Oxytocinergic modulation of social information use in threat perception

Ibukun Dorcas Fatunke Akinrinade

Dissertation presented to obtain the Ph.D degree
in Integrative Biology and Biomedicine

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
July, 2020

UNIVERSIDADE NOVA DE LISBOA

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ITQB UNIVERSIDADE NOVA DE LISBOA

Cover image:

A depiction of the zebrafish with deficits in perceiving the information provided by its conspecifics.

Image design by Rodrigo Abreu.

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This work was conducted at Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal, under the supervision of Prof Rui Filipe Oliveira.

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Summary

Adaptive behaviour relies on the appropriate response to information received from a variety of environmental sources.

The social environment provides individuals with the opportunity to gather and use information resulting from cues or signals provided by the same or different species.

In the context of predator-prey interactions, individuals use information from the environment to perceive whether there is an imminent predator threat. Information on predator threats can be detected directly by the individual, and/or by observing the behaviour of others. Regardless of the source of information, making correct decisions about what defensive behaviour to adopt is essential for animal survival.

Gathering information individually, however, may entail costs because individually assessing the safety of the environment can be time and energy-consuming. Moreover, threat detection by an individual can be risky and can even result in the individual's demise.

On the other hand, acquiring information by observing the behaviour of others has been suggested to improve the efficiency of individual decision-making because observing others reduces the probability of erroneous individual decisions and has a lower cost. For example, animals in groups detect predators earlier than animals living solitarily, and therefore they can make the more timely decisions.

Although social information use has been suggested to be a ubiquitous factor in group-living animals, the underlying neurobiological substrates regulating this phenomenon are largely unknown, namely for threat perception.

In the set of studies that make up this thesis, we sort to explore the role of oxytocin – a neurotransmitter known to be involved in the modulation of social behaviour – in the perception of social information about predator threats using zebrafish (*Danio rerio*), a social species that has a repertoire of behavioural phenotypes, with highly conserved genes and is an emerging experimental

model in social neuroscience and neuroethology due to availability of several behavioural, genetic and imaging tools.

In the first study, we implemented and tested the experimental protocols for social information use in threat perception in zebrafish; we used the alarm substance – a chemical released by injured fish after an attack by a predator – to simulate a predator threat; we focused on the three processes of social information use in threat perception: the process of social buffering of fear, the process of social contagion of fear and the process of social facilitation of fear; we measured zebrafish responses to fear using two behaviours: freezing and erratic movement; and, finally, we investigated possible sex differences in zebrafish responsiveness to these processes. Our results described zebrafish responses to the different processes of social information use in predator threat detection and revealed sex differences.

Our next study aimed at exploring the role of oxytocin in social contagion of fear using zebrafish with constitutive deletions of the two oxytocin receptors and the ligand. Our results showed a deficit in freezing response in the three mutants. This deficit was restored in the ligand mutants upon pharmacological treatment with isotocin drug (the oxytocin-like peptide found in teleosts), confirming the role of oxytocin in social contagion of fear. Further investigation of possible brain areas involved in the regulation of this behaviour – among the brain areas of the Social Decision Making Network (SDMN) – implicated more significantly the ventral telencephalon, precisely the ventral nucleus of the ventral telencephalon (Vv) and central nucleus of the ventral telencephalon (Vc). Our results also showed differences in the SDMN functional connectivity in wild types and mutants during exposure to the sight of alarmed conspecifics. We also confirmed the presence of oxytocin receptors in the SDMN brain regions, particularly in the brain areas of the ventral telencephalon, and we confirmed the projection of oxytocin neurons into the ventral telencephalon by visualization of the projection patterns.

Finally, we explored the role of oxytocin in social buffering of fear in zebrafish with constitutive deletion of the oxytocin receptor. Our results showed a deficit in freezing response in mutants, suggesting the role of oxytocin in social buffering of fear. Investigation of possible brain areas, among the SDMN, involved in the regulation of this process implicated the dorsal nucleus of the ventral telencephalon (Vd) and the central nucleus of the ventral telencephalon (Vc). In addition, we showed that the ventral nucleus of the ventral telencephalon (Vv), the lateral zone of the dorsal telencephalon (Dl), the anterolateral zone of the parvocellular preoptic nucleus (Ppal), the posterior part of the parvocellular preoptic nucleus (Ppp) and the dorsal habenular nucleus (Had) showed differences in the neural activation pattern between wildtypes and mutants. We then showed that the loss of oxytocin receptor caused changes in the functional connectivity of the SDMN such that while wild type test fish showed a general activation in the SDMN, the mutants showed a general inhibitory connectivity pattern.

In summary, this thesis improves our current understanding of the social information use in threat perception and the neurobiological mechanisms regulating this phenomenon; in particular, the role of oxytocin. It also opens up the possibility of answering new questions involving the complexity of this phenomenon and other possible neurobiological mechanisms implicated.

Resumo

O comportamento adaptativo depende da resposta apropriada aos diversos tipos de informação disponível no ambiente.

O ambiente social oferece aos indivíduos a oportunidade de adquirir e usar informação proveniente de sinais ou pistas transmitidas pela mesma ou por diferentes espécies.

No contexto das interacções predador-presa, os indivíduos usam informação do ambiente para saber se há uma ameaça de predação iminente. A informação sobre ameaças de predação pode ser detectada directamente por cada indivíduo e/ou pela observação do comportamento de outros indivíduos. Independentemente da fonte de informação, tomar decisões correctas sobre que comportamento defensivo adoptar é essencial para a sobrevivência dos animais.

Adquirir informação individualmente pode, no entanto, acarretar custos, pois avaliar individualmente a segurança do ambiente pode requerer gastos elevados de tempo e energia. Para além disso, a detecção de ameaças de predação por parte apenas de um indivíduo pode ser arriscada e até resultar na sua morte.

Por outro lado, tem sido sugerido que a aquisição de informação pela observação do comportamento de outros indivíduos pode melhorar a tomada individual de decisões, porque observar as decisões de outros reduz a probabilidade de erro individual e tem um custo menor. Por exemplo, os animais em grupo detectam os predadores mais cedo do que os animais solitários e assim podem tomar as melhores decisões mais atempadamente.

Embora se pense que o uso da informação social seja um fator omnipresente na vida em grupo, os substratos neurobiológicos subjacentes a fenómeno são amplamente desconhecidos, nomeadamente os da detecção de ameaças de predação.

No conjunto de trabalhos que compõem esta tese, analisámos o papel da oxitocina – um neurotransmissor conhecido por estar envolvido na modulação

do comportamento social – na detecção de informação social sobre ameaças de predação em peixe-zebra (*Danio rerio*), uma espécie social que possui um repertório de fenótipos comportamentais com genes altamente conservados e que é um modelo experimental emergente em neurociência social e neuroetologia devido à disponibilidade de várias ferramentas comportamentais, genéticas e de imagem para microscopia.

No primeiro estudo, implementámos e testámos os protocolos experimentais para o uso de informação social na detecção de ameaças de predação em peixe-zebra; usámos a substância de alarme – um composto químico libertado por peixes feridos após o ataque de um predador – para simular uma ameaça de predação; focámo-nos nos três possíveis efeitos da utilização de informação social na detecção de ameaças: o efeito de tampão ou de proteção social contra o medo, o efeito de contágio social do medo e o efeito da facilitação social do medo; medimos as respostas do peixe-zebra ao medo por meio de dois comportamentos: a imobilidade (ou congelamento) e o movimento errático; e, por fim, investigámos possíveis diferenças sexuais na capacidade de resposta do peixe-zebra a esses efeitos. Os nossos resultados descrevem a resposta do peixe-zebra aos diferentes efeitos da utilização de informação social na detecção de ameaças de predação e revela diferenças entre os sexos.

O estudo seguinte teve como objetivo explorar o papel da oxitocina no contágio social do medo usando linhas mutantes de peixe-zebra com deleções constitutivas nos dois receptores e no ligante da oxitocina. Os nossos resultados mostraram um déficit no comportamento de congelamento dos três mutantes. Esse déficit foi restaurado nos mutantes do ligante da oxitocina com um tratamento farmacológico de isotocina (o nome do peptídeo de oxitocina encontrado nos peixes actinopterígeos), confirmando o papel da oxitocina no contágio social do medo. Uma investigação mais aprofundada das possíveis áreas cerebrais envolvidas na regulação deste comportamento – que abrangeu as áreas da Social Decision Making Network (SDMN) – implicou especialmente o telencéfalo ventral, mais precisamente o núcleo ventral do telencéfalo ventral

(Vv) e o núcleo central do telencéfalo ventral (Vc). Os nossos resultados também mostraram diferenças na conectividade funcional da SDMN entre peixes do fenóptipo selvagem e os mutantes. Também confirmámos a presença de receptores de ocitocina nas regiões cerebrais da SDMN, particularmente nas áreas cerebrais do telencéfalo ventral, e confirmámos a projeção de neurónios de oxitocina para o telencéfalo ventral através da visualização dos padrões de projecção.

Por fim, explorámos o papel da oxitocina no efeito de protecção social contra o medo em peixe-zebra com a deleção constitutiva do receptor principal de oxitocina. Os nossos resultados mostraram um déficit no comportamento de congelamento dos mutantes, sugerindo o papel da oxitocina na protecção social contra o medo. A investigação de possíveis áreas cerebrais, de entre as da SDMN, envolvidas na regulação deste efeito implicou o núcleo dorsal do telencéfalo ventral (Vd) e o núcleo central do telencéfalo ventral (Vc). Adicionalmente, mostrámos que o núcleo ventral do telencéfalo ventral (Vv), a zona lateral do telencéfalo dorsal (Dl), a zona anterolateral do núcleo pré-óptico parvocelular (Ppal), a parte posterior do núcleo pré-óptico parvocelular (Ppp) e o núcleo habenular dorsal (Had) apresentaram diferenças no padrão de ativação neural entre peixes de fenóptipo selvagem e os mutantes. Mostrámos, então, que a perda do receptor de oxitocina causava alterações na conectividade funcional da SDMN, de tal modo que, enquanto os peixes de fenótipo selvagem mostravam uma ativação geral da SDMN, os mutantes mostravam um padrão geral de conectividade inibitória.

Em suma, esta tese melhora a nossa compreensão dos mecanismos neurobiológicos da oxitocina envolvidos no uso da informação social durante a percepção de ameaças. Também abre a possibilidade de responder a novas perguntas sobre outros níveis de detalhe e de complexidade do mecanismo da oxitocina assim como de outros mecanismos neurobiológicos.

Declaration

I declare that this dissertation and the data presented are the results of my work developed between 2015 and 2020 in the laboratory of Prof Rui F. Oliveira at the Instituto Gulbenkian de Ciência in Oeiras, Portugal. Specific author contributions are indicated in each chapter, in the author contributions section.

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Declaração

Declaro que esta dissertação de doutoramento e os dados nela apresentados são o resultado do meu trabalho, desenvolvido entre 2015 e 2020 no laboratório do Prof Rui F.Oliveira no Instituto Gulbenkian de Ciência em Oeiras, Portugal. As contribuições de cada autor são indicadas em cada capítulo na secção das contribuições de cada autor.

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1. Chapter one

General Introduction

1.1. **Social information use**

Information acquisition and processing are fundamental in decision making for group-living animals. When moving through their habitat, individuals can obtain information either by individual sampling or by observing others or the consequences of their behaviour in order to make decisions about fitness-affecting resources (Laland, Atton, & Webster, 2011; Morand-Ferron, Doligez, Dall, & Reader, 2010). Examples of the information acquired include foraging locations (Galef, 1977; Gil, Emberts, Jones, & St. Mary, 2017; Rauber & Manser, 2018), mate choice (Mery et al., 2009), breeding sites (Danchin, Giraldeau, Valone, & Wagner, 2004) and threat perception (Gomes Silva, Liu, Chen, Plath, & Trembo, 2017; King & Cowlishaw, 2007). Making correct decisions regarding these fitness-affecting resources increases survival benefits. (Oliveira & Faustino, 2017; Tremblay, Sharika, & Platt, 2017).

Generally, individually acquired information is said to be more accurate and reliable because it informs the individual about its personal limitations or preferences (Varela, Teles, & Oliveira, 2020); however, a downside to this is that it can be expensive to acquire both in terms of the time and energy that is invested, and also it increases the likelihood of encountering predators or exposure to other environmental risks while gathering it (Allen, Weinrich, Hoppitt, & Rendell, 2013; Webster & Laland, 2008). On the other hand, social information use is an adaptive strategy that reduces the costs of personal information gathering; this agrees with the 'costly information hypothesis' that predicts that individuals will use individually acquired information when the costs associated with doing so are low, but they should increasingly use social information as the costs of using individually acquired information rise (Oliveira & Faustino, 2017; Varela et al., 2020; Webster & Laland, 2008).

Implications of environmental spatial and temporal heterogeneity can result in conflicts in available individually acquired information and social information. The balance between individually acquired information and social information use of information reflects in individuals adjusting their decision-making process

in order to fully take advantage of the most reliable information available (King & Cowlishaw, 2007).

Social information can be acquired in two ways: social cues which are inadvertent behaviours or characteristics produced by individuals not specialized for communication; and signals which are traits that are evolutionarily selected to convey information (Bonnie & Earley, 2007; Danchin et al., 2004). In other words, cues refer to information produced incidentally by individuals, and signals refer to intentional communication (Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005).

It is important to note that in a continually changing environment, information diffused socially can be delayed due to environmental changes and so result in the transmission of out-dated and perhaps maladaptive information. In this situation, individual information may contrast with information from conspecifics (Rendell et al., 2010). In a study to determine if natural selection will tune individual behaviour to optimize information processing at the collective level, the model reported that group-living individuals would mostly rely on information from conspecifics and evolve to be influenced by them even under environmental uncertainty (Torney, Lorenzi, Couzin, & Levin, 2015). However, it remains to be investigated if this response occurs in all taxa especially in threat detection.

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1.2. **Oxytocin and social behaviour**

Understanding the neural basis of social behaviour has been an on-going question for several decades. Social behaviour can be referred to as all behaviours that influence, or are influenced by social agents. Social agents are individuals that share ecological requirements (Avarguès-Weber, Dawson, & Chittka, 2013; Varela et al., 2020). Social behaviour thus covers a wide repertoire of behaviours, from sexual and reproductive activities to all behaviour

that tends to bring individuals together as well as all forms of aggressive behaviour (Grant, 1963).

In 1974, Richard D. Alexander proposed that natural selection likely favours the evolution of sociality due to the possibility of having access to increased resources as well as protection from predators (Alexander, 1974; Smith, Petelle, Jerome, Cristofari, & Blumstein, 2017).

Likewise, specialized cognitive mechanisms may have evolved to perceive, attend, process, store and act efficiently on social information (Oliveira, 2013). Several reports have implicated the involvement of oxytocin in the modulation of various social behavioural phenotypes in different species. For example, studies in humans report that oxytocin increased perceived facial trustworthiness and attractiveness (Theodoridou, Rowe, Penton-Voak, & Rogers, 2009); intranasal administration of oxytocin to male and female marmosets *(Callithrix penicillata)* triggered the initiation of more huddling behaviour with their social partner (Smith A.S, Agmo A, Birnie A.K, 2010); intracerebroventricular administration of oxytocin to dominant squirrel monkeys (*Suimiri sciureus)*) triggered an increase in sexual and aggressive behaviours (Winslow & Insel, 1991); oxytocin spray on sixteen dogs (*Canis lupus familiaris*) caused a higher social orientation and affiliation toward their owners and higher affiliation and approach behaviours toward dog partners (Romero, Nagasawa, Mogi, Hasegawa, & Kikusui, 2014); oxytocin injected into the ventral tegmental area (VTA) induced a significant two-fold reduction in place avoidance for the social interaction chamber of male Syrian hamsters (*Mesocricetus auratus*) (Song, Larkin, Malley, & Albers, 2016); the antagonism of oxytocin receptors impaired the formation of pair bonds in prairie voles (*Microtus ochrogaster*) and zebra finches (*Taenioypygia guttata*) (Kelly & Goodson, 2014); the chemogenetic activation of oxytocin neurons within the paraventricular nucleus of hypothalamus (PVN) of male mice (*Mus musculus*) (OT-Ires-Cre) enhanced social investigation during a social choice test, while the chemogenetic inhibition of the neurons abolished typical social preferences (Resendez et al., 2020); oxytocin antagonist (OTA) chronically infused into the posterior paraventricular nucleus (PVN) of lactating dams (*Mus*

musculus) specifically reduced the duration of crouching behaviour over pups (Watarai et al., 2020); oxytocin enabled pup retrieval behaviour in female mice (*Mus musculus*) by enhancing auditory cortical pup call responses (Marlin, Mitre, D'Amour, Chao, & Froemke, 2015); the mutation of oxytocin receptor (*oxtr)* caused deficits in social and object recognition but not in shoal preference and object approach in zebrafish (*Danio rerio*) (Ribeiro et al., 2020).

Sir Henry Dale discovered oxytocin at the beginning of the 20th century, noting that it stimulated contractions of both the uterine muscles and the myoepithelial cells of the mammary gland, thus establishing its important role during labour and lactation. Since then, oxytocin has been implicated in regulating prosocial and aggressive behaviours as well as physiological mechanisms (Carter, 2017; Dölen, 2015; Neumann & Landgraf, 2019).

Oxytocin is a highly conserved nonapeptide that appeared at least 700 million years ago and has been identified in a wide diversity of taxa from hydra, worms, snails and insects to vertebrates, including humans (Donaldson & Young, 2008; Gimpl & Fahrenholz, 2001). Oxytocin is the name used for the peptide sequence found in mammals, mesotocin is the name for the peptide found in non-mammalian vertebrates, while annepressin, conopressin and inotocin are the names used for the peptide found in different classes of invertebrates (Fig. 1.1), isotocin is used for the peptide found in teleosts (Table 1.1), with the exception of zebrafish, where it is called oxytocin (even though the sequence is identical to isotocin) (Nunes, Carreira, Anbalagan, Blechman, & Levkowitz, 2020; Wee et al., 2019; Wircer, Ben-Dor, & Levkowitz, 2016).

Table 1.1 Amino acid sequences of oxytocin homologs.

(Hoyle, 1999)

*Oxytocin orthologs**										
Oxytocin	C	Y	I	Q	N	C	P	L	G	A
Mesotocin	–	–	–	–	–	–	–	I	–	–
Isotocin	–	–	–	S	–	–	–	I	–	–

**Amino acid sequences are given using single-letter codes. '-' indicates homology with the amino acid in the oxytocin sequences. 'A' denotes C-terminal amide. Oxytocin is cysteine - tyrosine - isoleucine - glutamine - asparagine - cysteine - proline - leucine – glycine, isotocin has a substitution of serine and isoleucine amino acids in position '4' and '8' (Hoyle, 1999).*

Oxytocin arose from a similar neuropeptide called vasopressin due to a gene-duplication event before vertebrate divergence (Hoyle, 1999). The oxytocin gene is located at chromosome position 20p12.21 in humans and zebrafish in Chromosome 5 - NC_007116.7. The precursors pre pro-oxytocin consist of a signal peptide, followed by the neuropeptide, then a Lys-Arg dibasic amino acid cleavage site, and neurophysin (Hoyle, 1999). In the genes that have been examined, there always appear to be three exons that code for all the translated peptide, although the lengths of the neurophysin and the presence of the glycoprotein are variable, being approximately 100 and 40 amino acids long, respectively. Oxytocin moiety is coded by exon 1, neurophysin by exons 1, 2, and 3, and copeptin by exon 3 (Hoyle, 1999; Wircer et al., 2016).

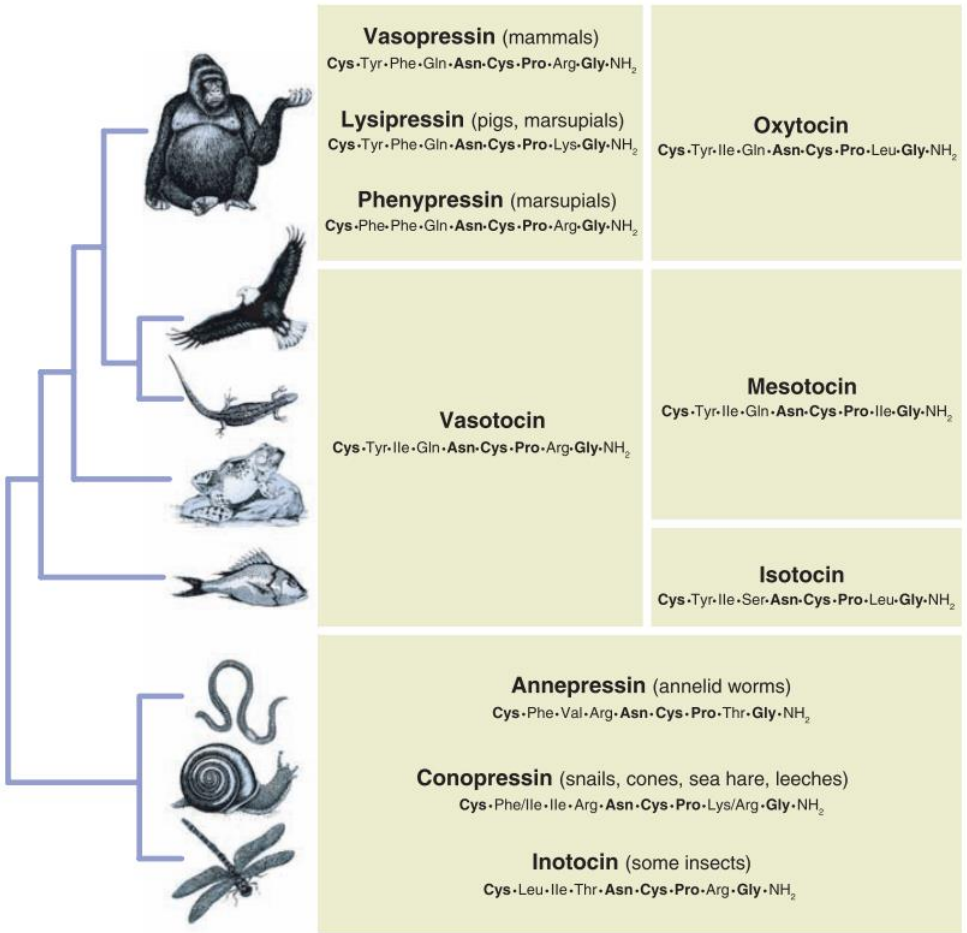

Figure 1.1 ***Oxytocin and vasopressin homologs***
(Donaldson & Young, 2008).

The receptors for oxytocin are 7-transmembrane proteins, and members of the G protein-coupled receptor (GPCR) Class A Rhodopsin-like family. Oxytocin receptors are functionally coupled to G_q and G_o/G_i protein subunits (Fig 1.2) that stimulate the activity of variable phosphorylation of PLCβ and PKC, activation of EGFR and MAPK cascade (Gimpl & Fahrenholz, 2001).

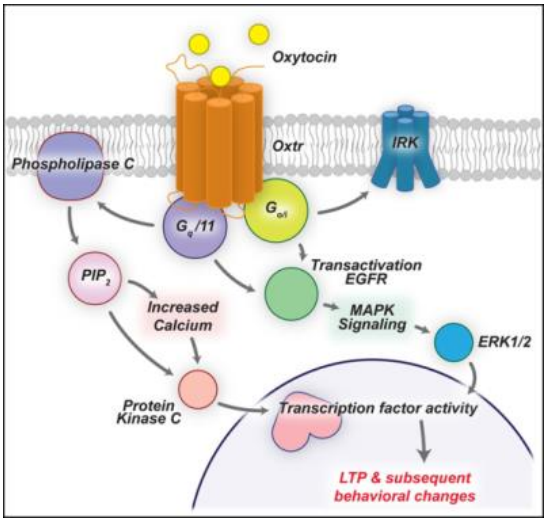

Figure 1.2 ***Oxytocin signalling cascades.***

Oxytocin receptors are G-protein-coupled-receptors that can activate some downstream pathways upon ligand binding (Gimpl & Fahrenholz, 2001; Pekarek, Hunt, & Arenkiel, 2020).

All vertebrate species have at least one oxytocin receptor (*oxtr*); due to a gene duplication event (Fig 1.3), teleosts have two oxytocin receptors called oxytocin receptor 1 (*oxtr*1) and 2 (*oxtr2*) or oxytocin receptor (*oxtr*) and oxytocin receptor-like (*oxtrl*) (in the case of zebrafish). The functional implication of this receptor duplication is still largely unknown. It is not clear if these receptors developed distinct functions or if there is functional redundancy. Recently, studies on the role of the sexually dimorphic roles of oxytocin on mate choice in medaka fish (*Oryzias latipes*) (where female fish choose familiar males while male fish mate indiscriminately) was explored; it was shown that *oxt-oxtr1* but not *oxt-oxtr2* signalling pathway was essential for eliciting female mate preference for familiar males, and males with these mutations exhibited enhanced mate-guarding behaviours toward familiar females, but not toward novel females (Yokoi et al., 2020). This suggests that the receptors may have distinct functions. Although, more studies are required to determine if they also have distinct functions in other components of social behaviour, and across teleosts in general.

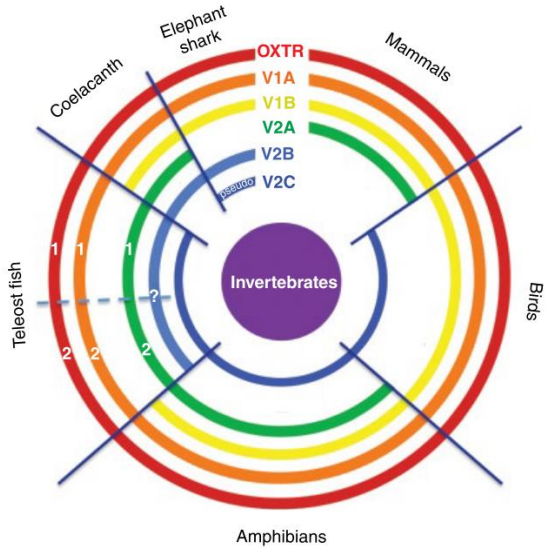

Figure 1.3*. Evolutionary development of the oxytocin and vasopressin receptors.*

Each concentric circle represents one family of receptors. The dashed line in the teleosts represents a lineage-specific duplication generating two different paralog genes designated 1 and 2. (Wircer et al., 2016).

In mammals, oxytocin is produced primarily within hypothalamic brain regions and then shuttled to the pituitary for peripheral release or projected to various brain regions. In like manner, oxytocin homologs are expressed in neurosecretory brain regions of organisms as diverse as worms and fish (Donaldson & Young, 2008; Knobloch & Grinevich, 2014; Wircer et al., 2016).

In most teleosts, oxytocin is produced in neurons located bilaterally along the third ventricle in the hypothalamic neurosecretory preoptic area (NPO) (Grinevich, Knobloch-Bollmann, Eliava, Busnelli, & Chini, 2016). Cells of the NPO vary in size; the rostral-ventral preoptic area contains relatively small (parvocellular) neurons (Fig1.4) and is thought to be the homolog of the supraoptic nucleus (SON) of higher vertebrates. The caudal dorsal region contains large (magnocellular and gigantocellular) neurons and is thought to be

the homolog of the paraventricular nucleus (PVN) (Godwin & Thompson, 2012; Goodson, 2008; Wircer et al., 2016).

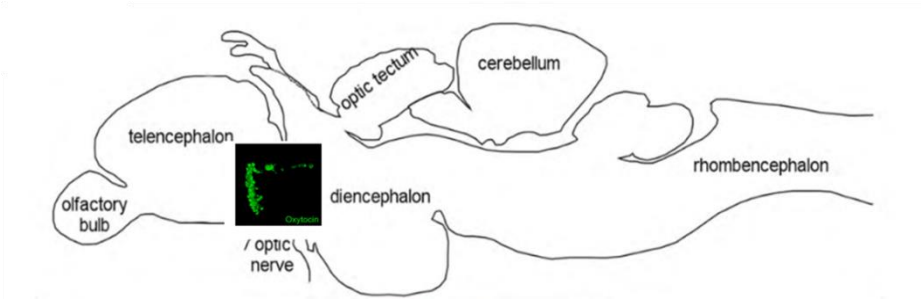

Figure 1.4*. Expression of oxytocin cells in the adult zebrafish brain.*
The schematic representation of a mid-sagittal section through the adult zebrafish brain showing the location of oxytocin cells (green) in the neurosecretory preoptic area (NPO) acquired by fluorescent in situ hybridization (Wircer et al., 2016).

Although oxytocin has been implicated in the modulation of social behaviour, there is a debate regarding what components of social behaviour oxytocin modulates; this is because behavioural neuroendocrinologists often discuss "sociality" as a unitary variable, even though it encompasses diverse behavioural traits that may not have evolved in a related fashion across species. This is because nonapeptide systems may tend to have evolved in very species-specific ways, depending upon the evolutionary background of the species (Goodson, 2013). This could affect the function of the oxytocin cell groups or the functions of the receptors distributed in the different brain regions. For instance, in common marmosets (*Callithrix penicillatata*), peripheral injections of oxytocin and an *oxtr* antagonist altered some aspects of affiliation but did not alter pair bond formation or subsequent partner preference behaviour (Smith A.S, Agmo

A, Birnie A.K, 2010), suggesting that oxytocin may modulate only specific components of social behaviour and this may occur in a species-specific manner.

Additionally, given that oxytocin bears a close similarity to vasopressin, it is possible that even though they have different physiological activities, gene-structures and evolutionary lineages, they may still retain some ability to bind with one another although it may be with differing affinities; making it difficult to draw conclusions based on pharmacological studies alone. For instance, oxytocin receptor binds to both oxytocin and vasopressin ligand with nearly equal affinity, but 100-fold more vasopressin is required for downstream activation of oxytocin; and the vasopressin receptors are more specific, to begin with, binding vasopressin with 400-fold higher affinity than oxytocin (Postina, Kojro, & Fahrenholz, 1996; Wircer et al., 2016). Therefore, to determine the specific social functions of oxytocin, it is important to utilize techniques that will specifically target each neuropeptide. The use of genetic models as zebrafish thus proffers a solution; here, we can specifically target oxytocin using reverse genetics to better understand its functions.

1.3. **Zebrafish as a model organism**

Zebrafish (*Danio rerio*) has emerged as a major model organism for biomedicine, developmental genetics, neuroethology and social neuroscience research (Engeszer, Patterson, Rao, & Parichy, 2007; Meyers, 2018).

It is a small-bodied, tropical, fresh-water fish from the *Cyprinidae* family, native to the floodplains of South Asia (Meyers, 2018; Spence, Gerlach, Lawrence, & Smith, 2008).

It feeds on organisms such as zooplankton, nematodes and insects; in turn, it can be preyed upon by larger fish in their environments such as snakeheads, needlefish, catfish and knife fish (Engeszer et al., 2007; Meyers, 2018; Spence et al., 2008).

They have a very high thermal tolerance with the ability to survive in temperatures between 24.6°C and 38.6°C with no signs of heat-induced stress (Engeszer et al., 2007).

Field investigations show that zebrafish can survive in a wide range of environments. Predominantly, they are found in relatively clear, slow-moving, warm waters with some vegetation and a silty bottom (Meyers, 2018; Spence et al., 2008; Suriyampola et al., 2016).

Zebrafish are group spawners and egg scatterers. The female zebrafish can be quite choosy regarding choosing sites for oviposition and the males tend to defend the territories around such sites (Spence et al., 2008).

Zebrafish are social species capable of forming mixed shoals of varying group sizes. This depends mostly on the habitat they are found; for example, a study found groups of 6-7 fish in a slow-flowing river, other groups of up to 22 individuals were found in still waters, while, groups up to 300 individuals found in a fast-flowing river (Suriyampola et al., 2016).

Zebrafish exhibit a wide range of behavioural repertoire that has been studied by our lab as well as others; this includes social tendency (Dreosti, Lopes, Kampff, & Wilson, 2015; Kalueff et al., 2013; Miller & Gerlai, 2012), social recognition (Gerlach, Hodgins-Davis, Avolio, & Schunter, 2008; Madeira & Oliveira, 2017), aggression (Cruz & Oliveira, 2015; Teles M.and Oliveira R.F, 2016), social eavesdropping (Abril-De-Abreu, Cruz, & Oliveira, 2015) social learning (Gerlai, 2011), social buffering of fear (Faustino, Tacão-Monteiro, & Oliveira, 2017), social transmission of fear (David Hall and Milton D Suboski, 1995).

Thanks to the zebrafish genome project, we now have the whole zebrafish genome completely sequenced and a reference genome allowing the possibility of targeting specific genes to increase our understanding of the molecular mechanisms regulating zebrafish behaviour (Howe et al., 2013). The use of forward and reverse genetics has helped in uncovering the functional roles of several genes (Driever et al., 1996; Koster & Sassen, 2015; Lawson & Wolfe, 2011; Skromne & Prince, 2008).

Therefore, with the vast array of social behavioural repertoires, and the availability of genetic tools which allows better visualization and manipulation of selected neural circuits, zebrafish appears to be an exemplary model to tackle the questions relating to social behaviour and their underlying neural mechanisms.

1.4. **Threat perception**

The ability to make correct decisions is fundamental to animal reproduction and survival. Decision-making is a process in which environmental information must be processed in order for animals to choose between two or more alternatives sometimes with uncertainty about their differences (Marshall et al., 2009). In the context of threats of predation, individuals are frequently faced with the dilemma of making correct decisions. On the one hand, they could activate a predator avoidance response to prevent their demise if there is the slightest indication of the presence of a predator; while on the other hand, they could risk losing energy and foraging or mating opportunities that increase their fitness if they activate a predator avoidance response when the apparent threat is low (Budaev, Jørgensen, Mangel, Eliassen, & Giske, 2019).

If the individual knows the environmental state with certainty, the optimal decision strategy is simply to flee whenever a threat is present. However, given that there is always a level of uncertainty when making decisions regarding threat caused by noise in the environment, one has to determine when to activate or ignore a cue regarding threat (Wiley, 2016; Zylberberg & DeWeese, 2011); hence the ability to detect an apparent threat and decide appropriately constitutes threat perception. For example, an individual may have to decide if a rustle in leaves depicts the presence of an apparent predator or if it is just the effect of the wind (Oliveira & Faustino, 2017; Zylberberg & DeWeese, 2011).

Following the signal detection theory, one has to set some criteria to determine the presence of threat; a conservative criterion minimizes false alarms but

increases exposure to missed detections, while a liberal criterion minimizes missed detections but increases exposure to false alarms (Wiley, 2006).

Hence there can be four possible outcomes:

(i) The individual responds to the threat cue when it is present (hit);

(ii) The individual responds to the threat cue when it is absent (false alarm);

(iii) The individual does not respond to the threat cue when it is present (miss);

(iv) The individual does not respond to the threat cue when it is absent (correct rejection) (Fig 1.5).

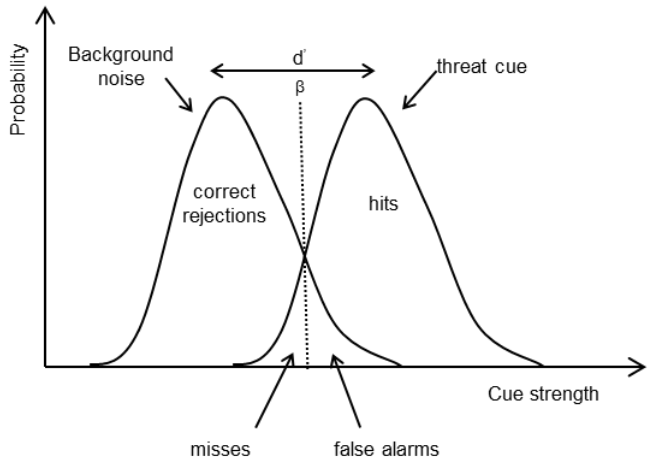

Figure 1.5 Application of signal detection theory in animal threat detection. The distributions of the threat cue and background noise within the decision-maker overlap, and the difference in position between the two distributions shows the sensitivity (d') of the individual to the threat cue; the individual uses a threat detection threshold, β, to decide when a threat is present (Oliveira & Faustino, 2017).

These options, however, should be chosen taking into account the state of the environment (Budaev et al., 2019; Oliveira & Faustino, 2017; Zylberberg &

DeWeese, 2011). Interestingly, the presence of conspecifics in the environment may either increase or decrease the threat detection threshold of an individual, depending on the state of the conspecifics (Morozov & Ito, 2019; Oliveira & Faustino, 2017).

Given that social behaviours mainly constitute mutual exchanges of social cues from conspecifics and the responses to them, individuals can combine individually acquired information with social information to assess the presence of threat in the environment and respond accordingly with the outcomes resulting in different social phenomena.

The individual could shift its detection threshold to a higher value when the relaxed behaviour of conspecifics contradicts the threat detected by the individual, hence social buffering of fear. On the other hand, the sight of the alarmed behaviour of conspecifics could signal the presence of an undetected threat by the individual causing a shift in the detection threshold to a lower value, hence social contagion of fear. And the sight of the alarmed behaviour of conspecifics could signal the presence of a detected threat by the individual causing a shift in the detection threshold to an even lower value, hence social facilitation of fear (Table 1.2) (Oliveira & Faustino, 2017). In all three situations, since a decision making step is required that involves the use of social information; it can be hypothesized that a shared neuronal mechanism may regulate them. Therefore, understanding the behavioural and neural mechanism underlying these phenomena may provide fundamental insights into the evolution of social behaviour.

Table 1.2 ***Social modulation of threat responses.***

The possible outcomes that could occur depending on environmental situations the individual is exposed to, giving rise to different social phenomena.

Individual information	Social information	Threat response	Phenomena
+	0	++	Individual threat response
+	-	+	Social buffering
-	+	+	Social contagion
+	+	+++	Social facilitation

1.5. **Social decision-making network in zebrafish**

Social decision-making (SDM) network is described as a network of nodes that regulate and implement responses to salient stimuli within the social domain (Almeida, Félix, Oliveira, Lopes, & Oliveira, 2019; O'Connell & Hofmann, 2011; Teles, Almeida, Lopes, & Oliveira, 2015).

This is a highly conserved network (J.L, 2005) present in fish, mammals, that is made up of the social behaviour network and the mesolimbic reward system (Fig 1.6, 1.7), and is generally assumed to evaluate stimulus salience via dopaminergic signalling (O'Connell & Hofmann, 2012).

The nodes of this network are reciprocally connected and they are involved in multiple forms of social behaviour such as aggression, appetitive and sexual behaviour, communication, social recognition, social affiliation, social bonding, parental care, and responses to social stressors (Teles et al., 2015).

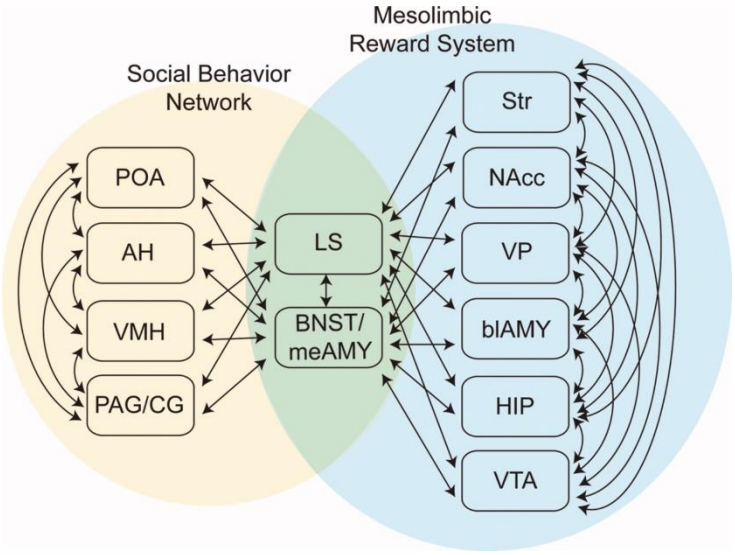

Figure 1.6. ***The social decision-making network.***

The brain regions in the social behaviour network (left), the mesolimbic reward system (right), as well as brain regions that interact with both systems (centre) are shown. Arrows indicate anatomical connections between the brain regions. POA= preoptic area, AH= anterior hypothalamus; VMH= ventromedial hypothalamus; PAG/CG, periaqueductal grey/central grey; LS, lateral septum; BNST/meAMY, bed nucleus of the stria terminalis/medial amygdala; Str, striatum; NAcc, nucleus accumbens; VP, ventral pallidum; blAMY, basolateral amygdala; HIP, hippocampus; VTA, ventral tegmental area (O'Connell & Hofmann, 2011).

Because social decision-making involves the integration of multimodal sensory information about social stimuli to produce an appropriate behavioural response, social decisions are expected to rely on multiple neural circuits, rather than being regulated by only one specific brain region, such that a social behavioural phenotype is reflected by the overall pattern of neuronal activation across nodes and variation in the strength of the connections among them (Goodson & Kabelik, 2009; Teles et al., 2015).

Studies have, indeed, established that the SDM network functions as an integrated circuit in regulating social behaviour (Fernald & Maruska, 2012; O'Connell & Hofmann, 2011). For example, hypothalamic stimulation elicits an aggressive response when the forebrain nodes of the social behaviour network (the BNST, LS, and meAMY) are activated (Halász, Liposits, Meelis, Kruk, & Haller, 2002; O'Connell & Hofmann, 2012). Also, short-term social interactions elicit distinct patterns in the SDM network in African cichlid fish (*Oreochromis mossambicus*) whose perception of outcomes of agonistic interactions were manipulated (Almeida et al., 2019). Similarly, the expression of socially driven behavioural states (dominant versus subordinate individuals) was associated with specific patterns of functional connectivity across the SDM network in a zebrafish fighting paradigm (Teles et al., 2015).

Additionally, neuromodulators may regulate social interactions by 'tuning' the social brain (Tremblay et al., 2017). Studies on the involvement of neuropeptides in the modulation of the SDM network are now coming to light. For instance, the blockade of endogenous oxytocin receptor *(oxtr)* signalling in the nucleus accumbens (NAc) during sexual interaction and mating of prairie voles (*Microtus ochrogaster*) does not strongly modulate levels of Fos expression (a marker of neural activation) in individual nodes of the network, but strongly modulates patterns of correlated Fos expression between the NAc and other nodes of the SDM network (Johnson, Z. V., Walum H, Xiao Y, Riefkohl P.C, 2017). It is therefore possible that neuropeptides such as oxytocin act on the SDM network to modulate the salience of social cues.

