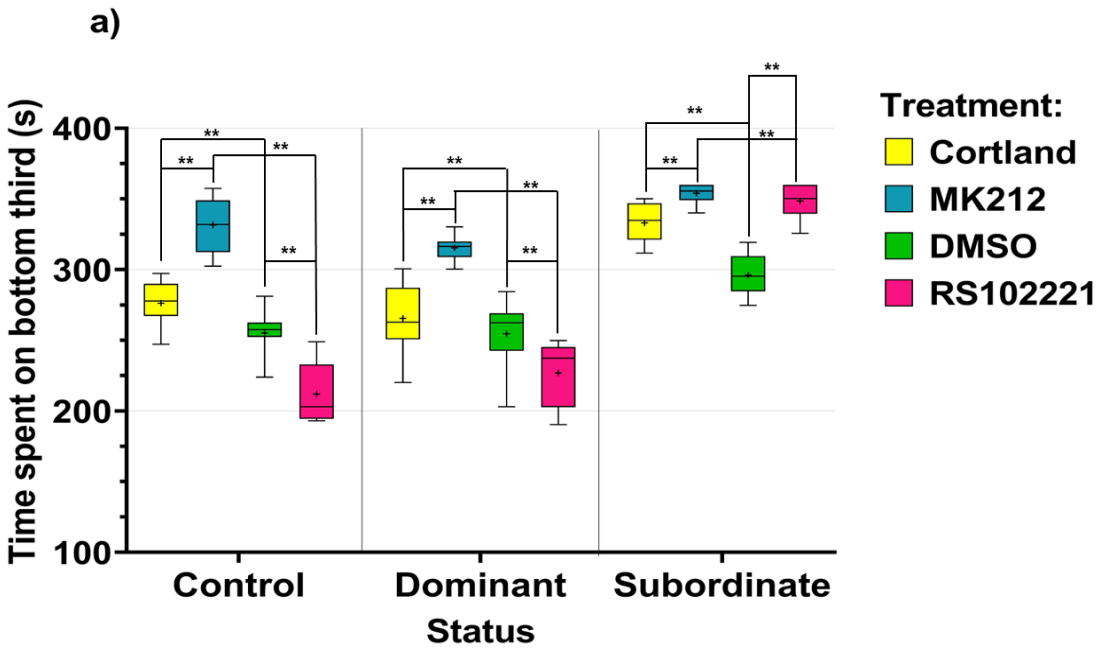

Figure 9. Effects of social status and drug treatments on Time spent in bottom third. Box plots show median and quartiles, whiskers represent the lowest and highest value. Means are represented by "+". **P<0.001. Graph obtained in GraphPad Prism v.9.3.1. (GraphPad Software, n.d.).

The post-hoc tests also revealed similar results for Cortland's solution vs agonist. The difference in the time spent on the bottom did not change between control and dominant individuals (p=0.071) nor between control and subordinates (p=0.189), but changed significantly between dominant and subordinates (p=0.011), which suggests that the 5-HT_{2C} agonist also impacts differently depending on the status.

Visual analysis of Fig.9 reveals a difference between the treatments in dominant individuals compared to subordinates.

The post-hoc tests showed significant differences in the time spent in the bottom third in DMSO vs antagonist for all social status comparisons ($p=0.012$ in controls vs dominants; $p=0.006$ in controls vs subordinates; $p<0.001$ in dominants vs subordinates). In subordinates, the 5-HT_{2C} antagonist presents higher times spent in the bottom third in comparison to the respective control-treated individuals.

No significant differences were found in agonist vs antagonist between controls and dominant individuals ($p=0.614$). However, significant differences were found in agonist vs antagonist between subordinates and control individual ($p<0.001$), as well as between subordinates and dominant individuals ($p<0.001$). Visual analysis of Fig.9 for comparisons between control and dominant individuals, reveals a difference in the time spent between agonist and antagonist, with the former having the highest values and the latter the lowest. Interestingly, this contrast is small in subordinates, with little difference between the means. In reality, regardless of the treatment, subordinates exhibited higher times spent in comparison to controls and dominant individuals.

