Social Eavesdropping in Zebrafish

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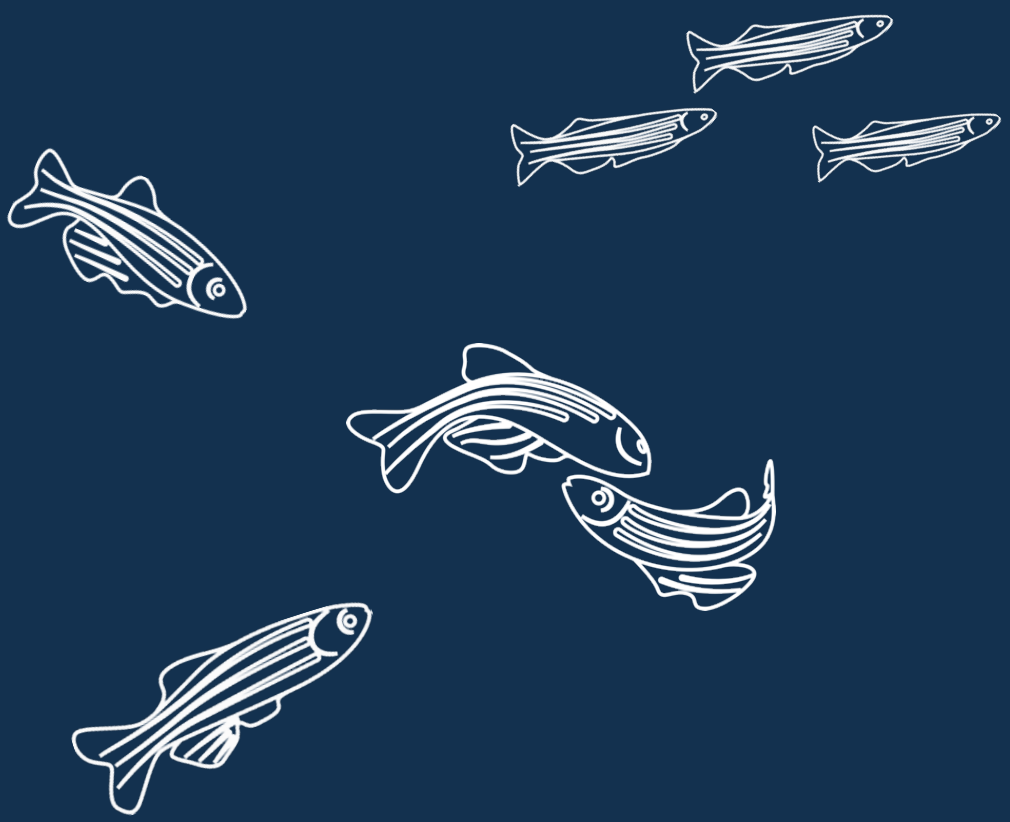

Dissertation presented to obtain the Ph.D degree in Biology | Neuroscience

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

Oeiras,
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Knowledge Creation

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A Dissertation

Presented to Universidade Nova de Lisboa

in Candidacy for the Degree

of Doctor of Philosophy

in Biology | Neuroscience

Advisor: Professor Doutor Rui F. Oliveira

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Summary

Group living animals may eavesdrop on signalling interactions between conspecifics. This enables them to collect adaptively relevant information about others, without incurring in the costs of first-hand information acquisition. Such ability, aka social eavesdropping, is expected to impact Darwinian fitness and hence predicts the evolution of cognitive processes that enable social animals to use social information available in the environment. Such adaptive specializations in cognition may have evolved both at the level of learning and memory mechanisms, and at the level of input mechanisms such as attention, which selects the information that is available for learning. Moreover, it is expected that social animals might integrate eavesdropped information with their own direct social experience in order to optimize the use of information from others. However, very little is known about the behavioural and neural mechanisms underlying social eavesdropping processes, and the interplay between eavesdropped and private social information.

The research presented in this thesis aimed to address these questions using zebrafish (*Danio rerio*), a highly social model organism that lives in communication networks and is an emerging experimental model in social neuroscience and neuroethology. A first set of studies aimed to test if attention in zebrafish is tuned to the exchange of information between conspecifics. Our results revealed that bystander zebrafish are more attentive towards interacting (i.e. fighting) than towards non-interacting pairs of conspecifics. Moreover, using video

playbacks as stimulus in order to manipulate form features of the interacting fish, we showed that bystanders' attention is higher when observing the assessment stage of a fighting interaction and more dependent on form features of the opponents; whereas during the post-resolution stage it is more driven by biological movement features of the dominant fish chasing the subordinate fish.

Following up on the first set of results, a second study aimed to start exploring the genetic basis of social eavesdropping. The goal was to analyse and compare the brain gene expression profiles of bystander zebrafish that exhibited different behavioural attentional profiles towards conspecifics, involved or not in fighting interactions. In order to achieve it, we used microarray gene chips to characterize their brain transcriptomes based on differentially expressed genes. This analysis was complemented by an analysis of the promoter regions of those genes. Using data from both approaches, protein interaction networks were further drafted. The obtained results suggest that attentiveness towards conspecifics, whether interacting or not, activates pathways linked to neuronal plasticity and memory formation. Moreover, specifically observing fighting interactions further triggers specific pathways. This suggests that the acquisition of eavesdropped information about social relationships might activate specific processes on top of those already activated just by observing conspecifics.

Finally, we designed a study to demonstrate the occurrence of social eavesdropping in zebrafish and its integration with the eavesdroppers' own past direct social experience. To investigate it, we first manipulated the dominance status of bystander zebrafish.

Next, bystanders were either allowed or prevented from observing a fight. Lastly, their behaviour towards the winners and losers of the interaction was assessed using a custom-made video-tracking system and directional analysis. Our results showed that only dominant bystanders who had seen the fight, revealed a significant increase in directional focus (a measure of attention) towards the losers of the fights. Furthermore, results indicated that information about the fighters' acquired status was collected from the signalling interaction itself and not from post-interaction cues, which implies the existence of individual recognition in zebrafish. Hence, our results showed for the first time that zebrafish eavesdrop on conspecific fighting interactions and that this process is modulated by the eavesdropper's dominance status.

In summary, we showed that zebrafish are tuned to attend and eavesdrop on social agonistic interactions between conspecifics. This attention is more focused on specific stages of the interactions and on form and movement features of the observed conspecifics. We further verified that attentiveness to the interactions has an impact at the brain gene expression level. Moreover, we showed that the use of eavesdropped information is modulated by the eavesdropper's past social experience. This thesis further advances the current understanding of the mechanisms of social eavesdropping, encouraging further venues of research and setting the stage for the study of its underlying neural mechanisms in a model organism.

Resumo

Animais sociais que vivem em grupos têm a possibilidade de observar trocas de sinais ocorrentes de interações entre conspecíficos. Esta capacidade permite-lhes recolher informação adaptativa relevante acerca dos outros, sem incorrerem em custos associados à aquisição de informação em primeira mão. É esperado que esta capacidade, conhecida por 'social eavesdropping', tenha impacto a nível da sua aptidão Darwiniana e consequentemente prevê a evolução de processos cognitivos que possibilitem o uso de informação social presente no seu ambiente. Estas especializações adaptativas na cognição poderão ter evoluído tanto ao nível de mecanismos de aprendizagem e memória, como ao nível de mecanismos de entrada tais como a atenção, que seleciona a informação disponível para aprendizagem. É também esperado que os animais sociais possam integrar a informação obtida por 'eavesdropping' com a sua própria experiência social direta, de modo a optimizar a informação obtida dos outros. No entanto muito pouco é ainda conhecido sobre os mecanismos comportamentais e neurais na base do 'social eavesdropping', assim como a inter-relação entre esta informação e informação social privada.

A investigação apresentada nesta tese abordou estas questões utilizando o peixe-zebra (*Danio rerio*), um organismo altamente social que vive em redes de comunicação e é um modelo experimental emergente em neurociências sociais e neuroetologia. Um primeiro conjunto de estudos experimentais procurou testar se a atenção no peixe-zebra está sintonizada para a troca de informação entre

conspecíficos. Os nossos resultados revelaram que os peixe-zebra são mais atentos a pares de conspecíficos quando estes interagem (i.e. lutam) do que quando não interagem. Seguidamente, usámos como estímulo imagens gravadas em vídeo de conspecíficos a lutar de forma a manipular a forma dos peixes apresentados num ecrã. Isto permitiu-nos mostrar que a atenção dos peixes-zebra espectadores é maior quando observam a fase de avaliação da luta (pré-resolução) e é mais dirigida por características de forma dos oponentes; enquanto que na fase de pós-resolução, a atenção é mais dirigida pelas características de movimento biológico do peixe dominante a perseguir o peixe subordinado.

A partir deste primeiro conjunto de resultados, começámos a explorar num segundo estudo, as bases genéticas do 'social eavesdropping'. O objectivo foi o de analisar e comparar os perfis de expressão génica cerebrais de peixes-zebra que apresentaram diferentes perfis comportamentais de atenção em relação aos conspecíficos, envolvidos ou não nas interações agonísticas. Para tal, usámos 'microarray gene chips' para caracterizar os seus transcriptomas cerebrais, baseando-nos na expressão diferencial de genes. Esta análise foi complementada por uma análise das regiões promotoras dos genes diferencialmente expressos. Usando dados das duas abordagens, esboçámos ainda redes de interação de proteínas. Os resultados obtidos sugerem que estados de atenção em relação a conspecíficos, interagindo ou não, ativam vias ligadas a plasticidade neuronal e formação de memória. Sugerem também que a observação de interações de luta aciona adicionalmente vias específicas, o que sugere que a aquisição de

informação por 'eavesdropping' sobre relações sociais possa ativar processos específicos para além dos já ativados apenas pela observação de conspecíficos.

Por fim, desenvolvemos um estudo com o objectivo de demonstrar a ocorrência e uso de informação por 'social eavesdropping' por parte dos peixe-zebra e a integração com a sua própria experiência direta social passada. Para abordar esta questão, manipulámos primeiramente o estatuto de dominância dos peixes espectadores. Em seguida, foi-lhes permitida ou impedida a observação de uma luta. Finalmente, o seu comportamento em relação aos vencedores e derrotados da luta foi avaliado, usando um sistema de video-tracking e análise direcional. Os nossos resultados mostraram que apenas os espectadores dominantes, que tinham observado a luta, revelaram um aumento significativo de foco direcional (uma medida de atenção) em relação aos derrotados das lutas. Adicionalmente, indicaram que a informação acerca do estatuto dos lutadores foi adquirida por observação da troca de sinais durante a luta e não por alguma pista obtida durante a pós-interacção, o que sugere a existência de reconhecimento individual em peixe-zebra.

Em resumo, neste trabalho mostrámos que os peixe-zebra estão sintonizados para observar e realizarem 'eavesdropping' de interações agonísticas entre conspecíficos. Descobrimos que esta atenção está focada em fases específicas da interação e em características de forma e movimento dos conspecíficos observados. Adicionalmente verificámos que observar interações sociais tem impacto ao nível de expressão génica no cérebro do peixe-zebra. Mostrámos ainda que o uso de

informação obtida por ‘eavesdropping’ é modulada pela experiência social passada do próprio observador. Esta tese contribui para o avanço do presente conhecimento sobre os mecanismos do fenómeno de ‘social eavesdropping’, encorajando novas direções de investigação e preparando as bases para o estudo dos seus mecanismos neurais num organismo modelo.

Author Contributions

Rodrigo Abril de Abreu (R.A.) and Rui Oliveira (R.O.) designed all studies presented in this thesis, together with Ana Sofia Cruz (A.S.C.) in the social eavesdropping experiment (Chapter 4). R.A. performed all studies, together with A.S.C. in the social eavesdropping experiment (Chapter 4). R.A. analysed all data, together with João Sollari Lopes (J.S.L.) who performed the microarrays analysis (Chapter 3). José Cruz (J.C.) wrote the tracking software. R.A. wrote the tracking data analysis scripts used in the experiments.

Part of the studies presented in Chapter 2 have been published in the article Abril-de-Abreu et al. 2015a and have been submitted in the article Abril-de-Abreu et al. 2015b, written by R.A. and R.O. The study presented in Chapter 3 has been submitted in the article Abril-de-Abreu et al. 2015c, written by R.A and J.S.L. with the assistance of R.O. Part of the study presented in Chapter 4 has been published in the article Abril-de-Abreu et al. 2015d, written by R.A. with the assistance of R.O.

Contents

Chapter 1
General Introduction

Everybody should eavesdrop once in a while. There's nothing like eavesdropping to show you that the world outside your head is different from the world inside your head.

— Thornton Wilder, *The Matchmaker*

Decision-making in social animals is inexorably interlinked with the behaviours of others. We interpret, evaluate and respond to our world based not only in prior personal experience, expectations, affective or motivational states; we do it alongside a constant interchange and monitoring of information with others. This ability allows us to better and faster deal with information in order to reduce uncertainty in a constantly changing world. However, successful group living requires a constant balance between the added advantages provided by social information, that would otherwise not be available or costly for a single individual, and the disadvantages that rise from conflicts of interest. Consequently, successful acquisition of relevant and reliable information from others and their interactions, i.e. social eavesdropping, becomes a fundamental aspect for flexible, efficient adjusting in complex social environments, and it hence predicts the evolution of cognitive specializations that enable social animals to achieve it. Understanding the behavioural and neural mechanisms underlying these processes may provide fundamental insight on the evolution and remarkable success of sociality.

1.1 Chapter summary

The present work has focused on investigating social eavesdropping in the zebrafish (*Danio rerio*). In this chapter we will review:

- A conceptual framework for the study of social eavesdropping by addressing the use of social information in the context of communication networks.
- The main research to date on social eavesdropping and its relationship with public and private information use.
- The model organism zebrafish; its social behaviour, cognitive abilities and currently available neurogenetic tools for its study.

1.2 Social information use in communication networks

Social animals can acquire information by direct trial-and-error strategies of interaction with the environment (i.e. personal information), or by observation of other individuals and their interactions (i.e. social information) (Danchin et al. 2004). This ability to acquire and use social information seems to be ubiquitous across taxa, both in vertebrates and invertebrates (e.g. Leadbeater & Chittka 2007), suggesting ancient evolutionary origins or multiple convergence events (Earley 2010).

There are two major types of social information: social cues, which are inadvertent behaviours or characteristics produced by individuals not specialized for communication; and signals, which are traits that have been evolutionarily selected to convey information (Danchin et al. 2004; Bonnie & Earley 2007). Social cues and signals only gain meaning in the presence of at least another individual capable of perceiving and processing them. However, in social groups most communication occurs not only between two individuals alone but in a network of several individuals within signalling and receiving range of each other; that is, a communication network (McGregor & Peake 2000; McGregor 2005). Therefore signalling exchanges (and social cues) are potentially accessible not only to signallers and receivers but also to bystanders. This provides individuals within a communication network the opportunity to detect and extract valuable social information by observing others and their signalling interactions (i.e. eavesdropping).

It is therefore possible that specialized cognitive mechanisms with social domain-specific modules at the neural network level have evolved to efficiently perceive, attend, process, store and act on social information available in such environments (Oliveira 2013). In fact, in order to successfully learn from social information an animal must first be able to detect, select and attend to relevant sources of such information (e.g. an agonistic interaction between conspecifics)[1] from a multitude of other stimuli in the environment, with consequent fitness impacts (e.g. deciding whom to subsequently avoid or attack)

[1] This example will be thoroughly addressed in chapter 2.

(see Shettleworth 1999 for a detailed review). Examples of such social domain-specific modules might be found in the face-selective areas specialized for the recognition of faces in humans and macaques (Kanwisher et al. 1997; Tsao et al. 2008), or distinct classes of visual neurons in the amygdala that selectively respond when monkeys make direct eye contact with others (Mosher et al. 2014).

Subsequently, once an animal attends to social information (Bushnell 1998), the next step will be to optimize learning about properties of the environment, how to manage it, how others interact with it, or about the relative qualities of others (Bonnie & Earley 2007). Three main fields of research have addressed these different sources and forms of social information use rather independently, although often overlapping to a large extent. Namely research on social learning, public information use and social eavesdropping (see Bonnie & Earley 2007 for a review).