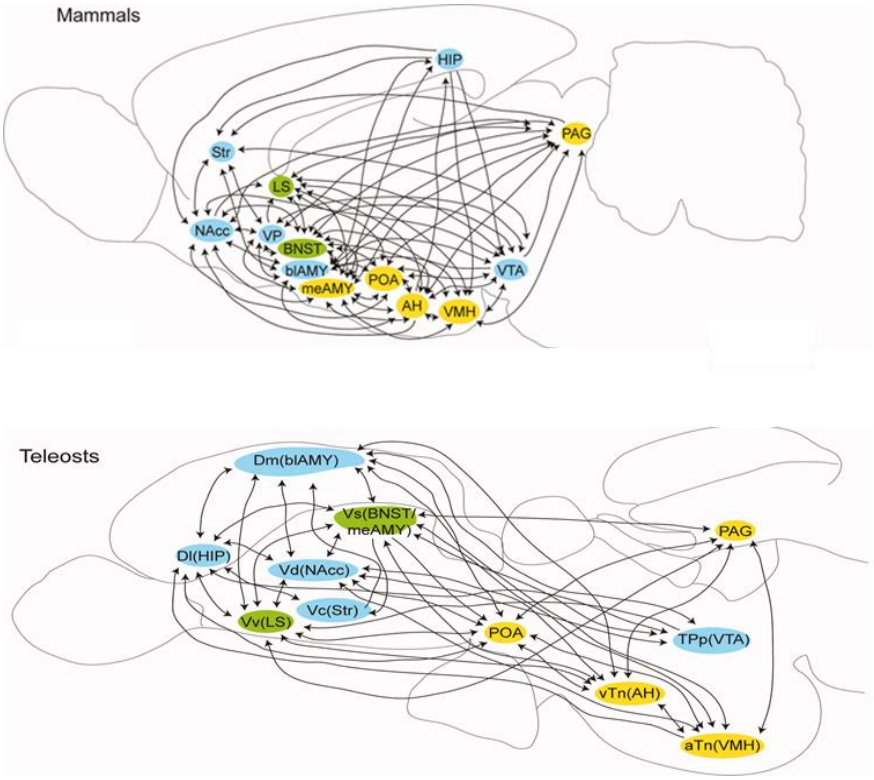

Figure 1.7*. Evidence for homologies by connectivity pattern.*

The sagittal view of the projection patterns of a social decision-making circuit is presented for mammals and teleosts. Arrows reflect the directions of the connection. Brain regions within the social behaviour network are coloured yellow, brain regions in the mesolimbic reward system are coloured blue, and brain regions shared by both networks are coloured green (O'Connell & Hofmann, 2011)***.***

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2. Chapter 2

Sex differences in social information use in threat perception in zebrafish (*Danio rerio*)

Abstract

Adaptive behaviour relies upon the appropriate response to information from a variety of sources, particularly when the information is related to the possible presence of a predator.

Information, mainly chemical information on predation, may persist in the environment long after the predator and the injured prey have gone, and this can affect an individual's accuracy of threat assessment. In such contexts, the information provided by the behaviour of conspecifics should aid decision-making on whether to respond or not, to the predator's cue with alarmed, defensive behaviours. The information provided by conspecifics may conflict with the information detected by the individual, reducing (social buffering) or triggering (social contagion) the alarmed response; or the information provided by conspecifics may confirm the information detected by the individual, increasing (social facilitation) the alarmed response.

Individual responses to social information use in threat perception are also likely to depend on factors such as sex; however, there has been no formal experimental investigation of sex differences in responses to predation threats.

We used the response to alarm substance (AS) – a chemical cue released by injured fish – to explore how male and female zebrafish (*Danio rerio*) respond to information about predation threats and how this is modified by the behaviour of an adjacent shoal of conspecifics. We quantified zebrafish alarmed responses as the amount of time spent freezing or in erratic movement after exposure to the AS.

We showed that conflicting threat information elicited different responses from males and females; less percentage of freezing by males when the shoal was not alarmed (social buffering) and more percentage of freezing by males when the shoal was alarmed (social contagion). Therefore, when the information provided by conspecifics conflicted with the information detected by the test fish, males reacted more to the behaviour of the shoal than did females. However,

congruent threat information (social facilitation) elicited more erratic movement responses from both males and females.

These sex differences provide insight into the effect of the social environment on individual behaviours.

2.1. **Introduction**

With a continually changing environment, individuals are oftentimes faced with unexpected opportunities and danger causing a need to alter their behaviour to react to such changes. The social environment provides individuals with the opportunity to gather and use information resulting from cues provided by other individuals from the same or different species (Brown & Laland, 2003; Dall et al., 2005). Adaptive behaviour relies upon the accurate appraisal of relevant ecological parameters; this means that the more information an individual has, the better it can develop and adjust its behaviour to meet the demands of a variable world (Dall et al., 2005).

In the context of predation threats, individuals use information from the environment to evaluate whether there is an imminent threat or not. Animals can detect predator cues individually or by observing the behaviour of others. Regardless of the source of information, deciding correctly on whether to adopt a defensive behaviour or not is essential for survival (Smith, 1992, Dayan & Daw, 2008; Pérez-Escudero & De Polavieja, 2017). Gathering information individually, however, may entail costs because the investment on valuable resources, such as energy, time and attention will occur at the expense of other biological demands such as growth and reproduction. Also, inspecting the environment for threat alone can be risky and can even result in the demise of the individual (Giraldeau, Valone, & Templeton, 2002). Acquiring information by observing the behaviour of others has been suggested to improve the efficiency of decision making because observing others has a lower cost than acquiring the information individually (Morand-Ferron et al., 2010): for example, individuals in groups detect predators faster and the group dilutes individual risk. A fundamental trade-off in any information detection context, including threat perception, arises from the consequences of altering thresholds for failed detections and false alarms (Marshall, Kurvers, Krause, & Wolf, 2019). If individuals set a high threshold for detection of threat, they will frequently fail to detect a real threat (miss), but they will respond to few false alarms. Conversely,

if they set a low threshold for threat detection, they will miss fewer real threats, at the expense of more frequent false alarms. In threat perception, a missed real threat might be fatal, whereas a false alarm might waste some resources; the more dangerous a threat, the stronger the selection pressure to efficiently detect it (Lima & Dill, 1990; Wolf, Kurvers, Ward, Krause, & Krause, 2013; Oliveira & Faustino, 2017; Marshall et al., 2019).

Information on predation, mainly chemical information, may persist in the environment long after the predator and the injured prey have gone, thereby leading to variation in an individual's potential to correctly assess danger (Duboscq, Romano, MacIntosh, & Sueur, 2016). When living in groups, individuals may use information from the behaviour of conspecifics to reduce uncertainty and improve their threat detection threshold. However, individuals can vary in their propensity to use social information, and the causes of such differences remain mostly unclear (Brown & Laland, 2003; Laland, 2004; Rendell et al., 2010; Dukas, Ratcliffe, Kendal, Coolen, & Laland, 2013).

Given that individuals often live in mixed groups of different sexes (Spence, Gerlach, Lawrence, & Smith, 2008), dominance ranks (Paull et al., 2010) and personalities (Collignon, Séguret, Chemtob, Cazenille, & Halloy, 2019; Heloysa, Jaquelinne, Silva, & Luchiari, 2018; Vonk, Weiss, & Kuczaj, 2017), the perception or dependency on social information may vary. However, there has been no formal experimental investigation of such differences, namely sex differences, in response to predation threats.

Alarm substance (AS), which is released from epidermal club cells upon injury of the skin, is a threat cue known to be used by many species of *Ostaryiophysi* fish, including zebrafish (*Danio rerio*) (Pfeiffer, 1977; v. Frisch, 1942). Upon sensing AS, zebrafish display a characteristic fear response that includes erratic movement (making multiple darts and fast acceleration bouts in rapid succession in which the direction of movement also changes in a stochastic manner between the rapid darts) and freezing (complete cessation of movement (except for gills and eyes) by the fish while at the bottom of the tank) (R. Gerlai, Lahav, Guo, & Rosenthal, 2000; Robert Gerlai, Lee, & Blaser, 2006; Kalueff et

al., 2013; Oliveira & Faustino, 2017; Speedie & Gerlai, 2008; Waldman, 1937). Erratic movement has been suggested to act as a predator deterrent by visually confusing predators (also known as the “confusion effect” (Landeau & Terborgh, 1986)) due to the high speed and stochastic swimming associated with these motor patterns (Parra, Adrian, & Gerlai, 2009). Freezing has been reported to be a form of attentive immobility serving to avoid detection by predators and to enhance perception (Roelofs, 2017). Moreover, considering that AS is a cue that does not deliberately signal the presence of threat (R. J. F. Smith, 1992a); it could be released in other conditions such as during fights or during accidents that cause damage to the skin which does not necessarily mean that a predator is present. Hence, individuals should set a threshold for determining when to activate a full array of predator defence responses after sensing the AS.

Observing conspecific responses to AS can help in adjusting the threat detection threshold to each situation. The information provided by conspecifics may conflict or confirm the information acquired individually: the unalarmed behaviour of other individuals can have a reduction effect on an individual’s response to a detected AS (social buffering of fear) by increasing its threat detection threshold; the alarmed behaviour of other individuals can act as a stressor (social contagion of fear), triggering an individual’s response to an undetected AS by decreasing its threat detection threshold; and the alarmed behaviour of other individuals can also act as an additional stressor (social facilitation of fear), increasing an individual’s response to a detected AS to the highest level by increasing certainty about a perceived threat

Social buffering of fear has been reported to occur in zebrafish (Faustino et al., 2017; Mathuru et al., 2017); and in males occurs independently of shoal size (two, four or eight) (Faustino et al., 2017). Groups of zebrafish that could see a group of alarmed demonstrators were also reported to show intense and prolonged alarm reactions (Hall & Suboski, 1995; Suboski et al., 1990). Here, alarm reactions were inferred from the depth in the tank of the test fish. However, this index of alarm reaction is confounding since it can also occur during other activities like regular swimming. To our knowledge, social

facilitation of fear has not yet been tested in zebrafish or other species, nor sex differences of zebrafish responses to all three social information scenarios. In the present work, we have used an experimental approach that allowed the investigation of sex differences in social buffering, social contagion and social facilitation of fear in zebrafish.

2.2. **Methods**

2.2.1. Animals and housing

TU strain adult wild-type zebrafish 6-12 months old were raised in mixed-sex 5 L tanks (n=35 per tank) in a recirculating system (ZebTEC active-blue, Tecniplast) under 14L: 10D photoperiod, in the fish facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). The tank water was maintained at 28ºC, 900 µS, pH 7.0, <0.2 ppm nitrites, <50 ppm nitrates and 0.01-0.1 ppm ammonia.
Experimental animals were fed with a combination of live food (*Paramecium caudatum, Artemia salina, and Rotifers)* and commercial processed dry food (Gemma). The Instituto Gulbenkian de Ciência ethics committee approved all animal protocols.

2.2.2. Alarm substance extraction

Alarm substance (AS) was extracted using a modified version of the protocol described by Speedie and Gerlai (Speedie & Gerlai, 2008) with ten donor zebrafish (five males and five females). Retrieval of donor fish was done with a fishing net, and then quickly sacrificed. Each donor fish was placed in a Petri dish on ice and 15 shallow cuts were made on both sides of the fish using a sterile surgical blade. Adequate release of AS was ensured by washing the cuts with 50ml of distilled water (vehicle) using a Terumo® syringe (8SS50L1) without the needle. Impurities were removed from the AS solution using 0.22 µm sterile vacuum filtering (Filtropur V50- 83.3940) and AS aliquots were stored at -20^{o}C. 0.8ml of AS and distilled water was given per trial.

2.2.3. Behavioural setup

The behavioural set up established by Faustino et al. (Faustino et al., 2017) was adopted, with two adjacent tanks (12 x 12 x 15 cm) each filled with 1.3L of water. The test fish was placed in one tank, and the other tank contained water with or without a shoal of two males and two females (Fig. 2.1). The tanks had opaque white bases, opposite and rear walls to prevent interference by external

environmental cues. AS was administered with a flexible and transparent PVC tubing (0.8 mm internal diameter; 2.4 mm external diameter). Two video cameras (either a pair of B&W mini surveillance cameras (Henelec 300B) or two webcams (Logitech B 525 high definition camcorder, Ref 960-000842) were placed above and to the side of the tanks. Eyeline surveillance software (www.nchsoftware.com) was used for video acquisition.

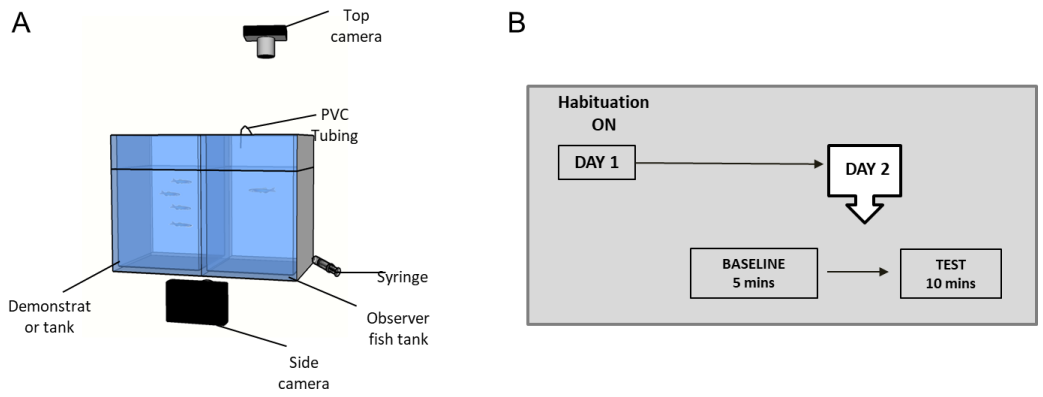

Figure 2.1. The behavioural paradigm.
A. The behavioural setup consists of the test fish tank placed adjacent to the shoal tank. AS or vehicle administered with PVC tubing; two cameras- side and top were used for synchronous video recordings. B. Schematic representation of the behavioural protocol; On day 1, test fish were left to habituate overnight either alone or with shoal depending on treatment group and the next day, behavioural recordings are made with 5 mins to establish baseline and 10 mins test recording.

2.2.4. Experimental procedure

In the social buffering experiment, test fish were randomly assigned to one of four treatments: Test fish alone exposed to vehicle (Alone_Control-AC); test fish alone exposed to AS (Alone_AS-AAs); test fish exposed to vehicle in the presence of a shoal (Obs+Shoal_Control-OSC); and test fish exposed to AS in the presence of a shoal (Obs_AS + Shoal-OAsS) (Fig. 2.2a). In the social contagion experiment, test fish were randomly assigned to one of two

treatments: test fish with shoal exposed to vehicle (Obs + Shoal_Control-OSC); and test fish with shoal exposed to AS (Obs +Shoal_AS-OSAs) (Fig. 2.3a). In the social facilitation experiment, test fish were randomly assigned to one of two groups: test fish alone exposed to AS (Alone_AS-AAs); and test fish and shoal both exposed to AS (Obs_AS + Shoal_AS-OAsSAs) (Fig. 2.4a). The order of testing was randomized in each experiment, and all experiments were conducted between 10:00 and 19:00.

In all three experiments, test fish included males and females for analysis of sex effects. On the day before the experiment, test fish were removed from home tanks and randomly assigned to treatment groups for acclimatization. On the test day, each trial lasted for 15mins and was divided into two phases: the first 5mins to establish a baseline and the subsequent 10 mins to measure the response to the treatment.

AS was kept in ice to avoid AS degradation during the trial and therefore the control (vehicle, distilled water) was kept in the same conditions to ensure comparability of treatments. The arena dimensions for top and side views were 10.5 x 10.5 cm and 11 x 10.5 cm, respectively. Two videos corresponding to the top and side view recordings were analysed per test fish. The x,y,z coordinates extracted from automated video tracking by Ethovision software (Ethovision XT 12, Noldus Technology) were analysed using an updated version of custom made xyz2b Python scripts. Percentage of time spent in erratic movement and freezing was used as a measure of the fear response in the experiments. Two criteria defined erratic movement; if the test fish accelerated >8 cm/s^2 and >5 changes in direction/sec. Two criteria defined freezing: if the test fish velocity was <0.2 cm/s; and if the test fish position on y-axis was below the bottom quarter of the side view of the arena.

2.2.5. Statistical analysis

In the social buffering experiment, we did logarithmic transformation [log10 (x+1)] of behavioural variables (the percentage (%) of time spent freezing and the % of time spent in erratic movement) to meet parametric test assumptions

after which we analysed them separately with Linear Models (LM) with sex, presence/absence of alarm substance, and presence/absence of shoal as fixed effects including their triple interaction. In social contagion and social facilitation experiments, logarithmic transformations did not normalize the data, so raw values of the behavioural variables (the percentage (%) of time spent freezing and the % of time spent in erratic movement) were analysed separately using Generalized Linear Models (GLM) with beta regression (Cribari-Neto & Zeileis, 2010; Ferrari & Cribari-Neto, 2004) with sex and alarm substance or sex and shoal as fixed effects respectively including their double interactions. In all models, a backward stepwise procedure was used to exclude non-significant interactions and fixed effects.

Normality was tested using the Shapiro (Royston, 1982) and D'Agostino & Pearson test (D'Agostino RB, 1986). Statistical significance was $p < 0.05$. Statistical analysis was performed using Graphpad Prism (version 8) and R version 3.6.1 (R Core Team, 2015) with the following packages: R base package (for LM, Linear Modeling), betareg (for GLM with beta regression), lm test (for model significance within the betareg), emmeans (for planned comparisons).

2.3. **Results**

2.3.1. Sex differences in social buffering

The freezing response of test fish was significantly affected by the presence / absence of Alarm Substance (AS), the sex of the test fish and the presence / absence of a shoal in the adjacent tank (Linear Model (LM); Main effects: sex: $F_{(1,154)}$= 7.777, p=0.006; AS: $F_{(1,154)}$= 209.750, p<0.001; shoal: $F_{(1,154)}$= 47.513, p<0.0001: Fig. 2.2F, Table 2.1). In addition, there was a significant interaction of sex and AS and of shoal and AS (LM; Interactions: sex x AS: $F_{(1,154)}$= 8.544, p<0.0001; shoal x AS: $F_{(1,154)}$= 38.315, p<0.0001: Fig. 2.2F, Table 2.1). Male test fish spent significantly less time freezing than female test fish when exposed to AS in the presence of a shoal (LM planned comparison: Male Obs_AS + Shoal vs Female Obs_AS + Shoal: $t_{(1,152)}$ = 4.469, p<0.0001: Fig.2.2F, Table 2.1). AS significantly increased the freezing response independent of the sex of the test fish when they were alone compared to when they were in the presence of a shoal (LM planned comparison: Male Alone_AS vs Male Obs_AS + Shoal: $t_{(1,152)}$ = -8.191, p<0.0001; Female Alone_AS vs Female Obs_AS + Shoal: $t_{(1,152)}$ = -5.020, p<0.0001 : Fig.2.2F, Table 2.1), and control groups showed no significant difference in freezing response relative to sex (LM planned comparisons: Male Alone_Control vs Male Obs + Shoal_Control: $t_{(1,152)}$ = -0.452, p<0.6519; Female Alone_Control vs Female Obs + Shoal_Control: $t_{(1,152)}$ = -0.258, p<0.7968; Fig. 2.2F, Table 2.1).

The erratic movement response of test fish was significantly affected by the presence/absence of AS and the presence/absence of a shoal in the adjacent tank but not by the sex of the test fish (LM; Main effects: AS: $F_{(1,156)}$ = 5.182, p=0.0242; shoal: $F_{(1,156)}$ = 16.500, p<0.0001 Fig. 2.2F, Table 2.1). There was a significant interaction of shoal and AS (LM: shoal x AS: $F_{(1,156)}$ = 6.444, p=0.0121 Fig. 2.2F, Table 2.1). Male and female test fish spent significantly more time in erratic movement when exposed to AS in the presence of shoal compared to when they were alone (LM planned comparison: Male Obs_AS + Shoal vs Male Alone_AS: $t_{(1,152)}$ = 3.319, p=0.0011; Female Obs_AS + Shoal vs Female

Alone_AS: $t_{(1,152)}$ = 3.235, p=0.0015 Fig. 2.2F, Table 2.1). AS significantly increased the erratic movement response independent of the sex of the test fish when they were alone compared to when they were in the presence of a shoal (LM planned comparison: Male Alone_AS vs Male Obs_AS + Shoal: $t_{(1,152)}$ = 3.319, p<0.0011; Female Alone_AS vs Female Obs_AS + Shoal: $t_{(1,152)}$ = 3.235, p<0.0015 : Fig.2.2F, Table 2.1), and control groups showed no significant difference in erratic movement relative to sex (LM planned comparisons: Male Alone_Control vs Male Obs + Shoal_Control: $t_{(1,152)}$ = 0.242, p<0.8090; Female Alone_Control vs Female Obs + Shoal_Control: $t_{(1,152)}$ = 1.271, p<0.2058; Fig. 2.2F, Table 2.1).

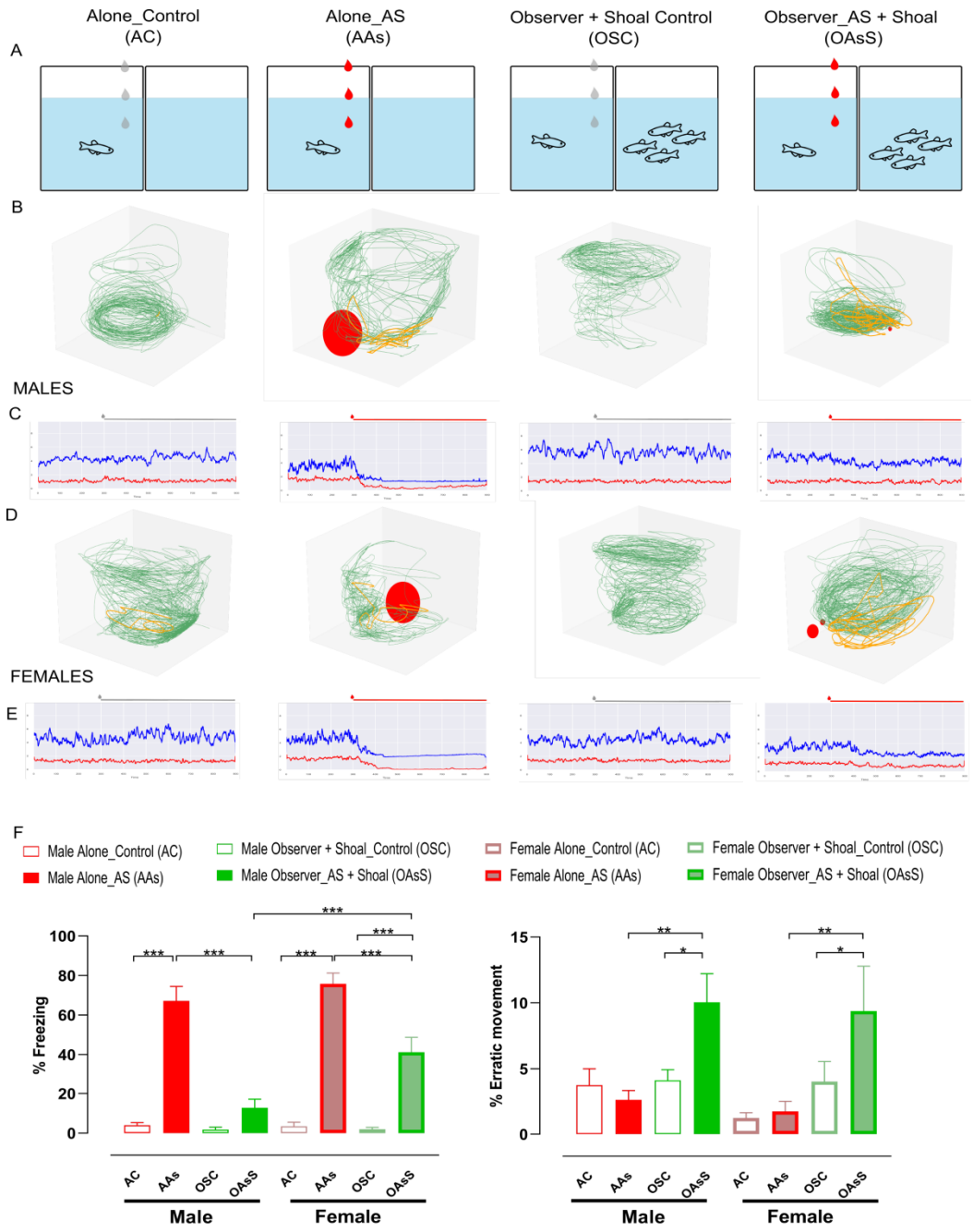

Figure 2.2 Fear response in social buffering.

(A) Schematic representation of the behavioural treatments. Droplets represent administration of vehicle (grey) and alarm substance (AS; red) to control and experimental groups. (B,D) Representative 3D swimming behaviour for each treatment group in males and females; normal swimming pattern (green), erratic movement (orange), and freezing episodes (red dot, size is proportional to

freezing time). (C,E) Mean velocity (red) and mean depth (blue) of each treatment group in males and females. (F) Percentage of time in freezing and erratic movement (mean±SEM) during test phase. The legend identifies sex, presence of AS and the presence of shoal for each treatment. Asterisks indicate statistical significance; *p < 0.05, **p < 0.01 and *** p < 0.001.

2.3.2. Sex differences in social contagion

The freezing response of test fish was significantly affected by the presence / absence of AS and by the sex of the test fish (Betareg: sex: $F_{(1,85)}$= 5.781, p=0.0162; AS: $F_{(1,85)}$= 81.286, p<0.0001: Fig. 2.3F Table 2.2). In addition, there was a significant interaction of sex and AS (Betareg: sex x AS: $F_{(1,85)}$= 7.685, p=0.005: Fig. 2.3F Table 2.2). Male test fish spent significantly more time freezing than female test fish upon sight of an alarmed shoal (Betareg planned comparison: Male Obs + Shoal_AS vs Female Obs + Shoal_AS: $z_{(1,85)}$ =-2.640, p=0.0083: Fig. 2.3F Table 2.2). There were no differences in erratic movement across treatments. (Betareg planned comparisons: Fig. 2.3F, Table 2.2).

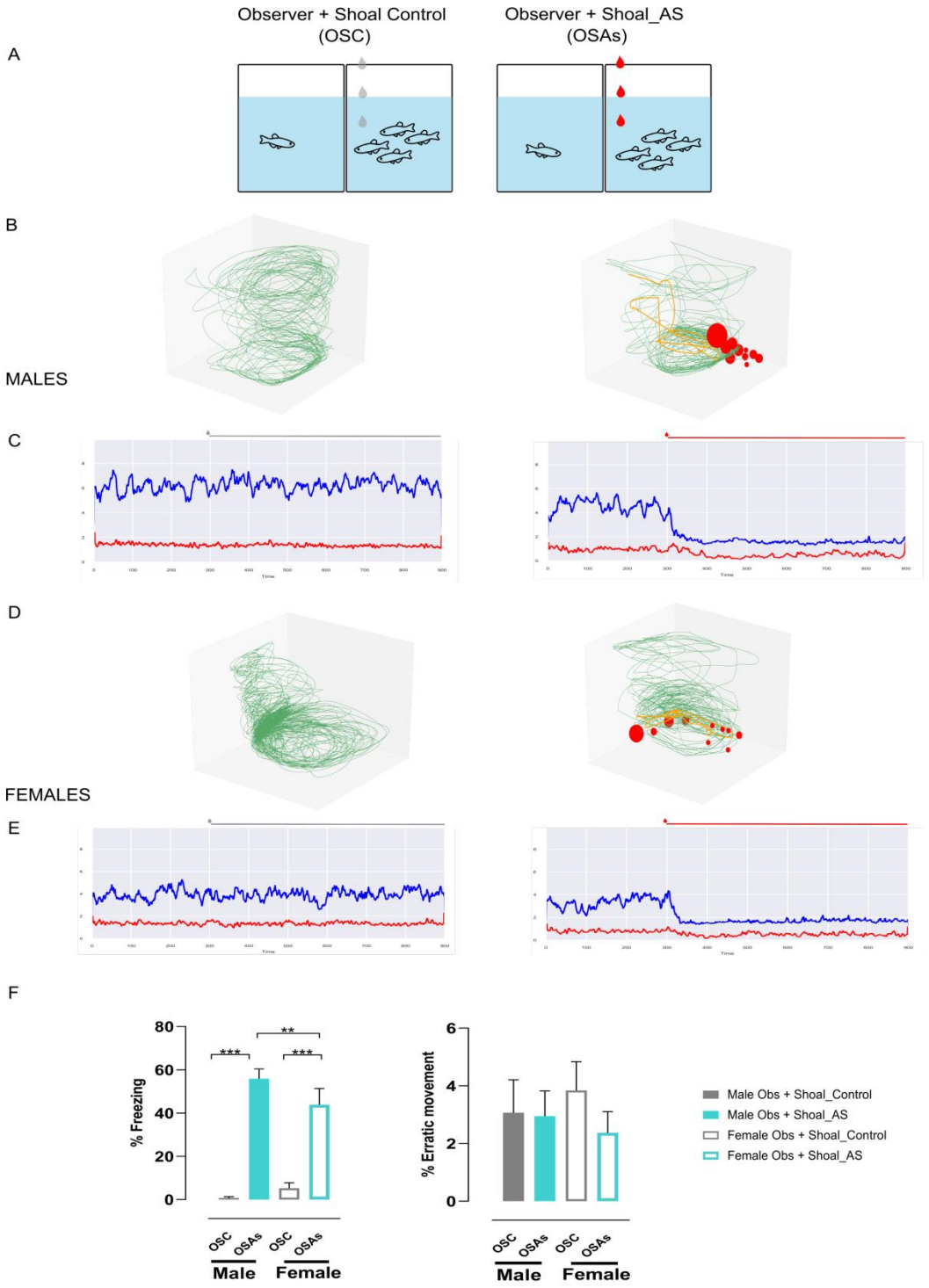

Figure 2.3. Fear response in social contagion.

(A) Schematic representation of the behavioural treatments. Droplets represent administration of vehicle (grey) and alarm substance (AS; red) to control and experimental groups. (B,D) Representative 3D swimming behaviour for each treatment group in males and females; normal swimming pattern (green), erratic movement (orange), and freezing episodes (red dot, size is proportional to freezing time). (C,E) Mean velocity (red) and mean depth (blue) of each treatment group in males and females. (F) Percentage of time in freezing and erratic movement (mean±SEM) during the test phase. The legend identifies sex, presence of AS and the presence of shoal for each treatment. Asterisks indicate statistical significance; **p < 0.01 and *** p < 0.001.

2.3.3. Sex differences in social facilitation

The freezing response of test fish was significantly affected by the presence/absence of a shoal in the adjacent tank but not by the sex of the test fish (Betareg: shoal: $F_{(1,76)}$ = 9.069, p=0.0026: Fig. 2.4F, Table 2.3). Test fish spent significantly less time freezing when both test fish and shoal were exposed to AS compared to when test fish were alone (Betareg planned comparison: Male Obs_AS + Shoal_AS vs Male Alone_AS: $z_{(1,76)}$ = -2.047, p=0.0407; Female Obs_AS + Shoal_AS vs Female Alone_AS: $z_{(1,76)}$ = -2.252, p=0.0243: Fig. 2.4F, Table 2.3).

The erratic movement response of test fish was significantly affected by the presence/absence of a shoal in the adjacent tank but not by the sex of the test fish (Betareg: shoal: $F_{(1, 76)}$ = 12.729, p=0.0004: Fig. 2.4F, Table 2.3). Test fish spent significantly longer time in erratic movement when both test fish and shoal were exposed to AS compared to when test fish were alone (Betareg planned comparison; Male Obs_AS + Shoal_AS vs Male Alone_AS: $z_{(1,76)}$ = 2.781, p=0.0054; Female Obs_AS + Shoal_AS vs Female Alone_AS: $z_{(1,76)}$ = 2.177, p=0.0295: Fig. 4F, Table 2.3).

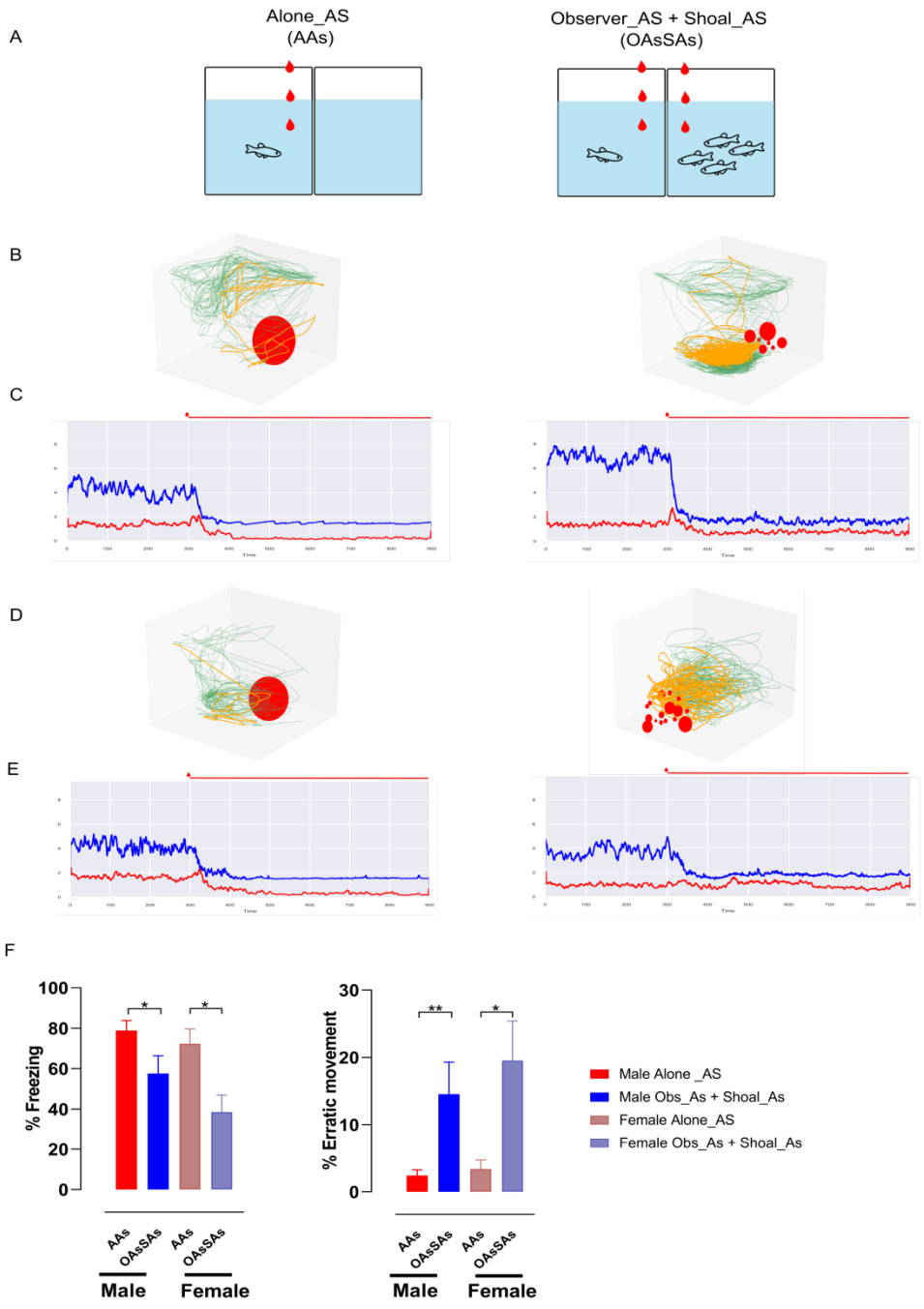

Figure 2.4. Fear response in social facilitation.

(A) Schematic representation of the behavioural treatments. Droplets represent administration of vehicle (grey) and alarm substance (AS; red) to control and

experimental groups. (B,D) Representative 3D swimming behaviour for each treatment group in males and females; normal swimming pattern (green), erratic movement (orange), and freezing episodes (red dot, size is proportional to freezing time). (C,E) Mean velocity (red) and mean depth (blue) of each treatment group in males and females. (F) Percentage of time in freezing and erratic movement (mean+SEM) during the test phase. The legend identifies sex, presence of AS and the presence of shoal for each treatment. Asterisks indicate statistical significance; *$p < 0.05$ and **$p < 0.01$.

2.4. **Discussion**

Our study demonstrates sex differences in social information use in two aspects of threat perception in zebrafish: social buffering and social contagion of fear. We show that both males and females reduced freezing and increased erratic movement when exposed to the alarm substance (AS) in the presence of unalarmed conspecifics (social buffering) but males spend significantly less time freezing than females (Fig. 2.2F, Table 2.1); also, males and females increased freezing when they saw alarmed conspecifics (social contagion) but males spend significantly more time freezing than females (Fig.2.3F, Table 2.2). On the other hand, there was no sex difference in fear response when they received congruent threat information from conspecifics (social facilitation) (Fig. 2.4A); both males and females significantly decreased freezing and increased erratic movement when both they and the shoal received AS but males did not respond significantly more or less than females (Fig 2.4F, Table 2.3). These results show that in threatening situations, zebrafish use information from conspecifics and that males respond strongly than females.

We can exclude the possibility that the sex differences we report in social buffering and fear contagion could have resulted from differences in how males and females were raised and treated during data collection. Test fish were of the same age and were raised in the same conditions (see Methods); also, both males and females followed the same experimental design (Fig. 2.2A, Fig. 2.3A). Previous studies of social buffering in zebrafish have not reported sex differences, but this is not surprising given that these experiments were performed using either only males (Faustino et al., 2017) or a group of mixed-sex test fish (Suboski et al., 1990, Hall & Suboski, 1995; Mathuru et al., 2017). However, sex differences in zebrafish behaviour have been indeed reported in other kinds of studies (Genario, de Abreu, Giacomini, Demin, & Kalueff, 2019); such behaviours include territoriality (Spence et al., 2008), shoaling decisions (Ruhl & McRobert, 2005), lateralization (Ariyomo & Watt, 2013), activity (Tran &

Gerlai, 2013), anxiety-like behaviour (Fontana, Cleal, & Parker, 2019) and courtship behaviour (Spence et al., 2008).

The phenomena of social buffering and contagion of fear are highly conserved, and have been reported in birds (Edgar et al., 2015), mice (Gutzeit et al., 2020; Panksepp, Lane, Solms, & Smith, 2017), rats (Davitz & Mason, 1955; Kim, Kim, Covey, & Kim, 2010; Jones, Riha, Gore, & Monfils, 2014; Kiyokawa, Hiroshima, Takeuchi, & Mori, 2014; Fuzzo et al., 2015; Kiyokawa, Kawai, & Takeuchi, 2018), sheep (Da Costa, Leigh, Man, & Kendrick, 2004; González et al., 2013), monkeys (Kalin & Shelton, 1989; Mineka, Davidson, Cook, & Keir, 1984), and humans (Haaker, Golkar, Selbing, & Olsson, 2017; Megan R. Gunnar, 2017). Some of these studies have used similar or opposite-sex dyads as test subjects and demonstrators (Mineka et al., 1984; Kiyokawa et al., 2014). In our study, we have used test subjects and mixed-sex demonstrators to simulate zebrafish behaviour in its natural environment, thus adding an ecological insight.

Our study demonstrates that males showed less freezing in social buffering and more freezing in social contagion of fear than females, which raises the question of why should males rely more on cues from conspecifics for decision-making under threatening situations than females. The answer could lie in the different behavioural ecologies of the sexes. Male zebrafish have been reported to engage in territorial and courtship behaviour (Spence et al., 2008). Hence, they may need to move to different sites in order to acquire and defend new territories or to search for potential mates. Such relatively unfamiliar environments may impose selection pressures to rely more on information from resident conspecifics (social cues) to evade predators than on information acquired individually. When defending breeding territories males often engage in fights during which synchrony with the behaviour of the opponent is very important. So it is also possible that males are more prone to synchronise with conspecifics behaviours than females, independent of context or information certainty.

In species in which females have high energetic demands imposed by pregnancy, lactation and parental care, they are more risk aversive, thereby

reducing the risk of predation and energetic shortfall, which could be fatal to themselves and their offspring (Reader & Laland, 2000; Smolla, Rosher, Gilman, & Shultz, 2019). However, zebrafish females are not constrained by these behaviours and therefore need not rely so much on social cues under threat. Hence, in zebrafish, the adaptation to both ecological and sexually selected traits may influence sex differences in social buffering and social contagion of fear.

Our finding that both male and female zebrafish display less freezing but more erratic movement in social facilitation indicates that both sexes adopt a similar defence response when there is congruency of information received by both test fish and shoal. When individually acquired information and social cues are in agreement, the behaviour of the group increases the individual's certainties regarding the presence of the predator, reinforcing the decision to respond to the cue in both males and females (Oliveira & Faustino, 2017). However, only the freezing and not the erratic movement response was higher when fish were exposed to AS alone. This could be due to a ceiling effect such that there is a maximum high level of freezing response that can be achieved and this is only reached when the test fish senses the threat in the absence of conspecifics. More so, adopting the erratic movement response strategy rather than freezing when alone could be riskier, because erratic movement could increase the attention of the predator to the location of the individual. On the other hand, engaging in erratic movement in a shoal should be adaptive because typically, zebrafish lives in sediment-rich slowly moving streams and ponds in its geographic origin (Engeszer et al., 2007). Erratic movement performed by a group of zebrafish in these habitats would cause the disturbance of the debris, creating a cloud of “dust” under which zebrafish could not only hide but visually confuse predators due to the high speed and unexpected swim direction changes associated with these motor patterns (Parra et al., 2009).

In conclusion, our study demonstrates that under threatening conditions, such as when predators are present, conflicting information regarding threat cues causes sexually dimorphic fear responses. In contrast, the perception of

congruent information on threat cues causes similar fear responses in both sexes. Together, our findings provide more insight into studying how the ecological context modifies the balance of reliance on information acquired individually and socially. Future studies should now focus on understanding the causes of sexually dimorphic fear responses, both in terms of behavioural ecology and neural regulation.

2.5. **Author Contributions**

Ibukun Akinrinade (I.A) and Rui Filipe Oliveira (R.F.O) designed the experiments and established the behavioural protocols. I.A performed the behavioural experiments; I.A and RFO analysed and discussed the results. We thank Susana Varela for help with statistical analysis and reviewing chapter. We thank Jose Cruz and Abisola Akinrinade for assistance with data analysis in Python.

The study in this chapter is in preparation to be submitted in an international peer-reviewed journal.