6.3. Erratic swimming

The post-hoc tests revealed significant differences between social status ($p<0.001$ in control vs subordinates; $p<0.001$ in dominants vs subordinates; $p=0.226$ in controls vs dominants) in erratic swimming. The post-hoc tests also revealed significant differences between Cortland and agonist ($p<0.001$), DMSO and antagonist ($p=0.047$), and agonist and antagonist ($p<0.001$) but not between controls ($p=0.064$). Subordinate individuals displayed more erratic swimming compared to control and dominant individuals, and MK-212 increased erratic swimming across all statuses. RS-102221 decreased erratic swimming in both controls and dominant individuals, but not in subordinates (Fig.10).

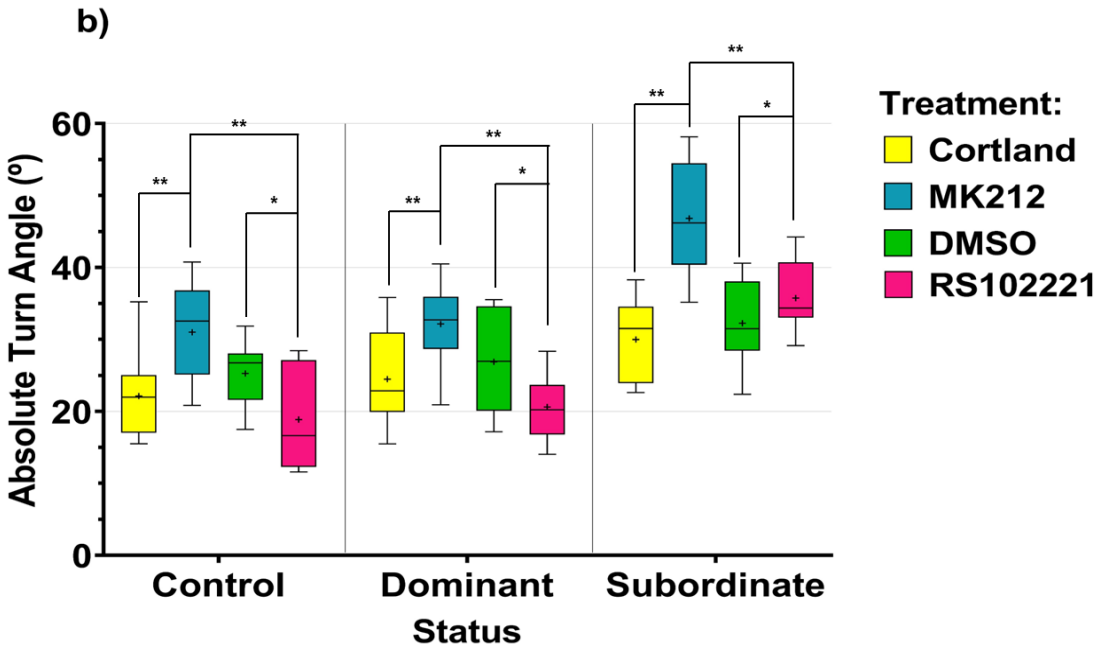

Figure 10. Effects of social status and drug treatments on Absolute turn angle. Box plots show median and quartiles, whiskers represent the lowest and highest value. Means are represented by "+". *P<0.05. **P<0.001. Graph obtained in GraphPad Prism v.9.3.1. (GraphPad Software, n.d.).

6.4. Freezing

The post-hoc tests revealed significant differences between individuals of different social statuses (p<0.001 in control vs subordinates; p<0.001 in dominants vs subordinates). The test also showed significant differences between Cortland's salt solution and MK-212 (p<0.001) and MK-212 and RS-102221 (p<0.001), but not between controls (p=0.775) nor between DMSO and RS-102221 (p=0.071; Fig.11).

