ISPA
INSTITUTO UNIVERSITÁRIO
CIÊNCIAS PSICOLÓGICAS, SOCIAIS E DA VIDA

SOCIAL BUFFERING OF FEAR IN ZEBRAFISH

Ana Isabel Soares Faustino

Dissertation presented to obtain the Ph.D. degree in
Behavioural Biology

2016

ISPA
INSTITUTO UNIVERSITÁRIO
CIÊNCIAS PSICOLÓGICAS, SOCIAIS E DA VIDA

SOCIAL BUFFERING OF FEAR IN ZEBRAFISH

Ana Isabel Soares Faustino

Dissertation supervised by Rui Filipe Nunes Pais de Oliveira, Ph.D. and Professor at ISPA – Instituto Universitário, principal investigator at Instituto Gulbenkian de Ciência and associate researcher at Champalimaud Neuroscience Programme

Dissertation presented to obtain the Ph.D. degree in Behavioural Biology

2016

2016

Dissertation presented to obtain the Ph.D. degree in Behavioural Biology, under the supervision of Rui Filipe Nunes Pais de Oliveira, Ph.D. and Professor at ISPA – Instituto Universitário, presented at ISPA – Instituto Universitário in the year of 2016.

This work was funded by a FCT research grant (EXCL/BIA-ANM/0549/2012 to Rui Filipe Nunes Pais de Oliveira).

Ana Isabel Soares Faustino was funded by an individual doctoral fellowship attributed by Fundação para a Ciência e a Tecnologia (SFRH/BD/79087/2011).

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

Acknowledgments

Doing a PhD was a process of tremendous professional and personal growth. For me, the very essence of this journey relies in the discovery of what it means to be a scientist. The next few words are dedicated to those that were by my side during this quest and in some way contributed to this revelation.

At first, I would like to thank Rui Oliveira for giving me the opportunity to learn about social behaviour, allowing me to be creative and explore my ideas. Also, thank you for teaching me the importance of being clear and confident when expressing my thoughts and for making me strive for more.

Secondly, I would like to thank my lab colleagues and friends. Specially, to David, Sara and Ana Félix for receiving me so well in the very beginning and for always caring for me. To Ana Isa for her smile and support. Thank you Tonia, the cute Greek friend with whom I shared my work and made my lab days brighter. I also want to thank my friend Gonçalo. Working with you was one of the most rewarding experiences of this PhD. You've helped me to be more autonomous and appreciate more the process of reading and writing.

I am deeply grateful to Banas, Sofia and Rute for their friendship and never failing encouragement. Also, thank you for understanding when I couldn´t be there sometimes.

My sincere gratitude goes to Instituto Gulbenkian de Ciência (IGC), my hosting institution, for the great scientific and working environment. Working surrounded by trees, flowers and wonderful persons was a privilege. Thanks to Maysa, Liliana, Monteiro, the "dream team" Tiago e Pedro, Sofia, Margarida and Sandra. I will miss you all.

I would like to thank the Champalimaud Neuroscience Program (CNP) and the persons that are part of it. The CNP was always a source of inspiration and possibility. Particularly I would like to thank Marta Moita and Susana Lima for the fruitful discussions. Susana, thank you for believing in me and for your incentive.

A big thank you goes to my family. To Dad for "planting" the ambition. Mum, thank you for your unconditional love and sense of justice. Mana, thanks for your funny way and optimism.

Thank you André, for running the last miles of the "marathon" with me. Your curiosity is motivating and working with you was a pure joy every day.

At last, I want to thank my colleague and friend Rodrigo. I will be forever grateful for your support. Thank you for giving me your hand and pushing me forward. I've grown the most since

then. Thank you for your mentoring, guidance, creativity, honesty, for the passionate brainstormings and all the fun we had doing them. Thank you for having my back, this thesis is also yours.

Palavras-chave

"Social buffering"; Medo; Stress; Peixe-zebra

Keywords

Social buffering; Fear; Stress; Zebrafish

PsycINFO Classification Categories and Codes

2400 Animal Experimental & Comparative Psychology

2440 Social & Instinctive Behaviour

2500 Physiological Psychology & Neuroscience

2510 Genetics

2540 Physiological Processes

RESUMO

A ubiquidade da formação de grupos em animais tem sido explicada, entre outros aspectos, pelos seus benefícios na proteção contra predadores, nomeadamente devido ao aumento global do estado de vigilância, diluição do risco e capacidade de confundir o predador. Assim, os grupos sociais proporcionam um ambiente mais seguro na presença de ameaças, onde a presença de conspecíficos diminui o medo desencadeado por eventos aversivos que surjam no ambiente, um fenómeno designado por "social buffering".

Este fenómeno social tem sido verificado em mamíferos, onde já existe alguma evidência sobre os mecanismos neurais envolvidos, mas o seu estudo em outros vertebrados ainda é escasso. Por esta razão, são necessários mais estudos comparativos para melhor compreender a evolução do "social buffering" entre animais sociais e quão evolutivamente conservados são os mecanismos subjacentes a este comportamento social.

Diferentes modalidades sensoriais (através de "cues") podem promover este efeito de "buffering" e a eficácia de cada canal sensorial neste fenómeno comportamental pode variar entre espécies. Além disso, do que é sabido, a eficácia ao longo do tempo de diferentes "cues" sensoriais, bem como a eficiência de grupos sociais que divirjam no seu número de elementos, nunca foi testada no contexto de "social buffering". Estas questões adquirem especial importância se considerarmos que exposições mais prolongadas a ameaças e grupos sociais maiores, influenciam as probabilidades de sobrevivência do indivíduo.

Dada a sua posição filogenética, os peixes teleósteos permitem explorar o "social buffering" e os mecanismos envolvidos neste comportamento social, na radiação evolutiva de maior sucesso entre os vertebrados (paralela à dos tetrápodes). O peixe-zebra é uma espécie pertencente aos teleósteos que apresenta comportamentos de medo quando submetido a situações ameaçadoras em contextos de isolamento social, no entanto a ocorrência de "social buffering" nesta espécie permanece praticamente inexplorada.

Nesta tese, investigámos a ocorrência de "social buffering" no peixe-zebra, submetendo peixes focais a um estímulo aversivo (substância de alarme) quer na presença ou ausência de "cues" sensoriais sociais. Numa primeira experiência, mostrámos que quando submetidos à substância de alarme na presença de "cues" sociais olfactivas (água do cardume) e visuais (visualização do cardume), os peixe-zebra apresentaram uma resposta de medo inferior do que quando submetidos à substância de alarme sozinhos (durante 30 minutos de exposição) revelando a ocorrência de "social buffering" continuado da resposta de medo. No entanto, análises ao cortisol ou RNA mensageiro da hormona libertadora de corticotrofina (CRF), dos receptores de glucocorticóides, e receptores de mineralocorticóides, não revelaram evidências de "buffering" da resposta de stress. Numa segunda experiência, testámos separadamente a eficácia de "cues" olfactivas e visuais no fenómeno de "social buffering", e verificámos que a visualização do cardume de peixes-zebra se revelou a "cue" mais eficaz na diminuição da resposta de medo provocada pela substância de alarme, quando num cenário de exposição constante à ameaça (30 minutos). Numa terceira experiência, demonstrámos que este "buffering" social não depende do número de conspecíficos presentes no cardume, uma vez que cardumes mais pequenos se revelaram igualmente eficazes em diminuir a resposta de medo provocada pela substância de alarme. Finalmente, usando a expressão génica de um "immediate early gene" (*c-fos*) como indicador de atividade neuronal, verificámos que o fenómeno de "social buffering" em peixe-zebra desencadeia um padrão específico de co-activação de áreas cerebrais conhecidas pelo seu envolvimento em respostas de medo e "buffering" em mamíferos.

Concluindo, o conjunto de estudos apresentados nesta tese sugerem uma origem evolutiva comum nos vertebrados para o comportamento de "social buffering", providenciando novas perspectivas sobre os mecanismos comportamentais e neurais envolvidos neste comportamento social.

ABSTRACT

The ubiquity of group formation among animals has been explained among several aspects by its anti-predatory benefits, including an overall increase in vigilance, the dilution of risk and predator confusion. Thus, social groups offer a safer environment in the presence of threats, and the presence of conspecifics is known to decrease the fear response to a detected threatening event, a phenomenon named social buffering.

This social phenomenon has been documented in mammals, where there is already some evidence about its neural mechanisms, but its study in other vertebrate taxa is still scarce. Thus, more comparative studies are needed to better understand the evolution of social buffering among social animals and how evolutionary conserved are its underlying mechanisms. For instance, different sensory modalities can convey relevant conspecific social cues used in buffering and the efficiency of each sensory channel may vary across species. Furthermore, to our knowledge, the effectiveness of these sensory cues over time and different conspecific group sizes has never been tested in the context of social buffering, which is of great relevance considering that long exposures to threat and bigger group sizes may influence individual chances of survival.

Given their phylogenetic position, teleost fish offer the possibility to investigate the occurrence of social buffering and its underlying mechanisms in the most successful evolutionary radiation among vertebrates, parallel to that of tetrapods. Zebrafish is a teleost species that expresses fear behaviour when individually exposed to threatening situations, however the occurrence of social buffering in this species has been virtually unexplored.

In this thesis we investigated social buffering in zebrafish, by exposing focal fish to an aversive stimulus (alarm substance – AS) either in the presence or absence of conspecific cues. In a first experiment, we showed that when exposed to AS in the presence of both olfactory (shoal water) and visual (sight of shoal) conspecific cues, zebrafish exhibited a lower fear response over the 30 min test than when tested alone, indicating sustained social buffering of fear. Nonetheless, analysis of cortisol or mRNA expression of corticotropin-releasing factor, glucocorticoid receptors and mineralocorticoid receptors did not reveal buffering of the stress response. In a second experiment, we separately tested olfactory and visual cues effectiveness on the AS-elicited fear response, and verified that the sight of shoal was more effective in reducing fear responses in a persistent threat scenario (30 min test). In a third experiment, we found that this effect was independent of conspecific number, as smaller shoals were equally efficient as larger shoals at inducing social buffering. Finally, by using the expression of an immediate early gene (*c-fos*) as a reporter of neuronal activity, we showed that social buffering elicits a distinct pattern of functional connectivity among a set of brain regions known to be involved in fear-like responses and buffering processes in mammals.

To conclude, the set of studies on this thesis suggests a shared evolutionary origin for social buffering in vertebrates, bringing new insights into the behavioural and neural mechanisms of this phenomenon.

Author Contributions

Ana Isabel Faustino (A.I.F.) and Rui Filipe Oliveira (R.F.O.) designed the experiments and established the behavioural protocols. A.I.F. validated the xyz2b software, performed all behavioural experiments, quantified cortisol and executed the data analysis. André Tacão-Monteiro (A.T.M.) processed all brain samples, quantified and analysed gene expression.

The studies presented in chapters 2, 3 and 4 will be part of a paper in preparation to be submitted. A.I.F. and R.F.O. wrote the paper with contributions from A.T.M.

Table index

Figure index

Contents

Chapter 1 |
General introduction

"We (…) are social beings. We come into the world as the result of others' actions. We survive here in dependence on others. Whether we like it or not, there is hardly a moment of our lives when we do not benefit from others' activities."

Tenzin Gyatso

It has been empirically shown that some humans thrive, whereas others surrender when exposed to threatening or challenging situations throughout life. Social support, defined as information leading the subject to believe that he (she) is cared for and loved, esteemed, and a member of a network of mutual obligations (see Hostinar, Sullivan, & Gunnar, 2014 for a review) as been identified as an important modulator of these discrepancies in human behaviour. Although social support has been extensively studied in humans, other social species also exhibit phenomena in which individuals rely on social information to adjust their behaviour. For instance, individuals lessen their fear response by relying on social information of "safety"/"calmness". This dampened fear response to aversive events when in the presence of conspecifics has been termed social buffering (Davitz & Mason, 1955; Fuzzo et al., 2015; Kiyokawa, Honda, Takeuchi, & Mori, 2014).

In the context of this thesis, social buffering will be investigated in zebrafish (*Danio rerio*), through the dissection of the behavioural, endocrine, sensory and neural mechanisms underlying it. Given their phylogenetic position, teleost fish offer the possibility to investigate the occurrence of the buffering process in the most successful evolutionary radiation among vertebrates, parallel to that of tetrapods (Amores, 1998). Zebrafish are a teleost species that offers a unique opportunity for such studies, not only because of their conspicuous social (Abril-de-abreu, Cruz, & Oliveira, 2015; Oliveira et al., 2016) and fear behaviours (Jesuthasan & Mathuru, 2008; Speedie & Gerlai, 2008), but also due to the great amount of neurogenetic and neuroimaging tools already available, enabling the study of the neurobiological basis of their behaviour both in adult and larval stages (Agetsuma et al., 2010; De Marco, Groneberg, Yeh, Castillo Ramírez, & Ryu, 2013; Jetti, Vendrell-Llopis, & Yaksi, 2014; Teles, Almeida, & Oliveira, 2015; Wyart et al., 2009).

1.1 Thesis scope

Communication among social animals is an essential process that not only allows the expression of personal information, but also enables acquiring public information from environmental threats (Bourdeau, 2010; Brown & Bongiorno, 2006; Hoare, Couzin, Godin, & Krause, 2004; Manassa, Dixson, McCormick, & Chivers, 2013; M. Smith & Belk, 2001). As a defence mechanism, social individuals tend to organize themselves in large groups, where protection from

probable dangerous events (e.g. predator attacks) is greater and sensory information about incoming threats can be easily disseminated (Hoare et al., 2004; Krause & Ruxton, 2002; Magurran & Pitcher, 1987). Therefore, living in groups enables individuals to more accurately assess potential environmental threats by using social information and to adjust their behaviour accordingly. Thus, the evolution of phenomena like social buffering of fear is expected in social animals.

In the context of social buffering, individuals use the presence of conspecifics to decrease their fear response to a detected imminent threat (Edgar et al., 2015; Fuzzo et al., 2015; Kiyokawa et al., 2014). The majority of studies addressing social buffering have been conducted in mammals, where the neural mechanisms underlying this phenomenon are still poorly understood (Fuzzo et al., 2015; Kiyokawa et al., 2014; A. S. Smith & Wang, 2014). The investigation of this behavioural process and underlying mechanisms in zebrafish will allow the demonstration of social buffering in fish for the first time (but see Ziv et al., 2013), providing insight into the evolution of this social behaviour. Moreover, different sensory modalities can convey relevant social cues (da Costa, Leigh, Man, & Kendrick, 2004; Rukstalis & French, 2005; Takahashi et al., 2013) and the effectiveness of each sensory channel in a persistent threat scenario have not yet been investigated in the context of social buffering. Furthermore, in other species, group size has been shown to modulate responses to threatening events (Brown & Bongiorno, 2006; Magurran & Pitcher, 1987), therefore it is relevant to explore if shoal size also mediates the buffering effect in zebrafish. Thus, the validation of social buffering in this model organism, together with the development of new paradigms that enable sensory and group size manipulations will be of great relevance for the understanding of the behavioural, endocrine, sensory and neural mechanisms underlying social buffering.

1.2 Chapter summary

The work presented in this thesis focused on investigating social buffering of fear using zebrafish as an experimental model and aimed to explore the behavioural, endocrine, sensory and neural mechanisms underlying it. Accordingly, this chapter will review:

- The conceptual framework for the study of social buffering, emphasizing the current behavioural findings and the influence of social context for the buffering phenomenon.
- Effects of social buffering at the neuroendocrine level, specifically addressing: the stress response and the Hypothalamic-Pituitary-Adrenal (HPA) axis; HPA axis regulation; and research targeting social buffering of the stress response.

- The main studies conducted to date investigating the sensory cues responsible for social buffering and studies supporting shoal size modulation of the buffering phenomenon.
- The main research to date on the neural mechanisms underlying social buffering.
- The state of the art of zebrafish studies regarding social and fear behaviours, as well as: this species ability to distinguish sensory cues of fear; response to stress (particularly acute stressors); the development of automated video-tracking tools to measure fear behaviour in zebrafish adults; and lastly, an overview of the neural tools available for assessing the neural circuits underlying behaviour in this model organism.

1.3 Social Buffering: behavioural evidence and the influence of social context

1.3.1 Behavioural evidence

Group formation among animals has been explained by its anti-predatory benefits, including an overall increase in vigilance ("many-eyes" hypothesis, Krause & Ruxton,. 2002; Roberts, 1996), the dilution of risk ("selfish herd" hypothesis, Krause & Ruxton, 2002; Viscido & Wethey, 2002) and predator confusion (Krause & Ruxton, 2002; Olson, Hintze, Dyer, Knoester, & Adami, 2013). Additionally, group living also offers the possibility for animals to use social information to assess potential threats in the environment, promoting better adaptation to dynamic contexts.

Hence, the evolution of both social transmission and social buffering of fear are expected in social animals. In the case of social transmission of fear, animals can use social signals (e.g. alarm calls in mammals and birds; see Hollén & Radford, 2009) or fear cues (e.g. cessation of movement in rats; see Pereira, Cruz, Lima, & Moita, 2012) to infer the presence of a threat in the environment, even when they have not detected it directly. On the other hand, in the case of social buffering of fear, animals can use social information of conspecifics to diminish their fear response to a threat that they have detected themselves (Edgar et al., 2015; Kiyokawa et al., 2014; A. S. Smith & Wang, 2014).

Social buffering was reported in a seminal study from Davitz & Mason (1955), which showed that rats exhibited lower fear responses when in the presence of non-fearful conspecifics to an aversive stimulus. In this experiment, the authors used a fear conditioning paradigm by establishing an association between a conditioned stimulus (CS - blinking light) and an unconditioned stimulus (US - electrical shock). Contrary to the CS, the US individually elicited a fear response - freezing – defined as the absence of movement, except for respiration (Buccafusco, 2000; Pereira et al., 2012). After the association was learnt (fear conditioning), subject rats responded with freezing behaviour to the CS itself. In this study, the fear conditioned

rats displayed decreased mean locomotor activity (the authors used this measure as a readout for the fear response and assessed it by quantifying the mean number of spaces traversed by the subject rats) when exposed alone to the conditioned stimulus. However, the presence of a non-fearful conspecific increased subject rats mean locomotor activity, demonstrating that the presence of conspecifics lowers the fear response in rats - social buffering (Davitz & Mason, 1955). More recently, other studies have corroborated these findings and added knowledge regarding the social buffering phenomenon in several species, namely humans (Thorsteinsson, James, & Gregg, 1998), non-human primates (Levine, Coe, Smotherman, & Kaplan, 1978), rats (Fuzzo et al., 2015; Kiyokawa, Kikusui, Takeuchi, & Mori, 2004), guinea pigs (Hennessy, Maken, & C. Graves, 2000) and birds (Edgar et al., 2015).

Nevertheless, apart from triggering fear behaviour, threatening events may also evoke a stress response (Livia Terranova, Cirulli, & Laviola, 1999; A. S. Smith & Wang, 2014). Thus, it is important to note that several studies addressing social buffering have been using other paradigms besides fear conditioning, particularly experimental designs established to induce stress activation, rather than explicit fear responses (Livia Terranova et al., 1999; Wilson, 2000). For example, Terranova and colleagues found that when in the presence of conspecifics, rats presented lower levels of corticosterone (stress hormone) when exposed to a novel cage, than when animals were placed alone in the novel environment (Livia Terranova et al., 1999). Accordingly, another study showed that acute exposure to an open field (novel environment used as a stress inducing stimulus) induced lower levels of prolactin (hormone released upon stress exposure) when rats were exposed to the open field with a same-sex conspecific, comparatively to when rats were alone (Wilson, 2000).

Importantly, the majority of behavioural paradigms assessing social buffering, both in the case of fear conditioning and novel environment contexts, use a "pair-exposure" context, where an individual is exposed to an aversive stimulus in the presence of a conspecific (Fuzzo et al., 2015; Livia Terranova et al., 1999). Thus, it is relevant to note that studies where animals are exposed in social isolation to an aversive event, but were previously "pair-housed" with a conspecific (Kiyokawa, Takeuchi, & Mori, 2007) do not reflect the social buffering phenomenon as defined in the literature - when conspecific individuals are present, individuals present a better recovery from aversive situations (see Kikusui, Winslow, & Mori, 2006 for a review).

As mentioned above, the presence of conspecifics alleviates fear/stress responses to threatening events and this is called social buffering. Yet, it seems likely that certain social context specificities (e.g. conspecifics' familiarity) may influence the buffering phenomenon. This subject will be further discussed in the section below.

1.3.2 Influence of social context

The presence of a conspecific can have different behavioural outcomes depending on the context in which the dyad's encounter occurs. For example, male rats exhibit aggressive behaviour against other males in situations of dominant-subordinate relationships and territorial intrusions (Adams, 1971; Blanchard & Blanchard, 1989). However, if a rat is paired together with another conspecific in an aversive situation, the presence of a second party may have a threat mitigating effect (Davitz & Mason, 1955; Fuzzo et al., 2015). Therefore, rats exhibiting social buffering rather than aggressive or territorial behaviours towards male conspecifics, when in a threatening environment, show the importance of the social context in which these encounters take place (Kiyokawa et al., 2014; Winslow, Noble, Lyons, Sterk, & Insel, 2003).

For instance, recent studies have shown that the buffering phenomenon is modulated by familiarity. That is, a familiar conspecific is more effective at inducing social buffering than an unfamiliar one (Hennessy, Zate, & Maken, 2008; Kiyokawa et al., 2014)[1]. To test the influence of familiarity in decreasing the subject's aversive responses to a given threatening stimulus, Kiyokawa and colleagues used a fear conditioning paradigm to show that the odour of a familiar rat was more efficient at decreasing fear responses than an unfamiliar scent (Kiyokawa et al., 2014). They did so by odourising the experimental cages either with familiar or unfamiliar odour and verified that the scent of familiar rats lowered freezing responses[2]. The study of Hennessy and colleagues corroborates the influence of familiarity in social buffering, this time using guinea pigs (*Cavia porcellus*) as an experimental animal. The authors verifyed that familiar males were more effective at decreasing stress responses - lower cortisol (stress hormone) - than unfamiliar males, after just one hour of being exposed to a novel environment (Hennessy et al., 2008).

However, other studies have shown conflicting results to the ones mentioned above, reporting no differences in the social buffering effect elicited by familiar and unfamiliar individuals (Cirulli, Terranova, & Laviola, 1996; Livia Terranova et al., 1999), or even that an unfamiliar individual was more efficient in decreasing stress responses than a familiar conspecific (Armario, Ortiz, & Balasch, 1983). Since the dynamics of social interactions are so diverse,

---

[1] It is worth mentioning that in the studies cited, familiarity between conspecifics was established by housing subject individuals with their companions some weeks prior to the social buffering test (e.g. 2 weeks, Hennessy et al., 2008; 3 weeks, Kiyokawa et al., 2014). Therefore, it is relevant to add that in the specific case of these studies, familiarity does not necessarily imply kin relationships among subjects and their partners. This is often the case in the research addressing the influence of familiarity in the social buffering phenomenon, thus the influence of kinship in the buffering process should be properly explored in the future.

[2] This study also raises the relevance of specific sensory conspecific cues as contributors to the social buffering phenomenon. This topic will be further discussed in section 1.5.

several factors may have influenced these results, namely the individuals' age, type of stressor used, experimental context and rearing conditions (see Kikusui et al., 2006 for a review).

The rearing conditions also appear to be a significant social factor influencing the buffering phenomenon (Winslow et al., 2003). Winslow and colleagues showed that mother-reared individuals showed lower stress responses than nursery-reared ones, when exposed to a novel environment in the presence of a familiar companion (Winslow et al., 2003). In this experiment, rhesus monkeys (*Macaca mulatta*) that were either mother-reared (kept with the mother until 1 year of age) or nursery-reared (separated from the mother 48 hours after birth and reared in a nursery until 1 year of age), were exposed to a stressor (30 min novel cage test) together with a familiar conspecific or alone. Results showed that monkeys that were mother-reared showed lower cortisol responses than those that were nursery-reared (Winslow et al., 2003).

Altogether, these results emphasize the influence of social context in the social buffering process. Moreover, as described in the previous sections, aversive events often activate a stress response that seems to be lessened by the social buffering effect. Hence, a detailed review of the literature on social buffering of the stress response will be further adressed in the section below.

1.4 Social Buffering – Neuroendocrine response

1.4.1 Stress response and the HPA axis

Studies investigating the social buffering effects on stress-endocrine activity have focused on the HPA axis (Kiyokawa et al., 2004; Levine et al., 1978; A. S. Smith & Wang, 2014). Stress has been defined as a perturbation of an organism's physiological and/or behavioural homeostasis as a result of exposure to certain events or situations (named stressors) (Novak, Hamel, Kelly, Dettmer, & Meyer, 2013; Selye, 1975). These perturbations can occur in response to rewarding events - called eustress; however most stress research have been focusing on disturbances resulting from aversive events – called distress (Novak et al., 2013; Selye, 1975). In the context of this thesis, aversive events that induce a distress response are referred as stressors, thus evoking a stress response.

When threatened by a stressor, animals' sympathetic nervous system is activated by neurons coming from the paraventricular nucleus (PVN) of the hypothalamus, inducing a "fight-or-flight" response (Galhardo & Oliveira, 2009). Besides the sympathetic activation, other structures contained both in the central nervous system and in peripheral tissues are involved in the stress response, namely the PVN, the anterior lobe of the pituitary gland, and the adrenal

gland. These structures and the intercommunications between them have been named the Hypothalamic-Pituitary-Adrenal (HPA[3]) axis (Hostinar et al., 2014; Kikusui et al., 2006; S. M. Smith & Vale, 2006). The activation of the HPA axis ultimately results in the production of glucocorticoids (GCs) - namely cortisol in humans and corticosterone in rodents (Hostinar et al., 2014) - known as the stress hormones that are released in response to an aversive event. Based on rodent research that has been confirmed in human and non-human primate investigation, the HPA axis mechanisms and function have been identified. Neurons in the parvocellular region of the PVN secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), that are released into the blood flow targeting the anterior pituitary and cause the release of adrenocorticotropic hormone (ACTH). ACTH will consequently bind to its receptors in the cortex of the adrenal glands, leading to the release of the glucocorticoids. These hormones circulate in the blood flow, binding to receptors distributed throughout the brain and the body, and under normal conditions exert negative feedback at several levels of the HPA axis, so that the axis can cease its own release when glucocorticoid levels are high (Hostinar et al., 2014; S. M. Smith & Vale, 2006). There is evidence suggesting that this negative feedback involves glucocorticoid receptors in areas not included in the HPA, particularly the amygdala, the bed nucleus of stria terminalis (BNST), the hippocampus and the prefrontal cortex (PFC) (Hostinar et al., 2014; Oitzl, Champagne, van der Veen, & de Kloet, 2010). Their role will be discussed in more detail below. Production of glucocorticoids is not only induced in response to stressors, as these hormones have numerous effects on the body (e.g. mobilization of energy to muscles), and its release in pulses (following a circadian clock) is required to ensure basal levels throughout the day for energy and organism optimal functioning (Fries, Dettenborn, & Kirschbaum, 2009; Hostinar et al., 2014; Sapolsky, Romero, & Munck, 2000).

Glucocorticoids differentially bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) when released in response to a stressful event comparatively to their basal circadian release. Glucocorticoids have 10 times higher affinity to MRs and act on them first, before occupying GRs (Hostinar et al., 2014; Oitzl et al., 2010). Therefore, in basal circumstances MRs are greatly saturated comparatively to GRs, which only start being occupied when stressors elevate glucocorticoids concentrations above basal levels. Accumulating evidence suggests that the balance between MR to GR is critical for an effective regulation of the stress response (Hostinar et al., 2014; Oitzl et al., 2010).

[3] HPI (Hypothalamic-Pituitary-Interrenal) axis is the homologous system in zebrafish. A brief description of the HPI axis and its specific features is available in section 1.7 (see also Félix et al., 2013; Pavlidis et al., 2013; Ramsay et al., 2009).

1.4.2 Regulation of the HPA axis

As mentioned previously the HPA axis is not an isolated system, as different brain regions regulate its activation. Limbic and cortical regions (e.g. amygdala, BNST, hippocampus, PFC) can activate or terminate stress responses in response to threatening events (Hostinar et al., 2014). This section will focus on the role of amygdala and BNST in the regulation of the stress response, given the well established role of these brain structures in fear and anxiety responses and its importance for the work presented in this thesis (Tovote, Fadok, & Lüthi, 2015).

The behavioural paradigm used in the context of this thesis (see chapter 2.1) does not specifically address fear learning and extinction, which involve the basolateral amygdala – BLA (one of the two main sub-areas of the amygdala, Tovote et al., 2015); or anxiety behaviours, which involve the BNST (Tovote et al., 2015). However, it seemed of great relevance to study the influence of these nuclei also in the social buffering phenomenon, given their importance in the regulation of the stress response and relevance for defense behaviours and survival (Hostinar et al., 2014; Tovote et al., 2015). Both BLA and the medial amygdala are known to activate the HPA axis in response to stressful events (Ulrich-Lai & Herman, 2009). Besides influencing the HPA axis, the amygdala is itself influenced by glucocorticoid release: for instance, stress-induced glucocorticoid release, enables amygdala activation and facilitates fear learning in infant rats (Moriceau, Roth, & Sullivan, 2010), and modulates fear learning in adults (Joels, Fernandez, & Roozendaal, 2011; Luksys & Sandi, 2011). Of similar importance is the involvement of the BNST in the HPA axis modulation, yet different subdivisions of the BNST seem to have contradictory effects -– antero-ventral nuclei seem to be have excitatory influence on the axis, while posterior regions appear to have inhibitory control (Choi et al., 2007).

1.4.3 Social buffering of the stress response

Stressful life events result in stress-associated dysfunctions both in humans (e.g. depression, Kendler, Karkowski, & Prescott, 1999; Pine, Cohen, Johnson, & Brook, 2002; posttraumatic stress disorder - PTSD, Rauch et al., 2000) and non-human animals (e.g. anxiety and depression-like behaviours, Chiba et al., 2012; chronic stress, Piato et al., 2011). The presence of conspecifics may attenuate the consequences of negative life events (Dalgard et al., 2006; A. S. Smith & Wang, 2014). Social buffering can thus be seen as a social therapeutic treatment for negative/stressful episodes, therefore the understanding of its mechanisms, particularly those related to stress response, is of great interest. Besides its mitigating effects on fear responses, the buffering phenomenon is also responsible for the diminishment of the stress response in several studies at the behavioural, endocrine and neural level (Edgar et al., 2015; Levine et al., 1978; A. S. Smith &

Wang, 2014). This was primarily shown in a seminal study by Levine and co-workers, which verified that squirrel monkeys (*Saimiri sciureus*) exhibited a lower stress response when reunited in mother-infant dyads following a stressful separation between both, comparatively to when both mother and infant where left alone after separation (Levine et al., 1978). In this experiment, mother and infant dyads that were housed together were submitted to three different treatments: baseline treatment, where mother and infant squirrels were separated and immediately sampled for plasma cortisol; separation treatment, in which mother and infant squirrels where separated and isolated for 30 min in individual cages and sampled for plasma cortisol; and finally, the reunion post-separation treatment, where mother and infants were separated for 30 min in individual cages and then reunited together and sampled 30 min after reunion for plasma cortisol (Levine et al., 1978). The bonding between mother and infant primates have been studied extensively and a mother-infant separation is known to be a stressful event (Broad, Curley, & Keverne, 2006; Levine et al., 1978). The results from this experiment have shown that 30 min after mother-infant reunion, plasma cortisol values were lower comparatively to the treatment where mothers and infants were kept isolated and higher than the treatment were mothers and infants were not exposed to the stressful separation (Levine et al., 1978). These results suggest that the presence of a conspecific (in this case, mother-infant dyad) has a diminishing effect on the stress response.