Social learning has been so far the most comprehensively studied field. The term can be broadly defined as 'learning about other agents or the inanimate world that is influenced by observation of, or interaction with another individual or its products' (Heyes 2012); and it has been found to be ubiquitous in animals, from insects to mammals, that routinely use it to successfully 'navigate' their social environment. Also, while traditionally it has been considered to depend on social-domain specific modules (but see Heyes 2012), little is still known about the cognitive and neural mechanisms underlying it (e.g. Burke et al. 2010). On the other hand substantial research exists about its adaptive function, and typically social learning studies have focused on 'how'

and 'what' is learned by observation of other individuals regarding the existence and obtainment of resources, mainly in the physical environment (e.g. how to obtain food) (Heyes 2012). To better address it, different categories have been created such as local and stimulus enhancement, observational conditioning, social facilitation, emulation and imitation; with studies usually measuring the subject animal's changes in attention, behaviour and motor skills resulting from the acquisition of knowledge from those observations (see Hoppitt & Laland 2013 for a detailed review; Bonnie & Earley 2007).

Social information however can also be about the characteristics of an environmental parameter, such as the quality of a location, a food resource, or even a conspecific. This information can often be conveyed unintentionally to a bystander as a by-product of the regular activities of other individuals within the group while optimizing their own performance. This type of inadvertent information acquired from the performance and decisions of others is defined as 'public information' (Valone 2007). It was originally introduced as a theory based on Bayesian updating in the context of group foraging animals and the influences of observing group members' foraging success (Valone 1989), but it has since developed into a field of research with a large body of work produced across different species. Consequently the more restrictive original definition has expanded to include different sources of social information regarding both physical (e.g. energy resources, habitat, breeding locations) and social (e.g. prospective mates, rivals, allies, predators) aspects of the environment that can affect the fitness

of an individual (see Danchin et al. 2004 and Valone 2007 for detailed reviews).

Lastly, as mentioned before, a unique source of social information that can only be obtained within a communication network is information extracted from signalling interactions between individuals. Now commonly referred as social eavesdropping, its study has also developed into a field of research of its own (Bonnie & Earley 2007). Here, information 'is not encoded in the cues and features of signals themselves, but in how signals are used in an interaction' (McGregor & Peake 2000), thus providing relative information about the signallers (e.g. hierarchy, mating success) that would not be available just from individual signals or cues (Bonnie & Earley 2007). We will address this topic it detail in the next section.

In brief, while social learning, public information use and social eavesdropping have been traditionally approached distinctively in the literature and its integration is subject to debate (e.g. Bonnie & Earley 2007), they can all be considered different forms and (often overlapping) aspects of social information acquisition and use, which are available to individuals within their social environment. Particularly, the focus of the present thesis — social eavesdropping — can be considered and approached as a form of social learning, which is based under certain circumstances on the acquisition of public information; and whose research (as we will see next) has much to gain from the conceptual similarities and advances already made in these fields.

1.3 Social eavesdropping

The term 'eavesdropping'[2] was originally introduced by McGregor (1993) in a communication network's context (particularly in territorial systems) to refer to information gathering from a signalling interaction by individuals that were not directly involved in the interaction. This definition was originally considered equivalent to situations where broadcasted signals were intercepted (usually by heterospecifics) to acquire absolute information about the signaller (e.g. localizing prey by using their mating signals). This concept was subsequently refined by Mcgregor & Dabelsteen (1996) to include a gain obtained from information contained in the interaction that could not be acquired from the individuals' signals alone. Later on, Peake (2005) separated eavesdropping in two classes: the prior he labelled 'interceptive eavesdropping' and the latter 'social eavesdropping' to denote specifically circumstances where bystanders acquire information on the 'relative performance of interacting signallers' (usually conspecifics) by attending to their signalling interactions, 'allowing both direct comparison of interactants and assessment of relationships between them' (Peake 2005)[3]. Social eavesdropping hence potentially allows a bystander the acquisition through different sensory modalities

[2] In the English language the word *eavesdropping* commonly refers to 'listening to someone's private conversation without them knowing' (Cambridge Advanced Learners Dictionary & Thesaurus, Cambridge University Press).

[3] An excellent historical perspective on social eavesdropping research and some of its experimental studies can be found in McGregor (2005) and Peake (2005). We will follow up on it, incorporating the most recent advances to the field.

(e.g. auditory, visual, chemical) of information about other individuals within a communication network, without the costs of first-hand experience (e.g. fighting). This suggests the existence of specialized cognitive mechanisms such as attention to social interactions (addressed in chapter 2), transitive inference (e.g. Grosenick et al. 2007), individual recognition (Beecher 1989; Tibbetts & Dale 2007) and social memory (e.g. Winslow et al. 2000; Hitti & Siegelbaum 2014), in order to perform it.

Social eavesdropping on aggressive interactions

Although some theoretical work has been developed regarding the impact of eavesdropping on aggressive interactions and its evolution in the framework of game theory (Johnstone 2001; McElreath 2003; Mesterton-Gibbons & Sherratt 2007), the majority of research to date has focused on behavioural studies in the context of acoustic and visual aggressive interactions, using birds and fish respectively. However a few studies have also tested eavesdropping in other species (including invertebrates) and in non-aggressive contexts (e.g. courtship and cooperation). The reason for this bias probably lays in the fact that using aggressive interactions as an experimental stimulus provides several advantages: (1) it is a ubiquitous, highly salient type of interaction in social species (e.g. fighting for territory, mates, food); (2) it can provide eavesdroppers accurate and reliable information on the relative competitive ability, condition, motivation, social status of future opponents; and (3) it is often a stereotyped behaviour, potentially

allowing the analysis, decoupling (e.g. signals, cues) and experimental manipulation of its features (see Peake & Mcgregor 2004 for a review).

The first experimental studies to directly test social eavesdropping using acoustic interactions were field studies in songbirds, using interactive playbacks of song contests simulating territorial intrusions (McGregor et al. 1997). They relied on prior research showing that overlapping or alternated singing interactions, and increasing or decreasing song lengths, encoded for willingness to escalate aggression (Dabelsteen et al. 1996). One of the first studies by Otter et al. (1999) used audio playbacks in a field study to instigate singing contests against two neighbouring male great tits (*Parus major*), each mated with a female. The playbacks (using speakers) simulated an intruder visiting the two males. One 'intruder' was aggressive (overlap singing) while the other was submissive (alternate singing) aiming to influence the female's assessment of the relative quality of the males. The bystander females paired with overlapped (challenged) males were more likely to intrude into the neighbouring male's territory in the following days than females paired with the alternated treatment's males (dominant). This suggested that females eavesdropped on the singing interactions and made a transitive inference about the relative quality of the males. Using a similar paradigm with black-capped chickadees (*Poecile atricapilla*), Mennill et al. (2002) played interactive song playbacks to engage high-ranking males with aggressive singing and low-ranking males with submissive singing, in order to alter the information possessed by female eavesdroppers about their mates. Microsatellite paternity analysis of offspring revealed that high-ranking

males that lost song contests with the simulated intruders showed a significantly higher level of paternity loss (by extra-pair copulations) than controls, while no effect was detected for low-ranking males. These results showed that females were strongly tuned to pay attention to the signalling interactions and suggested that information about relative quality was contained in the interaction. Furthermore, it implied that even short-term interactions could have significant fitness costs to the observed individuals.

However these experimental designs did not exclude possible effects from the playbacks on the interactants subsequent behaviour with the females, or controlled for prior social experience. Thus they could not demonstrate that the acquired information was restricted to the interaction. Two similar studies (Naguib & Todt 1997; Naguib et al. 1999) using male nightingales (*Luscinia megarhynchos)* avoided this problem by using speakers at two locations (inside territorial borders) to simulate both interactants performing overlapping/overlapped or leader/follower singing to a bystander male subject. They used the subject's location and its response-singing activity towards each speaker as preference measures. Subjects spent more time and sang more near the 'dominant' speaker after the interaction, even when the speaker was silent. This showed that social eavesdropping occurred on the information contained in the interaction (i.e. relative differences in song timing) and that the relative quality of interactants (dominant or submissive intruders) was associated with the locations of the speakers. Again using great tits, Peake et al. (2001) introduced a third speaker to this type of design to simulate a subsequent territorial intrusion by the

'winner' or 'loser' of a contest situated outside the subject's territory. The subject bystander males reduced their song output and switched song types more often to 'loser' intruders but not to 'winner' intruders, demonstrating the ability to use eavesdropped information in a subsequent direct 'aggressive' encounter.

Nevertheless, in natural conditions where multiple interactions are frequent among the members of a group, it should be unlikely that the only source of information about third parties comes from social eavesdropping. Both 'relative' information from prior eavesdropped interactions and direct interactions with others, as well as 'absolute' information from individual social cues and signals should also play important roles. Two other subsequent studies by Peake and colleagues demonstrated this. In the first study (Peake et al. 2002), subjects acquired prior relative information by direct social experience with a simulated male intruder A (high-aggressive or low-aggressive) and afterwards acquired further information by eavesdropping on a subsequent song contest between male A and an unknown male B. Subjects adapted their response to a later intrusion by male B according to their prior personal experience with A, showing that subjects integrated the two sources of information. In the second study (Peake et al. 2005) subjects had access to both absolute information about the individual quality of two simulated interactants (i.e. possessing a song repertoire of one or two songs) and to relative eavesdropped information obtained from the song matching contests between them. Subjects responded with reduced length songs to simulated 'two-songs' intruders compared to 'one-song' ones but on the other hand did not

approach or spend time near 'one-song' intruders that challenged 'two-songs' opponents, showing the use of both absolute information and relative eavesdropped information.

While acoustic signals are advantageous for long range signalling or when visual information is impaired (e.g. dense foliage), they convey different informational aspects than for instance visual signals at closer ranges, as is often the case with cohesive groups such as fish shoals. Alongside the acoustic interactions studies described above, several studies using visual and direct real interactions were conducted in the last two decades, mainly in fish but also in birds. The first study conducted in fish (Oliveira et al. 1998) used male Siamese fighting fish (*Betta splendens*), a highly territorial species with stereotyped visual aggressive displays. Bystander subjects were isolated in a central tank and allowed to observe (without themselves being seen) at one side of the tank an agonistic displaying interaction (fish visually interacting through a clear partition) between two male opponents (demonstrators). At the other side another similar interaction occurred but without being seen. Each of the seen and unseen 'winners' (i.e. those that displayed erect gill covers for longer while in close proximity) and 'losers' of the interactions were then introduced into a clear box in the central compartment simulating a territorial intrusion. Subjects took significantly longer to approach and display to seen 'winners' than to seen 'losers' but no differences were found with the unseen 'winners' and 'losers'. There were no differences in the demonstrators' size, colouration, competitive ability or intensity of displays. This strongly suggested that Siamese fighting fish assessed the relative fighting ability

of potential opponents by eavesdropping on the interactions and not as a consequence of other sources of information. It also suggested they were capable of individual recognition.

Unfortunately, unlike acoustic interactions studies, visual signalling interactions using real demonstrators are difficult to control and thus it is difficult to assess explicitly what is the nature of the relative information being acquired, or if other individual cues and signals may be contributing. Aiming to better confine the available information to the interaction, McGregor et al. (2001) extended the previous paradigm by decoupling the relative information in the interaction from the interaction itself. Subjects were presented with either real or apparent interactions where each demonstrator actually displayed to different opponents occluded inside an in-between small gap. After the interaction each demonstrator was visually presented to the subject sequentially (without any territorial intrusion). Subjects responded more aggressively to the apparent 'winners' than 'losers' of the fake interactions (regardless of the actual outcome), further suggesting that what was acquired was relative information from the perceived interaction. Peake et al. (2006) further attempted to control the signalling interactions by having bystander male Siamese fighting fish also observing an apparent interaction, this time between two male fish who were actually aggressively displaying against their own image in a mirror (i.e. an 'opponent' with exactly the same behaviour). Asymmetries in aggressiveness were manipulated by varying the distance of the mirror (distant mirror — lower aggressive behaviour) of one of the interactants while leaving the other constant; or by pre-test

exposure (which increases aggressive behaviour) of one of the interactants to another male. Afterwards, demonstrators were presented sequentially to the subject. Subjects responded more aggressively to 'winners' than 'losers' from the mirror interactions where losers (distant mirror) had decreased aggression. However they responded equally for mirror interactions where winners (pre-exposed) had increased aggression. The authors suggested that one possible explanation was that lower-than-normal levels of aggression may have been easier to discriminate or more relevant to eavesdroppers.

It should be noted however that in the described paradigms what was being assessed by eavesdroppers was not the actual winner and loser of an interaction (because an actual outcome never occurred) but the 'willingness' to engage or escalate a fight. Another aspect to consider when testing eavesdropping is that the demonstrators' behaviour can be affected by winner-loser effects (Rutte et al. 2006; Oliveira et al. 2011) resulting from the interaction or by audience effects (Marler et al. 1986; Zuberbühler 2008) resulting from previous mutual assessment between the eavesdropper and the demonstrators before the test. So for example, although no winner-loser effects were found in Oliveira et al. (1998) and audience effects were prevented by experimental design, in that paradigm there were also no outcomes from the agonistic interactions. Earley & Dugatkin (2002) aimed to tease apart these effects by analysing contest dynamics using actual fights between male green swordtail fish (*Xiphophorus helleri*). They also introduced an additional treatment where a bystander and interactants could see each other and interact during the fighting stage

through a clear glass. The results confirmed that swordtail fish eavesdropped on the interactions, similarly to Siamese fighting fish. Bystanders showed lower probability to initiate fights with observed winners than losers and significantly reduced their probability of winning those interactions. However, when prior direct assessment was available (clear glass) the eavesdropping effect disappeared and the probability of winning increased to control levels. This suggested that the prior direct experience with the interactants overrode the eavesdropped information. Interestingly, when losers performed well (more aggressively), subjects responded less strongly suggesting that additionally to relative information, bystanders acquired absolute information on the individual performance of each interactant (see 'good losers' hypothesis in Peake & McGregor 2004).

Also, although for practical reasons most eavesdropping studies focus on one sensory modality, there is no reason eavesdropping cannot use more than one, depending on its availability or social context. Another interesting set of studies tested the ability to use different sensory modalities for eavesdropping when in different contexts: using visual interactions in male domestic canaries (*Serinus canaria*), Amy & Leboucher (2007) first tested eavesdropping by allowing a male subject to see or not see two other male canaries in a contest for food and subsequently allowing it to interact for food with the winners or losers of the interactions. Subjects initiated less attacks and spent less time foraging to seen winners but not when they did not had visual access to the fighting interaction, which indicated the ability of canaries for visual social eavesdropping. In a follow-up study, Amy et al. (2008), first

exposed female subjects to simulated overlap singing male-male interactions and tested their subsequent sexual displays when exposed to the winners and losers. In a second experiment, with a similar interaction protocol to Amy & Leboucher (2007), female subjects observed male-male contests for food and were subsequently tested for proximity preference between the winners and losers. Females showed more sexual displays to the simulated winners of overheard song contests but avoided the seen winners of food contests, compared to losers. This suggested the ability of female canaries to eavesdrop using two different sensory modalities and to react differentially to dominance displays when in different contexts (i.e. fighting for mates or food). Still it should be noted that this study did not use the same demonstrator males in both experiments, so we do not know if females would react differentially to the same eavesdropped male.

Additionally, two other recent studies showed both the first and only[4] (to our knowledge) indication of social eaveadropping in invertebrates, and the use of two combined sensory modalities when eaveadropping. In the first study, Aquiloni et al. (2008) used crayfish (*Procambarus clarkii*) to conduct a mate choice paradigm where female crayfish were allowed or prevented from attending (visually and chemically) a male-male agonistic interaction. Similarly to the previously described paradigms, females that had observed the interaction visited more often and stayed longer with the winner, but

[4] Chan et al. (2008) performed a visual social eavesdropping test using jumping spiders (*Thiania bhamoensis*) but with less clear results.

not naïve females. The possibility of visual and chemical post-fight cues and individual signals was excluded and thus it strongly indicated the occurrence of social eaveadropping. In the second study, Aquiloni & Gherardi (2010) introduced new treatments in order to test the different sensory modalities (visual or chemical) separately and also effects of familiarity. Results revealed that females eavesdropped when interactants were familiar, and only when visual or chemical signals were available simultaneously, indicating both individual recognition mechanisms and that the integration of multimodal sensory information was necessary to allow the detection and recognition of the male conspecifics.