Table 2.1 Full LM output: Main effects, interaction, and planned comparisons between treatments for freezing and erratic movement responses of test fish in the social buffering experiment (Fig. 2.2A) # of animals per group=20

			F	p	t	p
Social Buffering Variables						
Freezing	Interaction	SEX:AS	8.544	0.0040*		
		SHOAL:AS	38.315	<.0001*		
	Main effect	SEX	7.777	0.0060*		
		AS	209.750	<.0001*		
		SHOAL	47.513	<.0001*		
	Planned comparisons	Male OAsS vs Female OAsS			4.469	0.0001***
		Male AAs vs Female AAs			1.299	0.1961
		Male OAsS vs Male AAs			-8.191	<.0001***
		Male AAs vs Male AC			9.706	<.0001***
		Male AC vs Male OSC			-0.452	0.6519
		Male OAsS vs Male OSC			1.967	0.0510
		Female OasS vs Female AAs			-5.020	<0.0001***
		Female AAs vs Female AC			11.170	<0.0001***
		Female AC vs Female OSC			-0.258	0.7968
		Female OAsS vs Female OSC			6.407	<0.0001***
Erratic movement	Interaction	SHOAL:AS	6.444	0.0121*		
	Main effect	AS	5.182	0.0242*		
		SHOAL	16.500	0.0001*		
	Planned comparisons	Male OAsS vs Female OAsS			-0.513	0.6084
		Male AAs vs Female AAs			-0.430	0.6681
		Male OAsS vs Male AAs			3.319	0.0011**
		Male AAs vs Male AC			-0.486	0.6276
		Male AC vs Male OSC			0.242	0.8090
		Male OAsS vs Male OSC			2.590	0.0105*
		Female OAsS vs Female AAs			3.235	0.0015**
		Female AAs vs Female AC			0.226	0.8215
		Female AC vs Female OSC			1.271	0.2058
		Female OAsS vs Female OSC			2.190	0.0300*

Treatments: AC, Alone_Control; AAs, Alone_Alarm substance; OSC, Observer + Shoal_Control; OAsS, Observer_AS + Shoal; F: F statistic; t: t-t statistic

*estimate; p: p-value; *significant difference for p < 0.05; **significant difference for p < 0.01; ***significant difference for p < 0.001.*

Table 2.2 Full GLM with Betareg output: Main effects, interaction, and planned comparisons between treatments for freezing and erratic movement responses of test fish in social contagion experiment (Fig. 2.3A).) # of animals per group: Male:OSC=22, OSAs=20; Female: OSC=25,OSAs=20

			F	p	z	p
Social Contagion Variables						
Freezing	Interaction	SEX:AS	7.685	0.0056**		
	Main effect	SEX	5.781	0.0162*		
		AS	81.286	<.0001***		
	Planned comparisons	Male OSAs vs Female OSAs			-2.640	0.0083**
		Male OSAs vs Male OSC			7.873	<.0001***
		Female OSAs vs Female OSC			4.650	<.0001***
		Male OSC vs Female OSC			0.875	0.3814
Erratic movement	Interaction	SEX:AS	0.005	0.9428		
	Main effect	SEX	0.029	0.8651		
		AS	2.143	0.1433		
	Planned comparisons	Male OSAs vs Female OSAs			-1.104	0.6870
		Male OSAs vs Male OSC			1.048	0.7209
		Female OSAs vs Female OSC			-1.034	0.7294
		Male OSC vs Female OSC			0.973	0.7649

*Treatments: OSC, Observer +Shoal_control; OSAs, Observer + Shoal_AS; F: F statistic; z: z estimate; p: p-value; *significant difference for p < 0.05; **significant difference for p < 0.01; ***significant difference for p < 0.001.*

Table 2.3 Full GLM with Betareg output: Main effects and planned comparisons between treatments for freezing and erratic movement responses of test fish in social facilitation experiment (Fig. 2.4A).) # of animals per group: Male:AAs=22,OAsAs=22; Female: AAs=20, OAsAs=20

			F	p	z	p
Social Facilitation Variables						
Freezing	Main effect	SHOAL	9.069	0.0026**		
	Planned comparisons	Male AAs vs Female AAs			-0.789	0.4304
		Male OAsSAs vs Female OAsSAs			-1.021	0.3072
		Male AAs vs Male OAsSAs			-2.047	0.0407*
		Female AAs vs Female OAsSAs			-2.252	0.0243*
Erratic movement	Main effect	SHOAL	12.729	0.0004***		
	Planned comparisons	Male AAs vs Female AAs			1.081	0.2796
		Male OAsSAs vs Female OAsSAs			0.307	0.7586
		Male AAs vs Male OAsSAs			2.781	0.0054**
		Female AAs vs Female OAsSAs			2.177	0.0295*

*Treatments: AAs, Alone_Alarm substance; OAsSAs, Observer_AS + Shoal_AS; F: F statistic; z: z estimate; p: p-value; *significant difference for p < 0.05; **significant difference for p < 0.01; ***significant difference for p < 0.001.*

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3. Chapter 3

Role of oxytocin in social contagion of fear in zebrafish

Summary

In this chapter, we present a set of studies aimed at investigating the role of oxytocin in social contagion of fear using oxytocin ligand (*oxt*) and receptor (*oxtr* and *oxtrl*) mutant (MUT) lines with their wildtype (WT) background- control line. We used the behavioural paradigm for social contagion adopted in chapter 1 (hereafter mentioned as social contagion paradigm), where test fish placed in a test tank can observe either an alarmed or an unalarmed shoal placed in an adjacent tank. The alarmed shoal was administered a threatening stimulus (alarm substance-AS) and the unalarmed shoal was administered a vehicle (distilled water). As an additional control, we also placed test fish in test tanks with AS administered directly and without a shoal in the adjacent tank.

- In a first experiment, we tested the role of oxytocin in the social contagion of fear using the oxytocin receptor (*oxtr*) line. Our results revealed that zebrafish WT displayed increased freezing in the presence of alarmed conspecifics. Conversely, $oxtr^{-/-}$ showed a deficit in freezing response; thus suggesting an effect of the oxytocin receptor in this behaviour.
- In a second experiment, we evaluated the role of oxytocin receptor-like (*oxtrl*) in the social contagion of fear. Our results showed that $oxtrl^{+/+}$ also displayed increased freezing response in the presence of alarmed conspecifics while $oxtrl^{-/-}$ group did not; thus suggesting an effect of the oxytocin receptor-like in this behaviour.
- In a third experiment, we tested the role of the oxytocin ligand (*oxt*) in social contagion of fear. Our results showed that $oxt^{+/+}$ displayed increased freezing response in the presence of alarmed conspecifics and on the contrary, $oxt^{-/-}$ did not show any freezing response to the sight of the alarmed shoal.
- In the fourth experiment, we show that the acute administration of isotocin (the oxytocin-like peptide found in ray-finned fish) to $oxt^{-/-}$ rescues the freezing-specific behaviour of oxytocin neurons; thus

demonstrating it is indeed oxytocin and not another neurotransmitter that binds to the oxytocin receptors that modulates social contagion of fear in zebrafish.

- In all cases, the deletion of oxytocin receptors and ligand did not affect the response to fear that occurred as a result of direct exposure to the alarm substance; thus, indicating a function of oxytocin in regulating a social component of behaviour (social contagion of fear).
- In the fifth experiment, we analysed brain samples of *oxtr* line to investigate the role of oxytocin during social contagion of fear in the brain regions involved in the Social Decision Making Network (SDMN) and evaluated the pattern of functional connectivity using the expression of pS6 – a marker of neuronal activation. Results showed changes in specific brain areas (Vv, Vc) in the ventral telencephalon as well as changes in the pattern of functional connectivity between social treatments.
- In the sixth experiment, we determined the presence of both oxytocin receptors in some SDMN brain regions. Results showed that oxytocin receptor is present in the Vv, Vc, Vd, Vs/Vp, POA, Dm, and Ob; and oxytocin receptor-like is present in the Vv, Vc, Vd, and Ob. We also examined the neural projection pattern of oxytocin to the ventral telencephalon in zebrafish adult and larvae. We show oxytocin projects to the ventral telencephalon in both zebrafish adult and larvae.
- Taken together, our results provide compelling evidence that indicates that oxytocin plays a role in the regulation of social contagion of fear in zebrafish and that this modulation occurs in specialized regions of the brain.

3.1. **Introduction**

The appropriate response to environmental stimuli that predict potentially harmful events is an adaptive mechanism crucial to the survival of any organism.

The evolution of predator avoidance requires the development of mechanisms to detect and avoid predator threats in order to increase fitness.

Threat perception can be seen as the act of decision making regarding threat upon the acquisition of information about predators individually, or by observing the alarming behaviour of conspecifics (Mineka et al., 1984).

Social information use is a ubiquitous phenomenon in group-living animals. There is ample evidence suggesting that animals can use social information through the observation of the behaviour of others to modulate individual perception of threat. Observational fear acquisition is said to be an adaptive strategy induced by the transmission of the demonstrator's affective state or behavioural response to a threatening stimulus (Keum & Shin, 2019; Olsson, Nearing, & Phelps, 2007). This phenomenon has been reported to occur in a wide range of species such as fish (Hall & Suboski, 1995), birds (Curio, Ernst, & Vieth, 1978), rodents (Bruchey, Jones, & Monfils, 2010; Jones et al., 2014; M. Kavaliers, Choleris, & Colwell, 2001; Monfils & Agee, 2019), cats (John et al., 1968), primates (Mineka et al., 1984) and humans (Haaker et al., 2017; Olsson & Phelps, 2007). Nevertheless, the underlying neurobiological substrates phenomenon are still not clear.

The highly conserved nine-amino-acid neuropeptide- oxytocin (OT) has been reported to play a role in the modulation of social behaviours such as approach, recognition, reward, learning, anxiety, aggression, mating and others (Bethlehem, Baron-Cohen, van Honk, Auyeung, & Bos, 2014; Dölen, 2015; Fineberg & Ross, n.d.; Goodson & Kingsbury, 2013; Grinevich & Stoop, 2018; Marlin et al., 2015; L. A. O'Connell, Matthews, & Hofmann, 2012), including fear (Braida et al., 2012).

Although most vertebrates have at least one receptor for oxytocin, teleosts have two oxytocin receptors; this occurred due to a gene duplication event (Wircer et al., 2016). There has been much speculation on the exact roles of oxytocin in the regulation of social behaviour as well as the functional implications of the duplication of its receptors in teleosts and zebrafish in particular. Also, the distribution pattern of oxytocin projection and receptors in zebrafish remains unclear.

The Social decision-making network (SDMN) is described as a network of nodes that regulate and implement responses to salient stimuli (Almeida, Félix, Oliveira, Lopes, & Oliveira, 2019; O'Connell & Hofmann, 2011; Teles, Almeida, Lopes, & Oliveira, 2015). Given that social decision-making has been described to involve the integration of multimodal sensory information about a social context to produce an appropriate behavioural response (Goodson & Kabelik, 2009; Teles et al., 2015), it could be hypothesized that social decisions should rely on multiple neural circuits, rather than being regulated by only one specific brain region, such that a social behavioural phenotype is reflected by the overall pattern of neuronal activation across nodes and variation in the strength of the connections among them.

In the present work, we investigated the role of oxytocin, a neurobiological substrate of social behaviour in the modulation of social contagion of fear in zebrafish. We used mutant zebrafish lines with the constitutive deletion of both oxytocin receptors to find out whether the receptors are functionally homologous or not. We also used mutant zebrafish lines with the constitutive deletion of the oxytocin ligand and a deficit recovery technique to investigate if it is oxytocin indeed that binds to the oxytocin receptors and modulates social contagion of fear. Next, we investigated which brain regions are modulated by oxytocin during social contagion, and examined the functional connectivity patterns between those regions. Finally, we investigated the presence of oxytocin receptors and oxytocinergic projections in some of the brain regions indentified before. With this complete approach, we aimed to wholly understand the role of

oxytocin in the modulation of social contagion of fear in a model species such as the zebrafish.

3.2. **Materials and methods**

3.2.1. Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) 6-12 months old TU strain wild-type (WT) were raised in mixed-sex 5 L tanks (n=35 per tank). Mutant (MUT) zebrafish 3-6 months old *oxtr*$^{-/-(wz16)}$ (ZFIN ID: ZDB-ALT-190830-1) also known as oxytocin receptor (*oxtr*), *oxtrl*$^{-/-(wz17Tg)}$ **(**ZFIN ID: ZBD-ALT-190819-1) also known as oxytocin receptor-like (*oxtrl*), *oxt*$^{-/-(wz13)}$ (ZFIN ID: ZBD-ALT-180904-7) also known as oxytocin ligand (*oxt*), and transgenic oxt:EGFP reporter [Tg(oxt:egfp)] lines were raised in a TL-mixed background and generated in the laboratory of Dr. Gil Levkowitz (Weizmann Institute of Science, Rehovot, Israel).

The *oxtr*$^{-/-}$ line was generated using the transcription activator-like effector nuclease (TALEN) genome-editing technique that causes a small deletion (1bp) due to a frameshift mutation leading to a premature stop codon and a truncated oxtr protein (Nunes et al., 2020; Ribeiro et al., 2020; Sander et al., 2011).

The *oxtrl*$^{-/-}$ line was generated using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - cas 9 technique which involves the insertion of a multi-frame stop cassette (total 83bp) at the ATG+260 position leading to a stop codon formation after the 89th amino acid (Irion, Krauss, & Nüsslein-Volhard, 2014).

The *oxt*$^{-/-}$ line was also generated using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - cas 9 gene-editing technique which involves a small deletion (7bp) in the exon 2 of the *oxt* gene causing a mutation that leads to an early frameshift leading to a novel sequence followed by a premature stop codon.

Transgenic oxt:EGFP reporter [Tg(oxt:egfp)] line was generated using the Tol2kit transposon-based vector system in which site-specific recombination-based cloning of cDNAs encoding the *oxt* gene was done downstream of the Gal4-responsive 10XUAS element and basal promoter (Blechman, Amir-Zilberstein, Gutnick, Ben-Dor, & Levkowitz, 2011; Gutnick et al., 2011; Kwan et

al., 2007) This line provides an endogenous expression of *oxt* mRNA and protein (Wircer et al., 2017).

The lines were raised and bred in a recirculating system (ZebTEC active-blue, Tecniplast) under 14L: 10D photoperiod, in the fish facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Husbandry protocols, water chemistry and health program have been described previously (Borges et al., 2016). In particular, tank water was maintained at 28ºC, 900-1300 µS, pH 7.0, <0.2 ppm nitrites, <50 ppm nitrates and 0.01-0.1 ppm ammonia, and the colony has been free from all known pathogens. Also, pre-filter sentinels tested negative for Mycobacteria and *Pseudoloma neurophilia* since 2016. Experimental animals were fed with a combination of live food (*Paramecium caudatum, Artemia salina, and Rotifers)* and commercial processed dry food (Gemma).

Male zebrafish were used as test fish in all the experiments. All test fish were drug naïve, and each fish was used only once. All experiments were performed following the standard operating procedures of the Instituto Gulbenkian de Ciência Ethics Committee.

3.2.2. Genotyping

We performed zebrafish genotyping by PCR of the genomic region of interest from clipped fins of adult zebrafish, followed by sequencing (Ribeiro et al., 2020; Xing, Quist, Stevenson, Dahlem, & Bonkowsky, 2014).We designed specific primer pairs to target ligand and receptors' deletion sites (Table 3.1).

3.2.3. Alarm substance extraction

Alarm substance (AS) (previously described in chapter one), a chemical compound that is released from epidermal club cells upon injury of the skin, was used to induce a fear response in this experiment. AS was extracted using a modified version of the protocol described by Speedie and Gerlai (Speedie & Gerlai, 2008). Ten donor zebrafish (five males and five females) were used to

prepare the alarm substance for the experiment. Donor fish were retrieved from the holding tank with a fishing net, and then quickly sacrificed by cervical transection, placed in a Petri dish on ice and 15 shallow cuts were made on both sides of the fish using a sterile surgical blade (Swann Morton #12536518). Using a Terumo® syringe (8SS50L1) without needle 50ml of distilled water (vehicle) was used to wash the cuts ensuring the adequate release of AS. Impurities were removed from the AS solution using 0.22 µm sterile vacuum filtering (Filtropur V50- 83.3940) and AS aliquots were stored at -20^{o}C.

3.2.4. Experimental procedure

We performed two types of experiments: a control experiment, where WT and MUT test fish were exposed directly to the AS, and social contagion experiments where WT and MUT test fish were exposed indirectly to the AS (they could observe a shoal of conspecifics that were exposed to the AS).

For the experiments on social contagion, WT and MUT test fish were randomly assigned to one of two treatments: test fish with shoal exposed to vehicle (distilled water) (Obs + Shoal_Control); and test fish with shoal exposed to AS (Obs +Shoal_AS). For the control experiment, WT and MUT test fish were assigned to only one treatment: test fish alone exposed to AS. This experiment allowed to measure the effect of the oxytocin mutations on individual perception of threat and therefore disentangle whether oxytocin acts on fear perception in general or exclusively on social contagion of fear.

The order of testing was randomized in each experiment, and all experiments were conducted between 10:00 and 19:00. On the day before the experiment, test fish were removed from home tanks and randomly assigned to treatment groups for acclimatization. On the test day, each trial lasted for 15 mins and was divided into two phases: the first 5mins to establish baseline and the subsequent 10 mins to measure the response to the treatment.

The behavioural setup was performed similarly to chapter two. Briefly, we used two adjacent tanks (12 x 12 x 15 cm) each filled with 1.3L of water. The test fish

(males) were placed in one tank, and the other tank contained a shoal of two males and two females. For the control experiment, the second tank remained empty. The tanks had opaque white bases, opposite and rear walls to prevent interference by external environmental cues.

AS was kept in ice to avoid degradation during the trials and therefore distilled water was kept in the same conditions to ensure comparability of treatments. AS and distilled water were administered with a flexible and transparent PVC tubing (0.8 mm internal diameter; 2.4 mm external diameter).

The arena dimensions for top and side views were 10.5 x 10.5 cm and 11 x 10.5 cm, respectively. Two video cameras (two webcams (Logitech B 525 high definition camcorder, Ref 960-000842)) were placed above and to the side of the tanks. Eyeline surveillance software (www.nchsoftware.com) was used for video acquisition. The x,y,z coordinates extracted from automated video tracking by Ethovision software (Ethovision XT 12, Noldus Technology), were analysed using an updated version of custom made xyz2b Python scripts. Percentage of time spent in erratic movement and freezing was used as a measure of the fear response in the experiments. Two criteria defined erratic movement; if the test fish accelerated >8 cm/s^2 and >5 changes in direction/sec. Two criteria defined freezing: if the test fish velocity was <0.2 cm/s; and if the test fish position on y-axis was below the bottom quarter of the side view of the arena.

3.2.5. Drug treatment

In one of the social contagion experiments we perform the acute administration of isotocin (the oxytocin-like peptide found in ray-finned fish) to *oxt*$^{-/-}$ mutants. Isotocin ((Ser4, Ile8)- oxytocin) (IT), (4030890.0005, Bachem, Germany) was dissolved in saline (0.9%) and stored at -20^{o}C until use. 2ng/kg isotocin was administered by intraperitoneal (i.p) injection. Doses were based on previous studies in zebrafish (Braida et al., 2012; Zimmermann, Gaspary, Siebel, & Bonan, 2016). Test fish were anaesthetized using 100mg/L tricaine solution (MS-222) before injection until the animals showed a lack of motor coordination

and reduced respiration rate. Following anaesthesia, body weights were measured, and i.p injection was done with the volume of drug adjusted to the weight of the fish (2ul/g) using a 10 ul Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). The administration period was approximately 20s. Test fish were subsequently individually returned to the test tank previously used for acclimatization to facilitate recovery and then returned to the experimental arena. Testing resumed after a total of 5mins of recovery.

3.2.6. Tissue collection and preparation

To determine how oxytocin modifies social contagion of fear, we evaluated the brain activation patterns and patterns of functional connectivity in *oxtr*$^{+/+}$ and *oxtr*$^{-/-}$ test fish brains. Animals were sacrificed 1hr after the conclusion of the behavioural trials to allow for the expression of the pS6 (phospho-S6 ribosomal protein) neural activation marker.

oxt:GFP zebrafish for visualizing oxytocinergic projections and oxtr test fish from social contagion experiment were anaesthesized in ice cold water followed by cervical transection.

Heads of the test animals from both experiments were subsequently fixed in 10% buffered formalin for three days at room temperature, rinsed in 1x PBS (2X, 30min) at room temperature and kept in EDTA (0.5 M, pH=8) for 2 days at room temperature.

We then performed immunohistochemical staining and microscopy and image analysis on these samples (see details below).

Samples were then processed for histological analysis where samples were incorporated in paraffin, and coronal sections were cut at 5µm thickness for pS6^{+} cell count and sagittal cuts at 6µm were made for visualizing oxytocin projection in adult zebrafish. Slides were subsequently stored in boxes at room temperature until immunohistochemical processing.

We also determined the *oxtr* and *oxtrl* receptors expression patterns in adult zebrafish using adult zebrafish TU line. Upon anaesthesia in ice cold water, animals were sacrificed by cervical transection and embedded in cryomoulds using tissuetek O.C.T. Compound (Sakura), for cryostat sectioning at 150 µm thickness.

For larvae processing, at 8dpf (days post fertilization) oxt:EGFP embryos were collected and fixed in 4% PFA in PBS overnight, rinsed in PBST, and then transferred through ascending grades of methanol/PBST and stored overnight. We then performed immunohistochemical staining, microscopy and image analysis

3.2.7. Immunohistochemical staining

For immunostaining, sectioned brains of adult oxt:EGFP fish were stained with oxytocin (inorder to enhance the visualization of the oxytocin projections) and isl1 (a neural progenitor marker that is expressed in the subpallium specifically in the Vv and Vs and serves as a landmark for these regions) for visualizing oxytocin projections and sectioned brains of WT and MUT oxtr, oxtrl and oxt fish exposed to the social contagion experiment were stained with phospho-S6 ribosomal protein (pS6) - a neural activation marker for determining neuronal activation. Slides were kept in Tris-EDTA at 95°C 20 min for antigen retrieval. Non-specific binding was blocked by incubating slides in TBS (0.025% Triton X-100) containing 1% BSA (albumin) during 1 hr at room temperature after which an overnight incubation at 4 °C in primary antibody prepared in blocking solution was done (Cell Signalling pS6 ribosomal protein Ser$^{235/236}$ antibody D57.2.2E Rabbit mAB #4858 1:400, GeneTex isl1 rabbit anti-Isl1 pAB GTX128201 1:200, Invitrogen chicken anti-eGFP pAB A10262; Life technologies/Thermo Fisher, Waltham, MA USA 1:200). Slides were then rinsed in TBS 0.025% Triton X-100 and incubated in secondary antibody (Alexa 594- Invitrogen goat anti-rabbit # A-11037 1:1000, Alexa 488- Invitrogen goat anti-chicken A-11039 1:1000) also prepared in blocking solution. Slides were subsequently washed in TBS 0.025% Triton X-100 and then TBS without triton before incubating in with DAPI (4',6-

diamidino-2-phenylindole) for nuclei counterstaining for 20 mins and then rinsed in TBS before mounting (Biotium, Everbrite- 23003).

For larvae processing for imaging, samples were rehydrated through descending grades of methanol/PBST for 5min and rinsed PBST (3x), DDWT-DDW, 0.1% TritonX-100 (2x), permeated with proteinase K (10 µg/ml in PBST), rinsed in PBST (3X), PBS-DT (1x PBS, 1%BSA, 1%DMSO,0.1%TritonX-100-2x), blocked with 5% goat serum in PBSDT for 2hrs at room temperature and then incubated in primary antibodies (peninsula laboratories Guinea-Pig anti-oxytocin antibody, T-5021 1:100, Invitrogen chicken anti-eGFP pAB A10262; Life technologies/Thermo Fisher, Waltham, MA USA 1:200, GeneTex isl1 rabbit anti-Isl1 pAB GTX128201 1:200) for 2 days, and then washed in PBSDT for 2hrs 3x, and incubated in secondary antibody (Alexa 488- Invitrogen goat anti-chicken A-11039 1:1000, Alexa 594- Invitrogen goat anti-rabbit # A-11037 1:1000) overnight, rinsed in PBSDT for 1hr 3x, and mounted in 80% glycerol.

3.2.8. Microscopy and image analysis

To quantify the number of pS6 positive cells, and visualize oxytocin projections in adult zebrafish brain, stained brain sections were acquired at 20x using the Zeiss Axioscan.Z1 slide scanner and analysed using the Zeiss Zen blue 2.1 imaging software. Images were acquired in .czi format and subsequently exported to Fiji software (Schindelin et al., 2012) for conversion to .tiff and .png format for panels arrangement.

Five consecutive coronal sections were quantified for each brain region selected (Fig. 3.1, Table 3.7). In each section, positive pS6 cells were counted in a rectangle with an area of at 1000 µm per area per section using the Zeiss Zen blue 2.1 imaging software. The nomenclature of brain nuclei was identified using the zebrafish atlas (Wullimann MF, Rupp B and Reichert Vogel, 1996).

Following larval immunostaining, embryos were mounted in 80% glycerol and Confocal Z-series stacks were acquired on a Leica SP5 confocal, using a 20x/0.7 multi-immersion objective, the 488nm and 561nm laser lines, and

spectral detection adjusted for the emission of the Alexa 488 and Alexa 568 fluorochromes, using HyD detectors in photon counting mode.

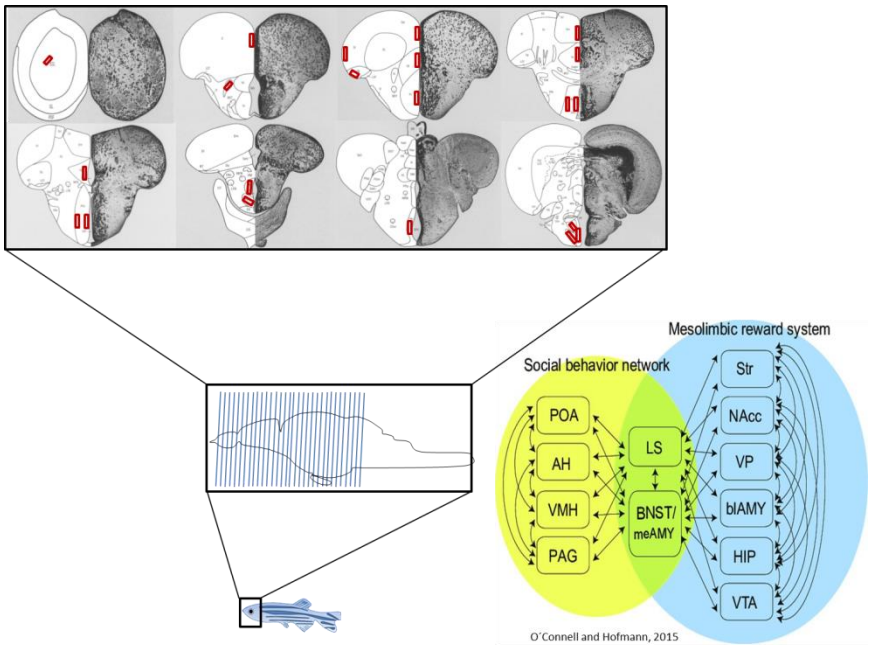

Figure 3.1. The identification of brain regions involved in the oxytocin modulation of social contagion of fear.
The social decision-making network; a combination of the social behaviour network and the reward system (O'Connell & Hofmann, 2011a). Red rectangles represent brain regions of interest for quantifying pS6 positive cells; brain regions of interest are identified using the zebrafish atlas (Wullimann MF, Rupp B and Reichert Vogel, 1996).

3.2.9. Microdissection of brain regions

In order to determine the expression pattern of *oxtr* and *oxtrl,* 11 TU zebrafish brains were microdissected to obtain micro areas for RNA extraction, cDNA production and qRT-PCR.

Microdissection method was adopted from Teles et al, (Teles et al., 2015). 11 naïve TU fish were sacrificed by rapid chilling method (Wilson, Bunte, & Carty, 2009), followed by rapid decapitation. The heads were embedded in mounting

media (OCT Compound, Tissue-Tek, Sakura 4583] and rapidly frozen on dry ice. Brains were sectioned in the coronal plane at 150 µm thickness in the cryostat (Leica CM 3050S) and brain sections were collected on cold regular glass slides previously cleaned with 70% ethanol and placed on a glass petri dish, filled with dry ice to maintain low temperatures.

Brain regions of interest were identified using the zebrafish brain atlas (Wullimann MF, Rupp B and ReichertVogel, 1996), and microdissected under a stereoscope (Zeiss Stemi 2000).

Tissues were collected with modified 27G needles (inner diameter = 210µm) with flattened and smoothened with an electric drill and then attached to a syringe. To prevent cross-contamination between brain regions, one needle per brain region was used and the needles were cleaned sequentially with distilled water and ethanol 70% between individuals.

We selected the following areas due to their involvement in social regulation and social decision making (Kermen, Franco, Wyatt, & Yaksi, 2013; Teles et al., 2015): the olfactory bulb (Ob) which projects to the ventral telencephalon (Rink & Wullimann, 2004), the medial zone of the dorsal telencephalic area (Dm, putative homologue of the mammalian basolateral amygdala), the preoptic area (POA) a node in the social behaviour network; and the ventral nucleus of the ventral telencephalic area (Vv, putative homologue of the mammalian lateral septum), the dorsal nucleus of the ventral telencephalic area (Vd, putative homologue of the mammalian striatum), the supracommissural nucleus of the ventral telencephalic area (Vs, putative homologue of the mammalian medial extended amygdala and the bed nucleus of the stria terminalis), and the postcommissural nucleus of ventral telencephalic area (Vp).

The Ob, Vv, Vd, Vs/Vp, and POA were collected from both hemispheres at a single sampling point, due to their small size when compared to the diameter of the microdissection, while the Dm was sampled from both hemispheres separately, and tissue from the two hemispheres was then pooled directly into lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen, 74804) and stored at -80 until

mRNA extraction. Also, the Vs/Vp brain region was pooled as one brain area due to their very close proximity to each other.

3.2.10. RNA extraction

Tissue was homogenized in 100 µl of qiazol (lysis buffer) and incubated for 7 min at room temperature (RT). 50 µl of Chloroform was then added and shaken vigorously for 15 s, after which the sample was left to incubate at RT for 5 min. Samples were subsequently centrifuged at 13000 g for 20 min at 4ºC, after which the upper aqueous phase was transferred to a new tube where 1 volume of 70% ethanol was added. This mixture was transferred to an RNEasy column and left to stand for 5 min at RT, and then was centrifuged for 1 min at 9000 g. Addition of a sequence of buffers (provided by the RNeasy Lipid Tissue Mini Kit) to the RNEasy column was done (700 µl of Buffer RW1, 500 µl of Buffer RPE and an additional 500 µl Buffer RPE). After each buffer, samples were centrifuged for 1 min at 9000 g and the flow-through was discarded. The RNeasy column was then placed in a new 2 ml tube and centrifuged for 3 min at 14000 g. The column was then transferred to a new 1.5 ml tube and RNA was eluted with 25 µl of RNAse-free water, and centrifuged for 2 min at 9000 g. The elution step was repeated with the same 25 µl of RNAse-free water to increase RNA recovery efficiency. RNA concentration and purity of all samples were estimated by spectrophotometric absorbance (260 nm and 280nm) in the Nanodrop (Thermo Scientific NanoDrop 2000), and the RNA integrity of the samples was determined using the Bioanalyzer (Agilent 2100 Bioanalyzer).

3.2.11. cDNA synthesis

RNA from a pool of each brain area was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Biorad, 1708890) following the manufacturer's instructions. Briefly, in a clean Eppendorf tube, nuclease-free water, 5x iScript reaction mix (4µl), iScript reverse transcriptase (1µl), and RNA template (100 fg to 1 µg total RNA) were added up to a total volume of 20µl and then incubated in a PCR thermocycler with the following protocol: Priming for 5 mins at 25 ºC, reverse

transcription for 60 min at 42 ºC, reverse transcription inactivation for 5 mins at 85 ºC, Hold at 4ºC. Samples were subsequently stored in -20 ºC until use.

3.2.12. Quantitative RT-PCR (qRT-PCR)

cDNA samples were diluted 1:10 before being used as a template for quantitative polymerase chain reactions (RT-PCR).

Primer sequences for oxytocin receptor (*oxtr*) and the eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*) -the reference gene were designed in Primer 3 software (Premier Biosoft International, Palo Alto, CA, USA) and primer sequences for oxytocin receptor-like (*oxtrl*) were received from Gil Levkowitz lab. qRT-PCR reactions were performed in the Applied Biosystems quantstudio 7 thermocycler (7900 HT, Thermofisher) in 8 µl triplicate reactions with SYBR Green PCR Master Mix (Applied Biosystems, Thermofisher) and primers at 50 µM for *oxtr* and *eef1a*; and 13.3 µM for *oxtrl.* Thermocycling conditions were 5 min at 95º C, followed by 40 cycles of 95ºC for 30 s, annealing temperature 60º C for 30 s, and extension at 72 ºC for 30 s.

After PCR, a melting curve program from 55 to 95ºC with 0.5 º C changes was applied. Fluorescence cycle thresholds (Ct) were automatically measured and the relative expression of the target genes calculated using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001; Teles et al., 2015) i.e $2^{Ct_{Ref}-Ct_{Target}}$, where Ct_{Ref} is the cycle threshold for the reference gene and Ct_{Target} is the cycle threshold for the target gene. Therefore, target gene expression is represented as relative expression to the reference gene. Mean of the Cts of the three technical replicates was used.

3.2.13. Statistical analysis

WT and MUT test fish response to AS alone was analysed using unpaired t-test for parametric data. When normality was confirmed but not homogeneity of variances, unpaired t-test with Welch's correction was used, and Mann-Whitney's non-parametric test was used for non-parametric data analysis.

Behavioural variables of *oxtr* and *oxtrl* experiments were logarithmically transformed [log10 (x+1)] to meet parametric test assumptions, and analysis was done using a two-way ANOVA method, with genotype and AS as fixed effects. Raw values of the experiment with *oxt* ligand were analysed using two-way ANOVA method, with genotype and AS as fixed effects and raw values of the oxytocin ligand recovery experiment were analysed using three-way ANOVA method with genotype, AS, and treatment as fixed effects. Following all ANOVA analysis, we performed planned comparisons corrected with False Discovery Rate (FDR) p-value adjustment (Benjamini and Hochberg's method) to assess differences between the experimental treatments. Normality was tested using the D'Agostino & Pearson test (D'Agostino RB, 1986).

For analysis of cell counts of pS6 positive cells, a generalized linear model (GLM) with quasi-Poisson regression was used, with AS and genotype as fixed effects. A backward stepwise procedure was used to exclude non-significant interactions and fixed effects, followed by planned comparisons corrected with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli method to give FDR-adjusted p-value (Glickman, Rao, & Schultz, 2014).

Analysis of functional connectivity was done with Pearson correlation matrices for comparison of pS6 cell count between each pair of brain nuclei (Batushansky, Toubiana, & Fait, 2016) and the correlations were used as a measure of co-activation between brain nuclei, such that positive correlations correspond to phasic activity and negative correlations to out-of-phase activity (Teles et al., 2015). Following this, statistical testing was done using a Quadratic Assignment Procedure (QAP) with 5000 permutations (So, Franks, Lim, & Curley, 2015). The null-hypothesis for QAP is that there is a non-random association between the tested matrices. Measures for network connectivity were analyzed using UCINET Version 6.102 (Borgatti, Everett, & Freeman, 2002), and brain nuclei representations of the SDMN network were produced using a custom-made Python script.

Assessment of network density between treatments was done using a t-test analysis and bootstrap was set at 5000 sub-samples to determine network

cohesion (Almeida et al., 2019; Schaefer et al., 2014).

Statistical significance was considered if $p < 0.05$ and data are presented as mean $\pm$ SEM. Graphpad Prism version 8 (Weiß, 2007) and R (R Core Team, 2015) statistical programs were used for data analysis.

3.3. **Results**

3.3.1. Response to alarm substance in wild type and oxytocin ligand and receptor mutants.

We measured freezing and erratic movement as parameters for assessing fear response in both WT and MUT oxytocin ligand and receptors. WT and MUT oxytocin ligand and receptors did not differ significantly in response to alarm substance. There was no variation in erratic movement and freezing response between the genotypes. (Fig. 3.2 A-F, Table 3.2).

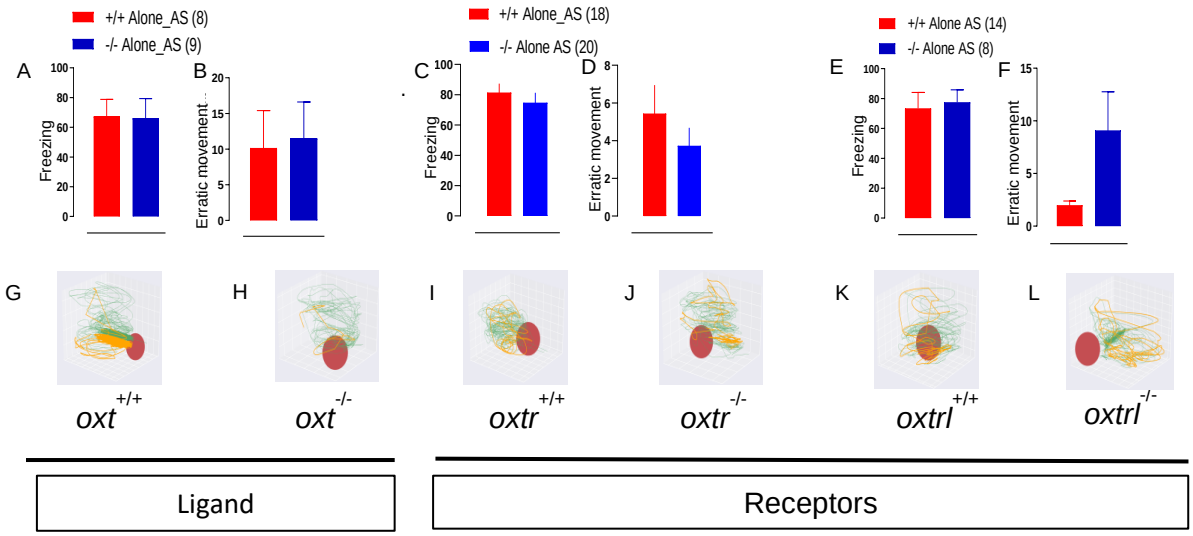

Figure 3.2. Response to alarm substance in oxytocin ligand and receptor WT and MUT.
(A-F) Percentage of time in freezing and erratic movement (mean±SEM) during the test phase. The legend identifies the genotype of each treatment. (G-L) Representative 3D swimming behaviour for each treatment group in males and

females; normal swimming pattern (green), erratic movement (orange), and freezing episodes (red dot, size is proportional to freezing time).

3.3.2. Oxytocin receptor mutants displayed freezing deficit upon sight of alarmed conspecifics

We assessed the response of oxytocin receptor mutants (*oxtr*) to the presence and absence of alarmed conspecifics. The freezing response of *oxtr* test fish was significantly affected by genotype and the presence of AS (two-way ANOVA: genotype: $F_{(1,76)}$= 4.113, p=0.0461; AS: $F_{(1,76)}$= 7.113, p=0.0093: Fig. 3.3E Table 3.3). But there was no significant interaction between genotype and AS (two-way ANOVA: genotype x AS: $F_{(1,76)}$= 2.342, p=0.1301: Fig. 3.3E Table 3.3).

When exposed to an alarmed shoal, the freezing response of $oxtr^{-/-}$ was significantly lower than $oxtr^{+/+}$ (planned comparisons: $oxtr^{-/-}$ Obs + Shoal_AS vs $oxtr^{+/+}$ Obs + Shoal_AS: $t_{(1,76)}$ =2.516, p=0.028). On the other hand, there was no significant difference in freezing response of $oxtr^{+/+}$ and $oxtr^{-/-}$ controls (planned comparisons: $oxtr^{-/-}$ Obs + Shoal_Control vs $oxtr^{+/+}$ Obs + Shoal_Control: $t_{(1,76)}$ =0.3518, p=0.7259) (Fig. 3.3E Table 3.3).

The freezing response of $oxtr^{+/+}$ test fish was significantly higher upon sight of alarmed conspecifics in contrast with control (planned comparisons: $oxtr^{+/+}$ Obs + Shoal_AS vs $oxtr^{+/+}$ Obs + Shoal_Control: $t_{(1,76)}$=2.968, p=0.016). On the contrary, there was no significant difference in the freezing response of $oxtr^{-/-}$ test fish exposed to an alarmed shoal when compared with control (planned comparisons: $oxtr^{-/-}$ Obs + Shoal_AS vs $oxtr^{-/-}$ Obs + Shoal_Control: $t_{(1,76)}$=0.8037, p=0.565).

Finally, there was no statistically significant difference in erratic movement response of $oxtr^{+/+}$ and $oxtr^{-/-}$ test fish exposed to an alarmed shoal (Fig. 3.3F Table 3.3).

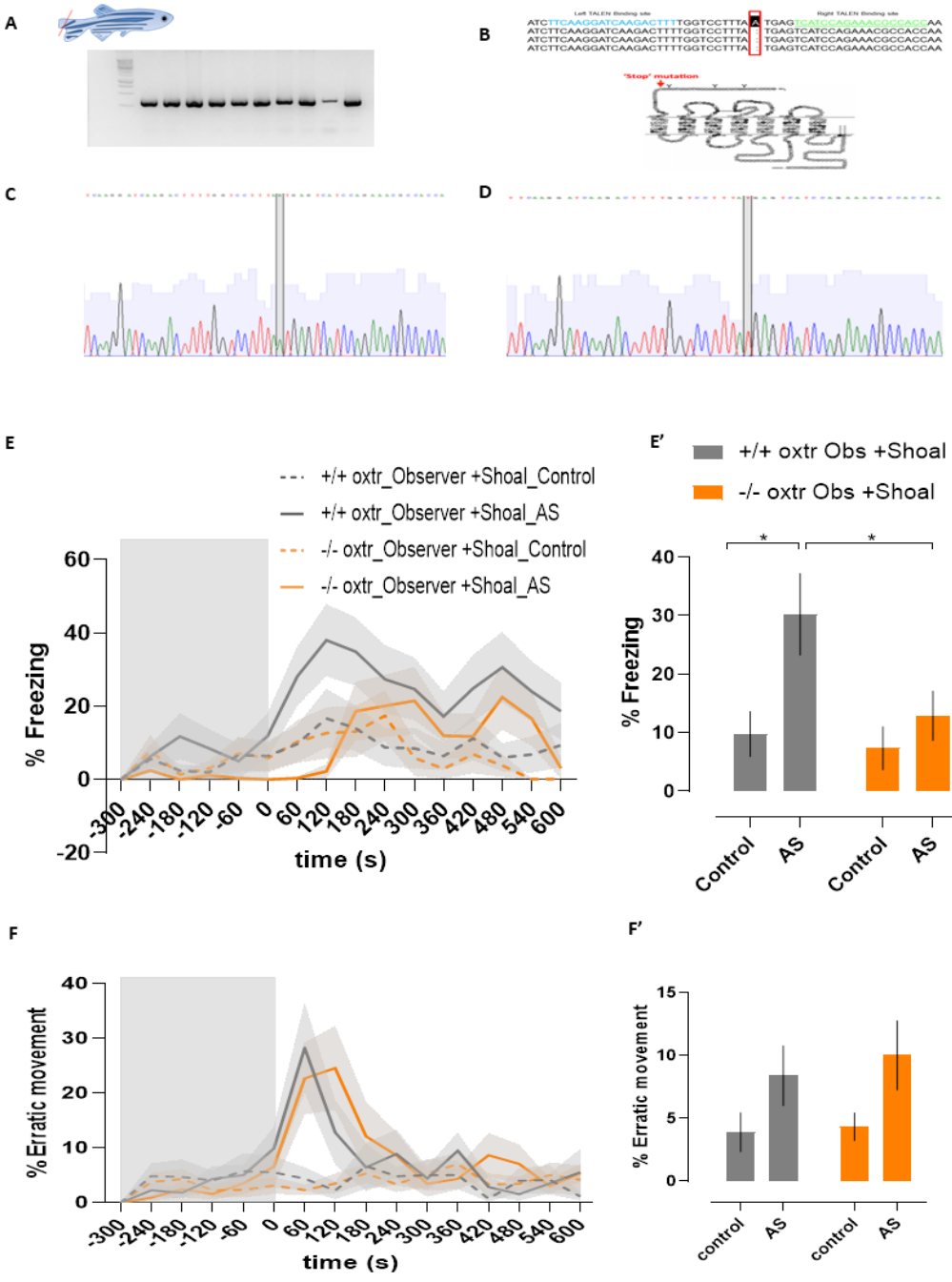
A
B
C
D
E
+/+ oxtr_Observer +Shoal_Control
+/+ oxtr_Observer +Shoal_AS
-/- oxtr_Observer +Shoal_Control
-/- oxtr_Observer +Shoal_AS
% Freezing
time (s)
E'
+/+ oxtr Obs +Shoal
-/- oxtr Obs +Shoal
% Freezing
Control
AS
Control
AS
F
% Erratic movement
time (s)
F'
% Erratic movement
control
AS
control
AS

Figure 3.3. Oxytocin receptor (oxtr) mutants' displayed altered freezing response in presence alarmed conspecifics.
(A) Genotyping of *oxtr* line using fin clip: gel electrophoresis showing DNA bands (B) Schematic of nucleotide deletion using TALEN approach (C-D) Ape file showing position of *oxtr* nucleotide deletion (E, F) Line graph showing temporal dynamics of freezing and erratic movement response across treatments. Shaded area indicates time before AS or vehicle administration (E',F'') Percentage of freezing and erratic movement (mean±SEM) upon treatment during the test phase. Asterisk indicate statistical significance; *p < 0.05. The legend identifies genotype, the number of test fish and treatment.