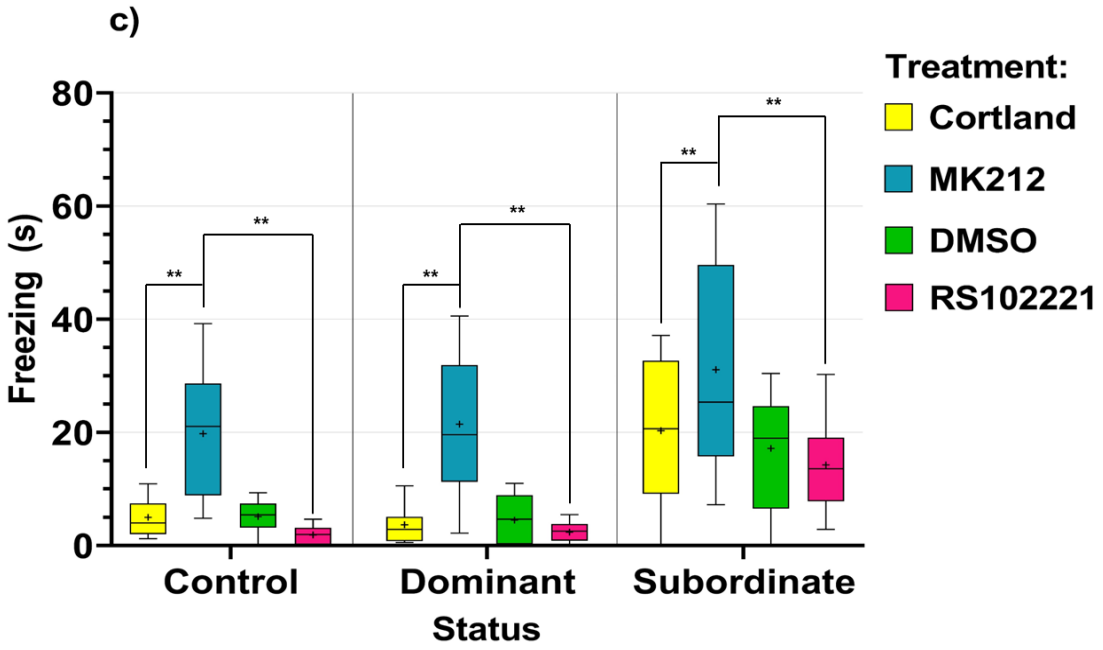

Figure 11. Effects of social status and drug treatments on Freezing. Box plots show median and quartiles, whiskers represent the lowest and highest value. Means are represented by "+". **P<0.001. Graph obtained in GraphPad Prism v.9.3.1. (GraphPad Software, n.d.).

In general, subordinate individuals showed more freezing, and MK-212 increased freezing in all individuals. RS-102221 decreased freezing in controls and dominant individuals, but not subordinates.

6.5. Swimming speed

As shown, the two-way ANOVA design employed with the *t2way()* function demonstrated that neither social status nor the drug treatments had significant effects on the swimming speed of the individuals (Table 2; Fig. 12). As a result, no post-hoc tests were conducted.