Surprisingly, studies in mammals (such as rodent models) are virtually absent. A recent study (Lai et al. 2014) tested social eavesdropping on aggressive interactions in golden hamsters, where subjects were allowed to simultaneously extract visual, chemical and auditory information from the fight interactions. Differences in behavioural parameters (latency and proximity) towards one of the demonstrators in a two arms (U-maze) choice paradigm (empty vs. demonstrator) were tested with the same demonstrators, prior to the interactions, immediately after and one day later. Results revealed the occurrence of social eaveadropping, with significantly more investigation time in the arm with a winner and lower latencies to approach, compared to neutral or loser demonstrators. Notably, and similarly to the studies presented further ahead in this thesis (see chapter 4), when the authors manipulated the subjects' social status by submitting them to a prior defeat experience, the eavesdropping

effects were the opposite, with subjects spending much less time in the winner's arm and showing significantly higher latencies to approach, while also exhibiting avoidance fleeing behaviours. These results suggest that the integration of past social experience with eavesdropped information affects subsequent behavioural responses.

Eavesdropping in non-aggressive contexts

Interactions in a social group are not only restricted to aggression and social eavesdropping is expected to occur in various contexts. Indirect evidence suggests that this is the case, namely in courtship and cooperative/altruistic interactions. For instance, social learning about mates (i.e. mate-choice copying) and about potential rivals by observing courtship interactions of others has been the subject of an extensive body of work (see White 2004 for a review). Although not specifically intended to test social eavesdropping at least two studies fall in this category and are worth mentioning. Galef and White (Galef jr & White 1998; White & Galef jr 1999) tested the reversal of female japanese quails' (*Coturnix coturnix japonica)* mate choice preferences by observing non-preferred males mate with another female. The used methods, similarly to social eavesdropping paradigms, tried to tease apart the influences of the interaction, individual social cues and of local cues. However, results did not allow to determine if both the interaction (copulation) and a social cue (female close to male) where equally important to this effect. Another interesting study (Crockford et al. 2007) was conducted in the field with baboons (*Papio hamadryas ursinus*), where opportunistic subordinate males eavesdropped on

simulated acoustic (similarly to the bird studies) mating signalling interactions between dominant males and their female consorts. They attended to the temporal and spatial relationship of the signals, responding when the social information was indicative of a mating opportunity.

In the context of cooperative/altruistic interactions, the ability to eavesdrop should potentially allow bystanders to assess the propensity of other individuals in the group to be 'helpers' or 'cheaters', and hence influence the decision to cooperate or not in future interactions with those individuals[5]. Similarly to eavesdropping on the relative dominance status of others (which requires the ability to attribute dominant/subordinate qualities to individual agents), eavesdropping in a cooperation context should require 'image scoring', i.e. attribution of 'reputations' to interacting third parties within a communication network (Nowak & Sigmund 1998). This in turn might act as a mechanism for the evolution of indirect reciprocity in social networks (Nowak & Sigmund 2005; Wedekind & Milinski 2000). That is, where it becomes advantageous for individuals to exhibit (to potential eavesdroppers) consistent altruistic or cooperative behaviours towards unrelated and random individuals, not restricted to kin or reciprocal altruism (Hamilton 1963; Trivers 1971). Although empirical evidence is scarce, a few suggestive examples can be found in studies by Bshary and colleagues using cleaner fish (*Labroides dimidiatus*), showing that

[5] Contrary to social eavesdropping studies on aggressive interactions, most work in the context of cooperation has been theoretical and not empirical, with some efforts to connect the two contexts (see Johnstone & Bshary 2004; Earley 2010 for reviews).

client fish prefer to associate with cleaner fish that cooperate with heterospecific clients (Bshary & Grutter 2006), avoid interacting cleaners that exhibit cheating behaviour (Pinto et al. 2011), and conversely that cleaner fish improve levels of cooperation in the presence of a bystander client fish (Pinto et al. 2011). Together these results suggest 'image scoring' of others' cooperative behaviours by eavesdropping on their interactions.

Eavesdropping on public information and audience effects

Social eavesdropping provides not only the possibility to learn about other individuals without the costs of trial-and-error learning but also the opportunity to obtain trustworthy information. This is the case because in general interacting individuals aim to optimize their own performance and decisions relative to the other in order to achieve the outcome with the highest gain (e.g. gaining a territory, getting the best mate).

Consequently any inadvertent social information picked up by eavesdroppers is expected to be 'honest' and reliable, unlike direct signalling that can be faked or manipulated. Accordingly, social eavesdropping can be considered a case of public information use if the interacting third parties are unaware of the eavesdroppers presence. Here, the inadvertent available information is not the quality of some physical parameter of the environment (e.g. best food patch) but the relative qualities of the interacting individuals themselves, obtained from their performance and decisions.

Most of the experimental studies on social eavesdropping presented in the previous section fall into this category because in the experimental designs used (e.g. use of speakers, one-way mirrors), interacting demonstrators are not aware of the bystander's presence in order to avoid confounding effects from possible subject-demonstrators signalling interactions.

However, circumstances often arise where eavesdropping is compromised, or simply not possible. For instance, if 'secrecy' is exposed audience effects may appear, as previously mentioned (Marler et al. 1986; Zuberbühler 2008). That is, interactants may manipulate their signalling behaviour (e.g. conspicuousness, intensity) to take into account the fact they are being observed, thus compromising the reliability of the (previously) acquired public information. As described, while experimental studies usually prevent for this, in natural environments where several individuals are simultaneously within signalling range from each other, going unnoticed is not always possible or the very least it is unlikely that audience effects do not eventually arise. Experimental evidence for this has been found in several contexts, which further indirectly implies the ubiquitousness of social eavesdropping processes: in mating, where signallers hide or provide misleading information about their mate choices to bystander rivals, specially to unfamiliar ones (Plath et al. 2008; Ziege et al. 2009; Bierbach et al. 2011); in agonistic interactions (both in males and females) where interacting signallers change their aggressive behaviours in the presence of bystanders (Doutrelant et al. 2001; Matos et al. 2003; Dzieweczynski et al. 2012; Fitzsimmons & Bertram 2013;

Cruz & Oliveira 2015) or manipulate information regarding the true levels of aggression sustained when potential help is present (Slocombe & Zuberbu 2007); or even in cooperative interactions, where cooperative behaviours increase when in the presence of bystanders (Pinto et al. 2011). Moreover, depending on factors such as group density and hierarchical structure (e.g. nested male alliances in dolphins; see Connor 2010), most interactions are not sustainably dyadic and are often subject to disruption by other individuals (personal observation in zebrafish).

Integrating eavesdropped with personal information

In the mentioned circumstances, an eavesdropper may only be able to obtain inaccurate, partial or no information at all. Consequently, in order to reduce uncertainty and optimize learning it should flexibly acquire and integrate eavesdropped information with simpler social cues (e.g. relative size of conspecifics) and personal information from direct interaction with others. Also the weights given to these different sources of information should vary with the trade-off between their reliability and costs/gains of considering it (or ignoring it).

To our knowledge these important questions have hardly been tested in social eavesdropping research (but see Earley & Dugatkin 2002 and Lai et al. 2014). Nonetheless, although sometimes contradictory, there is growing experimental evidence that integration between public and personal information occurs in animals with varying strategies. Theoretical and experimental studies suggest that when personal prior knowledge is uncertain or lacking, public information is a preferred

source of information (e.g. Boyd & Richerson 1988; Arganda et al. 2012). However, not necessarily when other sources of information are also available. For instance an experiment in guppies by Kendal et al. (2004) showed that when personal and public information regarding the location of a food source are both available and conflicting, bystanders will tend to use personal information (typically more reliable) if the costs are the same but will prefer public information if less costly. Conversely, other experiments show that the weight of personal information in the decision making process can decrease with its reliability and how recently it was updated (e.g. Bergen 2004). Moreover, in a communication network the use of personal information versus public information can be based on a quorum threshold (i.e. the number of individuals in the group that exhibit a particular decision), which is dynamically adjusted to the quality of the social information (e.g. Kurvers et al. 2014).

Care should be taken in generalizing these rules, as the ability and predisposition to choose social information over personal information may be evolutionary driven and constrained by the species ecology (e.g. Coolen et al. 2003). Also if the costs of making the wrong assessment are potentially dangerous or even lethal (e.g. fighting a stronger opponent; predation risk), decision making should be particularly sensitive to conflicting information, specially to specific aspects of the social information sources (e.g. number, size, age, familiarity, behaviour of conspecifics). Experimental studies suggest this is the case (e.g. Crane & Ferrari 2015) and future work integrating and expanding the existing empirical and theoretical research can provide further insight

in the context of social eavesdropping (see Kendal et al. 2005 for a detailed review).

In social eavesdropping the information being acquired concerns the social environment, i.e. quality parameters of the interacting third parties such as the status of rivals, mate quality, altruistic proneness. An alternative strategy to eavesdropping implies in this case direct interactions (social experience) with the observed individuals (e.g. fighting with different rivals). This can be expected to be more costly but also potentially more rewarding (e.g. conquering a better territory; mating with the best partner). As such, in communication networks where interactions between different individuals and bystanding is frequent, we should expect a constant interplay and information updating via eavesdropping and personal information from direct social experience. Indeed it is known that past social experience affects subsequent social behaviour, as is the case of winner-loser effects demonstrated to be widespread across species (Rutte et al. 2006). Moreover, most eavesdropping studies have shown that eavesdropped information affects subsequent direct social experience, as we have seen when eavesdroppers are faced with subsequent territorial intrusions or mate choices.

All together, these results suggest that an eavesdropper can use direct social interactions to update and improve the accuracy of information obtained via eavesdropping. Not only about the relative qualities of others but also as a state-dependent reference point (i.e. self-assessment), allowing an eavesdropper to make inferences about its own relationships with others (e.g. social status).

Neural correlates of eavesdropping

Finally, research directly addressing the neural mechanisms of social eavesdropping is still inexistent but a few studies are suggestive of the impact at this level. Oliveira et al. (2001) tested the androgen response of bystander Mozambique tilapia (*Oreochromis mossambicus*) when observing a pair of fighting conspecifics and found that both 11-ketotestosterone and testosterone levels significantly increased compared to when observing a non-interacting pair, suggesting a possible neuroendocrine mediator role of these hormones. Desjardins et al. (2010) used another cichlid fish (*Astatotilapia burtoni*) in a mate-choice paradigm to test the impact in the neural activity of females when observing a preferred male winning or loosing a fight. This was achieved by measuring immediate early genes (IEGs) expression in several brain nuclei. Reproduction related nuclei (preoptic area and ventromedial hypothalamus) were activated differentially when the female's preferred male won, while anxiety-like related nuclei (lateral septum) differentially activated when it lost the interaction. This indicates that specific information acquired from observing an interaction can have significant effects on the brain.

1.4 The zebrafish (*Danio rerio*)

The zebrafish is a small teleost fish native to the flood plains of South Asia, typically forming small mixed-sex shoals in the wild from two to 30 individuals (Engeszer et al. 2007; Pritchard et al. 2001; Spence et al.

2006; Parichy 2015). Although little is still known about its natural history, it has been widely used for decades as a model organism in development biology, genetics and translational study of human diseases (Stewart et al. 2014). It has also been rapidly emerging as a model organism in behavioural neuroscience and is a very promising candidate for the study of the proximate mechanisms underlying social cognition (Kalueff et al. 2013; Norton & Bally-Cuif 2010; Sumbre & de Polavieja 2014; Stewart et al. 2014; Oliveira 2013).

Indeed, the zebrafish is a highly social animal with sociality developing from an early age (Spence et al. 2008; Spence 2011). A strong visual preference for conspecifics together with a tendency to coordinate movements, gradually appears from one to three weeks post fertilization (Engeszer et al. 2007; Dreosti et al. 2015), and shoaling cohesion significantly increases between one to four months old (Buske & Gerlai 2011b). In adults the preference for conspecifics seems to be influenced by several factors such as overall activity or size of the group (Pritchard et al. 2001) and shoaling cohesion dynamically changes with the environmental context, such as the presence of a predator (Miller & Gerlai 2007) or strong vibrations (personal observation). The presence of conspecifics also seems to have rewarding properties, with the sight of conspecifics acting as a positive reinforcer in associative learning tasks and increasing brain dopamine levels (Al-Imari & Gerlai 2008; Saif et al. 2013). Moreover, social networks in zebrafish are dynamic and complex, with different individuals having distinct impacts on group dynamics and performance (Vital & Martins 2011; Vital & Martins 2013; Maaswinkel et al. 2013; Pérez-Escudero et al. 2014).

Zebrafish are also capable both of visual and chemical social recognition (Wiley 2013). Whether it is recognition of an individual[6] or a class of individuals, social recognition is expected to play an important role not only in the expression of social preferences (Oliveira 2013) but in the stability of a communication network (e.g. establishment of dominance hierarchies; Dugatkin & Earley 2004). For example juvenile zebrafish exhibit shoaling preferences for groups with the same colouration phenotype of the ones they were raised with (e.g. stripes vs. no pigmentation). This preference is socially learned early in development by exposure to the social environment and is mediated by visual cues (Engeszer et al. 2004). Zebrafish are also capable of kin recognition through phenotype matching by using an olfactory template that is imprinted on day six post-fertilization during a critical 24-hour window period (Gerlach & Lysiak 2006; Gerlach et al. 2008).

Although zebrafish show strong shoaling behaviours and preference for conspecifics they are also a territorial species and, depending on the environmental context, shoaling behaviour co-exists and dynamically switches to territorial behaviour with structured dominance hierarchies (Pérez-Escudero et al. 2014; Grant & Kramer 1992; Gerlach 2006). A zebrafish group is thus composed by territorial and non-territorial individuals (Spence et al. 2006) where agonistic interactions are common, both in males and females (Paull et al. 2010),

[6] To our knowledge no studies to date have directly tested individual recognition in zebrafish.

for the control of food, spawning sites, and possibly mating opportunities (Spence 2006). Dyadic fighting interactions in zebrafish, although never involving physical injuries, often show highly stereotyped behavioural patterns with a clear temporal structure and a winner-loser outcome. This behaviour has been thoroughly characterized by Oliveira et al. (2011) using a behavioural paradigm where male zebrafish consistently expressed fighting behaviours. In this paradigm two male zebrafish were allowed to interact in a confined space after a one-day isolation period, where they had the opportunity to establish their own territories. Under these circumstances, a fight interaction usually starts with each fish displaying symmetric aggressive behaviours to the opponent by erecting its fins, flaring its body flank and darkening body pigmentation, alternated with circling, strikes and bites. At a certain point in time a switching event is reached (i.e. fight resolution) where behaviour drastically changes, and one fish starts chasing and attacking (winner) while the other (loser) flees and assumes submissive postures (fins retracted, caudal region downwards), alternated with freezing periods at the bottom or surface of the water column. While fights may vary in duration length, display and aggression levels, the post-resolution asymmetry is never reversed (see Oliveira et al. 2011 for a detailed analysis). It should be noted however that the aggression levels and strong behavioural asymmetries that emerge in this type of experimental paradigm are probably magnified artificially by the confinement of the experimental arena (see 'desperado effect'; Grafen 1987), contrary to natural conditions where each individual can persist to engage or simply decide to quit and leave

the opponents' territory (personal observation). In the same study, winners significantly increased the probability of winning subsequent fights while losers decreased it, showing the existence of winner and loser effects in zebrafish and revealing behavioural flexibility dependent on past social experience. This was possibly achieved through mechanisms of self-assessment or exhibition of social cues that allow others to identify the acquired social status (see Fawcett & Mowles 2013 for a discussion).

In the face of such a rich and dynamic social environment, several other cognitive abilities are expected to exist in zebrafish for effective acquisition and use of social information, namely for social eavesdropping. While still an emerging field, behavioural studies in zebrafish have already addressed a wide array of relevant topics, such as perception (Engeszer et al. 2008; Neri 2012; Gori et al. 2014; Rosa Salva et al. 2014), visual attention (Braida et al. 2014; Parker et al. 2012), visual discrimination learning (Colwill et al. 2005), reversal learning (Parker et al. 2012), spatial associative learning (Sison & Gerlai 2010; Karnik & Gerlai 2012), memory (Lucon-Xiccato & Dadda 2014; Jia et al. 2014; Roberts et al. 2013), visual and olfactory observational conditioning (Suboski et al. 1990; Hall & Suboski 1995), social transmission (Lindeyer & Reader 2010) and others. Together with behavioural studies, the development of new tools for automated video-tracking and the creation of artificial stimuli using video playbacks (for the induction and manipulation of social behaviours), are also a growing focus of research in zebrafish (Cachat et al. 2011; Green et al.