3.3.3. Oxytocin receptor-like mutants displayed freezing deficit upon sight of alarmed conspecifics

We next assessed the response of oxytocin receptor-like mutants (*oxtrl*) to the presence of alarmed conspecifics. The freezing response of *oxtrl* test fish was significantly affected by AS but not by the genotype (two-way ANOVA: genotype: $F_{(1,77)}$= 0.3527, p=0.0641; AS: $F_{(1,77)}$= 9.951, p=0.0023: Fig. 3.4B Table 3.4); however, there was a significant interaction of genotype and AS (two-way ANOVA: genotype x AS: $F_{(1,77)}$= 8.659, p=0.0043: Fig. 3.4B Table 3.4). Accordingly, the freezing response of *oxtrl*$^{-/-}$ test fish was significantly less than *oxtrl*$^{+/+}$ test fish when both were exposed to an alarmed shoal (planned comparisons: *oxtrl*$^{-/-}$ Obs + Shoal_AS vs *oxtrl*$^{+/+}$ Obs + Shoal_AS: $t_{(1,77)}$ =3.388, p=0.0022; Fig. 3.4B Table 3.4). On the other hand, there was no significant difference in freezing response of *oxtrl*$^{+/+}$ and *oxtrl*$^{-/-}$ controls (planned comparisons: *oxtrl*$^{-/-}$ Obs + Shoal_Control vs *oxtrl*$^{+/+}$ Obs + Shoal_Control: $t_{(1,77)}$ =0.7572, p=0.6016: Fig. 3.4B Table 3.4).

The freezing response of *oxtrl*$^{+/+}$ test fish exposed to an alarmed shoal was significantly higher than control (planned comparisons: *oxtrl*$^{+/+}$ Obs + Shoal_AS vs *oxtrl*$^{+/+}$ Obs + Shoal_Control: $t_{(1,77)}$= 4.286, p=<0.0001; Fig. 3.4B Table 3.4).

Notably, there was no significant difference in the freezing response of *oxtrl*$^{-/-}$ test fish exposed to an alarmed shoal when compared with control (planned comparisons: *oxtrl*$^{-/-}$ Obs + Shoal_AS vs *oxtrl*$^{-/-}$ Obs + Shoal_Control: $t_{(1,77)}$=0.1508, p=0.8805; Fig. 3.4B Table 3.4).

The erratic movement response of test fish was significantly affected by AS but not by the genotype when exposed to an alarmed shoal (two-way ANOVA: genotype: $F_{(1,77)}$= 0.06361, p=0.8016; AS: $F_{(1,77)}$= 14.46, p=0.0003: Fig. 3.4B Table 3.4); and there was no significant interaction of genotype and AS (two-way ANOVA: genotype x AS: $F_{(1,77)}$=0.2028 , p=0.6537: Fig. 3.4C Table 3.4).

There was a significant increase in the erratic movement response of both WT and MUT test fish when exposed to an alarmed shoal (planned comparisons: *oxtrl*$^{+/+}$ Obs + Shoal_AS vs *oxtrl*$^{+/+}$ Obs + Shoal_Control: $t_{(1,77)}$ =1.547, p=0.042; *oxtrl*$^{-/-}$ Obs + Shoal_AS vs *oxtrl*$^{-/-}$ Obs + Shoal_Control: $t_{(1,77)}$ =1.956, p=0.0136; Fig. 3.4C Table 3.4); and there was no significant difference in erratic movement response of *oxtrl*$^{+/+}$ and *oxtrl*$^{-/-}$ controls (planned comparisons: *oxtrl*$^{+/+}$ vs *oxtrl*$^{-/-}$ $_{(1,77)}$ =1.489, p=0.8248: Fig. 3.4C Table 3.4).

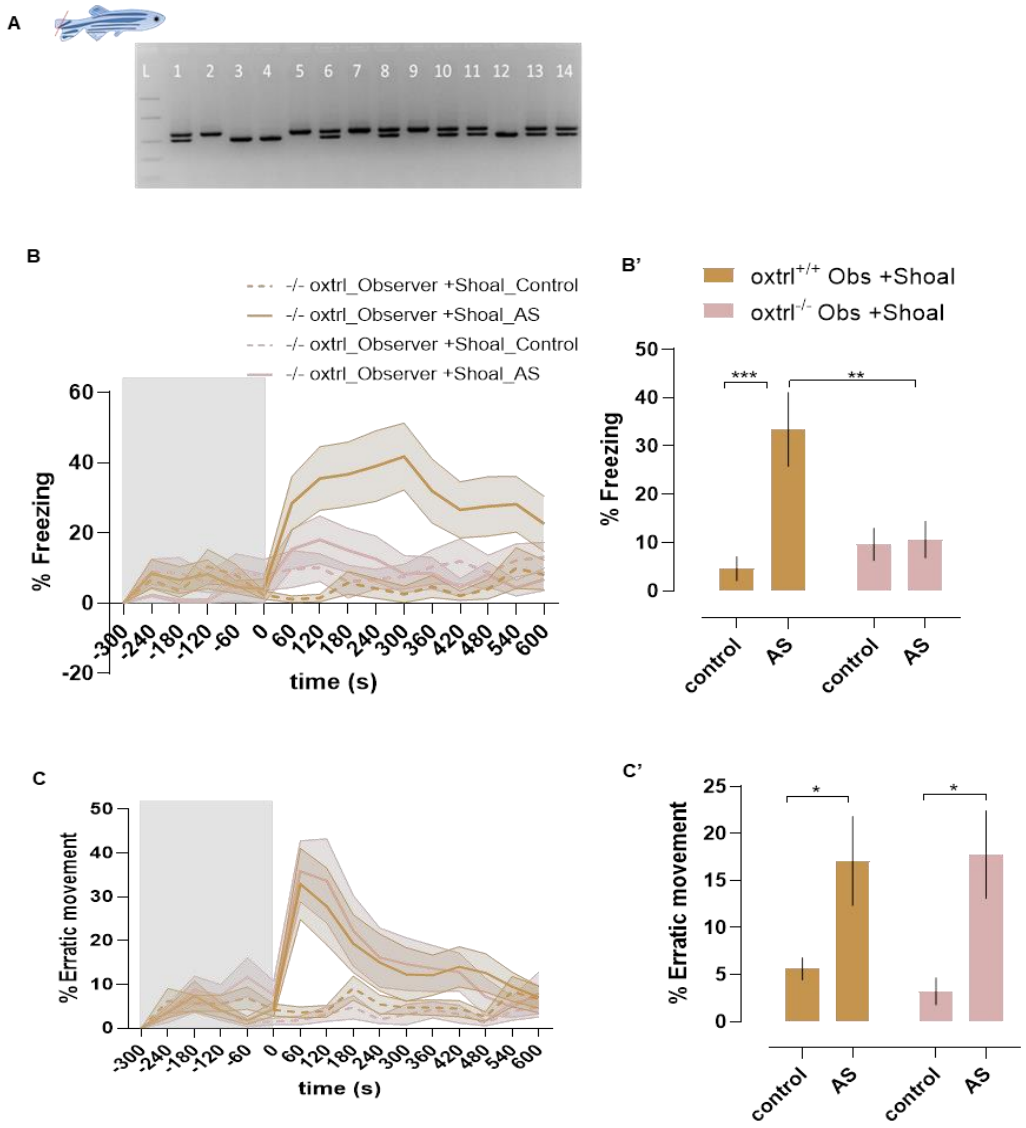

Figure 3.4. Oxytocin receptor-like mutants' displayed altered freezing response in presence alarmed conspecifics.

(A) Schematic of *oxtrl* genotyping using tail fin clip: gel electrophoresis showing ladder (L), and DNA bands of WT (3,4,12), MUT (2,5,7,9) and heterozygous-HET (1,6,8,10,11,13,14) (B, C) Temporal dynamics of freezing and erratic movement response across treatments. Shaded area represents the time before administration of AS or vehicle. (B',C')Percentage of freezing and erratic movement (mean±SEM) upon treatment during the test phase. Asterisk indicate statistical significance; *p < 0.05, **p < 0.01 and ***p < 0.001. The legend identifies genotype, the number of test fish and treatment.

3.3.4. Oxytocin ligand mutants displayed freezing deficit upon sight of alarmed conspecifics

We next assessed the response of oxytocin ligand mutants (*oxt*) to the presence of alarmed conspecifics. The freezing response of test fish was significantly affected by genotype and AS (two-way ANOVA: genotype: $F_{(1,73)}$= 38.42, p=<0.0001; AS: $F_{(1,73)}$= 20.18, p=<0.0001: Fig. 3.5B Table 3.5). There was also a significant interaction of genotype and AS (two-way ANOVA: genotype x AS: $F_{(1,73)}$=13.02, p=0.0006).

As demonstrated in the *oxt* receptors, the freezing response of $oxt^{-/-}$ test fish exposed to an alarmed shoal was significantly lower than $oxt^{+/+}$ test fish exposed to an alarmed shoal (planned comparisons: $oxt^{-/-}$ Obs + Shoal_AS vs $oxt^{+/+}$ Obs + Shoal_AS: $t_{(1,73)}$ =7.086, p=<0.0001); contrastingly, there was no difference in freezing response of $oxt^{+/+}$ and $oxt^{-/-}$ controls (planned comparisons: $oxt^{-/-}$ Obs + Shoal_Control vs $oxt^{+/+}$ Obs + Shoal_Control: $t_{(1,73)}$ =1.794, p=0.1027) (Fig. 3.5B Table 3.5).

The freezing response of $oxt^{+/+}$ test fish exposed to an alarmed shoal was significantly higher than control (planned comparisons: $oxt^{+/+}$ Obs + Shoal_AS vs $oxt^{+/+}$ Obs + Shoal_Control: $t_{(1,73)}$= 5.853, p=<0.0001). Contrastingly, there was no significant difference in the freezing response of $oxt^{-/-}$ test fish when exposed to an alarmed shoal compared with control (planned comparisons: $oxt^{-/-}$ Obs + Shoal_AS vs $oxt^{-/-}$ Obs + Shoal_Control: $t_{(1,73)}$= 0.612, p=0.5424: Fig. 3.5B Table 3.5).

There was no significant difference in erratic movement response of $oxt^{+/+}$ and $oxt^{-/-}$ test fish exposed to an alarmed shoal (Fig. 3.5C, Table 3.5).

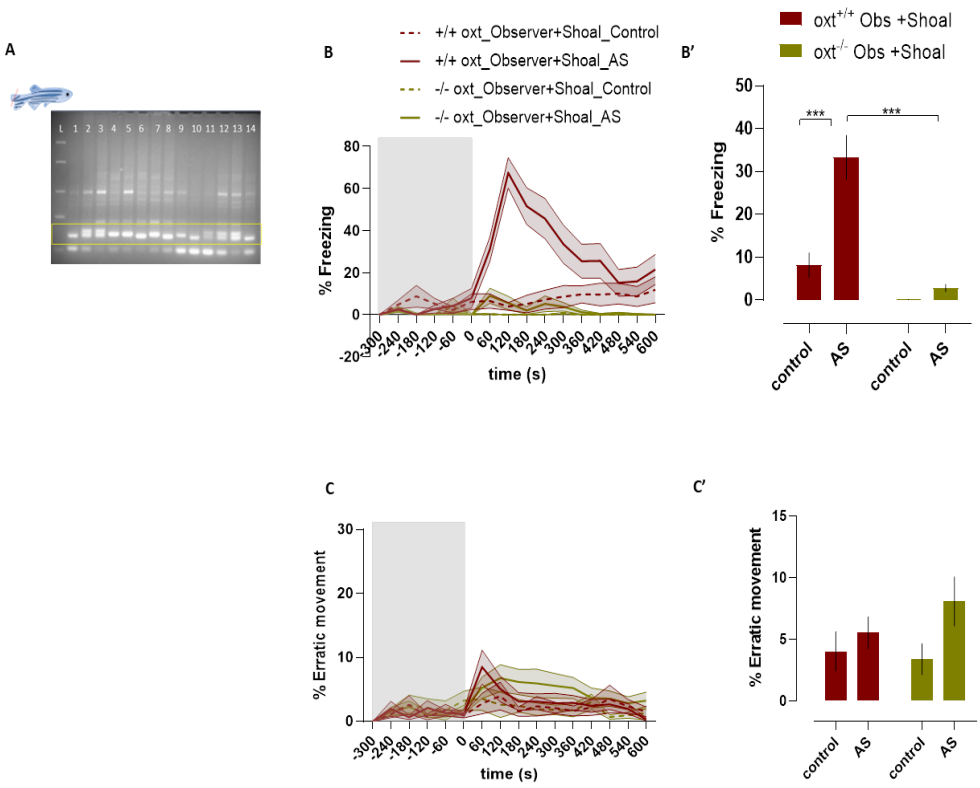

Figure 3.5 Oxytocin ligand mutants' displayed altered freezing response in presence alarmed conspecifics.

(A) Schematic of *OXT* ligand genotyping using fin clip: gel electrophoresis showing ladder (L) and DNA bands of MUT, WT and HET (B, C) Temporal dynamics showing freezing and erratic movement response across treatments (B',C') Percentage of freezing and erratic movement (mean±SEM) upon treatment during the test phase. Asterisk indicate statistical significance; ***p < 0.001. The legend identifies genotype, the number of test fish and treatment.

3.3.5. IP injection of oxt mutants rescued freezing phenotype

The freezing response of oxytocin ligand mutants (*oxt*) was significantly affected by AS but not by genotype or treatment with i.p injection of isotocin (three-way ANOVA: AS= $F_{(1,127)}$=43.45, p=<0.0001; Genotype= $F_{(1,127)}$=3.248, p=0.0739; Treatment= $F_{(1,127)}$=0.0107, p=0.9177).

There was a significantly higher freezing response in saline-treated $oxt^{+/+}$ compared with saline-treated $oxt^{-/-}$ when both were exposed to an alarmed shoal (planned comparisons: SAL $oxt^{+/+}$ Obs + Shoal_AS vs SAL $oxt^{-/-}$ Obs + Shoal_AS: $t_{(1,96)}$ =3.85, p=0.0005: Fig. 3.6B Table 3.6). However, the freezing response of $oxt^{-/-}$ test fish treated with isotocin i.p was not significantly less than $oxt^{+/+}$ test fish treated with saline i.p when both were exposed to an alarmed shoal (planned comparisons: OXT $oxt^{-/-}$ Obs + Shoal_AS vs SAL $oxt^{+/+}$ Obs + Shoal_AS: $t_{(1,96)}$ =1.643, p=0.128625: Fig. 3.6B Table 3.6).

The freezing response was not significantly increased in $oxt^{-/-}$ test fish that were saline-treated and exposed to an alarmed shoal compared with control, but the freezing response was significantly increased in $oxt^{-/-}$ test fish that received i.p injection of isotocin (oxytocin-like peptide) and were exposed to an alarmed shoal compared with control (planned comparisons: OXT $oxt^{-/-}$ Obs + Shoal_control vs OXT $oxt^{-/-}$ Obs + Shoal_AS: $t_{(1,96)}$ =3.386, p=0.0015: Fig. 3.6B Table 3.6). Contrastingly, there was a significant increase in freezing response when saline-treated $oxt^{+/+}$ test fish were exposed to an alarmed shoal compared to control (planned comparisons: SAL $oxt^{+/+}$ Obs + Shoal_Control vs SAL $oxt^{+/+}$ Obs + Shoal_AS: $t_{(1,96)}$ =4.924, p=<0.0001; Fig. 3.6B Table 3.6).), as well as a significant increase in freezing response when isotocin-treated $oxt^{+/+}$ test fish were exposed to an alarmed shoal compared to control.

There was no significant difference in erratic movement response (Fig. 3.6C, Table 3.6).

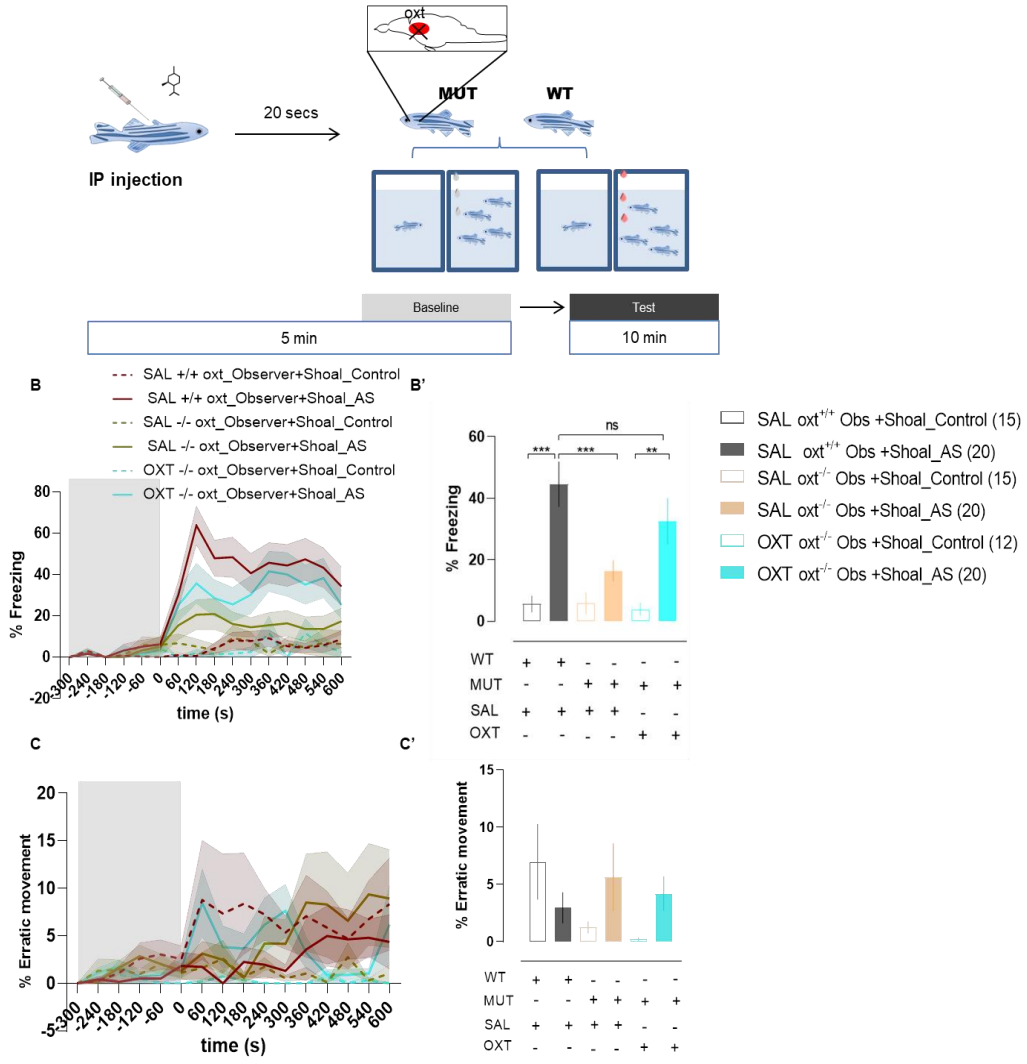

Figure 3.6. IP injection with isotocin rescued freezing phenotype in oxt lig mutant.

(A) schematic of oxytocin IP injection treatment of oxt$^{-/-}$ and oxt$^{+/+}$ (B, C) Temporal dynamics showing freezing and erratic movement response across treatments (B',C') Percentage of freezing and erratic movement (mean±SEM) upon treatment during the test phase. Asterisks indicates statistical significance: ** p < 0.01, *** p < 0.001, ns represents non-significance p>0.05. The legend identifies genotype, the number of test fish and treatment.

3.3.6. Comparison of freezing responses across all oxytocin mutants

The freezing response comparisons of oxytocin ligand and mutants exposed to an alarmed shoal revealed that the *oxt* ligand showed the lowest level of freezing and there was no difference in freezing response between WT exposed to alarmed shoal and *oxt* mut treated with isotocin i.p (Fig. 3.7a). Only *oxtrl*$^{-/-}$ showed a significant increase in erratic movement response compared to WT (Fig. 3.7b).

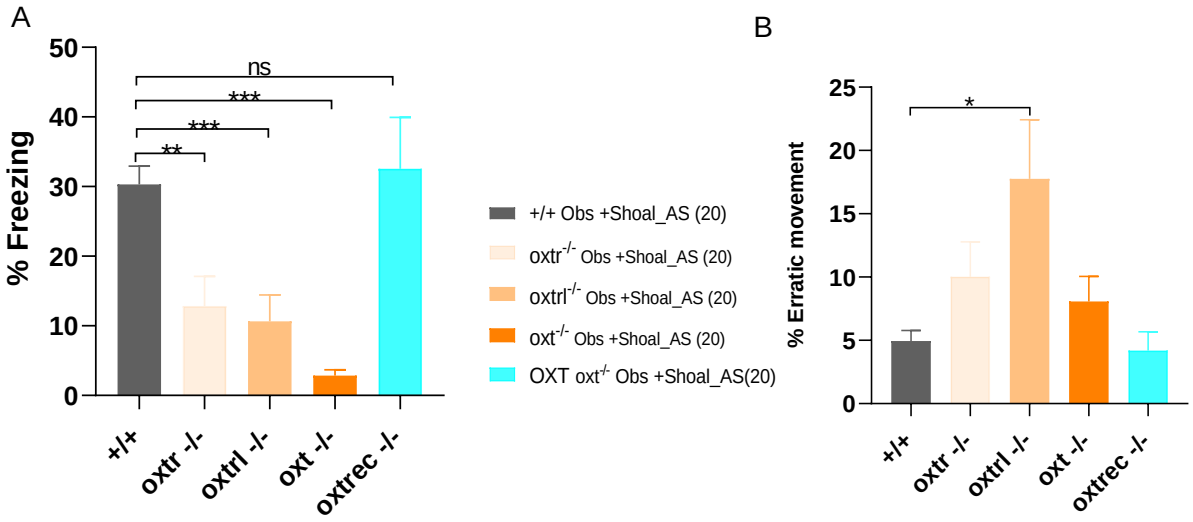

Figure 3.7 Loss of oxytocin ligand caused the most profound deficit in freezing response.
WT group was made by randomly selecting five WT animals from each experiment (*oxtr*$^{+/+}$, *oxtrl*$^{+/+}$, *oxt*$^{+/+}$, *oxtrec*$^{+/+}$). Percentage of freezing response (A) and erratic movement (B) (mean±SEM) in WT and MUT oxt ligand and receptors. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ns represents non-significance p>0.05. The legend identifies genotype, number of test fish and treatment.

3.3.7. Sight of alarmed shoal caused changes in oxtr$^{+/+}$ and oxtr$^{-/-}$ pS6 positive cell count

The brain regions involved in social contagion of fear and the role of oxytocin in this phenomenon was determined by quantifying the number of positive phosphorylated-S6 (pS6) cells in *oxtr*$^{+/+}$ and *oxtr*$^{-/-}$ treatments in selected SDMN brain areas.

There was a significant effect of genotype and AS in pS6 cell count in the Vv brain region (ANOVA: genotype: $F_{(1,33)}$= 42.147, p=<0.0001; AS: $F_{(1,33)}$= 12.127, p=0.001: Fig3.8 Table 3.8) and there was an interaction between genotype and AS (ANOVA: interaction: $F_{(1,33)}$= 39.316, p=<0.0001 Fig3.8 Table 3.8). There were significantly fewer pS6 positive cells in the Vv of *oxtr*$^{+/+}$ test fish exposed to an alarmed shoal (planned comparisons: *oxtr*$^{+/+}$ OBS + Shoal_AS vs *oxtr*$^{+/+}$ OBS + Shoal_control; $t_{(1,33)}$= -2.673, p= 0.01). Similarly, there were significantly fewer pS6 positive cells in the Vv of *oxtr*$^{+/+}$ compared to *oxtr*$^{-/-}$ test fish exposed to an alarmed shoal (planned comparisons: *oxtr*$^{+/+}$ OBS + Shoal_AS vs *oxtr*$^{-/-}$ OBS + Shoal_AS; $t_{(1,33)}$= 8.524, p=<0.0001). However, there were significantly higher number of pS6 positive cells in the Vv of *oxtr*$^{-/-}$ test fish exposed to an alarmed shoal (planned comparisons: *oxtr*$^{-/-}$OBS + Shoal_AS vs *oxtr*$^{-/-}$ OBS + Shoal_control; $t_{(1,33)}$= 6.427, p=<0.0001; Fig3.8 Table 3.8).

Likewise, there was a significant effect of genotype but not AS in pS6 cell count in the Vc brain region (ANOVA: genotype: $F_{(1,33)}$= 29.022, p=<0.0001; AS: $F_{(1,33)}$= 2.992, p=0.09: Fig3.8 Table 3.8) and there was an interaction between genotype and AS (ANOVA: interaction: $F_{(1,36)}$= 23.72, p=<0.0001; Fig3.8 Table 3.8). There were significantly fewer pS6 positive cells in the Vc of *oxtr*$^{+/+}$ test fish exposed to an alarmed shoal when compared to the control (planned comparisons: *oxtr*$^{+/+}$ OBS + Shoal_AS vs *oxtr*$^{+/+}$ OBS + Shoal_control; $t_{(1,33)}$= -2.674 , p= 0.01). Similarly, there were significantly fewer pS6 positive cells in the Vc of *oxtr*$^{+/+}$ compared to *oxtr*$^{-/-}$ test fish exposed to an alarmed shoal

(planned comparisons: *oxtr*$^{+/+}$ OBS + Shoal_AS vs *oxtr*$^{-/-}$ OBS + Shoal_AS; $t_{(1,33)}$= 6.86, p=<0.0001). However, there were significantly higher pS6 positive cells in the Vc of *oxtr*$^{-/-}$ test fish exposed to an alarmed shoal when compared to the control (planned comparisons: *oxtr*$^{-/-}$ OBS + Shoal_AS vs *oxtr*$^{-/-}$ OBS + Shoal_control; $t_{(1,33)}$= 4.317, p=<0.0001; Fig3.8 Table 3.8).

Among the remaining brain regions, the Vd, Vp, Dm, Dp, PPAM, PPAL and HAV in *oxtr*$^{-/-}$ test fish exposed to alarmed shoal also had significantly higher pS6 positive cells (Table 3.8) when compared with *oxtr*$^{+/+}$ test fish also exposed to alarmed shoal (planned comparisons: *oxtr*$^{-/-}$ OBS + Shoal_AS vs *oxtr*$^{+/+}$ OBS + Shoal_AS) (Table 3.8). There was no difference in the number of pS6 positive cells in *oxtr*$^{+/+}$ control test fish when compared with *oxtr*$^{-/-}$ control test fish (Table 3.8).

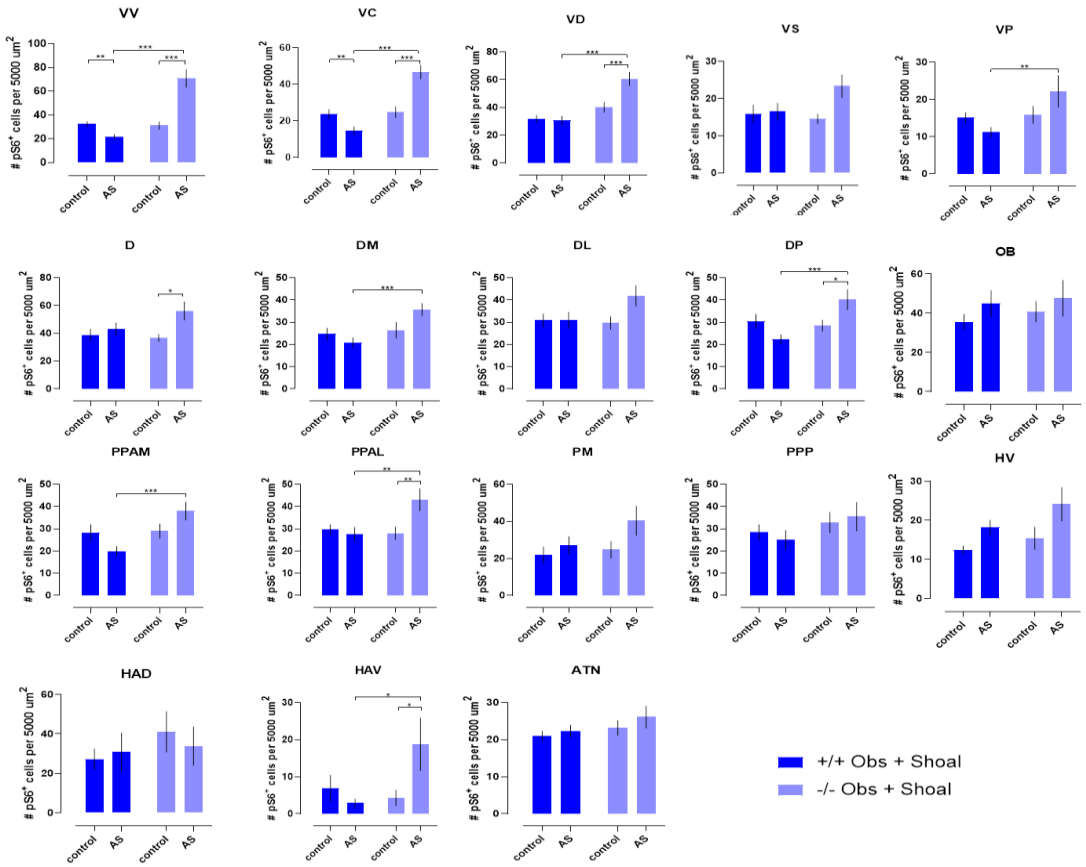

Figure 3.8 The number of pS6 positive cells in selected SDMN brain areas in social contagion test.

Bar plot shows the number of pS6 positive cells in each brain area (identified above the graph); Dark blue bars indicate oxtr$^{+/+}$ and light blue bars indicate *oxtr*$^{-/-}$; the legend identifies the genotype of test fish. The result is shown as Mean±SEM. *p < 0.05, **p < 0.01, *** p < 0.001.

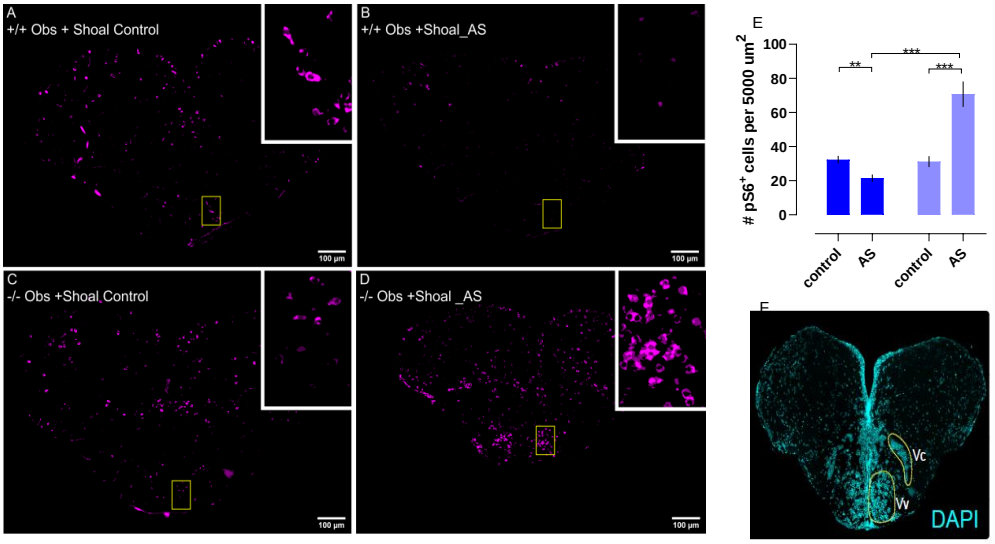

Figure 3.9 Changes in the number of pS6 positive cells in the Vv.

Representative coronal sections showing pS6 (magenta) antibody expression in the treatments (A-D). (E) Result showing number of ps6^{+} cells in the Vv across treatments. Dark blue bars indicate oxtr$^{+/+}$ and light blue bars indicate *oxtr*$^{-/-}$. (F) DAPI (cyan) staining to confirm landmark for brain area. Scale bar =20 µm for ROI.

E

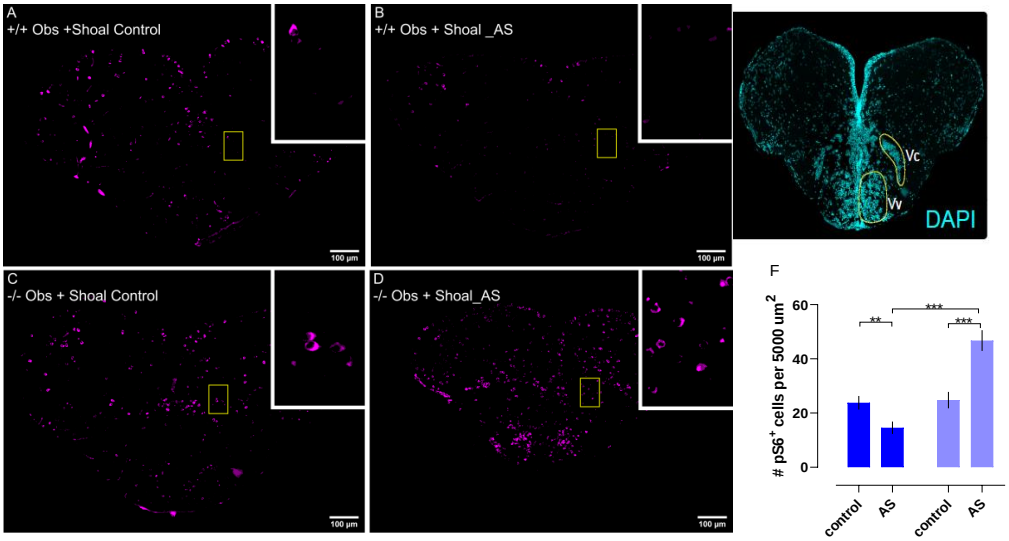

Figure 3.10 Changes in the number of pS6 positive cells in the Vc. Representative coronal sections showing pS6 (magenta) antibody expression in the Vc. (A-D). (E) Result showing number of ps6^{+} cells in the Vc across treatments. Dark blue bars indicate oxtr$^{+/+}$ and light blue bars indicate *oxtr*$^{-/-}$.(F) DAPI (cyan) staining to confirm landmark for brain area. Scale bars =20 µm for ROI.

3.3.8. Differences in patterns of functional connectivity among treatments in social contagion in zebrafish

Quadratic Assignment Procedure (QAP) correlation was used to assess the co-activation patterns between brain regions across treatments. There was no significant association between the correlation matrices of *oxtr*$^{+/+}$ test fish exposed to alarmed conspecifics and their control (*oxtr*$^{+/+}$ OBS + Shoal_AS vs *oxtr*$^{+/+}$ OBS + Shoal_Control: r=0.262, p=0.653). Likewise, there was no significant association between the correlation matrices of *oxtr*$^{+/+}$ and *oxtr*$^{-/-}$ controls (r=-0.462, p=0.245), suggesting that these networks have distinct co-activation patterns.

There was also no significant association between the correlation matrices of *oxtr*$^{-/-}$ test fish exposed to alarmed conspecifics compared with their control (*oxtr*$^{-}$

oxtr$^{-/-}$ OBS + Shoal_AS vs *oxtr*$^{-/-}$ OBS + Shoal_Control (r=-0.003, p=0.723); and there was no significant association between the correlation matrices in *oxtr*$^{+/+}$ and *oxtr*$^{-/-}$ test fish exposed to alarmed conspecifics (r=0.879, p=1.000), suggesting they have a distinct co-activation pattern. (Figure 3.9, Table 3.9, 3.11).

oxtr$^{+/+}$ OBS + Shoal_Control group had the highest network density (0.5222) and had significantly more dense network compared with *oxtr*$^{+/+}$ OBS + Shoal_AS (0.1378) (t=5.2238, p=0.0002). On the other hand, there was a significant increase in network density in *oxtr*$^{-/-}$ OBS + Shoal_AS (0.3071) in comparison with *oxtr*$^{-/-}$ OBS + Shoal_Control (0.1235) (t=2.682, p=0.009). *oxtr*$^{+/+}$ OBS + Shoal_AS had a significantly less dense network when compared with *oxtr*$^{-/-}$ OBS + Shoal_AS (t=2.8065, p=0.0056); and *oxtr*$^{+/+}$ OBS + Shoal_Control had a significantly denser network when compared with *oxtr*$^{-/-}$ OBS + Shoal_Control (t=4.854, p=0.0004) (Figure 3.9, Table 3.10).

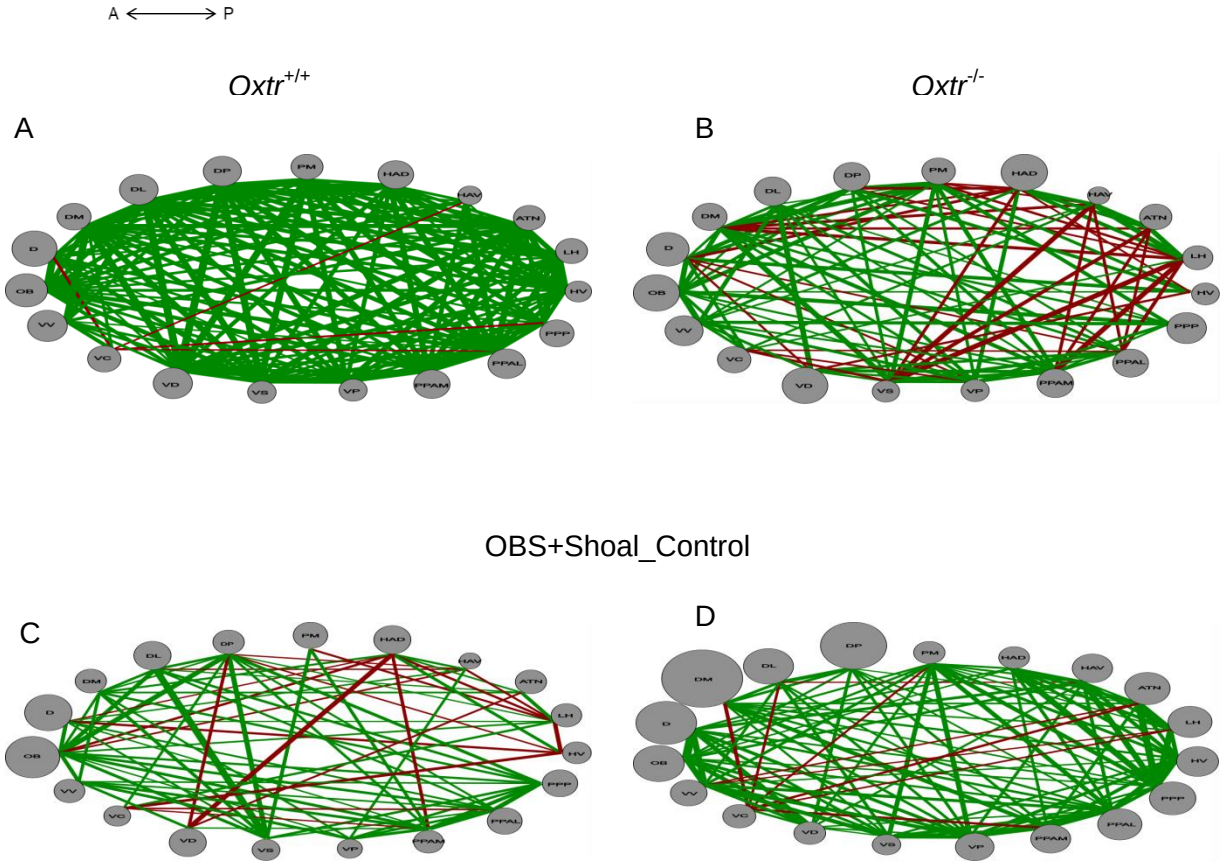

Figure 3.11 Functional connectivity of pS6 expression between pairs of brain nuclei for each experimental treatment of oxtr in social contagion test.

Networks represent *oxtr*$^{+/+}$ (A,C) and *oxtr*$^{-/-}$ (B,D) Observer + Shoal control and test groups; brain regions are identified in each node, and the size of the node represents the average number of pS6 positive cells for each brain region in each experimental group, nodes are arranged in the anterior (A) and posterior (P) direction; positive and negative correlations are shown in green and red respectively.

3.3.9. *Expression of oxytocin receptors in adult zebrafish*

Following differences in pS6 positive cell counts during social contagion of fear (Fig 3.9, 3.10) in some brain regions of the SDM network, we assessed the expression of oxytocin receptors in some of these brain regions.

The assessment of the relative expression pattern of oxtr and oxtrl in the Vv, Vc, Vd, Vs/Vp, POA, Dm, and Ob revealed the expression of oxtr all the seven areas while oxtrl was expressed although to a lesser extent in the Vv, Vc, Vd, and Ob only.