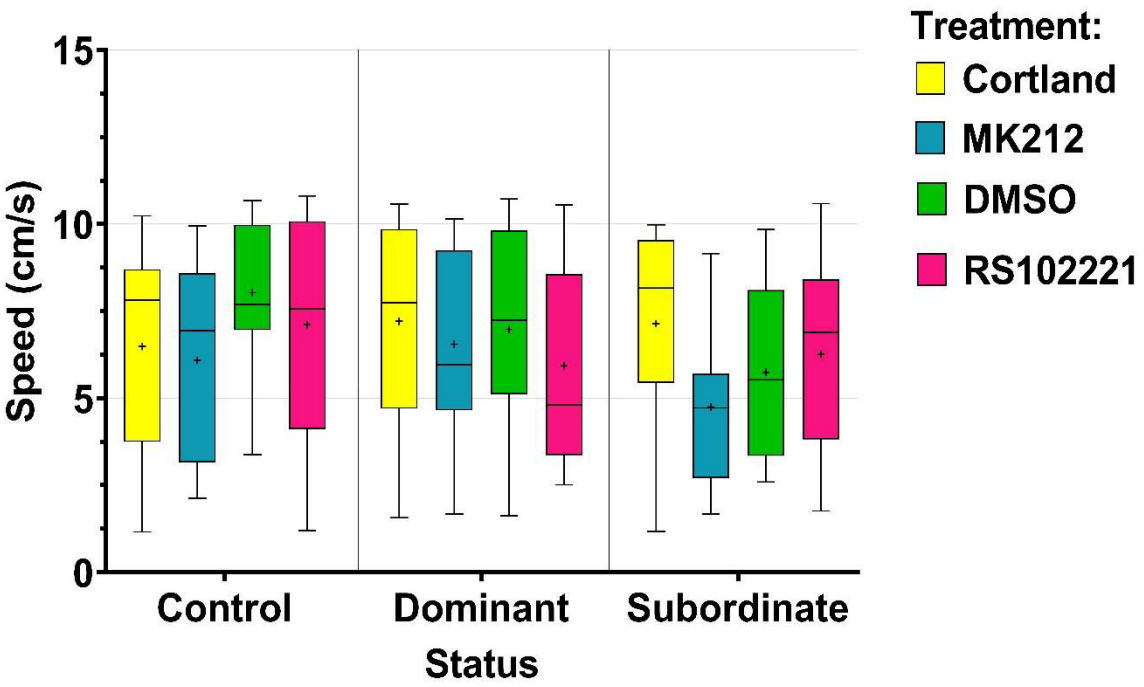

Figure 12. Effects of social status and drug treatments on Speed. Box plots show median and quartiles, whiskers represent the lowest and highest value. Means are represented by "+". Graph obtained in GraphPad Prism v.9.3.1. (GraphPad Software, n.d.)

7. Discussion

In the present study, the role of 5-HT and its relation with social status on zebrafish anxiety-like behaviours were studied using a novel tank test. For that end, social hierarchies were established and the 5-HT system was manipulated using a 5-HT_{2C} receptor agonist and antagonist (MK-212 and RS-102221, respectively).

In this study, no major differences were found between sexes after preliminary, qualitative observations in all behavioural variables studied. Similar results were observed in other studies in zebrafish. For instance, differences between sexes were found to be absent in regards to the effect of social status on anxiety (Bozi et al., 2021). Additionally, in Reolon et al., (2018) it was found that the time spent in zones divided vertically (which includes time spent on the bottom third of the tank) did not differ significantly between sexes. However, as mentioned before, the influence of sex in the behaviour of this species has not been consistent. For example, females have been recently shown to be more anxious than males (Fontana et al., 2020). Another study in which individuals were exposed to ethanol prior to behavioural testing reported sex differences in wild-type individuals chronically exposed (Dlugos, Brown, & Rabin, 2011). Moreover, Winberg & Thörnqvist, (2016) report an experiment in which 5-HT was modulated via treatment with fluoxetine and the results described therein indicate gender differences in several anxiety-like behaviours. Additionally, in a more recent study, male subordinate zebrafish were shown to have higher cortisol levels and reduced cell proliferation in the forebrain, which was not seen in female subordinates (Tea et al., 2019). There are several possible reasons for the disparities between studies in relation to the topic of the present study: i) sex has different impacts on anxiety depending on the stressor (e.g., basal anxiety vs. stress-induced increases in anxiety); ii) sex may have a factor in the physiological mechanisms of anxiety that are not detectable with sufficient sensitivity in the behavioural level; iii) differences between methodologies, as we employed a novel tank test and observed different behavioural variables whereas in Dlugos et al., (2011) clustering distance was used as the way to measure anxiety, and in Winberg & Thörnqvist, (2016) the open field test was used; iv) differences between strains, group sizes, age of the studied individuals or other factors that could have a significant interaction with sex in anxiety. Additionally, when comparing our results to those presented in Winberg & Thörnqvist, (2016), another possibility for the discrepancies observed, particularly for freezing/immobility, is for instance the different pharmacological compounds and different circuits aimed: in their study, fluoxetine was used, which as an SSRI, impacting 5-HT reuptake whereas we

targeted the 5-HT_{2C} receptors specifically. These overall discrepancies between studies should warrant further analyses, as it has also been suggested recently in Genario et al., (2020).

Subordination resulted in increased anxiety-like behaviours, with subordinates exhibiting, regardless of treatment, more time spent bottom-dwelling, more erratic swimming and more freezing behaviour. This is consistent with other studies. For example, in Bozi et al., (2021), hierarchies were formed through dyadic encounters, in the same manner as reported here. After the last encounter, a Novel Tank Test was also carried out with the individuals. In their study, they observed that subordinates spent more time bottom-dwelling than dominants, exhibited increased absolute turn angles and freezing behaviour. Moreover, increases in these anxiety-like behaviours have also been associated in the past with other stressors studied in zebrafish. For instance, acute restraint stress (Ghisleni et al., 2012; Piato, Rosemberg, et al., 2011) and unpredictable chronic stress (Piato, Capiotti, et al., 2011) have been shown to increase bottom-dwelling, acute restraint stress (Ghisleni et al., 2012) and use of CAS (Lima-Maximino et al., 2020; Silva et al., 2021) have been shown to result in increased absolute turn angles in stressed individuals and freezing has also been seen as a submissive behaviour in the presence of a dominant individual (Pavlidis et al., 2011; Theodoridi et al., 2017). This freezing behaviour has also been associated with a reactive or passive coping style, which is typical of subordinate individuals, and this association seems evolutionarily conserved, as subordinate rats in diverse social stress paradigms showcase passive defensive behaviour highlighted by freezing for most of the time (Motta et al., 2009; Nyuyki, Beiderbeck, Lukas, Neumann, & Reber, 2012). It is also important to note that the injection itself may be an acute stressor. Unfortunately, due to logistical constraints, it was not possible to have a negative control, in which individuals would not go through any treatment, which would enable us to see if the injection itself would significantly alter the time spent bottom-dwelling. However, it would be expected that subordinates not given any treatment to spend more time bottom-dwelling than dominants, as well.