2012; Pérez-Escudero et al. 2014; Qin et al. 2014; Turnell et al. 2003; Saverino & Gerlai 2008; Fernandes et al. 2015).

Moreover, neurobehavioural research is increasingly using the extensive, pharmacological, genetic and neuroanatomical toolbox available in this species. Zebrafish have a sequenced and annotated genome (Howe et al. 2013). Commercially available microarray gene chips (Affymetrix® 1.1 ST Array Strips) offer whole–transcriptome coverage and allow analysis of gene expression patterns. Detailed brained atlas (Wulliman et al. 2012) have been developed and homologies have been established with mammalian brain areas, namely with conserved neural networks seemingly implicated in the modulation of social behaviours (Mueller & Wullimann 2009; Wullimann & Mueller 2004; O'Connell & Hofmann 2011). In adults, *in situ* hybridization (Goto-kazeto et al. 2004; Lau et al. 2011; von Trotha et al. 2014), macroareas dissection and micropunches for sampling specific brain areas (Teles et al. 2015), combined with qPCR (quantitative polymerase chain reaction) have been used to analyse socially driven changes in neural activity, functional localization and also connectivity based on immediate early genes markers (Clayton 2000; Teles et al. 2015), and to measure differential gene expression of candidate genes (Ziv et al. 2012). Also, HPLC (high precision liquid chromatography) has been used to measure social modulation of neurotransmitters levels (Teles et al. 2013; Saif et al. 2013) and pharmacology has been used to test different mechanisms of social behaviour (Buske & Gerlai 2011a; Braida et al. 2012; Maaswinkel et al. 2013). Additionally, new techniques are being developed to allow non-

invasive endocrine measures of stress responses to social contexts and social status (Félix et al. 2013; Pavlidis et al. 2013; Pavlidis et al. 2011).

Functional studies of neural circuits in relation to social behaviour are currently somewhat restricted in adult zebrafish by the available techniques. On the other hand, studies using zebrafish larvae, benefiting from its small brain size and skull transparency, have recently been part of an amazing development of a wide array of imaging, optogenetic and transgenic tools, allowing real time visualization and manipulation of neural circuits and its activity in relation to behaviour (Agetsuma et al. 2010; Naumann et al. 2010; Ahrens et al. 2012; Okamoto et al. 2012; Muto et al. 2013; Bianco & Engert 2015). Unfortunately, contrary to adults, social behaviours in larvae are very limited. Extending the available optogenetic tools forward in zebrafish's development and also exploring the ontogeny of social behaviours in adult zebrafish, will potentially allow unmatched opportunities to access the neural basis of social information acquisition and use in a social species[7].

1.5 Aims and structure of the thesis

The present work has focused on investigating the occurrence and mechanisms of social eavesdropping in zebrafish, particularly in the context of agonistic social interactions.

[7] Recent studies tackling this challenge already show great promise (Dreosti et al. 2015; Oliveira and colleagues, unpublished).

- In chapter 2, we investigated if zebrafish are tuned to attend to social interactions (a requisite for social eavesdropping) and explored potential relevant features driving their attention. The first objective was to develop and validate a robust unforced-choice and adaptable behavioural paradigm, with automated video tracking and novel behavioural parameters. We further developed this paradigm using video playback stimuli amenable to manipulation in order to investigate relevant features of those interactions.

- In chapter 3, based on the previous results, we explored the impact of attending to agonistic social interactions at the zebrafish brain gene expression level by characterizing distinctive brain transcriptomic profiles and relevant candidate genes.

- In chapter 4, based on the established behavioural methodologies and results presented in chapter 2, we developed a social eavesdropping paradigm in order to test zebrafish's ability to eavesdrop on agonistic social interactions and how it might be modulated by its own past social experience.

Chapter 2
Tuning of attention to social interactions

2.1 Chapter summary

In this chapter we present a set of studies aimed at investigating if zebrafish are tuned to attend to social information exchanged between conspecifics and to explore its relevant features.

- In a first experiment, we designed a behavioural paradigm where bystander zebrafish could observe an agonistic interaction (fight) between two conspecifics, two non-interacting conspecifics, or an empty tank. We developed an automated video tracking software in order to facilitate analysis of attentional parameters such as sustained proximity, body orientation and directional focus. Our results show that zebrafish are more attentive towards interacting (i.e. fighting) than towards non-interacting pairs of conspecifics, with the exposure to fighting not increasing activity or stress levels.

- In a second experiment, we adapted the previous paradigm and used video playbacks instead of live stimuli to manipulate form features of the fighting fish. Our results showed that when observing the assessment stage of a video fight, bystanders' attention was not dependent on the fight's level of activity and was more driven by form features of the interacting opponents; whereas during the post-resolution stage it was more driven by biological movement features of the dominant fish chasing the subordinate fish.

- In a third experiment, we refined and extended the previous tasks using another wild-type strain of zebrafish (Tübingen), more amenable to experimental manipulation. Using video playback manipulations we tested the importance of the different stages of the fight interaction in eliciting bystanders' attention. Our results show that Tübingen zebrafish also reveal a strong tuning towards fighting conspecifics. Importantly, that the assessment stage of the observed fights elicits higher attentional responses than the post-resolution chasing stage, regardless of the fight's level of activity. Moreover, that the fight resolution event is potentially a relevant attentional switching point.

- Overall our results agree with the prediction that a social species such as the zebrafish, may possess adaptive specializations at the level of input mechanisms towards public information available in the social environment. In particular to the exchange of information between conspecifics.

2.2 Introduction

Animals may eavesdrop on agonistic signalling interactions between third parties in order to collect information on the relative competitive ability of the opponents, without incurring in the costs associated with fighting. Information which they may use on subsequent interactions with the observed individuals (Oliveira et al. 1998). This social eavesdropping ability may thus impact the Darwinian fitness of the animal (McGregor 1993; Peake 2005). Therefore, it has been proposed that group living has led to selection for the evolution of cognitive processes that enable animals to take advantage of the public information available in the social environment (Byrne & Whiten 1989; Dunbar & Shultz 2007; Humphrey 1976). Some authors have suggested that these cognitive adaptations for social living depend on a set of domain-specific modules that evolved specifically for this purpose, and consequently the mechanisms involved in social learning would differ from those of individual learning (Gigerenzer 1997). However, this hypothesis has been recently challenged by accumulating evidence which suggests that: (1) both social and individual (asocial) learning share general associative learning mechanisms (Heyes 2012); (2) social and asocial learning abilities co-vary across and within species, i.e. the better an animal performs in social learning tasks, the better it also performs in asocial learning tasks (Lefebvre & Giraldeau 1996; Munger et al. 2010; Shettleworth 1993); and (3) even solitary species can exhibit social learning (Fiorito & Scotto 1992; Wilkinson et al. 2010). Together these results have questioned the evolution of social learning as an

adaptive specialization for group living and suggest that social and asocial learning share the same underlying mechanisms (Heyes 1994; Heyes 2012).

As a consequence, it has recently been proposed that adaptive specializations in social cognition may have evolved at the level of input mechanisms, such as perception, attention or motivation, which select the information that becomes available for learning, rather than at the level of learning mechanisms (Heyes 2012). Despite the extensive literature on the adaptive function of social learning, research on its neural and cognitive mechanisms has been more scarce, and this "black-boxing" of mechanisms may limit our understanding of its functional role (Olsson & Phelps 2007). From the four basic cognitive processes involved in learning – acquisition, encoding, storage and retrieval of information – the former is related to the input mechanisms that select information available for learning, and the latter three are related to the long-term encoding of relevant information. Therefore, social and asocial information may share similar encoding, storage and retrieval mechanisms, but social species may be more tuned to social information available in the environment.

Input mechanisms are crucial for higher-level cognitive processes, since they determine which information is selected for subsequent processing. Indeed, each species sensory specializations define a species-specific perceptual space, i.e. the *umwelt* or "self-world" as proposed by Jakob von Uexküll (Von Uexküll 1934), which allows an individual to respond adaptively to their environment in terms of appropriate responses to its own food, mates, competitors and predators.

Subsequently, sensory information is filtered by perception and attention (Bushnell 1998), providing information that becomes available for learning and decision-making processes. The relevance of attentional processes for learning has been recently highlighted; in particular, individuals' selective attention efficacy has been shown to co-vary with performance on a wide range of cognitive tasks, which is taken as measure of a general intelligence trait (Matzel & Kolata 2010). Finally, motivation also plays a key role in the input mechanisms since it can direct attention to relevant stimuli in the environment by enhancing their salience, as exemplified by fear enhanced or hunger reduced vigilance towards predators in foraging fish (Milinski 1984; Godin & Smith 1988). In summary, adaptive specializations in input mechanisms can contribute as much as those in higher-level cognitive processes for the evolution of adaptive behaviours. Given that conspecifics are a significant component of the environment, it is expected that adaptive specializations have evolved to tune these input mechanisms towards relevant social information, in particular, to intercept the exchange of information between conspecifics.

2.3 A paradigm for testing attention to social interactions

In order to test if zebrafish males pay attention to social interactions between other conspecific males, we developed a one-trial, unforced preference task, where a bystander male zebrafish could observe without being observed: an agonistic interaction (fight) between two

male conspecifics; two non-interacting male conspecifics; or an empty tank (reference treatment). A set of behavioural parameters was used as a proxy for attention, here represented by a combination of measures, such as sustained proximity, body orientation and directional focus.

Methods

Animals and housing. Wild-type (AB) zebrafish (*Danio rerio*), 11 months old, bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used. Fish were kept in mixed sex shoals in environmentally enriched (gravel substrate, artificial plants and rocks) stock tanks with 50 × 25 × 30 cm (30 l) at 28 ºC, under a 14L:10D photoperiod. Water was filtered and monitored for nitrites (< 0.2 ppm), nitrates (< 50 ppm) and ammonia (0.01 – 0.1 ppm). Conductivity and pH were maintained at 700 μSm and pH 7.5 respectively. Fish were fed twice a day with commercial food flakes in the morning and with freshly hatched *Artemia salina* in the afternoon, except on the day of the experiments. No fish was injured as result of the expression of agonistic behaviours. All procedures were reviewed by the Instituto Gulbenkian de Ciência Ethics Committee and approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária permit 008955).

Behavioural Setup. The experimental setup consisted of three side-by-side test tanks (13 × 13 × 17 cm each) and three demonstrator tanks (15 × 15 × 17 cm), one for each experimental treatment (Figure 2.1). The observation glass side of each test tank was positioned head-to-head to

the end glass side of a demonstrator tank, which was divided in two by an opaque removable partition. A one-way mirror was placed in-between the tanks. This allowed each focal fish (bystander) full view of the demonstrator fish, without itself being seen. It also prevented interactions between demonstrators and bystanders. All tanks were filled up to a 9 cm water depth and no chemical communication was possible as the tanks were self-contained. A fluorescent light was placed over the demonstrator tanks, creating differential lighting required for the mirror effect. To further enhance this effect and also avoid interference of external visual cues, the demonstrator tanks had white opaque walls and the test tanks had black walls, with the exception of the transparent glass observation side. Three B&W mini surveillance cameras (Henelec 300B) with infrared sensitivity (IRs) were placed above each test tank and connected to a laptop (HP Pavilion g6). This allowed a top view video recording of the focal fish and demonstrator fish simultaneously. The setup was placed over an infrared LED (850 nm) custom built lightbox to increase contrast between the background of the test tanks and the focal fish (when video recording from above) without interfering with their vision, as IR light falls outside zebrafish's wavelength sensitivity (Fleisch & Neuhauss 2006). This increased image quality and optimized subsequent video tracking of the fishes' behaviour, using a custom made video-tracking system. A black curtain separated the setup from the rest of the behavioural room during the experiment and no person was allowed inside during the testing period.

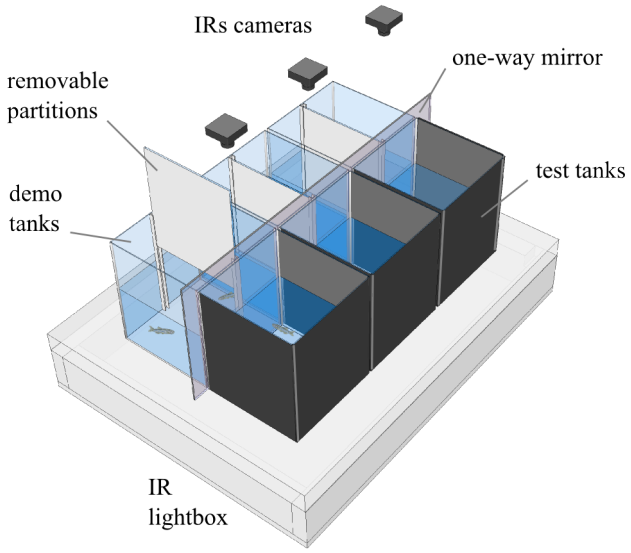

Figure 2.1 | Behavioural setup. 3D schematic of the experimental setup.

Experimental procedure. A total of 39 focal naïve male zebrafish were used (13 per treatment). Each fish was subjected to a single test corresponding to one of three treatments (Figure 2.2): (1) bystander to male fighting conspecifics (BIC); (2) bystander to non-interacting conspecifics (BNIC); (3) socially isolated (ISOL).

The behavioural setup allowed testing three different bystanders per day. On the day prior to the test, three fish of similar size were randomly removed from the stock tanks and isolated in each test tank overnight. This produced an isolation baseline effect and allowed for setup acclimatization. The order of the treatments attributed to each tank was randomized for each session. To prepare the BIC and BNIC demonstrators, two pairs of unfamiliar zebrafish matched in size, where placed in the corresponding demonstrator tanks. A removable white

opaque partition was placed between each pair overnight, allowing chemical but no visual communication. The ISOL treatment was prepared by keeping a demonstrator tank empty, with an opaque partition also placed in the middle to match the other tanks.

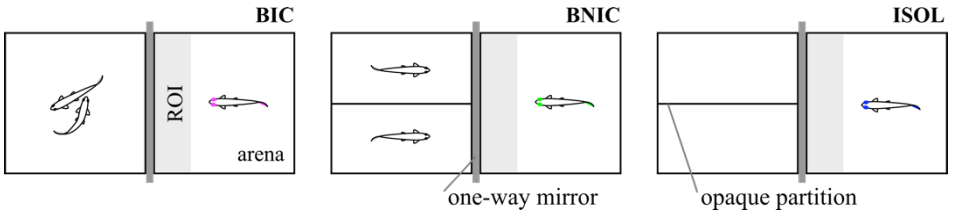

Figure 2.2 | Schematic of the experimental treatments. Bystander to fighting conspecifics (BIC); bystander to non-interacting conspecifics (BNIC); and socially isolated (ISOL). Focal fish represented with colour (BIC — magenta; BNIC — lime; ISOL — blue) and demonstrator fish (stimuli) represented in black. Region of interest (ROI) represented in light grey and one-way mirror in dark grey.

Removable opaque partitions were additionally placed between each test tank and the one-way mirror to prevent visual contact between demonstrators and bystanders during the isolation period. The demonstrators were allowed to habituate to the one-way mirror reflection overnight. This avoided interactions with the mirror during the tests. On the following day, at the beginning of each test, the opaque partition that visually separated each test tank from the corresponding demonstrator tank was removed. Each focal fish could then visually observe the corresponding demonstrator tank for 30 min. For the BIC treatment, the middle opaque partition separating the demonstrator dyad was also removed simultaneously, prompting the demonstrators to

fight. For the BNIC treatment, the middle partition remained in place, preventing the two demonstrators to interact. For the ISOL treatment, the middle partition also remained in place. All focal fish behaviours were video recorded for posterior offline behavioural tracking and analysis. On rare occasions video recordings malfunctioned or a focal fish exhibited abnormal stress behaviour from the beginning in the test tank. In such cases the corresponding fish were discarded prior to tracking.

Immediately after the test, each focal fish was euthanized with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L) and sectioning of the spinal cord. Gender was confirmed by dissection of the gonads. Body samples were stored at -80 ºC for posterior whole-body hormonal analysis.