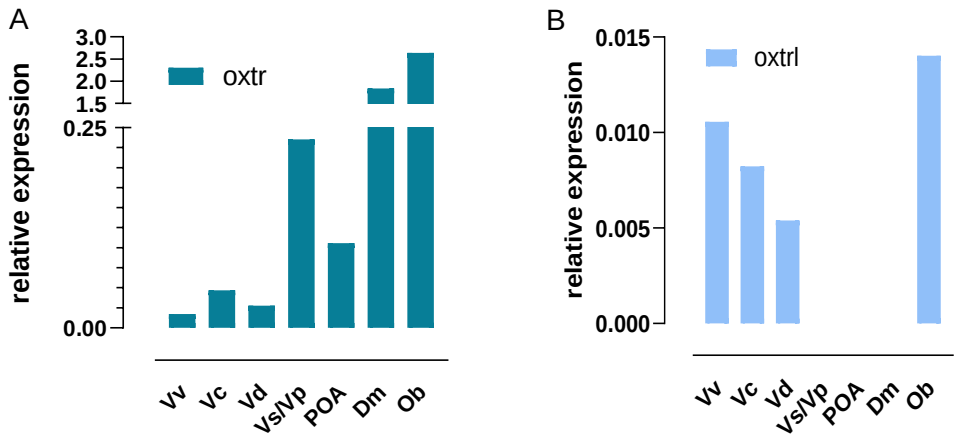

Figure 3.12 Relative expression of oxtr and oxtrl in selected regions of the adult zebrafish brain.
Raw values of (A) *oxtr* expression; (B) *oxtrl* expression (normalized to *eef1a1l1* in both cases).

3.3.10. *Visualization of oxytocinergic projection to the ventral telencephalon of adult and larvae zebrafish brains*

Following differences in pS6 positive cell counts during social contagion of fear (Fig 3.9, 3.10) in the ventral telencephalon, specifically in the Vv and Vc (Fig 3.10), we determined the pattern of oxytocin projections to the ventral telencephalon of both adult and larvae zebrafish brain using the double immunostaining of oxt:GFP and Isl1, [a neural progenitor marker that is

expressed in the Vv and Vs and more recently showed to be expressed also in the ventral part of the Vd in adults (Baeuml, Biechl, & Wullimann, 2019; Ganz et al., 2012)] as a landmark to determine the brain regions, in adults and oxytocin, GFP and Isl1 in larvae.

The results show projections of oxytocin to the Ventral telencephalon in both adult and larvae, particularly to Vv.

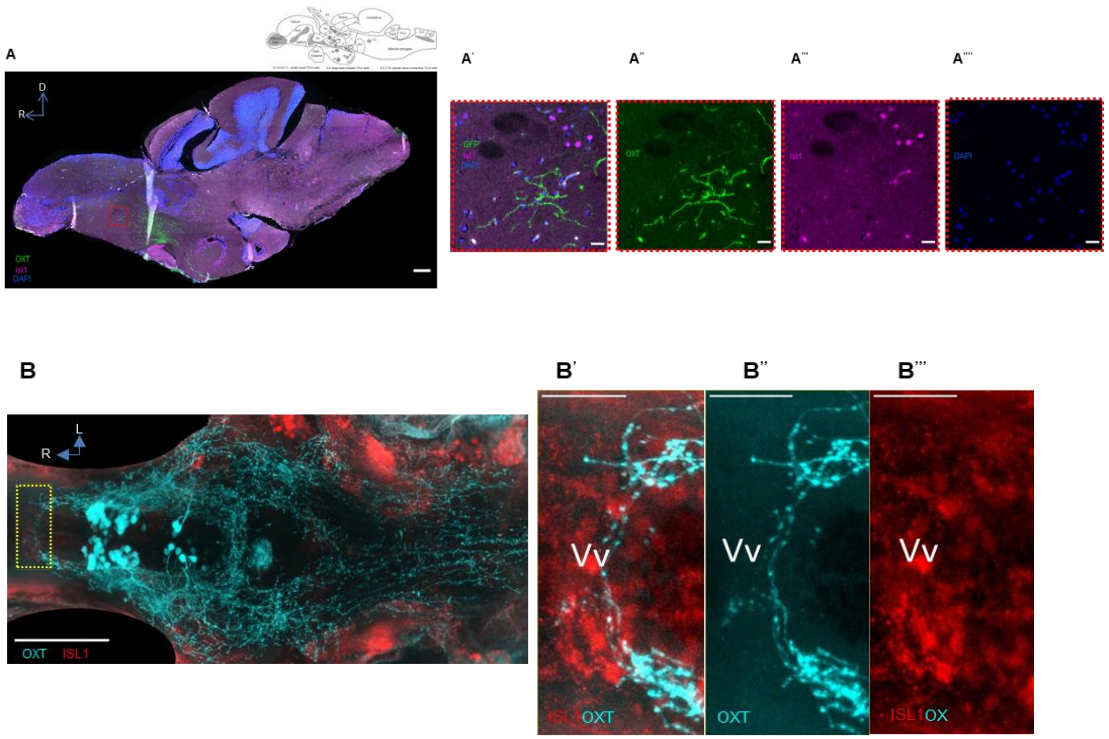

Figure 3.13 Oxytocin projects to the ventral telencephalon.
(A) Sagittal section of adult zebrafish, oxt:eGFP reporter line showing oxytocin projections (green) to the subpallium. Isl1 (magenta) a neural progenitor marker is used as a landmark for the subpallium; Scale bar = 200 µm. A' reveals subpallium with oxytocin projections (green), isl1 (magenta), and DAPI (blue), oxytocin projections (green) A'', Isl1 (magenta) A''', DAPI (blue) A''''; scale bar=20 µm B. Oxytocinergic projections (cyan) in larvae of oxt:eGFP reporter line with Isl1 (red) positive cells as landmark for the Vv. Scale bar = 100 µm B'

shows oxytocin projections (cyan) and isl1 (red) positive cells; Scale bar = 20 µm. D= dorsal, R= rostral, L=Lateral.

3.4. **Discussion**

Our findings provide genetic evidence for the oxytocinergic regulation of social contagion of fear in zebrafish using both *oxt-oxtr* and *oxt-oxtrl* signalling. Such that the loss either *oxtr, oxtrl,* or *oxt* caused deficits is in response to social contagion of fear with the wild types exhibiting freezing response when exposed to the sight of alarmed conspecifics in contrast to mutants. Importantly, the pharmacological treatment of oxytocin ligand mutants with isotocin (oxytocin-like peptide) abolished the observed deficits, thus confirming the role of oxytocin in this phenomenon.

Previous correlational studies in mammalian models such as humans (Hurlemann et al., 2010; Y. R. Kim, Kim, Park, Pyo, & Treasure, 2014), non-human primates (Y. R. Kim et al., 2014) and rodents (Pisansky, Hanson, Gottesman, & Gewirtz, 2017) have suggested the possible role of oxytocin in this social transmission of fear regulation. Together, these reports are in congruence with our findings and provide insights into the evolutionarily conserved role of oxytocin ligand and its receptors in regulating a component of social behaviour.

Social information that signals impending danger has been shown to increase both alertness and trigger defence behaviours in observer animals (Pereira, Cruz, Lima, & Moita, 2012; Seyfarth & Cheney, 2003; Smith, 1992). Besides, given that oxytocin has been reported to increase the salience of social cues thereby directing attention to socially relevant cues (Donaldson & Young, 2008a; Insel & Young, 2000; Reddon, O'Connor, Marsh-Rollo, & Balshine, 2012; Shamay-Tsoory & Abu-Akel, 2016; Theodoridou, Penton-Voak, & Rowe, 2013), our studies highlight the role of oxytocin in increasing the sensitivity to social cues; for example, human studies have reported a prosocial role of oxytocin by increasing trust and conformity in humans (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005; Stallen, De Dreu, Shalvi, Smidts, & Sanfey, 2012). Conversely, oxytocin was implicated in increasing mistrust and envy (Eckstein et al., 2014; Shamay-Tsoory et al., 2009). Also, studies with rodents implicate

oxytocin in the modulation of emotional state matching (Burkett et al., 2016; Zimbel, Pisansky, Kennedy, & Gewirtz, 2013).

It is interesting to note that freezing deficit observed in OXT mutant receptors and ligand was most heightened in the OXT ligand mutant (Fig 3.7 A). This could be because with the receptors, only one of the function of the receptors was truncated for each line and possible compensatory mechanisms might have occurred throughout development. Importantly, pharmacological treatment with OXT-like drug fully restored the freezing deficit.

The exposure to immediate environmental threats or cues that indicate the presence of a threatful stimulus activates multiple, interconnected neural networks to launch an innate behavioural program in order to maximize the individual's probability of survival. We explored the neural activation pattern of brain regions that could be involved in the social contagion phenomenon. We focused on some regions in the highly conserved SDMN network which consists of interconnected social behaviour and mesolimbic reward networks and has been proposed to regulate social behaviours across all vertebrates (L. O'Connell & Hofmann, 2011b, 2012; Teles et al., 2015).

Phosopho-S6 antibody targets ribosomes that become phosphorylated following changes in electrical activity in neurons and the same signalling pathways that trigger S6 phosphorylation correlates with neural activity (Flavell & Greenberg, 2008; Knight et al., 2012). Studies validating the activity of this neuronal activation marker are now emerging across different species including rodents and fish (Butler, Whitlow, Roberts, & Maruska, 2018; Fischer, Westrick, Hartsough, & Hoke, 2018; Knight et al., 2012). Interestingly, along with the behavioural changes observed were changes in the expression of phosopho-S6 (pS6)- a neural activation marker in the Vv and Vc; these two brain regions are relevant to emotional processing.

The ventral nucleus of ventral telencephalic area (Vv) and the central nucleus of ventral telencephalic area (Vc) are the putative homologues of the lateral septum and the nucleus accumbens/striatum respectively in mammalian models have been implicated in emotional regulation. For example, rodent studies

reveal that the loss of oxytocin receptor in the lateral septum reduced both social defeat-induced facilitation of freezing behaviour in response to contextual fear (Guzmán et al., 2013). Also, oxytocin injected into the dorsolateral septum has been shown to reduce social avoidance behaviour after social fear conditioning in which electric foot-shocks were applied during the investigation of a conspecific with social contact (Zoicas, Slattery, & Neumann, 2014). Similarly, the synthetic infusion of oxytocin or *oxtr* overexpression in the lateral septum diminished social fear expression in lactating mice (Menon et al., 2018). Zebrafish studies implicate Vv in the processing of social information such as social recognition (Ribeiro et al., 2020), and social orientation (Stednitz et al., 2018).

In mammalian models, the striatum is divided into two parts- the dorsal and the ventral region. The dorsal part is composed of the caudate and putamen has been implicated in the regulation of voluntary movement control. At the same time, the ventral striatum also called the striatum is integral for reward and emotional processing (Báez-Mendoza & Schultz, 2013), i.e. it aids in obtaining motivationally relevant goals by promoting likelihood, efficiency, and vigour of behaviours intended to obtain those goals, whether they be the procurement of things worth having (rewards), avoidance of aversive consequences, exploration of novel stimuli, or other objectives (Floresco, 2015; Reynolds & Berridge, 2002). For example, studies with Syrian hamsters (*Mesocricetus auratus*) show that the striatum is necessary for the expression of conditioned defeat (Luckett, Norvelle, & Huhman, 2012).

Although the complete homology of the striatum/Nucleus accumbens in the zebrafish has not been fully established, it is plausible to suggest that the Vc plays a role in regulating this component of social behaviour.

Indeed, we have shown that oxytocin projects to the ventral telencephalon in both adult and larvae zebrafish. This goes in line with other studies that suggest possible projections of oxytocin to this brain region (Herget, Gutierrez-Triana, Thula, Knerr, & Ryu, 2017; Wircer et al., 2017).

Likewise, we provide first evidence for the presence of oxytocin receptor (*oxtr*) and oxytocin receptor-like (*oxtrl)* in some key brain structures in the adult zebrafish. It is important to note that oxtr and oxtrl is present in the Vv and Vc, this provides additional evidence that oxytocin could be acting directly on these brain regions.

Given that magnocellular oxytocin neurones have been reported to project to the lateral septum (Knobloch et al., 2012; Onaka, Takayanagi, & Yoshida, 2012), we can argue that oxytocin acts on these regions to increase the salience of social information (Shamay-Tsoory & Abu-Akel, 2016).

It is interesting to note that the WT social control group had the highest network density when compared to the other treatments in our network connectivity analysis study. This may be because it is at the non-task state; this result agrees with the phenomenon has been described as a *default mode network* where a task-induced activity decreases functional connectivity in comparison with non-task states (Raichle, 2015; Teles et al., 2015).

It is also interesting to note that the loss of oxytocin receptor caused a change in the network density between the wild type and mutant control, and the network densities of the mutant groups were similar. The changes in network density and functional connectivity due to the loss of the oxytocin receptor agrees with reports that suggest the role of oxytocin in maintaining plasticity across nodes by enhancing the signal-to-noise ratio in brain connectivity (Pekarek et al., 2020); and interestingly, even though the Vv and Vc respond to social contagion of fear, the nodes in the SDMN analysed collaboratively respond in a distinct pattern to regulate different behavioural outputs.

In conclusion, our findings reveal a role of oxytocin in the regulation of social contagion of fear and highlight the possible roles of Vv and Vc in regulating this component of social behaviour in zebrafish.

3.5. **Author Contributions**

Ibukun Akinrinade (I.A) and Rui Filipe Oliveira (R.F.O) designed the experiments and established the behavioural protocols. I. A performed the behavioural experiments, processed all brains samples, quantified cell counts, and performed the functional connectivity analysis. I.A, R.F.O and G.L analysed and discussed results. Zebrafish lines were kindly donated by Prof Gil Levkowitz (G.L).

We thank Ana Rita Nunes and Michael Gliksberg for help in generating mutant zebrafish models for oxytocin. We thank Magda Teles for help with microdissection and qPCRs. We thank Susana Varela for help with statistical analysis. We thank Maria Hanalova from UIC for help with image acquisition and editing. We thank Abisola Akinrinade for assistance with data analysis in Python.

The study in this chapter is in preparation to be submitted to an international peer-reviewed journal.

TABLES

Table 3.1 Primer pairs for genotyping oxtr, oxtrl and oxt lines.

Gene	Forward primer	Reverse primer
oxtr: NM_001199370.1	5'-TGCGCGAGGAAAACTAGTT-3'	5'-AGCAGACACTCAGAATGGTCA-3'
oxtrl: NM_001199369.1	5'-TTTTACGCACAATGGAGAGCC-3'	5`-AGCATGTAAGTGGACGCGAA-3`
oxt NM_178291.2	5`-AGACACAAACACTAAGTAA-3`	5`-AGCAGACGGACAGCAGACACAGCA-3`

Table 3.2 Mann-Whitney test: comparisons between treatments for freezing and erratic movement of oxt ligand and receptors test fish in response to AS (Fig. 3.2A).

Genotype	Response to AS	Statistical Test	Comparison	U	p	t	p	# of animals
***oxt*$^{-/-}$**	Freezing	Mann Whitney test	+/+ vs -/-	36	>0.9999			*oxt*$^{+/+}$= 8
	Erratic movement			35	0.9626			*oxt*$^{-/-}$ = 9
***oxtr*$^{-/-}$**	Freezing			148	0.3572			*oxtr*$^{+/+}$= 18
	Erratic movement			148	0.3612			*oxtr*$^{-/-}$= 20
***oxtrl*$^{-/-}$**	Freezing	Unpaired t-test				0.265	0.7938	*oxtrl*$^{+/+}$= 13
	Erratic movement	Unpaired t-test (Welch's correction)				1.921	0.0952	*oxtrl*$^{-/-}$ = 13

Table 3.3 Full two-way ANOVA output: Main effects, interaction, and planned comparisons between treatments for freezing and erratic movement of oxt receptor (oxtr) test fish upon exposure to alarmed shoal (Fig. 3.3E,F).

oxtr							
Social contagion			**F**	**p**	**t**	**p**	**# of animals***
Freezing	Main effect	Genotype	4.113	0.0461*			*oxtr*$^{+/+}$OSC=20
		AS	7.113	0.0093*			*oxtr*$^{+/+}$ OSAs=20
	Interaction	Genotype x AS	2.342	0.1301			*oxtr*$^{-/-}$ OSC=20
	Planned comparisons	OBS +shoal_AS(*oxtr*$^{-/-}$ vs *oxtr*$^{+/+}$)			2.516	0.028*	*oxtr*$^{-/-}$ OSAs=20
		oxtr$^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			2.968	0.016*	
		oxtr$^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			0.8037	0.565	
		OBS +shoal_Contro l(*oxtr*$^{-/-}$ vs *oxtr*$^{+/+}$)			0.3518	0.7259	
Erratic movement	Main effect	Genotype	0.2501	0.6185			
		AS	6.134	0.0155*			
	Interaction	Genotype x AS	0.08379	0.773			
	Planned comparisons	OBS +shoal_AS(*oxtr*$^{-/-}$ vs *oxtr*$^{+/+}$)			0.5583	0.7712	
		oxtr$^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			1.547	0.2522	
		oxtr$^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			1.956	0.2161	
		OBS +shoal_Control(*oxtr*$^{-/-}$ vs *oxtr*$^{+/+}$)			0.1489	0.882	

*OBS +shoal_Control= OSC, OBS +Shoal_AS=OSAs

Table 3.4 Full two-way ANOVA output: Main effects, interaction, and planned comparisons between treatments for freezing and erratic movement of oxt receptor-like (oxtrl) test fish upon exposure to alarmed shoal (Fig. 3.4B,C).

oxtrl							
Social contagion			**F**	**p**	**t**	**p**	**# of animals***
Freezing	Main effect	Genotype	3.527	0.0641			*oxtrl*$^{+/+}$OSC=20
		AS	9.951	0.0023*			*oxtrl*$^{+/+}$ OSAs=20
	Interaction	Genotype x AS	8.659	0.0043*			*oxtrl*$^{-/-}$ OSC=21
	Planned comparisons	OBS +shoal_AS (*oxtrl*$^{-/-}$ *vs oxtrl*$^{+/+}$)			3.388	0.0022*	*oxtrl*$^{-/-}$ OSAs=20
		oxtrl$^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			4.286	<0.0001*	
		oxtrl$^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			0.1508	0..8805	
		OBS +shoal_Control (*oxtrl*$^{-/-}$ *vs oxtrl*$^{+/+}$)			0.7572	0.6016	
Erratic movement	Main effect	Genotype	0.06361	0.8016			
		AS	14.46	0.0003*			
	Interaction	Genotype x AS	0.2028	0.6537			
	Planned comparisons	OBS +shoal_AS (*oxtrl*$^{-/-}$ *vs oxtrl*$^{+/+}$)			0.5583	0.8896	
		oxtrl$^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			1.547	0.042*	
		oxtrl$^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			1.956	0.0136*	
		OBS +shoal_Control (*oxtrl*$^{-/-}$ *vs oxtrl*$^{+/+}$)			0.1489	0.8248	

*OBS +shoal_Control= OSC, OBS +Shoal_AS=OSAs

Table 3.5 Full two-way ANOVA output: Main effects, interaction, and planned comparisons between treatments for freezing and erratic movement of oxt ligand (oxt) test fish upon exposure to alarmed shoal (Fig. 3.5B,C).

Oxt							
Social contagion			**F**	**p**	**t**	**p**	**# of animals***
Freezing	Main effect	Genotype	38.42	<0.0001*			$oxt^{+/+}$OSC=20
		AS	20.18	<0.0001*			$oxt^{+/+}$ OSAs=20
	Interaction	Genotype x AS	13.02	0.0006*			$oxt^{-/-}$ OSC=17
	Planned comparisons	OBS +shoal_AS ($oxt^{-/-}$ *vs* $oxt^{+/+}$)			7.086	<0.0001*	$oxt^{-/-}$ OSAs=20
		$oxt^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			5.853	<0.0001*	
		$oxt^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			0.612	0.5424	
		OBS +shoal_Control($oxt^{-/-}$ *vs* $oxt^{+/+}$)			1.794	0.1027	
Erratic movement	Main effect	Genotype	0.3606	0.5501			
		AS	3.765	0.0562			
	Interaction	Genotype x AS	0.9985	0.321			
	Planned comparisons	OBS +shoal_AS ($oxt^{-/-}$ *vs* $oxt^{+/+}$)			1.156	0.503	
		$oxt^{+/+}$(OBS +shoal_AS vs OBS +shoal_Control)			0.68	0.665	
		$oxt^{-/-}$ (OBS +shoal_AS vs OBS +shoal_Control)			2.036	0.1816	
		OBS +shoal_Control($oxt^{-/-}$ *vs* $oxt^{+/+}$)			0.2762	0.7832	

*OBS +shoal_Control= OSC, OBS +Shoal_AS=OSAs

Table 3.6 Full three-way ANOVA output: Main effects and planned comparisons between treatments for freezing and erratic movement of oxt ligand recovery of test fish upon treatment with isotocin i.p (Fig. 3.6B,C).

oxt* ligand recovery			**F**	**p**	**t**	**p**
Freezing	Main effect	AS	43.45	<0.0001*		
		GENOTYPE	3.248	0.0739		
		TREATMENT	0.0107	0.9177		
	Interaction	GENOTYPE x AS	3.073	0.082		
		AS x TREATMENT	0.2296	0.6327		
		GENOTYPE X TREATMENT	2.733	0.1007		
		GENOTYPE x AS x TREATMENT	3.084	0.0815		
	Planned comparisons	OBS+Shoal_AS (SAL *oxt*$^{+/+}$ *vs OXT oxt*$^{-/-}$)			1.643	0.128625
		SAL *oxt*$^{+/+}$(OBS+Shoal_AS vs OBS +Shoal_Control)			4.924	<0.0001*
		SAL *oxt*$^{-/-}$ (OBS+shoal_AS vs OBS +Shoal_Control)			1.332	0.1852
		OXT *oxt*$^{-/-}$ (OBS+Shoal_AS vs OBS +Shoal_Control)			3.386	0.0015*
		SAL OBS+Shoal_AS (*oxt*$^{+/+}$ vs *oxt*$^{-/-}$)			3.85	0.0005*
Erratic movement	Main effect	AS	1.238	0.268		
		GENOTYPE	0.3	0.5848		
		TREATMENT	2.086	0.1512		
	Interaction	GENOTYPE x AS	3.62	0.0593		
		AS x TREATMENT	0.9703	0.3265		
		GENOTYPE X TREATMENT	0.3143	0.5761		
		GENOTYPE x AS x TREATMENT	1.282	0.2597		
	Planned comparisons	OBS+Shoal_AS (SAL *oxt*$^{+/+}$ vs OXT *oxt*$^{-/-}$)			0.4952	0.6213
		SAL *oxt*$^{+/+}$(OBS+Shoal_AS vs OBS +Shoal_Control)			1.488	0.1393
		SAL *oxt*$^{-/-}$ (OBS+shoal_AS vs OBS +Shoal_Control)			1.618	0.1081
		OXT *oxt*$^{-/-}$ (OBS+Shoal_AS vs OBS +Shoal_Control)			1.373	0.1722
		SAL OBS+Shoal_AS (*oxt*$^{+/+}$ vs *oxt*$^{-/-}$)			1.065	0.2889

*# of animals: SAL *oxt*$^{+/+}$ OBS+Shoal_Control= 15, SAL *oxt*$^{+/+}$OBS+Shoal_AS=20 , SAL *oxt*$^{-/-}$ OBS+Shoal_Control=15 , SAL *oxt*$^{-/-}$ OBS+Shoal_AS= 20, OXT *oxt*$^{-/-}$ OBS+Shoal_Control=12 , OXT *oxt*$^{-/-}$ OBS+Shoal_AS= 20

Table 3.7 List of brain regions used for cell counting, their abbreviations and their mammalian homologue (Figure 3.8,3.9).

#	Brain regions	Abbreviations
1	Olfactory bulb	OB
2	Ventral nucleus of ventral telencephalic area (lateral septum)	Vv
3	Central nucleus of ventral telencephalic area (Nucleus accumbens/striatum)	Vc
4	Dorsal nucleus of ventral telencephalic area (Nucleus accumbens/striatum)	Vd
5	Supracommissural nucleus of ventral telencephalic area (medial extended amygdala and the bed nucleus of the stria terminalis)	Vs
6	Postcommissural nucleus of ventral telencephalic area (part of the basal amygdala)	Vp
7	Dorsal telencephalic area	D
8	Medial zone of dorsal telencephalic area (Medial Amygdala)	Dm
9	Lateral zone of dorsal telencephalic area (Hippocampus)	Dl
10	Posterior zone of dorsal telencephalic area (Piriform cortex)	Dp
11	Anterioromedial part of parvocellular preoptic nucleus (SON)	PPAM
12	Anteriorlateral part of parvocellular preoptic nucleus (SON)	PPAL
13	Magnocellular preoptic nucleus (PVN)	PM
14	Posterior part of parvocellular preoptic nucleus (SON)	PPP
15	Dorsal habenular nucleus (Medial habenula)	HAD
16	Ventral habenular nucleus (Lateral habenula)	HAV
17	Ventral zone of periventricular hypothalamus (Arcuate nucleus of the hypothalamus)	HV
18	Anterior tuberal nucleus (Ventromedial hypothalamus- VMH)	ATN
19	Lateral hypothalamic nucleus (Lateral hypothalamus)	LH

Table 3.8 Full result of statistical analysis (GLM-quasi-poisson) of pS6 activity in selected brain regions of zebrafish SDMN in social contagion test (Figure 3.8).

Brain region	Factors						Planned comparisons							
	Genotype		AS		Genotype x AS		OBS + Shoal_AS: WT vs MUT		WT: OBS + Shoal_AS vs OBS + Shoal Control		MUT: OBS + Shoal_AS vs OBS + Shoal Control		OBS + Shoal_Control: WT vs MUT	
	F	p	F	p	F	p	t	p	t	p	t	p	t	p
OB	0.3734	0.5454	1.7632	0.1933	0.0723	0.7897	0.291	0.696	1.137	0.7711	0.73	0.696	0.64	0.696
VV	42.147	2.28E-07	12.127	0.001422	39.316	4.37E-07	8.524	<.0001	-2.673	0.01	6.427	<.0001	-0.26	0.7945
VC	29.022	5.88E-06	2.992	0.09302	23.72	2.70E-05	6.86	<.0001	-2.674	0.01	4.317	<.0001	0.249	0.8037
VD	28.0773	7.64E-06	6.3007	0.01715	6.268	0.01742	5.548	<.0001	-0.262	0.7936	3.513	0.0004	1.818	0.09213333
VS	1.1731	0.2866	3.5965	0.06669	2.6739	0.11151	1.94	0.8428	0.198	0.1048	2.473	0.0536	-0.445	0.8428
VP	6.4837	0.01574	0.1293	0.72147	4.9301	0.03337	3.321	0.0036	-1.398	0.216267	1.749	0.1604	0.255	0.799
D	ns	ns	5.6868	0.02264	ns	ns	1.864	0.62	0.731	0.1246	2.791	0.0208	-0.34	0.7336
DM	6.4213	0.01591	ns	ns	ns	ns	3.247	0.0012	-0.684	0.4938	2.13	0.0664	0.438	0.661
DL	1.6716	0.205	2.5936	0.1168	2.6645	0.1121	2.095	1	0	0.0724	2.284	0.0724	-0.265	1
DP	6.1355	0.018546	0.112	0.740049	10.0945	0.003221	3.965	0.0004	-2.056	0.053067	2.425	0.0306	-0.458	0.6466
PPAM	7.6324	0.009299	0.0123	0.912526	6.8904	0.013029	3.751	0.0004	-1.991	0.093	1.693	0.120533	0.172	0.8633
PPAL	4.018	0.05328	3.3712	0.07537	6.1621	0.01831	3.187	0.005	-0.485	0.6894	3.027	0.005	-0.4	0.6894
PM	2.0311	0.1635	3.5168	0.06962	0.5125	0.47911	1.584	0.590533	0.767	0.2264	1.835	0.2264	0.425	0.671
PPP	2.5179	0.1221	0.4282	0.5174	0.0238	0.8783	1.039	0.7153	0.564	0.5978	0.365	0.7153	1.232	0.5978
HAD	0.9677	0.3324	0.0242	0.8774	0.351	0.5576	0.245	0.8063	0.321	0.8063	-0.52	0.8063	1.11	0.8063
HAV	3.5091	0.351	1.8064	0.1881	5.8245	0.02152	2.54	0.0444	-1.081	0.372667	2.25	0.049	-0.641	0.5215
HV	ns	ns	7.3153	0.01049	ns	ns	1.515	0.104	1.83	0.173067	2.227	0.1344	0.987	0.3235
ATN	2.1721	0.15	1.0293	0.3177	0.1358	0.7148	1.33	0.6413	0.466	0.569333	0.974	0.569333	0.794	0.5693333
LH	1.3861	0.2475	2.0349	0.1631	2.4006	0.1308	1.943	0.104	-0.09	0.928	2.088	0.104	-0.306	0.928

*# of animals: $oxtr^{+/+}$ OBS +shoal_Control= 10, $oxtr^{+/+}$ OBS +Shoal_AS= 10, $oxtr^{-/-}$ OBS +shoal_Control=9 , $oxtr^{-/-}$ OBS +shoal_AS=8

Table 3.9 Pairwise correlations for pS6 cell count in selected SDMN brain regions in experimental groups of oxtr test fish in social contagion test(Figure 3.8)

oxtr$^{+/+}$ OBS+ Shoal_Control correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.826291	0.672763	0.631851	0.683113	0.655183	0.663032	0.590606	0.571118	0.505862	0.633141	0.456581	0.852436	0.791924	0.547992	0.786367	0.606328	-0.24193	0.206205
D			0.475029	0.495006	0.412716	0.273152	0.388157	0.577178	0.411237	0.109406	0.298994	0.204601	0.675496	0.465906	0.313923	0.660478	0.320808	-0.35445	0.196159
DM				0.826231	0.880189	0.707648	0.608757	0.253955	0.90317	0.649529	0.720532	0.405524	0.749077	0.698404	0.67481	0.814952	0.834733	0.329856	0.788909
DL					0.873537	0.830308	0.674302	0.14672	0.868644	0.809521	0.822209	0.689833	0.790442	0.548693	0.600216	0.756757	0.820354	-0.07915	0.510291
DP						0.870042	0.78322	0.401047	0.895229	0.75882	0.816981	0.612764	0.870113	0.769682	0.697835	0.767743	0.845326	0.038409	0.483949
PM							0.693256	0.13514	0.73715	0.85485	0.804794	0.76316	0.737789	0.647712	0.607021	0.714404	0.825814	-0.03399	0.254824
HAD								0.554325	0.615129	0.709188	0.885769	0.327538	0.738081	0.643218	0.483322	0.50443	0.586935	-0.13284	0.148683
HAV									0.118142	0.001358	0.30362	0.053453	0.612875	0.515044	0.156346	0.175015	-0.0016	-0.30274	-0.07855
ATN										0.704633	0.699078	0.466944	0.715103	0.691911	0.722137	0.862258	0.940403	0.144336	0.639176
LH											0.912525	0.726688	0.631848	0.630209	0.578769	0.540758	0.812328	-0.08039	0.266509
HV												0.554167	0.705851	0.666701	0.45147	0.519748	0.73769	-0.03181	0.282195
PPP													0.659852	0.490745	0.499296	0.451885	0.564124	-0.42012	0.05265
PPAL														0.78441	0.752051	0.760108	0.655178	-0.31406	0.338697
PPAM															0.646578	0.701478	0.762037	-0.11614	0.225587
VP																0.775947	0.691583	-0.08997	0.517174
VS																	0.853877	-0.00719	0.51872
VD																		0.112574	0.483823
VC																			0.610185
VV																			

oxtr$^{+/+}$ OBS+ Shoal_Control p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.003212	0.033028	0.050022	0.029455	0.039753	0.036646	0.072218	0.084622	0.135767	0.049412	0.184689	0.001729	0.006326	0.101019	0.006978	0.063124	0.500685	0.567622
D			0.165314	0.145768	0.235885	0.445102	0.267686	0.080628	0.237738	0.763518	0.401352	0.570704	0.032058	0.174737	0.377059	0.037638	0.366106	0.314931	0.587035
DM				0.003216	0.000778	0.022056	0.061789	0.478936	0.000342	0.042097	0.018738	0.244967	0.012648	0.024672	0.0323	0.004076	0.00266	0.351957	0.006674
DL					0.000958	0.00294	0.032479	0.685871	0.001108	0.004544	0.003506	0.027282	0.006495	0.100495	0.066564	0.011283	0.003646	0.827931	0.131811
DP						0.001064	0.007368	0.250717	0.000464	0.010934	0.003911	0.059629	0.001061	0.009224	0.02484	0.009515	0.002069	0.916103	0.156403
PM							0.026218	0.70972	0.014991	0.001624	0.004982	0.010226	0.014858	0.04287	0.062741	0.02027	0.003245	0.925739	0.47738
HAD								0.096344	0.058378	0.02164	0.000648	0.355555	0.014798	0.044822	0.15702	0.137061	0.074458	0.714477	0.681852
HAV									0.745141	0.997029	0.393745	0.883405	0.05957	0.127646	0.666231	0.628667	0.996496	0.395189	0.829222
ATN										0.022887	0.024475	0.173648	0.020091	0.026633	0.01835	0.001329	5.13E-05	0.690761	0.046627
LH											0.00023	0.017282	0.050023	0.050804	0.0796	0.106533	0.004298	0.825284	0.456684
HV												0.096459	0.022549	0.035252	0.190282	0.123606	0.014879	0.930488	0.429553
PPP													0.037884	0.149813	0.141763	0.189825	0.089385	0.226743	0.885147
PPAL														0.007219	0.012106	0.010721	0.039755	0.376841	0.338403
PPAM															0.043357	0.02378	0.010406	0.74935	0.530883
VP																0.008329	0.026734	0.804774	0.125807
VS																	0.001666	0.984264	0.124481
VD																		0.756841	0.156527
VC																			0.061014
VV																			

oxtr$^{+/+}$ OBS+ Shoal_AS correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.416988	0.30707	0.58101	0.794251	0.201306	-0.50361	-0.38952	0.178403	0.434931	-0.22176	0.552546	0.64318	0.659224	0.307079	0.563263	0.208749	0.198994	0.459097
D			-0.17703	0.09598	0.156503	-0.19567	-0.30801	0.3673	0.033212	0.305421	-0.5703	0.297799	0.43729	0.315159	0.01183	0.333923	0.198308	0.252386	0.219593
DM				0.481643	0.33466	0.148255	0.49503	0.164377	0.457143	-0.11069	0.278463	0.537454	0.227309	0.184853	0.349824	0.46756	-0.24416	0.235724	0.337193
DL					0.576289	0.247878	0.112522	-0.35333	0.316602	0.012984	0.175716	0.531169	0.418419	0.196868	-0.22379	0.792821	-0.20189	-0.14248	-0.1575
DP						0.061214	-0.23114	-0.32845	0.153048	0.301914	-0.3219	0.590623	0.34014	0.367601	0.281407	0.521837	-0.3402	0.305368	0.190449
PM							-0.29488	-0.19672	-0.22036	-0.43537	0.191793	0.224	0.234564	0.377281	0.011518	0.385502	0.267071	-0.26661	0.117878
HAD								0.500223	0.363503	-0.44138	0.488387	0.220489	-0.15173	-0.34237	0.02973	-0.06593	-0.64608	-0.11725	-0.2645
HAV									0.077023	-0.34709	-0.14589	0.279414	0.07506	0.021863	0.308628	-0.02785	-0.25485	0.107434	0.152459
ATN										0.490458	0.214113	0.163951	0.276514	0.021296	0.236218	-0.0042	-0.36624	0.171896	-0.28371
LH											-0.43997	-0.29849	0.019196	-0.05104	0.023726	-0.12071	0.131434	0.523474	-0.06408
HV												0.13414	0.232358	0.153863	0.13693	-0.21324	0.001157	-0.68364	-0.05559
PPP													0.745147	0.696556	0.544236	0.56351	-0.29328	-0.1526	0.406004
PPAL														0.91231	0.550416	0.386277	0.209114	-0.40792	0.41417
PPAM															0.704412	0.283822	0.347105	-0.32868	0.658129
VP																-0.1592	-0.04139	-0.00072	0.639103
VS																	0.000787	0.027463	0.135016
VD																		-0.17204	0.49387
VC																			0.138779
VV																			

oxtr$^{+/+}$ OBS+ Shoal_AS p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.230582	0.388119	0.078167	0.006066	0.577058	0.137802	0.265857	0.621929	0.209056	0.538063	0.097643	0.044838	0.038132	0.388104	0.089984	0.562746	0.581532	0.181971
D			0.624653	0.791968	0.665913	0.587987	0.386589	0.296435	0.927428	0.390804	0.085169	0.403328	0.206314	0.375081	0.974126	0.345689	0.582863	0.48175	0.542142
DM				0.158678	0.344559	0.682727	0.145746	0.649985	0.18408	0.76082	0.435939	0.109113	0.527668	0.60917	0.321731	0.173005	0.496609	0.512064	0.340689
DL					0.081206	0.489874	0.756952	0.316574	0.372778	0.971602	0.627271	0.114128	0.228821	0.585656	0.534243	0.006224	0.575932	0.694583	0.663891
DP						0.866595	0.52054	0.354143	0.672939	0.396542	0.364386	0.072208	0.336216	0.296008	0.430897	0.121837	0.336131	0.390891	0.598177
PM							0.408178	0.585938	0.540691	0.208542	0.595548	0.533855	0.514202	0.282479	0.974809	0.271257	0.4557	0.4565	0.745696
HAD								0.140907	0.301838	0.201608	0.15208	0.540452	0.675622	0.332849	0.935023	0.856405	0.043571	0.747008	0.460219
HAV									0.832508	0.32579	0.687562	0.434308	0.836728	0.952197	0.385591	0.939126	0.477333	0.767682	0.67414
ATN										0.150088	0.552516	0.650843	0.439291	0.953435	0.511154	0.990808	0.297935	0.634892	0.426972
LH											0.203221	0.402191	0.958024	0.888631	0.948128	0.739763	0.717404	0.120461	0.860403
HV												0.711791	0.518282	0.67128	0.706019	0.554172	0.997469	0.02928	0.878774
PPP													0.013389	0.02522	0.103859	0.089812	0.410838	0.673844	0.244354
PPAL														0.000232	0.099213	0.270212	0.562047	0.241923	0.234073
PPAM															0.022949	0.426782	0.325766	0.35378	0.038567
VP																0.66045	0.909621	0.998422	0.04666
VS																	0.998278	0.93997	0.709978
VD																		0.634613	0.14684
VC																			0.702202
VV																			

oxtr$^{-/-}$ OBS+ Shoal_Control correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.279476	0.606038	0.393658	0.414378	0.764997	-0.14546	0.031427	0.128737	-0.08289	0.364096	0.880555	-0.10295	0.47793	0.082263	0.052375	0.039125	0.387627	0.747723
D			-0.07919	-0.35057	0.672709	0.294705	-0.11152	0.028938	-0.15777	-0.02191	0.391791	0.100126	-0.45807	-0.25444	0.294868	-0.09707	0.106416	0.378188	-0.1986
DM				0.594755	0.494251	0.894925	-0.55531	-0.58162	-0.34944	-0.62025	0.359675	0.498368	0.553663	0.75203	-0.06568	0.653531	-0.04965	-0.25716	0.374081
DL					0.105262	0.370845	-0.07108	-0.2553	0.04627	-0.11824	0.069783	0.343327	0.58368	0.440226	0.022854	0.27248	0.291141	-0.07169	0.579079
DP						0.675209	-0.45374	-0.18575	-0.23457	-0.36875	0.574179	0.064867	0.270142	0.348243	0.116369	0.359336	0.211863	0.206614	0.008483
PM							-0.43763	-0.37046	-0.21666	-0.47255	0.570606	0.628105	0.258527	0.575647	0.094292	0.492139	-0.10789	-0.11822	0.376585
HAD								0.676386	0.531342	0.652867	-0.36553	0.093088	-0.20839	-0.39491	0.594307	-0.33931	0.271777	0.038118	0.333369
HAV									0.857448	0.819655	0.090153	-0.00795	-0.35018	-0.37569	0.091001	-0.69256	0.007614	0.42412	0.302387
ATN										0.681117	0.31097	0.147082	-0.23955	-0.2752	0.024715	-0.59802	-0.10404	0.159945	0.332084
LH											0.04955	-0.10683	-0.43963	-0.68049	-0.11852	-0.88771	-0.22218	0.37291	0.216548
HV												0.133206	-0.06654	-0.09384	-0.24874	-0.14231	-0.50544	-0.10628	-0.08436
PPP													-0.20841	0.396206	0.310493	0.118416	0.030885	0.117799	0.730285
PPAL														0.63791	0.027467	0.682393	0.350864	-0.35131	0.155054
PPAM															0.063553	0.728233	0.387688	-2E-17	0.471615
VP																0.38063	0.618343	-0.16568	0.202298
VS																	0.38538	-0.4942	-0.01695
VD																		0.311262	0.29356
VC																			0.374471
VV																			

oxtr$^{-/-}$ OBS+ Shoal_Control p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.466424	0.083649	0.294523	0.267489	0.016318	0.708848	0.93603	0.741335	0.832095	0.3354	0.001722	0.792115	0.193178	0.833358	0.893547	0.920398	0.302645	0.020535
D			0.839519	0.354977	0.047087	0.441403	0.775155	0.94109	0.685192	0.955373	0.297026	0.797721	0.21499	0.508813	0.44114	0.803801	0.785247	0.31558	0.608474
DM				0.091167	0.176231	0.001116	0.120624	0.100427	0.356648	0.074744	0.341741	0.172095	0.121966	0.019423	0.866669	0.056268	0.899071	0.504131	0.321296
DL					0.787533	0.325833	0.855802	0.507337	0.905908	0.761909	0.858415	0.365691	0.098936	0.235681	0.953463	0.478116	0.447206	0.854586	0.102279
DP						0.045966	0.219916	0.632298	0.543518	0.328793	0.105912	0.868317	0.482049	0.358406	0.765594	0.342229	0.584215	0.593777	0.98272
PM							0.238774	0.326371	0.57553	0.19896	0.10861	0.070083	0.501792	0.104816	0.809328	0.178374	0.78233	0.761958	0.317805
HAD								0.045444	0.14099	0.056605	0.333352	0.811727	0.590528	0.292855	0.091474	0.371698	0.479298	0.92244	0.380659
HAV									0.003124	0.006842	0.817581	0.983815	0.355553	0.319046	0.815889	0.038656	0.98449	0.255248	0.429012
ATN										0.043386	0.415353	0.705717	0.534724	0.473556	0.949677	0.088954	0.789946	0.681029	0.382611
LH											0.899264	0.784437	0.236387	0.043656	0.76136	0.001397	0.565595	0.322933	0.575729
HV												0.732616	0.864941	0.810219	0.518675	0.714938	0.165116	0.785511	0.82915
PPP													0.590504	0.291126	0.416106	0.761566	0.937132	0.76278	0.025467
PPAL														0.064529	0.944079	0.042841	0.35455	0.353894	0.690387
PPAM															0.870966	0.026094	0.302562	1	0.199972
VP																0.312207	0.0759	0.670107	0.601679
VS																	0.3057	0.176281	0.965469
VD																		0.414891	0.443264
VC																			0.32075
VV																			

oxtr$^{-/-}$ OBS+ Shoal_AS correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.951983	-0.0956	0.246695	0.388094	0.468771	0.3363	0.607646	-0.08735	-0.02493	0.189944	0.407828	0.529579	0.146784	0.690032	0.540566	0.766983	0.050058	0.754327
D			-0.13086	0.019078	0.222396	0.467265	0.466633	0.706184	-0.08027	-0.05623	0.276027	0.455206	0.589281	0.208471	0.677404	0.504778	0.685748	0.103927	0.704403
DM				0.577922	0.588309	0.068884	0.238102	-0.03714	0.459988	0.415079	0.564764	0.389336	0.500616	0.825631	0.478888	0.336296	-0.23295	-0.41341	0.118058
DL					0.890013	0.075401	-0.19162	-0.3293	0.059654	0.042167	-0.05843	0.089594	0.286435	0.356654	0.410489	0.410057	0.297798	-0.30087	0.36525
DP						0.099921	0.200892	-0.00301	0.301681	0.27674	-0.00295	0.371574	0.313006	0.407104	0.387874	0.22053	0.38776	-0.2346	0.255349
PM							0.509181	0.497829	0.395492	0.329594	0.608578	0.745927	0.672572	0.249853	0.573882	0.526539	0.347652	-0.32745	0.51936
HAD								0.840982	0.720044	0.689026	0.585367	0.915546	0.432026	0.329813	0.266467	-0.07819	0.187649	-0.04982	0.006768
HAV									0.419325	0.512443	0.575744	0.741175	0.39816	0.120605	0.381657	0.069729	0.384735	0.142881	0.258299
ATN										0.9542	0.47068	0.778173	0.107233	0.388132	-0.00035	-0.25499	-0.29278	-0.53679	-0.36876
LH											0.482568	0.732199	0.028199	0.264438	-0.00911	-0.29086	-0.214	-0.40238	-0.33978
HV												0.692733	0.744398	0.634797	0.66405	0.500717	-0.05288	-0.24115	0.38195
PPP													0.602358	0.451675	0.467818	0.178988	0.240652	-0.27569	0.201984
PPAL														0.683642	0.923869	0.832723	0.377308	-0.15249	0.784729
PPAM															0.671252	0.548019	-0.26144	-0.60727	0.284562
VP																0.917951	0.418249	-0.21378	0.885311
VS																	0.335824	-0.23844	0.912082
VD																		0.515946	0.675082
VC																			0.086043
VV																			

oxtr$^{-/-}$ OBS+ Shoal_AS p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.000267	0.821837	0.555871	0.342089	0.241329	0.415368	0.110052	0.837056	0.953279	0.652329	0.315881	0.177072	0.728708	0.058219	0.166579	0.02636	0.906298	0.030575
D			0.757417	0.964237	0.596553	0.243051	0.243776	0.050259	0.850136	0.894799	0.508136	0.257064	0.124238	0.620294	0.064934	0.202025	0.060448	0.806535	0.051102
DM				0.133499	0.125015	0.87125	0.570145	0.930434	0.25146	0.3065	0.144692	0.340411	0.206382	0.011581	0.229922	0.415373	0.578761	0.308643	0.78069
DL					0.003058	0.859159	0.649405	0.425745	0.888414	0.921031	0.89069	0.832908	0.491587	0.385819	0.312422	0.312983	0.473763	0.468989	0.373627
DP						0.813892	0.633339	0.994353	0.467731	0.506997	0.994476	0.36477	0.450319	0.316826	0.342387	0.599717	0.342541	0.575997	0.541625
PM							0.197467	0.209328	0.332149	0.425309	0.109358	0.033587	0.067619	0.550657	0.136882	0.180037	0.39877	0.42851	0.187142
HAD								0.008892	0.043982	0.058738	0.127387	0.001412	0.285102	0.424981	0.52352	0.853996	0.656331	0.906738	0.987311
HAV									0.301068	0.194126	0.135316	0.035368	0.328599	0.776048	0.350848	0.86968	0.346647	0.735723	0.5368
ATN										0.000232	0.239155	0.02295	0.800473	0.342037	0.999347	0.542222	0.481596	0.170151	0.368704
LH											0.225842	0.038887	0.947154	0.526808	0.982912	0.48462	0.610831	0.32302	0.410242
HV												0.056839	0.034154	0.090854	0.072511	0.206276	0.901038	0.565066	0.350446
PPP													0.114037	0.261243	0.242418	0.671496	0.565896	0.508678	0.631454
PPAL														0.061562	0.001041	0.010283	0.356823	0.71849	0.021087
PPAM															0.068363	0.159658	0.531678	0.110335	0.49455
VP																0.001297	0.30244	0.611208	0.003454
VS																	0.416071	0.569589	0.001589
VD																		0.190572	0.066216
VC																			0.839464
VV																			