Differences were found between control vehicles for the time spent in the bottom-third. In fact, the average time spent in the bottom third by DMSO-treated individuals is lower than the average time by Cortland's solution-treated individuals for all social status. Given that Cortland's solution is a saline solution, this could possibly be attributable to a relatively mild osmotic shock created by it that doesn't impact much dominant individuals, but, in subordinates, has an additive impact in this variable to the

effect caused by their social rank. However, this should mostly be associated to the effect of DMSO, as this solution has shown to induce reductions of anxiety-like behaviours in the novel tank test (Nowicki et al., 2014), as well as in the light/dark plus maze paradigm (Sackerman et al., 2010).

Overall, the treatment with the specific 5-HT_{2C} receptor agonist MK-212 resulted in increased anxiety across groups, showcased by a significant rise in time spent in the bottom third of the experimental tank, erratic swimming, and time spent freezing. This is consistent with a general anxiogenic effect of MK-212 independent of social status. For example, in Silva et al., (2021), MK-212 was shown to block defensive reactions elicited by a conspecific alarm substance during exposure. In another fish species, the rainbow trout, inhibited food intake, which is another anxiety-like behaviour (Pérez-Maceira, Mancebo, & Aldegunde, 2014; Pérez-Maceira, Otero-Rodiño, Mancebo, Soengas, & Aldegunde, 2016). This drug also shows anxiogenic effects in rodents (Alves et al., 2004; De Mello Cruz et al., 2005; Kshama et al., 1990), as well as in humans (Lowy & Meltzer, 1988), highlighting the general effect of MK-212 and the evolutionary conservation of the role in anxiety of the 5-HT_{2C} receptors. However, for the time spent in bottom-third of the tank, there was a significant interaction effect between drug treatment and social status, meaning that the differences between levels of one factor depends on the levels of the other. For this variable, there were differences between dominants and subordinates on the effects of MK-212, with the increases in geotaxis in the former being higher than the increases in the latter (in comparison with Cortland's solution-treated individuals). This could be explained either by an important interaction between social rank and the treatment, or a ceiling effect on subordinate individuals. In the first case, dominant individuals seem more affected by the receptor agonism than subordinates. However, the second hypothesis cannot be discarded since, in Cortland's solution-treated subordinates, the mean time spent in the bottom third is close to 360 seconds, which is the upper limit for the measurements in this variable. As such, the increases in the mean time spent in the bottom third caused by the treatment with MK-212 in subordinates could not be very large (ceiling effect).

The treatment with the specific 5-HT_{2C} receptor antagonist RS-102221 caused a reduction of the time spent bottom-dwelling and of erratic swimming in control and dominant individuals, which is in agreement with other studies reporting anxiolytic properties of this drug (Bell et al., 2014; Kuznetsova, Amstislavskaya, Shefer, et al., 2006; Scarpelli et al., 2008; Warhaftig et al., 2021), while, in subordinates, it actually resulted in increased time spent bottom-dwelling and increased erratic swimming,