Behavioural tracking and data acquisition. All focal fishes' behaviours were tracked from a top-down view perspective, using a custom made tracking software developed in Python (python™). For each behavioural video, a 2D region (arena) was defined for tracking (Figure 2.3A). The arena's position and size took into account the camera's perspective distortion caused by the water depth. It comprised the inner area of the bystander tank (12 x 12 cm), including the stimulus (demonstrators) observation side, where the lighting contrast between the white background and the fish was high. It excluded the black outer walls sidelines where contrast was low. The fish were tracked at a 29 fps (frames per second) rate. For each frame, the tracking software determined and extracted into data files the pixel coordinates of the

head, centroid, and tail (Figure 2.3B). This allowed determination of the position and orientation (Figure 2.3C) of the fish every 1/29 s. It also identified and counted all frames in which the fish was not detected. This only occurred at surface level, alongside the tank's black outer walls (on average 4% of the total time). After tracking, the head, centroid, and tail coordinates were projected over the video (see Results) allowing the manual inspection of the tracking quality and an easy early detection of possible tracking errors.

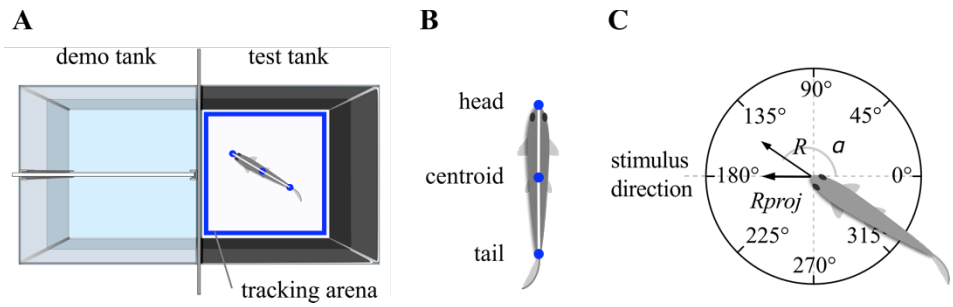

Figure 2.3 | Schematics of the behavioural tracking methods. (A) Top view schematic of demonstrator + test tank pair with focal fish. A tracking arena (blue rectangle) is defined post-test for offline tracking of the recorded videos. **(B)** Schematic of the tracking points (blue dots) used for coordinates extraction. **(C)** Schematic of the possible mean orientations of a focal fish, measured by its centroid-to-head axis angle α (0° opposite and 180° directed towards the demonstrator tank's direction). *R* represents the mean resultant vector's length and *Rproj* its projection onto the stimulus direction.

Behavioural data analysis. All tracked data files were imported to MATLAB (MathWorks®) and behavioural parameters were determined using a custom-made script. A region of interest (ROI) with 12 x 3 cm

(25% of the tank) corresponding to the width of the tank and the mean body length of an adult zebrafish, was defined in the area of the arena closest to the observation glass (Figure 2.2). The focal fish was considered in the ROI when its centroid point was inside that region.

Attentiveness of the focal fish at an individual and group level was inferred from their preferred positions in the arena and body orientation relative to the stimulus (Figure 2.3C). Four behavioural parameters, one qualitative and three quantitative, were used as read-outs: (1) the spatial distribution of the focal fish in the arena; (2) time spent in the vicinity (ROI) of the demonstrator fish; (3) mean orientation (α) towards the demonstrators; and (4) directional focus towards the demonstrators (*Rproj*). Locomotor activity of the focal fish was measured by their mean speed in the ROI and total distance covered in the arena. The determination of the total distance took into account an estimation of the distance covered when the fish could not be tracked by considering it proportional to the total distance covered when detected.

The mean resultant vector r was calculated by first transforming each orientation taken by the fish during the 30 min test into a unit vector $\hat{r}_i = (\cos\alpha_i, \sin\alpha_i)$, where α_i is the angle formed by the fish's centroid-to-head axis relative to the horizontal axis in each frame. The mean resultant vector was thus defined as the mean of all n frames' unit vectors, calculated by $r = \frac{1}{n}\sum \hat{r}_i$. The corresponding directional focus R was measured by the mean resultant vector's length $R = \|r\|$, which is defined by the vector's norm and inversely related to the angular standard deviation (Figure 2.3C). Its values range from 0 to 1. The closer

R is to one, the more concentrated are the n orientations around the mean direction. Lastly, the projection of R onto the demonstrator tank's direction (180°) was determined by $Rproj = -\cos\alpha$, where α is the mean resultant vector's angle relative to the horizontal axis (Figure 2.3C). This allowed measurement of the mean directional focus of each fish relative to the stimulus direction, using a linear scale ranging from 1 to -1. Positive values indicate directionality towards the stimulus direction, negative values away from it and null values no directional focus. For each treatment, a group mean resultant vector r_g, correspondent mean length R_g *and* angle α_g, was determined by the grand mean of all focal fishes' mean resultant vectors r, weighted by their individual lengths R.

The temporal dynamics and correlations of the BIC vs. ISOL treatments for the mean time spent in ROI and $R_g proj$ was analysed in 30 s bins.

Hormonal analysis. Measures of stress levels by cortisol whole-body concentrations were determined for each focal fish. For the hormone extraction, the collected whole-body samples kept at -80 °C, were first measured for body weight and length for normalization purposes. Each sample was partially thawed, weighed and dissected on ice into smaller parts for efficient homogenization. 500 µl of EIA Buffer (from Cayman EIA kit) were added and vortexed for 3 s. The samples were transferred to extraction glass tubes and homogenized using a mechanical homogenizer (IKA Labortechnik) for 30 s on ice. The homogenization rotor blade was washed with additional 500 µl of ice-cold EIA buffer

and collected in the glass tube containing the homogenate. Samples were sonicated for 30 s on ice, added 3 ml of diethyl ether, vortexed, stirred for 10 min in the orbital shaker and then centrifuged at 2000 rpm (4 ºC) for 15 min. Following centrifugation, samples were frozen at -80 ºC for 15 min and the organic layer (containing the hormones) was removed from each sample and placed in a separate test tube. Ether was evaporated with a speed vacuum centrifuge (Speedvac Savant SC 1101) equipped with a cryotrap. Samples were reconstituted in 1 ml of EIA buffer after evaporation and kept at -20 ºC until analysis. Cortisol levels were assayed using enzyme immunoassay (EIA) kits from Cayman Chemical Company (#500360) following the manufacturer's instructions. In the cases where samples were too concentrated, dilutions were performed and measurements repeated. The intra-assay coefficient of variation was 3.20% and inter-assay coefficient of variation was 8.79%.

Statistical analysis. Behavioural and hormonal results were represented as mean ± SEM unless stated otherwise. Statistical significance was considered for $p < .05$. For the behavioural parameters' comparisons between treatments, one-way ANOVAs were performed when normality and homogeneity of variances (Levene's test) was verified, followed by post-hoc Tukey HSD tests or contrasts for specific planned comparisons. When normality was verified but not homogeneity of variances, Welch's ANOVAs were used followed by Games-Howell post-hoc tests. When normality was not verified, non-parametric Kruskall-Wallis tests were used. Cortisol concentrations were first ln transformed to meet the assumption of a normal

distribution. Deviation from uniformity of the fishes' individual mean orientations' distribution was tested using the non-parametric Moore's Modified Rayleigh test, for each treatment. The group mean resultant vectors' angles were represented as mean and 95% C.I. when directionality was significant. Correlations were performed using a non-parametric Spearman rank correlation. All analyses were performed using MATLAB R2012b (MathWorks) with the CircStat toolbox (Berens 2009), STATISTICA 12 (Statsoft^{Inc}), SPSS Statistics 22 (IBM) and Oriana 4 (Kovach Computing Services).

Results

Bystander zebrafish pay attention to social interactions. Individual qualitative profiling of the time spent by the bystanders in each position of the arena, revealed different spatial distribution patterns for each treatment (Figure 2.4). On average BIC fish spent more time closer to the tank wall on the side of the demonstrator fish than did BNIC fish, which showed a more dispersed distribution in the arena. Some BNIC fish spent more time in the area closer to one of the two non-interacting demonstrator fish. ISOL fish showed on average a dispersed distribution in the arena. Analysis of the group mean percentage of time spent in the ROI confirmed that BIC fish spent significantly more time in the ROI than ISOL fish, whereas there were no differences between BNIC and ISOL fish. The differences between BIC and BNIC fish were also not significant (Figure 2.5; Table 2.1).

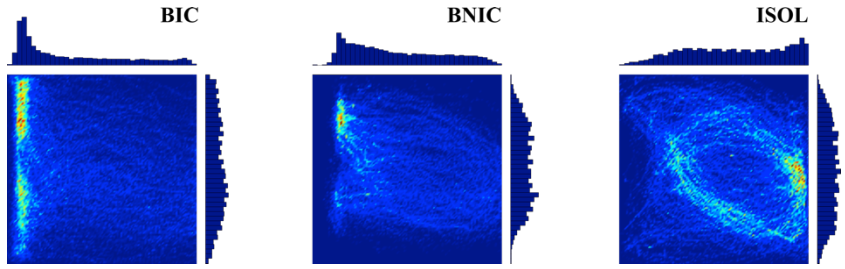

Figure 2.4 | Spatial distribution patterns. Two-dimensional heatmaps and linear histograms of the time spent in each position of the tracking arena by a representative focal fish (closest to the mean) from each treatment: BIC — bystander to fighting conspecifics; BNIC — bystander to non-interacting conspecifics; ISOL — socially isolated. Test tank observation glass on the left border. Heatmaps are scaled from maximum relative value (red) to minimum relative value (dark blue). Linear histograms represented in arbitrary scale.

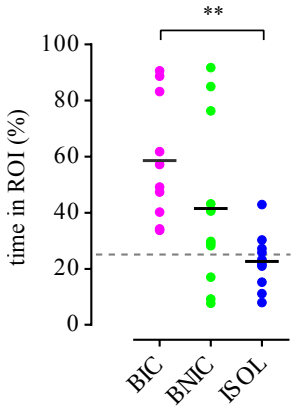

Figure 2.5 | Time in ROI. Scatter plot (n = 10 to 12 /treatment) of the individual (coloured dots) and mean (black lines) percentage of time spent in the ROI for each treatment. BIC — bystander to fighting conspecifics (magenta); BNIC — bystander to non-interacting conspecifics (lime); ISOL — socially isolated (blue). Dashed grey line represents the value expected from a random distribution in the arena (25%). ** $p < .01$.

Table 2.1 | Behavioural and hormonal results

	time ROI (%)	*Rproj* (-1 to 1)	total dist. (m)	speed ROI (m s^{-1})	cortisol (ng/g)
Mean ± SEM					
BIC	55.05 ± 7.22	0.25 ± 0.06	102.3 ± 5.25	0.04 ± 0.002	3.32 ± 1.40
BNIC	41.58 ± 8.20	0.14 ± 0.07	89.28 ± 3.85	0.04 ± 0.002	1.70 ± 0.53
ISOL	22.67 ± 3.17	0.04 ± 0.03	96.25 ± 7.54	0.04 ± 0.003	1.36 ± 0.26
ANOVA					
BIC× BNIC ×ISOL	$F_{2,30}$[a] = 9.31 p = .002	$F_{2,30}$[a] = 4.52 p = .03	$F_{2,30}$[a] = 1.86 p = .18	$F_{2,30}$[a] = 0.35 p = .71	$H_{2,33}$[b] =0.19 p = .91
Games-Howell					
BIC vs. ISOL	p = .003 d_s = 1.73	p = .03 d_s = 1.22	-	-	-
BNIC vs. ISOL	p = .12 d_s = 0.85	p = .40 d_s = 0.54	-	-	-
BIC vs. BNIC	p = .45 d_s = 0.50	p = .49 d_s = 0.48	-	-	-

BIC — bystander to fighting conspecifics (n=11); BNIC — bystander to non-interacting conspecifics (n=12); ISOL — socially isolated (n=10). [a] Welch's ANOVA; [b] Kruskal-Wallis non-parametric test.

The ISOL fish results (time in ROI = 22.67 ± 3.17%, n = 10) matched what would be expected from a uniform distribution in the arena, with the fish showing no particular preference for the ROI and spending on average 25% of the time in 25% (ROI) of the total area (one-sample *t*-test, t_9 = 0.73, p = .48). In the BNIC treatment, three (time in ROI = 84.39 ± 4.48%, n = 3) out of the 12 tested fish showed a strong proximity towards the demonstrators, which differed from the other nine fish (time in ROI = 27.03 ± 4.46%; n = 9), suggesting a possible bimodality of a subset of the sampled population.

Circular scatter plots of the individual mean orientations (α; see Figure 2.3C) and group directional focus (see Methods for details), revealed different distribution patterns for each treatment. We observed that BIC fishes' mean orientations, strongly clustered around the fighting conspecifics tank direction (180º). BNIC fish also oriented predominately towards the stimulus direction although scattered as well around other directions, whereas ISOL fish showed a dispersed distribution along different directions (Figure 2.6A).

Correspondingly, determination of the group mean resultant vector for each treatment (Figure 2.6A,B) revealed that all were oriented towards the demonstrator tanks at 180º with the corresponding mean vector's lengths R_g, a measure of directional focus, showing a higher value for the BIC treatment [α_g (BIC) = 182.59º, 95% C.I. = 158.43º–191.91º, R_g = 0.25, n = 11; α_g (BNIC) = 179.07º, R_g = 0.14, n = 12; α_g (ISOL) = 186.21º, R_g = 0.042, n = 10].

Likewise, the individual fish's directional focus (*Rproj*) towards the stimulus direction (Figure 2.6C) showed a significantly higher group mean for the BIC fish than ISOL fish, whereas there were no differences between BNIC and ISOL fish. The differences between BIC and BNIC fish were also not significant (see Table 2.1).

Reassuringly, circular uniformity analysis confirmed that only BIC fish showed a significant directional focus towards the stimulus, with the distribution of their individual mean orientations (Figure 2.6A) deviating significantly from uniformity and clustering around the corresponding group mean direction [Moore's test, (BIC): $p < .001$; (BNIC): $p > .1$; (ISOL): $p > .1$].

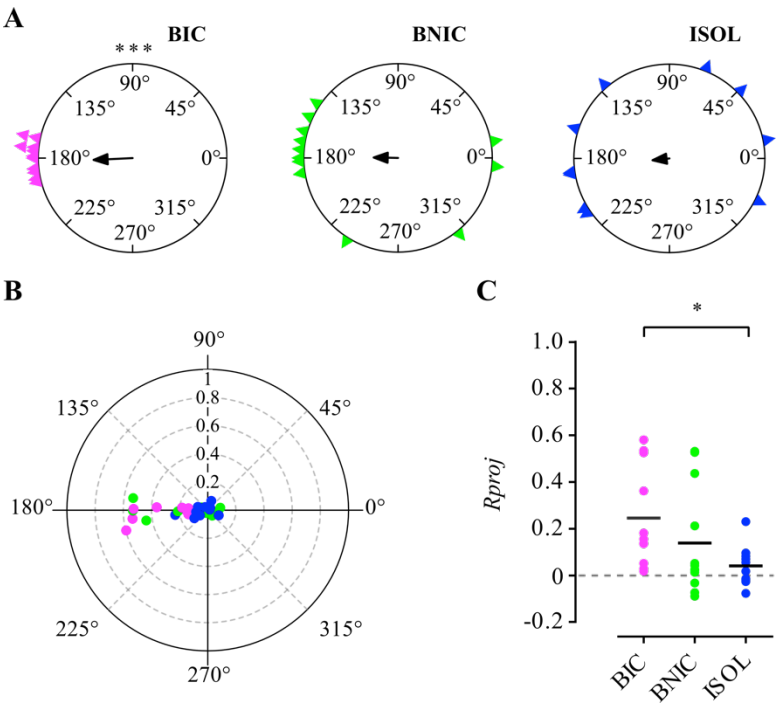

Figure 2.6 | Orientation and directional focus. (A) Circular plots of the focal fishes' individual mean resultant vectors' angles α for each treatment (BIC — magenta triangles, BNIC — lime triangles, ISOL — blue triangles) and corresponding group mean resultant vector (black arrows). Longer arrows indicate higher directional focus. BIC fish deviate significantly from a uniform distribution, clustering around its group mean resultant vector's direction. **(B)** Polar scatter plot of the focal fishes' (coloured dots) individual mean resultant vectors angles α (0° to 360°) combined with the corresponding vectors' lengths R (0 to 1), for each treatment. BIC — magenta; BNIC — lime; ISOL — blue. **(C)** Scatter plot of the individual (coloured dots) resultant vectors' lengths R projected (*Rproj*) onto the stimulus direction (180°) and corresponding group mean value $R_g proj$ (black lines), for each treatment. Positive values indicate directional focus towards the stimulus; zero value indicates no directionality (dashed grey line); negative values indicate directional focus opposite to the stimulus. * $p < .05$; *** $p < .001$.