Confirming the findings of the previous study, Smith and Wang found that female prairie voles (*Microtus ochrogaster*) exhibited lower levels of corticosterone in response to an aversive stimulus when allowed to recover from the threatening event in the presence of a male partner, showing that social buffering influences endocrine responses. In this experiment, female prairie voles were exposed to a stressful event (1 hour immobilization) and allowed to recover either alone or in the presence of a male partner. Results showed that female prairie voles allowed to recover with a male partner presented less anxiety-like behaviours and lower corticosterone levels, contrary to the female prairie voles recovering alone (Smith & Wang, 2014). Interestingly, it was verified that males' social behaviours (approaching, sniffing and grooming) towards females increased during the stress-recovery period (comparatively to pre-exposure to immobilization), suggesting that males' affiliative social displays contributed to the buffering phenomenon (Smith & Wang, 2014).

Other recent studies have shown that social buffering also influences the stress response in birds, by demonstrating diminishment of stress behaviours of chicks in the presence of their mother (Edgar et al., 2015). In this experiment, the authors showed for the first time the presence of social buffering in domestic hens (*Gallus gallus domesticus*), by exposing chicks to an

aversive stimulus (air puff) in the presence or absence of their mother (hen). They did this by placing pairs of chicks in boxes with or without their mother present and four treatments were established: without hen/control; with hen/control; without hen/air puff; and with hen/air puff (Edgar et al., 2015). In the control treatments chicks were left undisturbed for 10 min and in the air puff treatments chicks were sprayed with an air puff from a canister of inert compressed air into the box for 1 second every 30 sec (Edgar et al., 2015). Results showed that chicks spent more time preening and ground pecking when exposed to the air puff in the presence of their mother, comparatively to when alone, an indication of social buffering of the stress response - as preening and ground pecking behaviours are known to diminish in stressful circumstances (Edgar et al., 2015).

In sum, these results indicate that social buffering acts on several levels of the stress response, namely behavioural and endocrine. However, the experiments mentioned above used non-fearful/non-stressed conspecifics. Thus, the section below will focus on the influence of conspecifics' stress status in the social buffering phenomenon.

1.4.4 Conspecifics' stress status influence on Social Buffering

The stress status of conspecifics seems to be an important factor mediating the social buffering phenomenon. Another study by Kiyokawa and colleagues, verified that the partner's stress status modulates the response to an aversive stimulus (Kiyokawa et al., 2004). In this experiment, Kiyokawa and co-workers used a fear conditioning paradigm where subject rats learnt to associate a context (CS - test box) with electrical footshocks (US). After fear conditioning was established, subjects were exposed to the CS in three different conditions: alone; with a non-shocked partner (only exposed to CS during training); and with a shocked partner (fear conditioned) (Kiyokawa et al., 2004). Results showed that subjects that were exposed to the CS in the presence of a non-shocked partner exhibited the lowest fear responses (lowest freezing), indicating that a non-fearful conspecifc is more effective at inducing social buffering (Kiyokawa et al., 2004). Nontheless, the presence of a shocked-partner still evoked social buffering - freezing was significantly lower than when animals were alone and greater than when rats were with a non-shocked partner (Kiyokawa et al., 2004).

Despite the influence of conspecifics' stress status on social buffering, the sensory cues responsible for this process and the impact of larger groups of conspecifics are factors that may have an important influence in the buffering effect. These aspects will be addressed in more detail in the section below.

1.5 Sensory cues effectiveness & shoal size modulation of Social Buffering

The social buffering phenomenon is only possible due to the transmission of social information between conspecifics (da Costa et al., 2004; Rukstalis & French, 2005; Takahashi et al., 2013). Social information conveying "safety" is broadcasted by the partner animals (senders) and perceived by the receivers - the animals facing the threatening event (Davitz & Mason, 1955; Fuzzo et al., 2015; Kiyokawa et al., 2004). This social information is spread in the form of sensory cues and is varied among species (e.g. "voice signatures" of monkey marmosets, Rukstalis & French, 2005; visual cues in sheep, (da Costa et al., 2004) and experimental contexts, given that a single species may use different cues to communicate social information to conspecifics, depending on the environmental circumstances (e.g. rats can use both olfactory, Takahashi et al., 2013; and tactile cues, Wilson, 2001). Thus, it is important to consider the species' ecological and ethological context when assessing the cues responsible for social buffering.

There is evidence already for the involvement of several cues in the social buffering process, namely tactile (Wilson, 2001), olfactory (Takahashi et al., 2013), visual (da Costa et al., 2004) and vocal cues (Rukstalis & French, 2005). In relation to the involvement of tactile cues in the social buffering phenomenon, a study in rats has shown a reduction of the stress response due to tactile social information (Wilson, 2001). In a previous study, Wilson had shown a decrease in the levels of prolactin (an hormone that is known to be released upon exposure to stressful situations), when rats were exposed to an open field environment in the presence of a same-sex conspecific – an evidence of social buffering (Wilson, 2000). In a follow-up experiment, Wilson verified that the tactile contact with conspecifics was the cue responsible for the lowered levels of prolactin (Wilson, 2001). Wilson exposed rats to the open field environment in three different contexts: alone; separated from a same-sex conspecific by a perforated partition (allowing visual, olfactory and auditory cues); and in the presence of a conspecific (without partition). The results showed that the direct contact with a conspecific, significantly lowered prolactin levels, comparatively to being alone or with a conspecific separated by a partition. These findings indicate that the tactile cue is responsible for the social buffering in this context and that the visual, olfactory and auditory cues altogether are not sufficient to exert buffering, as these conditions showed the same prolactin levels as the alone treatment (Wilson, 2001).

Olfactory cues have also been shown to be involved in the social buffering process (Kikusui et al., 2006; Kiyokawa et al., 2014; Takahashi et al., 2013). For instance, recent work using an auditory fear conditioning paradigm in rats (CS - tone; US - electrical shock) has shown that conspecific odour elicited buffering of fear responses, revealing the contribution of olfaction for the social buffering phenomenon (Takahashi et al., 2013). In this experiment, the authors

verified that subject rats placed in cages that had been previously odorized with rat odour showed lower freezing responses, comparatively to rats that had been exposed to non-odorized cages (Takahashi et al., 2013).

In relation to visual cues, studies in sheep (*Ovis aries*) have shown evidence for a buffering effect relying only on the presence of visual information of conspecifics (see Kikusui et al., 2006 for a review). A previous study from the same laboratory had reported that pictures of sheep faces can be used in memory tasks successfully and that these mammals are capable of remembering facial pictures of 50 conspecifics for over a 2 years period (Kendrick, da Costa, Leigh, Hinton, & Peirce, 2001). In this experiment, da Costa and co-workers found that when sheep experience social isolation, the sight of conspecifics' pictures compared with those of inverted triangles, significantly decreased fear and stress responses in these animals.

Also, many species use vocal cues to communicate with other conspecifics (Kikusui et al., 2006; Rukstalis & French, 2005). Vocal cues are of special relevance in species that live in social groups, as is the case of the monkey marmosets (*Callithrix kuhlii*) that use unique "voice signatures" for individual identification (Jones, Harris, & Catchpole, 1993; Snowdon & Cleveland, 1980). Besides individual recognition, these vocalization calls have been shown to act as a sensory cue promoting social buffering in the context of social isolation (Rukstalis & French, 2005). Rukstalis and French exposed adult marmosets to short-term social isolation from their long-term pair mates and submitted them to three experimental conditions: 1) marmosets could listen to vocal cues from the pair mate; 2) marmosets could listen to vocal cues from an unfamiliar individual; 3) no auditory stimuli were presented to marmosets; and 4) undisturbed marmosets - control. Urinary cortisol levels were measured and results showed that marmosets which were socially isolated (no auditory stimuli presented) showed higher levels of cortisol than undisturbed conspecifics. Furthermore, cortisol levels were significantly lower when animals were exposed to vocal cues of pair mates, comparatively to when vocal signatures of unfamiliar conspecifics or no auditory stimuli were presented (Rukstalis & French, 2005).

Thus, understanding what kind of sensory cues drive social buffering is important to explore how different sensory modalities contribute to the buffering phenomenon and to investigate different sensory cues effectiveness in a long-term exposure to threatening events. In addition, understanding the most effective sensory channel in promoting social buffering allows a more accurate dissection of the neural mechanisms underlying the buffering effect.

Furthermore, it seems reasonable to suggest that conspecific number is a factor influencing the availability and reliability of sensory cues, that is larger numbers of group members generate greater sensory stimulation (e.g. more conspecifics - more visual information).

Thus, along with sensory cues contribution, also the number of conspecifics in a group may play a role in buffering the fear/stress responses to threatening events. Conspecifics' efficiency may depend on group size, since a larger group should be both more conspicuous and more reliable (i.e. a larger number of individuals conveying the same information). Indeed, species were shown to form larger groups in response to threat (Hoare et al., 2004) and group size was verified to modulate responses to threatening events (Brown & Bongiorno, 2006; Magurran & Pitcher, 1987). For instance, Hoare and colleagues reported that killifish (*Fundulus diaphanus*) formed larger shoals when exposed to a threat (alarm substance - AS[4]), than when food was administered or nothing was introduced in the water (control), indicating that individuals aggregate in larger groups when a dangerous stimulus is present.

Also, larger group sizes induce a faster recovery from aversive circumstances, as shown by Magurran and Pitcher (Magurran & Pitcher, 1987). In this experiment the authors accessed how fast different shoal sizes of minnows (*Phoxinus phoxinus*) recovered from exposure to a predator (pike). Three different shoal sizes (10, 20 and 50) were established and fish groups were confronted with a pike (*Esox lucius*). The results showed that smaller shoals (10) took longer to recover from exposure to the predator, comparatively to shoal sizes of 20 and 50 minnows (Magurran & Pitcher, 1987). The evidence documented above seems to highlight the anti-predatory benefits of larger groups, which in fact has been the main explanation in the literature for group living (Krause & Ruxton, 2002; Olson et al., 2013; Roberts, 1996; Viscido & Wethey, 2002). Given the advantages of larger groups of animals, it seems plausible to suggest that larger group sizes may in fact be more effective at buffering fear/stress responses.

This section enhanced the contribution of specific sensory cues for the buffering effect, besides presenting group size as a possible factor modulating social buffering. The next section will elaborate on the neural mechanisms underlying this social behaviour.

1.6 Neural mechanisms underlying Social Buffering

Apart from exploring the behavioural, endocrine and sensory mechanisms, some studies have focused on understanding the neural mechanisms underlying social buffering (Kiyokawa et al., 2014; A. S. Smith & Wang, 2014; Takahashi et al., 2013; Winslow et al., 2003). Given that familiarity and conspecifics' sensory cues (as seen in sections 1.3.2 and 1.5) are known to influence the buffering phenomenon (da Costa et al., 2004; Kiyokawa et al., 2014; Rukstalis &

[4] Alarm substance is a known fear-eliciting stimulus in several fish species, that is released into the water upon skin damage (Brown & Bongiorno, 2006; Speedie & Gerlai, 2008). Alarm substance effects will be described in more detail in section 1.7.3.

French, 2005), it is reasonable to suggest that several neural mechanisms are involved both at the sensory and social information processing levels. Also, it is expected that neural mechanisms involved in fear and stress responses are associated, since (has we have seen in sections 1.3.1 and 1.4.3) social buffering is known to reduce fearful and stress responses to aversive circumstances (Fuzzo et al., 2015; Kiyokawa et al., 2014; A. S. Smith & Wang, 2014).

Studies showing the social buffering effects of familiar conspecifics have been discussed previously at the behavioural level (see section 1.3.2). Interestingly, in one of the studies, familiarity was also shown to result in differential brain activity in stress and fear-related brain areas (Kiyokawa et al., 2014). As already described, Kiyokawa and colleagues, using a fear conditioning paradigm in rats, reported that the odour presence of a familiar conspecific was better at mitigating conditioned fear responses to auditory conditioned stimulus paired with foot shocks, comparatively to odour of an unfamiliar conspecific. In this experiment, besides verifying a significantly greater reduction in freezing responses when exposed to familiar conspecifics odour (comparatively to unfamiliar), the authors verified a down-regulation of *c-fos*[5] expression in the PVN, both when in the presence of unfamiliar and familiar conspecifics scent, as opposed to the condition were subject rats were exposed to the CS in a clean text box. However, the odour of familiar conspecifics was more effective at reducing *c-fos* expression, when compared to the unfamiliar smell (Kiyokawa et al., 2014). In addition to measuring PVN activity, the same study further showed a *c-fos* down-regulation in the lateral amygdala (LA) in response to the familiar and unfamiliar treatments (again greater when familiar conspecific odour was presented), and *c-fos* down-regulation in the central amygdala (CeA) only when familiar odour was present (Kiyokawa et al., 2014). Taken together, these results suggest that the scent of a familiar conspecific is more effective in promoting social buffering and that this effect promotes diminishment of *c-fos* expression both at the level of stress regulation (PVN) and fear associated (LA and CeA) brain nuclei.

So, familiarity seems to play an important role in regulating stress and fear responses, however transmission of social information is limited to the sensory channels available to receive and process it. Therefore, it is relevant to understand the sensory neural mechanisms involved in the buffering phenomenon. As explained above (see section 1.5), several sensory modalities were shown to be responsible for the buffering effect (da Costa et al., 2004; Rukstalis & French, 2005; Takahashi et al., 2013; Wilson, 2001). Some studies have reported the recruitment of sensory processing brain centres involved in social buffering (da Costa et al., 2004; Takahashi et al., 2013). As discussed previously, work from Takahashi and colleagues using an auditory fear conditioning

[5] *c-fos* is an immediate early gene used as a transient marker of neuronal activity (Lanahan & Worley, 1998; Okuno, 2011).

paradigm in rats showed that olfaction itself was enough to induce social buffering in rats. Moreover, this study showed that olfactory buffering also decreased *c-fos* expression in the PVN, suggesting that conspecific odour alone was sufficient to decrease the stress response elicited by a fearful stimulus. Furthermore, *c-fos* expression increased in the anterior olfactory nucleus (AON) and in the olfactory tubercule (Tu), but decreased in the basal amygdala (BA) and in the lateral amygdala (LA), indicating not only that sensory brain regions related to olfaction are recruited in the olfactory buffering process, but also that conspecific odour suppresses the activation of regions underlying fear conditioning in rats (Takahashi et al., 2013). In fact, another study conducted in rats, also using an auditory fear conditioning paradigm, showed that a bilateral lesion on the posteromedial region of the olfactory peduncle (pmOP) impaired social buffering (Kiyokawa, Wakabayashi, Takeuchi, & Mori, 2012). In this experiment, the authors performed lesions in the pmOP by injecting *N*-methyl-D-aspartic acid (NMDA) dissolved in phosphate-buffered saline (PBS) solution (Kiyokawa et al., 2012). The lesions in the pmOP resulted in increased levels of freezing in response to the fear-expression test (CS only), even in the presence of a conspecific (Kiyokawa et al., 2012). Furthermore, this study reported that the pmOP receives projections from the main olfactory bulb - MOB (Kiyokawa et al., 2012), which suggests an olfactory pathway connecting the MOB and the pmOP possibly involved in the olfactory buffering neural mechanism. In addition to olfactory buffering, we have mentioned also that the sight of conspecifics proved to be enough at inducing social buffering in sheep (da Costa et al., 2004; Kikusui et al., 2006). In this study, da Costa and co-workers verified that seeing conspecifics pictures significantly decreased fear and stress responses in sheep (see section 1.5). Along with the behavioural and endocrine effects, this experiment also showed that conspecific sight reduced *egr-1*[6] mRNA expression in the PVN, indicating that visual cues separately also lead to the decrease of the stress response at the brain level (da Costa et al., 2004). In sum, both olfactory and visual cues are sufficient to modulate neuromolecular mechanisms underlying social buffering in social animals. Thus, it is reasonable to suggest that other sensory modalities (tactile and vocal) have an identical role.

It is clear by the findings described in this section that neural mechanisms associated with fear responses are modulated by social buffering. Thus, studies showing direct neurophysiological evidence of decreased activity of specific fear-related brain regions during the buffering phenomenon are of great relevance. Accordingly, Fuzzo and colleagues demonstrated the first electrophysiological evidence that social buffering diminishes fear-induced activation in the LA, which in consequence reduced fear responses (Fuzzo et al., 2015). In this experiment, the authors

[6] *egr-1* is an immediate early gene used as a transient marker of neuronal activity (Lanahan & Worley, 1998; Okuno, 2011).

analysed freezing behaviour and local field potentials in the LA of auditory fear-conditioned rats in response to the CS (tone), either when a conspecific was present or absent. As well as reducing freezing responses, the presence of a conspecific significantly decreased mean peak amplitudes of auditory filed potentials, gamma oscillations and high frequency oscillations in the LA (Fuzzo et al., 2015). Several studies have thus shown the involvement of fear and stress related brain nuclei in the social buffering phenomenon, based on differential immediate early gene expression and electrophysiological evidence (da Costa et al., 2004; Fuzzo et al., 2015; Kiyokawa et al., 2014, 2004).

Moreover, recent research suggested oxytocin[7] as an important modulator of social buffering (Kikusui et al., 2006; A. S. Smith & Wang, 2014). In the face of threatening events animals stress response is activated, thus HPA axis stimulation induces CRH release, leading to an increase in circulating glucocorticoids (De Marco et al., 2013; Kikusui et al., 2006). Nonetheless, the PVN also contains oxytocinergic neurons that when activated mitigate the HPA axis function promoting social buffering (Kikusui et al., 2006; Neumann, Wigger, Torner, Holsboer, & Landgraf, 2000; A. S. Smith & Wang, 2014). It seems that the presence of social cues of conspecifics activates PVN oxytocin, mediating social buffering effects on fear and stress responses. In fact, in a study already described in section 1.4.3, Smith and Wang also studied the influence of social buffering on the stress response and respective modulation by the oxytocinergic neurons (Smith & Wang, 2014). In this study, female prairie voles were exposed to an aversive stimulus (1 hour immobilization) and then were allowed to recover alone or in the presence of a male conspecific. The aversive event increased corticosterone levels in the female prairie voles recovering alone, but not in the females recovering with their male partner. Also, the social buffering effect induced by the male partner lead to an increase in oxytocin release in the PVN. As these findings demonstrate PVN oxytocin as a social buffering modulator, it is reasonable to suggest that oxytocin may be a key neuropeptide acting on several other brain nuclei involved in social buffering such as: sensory processing nucleus (OB); fear-related brain areas (e.g. LA); or even brain regions directly connected to locomotion (as freezing behaviour is the absence of movement, it is possible that oxytocin is also modulating locomotor functions).

In conclusion, the neural mechanisms known to date underlying social buffering reveal the involvement of sensory processing brain areas, fear and stress-related centres and the neuropeptide oxytocin as an important modulator of the buffering effect. In the section below, zebrafish will be presented as a good candidate model organism to assess this social phenomenon, particularly due to: 1) zebrafish's social, fear and stress responses; 2) the

[7] Oxytocin is a neuropeptide secreted by the oxytocinergic neurons located in the paraventricular nucleus of the hypothalamus (see Kikusui et al., 2006 for a review).

neurogenetic and imaging tools available for assessing the neural circuits underlying social buffering in zebrafish; 3) the existence of mutant and transgenic lines of zebrafish enabling the dissection of neural regions and circuitry associated with the buffering effect.

1.7 Zebrafish as a candidate model to study Social Buffering

1.7.1 Ecological evidence & model organism

Zebrafish is a teleost fish native from South Asia with a reported tolerance for a wide range of temperatures, from 6 ºC in the winter to over 38 ºC in the summer. The temperature variation and the monsoon climate that characterizes zebrafish habitat, affects its distribution in a way that this species is most commonly encountered in shallow ponds and standing water bodies. Occasionally zebrafish can be found in streams and rivers with a diverse flow regime (i.e. low to fast regimes) (Rowena Spence, Gerlach, Lawrence, & Smith, 2008; Suriyampola et al., 2015). For this reason, zebrafish is described as a floodplain rather than a riverine species (Parichy, 2015; Rowena Spence et al., 2008; Rowena Spence, Jordan, & Smith, 2006). Due to the great seasonal variation and habitat diversity, zebrafish individuals form mixed-sex shoals ranging from 2 to 300 conspecifics in the wild (Pritchard, Lawrence, Butlin, & Krause, 2001; Rowena Spence et al., 2006; Suriyampola et al., 2015). In fact, a recent study has shown that group size variability seems to be habitat-dependent (i.e. groups up to 22 individuals found in still waters; groups of 6-7 fish found in a slow flowing river; groups up to 300 individuals found in a fast-flowing river), with the smaller shoals (6-7) exhibiting the higher levels of aggression (Suriyampola et al., 2015). These findings suggest that zebrafish collective and aggressive behaviours seem to be greatly influenced by the environmental conditions. However, given that zebrafish is most commonly found in floodplain areas with still waters, like shallow ponds and standing water bodies (Parichy, 2015; Rowena Spence et al., 2008, 2006), it is reasonable to suggest that zebrafish average shoal size ranges from 2 to 30 individuals (Rowena Spence et al., 2006).

Even though little is known about zebrafish ecology, this is not the case when it comes to its developmental biology, genetics and research focusing on anxiety or chronic stress - which may have applications in the treatment of certain human disorders, such as the generalized anxiety disorder and the post-traumatic stress disorder, respectively (Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). Moreover, zebrafish as been emerging as a model organism in the field of behavioural neuroscience, with particular relevance in the study of endocrine and neural mechanisms underlying social (Abril-de-Abreu, Cruz, & Oliveira, 2015; Oliveira et al., 2016; Teles et al., 2015), fear (Agetsuma et al., 2010; Speedie & Gerlai, 2008) and stress (Félix,

Faustino, Cabral, & Oliveira, 2013; Pavlidis, Theodoridi, & Tsalafouta, 2015; Piato et al., 2011) behaviours, that will be discussed in more detail below.

1.7.2 Social behaviour

Zebrafish is a shoaling species; a behaviour that seems to be innate, as shoal formation starts soon after hatching (Engeszer, Patterson, Rao, & Parichy, 2007; Rowena Spence et al., 2008). Recent studies using larval zebrafish stages have shown that the visual preference for conspecifics is very robust in 3 weeks old larvae (Dreosti, Lopes, Kampff, & Wilson, 2015; Engeszer, Barbiano, Ryan, & Parichy, 2007), although the occurrence of this behaviour starts to gradually appear from 1 week to 3 weeks post-fertilization larval zebrafish (Dreosti et al., 2015). Nonetheless, the average distance between fish within the shoal significantly decreases between one to four months old, indicating greater shoal cohesion over time (Buske & Gerlai, 2011). Several studies have reported shoaling behaviour in zebrafish adults in different environmental contexts (Al-Imari & Gerlai, 2008; Pritchard et al., 2001). In fact, the sight of conspecifics seems to have rewarding properties, as it was shown to act as a positive reinforcer in associative learning (Al-Imari & Gerlai, 2008). Additionally, shoaling behaviour seems to be affected by several factors, namely overall group activity, group size and the presence of an imminent threat (e.g. predator or alarm substance) (Brown & Bongiorno, 2006; Magurran & Pitcher, 1987; Miller & Gerlai, 2007; Pritchard et al., 2001). For instance, Pritchard and colleagues verified that zebrafish prefered to spend time with a larger shoal of conspecifics (e.g. prefer a shoal of 4 vs. a shoal of 1) when the water temperature of both shoals did not vary - warm water (as temperature was shown to influence fish activity, with colder temperatures leading to lower activity levels); however, with colder water zebrafish preferred more active shoals instead of larger ones (activity was measured as total distance moved), independently of the number of fish presented (Pritchard et al., 2001). These results indicate that zebrafish tend to display preference for larger shoals of fish, yet this is not the case when shoal activity decreases, since zebrafish may prefer to spend time with smaller shoals, if the group activity is greater. In addition to the influence of group size and overall group activity, other external environmental factors such as threatening events appear to modulate group behaviour. Accordingly, zebrafish shoal cohesion is influenced by the presence of a predator, which decreases shoal cohesion (Miller & Gerlai, 2007).

In agreement with the previous findings, sensory cues (olfactory or visual) are expected to play a role both in conspecifics recognition and phenotype matching. Indeed, studies have shown that the ability to recognize conspecifics is learnt early in zebrafish development by imprinting on odour cues of kin 6 days post-fertilization - dpf (Gerlach, Hodgins-Davis, Avolio, & Schunter, 2008). Also, Engeszer and co-workers showed that zebrafish exhibit a preference for shoaling

with fish that present the same phenotype as their raising mates, suggesting that the visual cues are important in determining social preferences later in life (Engeszer, Ryan, & Parichy, 2004).

1.7.3 Fear response

Moreover, additionally to being a social species, zebrafish is known to have a very robust fear response in response to aversive events (Agetsuma et al., 2010; Mathuru et al., 2012; Speedie & Gerlai, 2008). Fear has been defined as a defensive behavioural and physiological response that enables the organism to ensure its survival and adjust itself to a fluctuating environment (Tovote et al., 2015). Zebrafish has been shown to respond with stereotypical behaviours (e.g. erratic movement, freezing and flight, Kalueff et al., 2013) to different fear inducing stimulus, namely alarm substance (Speedie & Gerlai, 2008) and electrical shocks (Agetsuma et al., 2010).

For instance, Speedie and Gerlai showed that zebrafish significantly responded with erratic movement and freezing behaviours typical of an alarm reaction when in the presence of alarm substance (Kalueff et al., 2013). Erratic behaviour is characterized by multiple fast acceleration bouts in rapid succession, in which the direction of movement also changes in a seemingly stochastic manner between the rapid darts; freezing behaviour is characterized by a zebrafish remaining at the bottom of the tank in complete cessation of movement (except for gills and eyes) (Kalueff et al., 2013). More recently, other studies corroborated the efficacy of alarm substance in evoking fear responses in zebrafish and showed that this substance is a mixture of components that includes glycosaminoglycan (GAG) chondroitin (Mathuru et al., 2012). Like alarm substance, purified chondroitins were verified to activate the mediodorsal posterior olfactory bulb (Mathuru et al., 2012), setting the stage for future research exploring the neural circuits underlying the response to alarm substance.

As mentioned above, other experiments have addressed fear behaviours in zebrafish using other stimulus, namely electrical shocks (e.g. Agetsuma et al., 2010). In this study, Agetsuma and colleagues used a fear conditioning paradigm in which a red light (CS) was paired with an electrical shock (US). Conditioned zebrafish exhibited a flight response to the CS, which is also a fear-like behaviour characterized by an accelerated movement away from the stimulus - red light (Kalueff et al., 2013). The authors verified that genetic inactivation of the lateral subnucleus of the dorsal habenula (dHbl) decreased the normal fleeing behaviour and zebrafish exhibited instead higher levels of freezing behaviour, suggesting the role of the dHbl in modulating fear response strategies (Agetsuma et al., 2010).

Other studies have corroborated the importance of visual transmission of information and added on the relevance of the olfactory transmission of social information signalling

threating events (Hall & Suboski, 1995; Suboski, Bain, Carty, McQuoid, & et al, 1990). In two studies by Hall and Suboski, zebrafish learnt to respond to olfactory (morpholine - CS) and visual (red light - CS) stimulus associated with fear behaviour, after being placed in the same tank where conditioned mates were present. Conditioned zebrafish that learnt to associate the olfactory or visual stimulus to alarm substance (US), were put in the same tank as naïve zebrafish (that did not experience the conditioning task) and exposed to either morpholine or the red light. This lead to a generalized fear response, both from conditioned and naïve zebrafish. Naïve zebrafish were then separated from the conditioned fish and when tested alone to the olfactory and visual stimulus, responded with fear behaviour (Hall & Suboski, 1995; Suboski et al., 1990).

Alarm substance and cued conditioned fear responses have been used to address defense behaviours and its underlying mechanisms in zebrafish, however these behavioural paradigms use experimental setups in which the focal fish is tested alone to the aversive events (Agetsuma et al., 2010; Mathuru et al., 2012). Nonetheless, this is not often the case in zebrafish natural environment, as this species is known to form shoals in the wild (Pritchard et al., 2001; Rowena Spence et al., 2006). Therefore, paradigms addressing zebrafish fear response in the presence of conspecifics (social buffering) are still missing. The development of experimental setups to address social buffering of fear in zebrafish will greatly benefit the understanding of the neural mechanisms underlying fear behaviours in the presence of conspecifics.

1.7.4 Stress response

Apart from triggering fear responses, threatening events may also activate a stress response in order to preserve homeostasis (De Marco et al., 2013; Ramsay et al., 2009). Since the stress response is conserved across taxa, zebrafish may help dissecting the stress endocrine and neural mechanisms underlying the social buffering of fear. The zebrafish hypothalamic-pituitary-interrenal (HPI) axis shares several similar aspects with the HPA axis, with cortisol being the main glucocorticoid both in humans and teleosts (Bonga, 1997; Piato et al., 2011). Also, the preoptic nucleus in teleosts is considered to be the homologous structure to the mammalian PVN (De Marco et al., 2013; Peter, 1977). Adult zebrafish show behavioural stress reactions and elevated cortisol levels to several aversive stimulus (Pavlidis et al., 2015; Ramsay et al., 2009; Speedie & Gerlai, 2008). As well, the zebrafish stress system matures early in development and larval stages of this species are known to respond to threatening circumstances with increased cortisol levels (Alderman & Bernier, 2009; Alsop & Vijayan, 2008; De Marco et al., 2013).

Aside from responding to chronic stressors (Piato et al., 2011), most zebrafish stress research have been addressing stress responses to acute stimulus (Fuzzen, Van Der Kraak, &

Bernier, 2010; Pavlidis et al., 2015). Contrary to chronic exposures (e.g. stress protocols lasting 14 days (Piato et al., 2011), acute stressors are usually short (1-90 min) aversive events, such as restraint stress/confinement (Ghisleni et al., 2012), air exposure/netting (Alderman & Vijayan, 2012; Ramsay et al., 2009), lowered water level + chasing + air exposure (Pavlidis et al., 2015) and water vortex (Fuzzen et al., 2010). Both acute and chronic stress exposures induce cortisol elevation and differential expression of stress related genes (Pavlidis et al., 2015; Piato et al., 2011). In a seminal study, Ramsay and co-workers (Ramsay et al., 2009) have documented cortisol dynamics after stressor exposure (i.e. netting stress: 3 min netting + 3 min resting + 3 min netting) and verified a cortisol peak at 15 min post-exposure. Nonetheless, mean cortisol values among stressed fish were significantly greater than the ones of control groups from 3 to 30 min PS and no differences were found 60 min afterwards (Ramsay et al., 2009). Recently, other studies that have emerged investigating HPI axis response to threatening events at the endocrine and neural level, revealed some contradictory findings that are worth interpreting cautiously. For instance, Fuzzen and colleagues (Fuzzen et al., 2010) established a water vortex stressor (300 rpm's for 60 min) that induced a cortisol peak and corticotropin-releasing factor (CRF) mRNA (POA was used) elevation 10 min post-exposure. The same study also exposed zebrafish to the netting stressor (1 min followed by 60 min post-exposure) and showed a peak at 5 min, with cortisol values significantly higher than controls from 5 to 40 min post-exposure (Fuzzen et al., 2010), a result that clearly contrasts with what Ramsay and co-workers found (Ramsay et al., 2009). Additionally, another study reported a cortisol increase 15, 60 and 90 min after exposure to a restrain stress (confinement in 2 mL tubes) and CRF mRNA (whole-brain was used) decreased expression was only verified 90 min after exposure (Ghisleni et al., 2012). Furthermore, a different experiment using netting as a stressor (1 min netting followed by 24 hours sampling) documented a cortisol peak only 20 min after stressor exposure and no differences were found in the mRNA expression (whole-brain was used) of glucocorticoid receptors (Alderman & Vijayan, 2012).