suggesting that this drug may have an opposite effect in subordinate individuals of this species. Two possible reasons for such effect can be offered: the first is that there was an alteration (*i.e.,* reduction) in 5-HT levels in subordinates, since, as previously shown, subordinates spent more time in the bottom third of the tank than dominants and 5-HT levels have been shown to be negatively correlated with time spent bottom-dwelling (Maximino, Puty, et al., 2013). The second possible reason is that there was a desensitization of the receptors, which would be consistent with the results from MK-212. However, due to the limited number of studies conducted with this behavioural paradigm and with this drug, makes it difficult to compare to other results and to confidently associate a concrete reason for the loss of the anxiolytic property of this drug on this variable. As aforementioned, the interaction effect between drug treatment and social status was shown to be significant for time spent in bottom-third by the ANOVA, but it was not shown to be significant for the absolute turn angle, despite seemingly different effects in subordinates caused by the antagonist in comparison to the effects seen in the controls and dominants. The reason for this apparent difference in the effects of RS-102221 against those of DMSO between dominant and subordinates that is not picked up by the ANOVA could be due to the fact that ANOVA, simply stated, analyses the difference between the absolute values of two or more means and, as such, it does not take in consideration on which one is higher and which one is lower, meaning that it would not detect different effects of this drug between dominants and subordinates, or it could be due that control and dominant individuals exhibit similar measurements in this variable and, as such, could be influencing the result of the interaction. One way to observe if such is the case would be to do the ANOVA analysis without the control individuals and to check if the result for the interaction effect would change. Due to time constraints regarding the laboratory experiments and statistical analyses, as well as regarding the writing of the thesis, it was not possible to this second ANOVA and to analyse the results obtained from it.

The treatment with RS-102221 did not show decreases in freezing behaviour in any social status. For this variable, a reason for it is that the time spent freezing by individuals treated with DMSO was already relatively small, which meant that whatever changes in the behaviour as a result of 5-HT_{2c} receptor antagonism could not be very large. In subordinates, this could not be the case since the time spent freezing by subordinates treated with DMSO was noticeably higher but treatment with RS-102221 did not alter it significantly. In this case, this could mean that 5-HT_{2c} receptor antagonism with this drug was not sufficient, which could be due to pharmacological reasons inherent with the drug or due to the involvement of other 5-HT receptors. For

example, treatment with fluoxetine, a drug that, like RS-102221, has anxiolytic properties was shown to abolish this behaviour in subordinate individuals (Theodoridi et al., 2017), although in that last case freezing was observed in the presence of a dominant fish, which could change the behavioural meaning of this variable. Another possible explanation is that, similarly to the first two behavioural variables, there could have been a desensitization of 5-HT_{2C} receptors. Moreover, it is also possible that RS-102221 did have a significant effect in this variable but that effect was masked by the action of other 5-HT_{2C} or by the involvement of other neurotransmitters and modulators. Finally, it is possible that RS-102221 produces significant effects only in geotaxis and erratic swimming, and not freezing.

Conclusion

Results here demonstrate that individuals under social stress in the form of subordination show increased anxiety-like behaviours. Since this species is a social one, the establishment of social hierarchies and the effects subordination has on the behaviour and physiology of not just subordinates but also dominant individuals should be something to keep in mind when studying this species for a) welfare reasons and b) any potential influence it might have on any given study.

In addition, manipulation of the 5-HT system also impacted the behavioural output of the individuals, with the interaction between both factors being significant for the time spent in the bottom third of the tank. The treatment with the MK-212 with the selective 5-HT_{2C} agonist MK-212 increased the time spent in the bottom third of the tank, erratic behaviour (measured by the absolute turn angle) and freezing, with no significant changes in relation to swimming speed, while the treatment with the antagonist RS-102221 produced more complex results. The use of this drug had the expected anxiolytic results in the first two studied variables (bottom-dwelling and erratic swimming) but only in control and dominant individuals, whereas, in subordinates, it produced the opposite effect. In addition, no significant changes were found for freezing behaviour nor for swimming speed, this latter result signifying that the effects seen in the other variables are not likely due to changes in activity. The results obtained from the use of RS-102221 could indicate that this drug affects some but not all anxiety-like behaviours and that its action may be dependent on social status. Future work involving this drug is need to elucidate its effects on adult zebrafish and to possibly establish comparisons between fish and mammals.

Moreover, in this study, preliminary analyses involving the sex of the individuals and their effect on anxiety were conducted and no major differences between sexes were found. This is in accordance with recent papers but other studies in anxiety and its link with social status or 5-HT point to differences between males and females. In Genario et al., (2020), different open questions regarding sex differences in zebrafish were highlighted, with the discrepancy between tests regarding behavioural differences being one of them. This study presents itself as another reason for continued investment in this subject.

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