Next, we measured the locomotor activity of bystanders and their stress levels to make sure that the observed differences in attentional measures across treatments where not mediated by any of these variables. The total distance covered (Figure 2.7A) in the arena and the mean speed in the ROI (Figure 2.7B) values did not differ significantly across treatments (Table 2.1). Post-test whole-body cortisol levels were also not significantly different across treatments (Table 2.1; Figure 2.7C).

Finally we compared the temporal dynamics of the BIC group mean time in ROI and $R_g proj$, with the ISOL reference group. We observed that both mean values were sustained throughout the 30 min test (Figure 2.8A,B) and that the two parameters strongly correlated with each other (Spearman correlation: $r_s = 0.70$, $p < .001$; Figure 2.8C).

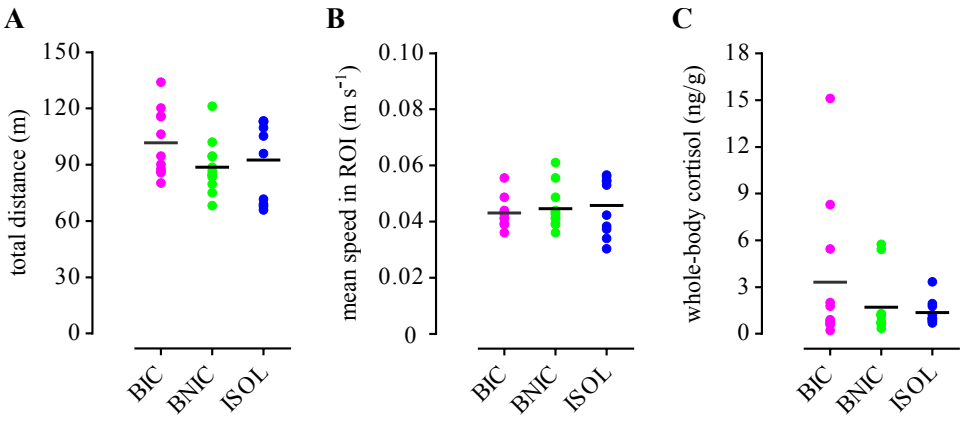

Figure 2.7 | Locomotor activity and cortisol levels. Scatter plots of the individual (coloured dots) and mean values (black lines) of the focal fishes' **(A)** total distance covered in the arena; **(B)** mean speed in the ROI; and **(C)** whole-body cortisol levels, for each treatment.

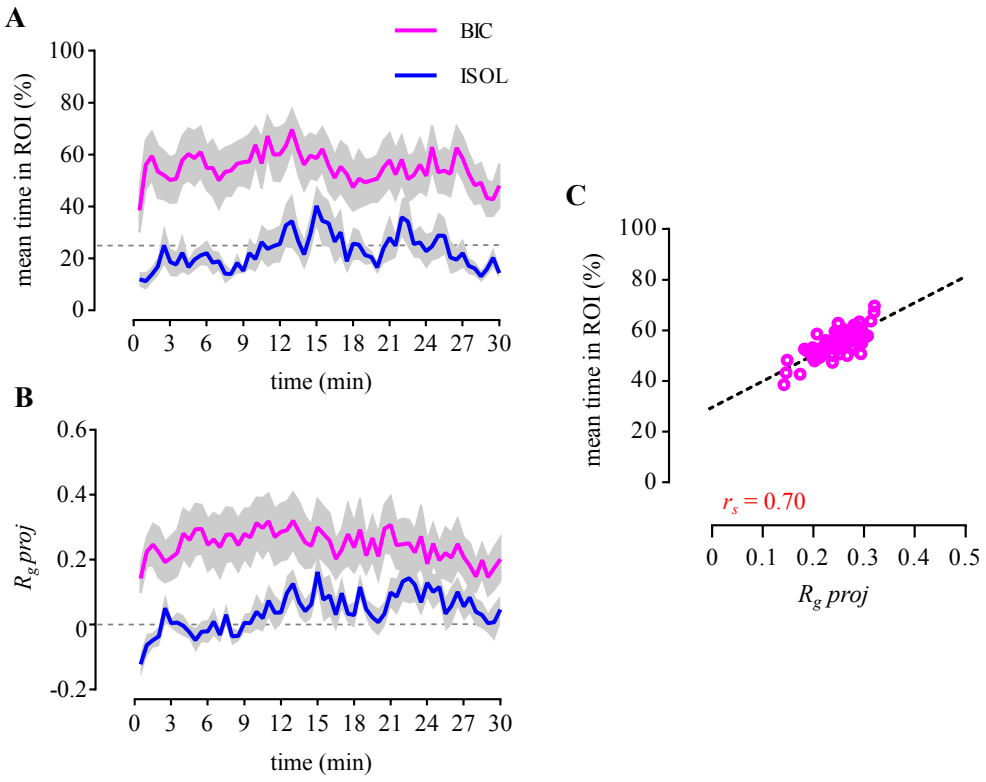

Figure 2.8 | Temporal dynamics of proximity and directional focus towards fighting conspecifics. (A) Comparison between the bystanders to fighting conspecifics' (BIC) mean time in the ROI and the socially isolated (ISOL) reference fish, measured in 30 s bins and throughout the 30 min test. **(B)** Comparison between the BIC and ISOL fishes' mean directional focus onto the stimulus direction ($R_g proj$), measured in 30 s bins and throughout the 30 min test. For both (A) and (B), the coloured thick lines (BIC — magenta; ISOL — blue) represent the mean values for each treatment. Grey shadows represent the standard error (SEM). The dashed grey line represents in (A) the value expected from a random distribution in the arena (25%) and in (B) no directionality ($R_g proj = 0$). **(C)** Scatter plot of the BIC fishes' mean time spent in the ROI as function of $R_g proj$. Open magenta circles represent the sampled (in 30 s bins) means, throughout the 30 min test. Spearman's correlation coefficient r_s is shown in red. Dashed black line indicates the regression line for easier visualization of trend.

Discussion

Together, the results presented here show that zebrafish were strongly attentive towards agonistic interactions between conspecifics and that this did not seem to influence the bystanders' mean levels of activity or stress. Also in our paradigm, the typical attentional parameter of proximity and the newly introduced directional focus towards the stimulus, strongly correlate when observing agonistic interactions, confirming the potential of including this second parameter as a reliable attentional measure. The fact that in our paradigm we were able to clearly discriminate the effects of observing an agonistic interaction using a one trial, unforced choice task, in such a small arena, supports the assumption of the high natural salience of this type of stimulus to zebrafish. Furthermore, when the interaction was prevented, the mean levels of attention were lower, although not significantly. This may be explained by the small sample size and that a subset of three bystanders was strongly attentive to one of the non-interacting conspecifics, which may have reduced the power to detect significant differences. Furthermore, it is not possible to control either the behaviour of an individual fish or the interaction dynamics between fish, which may be affecting individual bystander's levels of response. Thus, in order to standardize and manipulate the stimuli presented to the focal fish, in the next experiment we decided to test if video playbacks, which have been successfully used with zebrafish in other behavioural tasks, could also be used to test attention to these stimuli (Saverino & Gerlai 2008; Qin et al. 2014).

2.4 A video playback experiment

In this second experiment, we replaced the live stimuli used in the first experiment by video playbacks in order to manipulate social features present in the interaction and identify key features (e.g. form and movement) that may drive zebrafish's attention. First we replicated the previous experimental treatments using video playbacks to test the response of bystanders to videos. We then analysed the influence of the video fight's activity levels on bystanders that observed the fighting interaction. Finally, we compared the attentional response to an altered video fighting interaction, where the features of the interacting fish were edited such that the pattern of movement remained the same but body features were absent. This was achieved by replacing the fish on each frame by dots with the same surface area and mean colour (i.e. fighting fish vs. fighting dots) and allowed us to test if it is the type of movement present in the video images or specific form features present in the social interaction that drive zebrafish's attention.

Methods

Behavioural setup. The setup from the previous experiment (Figure 2.1) was adapted by replacing the demonstrator tanks with a 10-inch, 1024 × 768 LCD tablet, positioned adjoining the end glass side of a removable bystander tank (Figure 2.9). A camera was placed above the tank for a top-down view video recording and later tracking of the focal fish. The same lighting conditions were maintained to match the previous experimental settings.

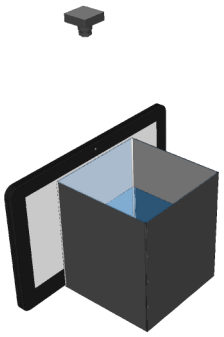

Figure 2.9 | Videoplayback setup. 3D schematic of the video experimental setup. A tablet display replaces the demonstrator tank from the original experimental setup (see section 2.3).

Experimental procedure. The procedures for animals and housing were the same as in the previous experiment. In this experiment the number of bystander focal fish was increased to 23 per treatment. Each focal fish was subjected to a single 30 min test corresponding to one of four new treatments: (1) bystander to a video of fighting conspecifics (BVIC), comprising a pre-resolution, resolution, and post-resolution stage (Oliveira et al. 2011); (2) bystander to a video of fighting dots (BVID), where the original fight video was manipulated by replacing the fighting fish by circles (dots) while maintaining the same original fish movements; (3) bystander to a video of non-interacting conspecifics (BVNIC); and (4) observing a video of an empty tank (VISOL) as control for the stimuli and any possible effects of the screen itself. Each stimulus video presented was previously recorded with a digital video camera (SONY Handycam DCR-SR58E) at a 25 fps and 720×576 pixel resolution, using the same conditions and settings of the previous

experiment. The videos were displayed on the tablet using real size images.

On the day prior to the test, fish of similar size were randomly removed from the stock tanks and isolated in each bystander test tank overnight, next to the experimental setup. This produced an isolation baseline effect and allowed for setup lighting acclimatization. Removable white opaque partitions were placed on the observation glass side of each test tank to prevent visual contact with the exterior. On the following day, prior to the beginning of each test, a test tank with an isolated focal fish inside was placed in the setup (with the opaque partition still in place), positioned in front of the tablet screen and allowed to habituate for 30 min. At the beginning of the test, the video started playing on the screen and the opaque partition was immediately removed. Each bystander focal fish could then visually observe a video for 30 min. The order of the treatments was randomized for each session. All bystanders' behaviours were video recorded for later offline behavioural tracking and analysis. Immediately after the test, each focal fish was euthanized. All samples were stored at -80 ºC for posterior analysis.

Manipulation of the fighting conspecifics' video. The replacement of the fighting fish by dots was achieved firstly by tracking and extracting both fighters' centroid coordinates, size, colour and contrast for each frame, using a custom-made tracking software. Two circles with the mean area, colour and contrast of the original fish were then placed at the corresponding centroid positions, over the fish-

subtracted background images of the tank (Figure 2.10). This allowed exact replication of the fighters' movement, while eliminating their form features.

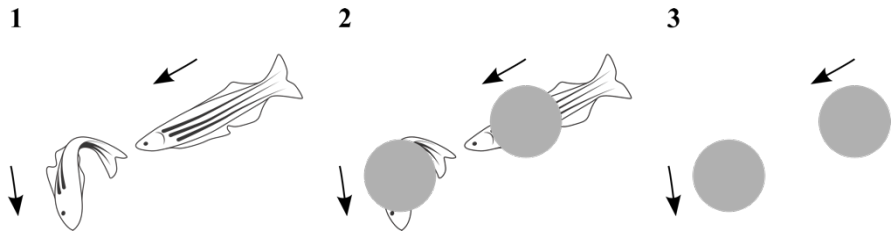

Figure 2.10 | Replacement of fish by dots. Schematic of the fighting fish by 'fighting' dots replacement procedure. (1) Centroid tracking; (2) dots overlapping; (3) fish subtraction. The areas, mean colours and movements of the original fish are maintained.

Activity analysis of the fighting conspecifics' video. In order to test if the bystanders' attention was correlated with a measure of activity on screen, we used the fighter's tracked data to determine the mean speed of the fighting dyad in the video throughout the 30 min test, in 30 s bins. This allowed profiling the temporal dynamics of the fight's activity levels and to compare it with the bystanders' time spent in close proximity to the screen (ROI) in 30 s bins, when observing the video fight and video dots fight.

Behavioural data analysis. The same behavioural analyses from the previous experiment were performed (see Methods in section 2.3), except for the temporal dynamics of the mean time spent in ROI and $R_g proj$. Correlations between the BVIC fishes' time spent in the ROI

and the video fight activity were performed. Additionally, comparisons between the BVIC and BVID fishes' time spent in the ROI were performed at specific pre-resolution and post-resolution time intervals of the fight.

Hormonal analysis. The procedures were the same as in the previous experiment. For this study, the intra-assay coefficient of variation was 5.10% and inter-assay coefficient of variation was 2.80%.

Statistical Analysis. The same statistical procedures from the previous experiment were used. Comparisons of the fighting fish vs. fighting dots at specific pre-resolution and post-resolution time intervals of the fight were performed using a Repeated Measures ANOVA, followed by contrasts for specific planned comparisons.

Results

Video playbacks of social stimuli confirm that zebrafish's attention is tuned to social interactions among third parties. Similarly to the results obtained using real stimuli (section 2.3), bystander focal fish spent more time in close proximity to the stimulus and showed higher directionality when presented with a video of fighting conspecifics. Specifically, BVIC fish spent significantly more time in the ROI than either BVNIC or VISOL fish. Moreover, there were no significant differences between BVNIC and VISOL fish (Figure 2.11; Table 2.2).

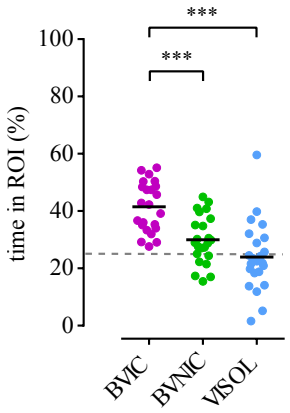

Figure 2.11 | Time in ROI. Scatter plot (n = 23 / treatment) of the individual (coloured dots) and mean (black lines) percentage of time spent in the ROI for each treatment. BVIC — bystander to video of fighting conspecifics (dark magenta); BVNIC — bystander to video of non-interacting conspecifics (green); VISOL — observing video of empty tank (light blue). Dashed grey line represents the value expected from a random distribution in the arena (25%). *** $p < .001$.

Both BVIC and BVNIC's group mean vectors were oriented towards the stimulus at 180° [α_g (BVIC) = 191.38°, 95% C.I. = 178.13° – 216.45°, R_g = 0.071, n = 23; α_g (BVNIC) = 185.71°, C.I. = 167.64° – 227.39°, R_g = 0.043, n = 23; α_g (VISOL) = 242.53°, R_g = 0.013, n = 23]. Although all group vector lengths R_g showed values proximate to zero (no focus), the individual fish's directional focus (*Rproj*) towards the stimulus showed a significantly higher group mean (R_g*proj*) for the BVIC treatment contrary to the BVNIC treatment, when compared to VISOL. However, there was no significant difference between BVIC and BVNIC (Figure 2.12; Table 2.2). Moreover, the distribution of the individual fish's mean orientations for the BVIC and BVNIC treatments

deviated significantly from uniformity towards the corresponding group mean direction (Moore's test: $p < .001$).