Lastly, Pavlidis and colleagues used an acute protocol (lowered water level + chasing for 5 min + netting 1 min, followed by resting in shoals of 6 for 120 min) to evoke a stress response in zebrafish and verified a cortisol increase from 15 to 60 min post-exposure, peaking at 30 min (Pavlidis et al., 2015). At the brain level (whole-brain was used), this study reported a significant mRNA increase in CRF (at 15 and 60 min) and GR (at 15 min) post-stressor administration; no differences were found in MR (Pavlidis et al., 2015). Furthermore, these authors explored *c-fos* mRNA expression and reported an increase in brain activity 15 and 30 min after exposure to stressor (Pavlidis et al., 2015).

Considering the results above and the great variation observed in zebrafish neuroendocrine response, it seems plausible to consider that cortisol and CRF responses to stress have an increase between 10 and 30 min after exposure to a stressor. Among other things, some factors such as strain, individuals' age and brain tissue collected may be influencing the variety reported. In fact, studies have already shown an effect of strain in response to novelty stress (Cachat et al., 2010; Egan et al., 2009). Besides, the age of zebrafish used in the studies above varied greatly (from 6 months in Ghisleni et al., 2012 to 13 months in Ramsay et al., 2009) and aging has already proven to affect cortisol response in other teleost species (Barcellos et al., 2012; Koakoski et al., 2012). Moreover, in the experiments described either whole-brain (Alderman & Vijayan, 2012; Ghisleni et al., 2012; Pavlidis et al., 2015) or tissue from specific brain structures (POA, Fuzzen et al., 2010) was used, which may be a source for the variation presented in gene expression data.

To conclude, in order to accurately access the stress response at the neural level, it seems important to control for possible sources of variation, such as: strain, individual's age and stress-inducing protocol. Furthermore, most studies have used whole-brain samples to investigate stress responses at the brain level, thus dissecting candidate brain regions known to be involved in the stress phenomenon in other species will greatly benefit questions regarding the neuromolecular mechanisms underlying the social buffering of the stress response.

1.7.5 Social buffering: candidate brain nuclei in zebrafish

Given the findings presented above, social buffering seems to rely on brain areas involved with sensory processing, fear and stress related centres, besides oxytocin-mediated mechanisms. Therefore, in order to investigate zebrafish social buffering responses at the neural level, besides the olfactory bulb (Mathuru et al., 2012) and the lateral dorsal habenula (Agetsuma et al., 2010), it will be relevant to explore the involvement of other brain structures known to be related with fear (Herry, Trifilieff, Micheau, Lüthi, & Mons, 2006; Quirk, Repa, & LeDoux, 1995; Sparta et al., 2014; Tovote et al., 2015): medial zone of the dorsal telencephalic area (Dm - putative homologue of the mammalian basolateral amygdala, see O'Connell & Hofmann, 2011); anxiety/sustained fear (Davis, Walker, Miles, & Grillon, 2010; Duvarci, Bauer, & Paré, 2009; Tovote et al., 2015): supracommissural nucleus of the ventral telencephalic area [Vs - putative homologue of the mammalian extended amygdala (comprising the medial amygdala and bed nucleus of stria terminalis, O'Connell & Hofmann, 2011); oxytocin secretion (Neumann et al., 2000; A. S. Smith & Wang, 2014): preoptic area (POA - putative homologue of the mammalian homonym/preoptic area, O'Connell & Hofmann, 2011); and regulation of affective states

(Sheehan, Chambers, & Russell, 2004; Tovote et al., 2015): ventral nucleus of the ventral telencephalic area (Vv - putative homologue of the mammalian lateral septum, O'Connell & Hofmann, 2011), which also shows high connectivity with the POA (O'Connell & Hofmann, 2011).

1.7.6 Behavioural quantification

The description of zebrafish rich repertoire of behavioural responses, not only at the level of its social features, but also due to the zebrafish robust fear responses and activation of stress mechanisms in the face of a threatening scenario, emphasized the need of establishing powerful quantification tools to measure behaviour. The development of automated video-tracking systems and custom-made software coding to quantify specific behaviours has become a growing focus in zebrafish research, specially due to this species promise for the field of behavioural neuroscience (Cachat et al., 2011; Green et al., 2012; Mirat, Sternberg, Severi, & Wyart, 2013; Pérez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014; Qin, Wong, Seguin, & Gerlai, 2014; Saverino & Gerlai, 2008). Rigorous and automated analysis of video recorded behaviour contributes to more reliable and consistent measurements of behaviour. New automated software have provided the research community the capability of quantifying specific aspects of zebrafish social behaviour (Green et al., 2012; Qin et al., 2014; Saverino & Gerlai, 2008); allowed computerized 3D reconstructions and clustering of behaviours (Cachat et al., 2011); tracking systems that quantify precise maneuvers of zebrafish larvae in a high-throughput format (Mirat et al., 2013) and enabled fingerprint extraction of individual zebrafish with multi-tracking algorithms, thus facilitating the tracking of large groups of animals (Pérez-Escudero et al., 2014). However, automated software allowing the quantification of complex fear responses of zebrafish (i.e. erratic movement and freezing) is still missing, therefore the development of such custom-made software will be highly profitable for the scientific community investigating fear and stress, using zebrafish as a model organism.

1.7.7 Zebrafish's neural "tool-box"

Lastly, a growing body of research in behavioural neuroscience have been using zebrafish as model organism, given the neurogenetic and imaging tools already available in this species (Agetsuma et al., 2010; Ahrens et al., 2012; De Marco et al., 2013; Jetti et al., 2014; Sergent et al., 2013; Teles et al., 2015). Zebrafish genome is fully sequenced and annotated (Howe et al., 2013) and brain atlas of zebrafish larval and adult brain stages are already available (Mueller &

Wullimann, 2015; Wullimann, Rupp, & Reichert, 1996). Also, homologies between zebrafish and the mammalian brain have been largely established (Ganz et al., 2012, 2015; O'Connell & Hofmann, 2011). Furthermore, several genetic techniques are enabling the understanding of zebrafish adult behaviour and its underlying neuromolecular mechanisms, namely: quantification of immediate early genes and target genes using *in situ hybridization* (Goto-Kazeto, Kight, Zohar, Place, & Trant, 2004; Lau, Mathur, Gould, & Guo, 2011) or qPCR (quantitative polymerase chain reaction), using whole-brain or candidate brain areas (microdissection technique) sampling (Pavlidis, Sundvik, Chen, & Panula, 2011b; Pavlidis et al., 2015; Teles et al., 2015); and whole-transcriptome analysis by using microarray gene chips, that allow the detection of differential gene expression patterns related to distinct behavioural profiles (Oliveira et al., 2016; Whitfield & Band, 2002). However, one of the limitations of the genetic techniques described above is the inability of providing *in vivo* information of the neural circuitry underlying behaviour. On the other hand, larval zebrafish stages possess small and transparent brains, which recently enabled a rapid and important development of optogenetic, imaging and transgenic tools that allowed *in vivo* visualization, recording and manipulation of the neural circuits underlying behaviour (Agetsuma et al., 2010; Ahrens et al., 2012; Bianco & Engert, 2014; De Marco et al., 2013; Jetti et al., 2014; Okamoto, Agetsuma, & Aizawa, 2012; Wyart et al., 2009). Yet, zebrafish larvae behaviours are usually restricted to very simple maneuvers (e.g. escapes, Wyart et al., 2009), thus studies unravelling the ontogeny of social behaviours (Dreosti et al., 2015; Engeszer, Barbiano, et al., 2007) and the development of optic tools that allow imaging in larger brains (Judkewitz, Wang, Horstmeyer, Mathy, & Yang, 2013) are setting the stage to further explore zebrafish social complexity at the neural level. Additionally, several zebrafish mutant and transgenic lines are already available (Ahrens et al., 2012; Jetti et al., 2014; Mirat et al., 2013), namely zebrafish lines allowing the genetic inactivation of fear-related brain regions (Agetsuma et al., 2010), enabling the dissection of brain nuclei and neuronal circuits possibly involved in social buffering.

Lastly, zebrafish larvae and juveniles social behaviour is poorly understood and investigating it at these stages will greatly benefit the understanding of zebrafish social repertoire and the neural mechanisms underlying it, as is the case of social buffering.

1.8 Thesis aims and structure

The present work focused on investigating the occurrence of social buffering of fear in zebrafish and aimed to explore the behavioural, endocrine, sensory and neural mechanisms underlying it.

- In chapter 2, the main goal was to test the occurrence of social buffering of fear in zebrafish. In order to do that the first aim was to establish a behavioural paradigm allowing the study of the social buffering phenomenon. The second aim was to develop an automated custom-made software (named xyz2b) to reliably quantify fear behaviour in zebrafish. Thirdly, we aimed at testing the occurrence of social buffering in zebrafish, both at the behavioural and stress response levels.
- In chapter 3, the main goal was to unravel the sensory cues promoting the social buffering effect. Furthermore, we aimed to verify the social buffering effectiveness of different sensory modalities in a persistent-threat scenario.
- In chapter 4 the goal was to investigate if the number of conspecifics modulates social buffering and explore the neuromolecular mechanisms underlying this social behaviour in zebrafish.

Chapter 2 |
Social Buffering in zebrafish - experiment I

2.1 Introduction

Communication among social animals not only enables the expression of personal information, but also facilitates the acquisition of public information from environmental threats (Brown & Bongiorno, 2006; Hoare et al., 2004). In fact, among other aspects, group living has been explained by its anti-predatory benefits, namely due to an overall increase in vigilance, the dilution of risk and predator confusion (Krause & Ruxton, 2002). Therefore, group formation allows individuals to infer the presence of threat in the environment, even when they have not detected it directly (social transmission of fear, Hollén & Radford, 2009; Pereira et al., 2012). On the other hand, animals can rely on conspecifics presence to lessen their fear response to a detected threat - social buffering (Edgar et al., 2015; Fuzzo et al., 2015; Kikusui et al., 2006).

Threatening events may elicit stress responses that can result in stress-derived disorders both in humans and non-human animals (Chiba et al., 2012; Kendler et al., 1999; Piato et al., 2011; Pine et al., 2002; Rauch et al., 2000). Thus the exploration of social buffering, a social behaviour responsible for the mitigation of threat-derived stress responses, and its underlying neuroendocrine mechanisms is of great interest. The social buffering phenomenon was first reported in a seminal study by Davitz & Mason (1955), in which rats were shown to exhibit lower fear responses to a fear-eliciting stimulus (fear conditioning paradigm: CS - blinking light; US: electrical shock) when in the presence of conspecifics, but not when alone. More recently studies have also been reporting social buffering of the stress response (Livia Terranova et al., 1999; A. S. Smith & Wang, 2014). For instance, Terranova and co-authors reported that when conspecific rats were present, subject rats presented lower levels of corticosterone, than when rats were placed alone in the novel context (Livia Terranova et al., 1999). Accordingly, Smith and Wang found that female prairie voles that recovered from a stressful event (1 hour immobilization) in the presence of a male partner showed lower corticosterone levels, comparatively to female prairie voles recovering alone (A. S. Smith & Wang, 2014). Studies have been addressing the social buffering of the stress response in other species, namely squirrel monkeys (Levine et al., 1978) and domestic hens (Edgar et al., 2015). However the social buffering effect is virtually unknown in fish (but see Ziv et al., 2013), thus its investigation in zebrafish is needed not only to better understand the evolution of social buffering in vertebrates, but also to bring robust evidence of the existence of social buffering in this model species and explore the behavioural and neuroendocrine mechanisms underlying it.

Zebrafish as been pointed as a highly social species, with a robust fear to a variety of aversive stimuli (Agetsuma et al., 2010; Pritchard et al., 2001; Speedie & Gerlai, 2008; Suriyampola et al., 2015). Besides living in shoals (Pritchard et al., 2001; Rowena Spence et al.,

2006; Suriyampola et al., 2015), zebrafish also display a very clear fear-like alarm response (Jesuthasan & Mathuru, 2008; Speedie & Gerlai, 2008). Alarm substance is produced in specialized epidermal cells and released into the water upon skin damage, hence warning for the presence of a potential threat in the environment (Speedie & Gerlai, 2008). In response to alarm substance zebrafish exhibit an alarm reaction response characterized by erratic movement and freezing behaviour (Kalueff et al., 2013). Erratic movement is defined by sharp changes in direction or velocity, as well as multiple darts (fast acceleration bouts in rapid succession in which movement direction also changes stochastically). On the contrary, freezing is characterized by the complete absence of movement (except for gills and eyes), while zebrafish is at the bottom of the tank (Kalueff et al., 2013). Erratic movement and freezing behaviours are normally quantified by multi event recorders, since their complexity limits automatic quantification by available commercial videotracker software (Speedie & Gerlai, 2008). Thus, the existence of codes that automatically and accurately quantify these behaviours are needed.

Aside from its well-established social and fear behaviour, zebrafish has also been used because of its strong stress response to acute stimuli (Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015). Thus, similarities between zebrafish and the mammalian stress axis, as well as brain homologies, have been largely established (Bonga, 1997; Ganz et al., 2012, 2015; O'Connell & Hofmann, 2011; Piato et al., 2011; Yeh, Glöck, & Ryu, 2013). Neurons in the parvocellular cell group of the zebrafish POA (putative homologous of the mammalian PVN) are known to secrete CRF in response to stress (De Marco et al., 2013; Fuzzen et al., 2010; O'Connell & Hofmann, 2011). Regarding glucocorticoid receptors, literature in mammals have shown considerable GR and MR expression in several brain regions, namely hippocampus, amygdala, lateral septum and some cortical areas (see de Kloet, Joëls, & Holsboer, 2005 for a review).

Besides eliciting a fear response, previous results in the lab (data not shown) have also revealed that the alarm substance evokes HPI axis activation, with a whole-body cortisol increase above basal levels verified 5 min after exposure to this aversive stimulus. Accordingly, zebrafish stress response to other acute stimuli have been shown to induce cortisol peaks between 5 and 30 min post-stress (Fuzzen et al., 2010; Pavlidis et al., 2015), CRF differential expression between 10 and 90 min post-stress (Fuzzen et al., 2010; Ghisleni et al., 2012) and GR differences at 15 min post-stress (Pavlidis et al., 2015).

Altogether, the findings presented above acknowledge zebrafish as a good candidate model for the study of social buffering, given its robust social, fear and stress behaviour. In this chapter, we present a novel behavioural paradigm to assess social buffering in adult zebrafish,

recurring to a known fear-inducing stimulus (AS) to induce fear and HPI axis activation in zebrafish. To this end, we automatically and accurately quantified zebrafish erratic and freezing behaviours using a custom made code (xyz2b) and assessed zebrafish stress response by quantifying cortisol and mRNA expression of stress-candidate genes encoding for CFR, GR and MR in the telencephalon and diencephalon. Thus, the main objectives of this chapter were: 1) explore the existence of social buffering of fear in zebrafish and 2) investigate the social buffering of the stress response (social buffering modulation of the HPI axis).

2.2 Methods

2.2.1 Fish and housing

All subjects used were 6-9 months old male wild-type (TU) zebrafish (*Danio rerio*) bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). All fish were raised in groups of 35 individuals (29 males, 6 females) in 3.5 L tanks (raising tanks), in a recirculating system (ZebTec, 93 Tecniplast), and kept at 28ºC, 750 μS, 7.0 pH in a 14L:10D photoperiod. Holding-water was monitored for nitrites (<0.2 ppm), nitrates (<50 ppm) and ammonia (0.01-0.1 ppm). At least one week before experiments fish were transferred to the behavioural room and housed in 6 L tanks (30 × 15 × 17 cm) under the same environmental conditions, in groups of 30 individuals (24 males, 6 females). This fish density was established to fulfil a 5 fish per liter criteria, thus decreasing agonistic interactions and lowering cortisol levels of fish in the stock tanks (Pavlidis et al., 2013). By implementing this density, the space for fish to establish territories (Pérez-Escudero et al., 2014) was diminished; therefore promoting environmental conditions for focal individuals to have less social status discrepancies as to control for the influence of hierarchical background in the response to alarm substance. The 6 L tanks were environmentally enriched with a 1 cm layer of crushed shells substrate. Fish were fed twice a day (except on the day of the experiments) with freshly hatched *Artemia salina* in the morning and commercial food flakes in the afternoon. All experiments were performed in accordance with the relevant guidelines and regulations, reviewed by the Instituto Gulbenkian de Ciência Ethics Committee, and approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária, permit 008955).

2.2.2 Alarm substance extraction

Alarm substance was extracted using a modified version of the protocol described in (Speedie & Gerlai, 2008). Ten month old donor zebrafish (8 females and 8 males) were used to prepare all three sets of alarm substance (one for each experiment). The total number, sex-ratio and age of

individuals used, allowed to control for possible variations in alarm substance content (see Figure 3D-E for alarm substance efficiency across experiments). Fish were retrieved from their raising tanks and placed in a small tank to proceed with alarm substance extraction. Fish were collected from the small tank, rinsed with distilled water and excess of water was removed from the skin with a paper towel. Fish were then quickly sacrificed by breaking the spinal cord with tweezers. Fish were fixed in a silicon bed and fifteen (fourteen vertically and one horizontally) shallow cuts were made on each side of the trunk with the help of a surgical scalpel blade. Fish were placed on a petri dish and 50 mL of distilled water (25 mL on each side) were used to wash the cuts (a 20 mL Terumo® syringe without needle was used to perform the washing). Superficial cuts are sufficient to extract alarm substance, as AS is known to be released upon skin damage (Speedie & Gerlai, 2008). There was no blood contamination in the alarm substance solution. The solution was then passed through filter paper to avoid solution contamination with residues. The same process was repeated for all 16 fish used. In the end, the alarm substance solution was filtered a second time. The 800 mL of solution were divided into 10 mL aliquots and stored at -20ºC until further use. During the collection process the solution was kept on ice. Alarm substance extraction was always done at least one day before experiments initiation, so that all aliquots would pass through the thawing process. The same solution of alarm substance was not used across different experiments to prevent possible degradation.

2.2.3 Experimental setup

The experimental setup (see Figure 1) consisted of a test and a demonstrators tank (12 × 12 × 15 each) placed side-by-side. This set-up was replicated eight times as to perform all the treatments in the same day, in a randomized manner. The observation side of each test tank faced the demonstrators tank and both test and demonstrators tanks had white opaque bottom and two white opaque walls (with the exception of the observation and the camera sides) to avoid visual contact with nearby tanks and interference of undesired visual cues from either the experimenter or the behavioural room. A 60 cm flexible and transparent PVC tubing (0.8 mm internal diameter; 2.4 mm external diameter) was introduced into each test tank (1 cm underwater) to enable the administration of the alarm substance. On the PVC tubing farmost extremity from the test tank, a 2-10 μL transparent pipette tip was attached to facilitate alarm substance administration. All tanks were filled with 1.3 L of water. Two B&W mini surveillance cameras (Henelec 300B), connected to a laptop (Samsung NP350V5C) through an USB 2.0 video capture device (ezcap), were placed on the side and on top of each test tank (see Figure 2) to acquire side and top view recordings of the test and demonstrator tanks simultaneously. Video synchrony

between side and top cameras was possible using Bonsai (https://bitbucket.org/horizongir/bonsai), an open source visual programming framework for data stream processing. A black curtain partially covered the top part of the setup to avoid light incidence and nobody was allowed to enter in the behavioural room during experiments (apart from the experimenter) to maintain quiet experimental conditions.

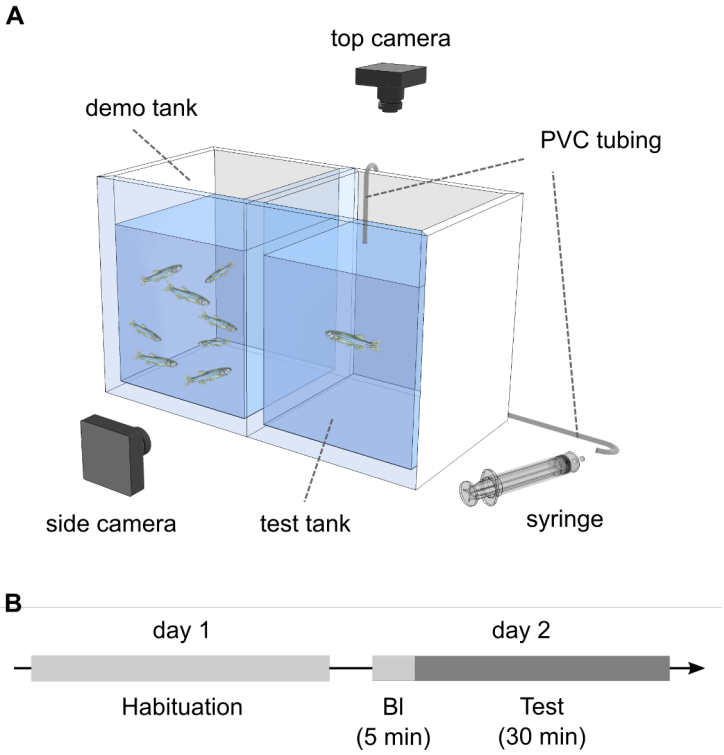

Figure 1 | Behavioural paradigm for the study of social buffering in zebrafish: experimental setup and behavioural protocol schematics.
(**A**) Schematic representation of the social buffering experimental setup. Test tank (focal fish) and demonstrator tank (with shoal present or absent depending on the treatment) were side-by-side and physically separated. AS or water (depending on treatment) were administered through a PVC tubing with the help of a syringe. Behaviour was video recorded with side and top cameras. (**B**) Schematic representation of the behavioural protocol. On day 1 focal fish were left to habituate overnight to the experimental setup. On the following day (day 2), behavioural video recording was initiated with 5 min of baseline (Bl), followed by 30 min of test.

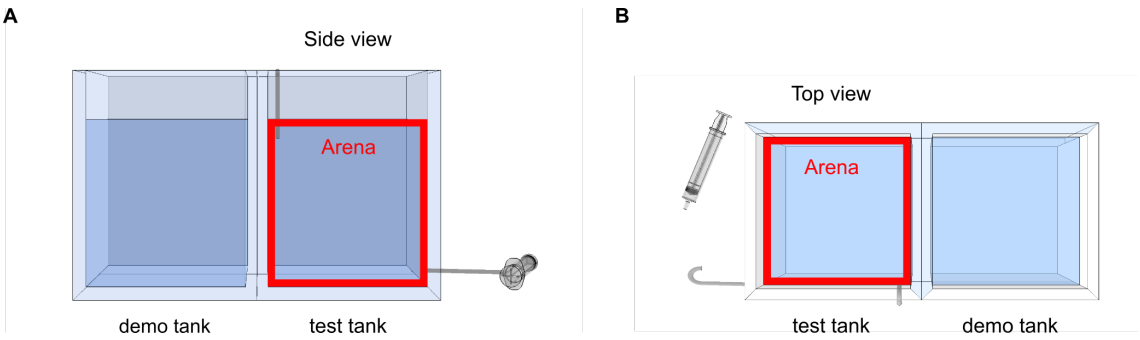

Figure 2 | Side and top view of 2D arenas.
For tracking, (**A**) a 10.5 × 11 cm 2D arena was defined for the side view and a (**B**) 10.5 × 10.5 cm 2D arena was defined for the top view. Both arenas excluded glass walls and all fish were tracked at 25 frames per second. The videotracking software EthoVision was used to determine and extract fish coordinates into excel files for posterior behavioural data analysis using xyz2b.

2.2.4 Experimental procedures

A total of 80 focal naïve male zebrafish were used (20 per treatment). Each focal fish was submitted to a single test corresponding to one of four possible treatments (Figure 4A): alone focal fish administered with water (Alone_Ctrl); alone focal fish administered with alarm substance (Alone_AS); focal fish administered with water and exposed simultaneously to shoal water and a shoal of 8 conspecifics [SB (O+V)_Ctrl] and focal fish administered with alarm substance and exposed simultaneously to shoal water and a shoal of 8 conspecifics [SB (O+V)_AS]. With our behavioural setup we were able to test eight focal fish per day (two fish per treatment). The control treatments allowed us to verify possible fear inducing responses introduced by the administration procedure in the experimental protocol. On the afternoon of the day before the test, fish were randomly removed from their stock tanks and isolated in each test tank overnight. This promoted setup habituation and produced a baseline effect. Per session, the order of the treatments attributed to each tank was done in a randomized fashion. To prepare the Alone_Ctrl and the Alone_AS treatments, 1.3 L of filtered water were placed both in the test and demonstrator tanks. To assemble the SB (O+V)_Ctrl and SB (O+V)_AS treatments, 800 mL of filtered water + 500 mL of shoal water (retrieved from the corresponding demonstrator tank) were placed in the test tank; and to the demonstrator tank (containing a mixed sex shoal of 4 females and 4 males zebrafish as for the case of these treatments) 500 mL of filtered water were added – this was done to re-establish the final volume of 1.3 L after the removal of the 500 mL of shoal water that were introduced in the test tank. The used 500 mL volume of shoal water was established to minimize the crowding stress induced to shoal due to lowering the water level - the same holds for experiment II (Ramsay et al., 2006). Shoals were composed by 8 conspecifics, as

this group size is within the range reported (2-10 and 2-30, as seen in (Pritchard et al., 2001) and (Rowena Spence et al., 2006), respectively) for number of fish integrating a shoal in zebrafish' floodplain habitats, which adds ecological relevance to the questions being formulated (the same holds for experiment II). Shoal members were always siblings and familiar to the focal fish (the same holds for experiment II and III) to avoid possible confounding effects of familiarity/unfamiliarity (Kiyokawa et al., 2014). We defined familiarity as being kin and tank mates (both in raising and stock tanks). Shoal assembly was done 2 days before testing (the same holds for experiments II and III), so that focal fish would have visual contact with a shoal with established hierarchies [after 3 hours of shoaling in a tank, zebrafish already exhibit distinct territories (Pérez-Escudero et al., 2014)]. Focal fish had visual contact with the demonstrator tank overnight to promote habituation and contribute to create the baseline effect. On the following day, all focal fish were tested and behaviour video recorded. Immediately after testing all focal fish were euthanized with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L) and brain macrodissection was performed as described in the section below. Zebrafish trunk samples were stored at -20ºC for posterior cortisol determination (see protocol below).

2.2.5 Behavioural trials

In all experiments 5 min (baseline) after video recording was initiated, 0.754 mL of filtered water (control treatments) or 0.754 mL of alarm substance (treatments with alarm substance) were delivered to the test tank through the PVC tubing with the help of a 10 mL Terumo® syringe. The alarm substance aliquot was thawed before testing was initiated and kept on ice throughout the entire experimental session to prevent AS degradation. The test lasted for 30 min, after which each focal fish was immediately euthanized with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L). Gender was confirmed by visual inspection of the gonads. Test and demonstrator tanks were sprayed with 70% ethanol and rinsed with filtered water between treatments, to eliminate hormones and odour residues. All test trials were conducted between 10:30 a.m. and 07:30 p.m. and the different experimental groups were intermixed throughout the day to account for possible diurnal variations in behaviour. A gap of 2.30 hours between lights onset and the start of the behavioural trials was used to prevent possible confounding effects of spawning behaviour that may have occurred in the shoals, since zebrafish are crepuscular breeders with a peak of mating at dawn (Engeszer, Patterson, et al., 2007; R. Spence et al., 2006).

2.2.6 Video tracking

All video files were analyzed with videotracker software (EthoVision® XT 8.0, Noldus) for extraction of the x, y, z coordinates of focal fish in each video frame. For each focal fish, 2 videos were analyzed corresponding to side and top views respectively (Figure 2). For tracking, a 10.5 × 11 cm 2D arena was defined for the side view and a 10.5 × 10.5 cm 2D arena was defined for the top view (Figure 2). Both arenas excluded glass walls and all fish were tracked at 25 frames sec^{-1} rate.

2.2.7 Behavioural analysis

Extracted x, y, z coordinates were subsequently analyzed using XYZ2b – Fish XYZ to Behaviour (see https://github.com/joseaccruz/xyz2b for detailed information and download), a custom-made code that combines a set of python scripts and infers erratic movement and freezing behaviour from x, y, z data produced by the EthoVision. Erratic movement is a complex behaviour characterized by sharp changes in direction or velocity, commonly associated with fast acceleration bouts and stochastic turns (Kalueff et al., 2013). Therefore, the xyz2b computes x,y,z data and decides on erratic movement behaviour if fish acceleration > 8 m/s^2. Freezing behaviour is described as a complete cessation of movement of zebrafish (except for opercula and eyes), while at the bottom of the tank (Kalueff et al., 2013). For this reason, the xyz2b computes x,y,z data and decides on freezing behaviour if two conditions apply: (1) fish velocity < 0.2 m/s; and (2) fish position on y axis is inside the bottom quarter of the arena (i.e. "freezing region" - as remaining in the bottom of the tank is one of the criteria for freezing behaviour, fish position on y axis was added to avoid false freezing computations when zebrafish is hovering (Kalueff et al., 2013), typically near the water surface). Because of camera's perspective distortion caused by water depth, the bottom quarter part of the tank was defined as threshold for the y position, as for the cases where fish exhibit freezing behaviour in the most distal area from the side camera, it is still included in the "freezing region". Erratic movement and freezing behaviours were only inferred when side and top views information was in accordance, as this contributed to a more accurate measurement of the behavioural parameters - avoiding false positives and false negatives (e.g. freezing was only considered when both side and top views data indicated velocity < 0.2 m/s). Total duration of erratic movement and freezing was calculated over 300 sec bins (5 min) and presented in percentage of total time. In the cases where 10 min bins are presented, data from the two corresponding 5 min bins was summed. The xyz2b generates a 3D plot (Figure 4B) representative of fish behaviour, in which the red dot size is proportional to freezing time and the grey line is representative of the remaining time in

swimming behaviour. In Figure 4B, the 3D plot for each treatment represents the behaviour of the fish that was the closest to the mean in the first 5 min after alarm substance onset. Since in our case we were focusing on freezing behaviour (Figure 3C), all remaining swimming behaviour not identified as freezing is grey coloured. However, xyz2b may be changed a priori for different colour and line thickness to be attributed to erratic movement and normal swimming independently. To test the performance of xyz2b detecting erratic movement and freezing behaviours, it was validated against human performance. For this validation, we used Observer® XT 7.0 (Noldus), commercial software for analysis of observational data. To test the performance of the xyz2b we applied it to 20 videos (first 5 min) of the treatment Alone_AS of the experiment I. We performed correlations (xyz2b vs. human) to validate xyz2b accuracy (Figures 3A-B). In order to test social buffering in zebrafish using alarm substance as a fearful event, we established the behavioural parameter (erratic movement or freezing) that better describes zebrafish response to this stimulus, using as reference the response to alarm substance when a fish is alone. Since in the case of our behavioural treatments we were exploring possible variations in fear behaviour due to absence or presence of conspecifics, we decided that the criteria for choosing the behavioural parameter were: 1) saliency of the behaviour - in terms of percentage of time; 2) consistency of the behaviour in time. Thus, the behaviour that was both salient and consistent in time, was the representative parameter of alarm reaction in our study.