Table 3.10 Network analysis: Density comparisons of oxtr experimental groups in social contagion test (Figure 3.9)

p-values		*oxtr*$^{+/+}$		*oxtr*$^{-/-}$	
		OBS+Shoal Control	OBS+Shoal_AS	OBS+Shoal Control	OBS+Shoal_AS
oxtr$^{+/+}$	OBS+Shoal Control	--------	0.0002	0.0004	--------
	OBS+Shoal_AS	0.0002	--------	--------	0.0056
oxtr$^{-/-}$	OBS+Shoal Control	0.0004	--------	--------	0.0090
	OBS+Shoal_AS	--------	0.0056	0.0090	--------
Density		0.5222	0.1378	0.1235	0.3071

t-statistic		*oxtr*$^{+/+}$		*oxtr*$^{-/-}$	
		OBS+Shoal Control	OBS+Shoal_AS	OBS+Shoal Control	OBS+Shoal_AS
oxtr$^{+/+}$	OBS+Shoal Control	--------	5.2238	4.854	--------
	OBS+Shoal_AS	5.2238	--------	--------	2.8065
oxtr$^{-/-}$	OBS+Shoal Control	4.854	--------	--------	2.6820
	OBS+Shoal_AS	--------	2.805	2.6820	--------

Table 3.11 Network analysis: QAP correlations and p-values of oxtr contagion experimental groups (Figure 3.9)

QAP correlations		*oxtr*$^{+/+}$		*oxtr*$^{-/-}$	
		OBS+Shoal Control	OBS+Shoal_AS	OBS+Shoal Control	OBS+Shoal_AS
oxtr$^{+/+}$	OBS+Shoal Control	--------	0.262	-0.462	--------
	OBS+Shoal_AS	0.262	--------	--------	1.000
oxtr$^{-/-}$	OBS+Shoal Control	-0.462	--------	--------	0.003
	OBS+Shoal_AS	--------	1.000	0.003	--------

QAP p-values		*oxtr*$^{+/+}$		*oxtr*$^{-/-}$	
		OBS+Shoal Control	OBS+Shoal_AS	OBS+Shoal Control	OBS+Shoal_AS
Oxtr$^{+/+}$	OBS+Shoal Control	--------	0.653	0.245	--------
	OBS+Shoal_AS	0.653	--------	--------	0.879
oxtr$^{-/-}$	OBS+Shoal Control	0.245	--------	--------	0.723
	OBS+Shoal_AS	--------	0.879	0.723	--------

3.6. **References**

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4. Chapter 4

Role of oxytocin in social buffering of fear in zebrafish

Summary

In this chapter, we present a set of studies aimed at investigating the role of oxytocin in social buffering of fear in zebrafish using a mutant line with constitutive deletion of the oxytocin receptor (*oxtr*).

- In the first experiment, we investigated the effects of oxytocin on zebrafish exposed to alarm substance-AS, a cue that indicates the presence of a threatening stimulus in the presence of an unalarmed shoal. Our results revealed that wild type (WT) zebrafish displayed reduced freezing response when exposed to AS in the presence of unalarmed shoal, indicating that they are capable of demonstrating social buffering of fear. Conversely, mutant zebrafish exhibited increased freezing response when exposed to AS in the presence of unalarmed shoal, suggesting an effect of the oxytocin receptor in this behaviour. .
- Next, we explored possible brain regions that could be involved in regulating the social buffering of fear in zebrafish using the quantification of the expression of pS6- a marker of neuronal activation. We showed that exposure of zebrafish to AS in the presence of unalarmed shoal causes changes in specific brain areas relevant to emotional regulation in WT zebrafish in contrast to the mutants.
- Overall, our results showed a conserved function of oxytocin in the regulation of social buffering of fear in zebrafish.

4.1. **Introduction**

The act of communicating is essential to individuals not only for the expression of personal information, but also for protection from environmental threats (Brown & Laland, 2003; Chivers et al., 2007; Kikusui, Winslow, & Mori, 2006).

The perception of threatening environmental stimuli triggers behavioural, physiological, and even phenotypic changes that imply high energetic costs on the individual (Anson, Dickman, Boonstra, & Jessop, 2013; Bedoya-Perez et al., 2019; Preisser & Bolnick, 2008; Ziv et al., 2013).

Social buffering of fear can be considered as a strategy in which individuals use social information indicating the absence of a threat to diminish their fear response to a threatful stimulus that they may have detected themselves in order to minimize the energy and life-threatening costs of making wrong individual decisions (Beauchamp & Ruxton, 2007; Oliveira & Faustino, 2017; Valone, 2007a; Wiley, 2006b).

Social buffering phenomena has long been acknowledged in other taxa especially primates and rodents (Davitz & Mason, 1955; Kikusui et al., 2006; Kiyokawa & Hennessy, 2018; Liddell, 1949; Mason, 1960); however, only recently has it began to be explored in other non-mammalian models such as teleosts (Faustino et al., 2017; Mathuru et al., 2017).

More importantly, considering the highly conserved nature of this phenomenon, very little is known about the underlying neurobiological substrates regulating this component of social behaviour.

The neuropeptide oxytocin has been implicated in the modulation of social behaviour across several species (Godwin & Thompson, 2012; Goodson & Thompson, 2010b) and there have been increasing reports suggesting the role of oxytocin in the regulation of social buffering in mammalian models. For example, in a placebo-controlled double-blind study, the intranasal treatment with oxytocin seemed to enhance the buffering effect of social support after healthy human subjects were exposed to stress (Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003a). Additionally, a study on prairie voles (*Microtus*

ochrogaster) reports that the presence of conspecifics facilitated recovery from stress and also increased oxytocin release in the paraventricular nucleus (PVN) (A. S. Smith & Wang, 2014). In a more recent study with mice (*Mus musculus*), the presence of cage mates reduced freezing in fear and anxiety-provoking contexts. Also, the optogenetic reactivation of neurons active during the social encoding of a new cagemate phenocopied the effects of cagemate presence in male and female mice in learned and innate fear contexts (Gutzeit et al., 2020).

Given that some studies in mammalian species have alluded to the role of neuropeptides in the regulation of this phenomena (Culbert, Gilmour, & Balshine, 2019), it remains unclear whether the role oxytocin in social buffering is conserved across taxa.

The Social decision-making network (SDMN) is described as a network of nodes in the brain that regulates and implements responses to salient stimuli (Almeida, Félix, Oliveira, Lopes, & Oliveira, 2019; O'Connell & Hofmann, 2011; Teles, Almeida, Lopes, & Oliveira, 2015). It integrates sensory information about a social context with previous experience in order to produce an appropriate behavioural response. Therefore, it is possible that social decisions rely on multiple nodes, rather than being controlled by one specific brain region.

In this study, we have provided an evolutionary framework to understanding the role of oxytocin in the regulation of social buffering of fear, using mutant zebrafish line with constitutive deletion of the oxytocin receptor (*oxtr*).

4.2. **Materials and methods**

4.2.1. Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) 3-6 months old *oxtr*$^{-/-(wz16)}$ (ZFIN ID: ZDB-ALT-190830-1), lines also known as oxytocin receptor mutants were generated in the laboratory of Dr Gil Levkowitz (Weizmann Institute of Science) using the transcription activator-like effector nuclease (TALEN) genome-editing technique that causes a small deletion (1bp) leading to a non-functional protein formation.

The line is of TL-mixed background, and was raised in a recirculating system (ZebTEC active-blue, Tecniplast) under 14L: 10D photoperiod, in the fish facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Tank water was maintained at 28ºC, 900 µS, pH 7.0, <0.2 ppm nitrites, <50 ppm nitrates and 0.01-0.1 ppm ammonia. Fish were fed with a combination of live food (*Paramecium caudatum, Artemia salina, and Rotifers)* and commercial processed dry food (Gemma). All test fish were drug naïve, and each fish was used only once. Twenty fish per group were used except otherwise stated. Male zebrafish were used as test fish in all the experiments.

All experiments were performed in accordance with the standard operating procedures of the Instituto Gulbenkian de Ciência Ethics Committee.

4.2.2. Genotyping

Zebrafish genotyping was performed by PCR followed by sequencing (Ribeiro et al., 2020; Xing et al., 2014). Specific primer pairs were designed to target the receptors' deletion sites (Table 4.1).

4.2.3. Alarm substance extraction

Alarm substance (AS) was extracted using a modified version of the protocol described by Speedie and Gerlai (Speedie & Gerlai, 2008). Ten donor zebrafish (five males and five females) were used to prepare the alarm substance for the experiment. Donor fish were retrieved from the holding tank with a fishing net, and then quickly sacrificed, placed in a Petri dish on ice and 15 shallow cuts

were made on both sides of the fish using a sterile surgical blade (Swann Morton #12536518). Using a Terumo® syringe (8SS50L1) without needle 50ml of distilled water (vehicle) was used to wash the cuts ensuring an adequate release of AS. Impurities were removed from the AS solution using 0.22 µm sterile vacuum filtering (Filtropur V50- 83.3940) and AS aliquots were stored at -20^{o}C.

4.2.4. Behavioural setup

The behavioural assay was performed similarly to chapter two. Briefly, using two adjacent tanks (12 x 12 x 15 cm- L x W x H) each filled with 1.3L of water. The test fish (males) were placed in one tank, and the other tank contained water with a shoal of two males and two females. The tanks had opaque white bases, opposite and rear walls to prevent interference by external environmental cues. AS was administered with a flexible and transparent PVC tubing (0.8 mm internal diameter; 2.4 mm external diameter). Two video cameras (two webcams-Logitech B 525 high definition camcorder, Ref 960-000842) were placed above and to the side of the tanks. Eyeline surveillance software (www.nchsoftware.com) was used for video acquisition.

4.2.5. Experimental procedure

Wildtype (WT) and mutant (MUT) test fish were randomly assigned to one of four treatments: test fish alone exposed to vehicle (Alone_Control); test fish alone exposed to AS (Alone_AS); test fish exposed to vehicle in the presence of a shoal (Obs + Shoal_Control); and test fish exposed to AS in the presence a shoal (Obs_AS + Shoal). The shoal used in the experiment was the WT background. The order of testing was randomized in each experiment and all experiments were conducted between 10:00 and 19:00. On the day before the experiment, test fish were removed from home tanks and randomly assigned to treatment groups for acclimatization.

On the test day, each trial lasted for 15mins and was divided into two phases: the first 5mins to establish baseline and the subsequent 10 mins to measure the response to the treatment.

Alarm substance (AS) (previously described in chapter one) was used to induce a fear response in this experiment. AS was kept in ice to avoid AS degradation during the trial and therefore distilled water was kept in the same conditions to ensure comparability of treatments. The arena dimensions for top and side views were 10.5 x 10.5 cm and 11 x 10.5 cm respectively. Two videos corresponding to the top and side view recordings were analysed per test fish. The x,y,z coordinates extracted from automated video tracking by Ethovision software (Ethovision XT 12, Noldus Technology), were analysed using an updated version of custom made xyz2b Python scripts. Percentage of time spent in erratic movement and freezing was used as a measure of the fear response in the experiments. Two criteria defined the erratic movement; if the test fish accelerated >8 cm/s^2 and >5 changes in direction/sec. Two criteria defined freezing: if the test fish velocity was <0.2 cm/s; and if the test fish position on y-axis was below the bottom quarter of the side view of the arena.

4.2.6. Tissue collection and preparation

To assess the brain activation patterns and to determine the patterns of functional connectivity in social buffering of fear experiment, as well as to further understand the role of oxytocin in this phenomena, $oxtr^{+/+}$ and $oxtr^{-/-}$ test fish brains were collected following the conclusion of the behavioural experiment. Animals were collected 1hr after the completion of the trial to allow for the expression of the pS6 neural activation maker. Test fish were anaesthetized in ice-cold water and sacrificed by rapid cervical transection. Heads of test fish were fixed in 10% buffered formalin for 3 days at room temperature, rinsed in 1x PBS (2X, 30min) at room temperature and kept in EDTA for decalcification (0.5 M, pH=8) for 2 days at room temperature. Samples were then processed for histological analysis where samples were incorporated in paraffin and coronal

sections were cut at 5um thickness. Slides were subsequently stored in boxes at room temperature until immunohistochemical processing.

4.2.7. Immunohistochemistry for pS6

Sectioned brains were stained for the neural activation marker phospho-S6 ribosomal protein (pS6). Slides were kept in Tris-EDTA at 95°C 20 min for antigen retrieval. Non-specific binding was blocked by incubating slides in TBS (0.025% Triton X-100) containing 1% BSA (albumin) during 1 hr at room temperature after which an overnight incubation at 4 °C in primary antibody (1:400; prepared in blocking solution was done (Cell Signalling pS6 ribosomal protein Ser$^{235/\mathit{236}}$ antibody D57.2.2E Rabbit mAB #4858). Slides were then rinsed in TBS 0.025% Triton X-100 and incubated in secondary antibody (Alexa 594-Invitrogen goat anti-rabbit # A-11037 (1:1000)) also prepared in blocking solution. Slides were subsequently washed in TBS 0.025% Triton X-100 and then TBS without triton before incubating in for DAPI (4',6-diamidino-2-phenylindole) for nuclei counterstaining for 20 mins and then rinsed in TBS before mounting (Biotium, Everbrite- 23003).

4.2.8. Microscopy and image analysis

To quantify the number pS6 positive cells, stained brain sections were acquired at 20x using the Zeiss Axioscan.Z1 slide scanner and processed using the Zeiss Zen blue 2.1 imaging software. Images were acquired in .czi format and subsequently exported to Fiji software (Schindelin et al., 2012) for conversion to .tiff and .png format for panels arrangement.

Five consecutive coronal sections were quantified for each brain region selected (Fig. 4.1, Table 4.7). In each section, positive pS6 cells were counted in a rectangle with an area of at 1000 µm per area per section using the Zeiss Zen blue 2.1 imaging software. The nomenclature of brain nuclei was identified using the zebrafish atlas (Wullimann MF, Rupp B and Reichert Vogel, 1996).

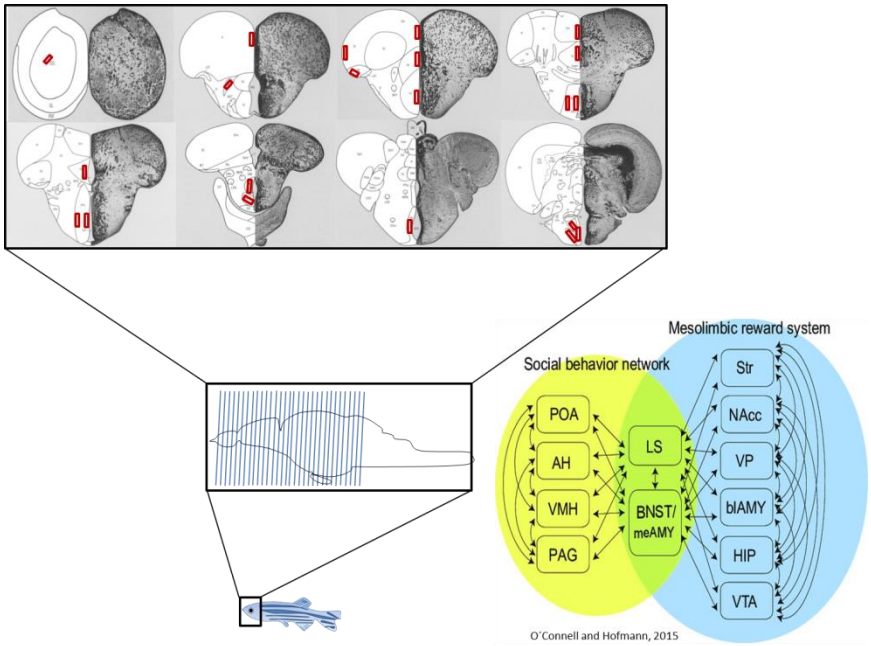


Figure 4.1 Identification of brain regions involved in social buffering of fear.
The social decision-making network; a combination of the social behaviour network and the reward system (O'Connell & Hofmann, 2011). Red rectangles represent brain regions of interest for quantifying pS6 positive cells (Table 4.7); brain regions of interest are identified using the zebrafish atlas (Wullimann MF, Rupp B and Reichert Vogel, 1996).

4.2.9. Statistical analysis

Analysis of the raw values of the behavioural variables was done using three-way ANOVA method with genotype, AS and shoal as fixed factors. Following which planned comparisons was done followed by corrections by False Discovery Rate (FDR) p-value adjustment (Benjamini and Hochberg's method). Normality was determined using the D'Agostino & Pearson test (D'Agostino RB, 1986).

For analysis of cell counts of pS6 positive cells, a generalized linear model (GLM) with quasi-Poisson regression was used, with AS, genotype and shoal as fixed effects. A backward stepwise procedure was used to exclude non-

significant interactions and fixed effects following which planned comparisons were done.

Statistical significance was considered if $p<0.05$ and data are presented as mean $\pm$ SEM. Graphpad Prism version 8 (Weiß, 2007) and R (R Core Team, 2015). (*p < .05; **p < 0.01; ***p < 0.001) statistical programs were used for data analysis.

Measures for network connectivity were analysed using UCINET Version 6.102 (Borgatti et al., 2002), and brain nuclei representations of the SDMN network were produced using a custom-made Python script. The Pearson product-moment correlation square matrices for comparison of ps6 cell count between each pair of brain nuclei was used as a measure of functional connectivity (Batushansky et al., 2016) and statistically tested using a Quadratic Assignment Procedure (QAP). QAP analysis tests the association between two square matrices (So et al., 2015) and the null-hypothesis is that there is a no association between the tested matrices. In the present study, QAP correlations were used with 5000 permutations, and two co-activation patterns were considered different when QAP p-value was higher than 0.05.

The density of the network was also measured to determine network cohesion, and it is given by the proportion of all possible connections that are present in the network (Faustino et al., 2017; Schaefer et al., 2014). Differences in network density between treatments were tested using a t-test (bootstrap set to 5000 sub-samples).

4.3. **Results**

4.3.1. Oxytocin receptor mutants displayed increased freezing response when exposed to Alarm substance (AS) in presence of unalarmed shoal

We assessed the response of oxytocin receptor mutants (*oxtr*) when exposed to AS in the presence of unalarmed conspecifics. The freezing response of *oxtr* test fish was significantly affected by AS and shoal but not by the genotype in social buffering (three-way ANOVA: AS: $F_{(1,161)}$= 35.27, p=<.0001; Shoal: $F_{(1,161)}$= 2.735, p=0.0043; Genotype: $F_{(1,161)}$= 0.256, p=0.3767: Fig. 4.2A Table 4.2); and there was a significant interaction of genotype x AS, genotype x shoal, shoal x AS but not genotype x shoal x AS (three-way ANOVA: genotype x AS: $F_{(1,161)}$= 2.93, p=0.0031; genotype x shoal: $F_{(1,161)}$= 3.139, p=0.0023; shoal x AS: $F_{(1,161)}$= 1.803, p=0.0199; genotype x shoal x AS: $F_{(1,161)}$= 2.791, p=0.0967: Fig. 4.2A Table 4.2).

The freezing response of $oxtr^{+/+}$ test fish exposed to AS in the presence of unalarmed shoal was significantly less than $oxtr^{+/+}$ test fish exposed to AS alone (planned comparisons: $oxtr^{+/+}$ Obs + Shoal_AS vs $oxtr^{+/+}$ Alone_AS: $t_{(1,161)}$= 5.017, p=<0.0001; Figure 4.2A, Table 4.2). Also, the freezing response of $oxtr^{+/+}$ test fish exposed to AS in the presence of unalarmed shoal was significantly less than $oxtr^{-/-}$ test fish exposed to AS in the presence of unalarmed shoal (planned comparisons: $oxtr^{+/+}$ Obs + Shoal_AS vs $oxtr^{-/-}$ Alone_AS: $t_{(1,161)}$= 4.662, p=<0.0001;Figure 4.2A, Table 4.2). Both $oxtr^{+/+}$ and $oxtr^{-/-}$ test fish exposed to AS alone significantly increased freezing response in the presence of AS (planned comparisons: $oxtr^{+/+}$ Alone_Control vs $oxtr^{+/+}$ Alone_AS: $t_{(1,161)}$= 5.588, p=<0.0001; $oxtr^{-/-}$ Alone_Control vs $oxtr^{-/-}$ Alone_AS: $t_{(1,161)}$= 6.886, p=<0.0001; Figure 4.2A, Table 4.2). There was no significant difference in the freezing response of controls (planned comparisms: $oxtr^{+/+}$ Alone_Control vs $oxtr^{+/+}$ Obs+shoal_Control: $t_{(1,161)}$= 0.967, p=0.5025; $oxtr^{+/+}$ Alone_Control vs

oxtr$^{-/-}$ Alone_Control: $t_{(1,161)}$= 1.733, p=0.1586 *oxtr*$^{-/-}$ Alone_Control vs *oxtr*$^{-/-}$ Obs+shoal_Control: $t_{(1,161)}$= 0.4345, p=0.7974; Figure 4.2A, Table 4.2).

In addition, there was no significant difference in erratic movement response (Figure 4.2B; Table 4.2).

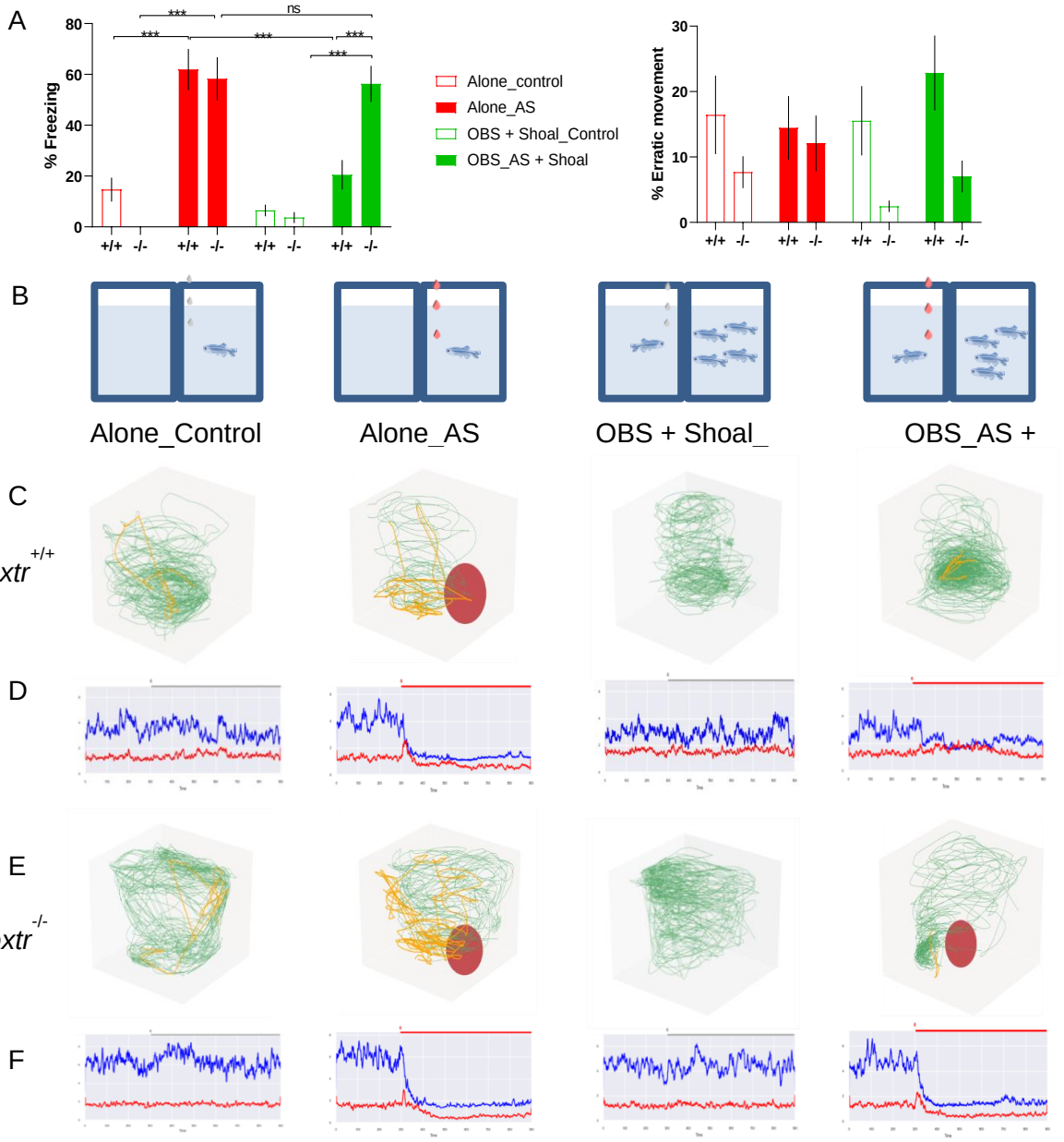


Figure 4.2 Loss of oxytocin receptor caused increased freezing in mutants.

(A) Percentage of time in freezing and erratic movement (mean+SEM) during the test phase. The legend identifies genotype, presence of AS and the presence of shoal for each treatment. (B) Schematic representation of the behavioural treatments. Droplets represent administration of vehicle (grey) and alarm substance (AS; red) to control and experimental groups. (C, E) Representative 3D swimming behaviour for each treatment group in males and females; normal swimming pattern (green), erratic movement (orange), and freezing episodes (red dot, size is proportional to freezing time). (D, F) Mean velocity (red) and mean depth (blue) of each treatment group in males and females. (F) Asterisks indicate statistical significance; ***p < 0.001, ns represents non-significance.

4.3.2. Sight of alarmed shoal caused changes in oxtr$^{+/+}$ and oxtr$^{-/-}$ pS6 positive cells count

The brain regions involved in social buffering of fear and the role of oxytocin in this phenomena was determined by quantifying the number of positive phosphorylated- S6 (pS6) cells in *oxtr*$^{+/+}$ and *oxtr*$^{-/-}$ experimental groups (Table 4.3).

There was a significant effect of AS in the number of pS6 positive cells in the Vd (Quasi-poisson: AS: $F_{(1,61)}$= 12.771, p=0.0006582). Planned comparisons showed a significantly fewer number of pS6 positive cells in the oxtr$^{+/+}$ Obs_AS + Shoal group compared to oxtr$^{+/+}$ Alone_AS group (planned comparisons: oxtr$^{+/+}$ Obs_AS + Shoal X oxtr$^{+/+}$ Alone_AS: t= 2.275, p=0.0229). In addition, there was a significantly higher number of pS6 positive cells in oxtr$^{+/+}$ Alone_AS compared to oxtr$^{+/+}$ Alone_Control (planned comparisons: oxtr$^{+/+}$ Alone_Control X oxtr$^{+/+}$ Alone_AS: t= 3.834, p=0.0001).

Similarly, there was a significant effect of AS in the number of pS6 positive cells in the Vc (Quasi-poisson: AS: $F_{(1,61)}$= 6.7581, p=0.01165) with an interaction of Genotype and AS (Quasi-poisson: Genotype X AS: $F_{(1,61)}$= 4.7212, p=0.03362).

Planned comparisons showed a significantly fewer number of pS6 positive cells in the oxtr$^{+/+}$ Obs_AS + Shoal group compared to oxtr$^{+/+}$ Alone_AS group (planned comparisons: oxtr$^{+/+}$ Obs_AS + Shoal X oxtr$^{+/+}$ Alone_AS: t= -2.553, p=0.0107). In addition, there was a significantly higher number of pS6 positive cells in oxtr$^{+/+}$ Alone_AS compared to oxtr$^{+/+}$ Alone_Control (planned comparisons: oxtr$^{+/+}$ Alone_Control X oxtr$^{+/+}$ Alone_AS: t= 3.693, p=0.0002).

There was a significant effect of AS and shoal in the number of pS6 positive cells in the Vv (Quasi-poisson: AS: $F_{(1,61)}$= 4.7776, p=0.032618, Shoal: $F_{(1,61)}$= 7.3357, p=0.008726) with an interaction of Genotype and AS (Quasi-poisson: Genotype X AS: $F_{(1,61)}$= 7.6691, p=0.0074). Planned comparisons showed a significantly higher number of pS6 positive cells in oxtr$^{+/+}$ Alone_AS compared to oxtr$^{+/+}$ Alone_Control (planned comparisons: oxtr$^{+/+}$ Alone_Control X oxtr$^{+/+}$ Alone_AS: t= 2.602, p=0.0093). There was also a significantly fewer number of pS6 positive cells in the oxtr$^{+/+}$ Obs_AS + Shoal compared to oxtr$^{-/-}$ Obs_AS + Shoal (planned comparisons: oxtr$^{+/+}$ Obs_AS + Shoal X oxtr$^{-/-}$ Obs_AS + Shoal: t= -2.702, p=0.0069).

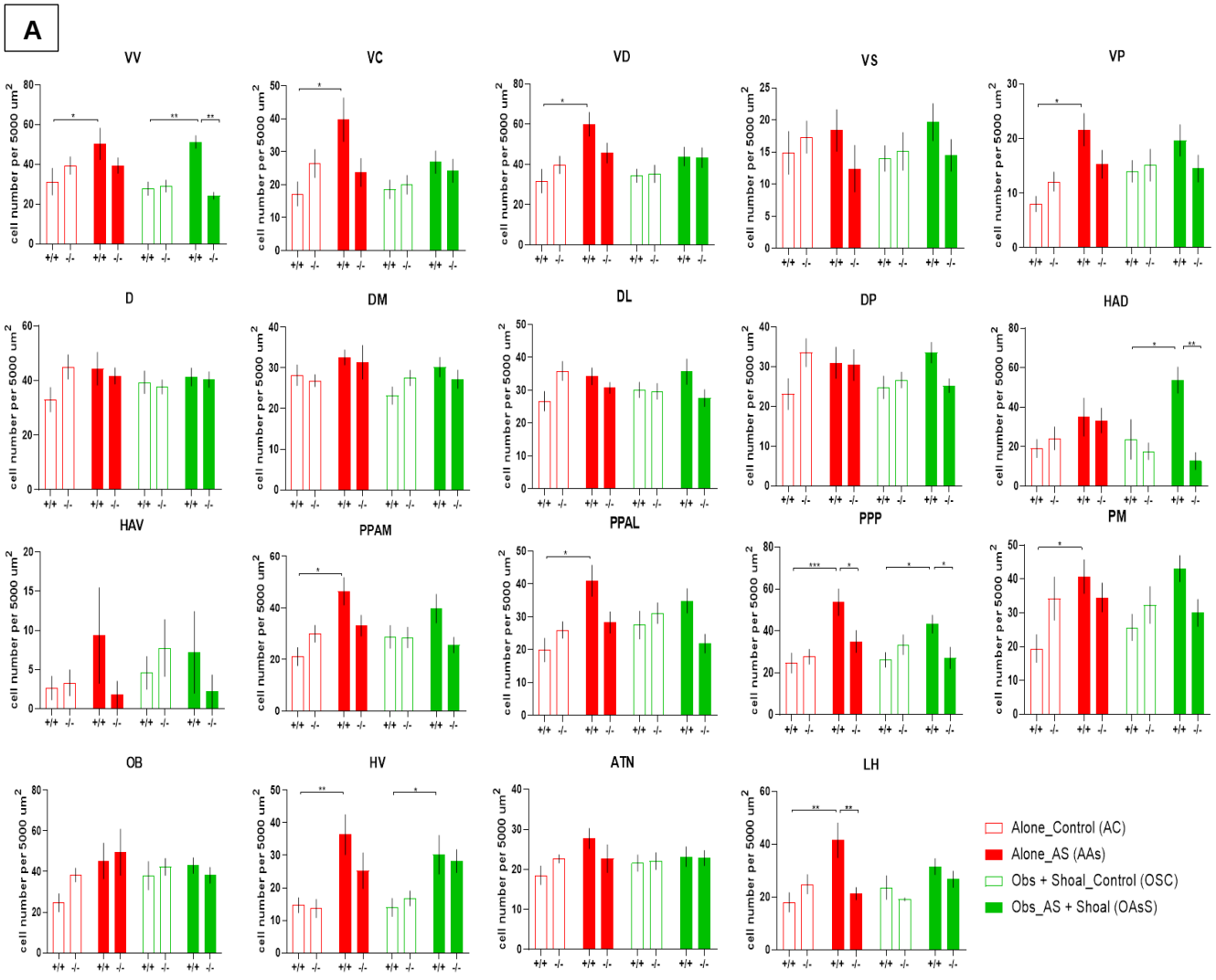
There was an interaction of Genotype and AS in the number of pS6 positive cells in the Dl (Quasi-poisson: Genotype X AS: $F_{(1,61)}$= 6.6823, p=0.01206). Planned comparisons showed a significantly fewer number of pS6 positive cells in the oxtr$^{+/+}$ Obs_AS + Shoal compared to oxtr$^{-/-}$ Obs_AS + Shoal (planned comparisons: oxtr$^{+/+}$ Obs_AS + Shoal X oxtr$^{-/-}$ Obs_AS + Shoal: t= -1.98, p=0.0477).

There was a significant effect of AS in the number of pS6 positive cells in the PPAL (Quasi-poisson: AS: $F_{(1,61)}$= 4.0378, p=0.04885) and there was an interaction of Genotype and AS (Quasi-poisson: Genotype X AS: $F_{(1,61)}$=10.1218, p=0.00229). Planned comparisons showed a significantly higher number of pS6 positive cells in oxtr$^{+/+}$ Alone_AS compared to oxtr$^{+/+}$ Alone_Control (planned comparisons: oxtr$^{+/+}$ Alone_Control X oxtr$^{+/+}$ Alone_AS: t= 3.847, p=0.0001). There was also a significantly higher number of pS6 positive cells in the oxtr$^{+/+}$ Obs_AS + Shoal compared to oxtr$^{-/-}$ Obs_AS + Shoal

(planned comparisons: $oxtr^{+/+}$ Obs_AS + Shoal X $oxtr^{-/-}$ Obs_AS + Shoal: t= -2.281, p=0.0225).

There was a significant effect of AS in the number of pS6 positive cells in the PPP (Quasi-poisson: AS: $F_{(1,61)}$= 11.8417, p=0.001042) and there was an interaction of Genotype and AS (Quasi-poisson: Genotype X AS: $F_{(1,61)}$= 8.8924, p=0.004089). Planned comparisons showed a significantly higher number of pS6 positive cells in $oxtr^{+/+}$ Alone_AS compared to $oxtr^{+/+}$ Alone_Control (planned comparisons: $oxtr^{+/+}$ Alone_Control X $oxtr^{+/+}$ Alone_AS: t= 3.854, p=0.0001). There was also a significantly fewer number of pS6 positive cells in the $oxtr^{+/+}$ Obs_AS + Shoal compared to $oxtr^{-/-}$ Obs_AS + Shoal (planned comparisons: $oxtr^{+/+}$ Obs_AS + Shoal X $oxtr^{-/-}$ Obs_AS + Shoal: t= -2.113, p=0.0346).

There was a significant effect of AS in the number of pS6 positive cells in the HAD (Quasi-poisson: AS: $F_{(1,61)}$= 4.4237, p=0.03944), and there was an interaction of Genotype and Shoal (Quasi-poisson: Genotype X Shoal: $F_{(1,61)}$= 4.5613, p=0.0366). Planned comparisons showed a significantly fewer number of pS6 positive cells in the $oxtr^{+/+}$ Obs_AS + Shoal compared to $oxtr^{-/-}$ Obs_AS + Shoal (planned comparisons: $oxtr^{+/+}$ Obs_AS + Shoal X $oxtr^{-/-}$ Obs_AS + Shoal: t= 2.771, p=0.0056) (Fig4.3, Table 4.3).