Table 2.2 | Behavioural and hormonal results

	time ROI (%)	*Rproj* (-1 to 1)	total dist. (m)	speed ROI (m s^{-1})	cortisol[a] (ng/g)
Mean ± SEM					
BVIC	41.46 ± 1.82	0.07 ± 0.01	126.89 ± 7.24	0.07 ± 0.004	3.02 ± 0.61
BVID	36.82 ± 3.37	0.05 ± 0.02	123.04 ± 8.74	0.07 ± 0.005	3.67 ± 1.02
BVNIC	30.03 ± 1.77	0.04 ± 0.01	116.09 ± 9.18	0.06 ± 0.005	2.92 ± 0.56
VISOL	24.00 ± 2.54	0.00 ± 0.02	112.83 ± 9.47	0.06 ± 0.005	2.93 ± 0.51
ANOVA					
BVIC × BVNIC ×VISOL	$F_{2,66} = 18.3$ $p < .001$	$F_{2,66} = 4.7$ $p = .01$	$F_{2,66} = 0.72$ $p = .49$	$F_{2,66} = 1.32$ $p = .28$	$F_{2,54} = 0.01$ $p = .99$
Tukey HSD					
BVIC vs. VISOL	$p < .001$ $d_s = 1.65$	$p = .009$ $d_s = 0.85$	- -	- -	- -
BVNIC vs. VISOL	$p = .11$ $d_s = 0.57$	$p = .19$ $d_s = 0.5$	- -	- -	- -
BVIC vs. BVNIC	$p = .001$ $d_s = 1.32$	$p = .40$ $d_s = 0.43$	- -	- -	- -
Planned comparisons					
BVIC vs. BVID	$t = 1.32$ $p = .19$	$t = 0.87$ $p = .39$	$t = 0.31$ $p = .75$	$t = 0.25$ $p = .80$	$t = 0.24$ $p = .80$

BVIC — bystander to video of fighting conspecifics (n=23); BVID — bystander to video of fighting dots (n=24); BVNIC — bystander to video of non-interacting conspecifics (n=23); VISOL — observing video of an empy tank (n=23). [a] Cortisol sample sizes: BVIC (n=18); BVID (n=19); BVNIC (n=20); VISOL (n=19).

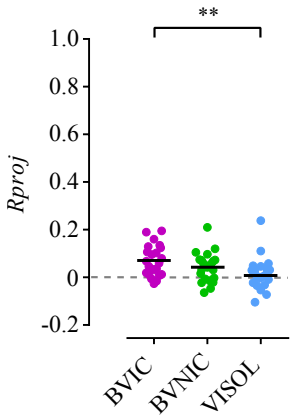

Figure 2.12 | Directional focus. Scatter plot of the individual (coloured dots) resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction (180˚) and corresponding group mean value $R_g proj$ (black lines), for each treatment. Positive values indicate directional focus towards the stimulus; zero value indicates no directionality (dashed grey line); negative values indicate directional focus opposite to the stimulus. ** $p < .01$.

Similarly to what happened in the first experiment neither differences in locomotor or stress levels across the three treatments explains the differences in attention between treatments. Analyses of the total distance covered and mean speed in the ROI did not reveal significant differences between treatments (Figure 2.13A,B; Table 2.2). Whole-body cortisol levels also did not show significant differences between treatments (Figure 2.13C; Table 2.2).

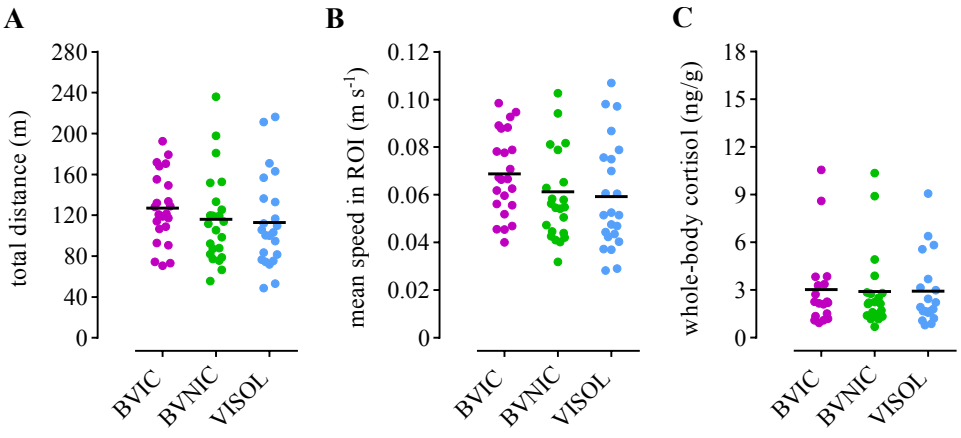

Figure 2.13 | Locomotor activity and cortisol levels. Scatter plots of the individual (coloured dots) and mean values (black lines) of the focal fishes' **(A)** total distance covered in the arena; **(B)** mean speed in the ROI; and **(C)** whole-body cortisol levels, for each treatment.

Zebrafish's attention towards social interactions is not merely associated with levels of activity of the stimuli. Analysis of the video fight used as stimulus showed that the fighters' activity profile was heterogeneous, revealing a steep increase in their mean speed after the fight resolution (i.e. time at which a dominant and a subordinate emerged in the fight), resulting from high speed chasing of the subordinate by the dominant followed by alternating periods of inactivity and chasing bouts (Figure 2.14A).

We performed a correlation analysis (Figure 2.14B) before (0 min to 3.5 min) and after (3.5 min to 7 min) the fight's resolution point, which occurred at 3.5 min into the video, using 30 s bins as samples units, in order to compare the BVIC fishes' mean time in the ROI with the activity in the video.

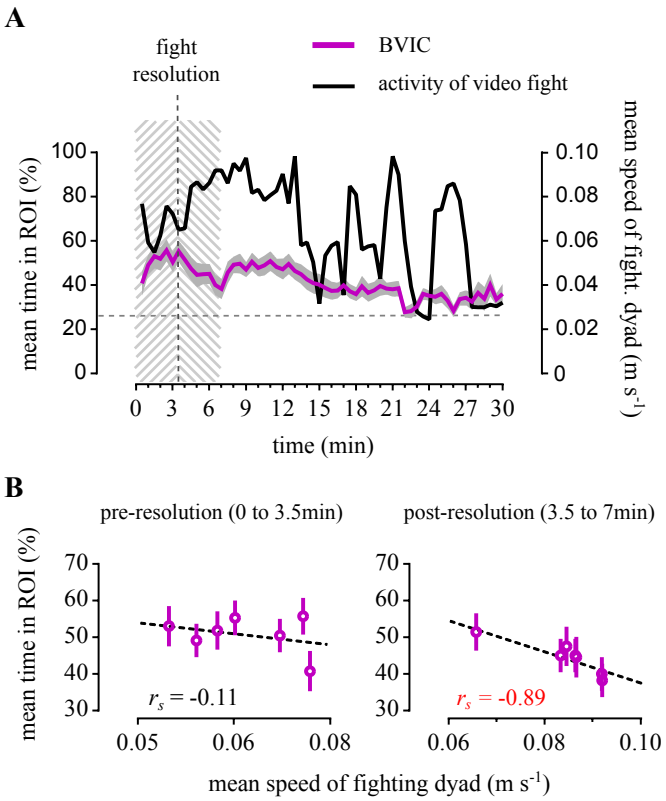

Figure 2.14 | Video fight activity vs. proximity of bystanders to video screen. (A) Temporal dynamics of the mean speed of the fighting dyad (black curve) in the video and the BVIC fishes' mean time spent in the ROI (dark magenta curve), measured in 30 s bins, throughout the 30 min test. Grey shadow represents the standard error (SEM); dashed grey horizontal line — value expected from a random distribution in the arena (25%); dashed black vertical line — video fight resolution time point (at 3.5 min); dashed grey areas — pre-resolution (0 to 3.5 min) and post-resolution (3.5 to 7 min) time intervals analysed. **(B)** Scatter plots of BVIC fishes' mean time spent in the ROI as function of the mean speed of the video's fighting dyad (video activity), before (0 min to 3.5 min) and after (3.5 min to 7 min) the fight resolution point. Open circles and error bars represent the sampled (in 30 s bins) mean ± SEM points. Spearman's correlation coefficient r_s is shown in red when significant ($p < .05$). Dashed black lines indicate the regression line for easier visualization of trends.

The results showed no correlation between the mean percentage of time in ROI and mean speed of the fighting dyad before the fight resolution (r_s = -0.11, p = .84), and showed a strong negative correlation after the resolution (r_s = -0.89, p = .012). Thus, the mean speed of the fighting dyad on screen during the time period around the fight resolution was either not correlated or negatively correlated with the bystanders' mean time spent in the ROI, suggesting that social features rather than conspicuousness of the conspecific dyad drive zebrafish's attention towards fighting interactions. We further investigated this hypothesis experimentally by editing the video clip of the fighting dyad used for the video playbacks.

Social features drive zebrafish's attention towards social interactions. Comparisons between the BVID and BVIC conditions did not reveal significance differences in the mean time spent in the ROI when considering the 30 min analysis, although BVID fish revealed twice the dispersion of BVIC (Figure 2.15A; Table 2.2). The BVID's group resultant mean vector also oriented towards the stimulus [α_g (BVID) = 176.75°, 95% C.I. = 219.87°–106.32°, R_g = 0.051, n = 24], with the distribution of the individual fish's mean orientations deviating significantly from uniformity (Moore's test, p < .005). The value of the group mean onto the stimulus direction ($R_g\hat{proj}$) was also low and not different from BVIC's (Figure 2.15B; Table 2.2). Analysis of the total distance covered and the mean speed in ROI did not reveal significant differences to the BVIC treatment (Figure 2.15C,D). Whole-body cortisol levels were also not significantly different (Figure 2.15E).

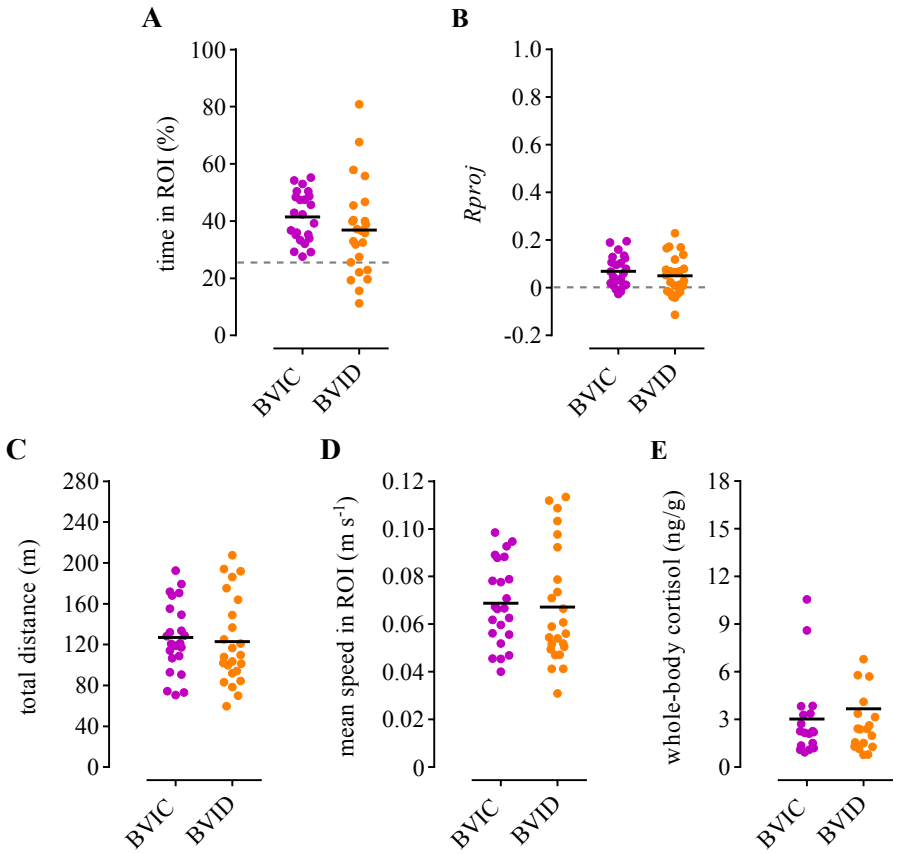

Figure 2.15 | Bystanders to fighting conspecifics vs. bystanders to fighting dots behavioural results. Scatter plots (n = 23 to 24 /treatment) of individual (coloured dots) and mean values (black lines) of the focal fishes' **(A)** time spent in the ROI; **(B)** resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction (180˚); **(C)** total distance covered; **(D)** mean speed in ROI; and **(E)** whole-body cortisol levels (n = 18 to 19 /treatment) for BVIC — bystander to video of fighting conspecifics (dark magenta) and BVID — bystander to video of fighting dots (orange) treatments. Dashed grey line represents in (A) the value expected from a random distribution in the arena (25%), and in (B) no directionality (*Rproj* = 0).

However, analysis of the temporal dynamics of both BVIC and BVID fishes' mean time spent in the ROI (Figure 2.16A), particularly

the adjacent time intervals before (0 min to 3.5 min) and after (3.5 min to 7 min) the fight resolution point, using two equal time bins of 3.5 min each, revealed a significant difference between the two treatments before the fight was resolved but not after [Repeated Measures ANOVA, interaction: $F_{1,45}$ = 5.23, p = .027; Contrasts (BVIC vs. BVID pre-resolution): t = 2.06, p = .04; Contrasts (BVIC vs. BVID post-resolution): t = 0.53, p = .60; Figure 2.16B].

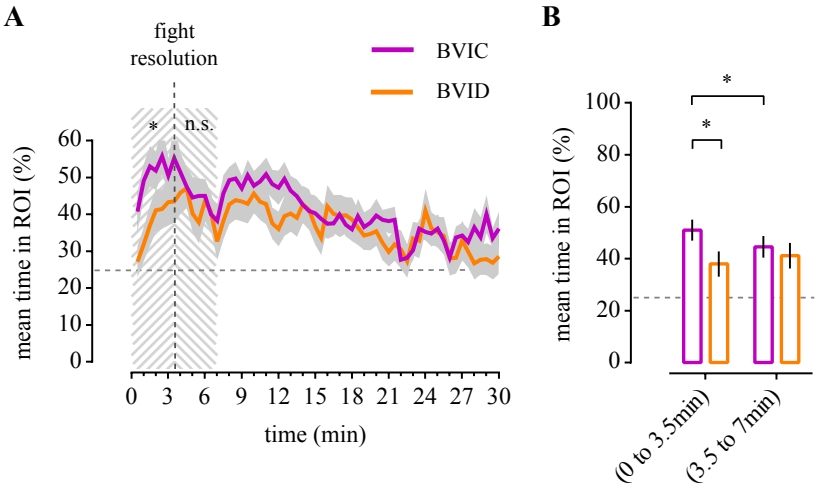

Figure 2.16 | Temporal dynamics of bystanders to fighting conspecifics vs. bystanders to fighting dots. (A) Comparison of the mean time spent in the ROI between BVIC (dark magenta) and BVID (orange) treatments. Grey shadows represent the standard error (SEM); dashed grey horizontal line — value expected from a random distribution in the arena (25%); dashed black vertical line — video fight resolution time point (at 3.5 min); dashed grey areas — analysed pre-resolution (0 to 3.5 min) and post-resolution (3.5 to 7 min) time intervals. **(B)** Bars plot of mean ± SEM comparison between the BVIC and BVID treatments, before and after the fight resolution event, in the previously defined time period. n.s. — non-significant; * p < .05.

Results also showed there was a significant decrease in the BVIC fishes' mean time spent in ROI [Contrasts (pre vs. post-resolution): t = 2.12, p = .039] after the fight was resolved, which did not happen in the BVID treatment [Contrasts (pre vs. post-resolution): t = 1.10, p = .27; Figure 2.16B].

Discussion

The results from this second experiment confirm those obtained with real conspecifics in the previous section, hence suggesting that zebrafish respond to video playbacks of conspecifics. Importantly, in this experiment where the sample size was increased and the stimuli standardized, the time spent in proximity to the fighting conspecifics compared to non-interacting conspecifics became significant as predicted, which supports the hypothesis that zebrafish attention is particularly tuned to social interactions. Notably the dispersion around the mean values was much lower for the attentional responses to video stimuli compared to the real stimuli, showing a strongly coherent response of the sampled individuals when faced with the same stimulus. The directional focus also showed the same pattern of response for the different treatments as in the first experiment. However its absolute values were very low. A possible explanation is that bystander fish lost track of the fish in the video when they got to close to the screen, contrary to the first experiment. If this was the case, a predicted effect would be of bystanders spending less time in sustained close proximity to the stimulus compared to the first experiment (as supported by the results) and also of a change in their directional behavioural patterns,

with bystanders increasing the amount of back and forth movement along the 0^{o} to 180^{o} axis, alternating from close proximity to the screen where they lose the visual signal to farther away where they may regain it, and back again. Complementary analysis of the increase in frequency of entries and exits in the ROI suggests that this was indeed the case (data not shown). An expected effect would thus be of a tendency for the bystander fish's directional vectors to cancel each other out on average, and consequently low values of directional focus towards the stimulus (as supported by the results). New pilot experimental setups are currently testing different focal distances between the observer and the screen. Importantly, it should be noted that only one video was used as stimulus for each tested condition and therefore we cannot at this point conclusively generalize the results to all fighting interactions and to all non-interacting conspecifics. However, the pattern of results is consistent with those obtained using real stimuli.