2.2.8 Cortisol determination

A modified version of the protocol described in (Pavlidis et al., 2011b) was used to perform cortisol extraction. Trunk samples were thawed on ice, weighed and dissected into smaller portions for efficient homogenization. Dissection was performed while trunks were inside a 2 mL microcentrifuge tube to avoid tissue losses. 500 μL of enzyme immunoassay buffer (Cayman Chemical) was added and samples were vortexed for 3 sec. Samples were transferred to glass extraction tubes and homogenized on ice for 30 sec using a mechanical homogenizer (IKA Labortechnik) at 200 W. Another 500 μL volume of enzyme immunoassay buffer (Cayman Chemical) was poured through the blade into the extraction tubes to wash possible remaining tissue (between samples, the homogenization probe was washed with ethanol 70% and ultrapure water). After sonicated on ice for 30 sec, 3 mL of diethyl ether were added to the samples, following vortex for 15 sec. Samples were placed in an orbital stirrer for 10 min - 1000 rpm and then centrifuged at 2000 rpm for 15 min at 4ºC. Following centrifugation samples were frozen at – 80 ºC for 15 min and the top layer was poured into small glass tubes. Ether was evaporated with a speed vacuum centrifuge equipped with a cryotrap. Samples were then reconstituted in 1

mL of enzyme immunoassay buffer (Cayman Chemical) and stored at -20 ºC until further processing. Cortisol was quantified by the use of a commercial enzyme immunoassay kit (Cayman Chemical, MI, USA). All samples were tested in duplicate.

2.2.9 Brain macrodissection

Twelve fish out of the 20 fish used for the behavioural experiments were selected. Individuals closer to the mean value of their respective treatment were chosen, since they were considered the best representatives of the average population response for each behavioural condition. A modified version of the protocol described in (Teles, Dahlbom, Winberg, & Oliveira, 2013) was used to perform brain macrodissection. Fish were sacrificed immediately after the experimental procedure with an overdose of tricaine solution (MS222, Pharmaq; 500 – 1000 mg/L) and placed in an ice cold silicon base (with ice underneath) for posterior dissection. Fish were fixed to the base and the scales covering the brain area were removed. The brain was macro dissected under a stereoscope (Zeiss; Stemi 2000) into two areas: Telencephalon (Tel) and Diencephalon (Di). To separate Tel and Di, a 70º vertical cut was performed, as the Preoptic Area (POA) tissue remained in the Di samples. Immediately after collection, the brain tissue was placed in 200 µL of QIAzol Lysis Reagent (QIAGEN) in 1,5 mL microcentrifuge tubes and stored at −80 ºC until further analysis.

2.2.10 RNA extraction and DNA Synthesis

RNA extraction was performed with RNeasy® Lipid Tissue Mini Kit using the manufacturer protocol with minor modifications. RNA integrity was measured in 10% of all samples processed (randomly chosen) using 2100 Bioanalyzer (Agilent Technologies). Samples were stored at -80°C until cDNA synthesis. iScript™ cDNA Synthesis Kit (Bio-Rad) was used to synthetize the DNA. Samples were incubated in a termocycler (T100™ Thermal Cycler, Bio-Rad) in accordance with manufacturer's instructions (5min at 25°C, 60min at 42°C and 5min at 85°C) and stored at -20°C.

2.2.11 Primer design-optimization-efficiency and normalization to the reference gene

Primers were designed using Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012) following standard parameters with some changes (changes are presented in parenthesis): primer size (Min:18, Opt:20, Max:22); primer Tm (Min:57.0, Opt:60.0, Max:63.0); max Tm difference (3.0); primer GC% (Min:45.0, Opt:50.0, Max:60.0); product size ranges (100-200); and max GC in primer 3' end (3). Primers were selected on the Quick Primer Test tool of the FastPCR v5.4

(Kalendar, Lee, & Schulman, 2011, 2014) by presenting the minimum number of primer dimers at maximum sensitivity and quality≥80. Thereafter, Primer-BLAST (Ye et al., 2012) was used to confirm if the selected primers were specifically amplifying the target gene, without amplifying any other gene in the *Danio rerio* genome. Primers were commercially synthesized (Sigma-Aldrich, Hamburg, Germany). To ascertain the optimal annealing temperature, a PCR was conducted (T100™ Thermal Cycler, Bio-Rad) with a gradient of temperatures. Moreover, to ascertain if primers were amplifying the right sequences, PCR products were sequenced. Primers efficiency was optimized through a two-fold dilution series, in order to guarantee an accurate quantification regardless of the DNA template concentration. Target gene expression was normalized to the reference gene through the equation: $2^{Ct_{Ref}-Ct_{Target}}$, 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 its relative expression to the reference gene. Mean of the Cts of the three technical replicates were used. A technical replicate was discarded whenever its Ct was 0.5 below or above the mean Ct of the replicates. A sample did not undergo further runs if: 1) at least two of the three technical replicates were not discarded; 2) In the case that only two replicates remained, the absolute value of the difference between them was less than 0.5. Samples that did not fulfil these parameters were repeated for a maximum of three times. At last, if after these three runs the parameters were not fulfilled, the sample was discarded and considered as a missing value.

Table 1 | Primer sequences, amplicons length, and annealing parameters for the genes used in qPCR - experiment I.

Gene	Accession Number	Primer Sequence (5'→3')	Amplicon length (bp)	Ta (°C)	Time of annealing (s)	Source
18s	NM_173234.1	For – GCACATCCTTCGTGTCCTCAA Rev – ACCCTCTCAACCTCATCCTCA	171	61	30	Current thesis
crh	NM_001007379.1	For – GGCAACAGAAACCCGACTT Rev – CAACTTTCCCCTCCAACAGA	118	61	30	Current thesis
gr	NM_001020711.3	For – GCTCAATGGCACAGCTTCTT Rev – CCGGTGTTCTCCTGTTTGAT	126	59	30	Current thesis
mr	NM_001100403.1	For – CAACAACCGCAAGTCAGAAA Rev – TGTTGGGAAAAGCCAAAGTC	111	62	60	Current thesis

For – primer forward; Rev – primer reverse; Ta – Annealing temperature

2.2.12 Real-time PCR (qPCR)

qPCR was used to determine mRNA expression levels in target and reference genes. Three stress genes were examined: corticotropin-releasing hormone (*crh*), glucocorticoid receptor (*gr*), and mineralocorticoid receptor (*mr*). *18s rRNA* was used as reference gene. qPCR assays were performed using SYBR® Green PCR Master Mix (Applied Biosystems™, Thermo Fisher). Each reaction mix consisted of: 1.7µL nuclease-free water, 4µL of SYBR Green, and 0.15µL of each

primer (with a concentration of 50pmol/μL). Reagents were added into the mix by the previous order. Both reaction mix preparation and plate filling were performed on ice. Individual wells were filled with 6μL of reaction mix and 2μL of cDNA template. Three technical replicates were performed for each sample, all run in the same plate. In order to remove any possible air bubble caused by pipetting or possible droplets in the wells walls, plates underwent a short spin (until reach 16000-18000g) in a plate centrifuge (Heraeus® Multifuge® 3S Plus Centrifuge, Thermo Scientific). Plates were transported on ice and covered with aluminum foil. Quantification was measured on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher) using the following reaction protocol: (i) denaturation (5min at 95°C); (ii) amplification and quantification (40 cycles; 30s at 95°C, primer-specific annealing time and temperature, 30s at 72°C with a single fluorescence measurement); and (iii) melting curve assessment (30s at 95°C; 30s at 55°C, followed by an 55–95°C with a heating rate of 0.05°C/s and a continuous fluorescence measurement; 30s at 95°C).

2.2.13 Statistical analysis

All statistical analyses were performed on the statistical software packages STATISTICA v. 10 (StatSoft, Inc.) and SPSS® Statistics v. 21 (IBM). Normality and homogeneity of variance of the data were tested, and the appropriate statistics were used as required. Correlations were performed using Pearson's correlation. Apart from correlations, all behavioural statistical analysis followed the same approach. Repeated measures ANOVA were performed, with treatment as categorical predictor and freezing in each time bin (5 or 10 min) as dependent variable, to compare freezing between: 1) baseline and the 1st 5 min after alarm substance administration; 2) 10 min bins over the 30 min test. When sphericity was not assumed, repeated measures ANOVA with Greenhouse-Geisser correction (SPSS) was used. Repeated measures ANOVA were followed by LSD posthoc. All pairwise comparisons extracted from the LSD posthoc matrix were corrected for multiple comparisons using the sequential bonferroni correction (see explanation of the method below). Differences in cortisol and gene expression between treatments were assessed using one-way ANOVA with treatment as categorical predictor and gene expression in the Tel and Di macroareas as dependent variables. When homogeneity of variance was not assumed, one-way ANOVA with Welch correction (SPSS) was used. Sequential bonferroni corrections were performed for all pairwise comparisons to correct for multiple comparisons and corrected p values (p') are reported. Statistical significance was set at adjusted p value - $p' < 0.05$. Uncorrected p-values were first rank-ordered by significance (from the smallest to the highest p value) and then corrected using the following algorithm (for m = number of

hypothesis tested, i = hypothesis number, j = ranked position of p value): $p'_{(i)} = \max_{j \le i}\{(m - j + 1)p_{(j)}\}_1$, where $\{x\}_1 \equiv \min(x,1)$. The procedure of correction was applied until the uncorrected p value stopped being significant, $p > 0.05$.

2.3 Results

2.3.1 Freezing is the most accurate behavioural parameter and freezing % is stable across experiments (independently of different alarm substance sets)

The performance of xyz2b detecting erratic movement and freezing behaviours was validated against a human observer for 20 videos (first 5 min) of the treatment Alone_AS of the experiment I. The validation of xyz2b against a human observer revealed that the custom-made code and human quantifications of erratic movement (Figure 3A) and freezing (Figure 3B) were positively correlated ($r = 0.84$, $p < 0.001$ and $r = 0.97$, $p < 0.001$, respectively), indicating that xyz2b accurately measures these behaviours. Also, erratic movement and freezing behaviours were compared during the 30 min test (10 min bins) to assess the parameter that better describes zebrafish response to alarm substance (20 videos from the treatment Alone_AS of the experiment I were used) and freezing was shown to be the most frequent and consistent parameter over time (Figure 3C), with higher percentages of freezing behaviour in all 10 min bins of the entire 30 min test ($p' < 0.001$). Repeated measures ANOVA showed differences for behaviour $F_{(1, 38)} = 77.189$; $p < 0.001$ and time $F_{(2, 76)} = 3.713$; $p < 0.05$. No differences were found for the interaction between behaviour and time $F_{(2, 76)} = 0.246$; $p = 0.783$. * $p' < 0.05$; ** $p' < 0.01$ and *** $p' < 0.001$. Furthermore, alarm substance efficiency across experiments was tested. Neither the acute [first 5 min (Figure 3D): $F_{(2, 57)} = 0.587$; $p = 0.560$] nor the sustained [30 min (Figure 3E), analyzed in 10 min bins: $F_{(2, 57)} = 0.182$; $p = 0.834$] freezing response to the alarm substance varied across experiments. These results indicate that different alarm substance sets do not exhibit significant variation between experiments. Again, the 20 videos from the Alone_AS treatment were used for this analysis.

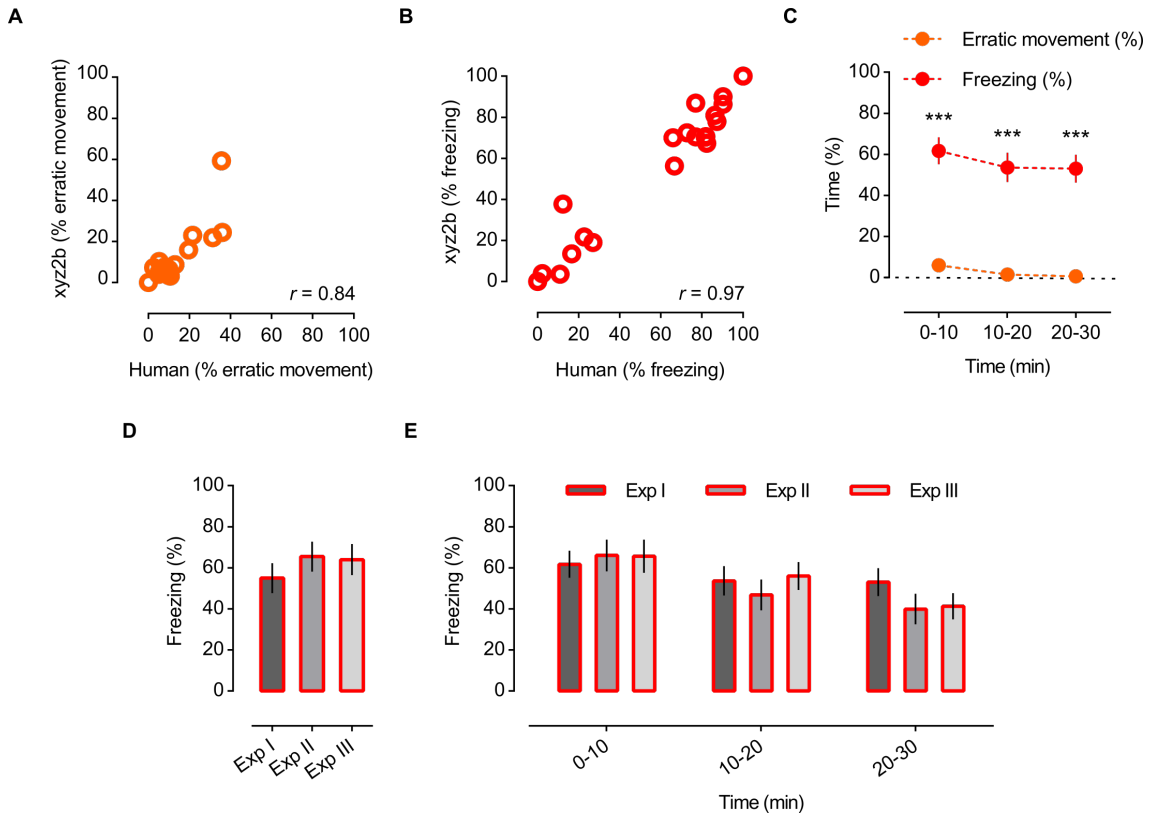

Figure 3| Freezing is the most accurate behavioural parameter and freezing % is stable across experiments (independently of different alarm substance sets).
(**A-B**) Validation of xyz2b against a human observer. xyz2b and human quantifications of erratic movement (**A**) and freezing (**B**) are shown in orange and red open circles, respectively. Pearson's correlation coefficients *r* are shown. (**C**) Erratic movement vs. Freezing. An analysis of the 30 min test in 10 min bins is displayed. Mean ± SEM values of erratic movement and freezing % are shown in orange and red dots, respectively. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001. (**D-E**) Alarm substance efficiency across experiments. (**D**) Acute response (first 5 min). Mean ± SEM values of freezing % are shown in the three different experiments. (**E**) Sustained response (30 min). Mean ± SEM values of freezing % are shown in the three different experiments.

2.3.2 Social Buffering in zebrafish

In the first 5 min of exposure to alarm substance (Figure 4C), focal fish in the SB (O+V)_AS treatment presented significantly lower freezing responses than fish in the Alone_AS, indicating social buffering. There were no differences between the treatments in baseline and water administration did not elicit freezing in the control treatments (Figure 4C). Repeated measures ANOVA with "time" as within-subject factor showed differences for time $F_{(1.0,\ 76.0)} = 68.418$, $p < 0.001$; treatment*time: $F_{(3.0,\ 76.0)} = 32.714$, $p < 0.001$; and treatment: $F_{(3,\ 76)} = 20.157$, $p < 0.001$. Corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc) are reported: * p' < 0.05; ** p' < 0.01 and *** p' < 0.001 (Figure 4C). A more detailed analysis over the 30 min test (0-10 min; 10-20 min; 20-30 min) revealed that focal fish in the SB (O+V)_AS treatment presented significantly lower freezing responses than fish in the Alone_AS

throughout time, indicating sustained social buffering (Figure 4D). Repeated measures ANOVA with "time" as within-subject factor showed differences for time: $F_{(2, 76)} = 4.141$, $p = 0.020$; treatment*time: $F_{(2, 76)} = 0.006$, $p = 0.994$; treatment: $F_{(1, 38)} = 38.798$, $p < 0.001$. Corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc) are reported: * p' < 0.05; ** p' < 0.01 and *** p' < 0.001 (Figure 4D).

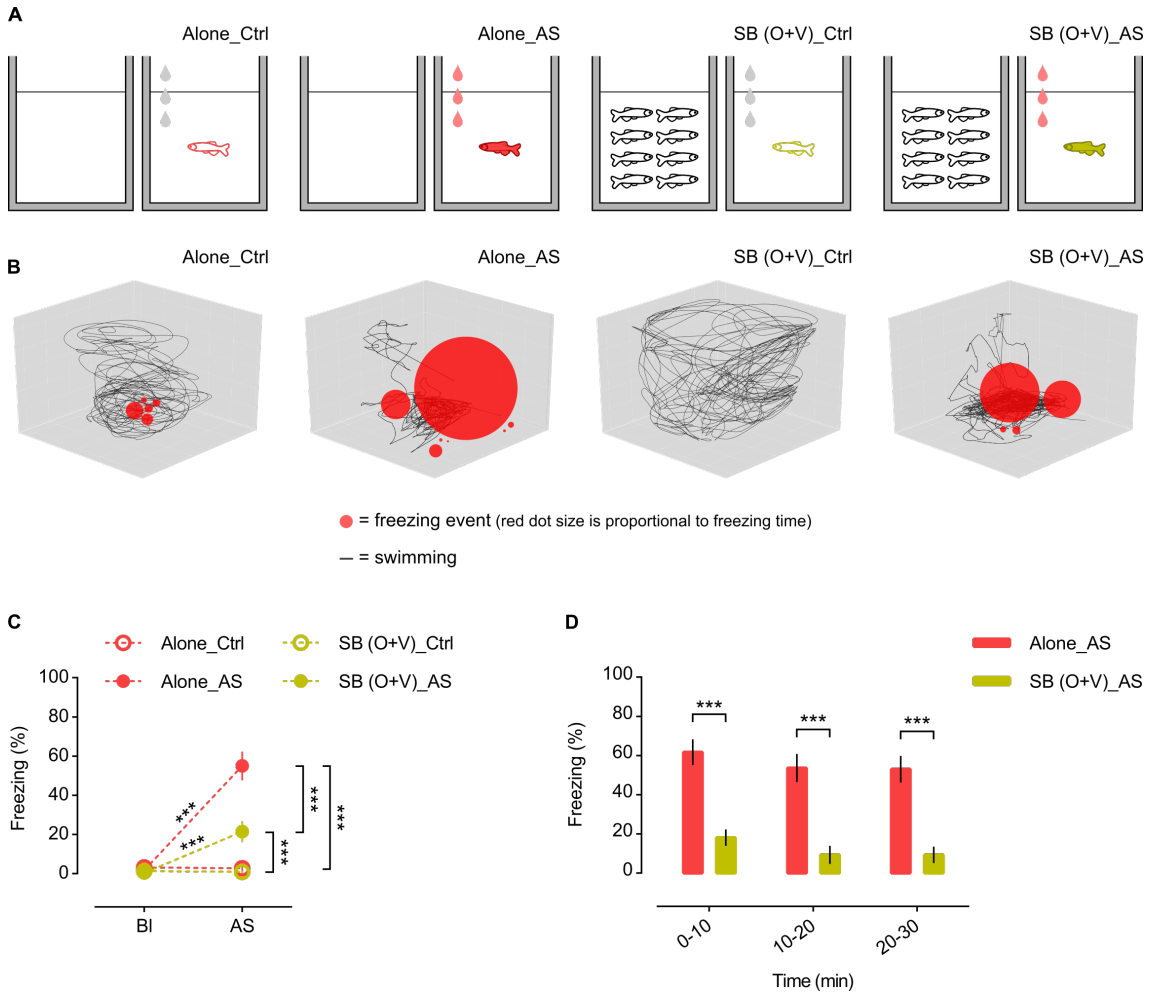

Figure 4 | Social buffering in zebrafish.
(**A**) Schematic representation of the behavioural treatments. From left to right: Alone_Ctrl - alone focal fish (red outline) administered with water; Alone_AS - alone focal fish (red filling) administered with alarm substance; SB (O+V)_Ctrl - focal fish (green outline) administered with water and exposed simultaneously to shoal water and a shoal of 8 conspecifics and SB (O+V)_AS - focal fish (green filling) administered with alarm substance and exposed simultaneously to shoal water and a shoal of 8 conspecifics. Grey and red drops represent water and alarm substance administration, respectively. (**B**) 3D plots representative of each behavioural treatment. From left to right: Alone_Ctrl; Alone_AS; SB (O+V)_Ctrl and SB (O+V)_AS. Each 3D plot exhibits a graphical representation of the first 5 min after AS onset for the focal fish closest to the mean in each treatment. Each freezing event is represented by a red dot. Red dot size is proportional to freezing time. The total freezing time for each treatment is: Alone_Ctrl - 1.95 %; Alone_AS - 52.24 %; SB (O+V)_Ctrl - 0.00 % and SB (O+V)_AS - 23.32 %. Grey line represents all the swimming behaviour besides freezing. (**C**) Freezing % in baseline (Bl) vs. first 5 min after AS onset (AS). Open red circles: Alone_Ctrl; filled red circles: Alone_AS; open green circles: SB (O+V)_Ctrl and filled

green circles: SB (O+V)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001. (**D**) Freezing % over the 30 min test in 10 min bins (0-10 min; 10-20 min; 20-30 min). Red bars: Alone_AS and green bars: SB (O+V)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001.

2.3.3 Social Buffering of stress in zebrafish

Cortisol quantification for the 30 min exposure to AS for each behavioural treatment was determined. One-way ANOVA with treatment as categorical predictor and gene expression in the Tel and Di macroareas as dependent variables showed no differences between the treatments: $F_{(3, 23.373)} = 0.939$, $p = 0.438$ (Figure 5A). *crh*, *mr* and *gr* gene expression and MR/GR ratio[8] in the Tel and Di (Figures 5B-E) revealed no differences between behavioural treatments [*crh*: $F_{(6, 84)} = 0.402$, $p = 0.876$; *mr:* $F_{(6, 84)} = 0.456$, $p = 0.839$; *gr:* $F_{(6, 84)} = 0.368$, $p = 0.898$; MR/GR ratio: $F_{(6, 84)} = 1.343$, $p = 0.247$]. These findings did not reveal an influence of the social buffering phenomenon in the AS-induced stress response. In fact these results showed that the 30 min of exposure to AS (when zebrafish are alone) were not sufficient to observe an HPI axis activation at the endocrine and neural level.

2.4 Discussion

At first, our findings demonstrate that our custom-made code (xyz2b) accurately measures erratic movement and freezing (Figures 3A-B) and that freezing is the most frequent and consistent behaviour over the 30 min exposure to alarm substance (Figure 3C). These results suggest that freezing is the behavioural parameter that better describes zebrafish response to AS in a 30 min context. Thus, freezing quantifications will support all behavioural results across experiments. Moreover, we show (Figures 3D-E) that the skin extract preparation that conveys the alarm substance is stable across experiments when prepared controlling for sex-ratio, age and total number of donor fish, enhancing the use of alarm substance as a stable natural threat-inducing stimulus.

Altogether, the results presented in this chapter demonstrate for the first time the occurrence of sustained social buffering of fear in zebrafish. The findings presented show that the presence of conspecific cues reduces the fear response to AS (Figure 4D). A previous study conducted with zebrafish GR mutants has already shown that the presence of a conspecific (WT) diminishes GR fish freezing behaviour when exposed to a stressful environment (novel tank), a phenomenon that was not observed when GR mutants were exposed alone to the novel tank (Ziv et al., 2013).

[8] MR/GR ratio is low when MR (but not GR) is fully occupied with cortisol; MR/GR ratio is high when cortisol levels are high, causing an increased occupation of GR and a desensitization of MR (Hyman, 2001).

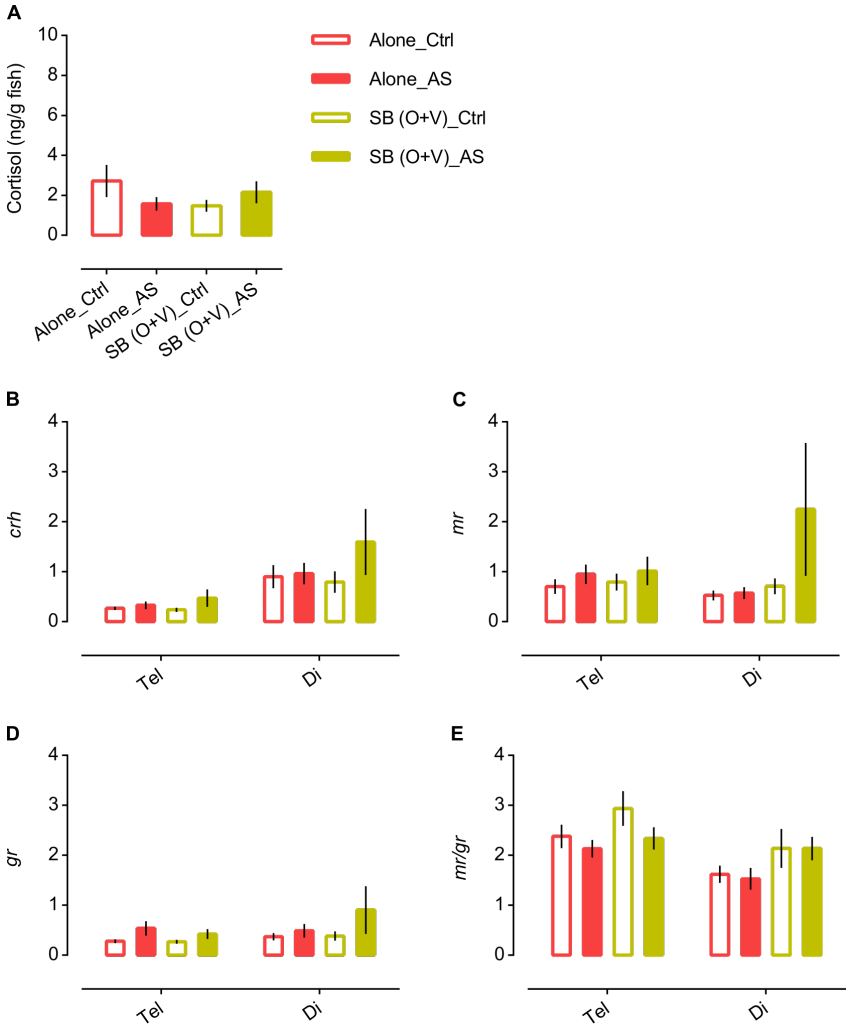

Figure 5 | Social buffering of the stress response in zebrafish.
(**A**) Whole-body cortisol (ng/g fish) quantification for the 30 min exposure to AS for each behavioural treatment. Legend from top to bottom: Alone_Ctrl - alone focal fish (red outline) administered with water; Alone_AS - alone focal fish (red filling) administered with alarm substance; SB (O+V)_Ctrl - focal fish (green outline) administered with water and exposed simultaneously to shoal water and a shoal of 8 conspecifics and SB (O+V)_AS - focal fish (green filling) administered with alarm substance and exposed simultaneously to shoal water and a shoal of 8 conspecifics. The same labelling applies for all the following graphs (B-E). Mean ± SEM values are shown. (**B**) *crh* gene expression in the Tel and Di for all behavioural treatments. Mean ± SEM values are shown. (**C**) *mr* gene expression in the Tel and Di for all behavioural treatments. Mean ± SEM values are shown. (**D**) *gr* gene expression in the Tel and Di for all behavioural treatments. Mean ± SEM values are shown. (**E**) MR/GR ratio in the Tel and Di for all behavioural treatments. Mean ± SEM values are shown.

Nevertheless, Ziv and co-workers did not report evidence for the buffering effect in the heterospecific or WT zebrafish lines (Ziv et al., 2013), suggesting that the results shown for the mutant line were presumably associated with the HPI axis hyperactivity promoted by the disruption of GR genomic activity. Thus, our findings provided more clear and robust evidence

for the existence of sustained buffering in a wild-derived strain and in an ecologically relevant context (alarm substance as threat-inducing stimulus).

Besides reducing fear, social buffering is also known to be responsible for lessening the stress response to threat-inducing stimulus (Edgar et al., 2015; Kiyokawa et al., 2004; Levine et al., 1978; A. S. Smith & Wang, 2014). However, our results did not show evidence for social buffering of the stress response (Figure 5). Neither cortisol, nor gene expression of genes involved in the stress response (*crh, mr, gr*) revealed an effect of conspecifics' presence on the response to AS. Also, results regarding *mr*/*gr* ratio did not show differences between the different behavioural treatments (Figure 5). Nonetheless, AS itself did not elicit a stress response in zebrafish that were alone, suggesting that the absence of buffering of the stress response should not necessarily be interpreted as a negative result. In fact, a previous study in the lab (data not shown) demonstrated a significant cortisol increase 5 min post-stress, corroborating the fast HPI axis activation in response to this natural stressor. Accordingly, most studies addressing acute stress response in zebrafish have observed that HPI axis activation (cortisol, CRF or GR differences) peaked until 15 min post-stress (Alderman & Vijayan, 2012; Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015; Ramsay et al., 2009). Thus, it seems plausible to suggest that HPI axis activation occurred shortly after alarm substance onset, as no differences in cortisol, *crh, mr, gr* or *mr*/*gr* ratio were verified 30 min post-stress exposure. Therefore, studies aiming at describing HPI axis dynamics (e.g. different periods of exposure to AS - 5, 10, 15, 20, 25, 30 min) with or without conspecific cues present are needed in order to better dissect the social buffering of stress response in zebrafish (please see 5.5.1 for a further discussion of this topic).