A
VV
VC
VD
VS
VP
D
DM
DL
DP
HAD
HAV
PPAM
PPAL
PPP
PM
OB
HV
ATN
LH
cell number per 5000 um2
+/+ -/-
Alone_Control (AC)
Alone_AS (AAs)
Obs + Shoal_Control (OSC)
Obs_AS + Shoal (OAsS)

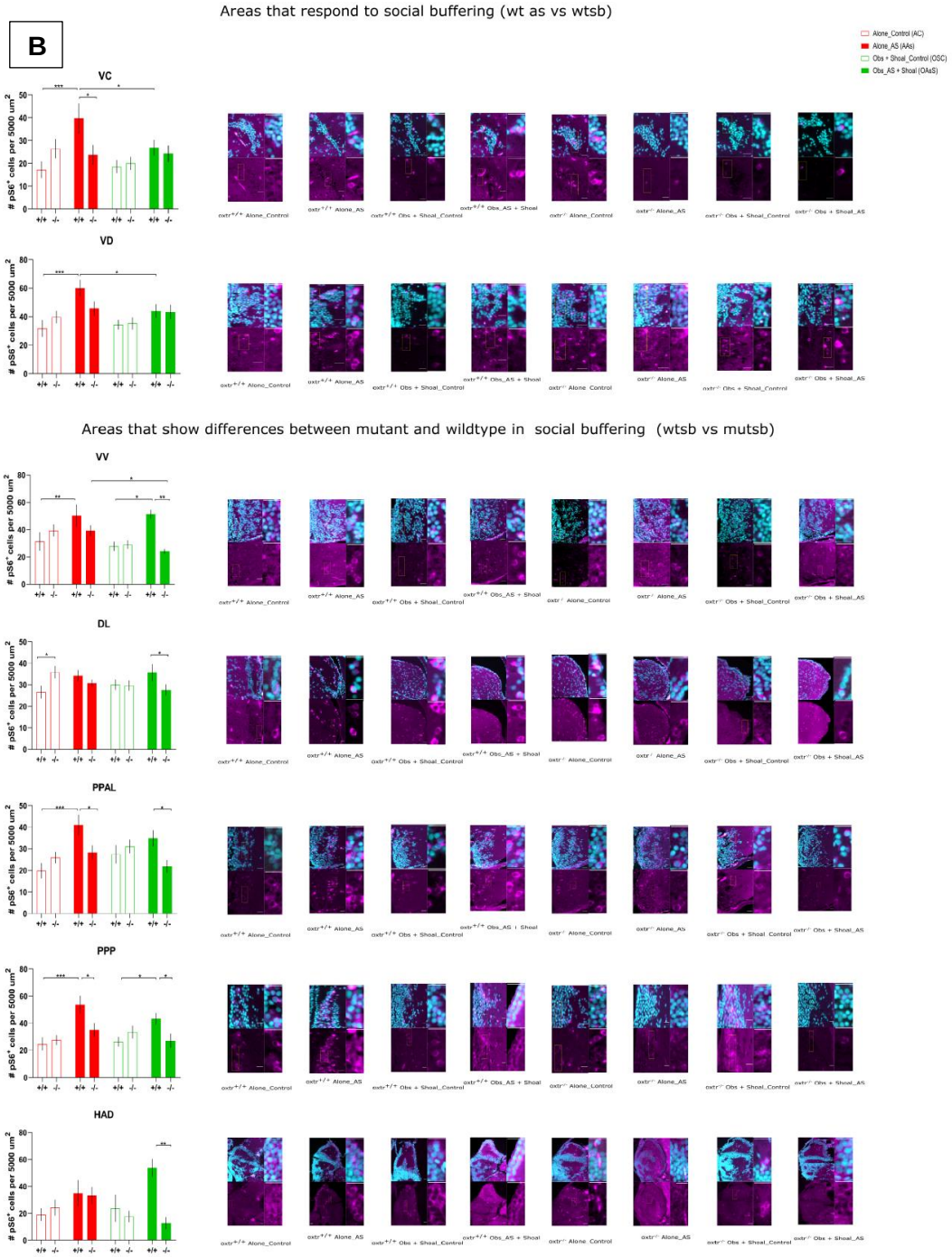

Areas that respond to social buffering (wt as vs wtsb)
B
Alone_Control (AC)
Alone_AS (AAs)
Obs + Shoal_Control (OSC)
Obs_AS + Shoal (OAsS)
VC
VD
Areas that show differences between mutant and wildtype in social buffering (wtsb vs mutsb)
VV
DL
PPAL
PPP
HAD
pS6+ cells per 5000 um2

Figure 4.3 Loss of oxytocin caused changes in the number of pS6 positive cells in selected SDMN brain areas in social buffering.
(A) pS6 positive cells across selected brain areasa in the sdm network. (B) Brain aeas that respond to social buffering and show changes between Wts and Muts exposed to social buffering with representative images showing these differences. Brain areas are identified above each graph and bar plot indicates the number of pS6 positive cells in each brain area; the legend identifies the genotype and treatment of test fish. The result is shown as Mean±SEM. *p < 0.05, **p < 0.01, *** p < 0.001. Scale bar=20 µm.

4.3.3.Differences in patterns of functional connectivity among treatments in social contagion in zebrafish

The Quadratic Assignment Procedure (QAP) correlation was used to assess the co-activation patterns between brain regions across treatments. There was no significant association between the correlation matrices of all WT treatments suggesting that these networks have distinct co-activation patterns (*oxtr*$^{+/+}$ Alone_Control vs Alone_AS: r=0.045, p=0.459; *oxtr*$^{+/+}$ Alone_Control vs OBS+Shoal_Control: r=-0.111, p=0.371; *oxtr*$^{+/+}$ OBS+Shoal_Control vs OBS_AS+Shoal: r=0.088, p=0.413; *oxtr*$^{+/+}$ Alone_AS vs OBS_AS+Shoal: r=0.313, p=0.289 Table 4.4).

Similarly, there was also no significant association between all correlation matrices in the MUT treatment (*oxtr*$^{-/-}$ Alone_Control vs Alone_AS: r=-0.056, p=0.383; *oxtr*$^{-/-}$ Alone_Control vs OBS+Shoal_Control: r=-1, p=0.126; *oxtr*$^{-/-}$ Alone_AS vs OBS_AS+Shoal: r=0.738, p=0.281; *oxtr*$^{-/-}$ OBS+Shoal_Control vs OBS_AS+Shoal: r=-0.282, p=0.255; Table 4.4), also suggesting that these networks have distinct co-activation patterns.

Additionally, there was no significant association between the correlation matrices of WT and MUT treatment groups (Alone _Control: *oxtr*$^{+/+}$ x *oxtr*$^{-/-}$ r=0.42, p=0.284; Alone _AS: *oxtr*$^{+/+}$ x *oxtr*$^{-/-}$ r=0.144, p=0.434; OBS+Shoal_Control *oxtr*$^{+/+}$ x *oxtr*$^{-/-}$ r=-0.539, p=0.237; OBS_AS+Shoal *oxtr*$^{+/+}$ x *oxtr*$^{-/-}$: r= 0.328, p=0.409) suggesting that the networks of WTs and MUTs have distinct co-activation patterns.

The network density of *oxtr*$^{+/+}$ was significantly higher than *oxtr*$^{-/-}$ across all treatment comparisons (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$ Alone_Control: t=3.2345, p=0.0026; *oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$ Alone_AS: t=2.148, p=0.0316; *oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$ OBS+Shoal_Control: t=5.7301, p=0.0002; *oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$ OBS+Shoal_AS: t=4.4307, p=0.0004; Table 4.4).

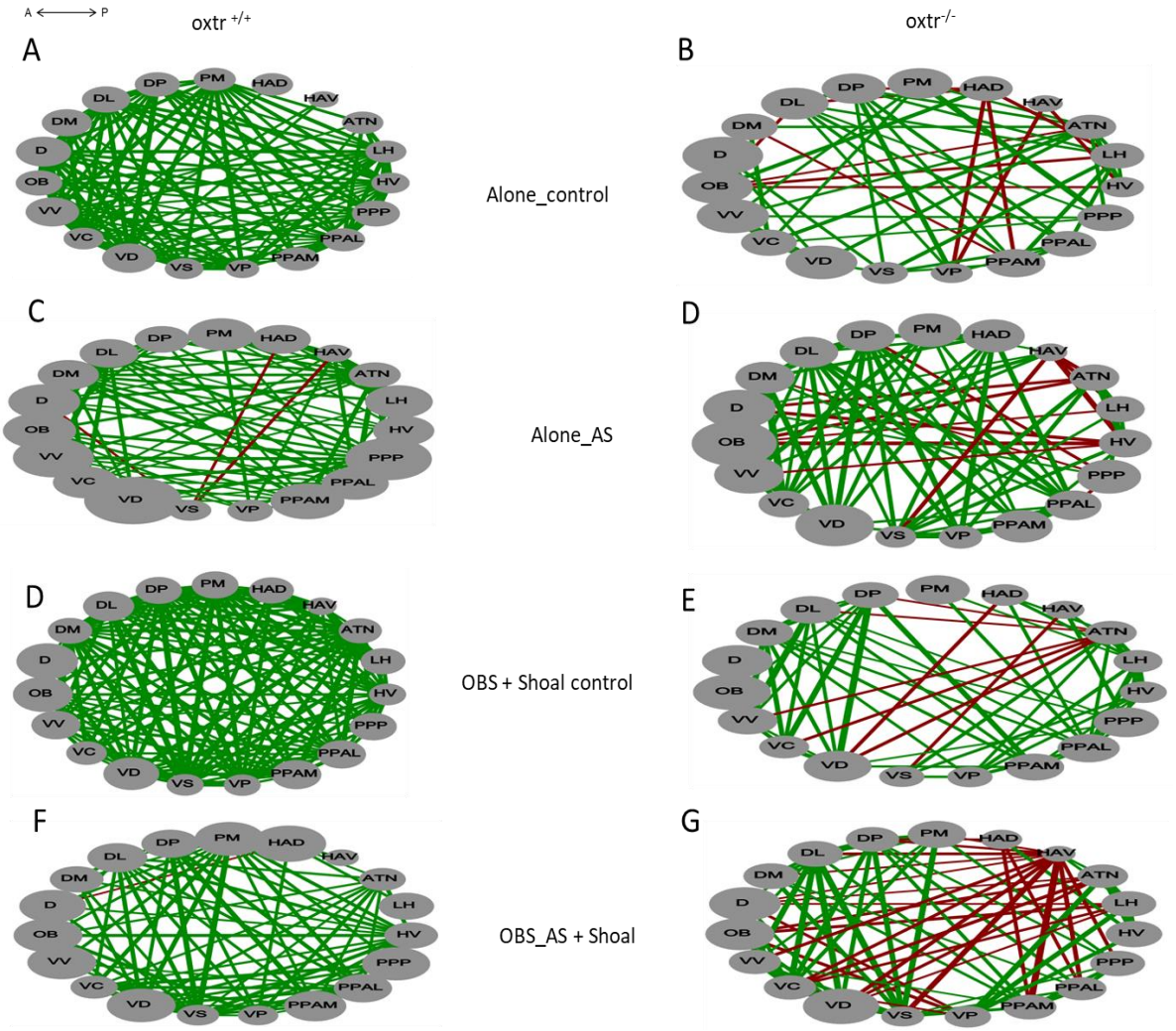


Figure 4.4 Functional connectivity of pS6 expression between pairs of brain nuclei for each experimental treatment in social buffering.
Networks represent oxtr$^{+/+}$ (A, C, E, G) and oxtr$^{-/-}$ (B, D, F, H) treatments; brain regions are identified in each node and the size of the node represents the

average number of pS6 positive cells for each brain region in each experimental group, nodes are arranged in the anterior (A) and posterior (P) direction; positive and negative correlations are shown in green and red respectively.

4.4. **Discussion**

Our studies demonstrate the role of oxytocin in the regulation of social buffering of fear in zebrafish. We show that threatening stimulus triggers increased freezing response in zebrafish, and the presence of unalarmed conspecifics decreases this heightened fear response. However, the loss of oxytocin receptor caused an increased freezing behaviour when test fish was exposed to threatening in the presence of their unalarmed conspecifics (Fig4.2). A further investigation of the neural activation pattern highlights the Vd and Vc as possible candidates for the regulation of social buffering of fear. Interestingly, our results also highlight the Vv, Dl, Ppal, Ppp and Had as regions involved in the oxytocinergic regulation of social buffering of fear (Fig4.3, Table 4.7). Additionally, network analysis showed a change in network connectivity in the regions of the SDM network upon deletion of the oxytocin receptor. These results highlight the role of oxytocin in the regulation of social buffering of fear in zebrafish.

To the best of our knowledge, our findings present the first clear evidence for the role of oxytocin in social buffering of fear responses in zebrafish. Previous studies have suggested possible role oxytocin in the regulation of social buffering in other species. However, the study on zebrafish have focused on the surge oxytocin upon recovery of stress in the presence of conspecifics; the changes in oxytocin levels could be as a result of the effects of the by-product(s) of other neuromodulators and not a direct consequence of the action of oxytocin. Indeed, reports on the possible role of other neuropeptides in social buffering of fear have been reported (Backström & Winberg, 2013).

Thus we have used genetic tools that can provide a more precise determination of the role of genes to demonstrate the role of oxytocin in social buffering of fear in zebrafish. We showed that *oxtr* mutant zebrafish displayed heightened freezing response when exposed to a threatful stimulus in the presence of unalarmed conspecifics highlighting the involvement of oxytocin in modulating social buffering of fear in zebrafish.

Although, some reports have shown the role of oxytocin in social buffering of fear in mammalian models (Gutzeit et al., 2020; Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003b); and recent experimental evidence suggests the effect of oxytocin in this phenomenon in vertebrates (Culbert et al., 2019; Mathuru et al., 2017), our study provides relevant evidence on the evolutionarily conserved role of oxytocin in modulating social buffering of fear in teleosts.

Group living animals have to adjust their behaviour to rapid changes in the social environment, and it has been hypothesized that the expression of social behaviour is better explained by the activity pattern of connectivity of the social decision-making network (SDMN) in the brain than by the activity of a single brain region (Almeida et al., 2019; Goodson & Thompson, 2010a; Newman, 2006; Teles et al., 2015)

The social decision-making network is said to consist of an integration of the evolutionarily conserved mesolimbic reward system and the social behaviour network; this network implements responses to salient stimuli through reciprocally interconnected nodes (J.L, 2005; L. O'Connell & Hofmann, 2011, 2012).

It is interesting to note that the controls had higher network connectivity compared to treatment groups, suggesting that the presence of a stimulus breaks apart the network connectivity and agrees with the "*default mode network*" hypothesis which proposes that a task-induced activity decreases functional connectivity in comparison with non-task states (Raichle, 2015). Similarly, wild type treatments showed higher network density in contrast with mutant treatments; a possible effect of the loss of the oxytocin receptor. The changes in network density and functional connectivity due to the loss of the oxytocin receptor agrees with reports that suggest the role of oxytocin in maintaining plasticity across nodes by enhancing the signal-to-noise ratio in brain connectivity (Pekarek, Hunt, & Arenkiel, 2020).

The Vc and Vd are putative homologues of the Nucleus accumbens and striatum respectively (Wullimann MF, Rupp B and ReichertVogel, 1996). These regions have been implicated in the regulation of fear. For example, a study

showed that deep brain stimulation of the ventral striatum leads to reduces fear expression and strengthened extinction of fear in rats (Rodriguez-Romaguera, Do Monte, & Quirk, 2012). Also, the selective ablation of striatal neurons strongly diminished contextual fear conditioning (Ikegami, Uemura, Kishioka, Sakimura, & Mishina, 2014). Our result provides supporting evidence to the role of striatum in and the nucleus accumbens in social buffering of fear.

The increased expression of pS6 in the ventral nucleus of the ventral telencephalic area (Vv), the dorso-lateral zone of the dorsal telencephalon (Dl), the dorsal habenula (Had), the anterolateral part of the parvocellular preoptic nucleus (Ppal), and the posterior part of the parvocellular preoptic nucleus (Ppp) of wildtypes in contrast to mutants suggests that oxytocin may be acting on these nodes to regulates its function. Future studies might explore the specific role each node in the regulation of social buffering of fear. Indeed, these brain regions are putative homologues for the lateral septum, hippocampus, medial habenula and paraventricular nucleus and supraoptic nucleus respectively ((Godwin & Thompson, 2012; O'Connell & Hofmann, 2012; Wircer, Ben-Dor, & Levkowitz, 2016) and have been linked to the regulation of emotional fear and memory (Mathuru & Jesuthasan, 2013; Okamoto, Agetsuma, & Aizawa, 2012; Yamaguchi, Danjo, Pastan, Hikida, & Nakanishi, 2013).

The habenula is a part of an evolutionarily highly conserved conduction pathway within the limbic system; the medial habenula (dorsal habenula in teleosts) connects the septal telencephalic nuclei to the interpeduncular nucleus (IPN) (Okamoto et al., 2012) and functions in the maintenance of appropriate affective states.

Moreover, since oxytocin has been reported to function in the modulation of nodes involved in the processing of social stimuli (Johnson, Z. V., Walum H, Xiao Y, Riefkohl P.C, 2017; Johnson & Young, 2017), It is plausible that oxytocin acts on different pathways to regulate social buffering. More studies are needed to further establish these connections.

Overall, our results provide more evidence on the conserved role of oxytocin in social buffering of fear in zebrafish.

4.5. **Author Contributions**

Ibukun Akinrinade (I.A) and Rui Filipe Oliveira (R.F.O) designed the experiments and established the behavioural protocols. I.A performed the behavioural tests, processed all brains samples, quantified cell counts, and performed the functional connectivity analysis. I.A, R.F.O, and G.L analysed and discussed results. Zebrafish line was kindly donated by Prof Gil Levkowitz (G.L). We thank Abisola Akinrinade for assistance with data analysis in Python.

The study in this chapter is in preparation to be submitted in an international peer-reviewed journal.

Table 4.1 Primer pairs for genotyping oxtr line

Gene	Forward primer	Reverse primer
oxtr: NM_001199370.1	5'-TGCGCGAGGAAAACTAGTT-3'	5'-AGCAGACACTCAGAATGGTCA-3'

Table 4.2 Full result of statistical analysis (three-way ANOVA) of behavioural variables to social buffering of fear in oxtr$^{+/+}$ and oxtr$^{-/-}$ (Fig4.2).

			F	p	t	q
Behavioural variables						
Freezing	Interaction	GENOTYPE*SHOAL*AS	2.791	0.0967		
		GENOTYPE*SHOAL	3.139	0.0023**		
		GENOTYPE*AS	2.93	0.0031**		
		SHOAL*AS	1.803	0.0199*		
	Main effect	GENOTYPE	0.256	0.3767		
		SHOAL	2.735	0.0043**		
		AS	35.27	<.0001***		
	Planned comparisons	*oxtr*$^{+/+}$(Alone_Control vs Alone_AS)			5.588	<0.0001
		oxtr$^{+/+}$(Alone_Control vs OBS+Shoal_Control)			0.967	0.5025
		oxtr$^{+/+}$(Alone_AS vs OBS+Shoal_AS)			5.017	<0.0001
		oxtr$^{+/+}$(OBS+Shoal_Control vs OBS+Shoal_AS)			1.692	0.1585714
		Alone_Control (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			1.733	0.1585714
		OBS+Shoal_Control (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			0.332	0.8037
		OBS+Shoal_AS (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			4.662	<0.0001
		Alone_AS (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			0.435	0.7974
		oxtr$^{-/-}$Alone_Control vs Alone_AS)			6.886	<0.0001
		oxtr$^{-/-}$Alone_Control vs OBS+Shoal_Control)			0.4345	0.7974
		oxtr$^{-/-}$Alone_AS vs OBS+Shoal_AS)			0.249	0.8037
		oxtr$^{-/-}$OBS+Shoal_Control vs OBS+Shoal_AS)			6.667	<0.0001
Erratic movement	Interaction	GENOTYPE*SHOAL*AS	0.5636	0.4539		
		GENOTYPE*SHOAL	2.138	0.1456		
		GENOTYPE*AS	0.08925	0.7655		
		SHOAL*AS	0.6031	0.4385		
	Main effect	GENOTYPE	10.87	0.0012**		
		SHOAL	0.05496	0.8149		
		AS	1.387	0.2407		
	Planned comparisons	*oxtr*$^{+/+}$(Alone_Control vs Alone_AS)			0.3171	0.819927
		oxtr$^{+/+}$(Alone_Control vs OBS+Shoal_Control)			0.1465	0.8837
		oxtr$^{+/+}$(Alone_AS vs OBS+Shoal_AS)			1.379	0.5097
		oxtr$^{+/+}$(OBS+Shoal_Control vs OBS+Shoal_AS)			1.204	0.55272
		Alone_Control (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			1.41	0.5097
		OBS+Shoal_Control (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			2.106	0.2202
		OBS+Shoal_AS (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			2.806	0.0672
		Alone_AS (*oxtr*$^{+/+}$ vs *oxtr*$^{-/-}$)			0.3842	0.819927
		oxtr$^{-/-}$Alone_Control vs Alone_AS)			0.7092	0.6389333
		oxtr$^{-/-}$Alone_Control vs OBS+Shoal_Control)			0.8422	0.6389333
		oxtr$^{-/-}$Alone_AS vs OBS+Shoal_AS)			0.8756	0.6389333
		oxtr$^{-/-}$OBS+Shoal_Control vs OBS+Shoal_AS)			0.7873	0.6389333

Table 4.3 Full result of statistical analysis (GLM-Quasi-Poisson) of pS6 activity in selected brain regions of zebrafish SDMN in social buffering test (Figure 4.3).

Brain region	Factor													
	Genotype		AS		Shoal		Genotype x AS		Genotype x Shoal		AS x Shoal		Genotype x AS x Shoal	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
OB	1.0244	0.31546	3.4455	0.06826	0.1369	0.71265	1.3618	0.24776	1.1143	0.29532	3.7448	0.05761	0.0882	0.76751
VV	1.7542	0.190209	4.7776	0.032618	7.3357	0.008726	7.6691	0.0074	1.4779	0.228708	0.3224	0.572198		
VC	0.049	0.82547	6.7581	0.01165	3.548	0.06431	4.7212	0.03362	0.3903	0.53442	0.4748	0.49334		
VD			12.771	0.0006582										
VS			0.1059	0.7459										
VP	0.2877	0.593656	12.0264	0.0009676	0.8288	0.3662048	1.9713	0.1653847	1.0512	0.3092837	4.0282	0.0491824	3.5277	0.0651331
D	0.424	0.5174	1.1478	0.2882	0.2122	0.6467	1.323	0.2545	1.2063	0.2764	0.1736	0.6784	1.9455	0.1681
DM			5.5866	0.02101										
DL	0.056	0.81371	0.697	0.40696	0.3024	0.58434	6.6823	0.01206						
DP	0.2259	0.63621	2.069	0.15526	0.7415	0.39243	5.9308	0.01772	3.3507	0.07191				
PPAM	1.3743	0.245481	7.2652	0.009004	0.4664	0.497165	6.1109	0.016148	0.4936	0.484902				
PPAL	1.645	0.20441	4.0378	0.04885	0.0042	0.94876	10.1218	0.00229	0.0206	0.88624	6.7723	0.01157		
PM	0.0691	0.79359	7.8002	0.006939	0.0407	0.840715	9.2923	0.00338	1.2393	0.269906	0.4946	0.484498		
PPP	2.4161	0.125181	11.8417	0.001042	0.6798	0.412826	8.8924	0.004089	0.3273	0.569316	3.1691	0.079942		
HAD	3.1995	0.07847	4.4237	0.03944	0.1816	0.67148	2.0475	0.1574	4.5613	0.0366				
HAV	2.0695	0.1554	0.6511	0.4228	2.0029	0.1621	0.0483	0.8268	0.3089	0.5804	0.1343	0.7153		
HV			32.767	2.66E-07										
ATN	0.0005	0.98272	3.1917	0.07898	0.0676	0.79579	2.134	0.14919	0.0084	0.92725	1.1837	0.28089	1.5663	0.21552
LH	3.8042	0.055723	11.5243	0.001213	0.2456	0.621941	4.6052	0.035862	0.0236	0.878484	0.1802	0.672693	5.9677	0.017481

Brain region	Planned comparisons																							
	WT: Alone_Control vs Alone_AS		WT: Alone_Control vs OBS + Shoal_Control		WT: OBS_AS + Shoal x OBS + Shoal_Control		WT: OBS_AS + Shoal vs Alone_AS		MUT: Alone_Control vs Alone_AS		MUT: Alone_Control vs OBS + Shoal_Control		MUT: OBS_AS + Shoal x OBS + Shoal_Control		MUT: OBS_AS + Shoal vs Alone_AS		Alone_Control: WT vs MUT		Alone_AS: WT vs MUT		OBS + Shoal_Control: WT vs MUT		OBS + Shoal_AS: WT vs MUT	
	z	p	z	p	z	p	z	p	z	p	z	p	z	p	z	p	z	p	z	p	z	p	z	p
OB	2.531	0.0114	1.819	0.0689	0.582	0.5603	-0.273	0.7849	1.202	0.2292	0.475	0.6349	-0.47	0.6385	-1.176	0.2397	1.822	0.0685	0.432	0.6659	0.53	0.5964	-0.519	0.6035
VV	2.602	0.0093	-0.602	0.5469	2.284	0.0224	-1.021	0.3074	-0.002	0.9983	-1.587	0.1125	-0.821	0.4118	-2.208	0.0273	1.228	0.2194	-1.334	0.1821	0.228	0.8198	-2.702	0.0069
VC	3.693	0.0002	0.306	0.7595	1.017	0.3092	-2.553	0.0107	-0.466	0.6411	-1.223	0.2213	0.805	0.4206	0.091	0.9272	1.797	0.0723	-2.333	0.0196	0.3	0.764	0.126	0.8993
VD	3.834	0.0001	-0.446	0.6553	1.274	0.2027	2.275	0.0229	0.815	0.4152	0.693	0.4884	1.19	0.234	0.305	0.7601	1.276	0.2021	-1.698	0.0896	0.155	0.8768	0.132	0.8952
VS	0.83	0.4067	0.24	0.8104	0.972	0.3309	0.136	0.8916	-1.171	0.2416	0.551	0.5817	-0.154	0.8777	-0.512	0.6087	0.608	0.5432	-1.362	0.1733	0.299	0.7651	-0.787	0.4311
VP	4.14	<0.0001	-2.061	0.0393	0.649	0.5164	1.759	0.0786	0.999	0.3179	-1.04	0.2983	0.995	0.3196	-0.91	0.3626	1.594	0.1108	-1.647	0.0995	0.613	0.54	0.961	0.3365
D	1.998	0.0457	1.224	0.2211	0.291	0.7707	-0.564	0.5725	-0.524	0.6004	-1.282	0.1999	0.474	0.6357	-0.214	0.8303	2.154	0.0313	-0.415	0.678	-0.322	0.7473	-0.107	0.9148
DM	1.253	0.2101	1.644	0.1001	2.255	0.0242	0.673	0.5011	1.276	0.2019	-0.207	0.8364	-0.092	0.9271	1.138	0.2553	-0.411	0.6814	-0.311	0.7557	1.435	0.1512	-0.868	0.3855
DL	1.918	0.0551	-0.948	0.3431	1.433	0.1519	-0.334	0.7385	-1.198	0.2308	1.56	0.1189	-0.531	0.5953	0.75	0.4534	2.361	0.0182	-0.81	0.4178	-0.117	0.907	-1.98	0.0477
DP	1.78	0.0751	-0.427	0.669	2.011	0.0444	-0.527	0.5984	-0.623	0.5333	1.514	0.13	-0.326	0.7446	1.082	0.2791	2.358	0.0184	-0.113	0.9096	0.457	0.6479	-1.783	0.0746
PPAM	3.807	0.0001	-1.445	0.1484	1.423	0.1548	1.203	0.2288	0.484	0.6284	0.246	0.8055	-0.503	0.615	1.167	0.2431	1.614	0.1065	-1.716	0.0862	-0.043	0.9655	-1.862	0.0625
PPAL	3.847	0.0001	-1.667	0.0954	1.241	0.2144	1.183	0.2366	0.431	0.6663	-1.001	0.3166	-1.806	0.0709	1.224	0.2209	1.333	0.1824	-2.038	0.0415	0.718	0.4726	-2.281	0.0225
PM	3.147	0.0017	-1.134	0.2567	2.578	0.0099	-0.313	0.7546	0.047	0.9624	0.277	0.7819	-0.342	0.7321	0.621	0.5349	2.38	0.0173	-0.772	0.4399	1.069	0.285	-1.773	0.0762
PPP	3.854	0.0001	-0.27	0.7869	2.416	0.0157	1.417	0.1566	1.069	0.2853	-0.905	0.3652	-0.962	0.3361	1.118	0.2634	0.509	0.6111	-2.192	0.0284	1.182	0.2373	-2.113	0.0346
HAD	1.478	0.1393	-0.509	0.6111	1.959	0.0502	-0.867	0.3859	0.798	0.4246	0.716	0.4738	-0.609	0.5428	1.913	0.0557	0.55	0.5823	-0.134	0.8934	-0.678	0.4975	-2.771	0.0056
HAV	1.364	0.1727	-0.527	0.5985	0.621	0.5349	0.392	0.6953	-0.448	0.6541	-0.217	0.8281	-0.534	0.5936	-0.134	0.8933	0.207	0.8358	-1.371	0.1704	-0.104	0.9175	-1.114	0.2653
HV	3.549	0.0004	0.157	0.8751	3.034	0.0024	0.901	0.3676	2.149	0.0316	-0.692	0.4887	2.033	0.0421	-0.453	0.6506	-0.231	0.8173	-1.564	0.1177	0.633	0.527	-0.307	0.7585
ATN	2.769	0.0056	-1.039	0.2988	0.526	0.599	1.302	0.1928	0.014	0.9888	0.175	0.8607	0.234	0.8152	-0.046	0.9634	1.351	0.1766	-1.358	0.1744	0.168	0.8668	-0.105	0.9165
LH	4.01	0.0001	-1.222	0.2219	1.518	0.1291	1.559	0.1189	-0.681	0.4961	1.199	0.2306	1.511	0.1308	-0.985	0.3244	1.446	0.1483	-3.115	0.0018	-0.968	0.3329	-0.84	0.401

Table 4.4 Network analysis: Network density comparisons in social buffering of fear (Figure 4.4)

Density comparisons p-values		*oxtr*$^{+/+}$				*oxtr*$^{-/-}$			
		Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal	Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal
oxtr$^{+/+}$	Alone_Control	--------	0.7878	0.5265	--------	0.0026	--------	--------	--------
	Alone_AS	0.7878	--------	--------	0.4493	--------	0.0316	--------	--------
	OBS+Shoal_ Control	0.5265	--------	--------	0.0304	--------	--------	0.0002	--------
	OBS_AS +Shoal	--------	0.4493	0.0304	--------	--------	---------	--------	0.0004
oxtr$^{-/-}$	Alone_Control	0.0026	--------	--------	--------	--------	0.7317	0.6507	--------
	Alone_AS	--------	0.0316	--------	--------	0.7317	---------	--------	0.0862
	OBS+Shoal_ Control	--------	--------	0.0002	--------	0.6507	--------	--------	0.1956
	OBS_AS +Shoal	--------	--------	-------	0.0004	--------	0.0862	0.1956	--------
Density		0.4672	0.4431	0.5128	0.372	0.2129	0.2378	0.1814	0.1101

Density comparisons t-statistic		*oxtr*$^{+/+}$				*oxtr*$^{-/-}$			
		Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal	Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal
oxtr$^{+/+}$	Alone_Control	--------	0.2621	-0.6164	--------	3.2345	--------	--------	--------
	Alone_AS	0.2621	--------	--------	-0.7581	--------	2.148	--------	--------
	OBS+Shoal_ Control	-0.6164	--------	--------	-2.1808	--------	--------	5.7301	--------
	OBS_AS +Shoal	--------	-0.7581	-2.1808	--------	--------	---------	--------	4.4307
oxtr$^{-/-}$	Alone_Control	3.2345	--------	--------	--------	--------	-0.3341	0.446	-------
	Alone_AS	--------	2.148	--------	--------	-0.3341	---------	--------	1.7005
	OBS+Shoal_ Control	--------	--------	5.7301	--------	0.446	--------	--------	1.2709
	OBS_AS +Shoal	--------	--------	-------	4.4307	--------	1.7005	1.2709	--------
Density		0.4672	0.4431	0.5128	0.372	0.2129	0.2378	0.1814	0.1101

Table 4.5 Network analysis: QAP correlations and p-values in social buffering of fear test (Figure 4.4)

QAP correlations		*oxtr*$^{+/+}$				*oxtr*$^{-/-}$			
		Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal	Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal
oxtr$^{+/+}$	Alone_Control	--------	0.045	-0.111	--------	0.42	--------	--------	--------
	Alone_AS	0.045	--------	--------	0.313	--------	0.144	----------	--------
	OBS+Shoal_ Control	-0.111	--------	----------	0.088	--------	----------	-0.539	--------
	OBS_AS +Shoal	--------	0.313	0.088	--------	--------	---------	--------	0.328
oxtr$^{-/-}$	Alone_Control	0.42	--------	--------	--------	--------	-0.056	-1	-------
	Alone_AS	--------	0.144	--------	--------	-0.056	---------	--------	0.738
	OBS+Shoal_ Control	--------	--------	-0.539	--------	-1	--------	--------	-0.282
	OBS_AS +Shoal	--------	--------	-------	0.328	--------	0.738	-0.282	--------

QAP p-values		*oxtr*$^{+/+}$				*oxtr*$^{-/-}$			
		Alone_ Control	Alone_AS	OBS+Shoal Control	OBS_AS +Shoal	Alone_ Control	Alone_A S	OBS+Shoal Control	OBS_AS +Shoal
oxtr$^{+/+}$	Alone_Control	--------	0.459	0.371	--------	0.284	--------	--------	--------
	Alone_AS	0.459	--------	--------	0.289	--------	0.434	----------	--------
	OBS+Shoal_ Control	0.371	--------	----------	0.413	--------	----------	0.237	--------
	OBS_AS +Shoal	--------	0.289	0.413	--------	--------	---------	--------	0.409
oxtr$^{-/-}$	Alone_Control	0.284	--------	--------	--------	--------	0.383	0.126	-------
	Alone_AS	--------	0.434	--------	--------	0.383	---------	--------	0.281
	OBS+Shoal_ Control	--------	--------	0.237	--------	0.126	--------	--------	0.255
	OBS_AS +Shoal	--------	--------	-------	0.409	--------	0.281	0.255	--------

Table 4.6 Analysis of pairwise correlations in social buffering of fear test (Figure 4.4)

oxtr$^{+/+}$ Alone_Control correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.846579	0.676192	0.906526	0.771523	0.533619	0.200188	0.492244	0.179005	0.624464	0.660148	0.287942	0.402127	0.39104	0.721113	0.551206	0.809648	0.761053	0.51579
D			0.45542	0.933504	0.705578	0.697807	0.15335	0.515239	0.369566	0.742007	0.717592	-0.06601	0.694413	0.486668	0.825838	0.752588	0.77671	0.69681	0.778605
DM				0.557252	0.763967	0.553582	0.427012	0.066437	0.284146	0.482401	0.509786	0.263511	0.287365	0.467632	0.490484	0.543984	0.630921	0.722394	0.539542
DL					0.841905	0.640919	0.233268	0.506788	0.375388	0.701966	0.759722	-0.00648	0.629313	0.448573	0.752097	0.617069	0.845753	0.779545	0.702822
DP						0.673194	0.469536	0.310771	0.399971	0.602324	0.76754	0.067508	0.558144	0.63868	0.532072	0.521486	0.878306	0.836784	0.730137
PM							0.375289	0.000736	0.770792	0.932597	0.869494	-0.51143	0.661127	0.661657	0.827385	0.841064	0.490011	0.44529	0.809259
HAD								0.303606	-0.0944	0.105328	0.167888	-0.04919	-0.20844	-0.08937	0.248794	0.031012	0.463615	0.555716	0.175707
HAV									-0.42488	-0.06148	-0.07429	0.318979	-0.07928	-0.22802	0.354886	-0.1098	0.71095	0.691427	0.203585
ATN										0.822224	0.774042	-0.72781	0.754662	0.663238	0.522591	0.698792	0.064005	0.006146	0.644966
LH											0.92587	-0.48655	0.717936	0.647773	0.856353	0.870195	0.398013	0.321974	0.7205
HV												-0.35866	0.751678	0.760061	0.678719	0.769823	0.501397	0.387818	0.703609
PPP													-0.37006	-0.12687	-0.33488	-0.31392	0.264203	0.296165	-0.29599
PPAL														0.803778	0.479135	0.804858	0.388692	0.281654	0.858149
PPAM															0.316525	0.688105	0.398615	0.271009	0.753793
VP																0.747445	0.556482	0.540975	0.66378
VS																	0.366753	0.33877	0.808092
VD																		0.965862	0.667902
VC																			0.622679
VV																			

oxtr$^{+/+}$ Alone_Control p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.003995	0.04553	0.00075	0.014889	0.138975	0.605555	0.178267	0.644931	0.072219	0.052982	0.452442	0.28331	0.298035	0.028348	0.123981	0.00818	0.017225	0.155213
D			0.217992	0.000234	0.033699	0.036605	0.693655	0.155731	0.327635	0.022075	0.029508	0.866003	0.037924	0.183995	0.006095	0.019282	0.013815	0.03699	0.013435
DM				0.119059	0.016552	0.122032	0.251674	0.865152	0.458688	0.188448	0.160917	0.493281	0.453388	0.204324	0.180064	0.130016	0.068459	0.027933	0.133812
DL					0.004416	0.062882	0.545814	0.163811	0.319471	0.035031	0.017538	0.9868	0.069383	0.22587	0.019406	0.076681	0.004067	0.01325	0.034712
DP						0.046868	0.202236	0.415667	0.286143	0.08608	0.015751	0.862996	0.118344	0.064105	0.140343	0.149911	0.001834	0.004911	0.025512
PM							0.319609	0.9985	0.015045	0.000245	0.002322	0.159342	0.052507	0.052251	0.005917	0.004495	0.180549	0.229703	0.008235
HAD								0.42706	0.809107	0.787401	0.665913	0.899987	0.590451	0.81914	0.518583	0.936874	0.208766	0.120299	0.651134
HAV									0.254301	0.875141	0.849345	0.402787	0.839328	0.555139	0.348676	0.778556	0.03178	0.03911	0.599318
ATN										0.006524	0.01436	0.026224	0.018764	0.051491	0.148895	0.036228	0.870055	0.98748	0.060709
LH											0.000339	0.184115	0.029393	0.05923	0.003205	0.00228	0.288728	0.398134	0.028548
HV												0.343209	0.019512	0.017458	0.044421	0.015253	0.169087	0.302386	0.034421
PPP													0.32694	0.744981	0.37837	0.410699	0.492104	0.439036	0.439323
PPAL														0.009042	0.191897	0.008879	0.301203	0.46281	0.003072
PPAM															0.406619	0.040456	0.287933	0.480588	0.01898
VP																0.020608	0.11968	0.13258	0.051232
VS																	0.331617	0.372505	0.008403
VD																		2.34E-05	0.04929
VC																			0.073281
VV																			

oxtr$^{-/-}$ Alone_Control correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.278035	-0.03144	-0.50295	-0.3742	-0.159	0.614499	0.189983	-0.43946	-0.69955	-0.6333	-0.20892	0.092044	-0.35055	-0.16714	-0.20558	-0.18054	-0.17944	0.062059
D			0.132757	-0.08213	-0.01115	0.550145	-0.05612	-0.17499	0.335985	0.157829	0.166447	0.299855	0.256594	-0.25007	0.065646	0.556838	0.553405	0.589997	0.676118
DM				0.059764	0.309463	0.042249	0.117371	-0.16456	0.614069	-0.14865	0.06427	0.078606	-0.37424	-0.41688	0.342301	-0.25241	0.285872	0.485524	0.449128
DL					0.868411	0.235671	-0.46751	0.209894	0.460657	0.636063	0.257293	0.508441	0.553196	0.66512	0.591725	0.204582	0.143362	0.219624	0.232871
DP						0.236122	-0.25974	-0.05979	0.661376	0.645853	0.302848	0.301054	0.578799	0.605127	0.815671	0.276842	0.196068	0.281426	0.237937
PM							0.164258	-0.03828	0.675915	0.524819	0.420111	0.792792	0.128066	0.018223	0.08166	0.623604	0.714134	0.743761	0.667289
HAD								0.323653	-0.03439	-0.42852	-0.4426	0.019347	-0.22522	-0.28046	-0.33869	-0.00062	0.234983	0.174528	0.189404
HAV									-0.25173	-0.23714	-0.5133	0.331701	0.02248	0.122352	-0.4989	-0.00656	0.247346	0.17697	0.313799
ATN										0.608627	0.546859	0.512541	0.051031	0.063484	0.501997	0.43788	0.668768	0.783298	0.618859
LH											0.553547	0.394773	0.525575	0.684846	0.45541	0.724761	0.434034	0.373229	0.212918
HV												0.078167	-0.10595	-0.07944	0.233106	0.320825	0.175459	0.240365	-0.038
PPP													0.145432	0.176537	0.080941	0.300152	0.568861	0.645294	0.706969
PPAL														0.788857	0.553945	0.500113	0.019648	-0.0526	0.085691
PPAM															0.444901	0.393811	-0.01337	-0.13438	-0.11167
VP																0.026946	-0.14851	-0.00996	0.009796
VS																	0.730899	0.577835	0.473013
VD																		0.956639	0.868541
VC																			0.930307
VV																			

oxtr$^{-/-}$ Alone_Control p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.468822	0.936009	0.167554	0.321129	0.682841	0.078271	0.624423	0.236597	0.035939	0.067105	0.589574	0.813809	0.355011	0.667332	0.595673	0.642051	0.644116	0.873979
D			0.73349	0.833614	0.977286	0.124857	0.885973	0.652493	0.376699	0.685072	0.668647	0.433079	0.505109	0.516364	0.866747	0.119393	0.122177	0.094458	0.045563
DM				0.878612	0.417736	0.91406	0.763623	0.67223	0.078539	0.702701	0.869521	0.840687	0.321073	0.264314	0.367219	0.512321	0.455844	0.185183	0.225226
DL					0.002387	0.541564	0.204457	0.587795	0.212071	0.065553	0.503908	0.162212	0.122348	0.050596	0.093255	0.597493	0.712901	0.570182	0.546517
DP						0.540768	0.499715	0.878555	0.052386	0.060239	0.428273	0.43115	0.102484	0.084241	0.007355	0.470812	0.613148	0.463187	0.537569
PM							0.67281	0.922115	0.045653	0.146858	0.260249	0.010816	0.742646	0.962888	0.834565	0.072729	0.030677	0.021594	0.049576
HAD								0.395536	0.930019	0.249825	0.232869	0.960599	0.560144	0.464786	0.372621	0.99873	0.54278	0.653357	0.625498
HAV									0.5135	0.538968	0.15756	0.383194	0.954223	0.753836	0.171569	0.986634	0.521099	0.648756	0.410894
ATN										0.08198	0.127593	0.158285	0.896266	0.871105	0.168495	0.238479	0.048889	0.012527	0.075586
LH											0.122061	0.293035	0.146172	0.041806	0.218004	0.027178	0.243105	0.322487	0.582302
HV												0.841569	0.786167	0.839021	0.546101	0.399917	0.651602	0.533299	0.922686
PPP													0.708901	0.649572	0.836006	0.432601	0.109943	0.060535	0.033195
PPAL														0.011505	0.121737	0.170358	0.959987	0.893089	0.826496
PPAM															0.230158	0.294319	0.972773	0.73033	0.774859
VP																0.94514	0.70297	0.979708	0.980046
VS																	0.025282	0.103194	0.198456
VD																		5.36E-05	0.002379
VC																			0.000275
VV																			

oxtr$^{+/+}$ Alone_AS correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.804136	0.182699	0.77999	0.432106	0.053326	0.060483	0.113	0.568775	-0.03205	0.247639	0.770135	0.368464	0.503329	0.363766	0.297364	0.581653	0.648088	0.813229
D			0.35264	0.918034	0.64273	0.240143	0.508367	0.619162	0.796153	0.349133	0.347903	0.78775	0.381464	0.270806	0.273179	-0.25607	0.642864	0.799715	0.916335
DM				0.574293	0.725821	0.478718	0.640482	0.656015	0.764584	0.70604	0.453823	0.625097	0.89671	0.761538	0.018775	-0.17262	0.500429	0.539455	0.383718
DL					0.726246	0.19395	0.468287	0.56191	0.815273	0.358669	0.32232	0.846009	0.572639	0.488437	0.096365	-0.23241	0.666462	0.788557	0.873464
DP						0.372893	0.803603	0.641648	0.850961	0.346059	0.107496	0.684994	0.529189	0.480297	-0.24453	-0.40715	0.420038	0.504518	0.495685
PM							0.617411	0.508327	0.434769	0.699349	0.590341	0.110309	0.481813	0.265701	0.348374	-0.06458	-0.00889	0.145769	0.043284
HAD								0.849075	0.790401	0.674472	0.37709	0.35136	0.454384	0.188953	-0.04015	-0.5764	0.170672	0.337295	0.289674
HAV									0.772984	0.822176	0.43038	0.539445	0.48622	0.164611	0.155537	-0.62157	0.54204	0.676532	0.557752
ATN										0.626076	0.54608	0.790067	0.749732	0.607406	0.244237	-0.20917	0.582091	0.718873	0.729813
LH											0.780277	0.314116	0.703294	0.342483	0.44647	-0.26164	0.337909	0.481863	0.330653
HV												0.228133	0.724351	0.480819	0.783197	0.270191	0.155142	0.325557	0.334105
PPP													0.616198	0.654739	0.209731	-0.03182	0.912882	0.924648	0.901231
PPAL														0.897126	0.377547	0.198424	0.473193	0.536753	0.452471
PPAM															0.292916	0.467428	0.477834	0.455643	0.40002
VP																0.547167	0.273352	0.360124	0.378893
VS																	-0.07688	-0.15666	-0.09586
VD																		0.962743	0.863086
VC																			0.941305
VV																			