Chapter 3 |
Effectiveness of visual and olfactory shoal cues at inducing Social Buffering - experiment II

3.1 Introduction

The social buffering phenomenon relies on the transmission of sensory information between conspecifics (da Costa et al., 2004; Rukstalis & French, 2005; Takahashi et al., 2013). Evidence for the contribution of different sensory channels in to the social buffering process has been shown in several species and across different experimental contexts (visual cues in sheep, da Costa et al., 2004; vocal cues in monkey marmosets, Rukstalis & French, 2005; olfactory cues in rats, Takahashi et al., 2013; tactile cues in rats, Wilson, 2001). Nonetheless, most studies have been focusing on the contribution of a specific sensory modality to the social buffering effect, rather than exploring the different sensory channels that may be carrying social information of "safety" and its effectiveness in short and long-term threatening scenarios. Thus, we set to explore the effectiveness of olfactory and visual cues in zebrafish social buffering and its separate efficiency in short or longer exposures to alarm substance. It is possible that other sensory modalities contribute to the social buffering process in zebrafish (e.g. tactile stimulation is known to reduce fear in this fish species (Schirmer, Jesuthasan, & Mathuru, 2013), nonetheless we focused on the olfactory and visual sensory channels, as they were easier to control experimentally. Furthermore, unravelling the most effective sensory channel contributing to the social buffering effect in zebrafish will enable a more precise dissection of the neuromolecular mechanisms underlying this social behaviour. Thus, the main goals of the work presented in this chapter were 1) to investigate the sensory cues promoting the buffering effect in zebrafish and 2) to explore the efficiency of these different sensory channels at inducing social buffering in short or long-lasting exposures to threat.

3.2 Methods

Fish and housing, alarm substance extraction, experimental setup, behavioural trials, video tracking and behavioural analysis information is available in section 2.2. The same procedures hold for this experiment.

3.2.1 Experimental procedures

A total of 80 focal naïve male zebrafish were used (20 per treatment). Each focal fish was submitted to a single test corresponding to one of four possible treatments (see Figure 6A): alone focal fish administered with alarm substance (Alone_AS); focal fish administered with alarm substance and exposed to shoal water [SB (O)_AS]; focal fish administered with alarm substance and exposed to a shoal of 8 conspecifics [SB (V)_AS] and focal fish administered with alarm

substance and exposed simultaneously to shoal water and a shoal of 8 conspecifics [SB (O+V)_AS]. As in experiment I, we were able to test eight focal fish per day (two fish per treatment). On the afternoon of the day before the test, fish were randomly removed from their stock tanks and isolated in each test tank overnight. This promoted setup habituation and produced a baseline effect. Per session, the order of the treatments attributed to each tank was done in a randomized fashion. To prepare the Alone_AS treatment, 1.3 L of filtered water were placed both in the test and demonstrator tanks. In the SB (O)_AS treatment, 800 mL of filtered water + 500 mL of shoal water (retrieved from a demonstrator tank from the SB (V)_AS treatment) were placed in the test tank and the demonstrator tank was filled with 1.3 L of filtered water. In the SB (V)_AS treatment, 1.3 L of filtered water were placed in the test tank and 500 mL of filtered water were added to the demonstrator tank (containing a mixed sex shoal of 4 females and 4 males zebrafish) to re-establish the final volume of 1.3 L after the removal of the 500 mL of shoal water that were included in the test tank of the SB (O)_AS treatment. In the SB (O+V)_AS treatment, 800 mL of filtered water + 500 mL of shoal water (retrieved from the corresponding demonstrator tank) were placed in the test tank, and 500 mL of filtered water were added to the demonstrator tank (containing a mixed sex shoal of 4 females and 4 males zebrafish) to re-establish the final volume of 1.3 L after the removal of the 500 mL of shoal water that were transferred to the test tank. Focal fish had visual contact with the demonstrator tank overnight to promote habituation and contribute to create the baseline effect. On the following day, all focal fish were tested and behaviour video recorded.

3.2.2 Statistical analysis

All statistical analyses were performed on the statistical software packages STATISTICA v. 10 (StatSoft, Inc.) and SPSS® Statistics v. 21 (IBM). Normality and homogeneity of variance of the data were tested, and the appropriate statistics were used as required. Repeated measures ANOVA were performed, with treatment as categorical predictor and freezing in each time bin (5 or 10 min) as dependent variable, to compare freezing between: 1) baseline and the 1st 5 min after alarm substance administration; 2) 10 min bins over the 30 min test. When sphericity was not assumed, repeated measures ANOVA with Greenhouse-Geisser correction (SPSS) was used. Repeated measures ANOVA were followed by LSD posthoc. All pairwise comparisons extracted from the LSD posthoc matrix were corrected for multiple comparisons using the sequential bonferroni correction (see explanation of the method in the section 2.2). Statistical significance was set at adjusted p value - $p' < 0.05$.

3.3 Results

3.3.1 Sensory cues effectiveness – olfactory vs. visual

Whereas there were no differences in baseline freezing levels (i.e. before exposure to AS, Figure 6B), either olfactory only, visual only, or both olfactory and visual shoal cues together were effective in reducing freezing behaviour after exposure to AS - 1st 5 min of exposure (repeated measures ANOVA with "time" as within-subject factor; time: $F_{(1,\ 76)} = 74.352$, $p < 0.001$; treatment*time: $F_{(3,\ 76)} = 8.016$, $p < 0.001$; treatment: $F_{(3,\ 76)} = 10.137$, $p < 0.001$; corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc): Alone vs. O: $p' < 0.001$; Alone vs. V: $p' < 0.001$; Alone vs. O+V: $p' < 0.001$; Figure 6B). However, a more detailed analysis over time (30 min exposure) showed that although both olfactory and visual cues were equally effective in the first 10 min of the test, the visual cue was more effective in decreasing the freezing response in the last 20 min (repeated measures ANOVA with "time" as within-subject factor; time: $F_{(1.6,\ 120.625)} = 11.723$, $p < 0.001$; treatment: $F_{(3,\ 76)} = 10.284$, $p < 0.001$; treatment*time: $F_{(4.8,\ 120.625)} = 3.871$, $p = 0.003$; corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc): O vs. V, 0-10 min: $p' = 0.194$; O vs. V, 10-20 min: $p' = 0.021$; O vs. V, 20-30 min: $p' = 0.009$; Figure 6C). Moreover, in the last 20 min the visual cue was as effective as the visual and olfactory cues combined, corroborating the higher effectiveness of the sight of the shoal in the buffering phenomenon [corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc): V vs. O+V, 0-10 min: $p' = 0.281$; V vs. O+V, 10-20 min: $p' = 0.338$; V vs. O+V, 20-30 min: $p' = 0.788$; O vs. O+V, 0-10 min: $p' = 0.019$; O vs. O+V, 10-20 min: $p' = 0.001$; O vs. O+V, 20-30 min: $p' = 0.004$; Figure 6C)].

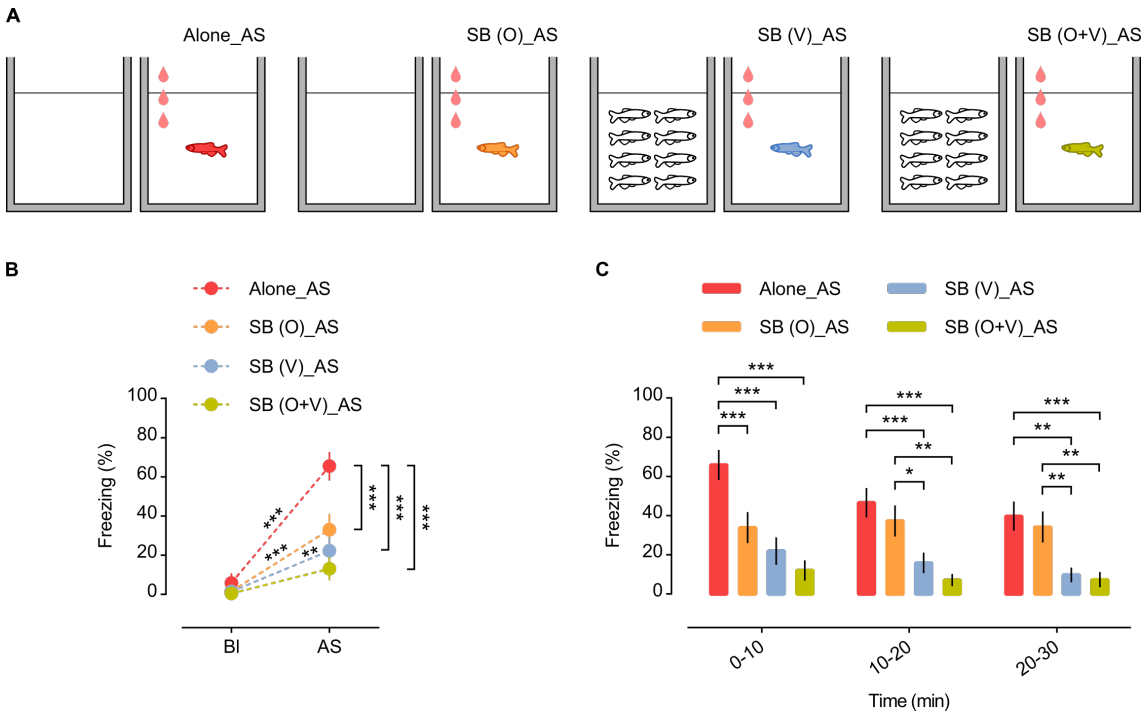

Figure 6 | Sensory cues effectiveness – olfactory vs. visual.
(**A**) Schematic representation of the behavioural treatments. From left to right: Alone_AS - alone focal fish (red filling) administered with alarm substance; SB (O)_AS - focal fish (orange filling) administered with alarm substance and exposed to shoal water; SB (V)_AS - focal fish (blue filling) administered with alarm substance and exposed to a shoal of 8 conspecifics and SB (O+V)_AS - focal fish (green filling) administered with alarm substance and exposed simultaneously to shoal water and a shoal of 8 conspecifics. Red drops represent alarm substance administration. (**B**) Freezing % in baseline (Bl) vs. first 5 min after AS onset (AS). Filled red circles: Alone_AS; filled orange circles: SB (O)_AS; filled blue circles: SB (V)_AS and filled green circles: SB (O+V)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001. (**C**) Freezing % over the 30 min test in 10 min bins (0-10 min; 10-20 min; 20-30 min). Red bars: Alone_AS; orange bars: SB (O)_AS; blue bars: SB (V)_AS and green bars: SB (O+V)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001.

3.4 Discussion

Our results showed that both olfactory and visual cues are equally effective in promoting social buffering in the first 10 min of exposure to alarm substance (Figure 6B). Nevertheless, when analyzing the response to AS over time, our results revealed that the visual cue was more effective in decreasing zebrafish' freezing behaviour (Figure 6C). In fact, the visual cue was as effective as the treatment where both olfactory and visual cues were present (Figure 6C), enhancing the efficiency of the sight of shoal in diminishing zebrafish fear responses.

Our findings corroborate the evidence in other animals for the contribution of different sensory cues in eliciting social buffering (da Costa et al., 2004; Rukstalis & French, 2005; Takahashi et al., 2013; Wilson, 2001). In addition, to our knowledge, we show for the first time the long-term effectiveness of different sensory modalities in the social buffering of AS-elicited

fear responses. These findings suggest that the long-term reliability of shoal cues signalling safety depend on sensory modality, and this may be due to the refresh rate of information typical of each sensory channel. The probability of a given information being correct or not has been defined as information reliability (Koops, 2004). Thus, in the initial phase of exposure to the AS, both visual and olfactory shoal cues are equally reliable, since both represent conspecifics presence. However, as time goes by available visual information from the shoal is constantly updated (that is, the shoal is a dynamic visual cue resembling safety, as non-fearful shoal mates indicate a secure environment), as for the case of olfactory cues, information will remain unaltered in the water, therefore preventing updates in the olfactory channel. Hence, after an initial phase visual cues are expected to be more reliable than olfactory ones in signalling a safe environment. Sensory cues effectiveness, the possible factors influencing sensory modalities efficiency and its influence in the social buffering effect, as well as future experiments addressing these questions will be further discussed in section 5.3 and 5.5.2.

Chapter 4 |
Shoal size modulation & neuromolecular mechanisms underlying Social Buffering

4.1 Introduction

Shoal cues efficiency may depend on shoal size, since a larger shoal should both be more conspicuous and more reliable (i.e. a larger number of individuals conveying the same information). In fact, species were shown to form larger group sizes in response to threatening stimulus (Hoare et al., 2004) and greater groups have been shown to modulate responses to aversive events (Brown & Bongiorno, 2006; Magurran & Pitcher, 1987). More specifically, a study by Magurran and Pitcher verified that minnows recover faster from a pike (predator) confrontation when shoalling in larger group sizes. Groups of 20 and 50 minnows were more efficient in aiding post-stress (predator confrontation) recovery than groups of 10 (Magurran & Pitcher, 1987). This study highlights the antipredatoty benefits of group living and suggests that larger collectives may be more efficient at inducing the social buffering phenomenon.

Thus, in the third experiment we tested if conspecific number modulates the effectiveness of the visual cue in the reduction of the fear response. In order to do this three different mixed-sex shoal sizes were used (2, 4 and 8). In this third experiment only the visual cue was used since in the previous experiment (II) it was shown that the sight of shoal was more effective at buffering the response to AS in a long-lasting exposure to threat. Finally, we used the expression of the immediate early gene *c-fos* as a marker of neuronal activity (Lanahan & Worley, 1998; Okuno, 2011) to characterize the pattern of neuronal activation during social buffering across a set of brain nuclei that are putative homologues to those in mammals (Ganz et al., 2012, 2015) involved in social buffering (Neumann et al., 2000; A. S. Smith & Wang, 2014), fear (Quirk et al., 1995; Sparta et al., 2014; Tovote et al., 2015), anxiety (Davis et al., 2010; Duvarci et al., 2009; Tovote et al., 2015) and affective states regulation (Sheehan et al., 2004; Tovote et al., 2015), namely the medial part of the dorsal telencephalon (Dm, homologue of the mammalian pallial amygdala), the supracommissural nucleus of the ventral telencephalon (Vs, homologue of the mammalian subpallial amygdala), the ventral nucleus of the ventral telencephalon (Vv, homologue of the mammalian septum), and the preoptic area (POA, homologue of the mammalian preoptic area / paraventricular nucleus of the hypothalamus) (Goodson & Kingsbury, 2013; Maximino, Lima, Oliveira, Batista, & Herculano, 2013; O'Connell & Hofmann, 2011). Since it has been recently shown that behavioural states in zebrafish are better associated with patterns of co-activation of relevant brain nuclei than with activation of specific brain regions (Teles et al., 2015), we have analyzed the effects of social buffering both on the activation levels of each of the brain nucleus per se and on the patterns of co-activation across nuclei. Differences in co-activation patterns between treatments were assessed by testing the association between the correlation matrices for *c-fos* expression across brain nuclei for each treatment, using

the quadratic assignment procedure (QAP) (Borgatti, Everett, & Johnson, 2013). Co-activation patterns reflect functional connectivity among areas, which was also characterized using cohesion and centrality network measures. Thus, the aims of the work reported in this chapter were 1) to investigate if the number of conspecifics modulates social buffering and 2) to explore the neuromolecular mechanisms underlying this social behaviour in zebrafish.

4.2 Methods

Fish and housing, alarm substance extraction, experimental setup, behavioural trials, video tracking, behavioural analysis, RNA extraction and DNA synthesis and primer design-optimization-efficiency and normalization to the reference gene information is available in section 2.2. The same procedures hold for this experiment.

4.2.1 Experimental procedures

A total of 160 focal naïve male zebrafish were used (20 per treatment). Each focal fish was submitted to a single test corresponding to one of eight possible treatments (Figure 8A): alone focal fish administered with water (Alone_Ctrl); alone focal fish administered with alarm substance (Alone_AS); focal fish administered with water and exposed to a shoal of 2 conspecifics [SB (2)_Ctrl]; focal fish administered with alarm substance and exposed to a shoal of 2 conspecifics [SB (2)_AS]; focal fish administered with water and exposed to a shoal of 4 conspecifics [SB (4)_Ctrl]; focal fish administered with alarm substance and exposed to a shoal of 4 conspecifics [SB (4)_AS]; focal fish administered with water and exposed to a shoal of 8 conspecifics [SB (8)_Ctrl] and focal fish administered with alarm substance and exposed to a shoal of 8 conspecifics [SB (8)_AS]. In the case of this experiment, shoals were defined as groups of 2 to 8 conspecifics, which is a fluctuation in terms of group size that is within the variation reported (2-10 and 2-30, as seen in (Pritchard et al., 2001) and (Rowena Spence et al., 2006), respectively) for number of fish integrating a shoal in the wild. We were able to test eight focal fish per day (one fish per treatment). On the afternoon of the day before the test, fish were randomly removed from their stock tanks and isolated in each test tank overnight. This promoted setup habituation and produced a baseline effect. Per session, the order of the treatments attributed to each tank was done in a randomized fashion. To prepare the Alone_Ctrl and Alone_AS treatments, 1.3 L of filtered water were placed both in the test and demonstrator tanks. To assemble the [SB (2)_Ctrl] and [SB (2)_AS] treatments, 1.3 L of filtered water were placed in the test tank. In these treatments, the demonstrator tanks contained a mixed sex shoal

of 1 female and 1 male zebrafish. To prepare the [SB (4)_Ctrl] and [SB (4)_AS] treatments, 1.3 L of filtered water were placed in the test tank. In the case of these treatments, the demonstrator tanks contained a mixed sex shoal of 2 females and 2 males zebrafish. To prepare the [SB (8)_Ctrl] and [SB (8)_AS] treatments, 1.3 L of filtered water were placed in the test tank. In the case of these treatments, the demonstrator tanks contained a mixed sex shoal of 4 females and 4 males zebrafish. Regarding the demonstrator tanks containing shoals, 500 mL of shoal water were removed and re-established by 500 mL of filtered water – this was done to replicate the shoal water removal of previous experiments (I and II), as to have all demonstrator tanks with shoals under the same experimental conditions. Focal fish had visual contact with the demonstrator tank during the overnight period to contribute to create the baseline effect.

4.2.2 Brain microdissection protocol

Only fish from the treatments Alone_Ctrl, Alone_AS, SB (8)_Ctrl and SB(8)_AS were subjected to the brain microdissection protocol. Twelve fish out of the 20 fish used for the behavioural experiments were selected. Individuals closer to the mean value of their respective treatment were chosen, since they were considered the best representatives of the average population response for each behavioural treatment. Zebrafish embedded heads were retrieved from -80°C and sliced on a cryostat (Leica CM 3050 S) set at -22°C, in serial 150μm-thick sections in the coronal plane, that were thaw mounted in regular glass slides, and refrozen in the cryostat. Sections were sequentially collected from the point where olfactory bulbs were visible until the tectal ventricles were evident. Once all sections of interest were sampled, they were microdissected under a stereoscopic microscope (Nikon SMZ745) on top of a cold plate. Brain nuclei were identified and classified as in (Wullimann et al., 1996), and harvested with a modified 27G needle (inner diameter = 210μm). To minimize the risk of cross-contamination a single needle was used per brain nucleus, and a new set of needles was used for each fish. To prevent RNA degradation, all needles were cleaned with RNaseZAP™ (Sigma-Aldrich, Hamburg, Germany), followed by 70% ethanol, and dried overnight in an incubator (VWR INCU-Line® IL10) at 70°C. With this technique we collected tissue from brain nucleus known to be involved in behavioural responses implicated in the social buffering phenomenon, namely: fear (Herry et al., 2006; Quirk et al., 1995; Sparta et al., 2014; Tovote et al., 2015): medial zone of the dorsal telencephalic area (Dm - putative homologue of the mammalian basolateral amygdala (O'Connell & Hofmann, 2011)); anxiety/sustained fear (Davis et al., 2010; Duvarci et al., 2009; Tovote et al., 2015): supracommissural nucleus of the ventral telencephalic area [Vs - putative homologue of the mammalian extended amygdala (comprising the medial amygdala and bed nucleus of stria

terminalis (O'Connell & Hofmann, 2011))]; oxytocin secretion (Neumann et al., 2000; A. S. Smith & Wang, 2014): preoptic area (POA - putative homologue of the mammalian homonym/preoptic area (O'Connell & Hofmann, 2011)); and regulation of affective states (Sheehan et al., 2004; Tovote et al., 2015): ventral nucleus of the ventral telencephalic area (Vv - putative homologue of the mammalian septum (O'Connell & Hofmann, 2011)), which also shows high connectivity with the POA (O'Connell & Hofmann, 2011). Oxytocin neurons are known to be related with social buffering in mammals (A. S. Smith & Wang, 2014) and are located in the PVN - paraventricular nucleus of the hypothalamus (Kikusui et al., 2006; Neumann et al., 2000), putative homologue of the parvocellular cell group of the teleost POA (O'Connell & Hofmann, 2011). All brain nuclei were sampled in both hemispheres at once (Figure 7). The harvested tissue was immediately injected with a 10 mL syringe into a 1,5 mL autoclaved microtube filled with 50 µL QIAzol Lysis Reagent (Qiagen). Except for Vs, more than one punch was harvested for each brain nucleus (Figure 7). Punches from the same brain nucleus were pooled into the same microtube. Samples were kept on ice and covered with aluminum foil until all slides were processed and stored at -80°C until RNA extraction.

4.2.3 Real-time PCR (qPCR)

qPCR was used to determine mRNA expression levels of an immediate early gene (*c-fos)* and the reference gene (*18s rRNA). C-fos* is a transient marker of neuronal activity and its expression has been used to characterize brain activation in response to behavioural manipulations (Teles et al., 2015). qPCR assays were performed using SYBR® Green PCR Master Mix (Applied Biosystems™, Thermo Fisher). Each reaction mix consisted of: 1.7 µL nuclease-free water, 4 µL of SYBR Green, and 0.15 µL of each primer (with a concentration of 50 pmol/µL). Three technical replicates were performed for each sample, all run in the same plate. Quantification was measured on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher) using the following reaction protocol: (i) denaturation (5 min at 95°C); (ii) amplification and quantification (40 cycles; 30 s at 95°C, primer-specific annealing time and temperature, 30 s at 72°C with a single fluorescence measurement); and (iii) melting curve assessment (30 s at 95°C; 30 s at 55°C, followed by an increase from 55–95°C with a heating rate of 0.05°C/s and a continuous fluorescence measurement; 30s at 95°C).

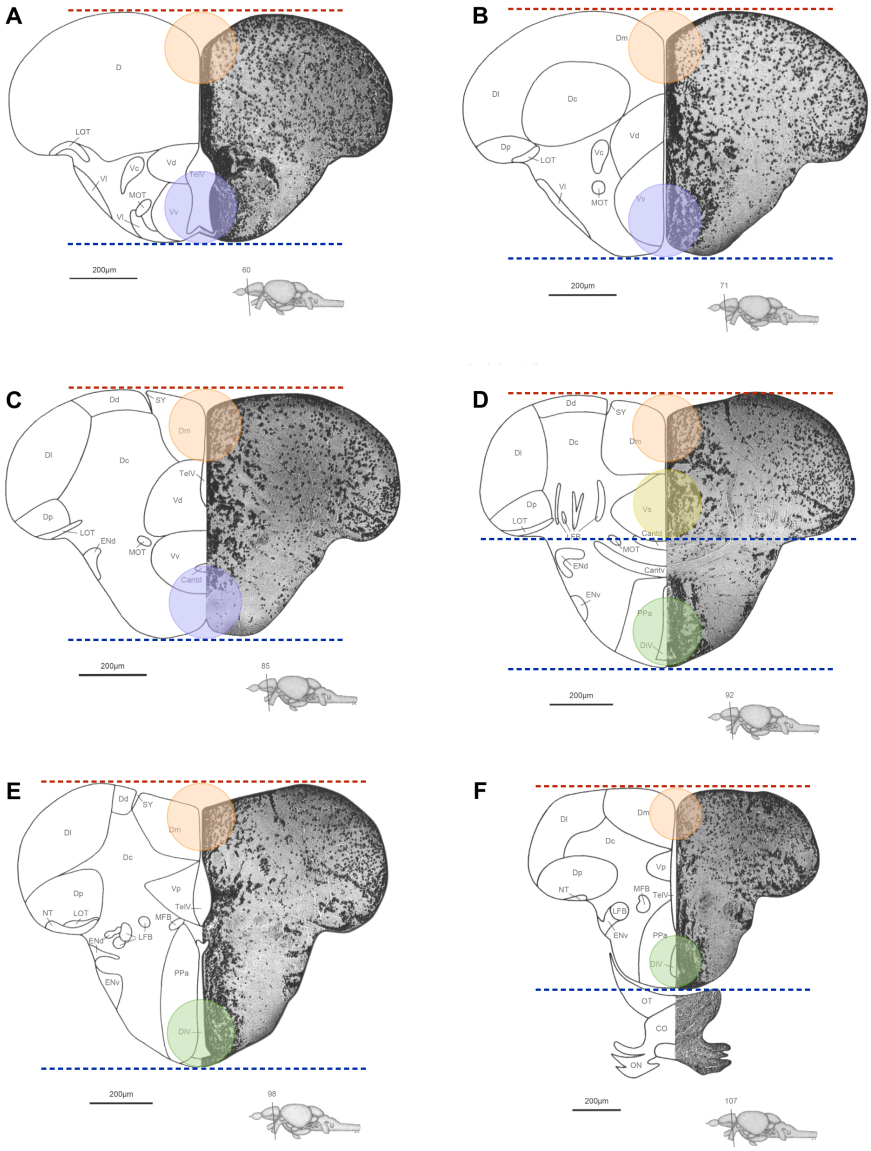

Figure 7 | Microdissection of regions of interest in the zebrafish brain.
(**A–F**) Coronal sections from the zebrafish brain are represented [adapted from (Wullimann et al., 1996)]. The right hemisphere shows tissue sections stained with Nissl-stain cresyl violet and the left hemisphere a schematic representation of the sections with each nucleus specified and delimitated. In the bottom right of each figure, a lateral view of the brain with the location of the respective section (numbers indicate the amount of 10µm sequential slices made to reach the observed image) is presented. Circles indicate the sampling points for the target brain nuclei in the various sections, and their diameter is scaled for the inner diameter of the microdissection needle; orange circles represent sampling points for the medial zone of the dorsal telencephalic area (Dm); purple circles represent sampling points for the ventral nucleus of the ventral telencephalic area (Vv); yellow circles represent sampling points for the supracommissural nucleus of the ventral telencephalic area (Vs) and green circles represent sampling points for the preoptic area (POA). Red and blue dashed lines indicate the top and bottom microdissection limits (guidelines on how to correctly perform the microdissection in order to more accurately sample each brain area), respectively. It is important to note that these images (**A–F**) correspond to 10µm sections and that the distance between two consecutive images is not necessarily the same. In this study, microdissections were performed in sections of 150 µm, therefore this figure is only a reference and each sampling point may comprise more than one of the sections represented.

Table 2 | Primer sequences, amplicons length, and annealing parameters for the genes used in qPCR - experiment III.

Gene	Accession Number	Primer Sequence (5'→3')	Amplicon length (bp)	Ta (°C)	Time of annealing (s)	Source
18s	NM_173234.1	For – GCACATCCTTCGTGTCCTCAA Rev – ACCCTCTCAACCTCATCCTCA	171	61	30	Current thesis
c-fos	NM_205569.1	For – CCGATACACTGCAAGCTGAA Rev – CGGCGAGGATGAACTCTAAC	111	58	60	(Teles et al., 2015)

For – primer forward; Rev – primer reverse; Ta – annealing temperature

4.2.4 Statistical analysis

All statistical analyses were performed on the statistical software packages STATISTICA v. 10 (StatSoft, Inc.) and SPSS® Statistics v. 21 (IBM). Normality and homogeneity of variance of the data were tested, and the appropriate statistics were used as required. All behavioural statistical analysis followed the same approach: repeated measures ANOVA were performed, with treatment as categorical predictor and freezing in each time bin (5 or 10 min) as dependent variable, to compare freezing between: 1) baseline and the 1st 5 min after alarm substance administration; 2) 10 min bins over the 30 min test. When sphericity was not assumed, repeated measures ANOVA with Greenhouse-Geisser correction (SPSS) was used. Differences in relative gene expression between treatments were assessed using one-way ANOVA with treatment as categorical predictor and gene expression in Dm, Vv, Vs and POA as dependent variables. When homogeneity of variance was not assumed, one-way ANOVA with Welch correction (SPSS) was used. Sequential bonferroni corrections were performed for all pairwise comparisons to correct for multiple comparisons and corrected p values (p') are reported. Statistical significance was set at adjusted p value - $p' < 0.05$. Brain co-activation patterns of the different treatments were assessed as in (Teles et al., 2015). Pearson product moment correlation square matrices for the brain nuclei were used as a proxy of functional connectivity such that Pearson $r > 0$ values represent co-activation, whereas Pearson $r < 0$ values represent co-inhibition. Differences between co-activation patterns of two treatments were assessed through the quadratic assignment procedure (QAP). This technique tests the association between two square matrices. In brief, Pearson coefficient is computed for the corresponding cells of the matrices, generating a new matrix with the same size as the original one, the observed matrix. Numerous independent matrices are generated by randomly rearranging the observed matrix in a manner that the proprieties of the later are preserved, creating a distribution of correlations with which the observed matrix will be compared (Barnett, 2011; Borgatti et al., 2013). A significant association between matrices occurs when the correlation values of the observed matrix are greater than 95% of the corresponding values of the generated matrices distribution, which indicates an association between matrices at a significance level of 0.05. Thus, the null hypothesis in QAP is that no

association between the two matrices is found (Borgatti et al., 2013). 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. Co-activation patterns were also structurally characterized using cohesion and centrality network measures, density and eigenvector centrality, respectively. Density is the proportion between the number of observed connections in a network and all the possible connections in the network (Borgatti et al., 2013). Comparisons of densities between treatments were performed using a bootstrap paired sample *t*-test with 5,000 sub-samples. Eigenvector centrality measurement is based not only on the number of connections a node has, but also on the different weights attributed to each connection, determining how well connected (i.e. how central) is the node (Borgatti et al., 2013). Network statistical analysis were performed using UCINET 6 (Borgatti, Everett, & Freeman, 2002). Brain nuclei co-activation network figures were produced using a custom-made python code.

4.3 Results

4.3.1 Smaller shoals are equally effective in promoting Social Buffering

We verified that conspecific number did not influence freezing responses neither during the acute response (repeated measures ANOVA with "time" as within-subject factor; time: $F_{(1.0,\ 152.0)} = 131.542$, $p < 0.001$; treatment*time: $F_{(7.0,\ 152.0)} = 22.358$, $p < 0.001$; treatment: $F_{(7,\ 152)} = 22.454$, $p < 0.001$; Figure 8B) nor over the 30 min duration of the test, and that a shoal of 2 conspecifics was enough to significantly decrease freezing behaviour in response to the AS (repeated measures ANOVA with "time" as within-subject factor; time: $F_{(1.6,\ 122.349)} = 19.234$, $p < 0.001$; treatment: $F_{(3,\ 76)} = 9.822$, $p < 0.001$; treatment*time: $F_{(4.8,\ 122.349)} = 1.232$, $p = 0.299$; corrected p values (p') after sequential Bonferroni correction for multiple comparisons (LSD post hoc): alone vs. shoal 2, 0-10 min: $p' < 0.001$; alone vs. shoal 2, 10-20 min: $p' = 0.003$; alone vs. shoal 2, 20-30 min: $p' = 0.016$; Figure 8C).

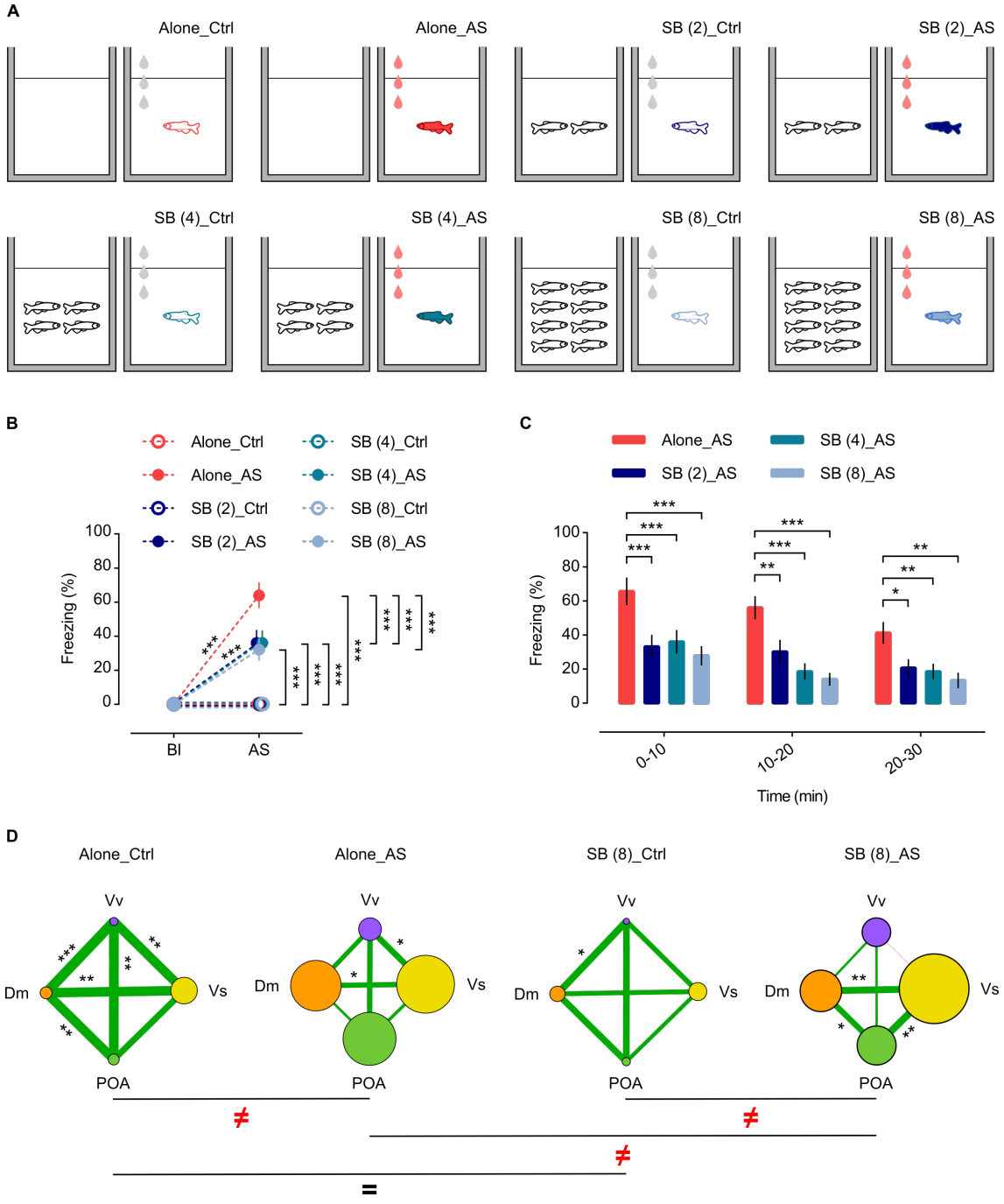

Figure 8 | Shoal size modulation and neuromolecular mechanisms underlying the buffering phenomenon.

(**A**) Schematic representation of the behavioural treatments. From left to right (top panel): Alone_Ctrl - alone focal fish (red outline) administered with water; Alone_AS - alone focal fish (red filling) administered with alarm substance; SB (2)_Ctrl - focal fish (dark blue outline) administered with water and exposed to a shoal of 8 conspecifics; SB (2)_AS - focal fish (dark blue filling) administered with alarm substance and exposed to a shoal of 8 conspecifics. From left to right (bottom panel): SB (4)_Ctrl - focal fish (sea blue outline) administered with water and exposed to a shoal of 4 conspecifics; SB (4)_AS - focal fish (sea blue filling) administered with alarm substance and exposed to a shoal of 4 conspecifics; SB (8)_Ctrl - focal fish (light blue outline) administered with water and exposed to a shoal of 8 conspecifics; SB (8)_AS - focal fish (light blue filling) administered with alarm substance and exposed to a shoal of 8 conspecifics. Grey and red drops represent water and alarm substance administration, respectively. (**B**) Freezing % in baseline (Bl) vs. first 5 min after AS onset (AS). Open red circles: Alone_Ctrl; filled red

circles: Alone_AS; open dark blue circles: SB (2)_Ctrl; filled dark blue circles: SB (2)_AS; open sea blue circles: SB (4)_Ctrl; filled sea blue circles: SB (4)_AS; open light blue circles: SB (8)_Ctrl; filled light blue circles: SB (8)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001. (**C**) Freezing % over the 30 min test in 10 min bins (0-10 min; 10-20 min; 20-30 min). Red bars: Alone_AS; dark blue bars: SB (2)_AS; sea blue bars: SB (4)_AS and light blue bars: SB (8)_AS. Mean ± SEM are shown. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001. (**D**) Brain co-activation patterns as measured by *c-fos* mRNA expression for each treatment. From left to right: Alone_Ctrl; Alone_AS; SB (8)_Ctrl and SB (8)_AS. Circle diameters represent the mean *c-fos* expression for each brain nucleus, which are colour-coded (orange, purple, yellow, and green represent Dm, Vv, Vs, and POA, respectively). Distinct co-activation patterns of *c-fos* mRNA expression between treatments, as revealed by significant QAP correlations, are indicated by ≠ (QAP p > 0.05). Similar co-activation patterns of *c-fos* mRNA expression between treatments are indicated by = (QAP p < 0.05). Lines linking brain nucleus represent the functional connectivity between them, as revealed by Pearson's correlation coefficients (*r*), with line thicknesses proportional to *r* value and positive/negative correlations indicated by line colour (green and red, respectively); asterisks indicate significant correlations: * p < 0.05; ** p < 0.01 and *** p < 0.001.

4.3.2 Neuromolecular mechanisms underlying Social Buffering

There was a significant increase in activity in all brain regions measured when the fish were exposed to the AS either alone or in the presence of conspecifics, but there were no significant differences in activation of any of the regions between fish exposed to AS alone or in the presence of the shoal [one-way ANOVA analysis revealed an overall effect for *c-fos:* $F_{(12,\ 90.25)}$ = 2.929, p < 0.01; however there was no evidence of social buffering in *c-fos* relative gene expression in any of the brain nuclei considered (Dm: p' = 0.802; Vv: p' = 0.725; Vs: p' = 0.361; POA: p' = 0.593); Figure 9]. In contrast, the co-activation patterns across brain nuclei were specific for each treatment except for the two control treatments (see Figure 8D). Thus, social buffering is paralleled by a specific pattern of brain co-activation, characterized by a significant functional connectivity between Dm-Vs-POA, rather than by a localized increase in activity in a single brain nucleus. Moreover, the social buffering treatment (i.e. shoal of 8 exposed to AS) was the only one where network measures were significantly different, with Vv presenting lower centrality than the other nuclei (see Table 3 for more information).

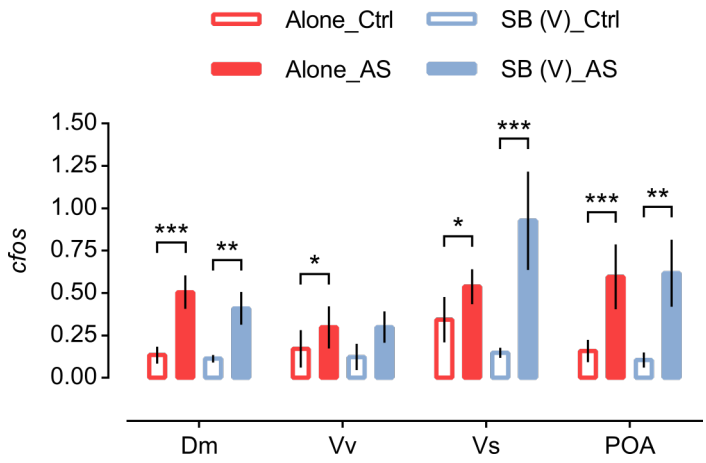

Figure 9 | c-fos gene expression in the four different brain areas.
c-fos gene expression in the four different brain areas (Dm: medial zone of the dorsal telencephalic area; Vv: ventral nucleus of the ventral telencephalic area; Vs: supracommissural nucleus of the ventral telencephalic area and POA: preoptic area) for different behavioural treatments [Alone_Ctrl, Alone_AS, SB (V)_Ctrl and SB (V)_AS]. *c-fos* gene expression was normalized for *18S*. Bars represent mean ± SEM. * p' < 0.05; ** p' < 0.01 and *** p' < 0.001.

Table 3 | Network characterization of the co-activation patterns of the four brain regions sampled.

	Brain Nucleus	Treatment			
		Alone_Ctrl	Alone_AS	SB (V)_Ctrl	SB (V)_AS
eigenvalue					
	Dm	0.524	0.472	0.529	0.557
	Vv	0.524	0.560	0.536	0.196
	Vs	0.473	0.529	0.424	0.575
	POA	0.476	0.429	0.503	0.566
density					
		0.813	0.506	0.501	0.448

Eigenvector centrality (eigenvalue) and density were used as centrality and cohesion measures, respectively. Alone_Ctrl: alone focal fish administered with water; Alone_AS: alone focal fish administered with alarm substance; SB (V)_Ctrl: focal fish administered with water and exposed to a shoal of 8 conspecifics; SB (V)_AS: focal fish administered with alarm substance and exposed to a shoal of 8 conspecifics; Dm: medial zone of the dorsal telencephalic area; Vv: ventral nucleus of the ventral telencephalic area; Vs: supracommissural nucleus of the ventral telencephalic area; POA: preoptic area.

4.4 Discussion

In this experiment we tested if conspecific number influences the social buffering phenomenon. We verified that different shoal sizes (2, 4 and 8) equally modulate the buffering effect, since smaller shoals equally contributed to a sustained buffering. In our experiment the larger shoal size tested consisted of 8 conspecifics. Considering that larger shoals of up to 30 individuals have been observed in the zebrafish' floodplain habitats (Pritchard et al., 2001; Rowena Spence et al.,

2006; Suriyampola et al., 2015), it is possible that much larger shoals (e.g. 20, 30) may be even more efficient in buffering responses to alarm substance, since fish in larger groups (i.e. 20, 50) are known to recover faster from a threatening situation (Magurran & Pitcher, 1987). Furthermore, a shoal of 8 conspecifics was shown to elicit a specific pattern of brain co-activation, characterized by a significant functional connectivity between Dm-Vs-POA, rather than by a localized increase in activity in a single brain nucleus. These results suggest that several brain nuclei are underlying the social buffering phenomenon and that its neural processing is not region specific. A recent study has also documented similar findings in a different behavioural context (agression paradigm, Teles et al., 2015), which may indicate that different social behaviours reflect an overall profile of activation across different brain nuclei instead of single node activation. At last, co-activation patterns across a set of zebrafish brain regions that are putative homologues of limbic regions in mammals and are important in activating a response to psychogenic threats (Hennessy, Kaiser, & Sachser, 2009; Hostinar et al., 2014; Kikusui et al., 2006; Tovote et al., 2015), suggest a substantially evolutionary conserved mechanism underlying social buffering in two distinct vertebrate lineages. A further discussion of these findings and possible future directions will be addressed in sections 5.4 and 5.5.3.

Chapter 5 |
General discussion

5.1 Overview of empirical findings

The main focus of this thesis was to explore the social buffering process in zebrafish, a phenomenon present in social animals. To achieve it, a behavioural paradigm was established, allowing not only the study of the buffering effect but also the exploration of the sensory modalities contributing to the this social behaviour, as well as the modulation of the buffering phenomenon by shoal size. The behavioural setup consisted of two side-by-side tanks preventing physical contact between test and demonstrator tanks, but enabling visual contact among focal (test tank) and stimuli fish (demonstrator tank). Stimuli fish were always zebrafish mixed-sex shoals, oscillating in number depending on the experiment[9]. Moreover, an automated code (named xyz2b) for erratic movement and freezing detection and quantification was developed. This allowed automatic and accurate quantification of both behaviours, with strong correlation values to human observations both for erratic movement and freezing, although revealing an even better performance in quantifying freezing. Furthermore, in order to determine the parameter that better described zebrafish response to alarm substance over the 30 min test, an analysis revealed that freezing was the most frequent and consistent behaviour in the 30 min time. Due to the better performance of xyz2b in quantifying freezing and the higher frequency and consistency of this behaviour over the erratic movement, freezing was the behavioural parameter used in all analysis.

We started by testing the presence of social buffering in zebrafish (experiment I, see chapter 2) and found that the presence of sensory cues from conspecifics lessened the fear response of focal fish to alarm substance. That is, the focal fish in the social buffering treatment presented significantly lower freezing than the fish exposed to the alarm substance alone. Additionally, it was observed that the buffering phenomenon was persistent over time, indicating sustained social buffering in zebrafish. Moreover, apart from lowering freezing responses, which is a behavioural measure known to indicate high levels of fear and anxiety stress (Speedie & Gerlai, 2008; Cachat et al., 2010), we found no evidence of social buffering of the stress response (HPI axis modulation) in the presence of conspecific cues, either at the endocrine or at the neural level. However, we also did not find any HPI axis activation in response to the alarm substance alone, which suggests that the absence of buffering of the stress response should not necessarily be interpreted as a negative result. In fact, a prior study (data not shown) in our lab showed a whole-body cortisol increase 5 min post-alarm substance administration. Thus, the absence of endocrine and neural activation (comparatively to control groups) and particularly the lack of change in basal cortisol levels in response to alarm substance, suggest that both cortisol and

[9] Shoal size modulation and respective results will be discussed further below.

glucocorticoids receptors may have peaked shortly after the stressor initiation, and that HPI activation had already ceased when the focal zebrafish were sacrificed. Future experiments addressing this question will be further discussed in the section 5.2.

Afterwards, we set to investigate which of the two sensory modalities tested (olfactory or visual) was more effective at inducing the social buffering phenomenon (experiment II, chapter 3). To achieve this, we tested each sensory cue separately by establishing treatments where they were presented individually to the focal fish. The results revealed that olfactory and visual cues were equally effective in decreasing the freezing responses in the first 10 min. However, further analysis of the freezing responses' temporal dynamics, showed that the sight of shoal was more efficient over time (30 min) at buffering the fear response to alarm substance. Thus, these results suggest that the long-term reliability of shoal cues signalling safety depend on sensory modality.

Next (experiment III, chapter 4), we explored shoal size modulation of the buffering effect by exposing focal fish to different mixed-sex shoal sizes (2, 4 and 8 individuals). In this experiment only the visual cue was used, since it was the most efficient sensory modality at inducing the buffering phenomenon. The results demonstrated that the number of conspecifics did not influence freezing responses during the entire duration of the tests and that 2 shoal mates were enough to significantly decrease fear responses to the alarm substance.

Finally we used brain tissue from the above experiment to characterize the co-activation patterns of brain nuclei (as well as brain activation of each brain nucleus per se) during social buffering, across a set of brain regions known to be involved in social buffering (Neumann et al., 2000; A. S. Smith & Wang, 2014), fear (Quirk et al., 1995; Sparta et al., 2014; Tovote et al., 2015), anxiety (Davis et al., 2010; Duvarci et al., 2009; Tovote et al., 2015) and affective states regulation (Sheehan et al., 2004; Tovote et al., 2015). Since all group sizes equally buffered fear responses, the treatment with a shoal size of 8 was selected for characterizing brain activation, as this group size is the closest to the average shoal size observed in zebrafish floodplain habitats (Pritchard et al., 2001; Rowena Spence et al., 2006; Suriyampola et al., 2015). Although *c-fos* mRNA expression in each brain nucleus did not reveal differential activation in the presence of conspecifics, social buffering elicited a specific co-activation pattern, characterized by a significant functional connectivity between Dm-Vs-POA, rather than by a localized increase in activity in a single brain nucleus. In fact, centrality measures revealed a decoupling of Vv from the other brain nuclei, emphasizing the role of Dm-Vs-POA in the social buffering phenomenon. These changes in co-activation patterns across a set of zebrafish brain regions that are putative homologues of limbic regions in mammals (Ganz et al., 2012, 2015) and are important in activating a response to psychogenic threats (Hennessy et al., 2009; Hostinar et al., 2014; Kikusui et al., 2006; Tovote et

al., 2015), suggest a substantial evolutionary conserved mechanism underlying social buffering in two distinct vertebrate lineages (i.e. mammals and teleosts).

In conclusion, we found that zebrafish decreased their fear responses to a threat in the presence of conspecific cues, and that the sight of conspecifics was more effective than their odour in promoting social buffering in a persistent threat scenario. Moreover, social buffering was independent of conspecific number. Finally, we also showed that social buffering was paralleled by a specific pattern of co-activation of brain regions homologue to those involved in the same phenomenon in mammals, suggesting that social buffering in vertebrates may share a common evolutionary origin.

In the remaining sections of this chapter, specific and general aspects, as well as future perspectives for the development of this line of research will be discussed. Furthermore, a view of the potential implications of studying social buffering will be presented.

5.2 Social Buffering of the stress response

Social buffering has been seen as a phenomenon in which social animals recover better from aversive experiences when in the presence of conspecifics (Davitz & Mason, 1955; Edgar et al., 2015; Fuzzo et al., 2015; Kikusui et al., 2006; A. S. Smith & Wang, 2014). Understanding the neuromolecular mechanisms underlying this phenomenon is a way to access how the brain processes this information. Therefore it is relevant to understand how social information resembling "safety" is interchanged between individuals, namely addressing the role of sensory cues and shoal size modulation, as well as focusing on the neuroendocrine response in the context of social buffering.

One way to start addressing the social buffering phenomenon is by focusing on the buffering of the stress response, namely how threatening events generate a stress response and specifically if conspecific presence buffers the response to these events (Edgar et al., 2015; Kiyokawa et al., 2004; Levine et al., 1978; A. S. Smith & Wang, 2014). Some studies have assessed how specific sensory cues of conspecifics contribute to lessen the stress response (da Costa et al., 2004; Takahashi et al., 2013). These experiments reported that the buffering effect diminishes responses to stress at different levels: behavioural responses (domestic hens, Edgar et al., 2015; rats, Kiyokawa et al., 2004); endocrine responses (squirrel monkeys, Levine et al., 1978; prairie voles, A. S. Smith & Wang, 2014); and neural responses (sheep, da Costa et al., 2004; rats, Takahashi et al., 2013).

Several studies in zebrafish have investigated responses to chronic (Piato et al., 2011) and acute stressors (Alderman & Vijayan, 2012; Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et

al., 2015; Ramsay et al., 2009); however social buffering of stress is still virtually unexplored in zebrafish. Interestingly, Ziv et al., 2013 verified that GR zebrafish mutants (GR genomic activity disrupted) presented significantly higher levels of cortisol when exposed to a confinement stressor (10 min) and significantly greater freezing levels comparatively to wild-type (WT) fish when exposed to a novel test tank. Notably, when exposed to the novel tank (10 min) in the view of a conspecific (WT), zebrafish GR mutants did reduce freezing behaviour comparatively to when alone (view of empty tank); nonetheless, conspecific presence did not result in lowered levels of cortisol (Ziv et al., 2013). Although these experiments were conducted in GR zebrafish mutants and no information regarding social buffering on the WT line was reported, these findings suggest the presence of the buffering phenomenon in a zebrafish mutant line at the behavioural level. Still, these results require cautious interpretations and conclusions, as the buffering effect was not verified in the heterozygous fish (Ziv et al., 2013). Thus, it is reasonable to hypothesize that the obtained buffering effect was probably associated with the HPI axis hyperactivity induced by the disruption of GR genomic activity. Accordingly, and given the unclear results provided by (Ziv et al., 2013), exploring this social behaviour in a wild-derived zebrafish strain and using an ecologically relevant approach (natural threat-inducing stimulus) allowed us to establish a robust and clear paradigm to investigate social buffering and its mechanisms in a promising model organism like zebrafish, as well as showing strong evidence for the presence of this social effect in fish for the first time.

Briefly, the results from the first experiment (chapter 2) demonstrated that conspecific cues (olfactory + visual) diminished zebrafish freezing behaviour when exposed to alarm substance. However this was not the case in terms of neuroendocrine response, since neither cortisol nor the glucocorticoid receptors presented significantly lower levels comparatively to the alone or the control conditions. Furthermore, the MR/GR ratio also did not show a social buffering effect. These results do not corroborate the findings reported in the mammals literature previously mentioned, that state a decreased activation of the HPA axis as a result of social buffering (da Costa et al., 2004; Levine et al., 1978; A. S. Smith & Wang, 2014; Takahashi et al., 2013). In all the experiments previously cited, subject animals were exposed to social isolation (stressor) and endocrine and brain activity at the PVN level was observed. In the behavioural paradigm presented in this thesis, focal zebrafish were either alone or in the presence of conspecifics when exposed to alarm substance (the threatening inducing stimulus/stressor). But social isolation itself did not induce freezing responses in zebrafish or a neuroendocrine response, indicating that acute social isolation in zebrafish does not act as an acute stressor. In fact, co-activation patterns results corroborated this conclusion, as being alone or in the presence

of conspecifics did not elicit different co-activation patterns of neural activity in the brain. Moreover, it is important to refer that in some of the studies presented (da Costa et al., 2004; Takahashi et al., 2013), decreases in neural activation in the PVN were detected by recurring to immediate early gene expression. Differently, in our first experiment, stress-candidate genes were used to assess HPI axis modulation by the buffering phenomenon. Several factors involved in zebrafish response to acute stressors (that may differ from mammals) and its contribution to the results found will be discussed next.

As mentioned, there was no HPI axis activation in response to stress, or social buffering of the stress response. However, it is known that adult zebrafish show behavioural stress reactions, elevated cortisol levels and neural activation of the PVN nucleus in response to acute stimuli (Alderman & Vijayan, 2012; Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015; Ramsay et al., 2009). Thus, it is questionable of whether or not the experimental design and protocol used impaired the detection of HPI axis activation in a stressful situation (exposure to alarm substance). Several factors may have contributed to the lack of HPI axis activation, namely the type of stressor used, the duration of exposure to alarm substance, strain and age. Studies focusing in the adult zebrafish response to acute stressors have been using acute stimuli, such as restraint stress/confinement (Ghisleni et al., 2012); air exposure/netting (Alderman & Vijayan, 2012; Ramsay et al., 2009); lowered water level + chasing + air exposure (Pavlidis et al., 2015) and water vortex (Fuzzen et al., 2010). In these studies authors reported cortisol peaks at different post-stress times: 5 min (Fuzzen et al., 2010); 10 min (Fuzzen et al., 2010); 15 min (Ramsay et al., 2009); 20 min (Alderman & Vijayan, 2012); and 30 min (Pavlidis et al., 2015). In relation to CRF and GR differential activation responses, these experiments observed: 10 min post-stress CRF increase (Fuzzen et al., 2010); 90 min post-stress CRF decrease (Ghisleni et al., 2012); 15 and 60 min post-stress CRF increase (Pavlidis et al., 2015); and 15 min post-stress GR increase (Pavlidis et al., 2015). Pavlidis et al., 2015 did not verify differences in MR's expression and in one of the studies referenced no differences in glucocorticoid receptors were verified (Alderman & Vijayan, 2012).

So, despite the rather scarce literature and the great variation in all stress protocols (not only in terms of the stressor used, but also on the duration of exposure to the stressful stimulus), it seems reasonable to suggest that adult zebrafish stress responses to acute events, at the cortisol and glucocorticoid receptors levels, happen shortly after stress induction, since most HPI axis activation described (cortisol, CRF or GR differences) peaked until 15 min post-stress (Alderman & Vijayan, 2012; Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015; Ramsay et al., 2009). Thus it seems likely that in the case of experiment I, HPI axis activation may have

occurred close to alarm substance administration, since no differences between controls and alarm substance treatments were found 30 min after exposure both in cortisol levels and glucocorticoids receptors mRNA expression. This lack of HPI axis activation was not consistent with the freezing behaviour verified in fish when alone; nonetheless, a similar situation has already been observed in zebrafish in response to an acute stressor, where the higher novelty stress behaviour levels reported did not coincide with HPI axis activation - high levels of cortisol and decreased CRF expression (Ghisleni et al., 2012). Additionally, in previous studies using alarm substance as an acute stimulus in the lab, a significant cortisol increase was verified 5 min post-stress (data not shown), corroborating the fast HPI axis activation in response to this natural stressor. Additionally, one could state that independently of the absence of cortisol increase 30 min post-stress, there could have been a differential expression of CRF and glucocorticoid receptors; however the research suggests that whole-body cortisol levels above baseline are usually associated with HPI axis activation at the brain level, as indicated by CRF, MR and GR differential expression (Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015).

Interestingly, functional connectivity among specific brain regions involved in the stress response (e.g. Dm, POA) were detected in response to alarm substance, both when zebrafish were alone or in the social buffering treatment in experiment III (chapter 4), with the buffering phenomenon eliciting a specific co-activation pattern, characterized by a significant functional connectivity between Dm-Vs-POA. This analysis was based on the individual brain regions' activation results by quantification of *c-fos* differential expression 30 min post-stress. Similarly, Pavlidis et al., 2015 observed *c-fos* differential expression 30 min after exposure to an acute stressor, strengthening the use of this timepoint to assess brain activation in response to threatening situations in zebrafish.

To conclude, despite the social buffering effect in terms of behavioural response, buffering of the stress response at the neuroendocrine level was not observed. In fact, zebrafish did not seem to respond to alarm substance with HPI axis activation, even when in social isolation. Yet, given that alarm substance is known to activate a stress response 5 min post alarm substance administration, we hypothesize that when zebrafish were sacrificed (30 min post alarm substance introduction) the stress response had already ceased, thus explaining the absence of differences between alone and social buffering treatments with that of controls. Nonetheless, functional connectivity between stress-related regions was verified in experiment III (chapter 4), indicating that brain centres involved with stress response were recruited in the social buffering phenomenon. Still, future experiments (see section 5.4) should be conducted to dissect HPI axis activation in the context of social buffering, in order to achieve a precise characterization of the

axis dynamics.

5.3 Sensory modalities effectiveness

As already mentioned, social buffering can be mediated by different sensory modalities, as distinct sensory channels convey relevant social information that contributes to the buffering phenomenon (da Costa et al., 2004; Kikusui et al., 2006; Rukstalis & French, 2005; Takahashi et al., 2013; Wilson, 2001). In the context of this thesis, we tested the contribution of olfactory and visual cues (experiment II, chapter 3) and their effectiveness in short-term/long-term threatening scenarios. We verified that both sensory cues were equally effective in the first 10 min after alarm substance administration. Nonetheless a more detailed analysis of the 30 min exposure to alarm substance showed that in the last 20 min of the test (30 min total), the sight of shoal was more efficient in decreasing the fear response. In fact, in the last 20 min the visual cue was as effective as the treatment with olfactory and visual cues combined, strengthening the evidence for a main role of the visual social information in the buffering phenomenon. These results suggest that the relative weights given to conspecific information acquired over different sensory channels are time dependent, as in an initial period of contact with alarm substance both olfactory and visual cues seem to be equally reliable, likely because both indicate the presence of conspecifics. Nevertheless, as the time of exposure to threat increases, the information given by the sight of shoal is updated consistently. As a shoal is a dynamic visual cue, relaxed shoal mates over time reinforce indication of a free from harm environment. On the contrary olfactory cues remain invariable in the tank (therefore limiting updates in the olfactory modality), have no directional specificity and are "competing" on the same sensory channel with the alarm substance's threat information. Accordingly, after an initial exposure to a threatening scenario, visual cues are expected to be more reliable than olfactory ones.

Information reliability has been defined as the probability of such information being correct or not (Koops, 2004). Additionally, it has been shown that the value of available information is dependent on both information reliability and environmental uncertainty (McLinn & Stephens, 2006). In this study, McLinn & Stephens, 2006 developed a model predicting that blue jays (*Cyanocitta cristata*) choose "signal following" over choosing the most common option, which in this case authors named "environmental tracking", when the reliability of the signal outperforms environmental certainty. To test the model, McLinn & Stephens developed an experimental design in which blue jays had to repeatedly choose between two stimuli (environmental tracking) presented with varying probabilities: one option was correct and led to food, and the other was incorrect and provided no reward. A third element (signal) provided

information about which choice stimulus was correct or not. This allowed to manipulate signal reliability — q (the probability that the signal matched the correct stimulus) and environmental uncertainty — p (the background probability that a given stimulus was correct) (McLinn & Stephens, 2006). The results of this experiment showed that blue jays followed the signal when the information provided was completely reliable (q = 1.0) and the environment was uncertain (p = 0.5). However, blue jays' strategy shifted to choosing the most frequently rewarded choice stimulus over the reliability of the signal provided when environmental uncertainty decreased (p = 0.75) and signal reliability stayed the same (q = 1.0). Thus, jays' behaviour resembled environmental tracking instead of signal following, as they preferred a 75% success rate, even when a 100% success rate was available. Therefore, the results did not predict the model since blue jays favoured environmental tracking over signal following. The authors suggest that this may have happened due to jays' prior experience with the environmental tracking task when they were tested for signal following, indicating that the prior experience with environmental tracking overcomes the advantages of signal following when there is a certain level of certainty in the environment (McLinn & Stephens, 2006).

In experiment II (chapter 3), where zebrafish were exposed to alarm substance in the presence of conspecific olfactory and visual cues separately, it is possible that some degree of environmental uncertainty (p) was present at the beginning of the test in both treatments, as in both cases contradictory information was given: threat/alarm substance vs. safety/olfactory cues of conspecifics; or threat/alarm substance vs. safety/visual cues of conspecifics. Nonetheless, it seems reasonable to assume that over time zebrafish relied increasingly on environmental information that was being updated, as was the case of the visual cue but not the olfactory. Our findings suggest that the superseding of an environmental tracking (conspecific presence) strategy over signal following (fear response to alarm substance) increased with time when zebrafish subjects could see the conspecifics (constant input of information), but not when they could only smell them. This makes sense given that olfactory cues remained invariable in the water and therefore do not provide real-time updating of information about the state of conspecifics (same degree of environmental uncertainty/certainty as in the beginning), hence leading to a more efficient buffering from the visual sensory channel[10] over time.

Several studies have reported the influence of environmental uncertainty on behaviour. For example, in some cases this environmental unpredictability leads animals to gather and use adaptively important information from third parties interactions (i.e. social eavesdropping) (Abril-de-abreu et al., 2015). For instance, social eavesdropping allows bystanders (individuals in the

[10] Future studies addressing this hypothesis will be further discussed in the section 5.5.

periphery of the social interaction) to collect information about conspecifics' fighting abilities (in zebrafish, Abril-de-abreu et al., 2015) and further use it in future contest dynamics with the winners and losers of the observed fights (in green swordtail, Earley & Dugatkin, 2002), thus making use of acquired social information to direct subsequent behaviour. On the other hand, research also shows that prior information may be less reliable, when the rate at which environment changes is too fast (Devenport & Devenport, 1994). Devenport and Devenport, performed field experiments with least chipmunks (*Tamias minimus*) and golden-mantled ground squirrels (*Spermophilus lateralis*) in which sunflower seeds patches (held stable or varied in quality) were manipulated to create an unstable environment. The patches changed constantly and the latency for allowing animals to choose them was systematically varied. As a result, animals developed temporally weighted estimates of patch quality, enabling them to select the patches with the highest probability of reward (Devenport & Devenport, 1994).