oxtr$^{+/+}$ Alone_AS p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.016133	0.664987	0.022424	0.285003	0.900204	0.886871	0.789922	0.141227	0.939944	0.554311	0.02537	0.369114	0.203536	0.375719	0.474439	0.130416	0.082221	0.014092
D			0.391571	0.001293	0.085641	0.566744	0.198305	0.101652	0.018071	0.396626	0.398407	0.020261	0.351112	0.516518	0.512701	0.540437	0.085554	0.017189	0.001374
DM				0.136536	0.041513	0.23011	0.0871	0.07731	0.02713	0.050327	0.258697	0.097471	0.002546	0.028126	0.964805	0.682708	0.206579	0.167625	0.348033
DL					0.041335	0.64536	0.241882	0.147185	0.013656	0.382945	0.436203	0.008107	0.137933	0.219414	0.82043	0.579671	0.071106	0.020044	0.004597
DP						0.362935	0.016258	0.086342	0.007379	0.401082	0.799992	0.060846	0.177451	0.228355	0.559451	0.316768	0.30016	0.202295	0.211609
PM							0.102905	0.198346	0.28171	0.053542	0.123393	0.794843	0.226676	0.524762	0.397725	0.879254	0.983339	0.730531	0.918943
HAD								0.007651	0.019553	0.066555	0.357123	0.393413	0.258034	0.654056	0.924791	0.134767	0.686149	0.413901	0.48648
HAV									0.024495	0.012249	0.287148	0.167634	0.221831	0.696885	0.713038	0.099944	0.165198	0.065414	0.150861
ATN										0.09679	0.161443	0.019641	0.032201	0.110231	0.559941	0.61909	0.130057	0.044492	0.039858
LH											0.022341	0.448627	0.051632	0.406292	0.267462	0.531352	0.412998	0.22662	0.423732
HV												0.58686	0.042133	0.227776	0.021514	0.517508	0.713742	0.43134	0.418611
PPP													0.103777	0.078088	0.618134	0.940377	0.001547	0.00101	0.002234
PPAL														0.002516	0.356493	0.637606	0.236308	0.170181	0.260297
PPAM															0.48139	0.242864	0.231097	0.25655	0.326134
VP																0.160441	0.512425	0.380876	0.354639
VS																	0.85641	0.711042	0.821357
VD																		0.000126	0.005775
VC																			0.000484
VV																			

oxtr$^{-/-}$ Alone_AS correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.662863	0.466448	-0.14212	0.136061	0.199446	0.401324	0.886836	-0.67265	-0.47652	-0.74403	-0.13222	-0.08952	-0.39958	-0.23687	-0.55309	-0.07439	0.298273	0.472783
D			0.487534	0.061889	0.124449	0.377518	0.452911	0.543602	-0.67374	0.178771	-0.80717	0.414295	-0.29152	-0.18009	-0.40885	-0.59655	0.241618	0.48379	0.577353
DM				0.496685	0.867692	0.597013	0.861426	0.483636	-0.11334	0.039001	-0.32656	-0.46561	0.35075	0.120337	0.187476	-0.00516	0.814628	0.955428	0.825819
DL					0.710375	0.757007	0.601281	0.079537	0.427582	0.273201	0.429566	-0.34706	0.853492	0.840976	0.676715	0.521619	0.700609	0.523148	0.510744
DP						0.504922	0.903516	0.165942	0.348982	0.197549	0.148571	-0.59224	0.67828	0.4212	0.583814	0.490347	0.861038	0.804755	0.559809
PM							0.436703	0.538673	0.012878	0.071929	-0.00415	-0.21626	0.513788	0.388637	0.345988	-0.09347	0.665614	0.698139	0.856429
HAD								0.303822	0.1125	0.262308	-0.12564	-0.32833	0.514626	0.295083	0.431269	0.31539	0.736287	0.752071	0.536686
HAV									-0.54416	-0.53779	-0.55409	-0.25773	0.108737	-0.26867	-0.06329	-0.5607	0.055515	0.395344	0.674964
ATN										0.454104	0.923657	-0.22216	0.588619	0.401399	0.844623	0.877894	0.327757	-0.063	-0.34667
LH											0.234188	0.573024	-0.03565	0.17978	0.237804	0.342599	0.479329	0.189062	-0.12473
HV												-0.26807	0.627539	0.548447	0.770181	0.84134	0.101836	-0.28048	-0.41303
PPP													-0.64214	-0.28036	-0.42509	-0.36365	-0.28033	-0.33549	-0.31924
PPAL														0.745565	0.868914	0.70694	0.451609	0.275014	0.25397
PPAM															0.490588	0.627403	0.352659	0.11922	0.143507
VP																0.797677	0.429503	0.155202	-0.00665
VS																	0.296071	-0.0612	-0.35046
VD																		0.903307	0.665579
VC																			0.871388
VV																			

oxtr$^{-/-}$ Alone_AS p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.104631	0.291389	0.761163	0.771148	0.668104	0.372205	0.007778	0.097777	0.27966	0.055161	0.777501	0.848638	0.374477	0.609062	0.197812	0.874053	0.515857	0.283987
D			0.267087	0.895135	0.790361	0.403782	0.307484	0.207227	0.097031	0.701335	0.028184	0.355444	0.525866	0.699201	0.362442	0.157392	0.601685	0.271332	0.174685
DM				0.256837	0.011374	0.156985	0.012724	0.271506	0.808825	0.933841	0.474694	0.292372	0.44049	0.797185	0.687294	0.991244	0.025646	0.000786	0.022089
DL					0.073639	0.048801	0.153266	0.865401	0.338608	0.553313	0.336124	0.445649	0.014559	0.017745	0.095003	0.229851	0.079541	0.228242	0.241451
DP						0.24777	0.005269	0.72215	0.442958	0.671136	0.750553	0.1612	0.093946	0.346651	0.168765	0.263916	0.012811	0.029033	0.191265
PM							0.327252	0.212203	0.978139	0.878206	0.992962	0.641385	0.238178	0.388904	0.447148	0.842019	0.102681	0.081072	0.013863
HAD								0.507688	0.810221	0.569853	0.788387	0.472163	0.23728	0.520573	0.333997	0.490804	0.059156	0.05117	0.214226
HAV									0.206672	0.213104	0.196825	0.576843	0.816492	0.560178	0.892765	0.190401	0.905899	0.38004	0.096194
ATN										0.306051	0.002967	0.632082	0.164428	0.372107	0.016779	0.00936	0.472981	0.89326	0.446189
LH											0.613243	0.178708	0.939512	0.699704	0.607609	0.451909	0.27643	0.684744	0.789896
HV												0.56109	0.131373	0.202391	0.04278	0.017647	0.828013	0.542353	0.357066
PPP													0.119943	0.542536	0.34174	0.422652	0.542577	0.461959	0.485236
PPAL														0.054385	0.011121	0.075687	0.309053	0.550575	0.582616
PPAM															0.263645	0.131482	0.437831	0.799042	0.758876
VP																0.031608	0.336202	0.739683	0.988715
VS																	0.51911	0.896297	0.440898
VD																		0.005297	0.102706
VC																			0.010618
VV																			

oxtr$^{+/+}$ OBS + Shoal_Control correlation

	OB	D	DM	DL	Dp	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.917996	0.489421	0.254884	0.313679	0.558928	0.055599	0.087302	0.722541	0.042734	0.584031	0.396375	0.556595	0.715122	0.369972	0.332148	0.84753	0.042183	0.219779
D			0.40726	0.165739	0.120445	0.335436	-0.14574	-0.11854	0.668578	-0.22113	0.322693	0.259391	0.570566	0.598292	0.164816	0.241628	0.726461	-0.02466	0.252218
DM				0.730303	0.813793	0.70673	0.410931	0.687176	0.833309	0.495175	0.559673	0.335921	0.654972	0.702036	0.425907	0.805931	0.736011	0.688157	0.833418
DL					0.883186	0.855763	0.763021	0.708225	0.646871	0.826463	0.623283	0.702534	0.461821	0.374757	0.617096	0.841066	0.552375	0.248153	0.533022
Dp						0.88208	0.775123	0.882759	0.703111	0.836746	0.656959	0.547004	0.462469	0.518373	0.631219	0.892795	0.64072	0.436986	0.473367
PM							0.752831	0.737933	0.725732	0.795873	0.859233	0.766984	0.584561	0.616887	0.655017	0.721913	0.741756	0.234821	0.367493
HAD								0.835849	0.381158	0.841152	0.66348	0.429606	0.171049	0.15817	0.588203	0.631856	0.349802	0.235115	0.179167
HAV									0.49268	0.735418	0.595181	0.242428	0.366372	0.369643	0.375924	0.703671	0.408429	0.589082	0.406948
ATN										0.375589	0.677322	0.481438	0.774217	0.88605	0.491743	0.651087	0.954962	0.512089	0.635947
LH											0.676316	0.659895	0.125736	0.229079	0.710876	0.677014	0.375564	0.196245	0.265697
HV												0.61572	0.461251	0.68108	0.527207	0.407266	0.768605	0.331953	0.334894
PPP													0.473059	0.436314	0.608149	0.412566	0.51261	-0.19183	0.147314
PPAL														0.820098	0.178401	0.353794	0.661906	0.455578	0.580671
PPAM															0.30573	0.337883	0.889289	0.562618	0.540947
VP																0.695854	0.539293	0.039538	0.258107
VS																	0.565405	0.318623	0.529274
VD																		0.378499	0.477205
VC																			0.773422
VV																			

oxtr$^{+/+}$ OBS + Shoal_Control p-values

	OB	D	DM	DL	Dp	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.000179	0.151083	0.477273	0.377451	0.093034	0.878752	0.810475	0.018254	0.90669	0.076261	0.256796	0.094703	0.020086	0.292663	0.348417	0.001959	0.907889	0.541791
D			0.242757	0.647242	0.740316	0.34337	0.687873	0.744311	0.034553	0.539238	0.363135	0.469243	0.084991	0.067672	0.6491	0.501232	0.017334	0.946081	0.482052
DM				0.016465	0.004173	0.022307	0.238121	0.028127	0.002748	0.145609	0.092505	0.34263	0.039839	0.02362	0.219732	0.004874	0.01523	0.027813	0.002741
DL					0.000706	0.001585	0.010249	0.0219	0.043231	0.0032	0.054196	0.023479	0.179056	0.285975	0.05735	0.002294	0.097769	0.489375	0.112634
Dp						0.000732	0.008443	0.000716	0.023315	0.002539	0.039035	0.101762	0.178366	0.124778	0.050322	0.000507	0.045932	0.206666	0.167008
PM							0.011967	0.014828	0.017503	0.005889	0.001444	0.00963	0.07593	0.057459	0.03982	0.018404	0.014053	0.513728	0.296161
HAD								0.002593	0.277157	0.002289	0.036474	0.215318	0.636586	0.66253	0.073679	0.05002	0.321764	0.513186	0.620412
HAV									0.147968	0.015355	0.069489	0.499773	0.29775	0.293127	0.284356	0.023157	0.241275	0.073142	0.243154
ATN										0.284819	0.03142	0.158881	0.00857	0.000641	0.148859	0.041442	1.7E-05	0.130226	0.048104
LH											0.03177	0.037867	0.729261	0.524369	0.02119	0.031527	0.284854	0.586867	0.45811
HV												0.058068	0.179664	0.030135	0.117363	0.242749	0.009385	0.348718	0.3442
PPP													0.167323	0.207446	0.062121	0.236073	0.129769	0.595479	0.684652
PPAL														0.003666	0.621933	0.31589	0.037082	0.185779	0.078382
PPAM															0.3903	0.33964	0.000574	0.090433	0.106387
VP																0.02543	0.107672	0.913646	0.471524
VS																	0.0885	0.369564	0.115668
VD																		0.280802	0.163114
VC																			0.008682
VV																			

oxtr$^{-/-}$ OBS + Shoal_Control correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.568951	0.201472	-0.24569	0.499928	-0.03438	-0.12657	0.430668	-0.10493	0.043688	0.270648	-0.2945	-0.04899	0.183854	0.045166	0.160503	0.319367	0.487247	-0.22583
D			-0.19513	-0.08866	0.399506	-0.14412	-0.01344	0.403493	-0.33677	-0.1499	-0.1304	-0.05755	-0.20059	0.298574	-0.24799	-0.47104	0.513606	0.264205	-0.38086
DM				0.619621	0.579469	0.455607	-0.33952	-0.01955	-0.14967	0.490111	0.534444	0.464838	0.705082	0.506444	0.678844	0.484987	0.297599	0.380317	0.654001
DL					0.30414	0.212765	-0.25602	-0.39947	-0.43727	0.124951	0.010637	0.471328	0.567926	0.439429	0.411015	0.062468	0.160047	0.152304	0.869585
DP						-0.1076	-0.49651	0.319276	-0.49591	0.097609	0.342589	0.44305	0.172477	0.77481	0.091884	-0.11048	0.782174	0.581359	0.190079
PM							0.153829	0.196923	0.457386	0.482863	0.567871	0.18956	0.695501	0.101415	0.599733	0.624626	0.050126	0.014061	0.12842
HAD								-0.30609	0.740309	0.612503	0.305645	0.09139	0.294641	-0.10003	0.17827	0.130286	-0.60419	-0.41022	-0.43404
HAV									-0.20641	-0.16118	0.013979	0.096239	-0.34876	-0.11455	-0.45966	-0.26205	0.720959	0.693822	-0.30993
ATN										0.580741	0.608766	-0.13747	0.400639	-0.09108	0.447228	0.622643	-0.60301	-0.55715	-0.49804
LH											0.767787	0.544295	0.776878	0.36186	0.657112	0.444043	-0.20137	-0.04407	-0.00766
HV												0.322386	0.75277	0.589707	0.693355	0.697472	0.036583	-0.01655	-0.12966
PPP													0.445461	0.504147	0.105894	-0.22596	0.413446	0.370827	0.32423
PPAL														0.555116	0.896333	0.658254	-0.11735	-0.12667	0.365069
PPAM															0.448253	0.114176	0.458533	0.05638	0.096217
VP																0.806587	-0.28182	-0.33546	0.282118
VS																	-0.38797	-0.26698	0.131254
VD																		0.701925	0.067102
VC																			0.30665
VV																			

oxtr$^{-/-}$ OBS + Shoal_Control p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.109874	0.603196	0.523981	0.170542	0.930037	0.745577	0.247192	0.788191	0.91114	0.481196	0.441739	0.900408	0.635846	0.908144	0.679963	0.402183	0.183396	0.559046
D			0.614887	0.820562	0.286757	0.711442	0.97262	0.281523	0.375522	0.700279	0.738081	0.88308	0.604824	0.435143	0.519973	0.200593	0.157275	0.4921	0.311891
DM				0.075123	0.101992	0.21778	0.371377	0.960196	0.700733	0.180446	0.138249	0.207408	0.03388	0.164144	0.044367	0.185742	0.436717	0.312639	0.056031
DL					0.426206	0.582579	0.506094	0.286806	0.239208	0.74874	0.978332	0.200283	0.110661	0.236629	0.271784	0.873154	0.680834	0.695665	0.002316
DP						0.782896	0.173951	0.402324	0.17456	0.802725	0.366789	0.232336	0.657227	0.014202	0.814128	0.77721	0.01274	0.100614	0.624244
PM							0.692737	0.611571	0.215759	0.187963	0.110704	0.625208	0.037498	0.795162	0.087801	0.072123	0.898097	0.97136	0.741954
HAD								0.423097	0.022547	0.07952	0.423803	0.815113	0.441507	0.79792	0.646312	0.73831	0.084854	0.272811	0.243092
HAV									0.594156	0.678674	0.971527	0.805451	0.357638	0.769173	0.213193	0.495771	0.028398	0.038157	0.416997
ATN										0.101063	0.081891	0.724319	0.285265	0.815728	0.227436	0.073303	0.085627	0.11914	0.172422
LH											0.015696	0.129752	0.013781	0.338599	0.054474	0.231167	0.603385	0.91036	0.984393
HV												0.397496	0.019236	0.09466	0.038342	0.036734	0.925557	0.966298	0.73953
PPP													0.229502	0.166383	0.786281	0.558816	0.268676	0.325859	0.394646
PPAL														0.120785	0.001066	0.05391	0.763657	0.745369	0.334012
PPAM															0.226242	0.769915	0.214462	0.885447	0.805495
VP																0.008622	0.462528	0.377485	0.46204
VS																	0.302173	0.487389	0.736421
VD																		0.035046	0.863813
VC																			0.422203
VV																			

oxtr$^{+/+}$ OBS_AS + Shoal correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.564274	0.571708	0.620484	0.40104	0.542652	0.308071	0.238405	0.604234	0.366241	0.772584	0.077948	0.482452	0.35459	0.705607	0.702181	0.716211	0.464918	0.262789
D			0.435724	0.225846	0.210184	0.385793	-0.23589	-0.12124	-0.10767	-0.28322	0.415375	-0.04998	0.1915	0.112062	0.21933	0.398	0.189954	0.150163	0.079358
DM				0.835542	0.213449	0.457754	0.021974	-0.04086	0.312205	-0.13344	0.772318	0.245473	0.531134	0.212395	0.183511	0.66161	0.703215	0.418134	0.131009
DL					0.50272	0.38411	0.166883	-0.18891	0.690638	-0.1339	0.905228	0.319786	0.698417	0.571423	0.571339	0.792167	0.751034	0.573319	0.401987
DP						0.296113	0.171628	0.051527	0.385881	-0.1181	0.559186	0.288134	0.760737	0.919684	0.66421	0.752046	0.49411	0.381057	0.578853
PM							-0.38088	-0.14448	0.196006	0.402381	0.671252	0.589386	0.632527	0.406201	0.341855	0.73655	0.648811	0.729937	0.572566
HAD								0.598706	0.258678	0.268702	-0.02177	-0.61222	-0.05648	0.001862	0.350524	-0.08026	0.02265	-0.42525	-0.21045
HAV									-0.01003	0.529231	-0.1479	-0.35394	-0.19444	-0.24897	0.014595	-0.05831	0.188176	-0.35927	-0.5075
ATN										0.275266	0.678002	0.351772	0.458107	0.496714	0.708263	0.569806	0.682851	0.623246	0.344402
LH											0.049561	0.134635	0.057381	-0.09175	0.180442	0.087205	0.355527	0.234994	0.076131
HV												0.428572	0.728112	0.610474	0.696487	0.934145	0.842899	0.719123	0.467018
PPP													0.673124	0.522684	0.005861	0.556683	0.613789	0.893853	0.650558
PPAL														0.879872	0.451699	0.863821	0.749631	0.766894	0.839729
PPAM															0.64117	0.771088	0.525467	0.619462	0.828323
VP																0.657424	0.457977	0.368055	0.405727
VS																	0.870077	0.755823	0.592161
VD																		0.777279	0.397417
VC																			0.732243
VV																			

oxtr$^{+/+}$ OBS_AS + Shoal p-values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.113493	0.107774	0.0746	0.284737	0.131147	0.419944	0.536744	0.084824	0.332344	0.014665	0.842007	0.188395	0.349106	0.033688	0.034951	0.029971	0.207319	0.49451
D			0.241067	0.559021	0.587269	0.305137	0.541179	0.75602	0.782773	0.460222	0.266222	0.8984	0.621605	0.774085	0.570711	0.288747	0.624476	0.69978	0.83918
DM				0.005037	0.581338	0.215343	0.955252	0.916879	0.413404	0.732156	0.014721	0.524361	0.141176	0.58325	0.636487	0.052273	0.034566	0.262734	0.736898
DL					0.167784	0.307433	0.66782	0.626426	0.039427	0.731267	0.000786	0.401531	0.036372	0.10799	0.108054	0.010924	0.019677	0.106558	0.283493
DP						0.439121	0.658832	0.895261	0.305017	0.762185	0.117511	0.452125	0.017299	0.000447	0.051027	0.019419	0.176373	0.31162	0.102444
PM							0.311864	0.710743	0.613264	0.282977	0.047748	0.094885	0.067543	0.277996	0.367885	0.023614	0.058689	0.025573	0.107125
HAD								0.088489	0.501534	0.484479	0.955667	0.079699	0.885238	0.996206	0.35505	0.837371	0.953878	0.253852	0.586782
HAV									0.979575	0.142874	0.704142	0.350051	0.616156	0.51827	0.970273	0.881549	0.627784	0.34233	0.163118
ATN										0.473446	0.044734	0.353221	0.214943	0.17375	0.032731	0.10922	0.042646	0.072943	0.364091
LH											0.899241	0.729833	0.883425	0.814402	0.642235	0.82347	0.347743	0.542761	0.845655
HV												0.249756	0.026131	0.080802	0.037115	0.000226	0.004324	0.029	0.204999
PPP													0.046899	0.14881	0.98806	0.119518	0.078714	0.001155	0.057785
PPAL														0.001756	0.222255	0.002679	0.020037	0.015893	0.004622
PPAM															0.062746	0.014981	0.146269	0.075219	0.005811
VP																0.054319	0.21509	0.329771	0.278612
VS																	0.002287	0.018477	0.092952
VD																		0.0137	0.289518
VC																			0.024878
VV																			

oxtr$^{-/-}$ OBS_AS + Shoal correlation

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.624618	-0.14735	0.481318	0.299961	0.387989	-0.28812	-0.47616	-0.77014	-0.85169	-0.37456	0.108437	-0.38001	-0.15685	-0.79863	0.209047	0.709921	0.765747	0.43367
D			0.10143	0.495805	0.23519	0.164879	-0.25654	-0.4539	-0.43687	-0.45064	-0.17698	0.041547	-0.14438	0.127777	-0.62975	0.302624	0.493032	0.528708	0.312211
DM				0.672943	0.543467	0.430045	-0.12838	-0.43348	-0.18286	0.115954	-0.13129	0.129002	0.40153	0.528326	0.177168	0.739356	0.382729	0.449329	0.437571
DL					0.760003	0.699515	-0.21222	-0.63579	-0.39981	-0.24697	-0.05427	-0.02203	0.192491	0.270248	-0.13559	0.867962	0.848915	0.858497	0.807891
DP						0.647734	-0.53615	-0.60453	-0.38705	-0.31547	-0.15728	0.331857	0.543536	0.460892	-0.05507	0.637123	0.578814	0.66458	0.417895
PM							0.160538	-0.43165	-0.17574	-0.24554	0.318405	0.450502	0.260562	0.219421	0.100717	0.817135	0.389811	0.6217	0.462227
HAD								0.621798	0.623918	0.567074	0.764413	-0.22608	-0.39314	-0.51487	0.499978	0.031205	-0.41245	-0.27924	-0.14347
HAV									0.560104	0.5896	0.376719	-0.42343	-0.52442	-0.75857	0.340727	-0.64326	-0.60372	-0.51004	-0.66955
ATN										0.901925	0.835909	-0.26087	0.211064	-0.12568	0.871347	-0.11026	-0.65717	-0.72973	-0.26412
LH											0.657061	-0.46124	0.138335	-0.15154	0.862589	-0.0479	-0.49072	-0.58134	-0.17981
HV												-0.08872	0.13192	-0.1792	0.741551	0.236141	-0.40242	-0.34316	-0.02723
PPP													0.49665	0.603529	-0.12637	0.219292	-0.24811	0.032677	-0.2477
PPAL														0.86904	0.455763	0.439752	-0.1135	-0.17358	0.145157
PPAM															0.095921	0.494425	0.036555	-0.01021	0.24271
VP																0.187958	-0.46899	-0.52128	-0.0515
VS																	0.535429	0.582848	0.747139
VD																		0.888632	0.817477
VC																			0.600918
VV																			

oxtr$^{-/-}$ OBS_AS + Shoal p values

	OB	D	DM	DL	DP	PM	HAD	HAV	ATN	LH	HV	PPP	PPAL	PPAM	VP	VS	VD	VC	VV
OB		0.097804	0.727698	0.227224	0.4704	0.342232	0.488925	0.232973	0.025367	0.007275	0.360615	0.798269	0.35311	0.710694	0.017454	0.619307	0.048517	0.026755	0.283067
D			0.811119	0.211481	0.57501	0.696409	0.539683	0.258606	0.279126	0.26247	0.675027	0.92219	0.733018	0.763013	0.094263	0.466271	0.214449	0.177919	0.451533
DM				0.06741	0.163866	0.287565	0.761926	0.283305	0.664707	0.784527	0.756654	0.760792	0.324139	0.178291	0.674696	0.036065	0.349382	0.264038	0.278265
DL					0.028637	0.053461	0.613876	0.090191	0.32641	0.555412	0.898438	0.958701	0.647896	0.517417	0.748863	0.0052	0.007674	0.006353	0.015269
DP						0.082444	0.170753	0.112392	0.3435	0.44657	0.709924	0.421943	0.163802	0.250408	0.896955	0.089307	0.132758	0.072201	0.302892
PM							0.704124	0.285566	0.677206	0.557782	0.442114	0.262638	0.533108	0.601599	0.812428	0.013267	0.33977	0.099851	0.248858
HAD								0.099782	0.098293	0.14269	0.027185	0.590316	0.335298	0.191662	0.207054	0.941529	0.30988	0.503005	0.734671
HAV									0.148776	0.123983	0.357635	0.295858	0.182117	0.029118	0.40886	0.085297	0.113	0.196581	0.069331
ATN										0.002188	0.009731	0.532612	0.615851	0.766818	0.004823	0.794926	0.076609	0.039892	0.527318
LH											0.076675	0.250002	0.743912	0.72018	0.005836	0.910324	0.216944	0.130676	0.670049
HV												0.834515	0.755505	0.671129	0.035225	0.57342	0.32297	0.405302	0.948961
PPP													0.21058	0.113148	0.765558	0.60182	0.553539	0.938773	0.554211
PPAL														0.005078	0.256409	0.275599	0.789	0.681025	0.731629
PPAM															0.821249	0.212955	0.93152	0.98086	0.562475
VP																0.655791	0.241078	0.185228	0.903603
VS																	0.171442	0.129437	0.033141
VD																		0.003171	0.013197
VC																			0.115136
VV																			

Table 4.7 List of brain regions used for cell counting, their abbreviations and their mammalian homologue (Figure 4.8,4.9).

#	Brain regions	Abbreviations
1	Olfactory bulb	OB
2	Ventral nucleus of ventral telencephalic area (lateral septum)	Vv
3	Central nucleus of ventral telencephalic area (Nucleus accumbens/striatum)	Vc
4	Dorsal nucleus of ventral telencephalic area (Nucleus accumbens/striatum)	Vd
5	Supracommissural nucleus of ventral telencephalic area (medial extended amygdala and the bed nucleus of the stria terminalis)	Vs
6	Postcommissural nucleus of ventral telencephalic area (part of the basal amygdala)	Vp
7	Dorsal telencephalic area	D
8	Medial zone of dorsal telencephalic area (Medial Amygdala)	Dm
9	Lateral zone of dorsal telencephalic area (Hippocampus)	Dl
10	Posterior zone of dorsal telencephalic area (Piriform cortex)	Dp
11	Anterioromedial part of parvocellular preoptic nucleus (SON)	PPAM
12	Anteriorlateral part of parvocellular preoptic nucleus (SON)	PPAL
13	Magnocellular preoptic nucleus (PVN)	PM
14	Posterior part of parvocellular preoptic nucleus (SON)	PPP
15	Dorsal habenular nucleus (Medial habenula)	HAD
16	Ventral habenular nucleus (Lateral habenula)	HAV
17	Ventral zone of periventricular hypothalamus (Arcuate nucleus of the hypothalamus)	HV
18	Anterior tuberal nucleus (Ventromedial hypothalamus-VMH)	ATN
19	Lateral hypothalamic nucleus (Lateral hypothalamus)	LH

4.6. **References**

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5. Chapter 5

General discussion

5.1. **Overview of empirical findings**

The main focus of this thesis was to explore the process of social information use in threat perception using zebrafish as our model organism. To accomplish this, we first explored social information use when zebrafish is exposed to a threatening stimulus (the alarm substance, AS) in different social contexts based on whether the test fish has conflicting or congruent social information available compared to the information it acquired individually. Therefore, we examined social buffering of fear (where the test fish was exposed to AS in the presence of an unalarmed shoal), social contagion of fear (where the test fish was exposed to the sight of an alarmed shoal), and social facilitation of fear (where both the test fish and the shoal were exposed to AS) (Oliveira & Faustino, 2017). We investigated possible sex differences in these phenomena, and we showed that male test fish were more sensitive to conflicting social information than females, showing higher social buffering (they spend significantly less time freezing when exposed to AS in the presence of unalarmed conspecifics) and social contagion (they spend significantly more time freezing when they see alarmed conspecifics) responses. However, there was no sex difference in fear response when they received congruent threat information, showing similar social facilitation responses (both males and females significantly decreased freezing and increased erratic movement when both test fish and the shoal received AS). These results showed that in threatening situations, zebrafish use information from conspecifics to make decisions and that males use social information more than females.

Next, in our second experiment, we set out to determine the role of oxytocin (OXT) – this highly conserved nonapeptide implicated in the regulation of social behaviours (Grinevich & Stoop, 2018)– in social contagion of fear in zebrafish. Here, we used zebrafish lines with constitutive deletions of oxytocin ligand and receptors in the behavioural paradigm where wildtype (WT) or mutant (MUT) test fish were exposed to alarmed shoals. We showed that OXT ligand along with the two receptor mutants showed freezing deficits upon sight of alarmed

conspecifics. Subsequently, we performed a pharmacological ligand rescue where OXT i.p caused a reinstatement of freezing response upon sight of alarmed conspecifics highlighting the role of OXT in the regulation of social contagion of fear.

It is important to note here that both WT and MUT display alarmed responses when exposed to AS alone indicating that oxytocin modulates the social component of the response to threat stimulus and not the response to the threat stimulus itself. Following the behavioural tests of our second experiment, we then sort to understand the brain regions involved in the regulation of social contagion of fear and we showed that two main regions were shown to be primarily involved in the regulation of social contagion – the ventral telencephalic area (Vv) and the central nucleus of ventral telencephalic area (Vc). The loss of oxytocin receptor caused a change in the Social Decision-Making (SDM) network which are a set of nodes involved in the regulation of social behaviour (Teles, Almeida, Lopes, & Oliveira, 2015) indicating that oxytocin acts on the SDM network primarily through the Vv and Vc to regulate social contagion of fear and the loss of OXT triggers disruption of the SDM network connectivity. We confirmed the projection of oxytocin neurons to ventral telencephalon and we also confirmed the presence of oxytocin receptors in these brain areas, thus providing further evidence to support the action of oxytocin in the regulation of this behaviour.

Next, using zebrafish with constitutive deletion of oxytocin receptors (*oxtr*), we explored the role of oxytocin in social buffering of fear and we found that loss of *oxtr* caused a deficit in social buffering of fear such that *oxtr* mutant showed more freezing response than wildtype when exposed to AS in presence of shoal. We further analysed possible brain regions oxytocin acts on to modulate social buffering and found the Vd, and Vc as the areas that respond to social buffering, while the Vv, Dl, Ppal Ppp, and Had showed differences between WT and MUT neuronal activation. The analysis of brain coactivation pattern revealed changes in brain connectivity in response to the loss of oxytocin receptor during social buffering.

In conclusion, we show that zebrafish exhibit social contagion of fear, social buffering of fear and social facilitation of fear with sex differences and oxytocin modulates social contagion and buffering of fear.

In the following sections, we will discuss specific and general aspects of these results, present future perspectives for the continuing development of studying social information use in threat perception.

5.2. **Social information use in threat perception**

Group-living individuals have the opportunity to acquire information about the environment through their personal experience as well as through social information gathered from others by observation or interaction with others or the products of their behaviour (Bonnie & Earley, 2007; Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005; Danchin, Giraldeau, Valone, & Wagner, 2004; Heyes, 1994; Mathiron, Crane, & Ferrari, 2015; Mathis & Chivers, 2002; Rendell et al., 2010). In threat perception, the use of social information is of uttermost importance because it aids in the assessment of potential threats without being directly exposed to a predator (Dall et al., 2005; Oliveira & Faustino, 2017; Valone, 2007).

Given that survival largely depends on the appropriate behavioural and physiological responses to danger (Rupia, Binning, Roche, & Lu, 2016), copying others' decisions may be the optimal strategy when reacting to an uncertain environment (Kikusui, Winslow, & Mori, 2006; Kiyokawa, Shimozuru, Kikusui, Takeuchi, & Mori, 2006; Pérez-Escudero & De Polavieja, 2017).

Indeed, the presence and behaviour of conspecifics can serve as cues for the presence of a threat or act as an ameliorating stimulus in the environment (Morozov & Ito, 2019; Oliveira & Faustino, 2017).

Cues can be received via chemical, visual or vocal sensory modalities (Barcellos et al., 2014; Mathis & Chivers, 2002; Mathis, Chivers, & Smith, 1995; Ord & Stamps, 2008; Wiley, 2006). In teleosts including zebrafish, a chemical cue known as "*Shreckstoff*" or alarm substance (AS) is released upon skin injury; and when this cue is detected by conspecifics, they exhibit anti-predator

responses that involve moving with increased speed in a non-directional manner (also referred to as erratic movement), and then moving to the bottom of their environment while ceasing any form of locomotion activity except in the operculum (Magurran, Irving, & Henderson, 1996; Mathis & Chivers, 2002; Speedie & Gerlai, 2008; v. Frisch, 1942).

Despite the benefits of AS, one can consider that in the social environment there is the possibility of constant exposure to this cue. AS is possibly released in other conditions. For example, during fights, or during accidents that damage the skin when moving in a turbid environment. These forms of exposure to AS does not necessarily mean that a predator is present. This could occur by its involuntary release during agonistic interactions or movements in turbid environments (Chakravarty et al., 2013; Huang, Butler, & Lubin, 2019; Lopez-Luna, Al-Jubouri, Al-Nuaimy, & Sneddon, 2017).

Indeed, two important factors that influence group decision-making are information uncertainty and conflicting preferences (Conradt, 2012). Decisions made under perceptual uncertainty form the basis of the signals framework of decision making (Lynn, Wormwood, Barrett, & Quigley, 2015). In order to decide what fear response strategy to use, group-living animals need to first decide if there is a true threat in the environment. This assessment is necessary due to the presence of ambient noise. If individuals set a high threshold for threat detection, the detection of real threat will be difficult thereby increasing the propensity of having “misses”, although they will have few “false alarms”. On the other hand, if they set a low threshold for detecting a threat, they will miss fewer real threats at the cost of more frequent false alarms. Therefore, there is a trade-off between misses and false alarms (Beauchamp & Ruxton, 2007; Jones & Monfils, 2016; Oliveira & Faustino, 2017; Wiley, 2006). Hence, if one considers the relative costs of misses vs. false alarms to be fixed, then the optimal threshold will depend exclusively on the probability of a threat being present and when the relaxed behaviour of conspecifics present in the environment signals the absence of threat, there will be a shift in the detection threshold to a higher value; invariably, when the calm behaviour of the group

contradicts the threat detected by the individual, the alarm response of the observer is buffered.

Therefore social information use in threat perception as shown in our results can be regarded as an adaptive strategy used by individuals when they have conflicting cues regarding threat such that social buffering of fear is activated when the behaviour of conspecifics depict the environment is safe and social contagion of fear is activated when the behaviour of conspecifics depict that the environment is unsafe (because this does not require the direct detection of the threat, but the use of information conveyed by others, it is likely to decrease the risk of direct encounters with a predator). Interestingly, we found that males are more sensitive to social information than females when individually acquired information and social information cues are conflicting. Because males are more territorial than females, they might need to be more flexible to changes in the social environment. On the other hand, when individually acquired information and social information are congruent regarding threat both males and females model their behaviour equally, demonstrating that females require high levels of information certainty to more strongly change their behaviour.

It is important to note that observations from our study were carried out in test fish physically separated from the shoal although provided visual access to shoal. This limits our ability to study the dynamics of group behaviour. Future experiments can, therefore, incorporate the extensive study of collective behaviour of zebrafish in the study of social information use in threat perception and how the presence of oxytocin mutants can affect the dynamics of this social phenomenon.

Risk assessment is a core process in the choice of defence response to fear; Upon increasing levels of threat, animals activate different defensive modes, including freezing and active fight-or-flight reactions in mammals and erratic movement in teleosts (Hermans, Henckens, Roelofs, & Fernández, 2013). Freezing is related to a behavioural inhibition that is accompanied with a parasympathetically dominated heart rate deceleration and a halt in mobility, while flight reactions or erratic movement are associated with sympathetically

driven heart rate acceleration with the movement away from the source of danger in the case of flight, and a hap-hazard movement to create confusion for the predator in the case of erratic movement- otherwise known as “the confusion effect” (Blanchard, Griebel, Pobbe, & Blanchard, 2011; Herbert-Read et al., 2017; Hermans et al., 2013; Landeau & Terborgh, 1986; Rupia et al., 2016). Although freezing is often seen as a passive state of immobility, reports are now starting to show that freezing is an active defence strategy that involves sustained muscle tension likely related to postural control and it requires the sustained activity of several brain regions (Koutsikou et al., 2014; Zacarias, Namiki, Card, Vasconcelos, & Moita, 2018). Indeed, our results showed that zebrafish can adopt specific defence strategies depending on the status of the environment supporting the conserved nature of defence strategies across taxa. Additionally, our experiments showed the activation of freezing strategy upon direct detection of a threat or upon the sight of alarmed conspecifics, a behaviour that was absent in the absence of threat information, therefore supporting freezing as an active defence strategy instead of a passive, random state of immobility.

Our results also demonstrate the role of oxytocin in regulating freezing in social information use in threat perception such that upon sight of alarmed conspecifics oxytocin triggers the activation of a freezing response through a specialized functional activation pattern involving different brain regions.

Therefore, what our studies clarified is that individuals need to set a threshold for determining when to activate a defence response. When the unalarmed behaviour of conspecifics signals the absence of a threat it is therefore of adaptive significance to activate a buffering mechanism of fear, with a shift in the detection threshold of the observer to a higher value. When the alarmed behaviour of conspecifics signals the presence of a threat, there can be a shift in the detection threshold of the observer to a lower value thus triggering a fear response in the observer even when there is an absence of direct information signalling a threat (Oliveira & Faustino, 2017).

5.3. Neural mechanisms underlying social information use in threat perception

In this thesis, we explored the neuromolecular mechanisms underlying social information use in threat perception in the context of two phenomena that can be regarded as bidirectional; social contagion of fear and social buffering of fear. We found out that oxytocin was implicated in the two phenomena such that the loss of oxytocin ligand and receptors caused deficits in social contagion of fear (i.e. lack of freezing response upon sight of alarmed conspecifics), while the pharmacological recovery with oxytocin i.p caused a restoration of the phenotype. Similarly, the loss of oxytocin receptor caused deficits in social buffering of fear (i.e. heightened freezing when exposed to AS in the presence of unalarmed conspecifics). This means that in both conditions the test individuals are unable to perceive the information provided by their conspecifics regarding the threat situation in the environment, indicating that oxytocin has a role in the perception of social information. This finding is indeed in agreement with studies in other species. For example, in a study to determine the role of oxytocin in regulating the female mice (*Mus musculus*) use of olfactory social cues to determine their choice and responses to males, it was shown that oxytocin gene-deficient (knockout) females were impaired in their use of social cues to modulate their responses to either uninfected males of differing sexual states or infected males (Kavaliers et al., 2006). Also, another study showed that septal oxytocin system enhanced memory of social interactions in mice regardless of their valence, reducing fear after positive and enhancing fear after negative social encounters (Guzman YF, Tronson NC, Sato K, Mesic I, Guedea AL, Nishimori K, 2014).

Another interesting finding from our study is that even though oxytocin modulates social contagion and buffering of fear, it acts via different connection of nodes in the SDMN network to modulate the respective social behaviours. In social contagion of fear, the neuronal activation pattern of oxtr mutants differed from wild types in the Vv, Vc, Vd, Vp, Dm, Dp, PPAM, PPAL, and HAV, and in social buffering of fear, the neuronal activation pattern of oxtr mutants differed

from wild types in the Vv, Dl, PPAL, PPP and HAD. Indeed, these bidirectionally connected nodes have been implicated in the control of multiple forms of social behaviour (O'Connell & Hofmann, 2012) and oxytocin operates through both synaptic and cellular plasticity mechanisms in the different nodes to rewire brain circuitry to increase the neuronal representation of sensory stimuli. This increased sensory salience facilitates both the formation and maintenance of complex social behaviours (Pekarek, Hunt, & Arenkiel, 2020). We performed these neuromolecular studies only in males, but since males and females respond to social buffering and social contagion with different intensities, it would now be interesting to perfom similar neuromolecular studies in females. The neuronal activation pattern of oxtr mutants are expected to be the same, but the functional connectivity patterns, although generally similar, might reveal differences in strength of connectivity.

It would also be interesting to determine, both in males and females, the cellular identities in the various regions implicated in social buffering and social contagion of fear to further understand how oxytocin functions in these behaviours especially in the case of Vv.

Also relevant is the study of the effects of epigenetic modifications in oxytocin function. For example, a study showed that in humans, high levels of *oxtr* methylation were associated with greater amounts of activity in regions associated with face and emotion processing including the amygdala, fusiform, and insula (Puglia, Lillard, Morris, & Connelly, 2015).

5.4. **Final remarks**

In this thesis, we proposed that the use of social information in threat perception as a phenomenon that could occur in different contexts – conflicting and congruent information between the individual and the social group – with three different processes: social buffering of fear, social contagion of fear and social facilitation of fear. We were able to establish that these three processes occur in zebrafish with sex differences in their fear response strategies, the freezing and erratic movement behaviours.

Additionally, we demonstrated the conserved nature of the role of oxytocin in regulating social contagion and buffering of fear in zebrafish. We showed that oxytocin modulates these processes by causing changes in specific nodes of the SDM network, affecting the overall network connectivity pattern. These results are relevant to better our understanding of how oxytocin modulates fear responses in different social contexts. It now opens up new avenues for answering other important questions such as studies that now focus on oxytocin-cell identities and on the molecular and synaptic changes occurring at the node level during the oxytocin mediated response.

5.5. **References**

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Oxytocinergic modulation of social information use in threat perception

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