Furthermore, recent literature showed that frightened fish (conspecific model) appeared to induce subject fish to ignore their prior information of safety, possibly indicating that learning from conspecifics about a threatening event is more persuasive than learning via assessment to alarm substance (Crane & Ferrari, 2015). In this experiment, Crane & Ferrari, 2015 exposed minnows several times to pike odour (predator) with no consequence, so that minnows could associate pike scent to safety. Next, the same individuals were either alarm substance conditioned (pike odour was administered at the same time that minnow's alarm substance was delivered) or conditioned with a live model (in this treatment a minnow conspecific previously fear conditioned with pike odour + alarm substance was introduced in the subject's tank 18-24h before the release of pike scent). At the test stage all subjects were tested alone either with pike odour or water. The results showed that fish that previously learned that pike odour was safe did not exhibit fearful responses after alarm substance conditioning, whereas those conditioned with the live frightened model exhibited subsequent fear behaviour in response to pike odour. This suggests that fish ignored the prior learning of safety and favoured learning from the live conspecifics (Crane & Ferrari, 2015).

5.4 Role of shoal size

Likewise, the number of conspecifics in a shoal is expected to influence social buffering, as a greater number of animals are conveying the same information of safety. Thus, in this thesis we also hypothesised that shoal size could potentially modulate social buffering in zebrafish. It is known from the literature that species form larger groups in response to threats (Hoare et al., 2004) and that bigger group sizes modulate responses to aversive events (Brown & Bongiorno,

2006; Magurran & Pitcher, 1987). Thus, larger groups may be more conspicuous and reliable. Accordingly, in experiment III (chapter 4) we tested if shoal size modulated the effectiveness of the visual cue in the reduction of the fear response. Different mixed-sex shoal sizes were used (2, 4 and 8 fish), but we found no influence on the focal fishes' fear responses, as smaller shoals equally contributed to a sustained buffering. Nevertheless, one can not rule out the possibility that much larger shoals of zebrafish - up to 20, 30 fish as observed in floodplain' habitats (Pritchard et al., 2001; Rowena Spence et al., 2006; Suriyampola et al., 2015) or 300 individuals in fast-flowing rivers (Suriyampola et al., 2015) - may be even more efficient in promoting the buffering effect. In fact, studies with minnows have shown that much larger shoals (e.g. 20, 50) than the larger shoal size used in our studies (8 conspecifics) are better at aiding in the recovery after a stressful event (exposure to a predator, Magurran & Pitcher, 1987). Also, Suriyampola et al., 2015 have shown that larger zebrafish groups (300 individuals) inhabiting a fast-flowing river are more cohesive, exhibit coordinated locomotion and practically inexistent levels of aggression towards other conspecifics. On the contrary smaller shoals (6-7 fish) found in a slow-flowing river formed less-cohesive groups and were much more aggressive than those in larger groups (Suriyampola et al., 2015). Formation of larger and more cohesive groups has been observed to be a common behavioural adaptation that reduces predation risk (Gerlotto, Bertrand, Bez, & Gutierrez, 2006) and improves signal detection (Chicoli, Butail, Lun, & Coombs, 2014). In sum, conspicuous social behaviours (e.g. cohesion) of larger groups is an advantage in threatening contexts by enhancing signal detection, decreasing the risk of predation and aiding in the recovery after threatening events. Thus, it is possible that larger zebrafish shoals than the ones used in the context of this thesis may be more efficient at buffering fear responses. Accordingly, in the future, studies addressing the effect of greater shoal sizes (e.g. 16, 24)[11] in the buffering phenomenon should be conducted.

5.5 Social Buffering in zebrafish - future directions

5.5.1 Stress response

Our results showed that the presence of conspecific cues (olfactory and visual) contributed to the lessening of the fear responses in zebrafish (i.e. social buffering) and that the sight of shoal was more efficient at decreasing freezing in a persistent threat scenario (long-lasting exposure to threat). Moreover, it was verified that smaller shoal sizes (2 or 4 individuals) are equally effective as larger shoals at inducing this social phenomenon. Furthermore, it was shown that social

[11] This hypothesis will be further discussed in the future directions section 5.5.

buffering elicits a distinct pattern of functional connectivity among a set of brain regions known to be involved in fear-like responses (LeDoux, 2003; Tovote et al., 2015) and buffering (Kiyokawa et al., 2014; Neumann et al., 2000; A. S. Smith & Wang, 2014) processes in mammals. However, even though the presence of conspecifics decreased behavioural fear responses, the buffering effect was not verified at the level of the neuroendocrine stress response (differences in cortisol and glucocorticoid receptors).

Although these findings already provide valuable and novel information not only about the social buffering phenomenon in zebrafish, but also about social buffering in general, several important questions arise from the experiments conducted that are worth addressing and considering for future research directions. One of which concerns the apparent lack of social buffering of the stress response and absence of zebrafish HPI axis activation as a result of exposure to alarm substance. As discussed in section 5.2, the lack of social buffering of the stress response does not necessarily imply that the presence of conspecifics is not inducing an HPI axis modulation. Rather, given that there was no HPI axis activation in response to alarm substance when zebrafish were alone, one may suggest that a different experimental design should have been used in order to accurately detect changes at the neuroendocrine level as a consequence of exposure to alarm substance and social buffering. Moreover, in section 5.2 we suggested that at 30 min post-AS administration, the stress response may have already ceased, particularly because previous data using alarm substance in the lab have shown a significant cortisol increase 5 min post-AS administration, suggesting that the cortisol peak in response to this stressor-inducing stimulus may occur shortly after alarm substance exposure. This hypothesis is corroborated by the literature since most HPI axis activation described (cortisol, CRF or GR differences) peaked until 15 min post exposure to the stressor (Alderman & Vijayan, 2012; Fuzzen et al., 2010; Ghisleni et al., 2012; Pavlidis et al., 2015; Ramsay et al., 2009).

Nonetheless, these studies also show cortisol, CRF or *c-fos* levels above baseline at 30 min. and therefore the 30 min test used in the context of this thesis was chosen based on these findings. Accordingly, the results from experiment III (chapter 4) verified a significant functional connectivity (using *c-fos* as an immediate early gene) between specific brain areas involved in the stress response - Dm and POA - 30 min post-AS administration. Thus, it seems plausible to suggest that different readouts (cortisol, CRF, GR, MR, *c-fos*) of HPI axis activation peak at distinct timepoints, hence encouraging a more accurate and characterized exploration of the stress axis. Since it was observed that the visual cue was the most efficient in a long-term exposure to alarm substance (30 min) and the sight of a shoal of 8 conspecifics induced a distinct pattern of functional connectivity among a set of brain regions known to be involved in fear and

buffering responses (Dm-Vs-POA) 30 min post-AS administration, a possible follow-up experiment would include 4 treatments: social buffering visual - shoal of 8 (control and alarm substance exposed) and alone (control and alarm substance exposed). Focal zebrafish would be sacrificed in different time points after exposure to alarm substance - 5; 10; 15; 20; 25 and 30 min post-AS administration. Whole-body cortisol would be quantified in each time point and all brain samples would be processed as in experiment III (chapter 4) (microdissection of Dm, Vs, POA and Vv, followed by CRF, GR, MR and *c-fos* mRNA quantification). *C-fos* data would be analysed using the quadratic assignment procedure (QAP) for assessment of functional connectivity between brain nuclei as in experiment III (chapter 4). This experiment would allow comparing HPI activation and *c-fos* expression in the same brain areas at different timepoints, allowing for a more precise characterization of the dynamics of social buffering of stress in adult zebrafish.

5.5.2 Environmental uncertainty vs. cue reliability

In experiment II (chapter 3) we verified that the visual cue was more effective than the olfactory in diminishing fear responses in a persistent threat context. As discussed in section 5.3, it is possible that this happened due to a greater reliability of the visual sensory modality. Over time zebrafish focal fish may have valued more the information provided by the sight of shoal (against their personal information of threat) possibly due to an increasing environmental certainty about safety provided by the visual channel, contrary to the olfactory one. As specified in section 5.4, the sight of the shoal provided a constant input of information resembling safety. Given the strength of this stimulus in buffering the fear behaviour effects of exposure to alarm substance, we also hypothesized that different conspecific numbers could modulate social buffering, as larger shoals have more conspecifics conveying the same information of safety. However, the results of the experiment III (chapter 4) showed that the shoal sizes used in the context of this thesis were equally effective in lessening freezing. These findings raise new questions worth further exploring in future experiments. First, it would be relevant to understand if larger shoal sizes than the ones used in the experiment III (chapter 4) are more efficient at inducing social buffering. Secondly, it would be important to investigate how environmental uncertainty (e.g. conflicting information - safety vs. threat) and cue reliability (e.g. alarm substance) may influence the buffering effect, which would contribute to better understand how zebrafish value social information over personal information in a threatening context.

In order to address the first question, one straightforward approach would be to test if larger shoal sizes would be more efficient at inducing social buffering. Thus, a follow-up experiment would be conducted. Six different treatments would be established: shoal of 8

(exposed to alarm substance or water); shoal of 16 (exposed to alarm substance or water); and a shoal of 24 (exposed to alarm substance or water). The shoal of 8 in the case of this follow-up experiment would be kept as a positive control for the results obtained in experiment III (chapter 4). Besides being multiple numbers of 8 (to ensure a linear increase in shoal size), the shoals of 16 and 24 are also group ranges that fit within the greater shoal sizes observed in zebrafish common floodplain habitats (2-10, Pritchard et al., 2001; 2-30, R. Spence et al., 2006; 22, Suriyampola et al., 2015).

In order to address the second question, that aims to understand how environmental uncertainty and cue reliability influence the buffering effect, we would need to experimentally establish and be able to manipulate these two factors. For instance, if results show that greater shoal sizes are more effective at buffering the response to alarm substance (i.e. 8 < 16 < 24), we could subsequently use this information to induce three conditions of environmental uncertainty (AS vs. 8; AS vs. 16; AS vs. 24), with the shoal of 24 being the most effective at inducing buffering, thus the one that promotes the greater level of environmental certainty. Correspondingly, another parallel follow-up experiment should be conducted focusing on establishing three conditions of increasing cue reliability. In previous experiments (data not shown), we verified that different concentrations of alarm substance (10% and 100%) induced significantly different levels of freezing, with the most concentrated alarm substance inducing greater freezing levels. In the studies encompassed in this thesis, the alarm substance used was the most concentrated version, thus an experiment establishing a response curve demonstrating that different concentrations induce different freezing levels would be needed (e.g. 10% AS < 50% AS < 100% AS).

Assuming that the previous hypotheses are corroborated by the results, we could then investigate how environmental uncertainty and cue reliability regulate the social buffering phenomenon. To do that a last experiment could be conducted with 12 treatments: shoal of 8 exposed to water, 10% AS, 50% AS, 100% AS; shoal of 16 exposed to water, 10% AS, 50% AS, 100% AS; and shoal of 24 exposed to water, 10% AS, 50% AS, 100% AS. In this manner, different environmental uncertainties would be confronted with distinct cue reliabilities (e.g. low uncertainty: 10% AS vs. shoal of 24; high uncertainty: 100% vs. shoal of 24), enabling to understand the weight of these factors in mediating the buffering process. A study exploring the effects of different group sizes when exposed to increasing concentrations of alarm substance in juvenile convict cichlids (*Archocentrus nigrofasciatus*) showed that increases in shoal size (1 to 6 individuals) results in higher levels of immobility when there is an increase in alarm substance concentration (Brown & Bongiorno, 2006), suggesting that larger shoals tend to have higher

thresholds to threat. However, in the experiment mentioned shoal numbers were 1, 3 and 6, as for alarm substance concentrations percentages were 12,5%, 25%, 50% and 100% (Brown & Bongiorno, 2006), thus the findings presented may not necessarily be confirmed by the results of the experiment suggested, since shoal sizes in Brown & Bongiorno, 2006 are significantly smaller and the potency and equidistance of the alarm substances' concentrations used is probably a factor influencing the results. Still, using zebrafish as model organism to address the influence of environmental uncertainty and cue reliability in the social buffering effect would greatly benefit the understanding of this social behaviour.

5.5.3 Neural mechanisms

In the context of this thesis, the neuromolecular mechanisms underlying social buffering were also explored. This social behaviour evoked a specific pattern of brain co-activation, characterized by a significant functional connectivity between Dm-Vs-POA, rather than by a localized increase in activity in a single brain nucleus. These findings suggest that the neural mechanisms underlying the social buffering phenomenon are not nucleus specific, thus techniques that promote *in vivo* recordings and manipulation of whole-brain neuronal circuits (Ahrens et al., 2012; Ahrens, Orger, Robson, Li, & Keller, 2013; Portugues, Feierstein, Engert, & Orger, 2014) would greatly benefit the understanding of this social behaviour at the brain level. However, these techniques are optimized for zebrafish larval stages and most studies in zebrafish addressing social behaviours (including the present one) have been performed in adults (Abril-de-abreu et al., 2015; Al-Imari & Gerlai, 2008; Oliveira et al., 2016; Pavlidis, Sundvik, Chen, & Panula, 2011a; Teles et al., 2015). Fortunately, recent studies have shown a strong visual preference for conspecifics in 3 weeks old larvae (Dreosti et al., 2015; Engeszer, Barbiano, et al., 2007), suggesting that social behaviours appear earlier in zebrafish development. Thus, some recent studies have started to use imaging techniques in 21-28 days (Jetti et al., 2014) and 3-5 weeks zebrafish larvae (Mathuru et al., 2012). Even though 3 weeks old fish already present skull ossification and some head pigmentation (that limit imaging techniques used in younger transparent brains), these experiments performed imaging in the zebrafish olfactory bulb (Jetti et al., 2014; Mathuru et al., 2012), which is a superficial brain structure, thus allowing good resolution brain imaging. Still, not all behaviours involve superficial brain structures, and optic techniques allowing imaging of larger brains and deeper structures (thicker layers of biological tissue) are now emerging (Judkewitz, Horstmeyer, Vellekoop, Papadopoulos, & Yang, 2015; Judkewitz et al., 2013). In fact, these tools are currently being improved to enable imaging in zebrafish juvenile brains (unpublished data, Benjamin Judkewitz Lab).

Accordingly, for future explorations of the neuronal mechanisms underlying this social phenomenon it would be relevant to validate the social buffering paradigm at 3-5 weeks old zebrafish, where zebrafish social behaviours seem to be appearing (Dreosti et al., 2015; Engeszer, Barbiano, et al., 2007). Thus, firstly it would be important to establish the onset of the fear response to alarm substance by performing an experiment with 3, 4 and 5 weeks old zebrafish and investigate the earliest age at which zebrafish start freezing when exposed to alarm substance. Assuming that zebrafish respond to alarm substance within the 3-5 weeks range, we would also need to validate the existence of social buffering in this time-window. In order to do that, the olfactory cues of the shoal would be the most feasible candidate cue to be used, given their efficiency in buffering the response to alarm substance (even tough less effective than the visual cue at longer exposures) and its easy manipulation (e.g. administration in a petri dish) in a more complex experimental setup where the focal fish needs to be immobilized (as is the case of experimental setups using imaging techniques in zebrafish larvae and juveniles). Once validated that 3 to 5 weeks zebrafish exhibit social buffering, we can start exploring the possible brain areas or circuits underlying this phenomenon.

As previously mentioned, it was observed that conspecific odour is sufficient to buffer the fear response elicited by the alarm substance. This result suggests the involvement of the olfactory system in the social buffering phenomenon, perhaps at the early processing of conspecific odour cues. Thus, it would be relevant to test the role of olfactory kin recognition in the social buffering phenomenon. According to Gerlach and co-workers, the zebrafish olfactory imprinting that occurs on day 6 post-fertilization is likely to be induced by a cell-cell recognition system involving the major histocompatibility complex (MHC). It is suggested that MHC transmembrane molecules are shed from the cell surface (chemical ligand) and appear in urine. Thus, it is possible that these molecules are assessed by other conspecifics through olfactory processing, as MHC peptides located on the cell surface of the olfactory receptors may be concerned with the neural processing underlying conspecific recognition (Gerlach et al., 2008). The MHC has been shown to be present in zebrafish (Dirscherl, McConnell, Yoder, & de Jong, 2014) and to be involved species recognition in other animals (Gerlach et al., 2008). Hence, it is also probable that MHC genes are influencing the social buggering effect. Yet, before assessing this hypothesis, a previous follow-up experiment showing that zebrafish conspecifics are more efficient than heterospecifics at promoting buffering needs to be carried, as this has never been shown in zebrafish[12]. Afterwards, and assuming that conspecifics would be more effective at

[12] It has already been shown that familiar rats are more effective at inducing buffering than unfamiliar ones (Kiyokawa et al., 2014). Nonetheless, to our knowledge, conspecific vs. heterospecific roles in the buffering phenomenon have never been tested.

evoking buffering than heterospecifics, one hypothesis would be to identify the MHC genes underlying conspecific recognition in zebrafish and use knockout zebrafish lines for the ligand and receptor to investigate the role of the MHC genes in social buffering. If MHC genes are involved in conspecific recognition, the zebrafish knockout line will exhibit more freezing behaviour comparatively to the wild-derived fish.

Still focusing on the early processing of conspecific odour and its influence on social buffering, it would be relevant to investigate the olfactory bulb computations in a social buffering context where environmental uncertainty is present. Specifically, how social odours conveying threat (e.g. alarm substance) and safety (e.g. shoal water) are simultaneously represented in the bulbar circuitry, and how these representations influence zebrafish behavioural outcomes – threat (freezing) vs. safety (normal swimming).

Some hypotheses arise from this question, one of which is based on odour complexity (the number of compounds that constitute a scent determines odour complexity, that in consequence is likely to elicit higher olfactory bulb activation, Kermen, Franco, Wyatt, & Yaksi, 2013) and its influence on the olfactory bulb's representation. Specifically, it is possible that higher odour complexity affects the strength of the signal output sent to locomotor brain centres, therefore controlling behaviour (e.g. a possible direct pathway linking the olfactory bulb and locomotor areas was already proposed in sea lamprey but lacks confirmation in zebrafish; Kermen, Franco, Wyatt, & Yaksi, 2013). In our case, and given that olfactory cues are enough to induce the buffering effect, it is probable that shoal odour is more complex than alarm substance, as shoal water contains bile acids, prostaglandins and steroids released through urine (Kermen et al., 2013). Thus, in the circumstances previously described, social buffering would elicit a characteristic pattern of neuronal activation that could potentially be detected at the bulbar level.

On the other hand, in the absence of a direct pathway connecting the olfactory bulb and locomotor areas, bulbar odour representations may be modulated by higher brain structures, such as fear (habenula - Hb; Agetsuma et al., 2010) or stress (PVN; A. S. Smith & Wang, 2014; Takahashi et al., 2013) related centres. Furthermore, projections from the olfactory bulb directly to the habenula and hypothalamus have already been documented (Kermen et al., 2013). Thus, it may be the case that feedback projections from the Hb and/or PVN to the olfactory bulb may be modulating the bulbar circuitry and generating a characteristic odour map representative of the social buffering phenomenon. Hb and PVN (Kiyokawa et al., 2014; Takahashi et al., 2013) activation in a buffering scenario should decrease (lessening of fear), therefore influencing the inputs sent to the olfactory bulb. To test the hypothesis that the Hb is modulating the bulbar circuitry, *elavl3*:GCAMP5G (Ahrens et al., 2013) and GAL4^{s1019t}/UAS:mCherry (Jetti et al., 2014)

zebrafish lines are available to assess neural activity and identity, respectively. To address the role of the PVN in the social buffering phenomenon in zebrafish, a different strategy can be used. It is known from studies in mammals (Neumann et al., 2000; A. S. Smith & Wang, 2014) that oxytocinergic neurons in the PVN seem to be underlying the buffering effect. In order to corroborate this in zebrafish, transgenic (GAL4/UAS system) and mutant (TALEN and CRISPR systems) lines are now being characterized (unpublished data, Rui Oliveira and Gil Levkowitz' labs), and there is already some evidence for the presence of oxytocin expressing neurons in the parvocellular cell group of the zebrafish POA, the putative homologous of the mammalian PVN (O'Connell & Hofmann, 2011). Also, there is new evidence reporting oxytocinergic projections from the hypothalamus down to the spinal cord (unpublished data, Gil Levkowitz and Florian Engert' labs). Altogether, these lines and results set the stage for the exploration of the oxytocin's role in zebrafish social buffering.

Therefore, one option would be to silence POA oxytocinergic neurons (using the zebrafish transgenic GAL4/UAS line) and/or knockout the oxytocin receptor and ligand (using the TALEN and CRISPR lines) and demonstrate that the silencing and/or knockouts supressed social buffering. Another possibility would be to investigate oxytocinergic projections from the POA to the olfactory bulb and understand if the oxytocinergic neurons are influencing bulbar activity in a buffering context (Magda et al. observed reasonable oxytocin levels in the zebrafish' olfactory bulbs, unpublished data). Finally, another direction would be to study spinal cord processing of alarm substance in the absence and presence of social buffering (olfactory conspecific cues) using the available zebrafish lines labelling oxytocinergic neurons. Specifically, as oxytocinergic projections to the spinal cord have been reported, one possible course (and more grounded on the current evidence) would be first to identify the oxytocinergic neurons projecting down the spinal cord and record their activity during threat exposure (alarm substance) with/without social buffering olfactory cues. Secondly, to map oxytocinergic projections in the spinal cord and identify their targets (using ChR2-mediated optogenetics established in Florian Engert' lab). Thirdly manipulate the corresponding oxytocinergic neurons using optogenetics (activate and silence) and: a) monitor behaviour in freely moving animals; b) monitor behaviour in fictive preparations and record the activity of targets in the spinal cord. Finally, ablate specific oxytocinergic neurons projecting down the spinal cord and monitor the corresponding behavioural response. If oxytocinergic neurons are underlying the buffering effect, their ablation will result in social buffering suppression.

5.6 Final remarks

The results provided in this thesis demonstrate that a fish species is capable of decreasing its fear responses in the presence of conspecifics, a social phenomenon that had been mainly observed in mammals until now. Our findings not only show for the first time clear and robust evidence for the existence of social behaviour in zebrafish, but also advance on the relevance of specific sensory channels (visual) and its contribution for the buffering effect at the behavioural and neural level. The existence of this social phenomenon in zebrafish, suggests not only that social buffering in vertebrates may share a common evolutionary origin, but also that social support during a threatening event seems to be a conserved process across species. Social support has been identified as an important modulator of human behaviour, also because of its relevance in diminishing stress responses when individuals face aversive circumstances. While zebrafish shoals certainly lack the complexity of human social support, investigating a common fundamental phenomenon such as social buffering and its mechanisms in this model species, may help us understand the neural basis of this process.

References

Abril-de-abreu, R., Cruz, A. S., & Oliveira, R. F. (2015). Social dominance modulates eavesdropping in zebrafish Subject Category. *Royal Society Open Science*, *2*, 150220. doi:10.1098/rsos.150220

Abril-de-Abreu, R., Cruz, J., & Oliveira, R. F. (2015). Social Eavesdropping in Zebrafish: Tuning of Attention to ocial Interactions. *Scientific Reports*, *5*, 1–14. doi:10.1038/srep12678

Adams, D. B. (1971). Nature Publishing Group. *Nature*, *232*(20), 573–574. doi:http://dx.doi.org/10.1038/232573a0

Agetsuma, M., Aizawa, H., Aoki, T., Nakayama, R., Takahoko, M., Goto, M., Okamoto, H. (2010). The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nature Neuroscience*, *13*(11), 1354–6. doi:10.1038/nn.2654

Ahrens, M. B., Li, J. M., Orger, M. B., Robson, D. N., Schier, A. F., Engert, F., & Portugues, R. (2012). Brain-wide neuronal dynamics during motor adaptation in zebrafish. *Nature*, *485*(7399), 471–477. doi:10.1038/nature11057

Ahrens, M. B., Orger, M. B., Robson, D. N., Li, J. M., & Keller, P. J. (2013). Whole-brain functional imaging at cellular resolution using light-sheet microscopy. *Nat Methods*, *10*(5), 413–420. doi:10.1038/nmeth.2434

Al-Imari, L., & Gerlai, R. (2008). Sight of conspecifics as reward in associative learning in zebrafish (Danio rerio). *Behavioural Brain Research*, *189*(1), 216–219. doi:10.1016/j.bbr.2007.12.007

Alderman, S. L., & Bernier, N. J. (2009). Ontogeny of the corticotropin-releasing factor system in zebrafish. *General and Comparative Endocrinology*, *164*(1), 61–69. doi:10.1016/j.ygcen.2009.04.007

Alderman, S. L., & Vijayan, M. M. (2012). 11 -Hydroxysteroid dehydrogenase type 2 in zebrafish brain: a functional role in hypothalamus-pituitary-interrenal axis regulation. *Journal of Endocrinology*, *215*(3), 393–402. doi:10.1530/JOE-12-0379

Alsop, D., & Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *294*(3), R711–R719. doi:10.1152/ajpregu.00671.2007

Amores, A. (1998). Zebrafish hox Clusters and Vertebrate Genome Evolution. *Science*, *282*(5394), 1711–1714. doi:10.1126/science.282.5394.1711

Armario, A., Ortiz, R., & Balasch, J. (1983). Corticoadrenal and behavioral response to open field

in pairs of male rats either familiar or non-familiar to each other. *Experientia*, *39*(11), 1316–1317. doi:10.1007/BF01990391

Barcellos, L. J. G., Kreutz, L. C., Koakoski, G., Oliveira, T. A., da Rosa, J. G. S., & Fagundes, M. (2012). Fish age, instead of weight and size, as a determining factor for time course differences in cortisol response to stress. *Physiology and Behavior*, *107*(3), 397–400. doi:10.1016/j.physbeh.2012.09.008

Barnett, G. A. (2011). *Encyclopedia of Social Networks*. (G. A. Barnett, Ed.). 2455 Teller Road, Thousand Oaks California 91320 United States: SAGE Publications, Inc. doi:10.4135/9781412994170

Bianco, I., & Engert, F. (2014). Visuomotor Transformations Underlying Hunting Behavior in Zebrafish. *Current Biology*, *25*(7), 831–846. doi:10.1016/j.cub.2015.01.042

Blanchard, R. J., & Blanchard, D. C. (1989). Attack and defense in rodents as ethoexperimental models for the study of emotion. *Progress in Neuropsychopharmacology and Biological Psychiatry*, *13*(SUPPL. 1). doi:10.1016/0278-5846(89)90105-X

Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews*, *77*(3), 591–625.

Borgatti, S. P., Everett, M. G., & Freeman, L. C. (2002). Ucinet for Windows: Software for social network analysis. *Analytic Technologies*.

Borgatti, S. P., Everett, M. G., & Johnson, J. C. (2013). *Analyzing Social Networks*. SAGE Publications Limited.

Bourdeau, P. E. (2010). Cue reliability, risk sensitivity and inducible morphological defense in a marine snail. *Oecologia*, *162*, 987–994. doi:10.1007/s00442-009-1488-5

Broad, K. D., Curley, J. P., & Keverne, E. B. (2006). Mother-infant bonding and the evolution of mammalian social relationships. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *361*(1476), 2199–214. doi:10.1098/rstb.2006.1940

Brown, G., & Bongiorno, T. (2006). Effects of group size on the threat-sensitive response to varying concentrations of chemical alarm cues by juvenile convict cichlids. *Canadian Journal of Zoology*, (84), 8. doi:10.1139/Z05-166

Buccafusco, J. J. (2000). *Methods of behavior analysis in neuroscience*. CRC Press.

Buske, C., & Gerlai, R. (2011). Shoaling develops with age in Zebrafish (Danio rerio). *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *35*(6), 1409–1415. doi:10.1016/j.pnpbp.2010.09.003

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols*, *5*(11), 1786–1799. doi:10.1038/nprot.2010.140

Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., Kalueff, A. V. (2011). Three-dimensional neurophenotyping of adult zebrafish behavior. *PLoS ONE*, *6*(3). doi:10.1371/journal.pone.0017597

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *39*(1), 112–119. doi:10.1016/j.pnpbp.2012.05.018

Chicoli, A., Butail, S., Lun, Y., & Coombs, S. (2014). The effects of flow on schooling Devario aequipinnatus : school structure , startle response and information, 1401–1421. doi:10.1111/jfb.12365

Choi, D. C., Furay, A. R., Evanson, N. K., Ostrander, M. M., Ulrich-lai, Y. M., & Herman, J. P. (2007). Bed Nucleus of the Stria Terminalis Subregions Differentially Regulate Hypothalamic – Pituitary – Adrenal Axis Activity : Implications for the Integration of Limbic Inputs. *Animals*, *27*(8), 2025–2034. doi:10.1523/JNEUROSCI.4301-06.2007

Cirulli, F., Terranova, M. L., & Laviola, G. (1996). Affiliation in periadolescent rats: Behavioral and corticosterone response to social reunion with familiar or unfamiliar partners. *Pharmacology Biochemistry and Behavior*, *54*(1), 99–105. doi:10.1016/0091-3057(95)02169-8

Crane, A. L., & Ferrari, M. C. O. (2015). Minnows trust conspecifics more than themselves when faced with conflicting information about predation risk. *Animal Behaviour*, *100*, 184–190. doi:10.1016/j.anbehav.2014.12.002

da Costa, A. P., Leigh, A. E., Man, M.-S., & Kendrick, K. M. (2004). Face pictures reduce behavioural, autonomic, endocrine and neural indices of stress and fear in sheep. *Proceedings of the Royal Society B: Biological Sciences*, *271*(1552), 2077–2084. doi:10.1098/rspb.2004.2831

Dalgard, O. S., Dowrick, C., Lehtinen, V., Vazquez-Barquero, J. L., Casey, P., Wilkinson, G., Dunn, G. (2006). Negative life events, social support and gender difference in depression. *Social Psychiatry and Psychiatric Epidemiology*, *41*(6), 444–451. doi:10.1007/s00127-006-0051-5

Davis, M., Walker, D. L., Miles, L., & Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology : Official*

Publication of the American College of Neuropsychopharmacology, *35*(1), 105–35. doi:10.1038/npp.2009.109

Davitz, J. R., & Mason, D. J. (1955). Socially Facilitated Reduction of a Fear Response in Rats. *Journal of Comparative and Physiological Psychology*, *48*(3), 149–151. doi:10.1037/h0046411

de Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. *Nature Reviews. Neuroscience*, *6*(6), 463–475. doi:10.1038/nrn1683

De Marco, R. J., Groneberg, A. H., Yeh, C.-M., Castillo Ramírez, L. a, & Ryu, S. (2013). Optogenetic elevation of endogenous glucocorticoid level in larval zebrafish. *Frontiers in Neural Circuits*, *7*(May), 82. doi:10.3389/fncir.2013.00082

Devenport, L. D., & Devenport, J. A. (1994). Time-dependent averaging of foraging information in least chipmunks and golden-mantled ground squirrels. *Animal Behaviour*, *47*(4), 787–802. doi:10.1006/anbe.1994.1111

Dirscherl, H., McConnell, S. C., Yoder, J. A., & de Jong, J. L. O. (2014). The MHC class I genes of zebrafish. *Developmental and Comparative Immunology*, *46*(1), 11–23. doi:10.1016/j.dci.2014.02.018

Dreosti, E., Lopes, G., Kampff, A. R., & Wilson, S. W. (2015). Development of social behaviour in young zebrafish. *bioRxiv*, *9*(August), 017863. doi:10.1101/017863

Duvarci, S., Bauer, E. P., & Paré, D. (2009). The bed nucleus of the stria terminalis mediates inter-individual variations in anxiety and fear. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(33), 10357–10361. doi:10.1523/JNEUROSCI.2119-09.2009

Earley, R. L., & Dugatkin, L. A. (2002). Eavesdropping on visual cues in green swordtail (Xiphophorus helleri) fights: a case for networking. *Proceedings. Biological Sciences / The Royal Society*, *269*(April), 943–952. doi:10.1098/rspb.2002.1973

Edgar, J., Held, S., Paul, E., Pettersson, I., I'Anson Price, R., & Nicol, C. (2015). Social buffering in a bird. *Animal Behaviour*, *105*, 11–19. doi:10.1016/j.anbehav.2015.04.007

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, *205*(1), 38–44. doi:10.1016/j.bbr.2009.06.022

Engeszer, R. E., Barbiano, L. A. DA, Ryan, M. J., & Parichy, D. M. (2007). Timing and plasticity of shoaling behaviour in the zebrafish, Danio rerio. *Animal Behaviour*, *74*(5), 1269–1275. doi:10.1016/j.anbehav.2007.01.032

Engeszer, R. E., Patterson, L. B., Rao, A. a, & Parichy, D. M. (2007). Zebrafish in the wild: a

review of natural history and new notes from the field. *Zebrafish*, *4*(1), 21–40. doi:10.1089/zeb.2006.9997

Engeszer, R. E., Ryan, M. J., & Parichy, D. M. (2004). Learned Social Preference in Zebrafish. *Current Biology*, *14*(10), 881–884. doi:10.1016/j.cub.2004.04.042

Félix, A. S., Faustino, A. I., Cabral, E. M., & Oliveira, R. F. (2013). Noninvasive Measurement of Steroid Hormones in Zebrafish Holding-Water. *Zebrafish*, *10*(1), 1 – 7. doi:10.1089/zeb.2012.0792

Fries, E., Dettenborn, L., & Kirschbaum, C. (2009). The cortisol awakening response (CAR): Facts and future directions. *International Journal of Psychophysiology*, *72*(1), 67–73. doi:10.1016/j.ijpsycho.2008.03.014

Fuzzen, M. L. M., Van Der Kraak, G., & Bernier, N. J. (2010). Stirring up new ideas about the regulation of the hypothalamic-pituitary-interrenal axis in zebrafish (Danio rerio). *Zebrafish*, *7*(4), 349–58. doi:10.1089/zeb.2010.0662

Fuzzo, F., Matsumoto, J., Kiyokawa, Y., Takeuchi, Y., Ono, T., & Nishijo, H. (2015). Social buffering suppresses fear-associated activation of the lateral amygdala in male rats: behavioral and neurophysiological evidence. *Frontiers in Neuroscience*, *9*(March), 1–8. doi:10.3389/fnins.2015.00099

Galhardo, L., & Oliveira, R. F. (2009). ARBS Annual Review of Biomedical Sciences Psychological Stress and Welfare in Fish, 1–20.

Ganz, J., Kaslin, J., Freudenreich, D., Machate, A., Geffarth, M., & Brand, M. (2012). Subdivisions of the adult zebrafish subpallium by molecular marker analysis. *Journal of Comparative Neurology*, *520*(3), 633–655. doi:10.1002/cne.22757

Ganz, J., Kroehne, V., Freudenreich, D., Machate, A., Geffarth, M., Braasch, I., Brand, M. (2015). Subdivisions of the adult zebrafish pallium based on molecular marker analysis. *F1000Research*, *520*(3), 633–655. doi:10.12688/f1000research.5595.2

Gerlach, G., Hodgins-Davis, A., Avolio, C., & Schunter, C. (2008). Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings. Biological Sciences / The Royal Society*, *275*(1647), 2165–2170. doi:10.1098/rspb.2008.0647

Gerlach, G., & Lysiak, N. (2006). Kin recognition and inbreeding avoidance in zebrafish, Danio rerio, is based on phenotype matching. *Animal Behaviour*, *71*(6), 1371–1377. doi:10.1016/j.anbehav.2005.10.010

Gerlotto, F., Bertrand, S., Bez, N., & Gutierrez, M. (2006). Waves of agitation inside anchovy schools observed with multibeam sonar: a way to transmit information in response to predation.

ICES Journal of Marine Science, *63*(8), 1405–1417. doi:10.1016/j.icesjms.2006.04.023

Ghisleni, G., Capiotti, K. M., Da Silva, R. S., Oses, J. P., Piato, Â. L., Soares, V., Bonan, C. D. (2012). The role of CRH in behavioral responses to acute restraint stress in zebrafish. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *36*(1), 176–182. doi:10.1016/j.pnpbp.2011.08.016

Goodson, J. L., & Kingsbury, M. A. (2013). What's in a name? Considerations of homologies and nomenclature for vertebrate social behavior networks. *Hormones and Behavior*, *64*(1), 103–112. doi:10.1016/j.yhbeh.2013.05.006

Goto-Kazeto, R., Kight, K. E., Zohar, Y., Place, A. R., & Trant, J. M. (2004). Localization and expression of aromatase mRNA in adult zebrafish. *General and Comparative Endocrinology*, *139*(1), 72–84. doi:10.1016/j.ygcen.2004.07.003

Green, J., Collins, C., Kyzar, E. J., Pham, M., Roth, A., Gaikwad, S., Kalueff, A. V. (2012). Automated high-throughput neurophenotyping of zebrafish social behavior. *Journal of Neuroscience Methods*, *210*(2), 266–271. doi:10.1016/j.jneumeth.2012.07.017

Hall, D., & Suboski, M. D. (1995). Visual and olfactory stimuli in learned release of alarm reactions by zebra danio fish (Brachydanio rerio). *Neurobiology of Learning and Memory*. doi:10.1037/0735-7036.109.1.76

Hennessy, M. B., Kaiser, S., & Sachser, N. (2009). Social buffering of the stress response: Diversity, mechanisms, and functions. *Frontiers in Neuroendocrinology*, *30*(4), 470–482. doi:10.1016/j.yfrne.2009.06.001

Hennessy, M. B., Maken, D. S., & C. Graves, F. (2000). Consequences of the presence of the mother or unfamiliar adult female on cortisol, ACTH, testosterone and behavioral responses of periadolescent guinea pigs during exposure to novelty. *Psychoneuroendocrinology*, *25*(6), 619–632. doi:10.1016/S0306-4530(00)00014-7

Hennessy, M. B., Zate, R., & Maken, D. S. (2008). Social buffering of the cortisol response of adult female guinea pigs. *Physiology & Behavior*, *93*(4-5), 883–8. doi:10.1016/j.physbeh.2007.12.005

Herry, C., Trifilieff, P., Micheau, J., Lüthi, A., & Mons, N. (2006). Extinction of auditory fear conditioning requires MAPK/ERK activation in the basolateral amygdala. *European Journal of Neuroscience*, *24*(1), 261–269. doi:10.1111/j.1460-9568.2006.04893.x

Hinz, C., Kobbenbring, S., Kress, S., Sigman, L., Müller, A., & Gerlach, G. (2013). Kin recognition in zebrafish, Danio rerio, is based on imprinting on olfactory and visual stimuli. *Animal Behaviour*, *85*(5), 925–930. doi:10.1016/j.anbehav.2013.02.010

Hoare, D. ., Couzin, I. ., Godin, J.-G. ., & Krause, J. (2004). Context-dependent group size choice in fish. *Animal Behaviour*, *67*(1), 155–164. doi:10.1016/j.anbehav.2003.04.004

Hollén, L. I., & Radford, A. N. (2009). The development of alarm call behaviour in mammals and birds. *Animal Behaviour*, *78*(4), 791–800. doi:10.1016/j.anbehav.2009.07.021

Hostinar, C. E., Sullivan, R. M., & Gunnar, M. R. (2014). Psychobiological mechanisms underlying the social buffering of the hypothalamic–pituitary–adrenocortical axis: A review of animal models and human studies across development. *Psychological Bulletin*, *140*(1), 256–282. doi:10.1037/a0032671

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. doi:10.1038/nature12111

Hyman, S. E. (2001). *The Science of Mental Health: Depression*. (S. E. Hyman, Ed.). Routledge.

Jesuthasan, S. J., & Mathuru, A. S. (2008). The alarm response in zebrafish: innate fear in a vertebrate genetic model. *Journal of Neurogenetics*, *22*(3), 211–228.

Jetti, S. K., Vendrell-Llopis, N., & Yaksi, E. (2014). Spontaneous activity governs olfactory representations in spatially organized habenular microcircuits. *Current Biology*, *24*(4), 434–439. doi:10.1016/j.cub.2014.01.015

Joels, M., Fernandez, G., & Roozendaal, B. (2011). Stress and emotional memory: A matter of timing. *Trends in Cognitive Sciences*, *15*(6), 280–288. doi:10.1016/j.tics.2011.04.004

Jones, B. S., Harris, D. H. R., & Catchpole, C. K. (1993). The stability of the vocal signature in phee calls of the common marmoset,Callithrix jacchus. *American Journal of Primatology*, *31*(1), 67–75. doi:10.1002/ajp.1350310107

Judkewitz, B., Horstmeyer, R., Vellekoop, I. M., Papadopoulos, I. N., & Yang, C. (2015). Translation correlations in anisotropically scattering media. *Nature Physics*, (June), 1–6. doi:10.1038/nphys3373

Judkewitz, B., Wang, Y. M., Horstmeyer, R., Mathy, A., & Yang, C. (2013). Speckle-scale focusing in the diffusive regime with time-reversal of variance-encoded light (TROVE). *Nature Photonics*, *7*(4), 300–305. doi:10.1038/nphoton.2013.31

Kalendar, R., Lee, D., & Schulman, A. H. (2011). Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. *Genomics*, *98*(2), 137–44. doi:10.1016/j.ygeno.2011.04.009

Kalendar, R., Lee, D., & Schulman, A. H. (2014). FastPCR Software for PCR, In Silico PCR, and

Oligonucleotide Assembly and Analysis. In S. Valla & R. Lale (Eds.), *DNA Cloning and Assembly Methods, Methods in Molecular Biology* (pp. 271–302). Humana Press.

Kalueff, A. V, Gebhardt, M., Stewart, A. M., Cachat, J. M., Brimmer, M., Chawla, J. S., Schneider, H. (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish*, *10*(1), 70–86. doi:10.1089/zeb.2012.0861

Kendler, K. S., Karkowski, L. M., & Prescott, C. A. (1999). Causal Relationship Between Stressful Life Events and the Onset of Major Depression. *Psychiatry Interpersonal and Biological Processes*, *156*(June), 837–841. doi:10.1176/ajp.156.6.837

Kendrick, K. M., da Costa, A. P., Leigh, A. E., Hinton, M. R., & Peirce, J. W. (2001). Sheep don't forget a face. *Nature*, *414*(6860), 165–166. doi:10.1038/35102669

Kermen, F., Franco, L. M., Wyatt, C., & Yaksi, E. (2013). Neural circuits mediating olfactory-driven behavior in fish. *Frontiers in Neural Circuits*, *7*(April), 62. doi:10.3389/fncir.2013.00062

Kikusui, T., Winslow, J. T., & Mori, Y. (2006). Social buffering: relief from stress and anxiety. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *361*(1476), 2215–2228. doi:10.1098/rstb.2006.1941

Kiyokawa, Y., Honda, A., Takeuchi, Y., & Mori, Y. (2014). A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behavioural Brain Research*, *267*, 189–93. doi:10.1016/j.bbr.2014.03.043

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., & Mori, Y. (2004). Partner's stress status influences social buffering effects in rats. *Behavioral Neuroscience*, *118*(4), 798–804. doi:10.1037/0735-7044.118.4.798

Kiyokawa, Y., Takeuchi, Y., & Mori, Y. (2007). Two types of social buffering differentially mitigate conditioned fear responses. *European Journal of Neuroscience*, *26*(12), 3606–3613. doi:10.1111/j.1460-9568.2007.05969.x

Kiyokawa, Y., Wakabayashi, Y., Takeuchi, Y., & Mori, Y. (2012). The neural pathway underlying social buffering of conditioned fear responses in male rats. *European Journal of Neuroscience*, *36*(10), 3429–3437. doi:10.1111/j.1460-9568.2012.08257.x

Koakoski, G., Oliveira, T. A., da Rosa, J. G. S., Fagundes, M., Kreutz, L. C., & Barcellos, L. J. G. (2012). Divergent time course of cortisol response to stress in fish of different ages. *Physiology and Behavior*, *106*(2), 129–132. doi:10.1016/j.physbeh.2012.01.013

Koops, M. a. (2004). Reliability and the value of information. *Animal Behaviour*, *67*(1), 103–111.

doi:10.1016/j.anbehav.2003.02.008

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics (Oxford, England)*, *23*(10), 1289–91. doi:10.1093/bioinformatics/btm091

Krause, J., & Ruxton, G. D. (2002). *Living in groups*. Oxford University Press.

Lanahan, A., & Worley, P. (1998). Immediate-Early Genes and Synaptic Function. *Neurobiology of Learning and Memory*, *70*(1-2), 37–43. doi:10.1006/nlme.1998.3836

Lau, B. Y. B., Mathur, P., Gould, G. G., & Guo, S. (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proceedings of the National Academy of Sciences*, *108*(6), 2581–2586. doi:10.1073/pnas.1018275108

LeDoux, J. (2003). The emotional brain, fear, and the amygdala. *Cellular and Molecular Neurobiology*, *23*(4-5), 727–738. doi:10.1023/A:1025048802629

Levine, S., Coe, C. L., Smotherman, W. P., & Kaplan, J. N. (1978). Prolonged cortisol elevation in the infant squirrel monkey after reunion with mother. *Physiology and Behavior*, *20*(1), 7–10. doi:10.1016/0031-9384(78)90194-4

Livia Terranova, M., Cirulli, F., & Laviola, G. (1999). Behavioral and hormonal effects of partner familiarity in periadolescent rat pairs upon novelty exposure. *Psychoneuroendocrinology*, *24*(6), 639–656. doi:10.1016/S0306-4530(99)00019-0

Luksys, G., & Sandi, C. (2011). Neural mechanisms and computations underlying stress effects on learning and memory. *Current Opinion in Neurobiology*, *21*(3), 502–508. doi:10.1016/j.conb.2011.03.003

Magurran, A. E., & Pitcher, T. J. (1987). Provenance, Shoal Size and the Sociobiology of Predator-Evasion Behaviour in Minnow Shoals. *Proceedings of the Royal Society B: Biological Sciences*, *229*(1257), 439–465. doi:10.1098/rspb.1987.0004

Manassa, R. P., Dixson, D. L., McCormick, M. I., & Chivers, D. P. (2013). Coral reef fish incorporate multiple sources of visual and chemical information to mediate predation risk. *Animal Behaviour*, *86*(4), 717–722. doi:10.1016/j.anbehav.2013.07.003

Mathuru, A. S., Kibat, C., Cheong, W. F., Shui, G., Wenk, M. R., Friedrich, R. W., & Jesuthasan, S. (2012). Chondroitin fragments are odorants that trigger fear behavior in fish. *Current Biology*, *22*(6), 538–544. doi:10.1016/j.cub.2012.01.061

Maximino, C., Lima, M. G., Oliveira, K. R. M., Batista, E. de J. O., & Herculano, A. M. (2013). " Limbic associative" and " autonomic" amygdala in teleosts: A review of the evidence. *Journal of*

Chemical Neuroanatomy, *48-49*, 1–13. doi:10.1016/j.jchemneu.2012.10.001

McLinn, C. M., & Stephens, D. W. (2006). What makes information valuable: signal reliability and environmental uncertainty. *Animal Behaviour*, *71*(5), 1119–1129. doi:10.1016/j.anbehav.2005.09.006

Miller, N., & Gerlai, R. (2007). Quantification of shoaling behaviour in zebrafish (Danio rerio). *Behavioural Brain Research*, *184*(2), 157–166. doi:10.1016/j.bbr.2007.07.007

Mirat, O., Sternberg, J. R., Severi, K. E., & Wyart, C. (2013). ZebraZoom: an automated program for high-throughput behavioral analysis and categorization. *Frontiers in Neural Circuits*, *7*(June), 1–12. doi:10.3389/fncir.2013.00107

Moriceau, S., Roth, T. L., & Sullivan, R. M. (2010). Rodent model of infant attachment learning and stress. *Developmental Psychobiology*, *52*(7), 651–660. doi:10.1002/dev.20482

Mueller, T., & Wullimann, M. (2015). Atlas of early zebrafish brain development: a tool for molecular neurogenetics. *Academic Press.*

Neumann, I. D., Wigger, a., Torner, L., Holsboer, F., & Landgraf, R. (2000). Brain oxytocin inhibits basal and stress-induced activity of the hypothalamo-pituitary-adrenal axis in male and female rats: Partial action within the paraventricular nucleus. *Journal of Neuroendocrinology*, *12*(3), 235–243. doi:10.1046/j.1365-2826.2000.00442.x

Novak, M. A., Hamel, A. F., Kelly, B. J., Dettmer, A. M., & Meyer, J. S. (2013). Stress, the HPA axis, and nonhuman primate well-being: A review. *Applied Animal Behaviour Science*, *143*(2-4), 135–149. doi:10.1016/j.applanim.2012.10.012

O'Connell, L. a, & Hofmann, H. a. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *The Journal of Comparative Neurology*, *519*(18), 3599–639. doi:10.1002/cne.22735

Oitzl, M. S., Champagne, D. L., van der Veen, R., & de Kloet, E. R. (2010). Brain development under stress: Hypotheses of glucocorticoid actions revisited. *Neuroscience and Biobehavioral Reviews*, *34*(6), 853–866. doi:10.1016/j.neubiorev.2009.07.006

Okamoto, H., Agetsuma, M., & Aizawa, H. (2012). Genetic dissection of the zebrafish habenula, a possible switching board for selection of behavioral strategy to cope with fear and anxiety. *Developmental Neurobiology*, *72*(3), 386–394. doi:10.1002/dneu.20913

Okuno, H. (2011). Regulation and function of immediate-early genes in the brain: Beyond neuronal activity markers. *Neuroscience Research*, *69*(3), 175–186. doi:10.1016/j.neures.2010.12.007

Oliveira, R. F., Simões, J. M., Teles, M. C., Oliveira, C. R., Becker, J. D., & Lopes, J. S. (2016). Assessment of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain. *Proceedings of the National Academy of Sciences*, 201514292. doi:10.1073/pnas.1514292113

Olson, R. S., Hintze, A., Dyer, F. C., Knoester, D. B., & Adami, C. (2013). Predator confusion is sufficient to evolve swarming behaviour. *Journal of the Royal Society, Interface / the Royal Society*, *10*(85), 20130305. doi:10.1098/rsif.2013.0305

Parichy, D. M. (2015). The natural history of model organisms: Advancing biology through a deeper understanding of zebrafish ecology and evolution. *eLife*, *2015*(4), 1–11. doi:10.7554/eLife.05635.001

Pavlidis, M., Digka, N., Theodoridi, A., Campo, A., Barsakis, K., Skouradakis, G., … Tsalafouta, A. (2013). Husbandry of zebrafish, Danio rerio, and the cortisol stress response. *Zebrafish*, *10*(4), 524–31. doi:10.1089/zeb.2012.0819

Pavlidis, M., Sundvik, M., Chen, Y. C., & Panula, P. (2011a). Adaptive changes in zebrafish brain in dominant-subordinate behavioral context. *Behavioural Brain Research*, *225*(2), 529–537.

Pavlidis, M., Sundvik, M., Chen, Y.-C., & Panula, P. (2011b). Adaptive changes in zebrafish brain in dominant–subordinate behavioral context. *Behavioural Brain Research*, *225*(2), 529–537. doi:10.1016/j.bbr.2011.08.022

Pavlidis, M., Theodoridi, A., & Tsalafouta, A. (2015). Neuroendocrine regulation of the stress response in adult zebrafish, Danio rerio. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *60*, 121–131. doi:10.1016/j.pnpbp.2015.02.014

Pereira, A. G., Cruz, A., Lima, S. Q., & Moita, M. A. (2012). Silence resulting from the cessation of movement signals danger. *Current Biology*, *22*(16), R627–R628. doi:10.1016/j.cub.2012.06.015

Pérez-Escudero, A., Vicente-Page, J., Hinz, R. C., Arganda, S., & de Polavieja, G. G. (2014). idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature Methods*, *11*(7), 743–748. doi:10.1038/nmeth.2994

Peter, R. E. (1977). The preoptic nucleus in fishes: A comparative discussion of function-activity relationships. *Integrative and Comparative Biology*, *17*(4), 775–785. doi:10.1093/icb/17.4.775

Piato, Â. L., Capiotti, K. M., Tamborski, A. R., Oses, J. P., Barcellos, L. J. G., Bogo, M. R., Bonan, C. D. (2011). Unpredictable chronic stress model in zebrafish (Danio rerio): behavioral and physiological responses. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *35*(2), 561–7.

doi:10.1016/j.pnpbp.2010.12.018

Pine, D. S., Cohen, P., Johnson, J. G., & Brook, J. S. (2002). Adolescent life events as predictors of adult depression. *Journal of Affective Disorders*, *68*(1), 49–57. doi:10.1016/S0165-0327(00)00331-1

Portugues, R., Feierstein, C. E., Engert, F., & Orger, M. B. (2014). Whole-brain activity maps reveal stereotyped, distributed networks for visuomotor behavior. *Neuron*, *81*(6), 1328–1343. doi:10.1016/j.neuron.2014.01.019

Pritchard, V., Lawrence, J., Butlin, R. K., & Krause, J. (2001). Shoal choice in zebrafish, Danio rerio: the influence of shoal size and activity. *Animal Behaviour*, *62*(6), 1085–1088. doi:10.1006/anbe.2001.1858

Qin, M., Wong, A., Seguin, D., & Gerlai, R. (2014). Induction of social behavior in zebrafish: live versus computer animated fish as stimuli. *Zebrafish*, *11*(3), 185–97. doi:10.1089/zeb.2013.0969

Quirk, G. J., Repa, J. C., & LeDoux, J. E. (1995). Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: Parallel recordings in the freely behaving rat. *Neuron*, *15*(5), 1029–1039. doi:10.1016/0896-6273(95)90092-6

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, Danio rerio. *Aquaculture*, *258*(1-4), 565–574. doi:10.1016/j.aquaculture.2006.04.020

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture*, *297*(1-4), 157–162. doi:10.1016/j.aquaculture.2009.08.035

Rauch, S. L., Whalen, P. J., Shin, L. M., McInerney, S. C., MacKlin, M. L., Lasko, N. B., Pitman, R. K. (2000). Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: A functional MRI study. *Biological Psychiatry*, *47*(9), 769–776. doi:10.1016/S0006-3223(00)00828-3

Roberts, G. (1996). Why individual vigilance declines as group size increases. *Animal Behaviour*, *51*(June 1995), 1077–1086. doi:10.1006/anbe.1996.0109

Rukstalis, M., & French, J. A. (2005). Vocal buffering of the stress response: Exposure to conspecific vocalizations moderates urinary cortisol excretion in isolated marmosets. *Hormones and Behavior*, *47*(1), 1–7. doi:10.1016/j.yhbeh.2004.09.004

Sapolsky, R. M., Romero, L. M., & Munck, a. U. (2000). How Do Glucocorticoids Influence Stress Responses? Preparative Actions *. *Endocrine Reviews*, *21*(April), 55–89.

doi:10.1210/er.21.1.55

Saverino, C., & Gerlai, R. (2008). The social zebrafish: Behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural Brain Research*, *191*(1), 77–87. doi:10.1016/j.bbr.2008.03.013

Schirmer, A., Jesuthasan, S., & Mathuru, A. S. (2013). Tactile stimulation reduces fear in fish. *Frontiers in Behavioral Neuroscience*, *7*(November), 167. doi:10.3389/fnbeh.2013.00167

Selye, H. (1975). Confusion and controversy in the stress field. *Journal of Human Stress*, *1*(2), 37–44.

Sergent, C., Wyart, V., Babo-Rebelo, M., Cohen, L., Naccache, L., & Tallon-Baudry, C. (2013). Cueing attention after the stimulus is gone can retrospectively trigger conscious perception. *Current Biology : CB*, *23*(2), 150–5. doi:10.1016/j.cub.2012.11.047

Sheehan, T. P., Chambers, R. A., & Russell, D. S. (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. *Brain Research Reviews*, *46*(1), 71–117. doi:10.1016/j.brainresrev.2004.04.009

Smith, A. S., & Wang, Z. (2014). Hypothalamic Oxytocin Mediates Social Buffering of the Stress Response. *Biological Psychiatry*, *76*(4), 281–288. doi:10.1016/j.biopsych.2013.09.017

Smith, M., & Belk, M. (2001). Risk assessment in western mosquitofish (Gambusia affinis): do multiple cues have additive effects? *Behavioral Ecology and Sociobiology*, *51*(1), 101–107. doi:10.1007/s002650100415

Smith, S. M., & Vale, W. W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues in Clinical Neuroscience*, *8*(4), 383–395. doi:10.1038/nrendo.2011.222

Snowdon, C. T., & Cleveland, J. (1980). Individual recognition of contact calls by pygmy marmosets. *Animal Behaviour*, *28*(3), 717–727. doi:10.1016/S0003-3472(80)80131-X

Sparta, D. R., Smithuis, J., Stamatakis, A. M., Jennings, J. H., Kantak, P. A., Ung, R. L., & Stuber, G. D. (2014). Inhibition of projections from the basolateral amygdala to the entorhinal cortex disrupts the acquisition of contextual fear. *Frontiers in Behavioral Neuroscience*, *8*(May), 6–11. doi:10.3389/fnbeh.2014.00129

Speedie, N., & Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (Danio rerio). *Behavioural Brain Research*, *188*(1), 168–177. doi:10.1016/j.bbr.2007.10.031

Spence, R., Fatema, M. K., Reichard, M., Huq, K. A., Wahab, M. A., Ahmed, Z. F., & Smith, C.

(2006). The distribution and habitat preferences of the zebrafish in Bangladesh. *Journal of Fish Biology*, *69*(5), 1435–1448. doi:10.1111/j.1095-8649.2006.01206.x

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews of the Cambridge Philosophical Society*, *83*(1), 13–34. doi:10.1111/j.1469-185X.2007.00030.x

Spence, R., Jordan, W. C., & Smith, C. (2006). Genetic analysis of male reproductive success in relation to density in the zebrafish, Danio rerio. *Frontiers in Zoology*, *3*, 5. doi:10.1186/1742-9994-3-5

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., & Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends in Neurosciences*, *37*(5), 264–278. doi:10.1016/j.tins.2014.02.011

Suboski, M. D., Bain, S., Carty, A. E., McQuoid, L. M., & et al. (1990). Alarm reaction in acquisition and social transmission of simulated-predator recognition by zebra danio fish (Brachydanio rerio). *Journal of Comparative Psychology*, *104*(1), 101–112. doi:10.1037/0735-7036.104.1.101

Suriyampola, P. S., Shelton, D. S., Shukla, R., Roy, T., Bhat, A., & Martins, E. P. (2015). Zebrafish Social Behavior in the Wild. *Zebrafish*, *00*(00), zeb.2015.1159. doi:10.1089/zeb.2015.1159

Takahashi, Y., Kiyokawa, Y., Kodama, Y., Arata, S., Takeuchi, Y., & Mori, Y. (2013). Olfactory signals mediate social buffering of conditioned fear responses in male rats. *Behavioural Brain Research*, *240*(1), 46–51. doi:10.1016/j.bbr.2012.11.017

Teles, M. C., Almeida, O., & Oliveira, R. F. (2015). Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish.

Teles, M. C., Dahlbom, S. J., Winberg, S., & Oliveira, R. F. (2013). Social modulation of brain monoamine levels in zebrafish. *Behavioural Brain Research*, *253*, 17–24. doi:10.1016/j.bbr.2013.07.012

Thorsteinsson, E. B., James, J. E., & Gregg, M. E. (1998). Effects of video-relayed social support on hemodynamic reactivity and salivary cortisol during laboratory-based behavioral challenge. *Health Psychology*, *17*(5), 436–444. doi:10.1037/0278-6133.17.5.436

Tovote, P., Fadok, J. P., & Lüthi, A. (2015). Neuronal circuits for fear and anxiety. *Nature Reviews Neuroscience*, *16*(6), 317–331. doi:10.1038/nrn3945

Ulrich-Lai, Y. M., & Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. *Nature Reviews. Neuroscience*, *10*(6), 397–409. doi:10.1038/nrn2647

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3--new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), e115. doi:10.1093/nar/gks596

Viscido, S. V., & Wethey, D. S. (2002). Quantitative analysis of fiddler crab flock movement: evidence for "selfish herd"behaviour. *Animal Behaviour*, *63*(4), 735–741. doi:10.1006/anbe.2001.1935

Whitfield, C., & Band, M. (2002). Annotated expressed sequence tags and cDNA microarrays for studies of brain and behavior in the honey bee. *Genome Research*, 555–566. doi:10.1101/gr.5302.information

Wilson, J. H. (2000). A conspecific attenuates prolactin responses to open-field exposure in rats. *Hormones and Behavior*, *38*(1), 39–43. doi:10.1006/hbeh.2000.1600

Wilson, J. H. (2001). Prolactin in rats is attenuated by conspecific touch in a novel environment. *Cognitive, Affective & Behavioral Neuroscience*, *1*(2), 199–205. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12467115

Winslow, J. T., Noble, P. L., Lyons, C. K., Sterk, S. M., & Insel, T. R. (2003). Rearing effects on cerebrospinal fluid oxytocin concentration and social buffering in rhesus monkeys. *Neuropsychopharmacology*, *28*(5), 910–918. doi:10.1038/sj.npp.1300128

Wullimann, M. F., Rupp, B., & Reichert, H. (1996). *Neuroanatomy of the Zebrafish Brain: A Topological Atlas*. (M. F. Wullimann & B. Rupp, Eds.). Birkhäuser Basel. doi:10.1007/978-3-0348-8979-7

Wyart, C., Del Bene, F., Warp, E., Scott, E. K., Trauner, D., Baier, H., & Isacoff, E. Y. (2009). Optogenetic dissection of a behavioural module in the vertebrate spinal cord. *Nature*, *461*(7262), 407–410. doi:10.1038/nature08323

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*(1), 134. doi:10.1186/1471-2105-13-134

Yeh, C. M., Glöck, M., & Ryu, S. (2013). An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish. *PLoS ONE*, *8*(11), 1–8. doi:10.1371/journal.pone.0079406

Ziv, L., Muto, A., Schoonheim, P. J., Meijsing, S. H., Strasser, D., Ingraham, H. A., Baier, H. (2013). An affective disorder in zebrafish with mutation of the glucocorticoid receptor. *Molecular Psychiatry*, *18*(6), 681–691. doi:10.1038/mp.2012.64