Additionally, replacement of the fighting fish by fighting dots, although not eliciting significantly different mean responses in the time spent in proximity to the stimulus, when considering the overall 30 minutes, revealed twice the dispersion around the mean value. This suggests that the video fishes' form features provide some sort of information specificity to bystanders which increases the homogeneity of their response levels, which was lost when this component was removed. Moreover, the temporal dynamics of the bystanders' proximity to the fighting fish and fighting dots, together with correlation analysis with the video fight's activity levels, indicates that

during the pre-resolution stage of the fight, during which interacting fish are signalling to each other their competitive ability using ritualized displays, bystanders' attention was not explained by the activity levels or structure of movement of the stimuli fish alone. The results suggest that attention is tuned to relevant form features present in signalling interactions at this stage. We further explore and confirm this possibility in the next experiment.

2.5 Testing attention at different stages of the interaction

In this third experiment we used Tübingen (TU) zebrafish, a wild-type strain more amenable to manipulation. We first replicated the results from the first experiment (section 2.3) using this strain. This validation was needed given the significant differences in behaviour and cognition that have been described across different zebrafish strains (Vignet et al. 2013). Next we tested the response of bystander TU fish not only to one video of fighting conspecifics (as in section 2.4) but to several video fights in order to better represent the variability of live stimuli, addressing the issue of pseudo-replication (Mcgregor 2000). It also allowed us to further analyse the attentional response of bystanders, specifically around different fight resolution time points. Finally we compared the attentional response of bystanders to manipulated looped samples of a video fight, specifically of a pre-resolution stage and post-resolution stage. This allowed us to analyse the influence of these two

stable, repeating stimuli independently, in order to better decouple the influence of activity levels from the structure of the movement.

Methods

Animals and housing. Wild-type Tübingen (TU) adult male zebrafish (*Danio rerio*), bred at Champalimaud Centre for the Unknown (CCU, Lisboa, Portugal) and Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used. Fish were kept in mixed sex shoals of 30 individuals in environmentally enriched stock tanks with 50 × 25 × 30 cm (30 l) at 25 ºC, under a 12L:12D photoperiod. The remaining procedures were similar to the previous experiments. No fish was injured as result of the expression of agonistic behaviours. Used animals were returned to stock tanks and reused in other pilot studies.

Tübingen's validation using real stimuli. The same setup, treatments and experimental procedures of the first experiment (see Methods in section 2.3) were followed with slight modifications. The demonstrator tanks were reduced in length from 15 cm to 7.5 cm. This confined the demonstrator fish closer to the bystander's tank side in order to increase the stimulus salience. The sample size of the focal fish was enlarged to an average of 18 per treatment.

Attention towards video playbacks of fighting conspecifics. The same setup and experimental procedures of the second experiment (see Methods in section 2.4) were followed with slight modifications. Each focal fish was subjected to a single 30 min test corresponding to

one of two treatments: (1) bystander to a video of fighting conspecifics (BVIC); and (2) observing a video of an empty tank (VISOL), as control for any possible effects of the screen itself. A sample size of 18 focal fish was used per treatment and 18 different video recorded fights, each comprising a clear pre-resolution, resolution, and post-resolution stage, were used as stimuli in the BVIC treatment. In order to increase video quality, a Gopro Hero3+ Silver camera recording at 120 fps (displayed at 60 fps) and 1280×720 pixel resolution was used. The videos were displayed on the tablet using real size images.

Attention towards video playbacks of looped assessment vs. looped chasing fight stages. The same setup and experimental procedures of the second experiment (see Methods in section 2.4) were followed with slight modifications. A sample size of 18 focal fish was used per treatment. Each focal fish was subjected to a single 30 min test corresponding to one of three treatments: (1) bystander to a pre-resolution looped video of fighting conspecifics (BVICpre); (2) bystander to a post-resolution looped video of fighting conspecifics (BVICpost).

Analysis of the video fight's activity curve. A typical video fight was chosen from the 18 previously recorded video fights. In order to characterize the overall levels of activity of the fight on the screen, we tracked the fighting fish in the video and used the tracked data to determine the mean speed of the fighting dyad in the video, throughout

the 30 min test, using 30 s bins (see Methods in section 2.4). This allowed profiling the temporal dynamics of the fight's activity levels.

Editing the video loops. A 5 min sample from the pre-resolution stage and another from the post-resolution stage were selected and edited based on the video fight's activity curve. For each edited sample, a 30 min video was prepared at 60 fps and 1280×720 pixel resolution by creating a sequence of 6 looped repetitions of the 5 min sample (Figure 2.17). To minimize cut effects, the transitions between loops were smoothed out by a dissolve overlap. The mean activity of the pre-resolution and post-resolution looped videos was determined as the mean speed of the fighting dyad in the correspondent 5 min video samples, using the video fighters' tracked data.

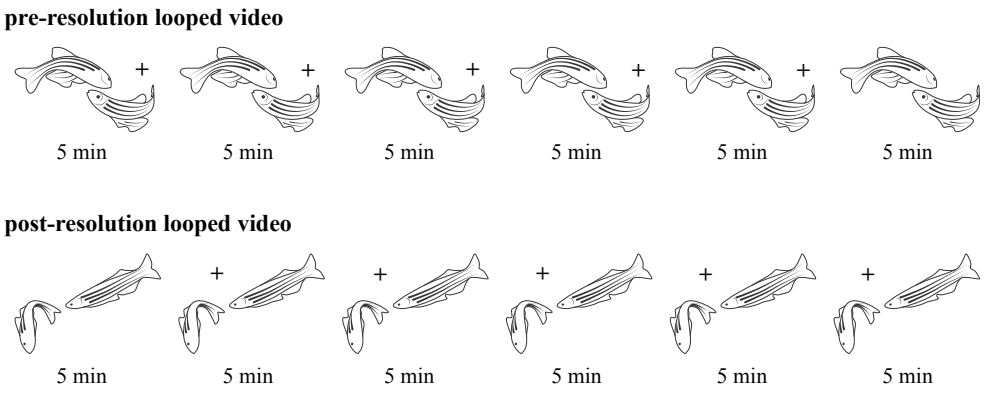

Figure 2.17 | Looped video fights. Schematic of the two looped videos used as stimulus and created from repeated sequences of 5 minutes samples from the pre-resolution and post-resolution stages of a selected video fight.

Behavioural data analysis. We focused on the two attentional parameters that revealed significant results in the previous experiments (time in ROI and *Rproj*). For the validation task an equivalent behavioural analysis to the first experiment was performed (see Methods, section 2.3). For the 18 video fights playback task, validation of the mean response for the 30 min test compared to a video of an empty tank was performed. Additionally, the difference in the bystanders' mean response between the period immediately before and after the fight resolution time point was analysed. This was achieved by aligning the individual temporal response curves of all bystanders by the fight's resolution time (using 15 s bins) and by determining the group's mean response curve. The time interval considered was the maximum amount of time, before and after the fight resolution, which allowed the inclusion of all sampled focal fish in the analysis. For the looped videos task, analysis of the mean values for both behavioural parameters was performed for the overall 30 min test. Additionally the temporal dynamics (time series) of both attentional behavioural parameters, measured in 30 s bins, for the 30 min test was also analysed for the looped video treatments. Comparison between the pre-resolution and post-resolution looped videos treatments was performed for every 5 min bin (corresponding to one loop).

Statistical Analysis. For the validation task using real stimuli, one-way ANOVAs were performed followed by post-hoc Tukey HSD tests after normality and homogeneity of variances (Levene's test) was verified. For the video stimuli validation task, *t*-tests were conducted

after normality and homogeneity of variances was verified. For the fight resolution time alignment analysis, dependent *t*-tests were used for adjacent bins comparisons within the time series. For the looped videos task analysis, *t*-tests were performed for all behavioural treatments' mean comparisons. A non-parametric Wilcoxon test was used for comparing the selected video samples activity. Mixed-design ANOVAs were performed for the time series analyses, followed by LSD Fisher post-hoc tests for bin comparisons. The obtained *p*-values were adjusted (*p'*) for multiple comparisons using sequential Bonferroni corrections. Effect sizes were determined by Cohen's *d*.

Results

TU zebrafish show equivalent attentional responses to AB zebrafish. Similarly to the first experimental results (see section 2.3), analysis of the group mean percentage of time spent in the ROI and *Rproj* in the 30 min test, confirmed that Tübingen (TU) bystanders to fighting conspecifics (BIC) also spent significantly more time in the ROI and with higher directional focus towards the stimulus, compared to bystanders to non-interacting conspecifics (BNIC) or socially isolated (ISOL) fish, Here the differences between BIC and BNIC became significant (Figure 2.18A,B; Table 2.3). The mean values for both treatments were also higher than in the first experiment. No differences were found across treatments for the total distance covered in the arena, although the mean speed in the ROI was lower for BNIC fish compared to BIC fish (Figure 2.18C,D; Table 2.3).

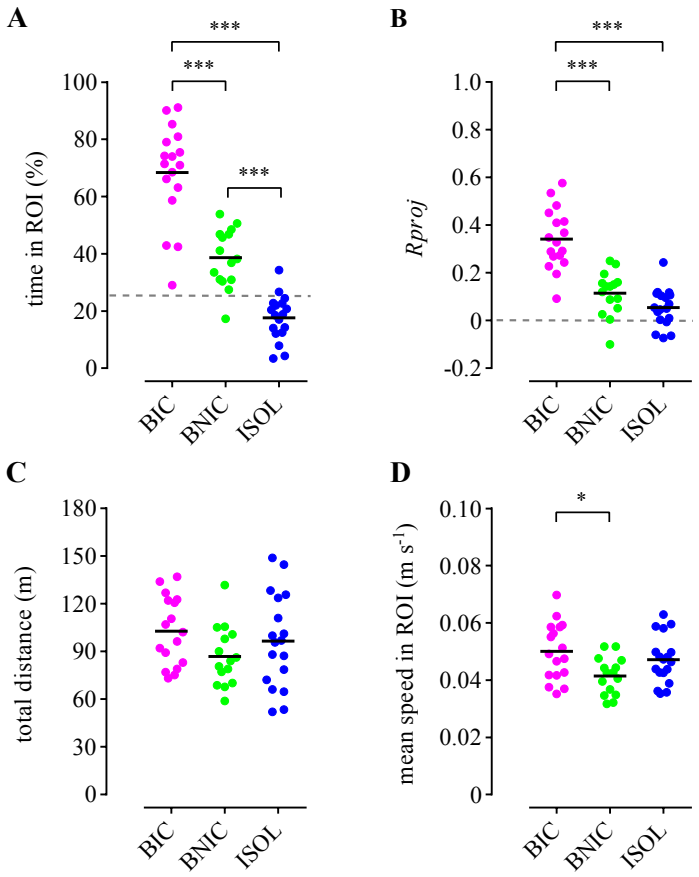

Figure 2.18 | Behavioural results of Tübingen's validation using real stimuli. Scatter plots (n = 15 to 18 /treatment) of the individual (coloured dots) and mean values (black lines) of the focal fishes': **(A)** time spent in the ROI; **(B)** resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction 180˚; **(C)** total distance covered; and **(D)** mean speed in ROI. BIC — bystander to fighting conspecifics (magenta); BNIC— bystander to non-interacting conspecifics (lime); ISOL — socially isolated (blue). Dashed grey line represents in (A) the value expected from a random distribution in the arena (25%) and in (B) no directionality (*Rproj* = 0). * $p < .05$; *** $p < .001$.

Table 2.3 | Behavioural results of Tübingen's validation experiment

	time ROI (%)	*Rproj* (-1 to 1)	total dist. (m)	speed ROI (m s^{-1})
Mean ± SEM				
BIC	68.43 ± 4.15	0.34 ± 0.03	102.86 ± 5.24	0.05 ± 0.002
BNIC	38.60 ± 2.64	0.11 ± 0.02	86.95 ± 4.88	0.04 ± 0.002
ISOL	17.62 ± 1.85	0.05 ± 0.02	96.60 ± 6.94	0.05 ± 0.002
ANOVA				
BIC × BNIC × ISOL	$F_{2,47} = 74.26$ $p < .001$	$F_{2,47} = 38.46$ $p < .001$	$F_{2,47} = 1.75$ $p = .18$	$F_{2,47} = 4.13$ $p = .02$
Tukey HSD				
BIC vs. ISOL	$p < .001$ $d_s = 3.85$	$p < .001$ $d_s = 2.74$	$p = .72$ $d_s = 0.24$	$p = .57$ $d_s = 0.95$
BNIC vs. ISOL	$p < .001$ $d_s = 2.33$	$p = .21$ $d_s = 0.71$	$p = .49$ $d_s = 0.38$	$p = .15$ $d_s = 0.74$
BIC vs. BNIC	$p < .001$ $d_s = 2.09$	$p < .001$ $d_s = 2.02$	$p = .16$ $d_s = 0.78$	$p = .02$ $d_s = 0.78$

BIC — bystander to fighting conspecifics (n=17); BNIC — bystander to non-interacting conspecifics (n=15); ISOL — socially isolated (n=18).

Zebrafish's attention towards video fighting interactions decreases once the fight is resolved. First, an analysis of the BVIC group's mean percentage of time spent in the ROI and *Rproj* for the 30 min, confirmed that Tübingen fish also responded to the 18 presented videos of fighting conspecifics, with the BVIC fishes' mean values significantly higher than VISOL fish (time in ROI: *t*-test, $t_{33} = 5.32$, $p < .001$, $d_s = 1.8$; *Rproj*: *t*-test, $t_{33} = 3.34$, $p = .002$, $d_s = 1.13$; Figure 2.19A,B).

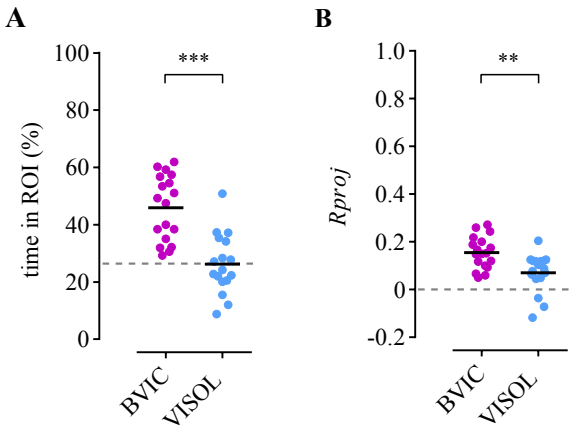

Figure 2.19 | Tübingen's attentional response to videos of fighting conspecifics. Scatter plots of the individual (coloured dots) and mean values (black lines) of the focal fishes': **(A)** time spent in the ROI; **(B)** resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction (180˚). BVIC (n = 18) — bystanders to video of fighting conspecifics (dark magenta); VISOL (n = 17) — observing video of empty tank (light blue). Dashed grey line represents in **(A)** the value expected from a random distribution in the arena (25%) and in **(B)** no directionality (*Rproj* = 0). ** $p < .01$; *** $p < .01$.

Next, the individual time series of the BVIC fishes' time spent in the ROI (Figure 2.20A) and directional focus (*Rproj*) were determined and aligned by the corresponding fight resolution times. The group's mean curves for both parameters were calculated in a 1.5 min interval before and after the fight resolution point (Figure 2.20B,C). Analysis of the two adjacent time intervals, revealed a significant decrease in the mean time spent in proximity to the video fights immediately after the fight was resolved (dependent *t*-test: $t_{17} = 3.43$, $p = .003$, $d_z = 0.80$, n = 18; Figure 2.20B). The mean curve of the BVIC fishes' directional focus towards the video fights (*Rproj*) also showed a decreasing trend

after the fight resolution point, although not significant (dependent *t*-test: t_{17} = 1.66, p = .11, d_z = 0.39, n = 18; Figure 2.20C).

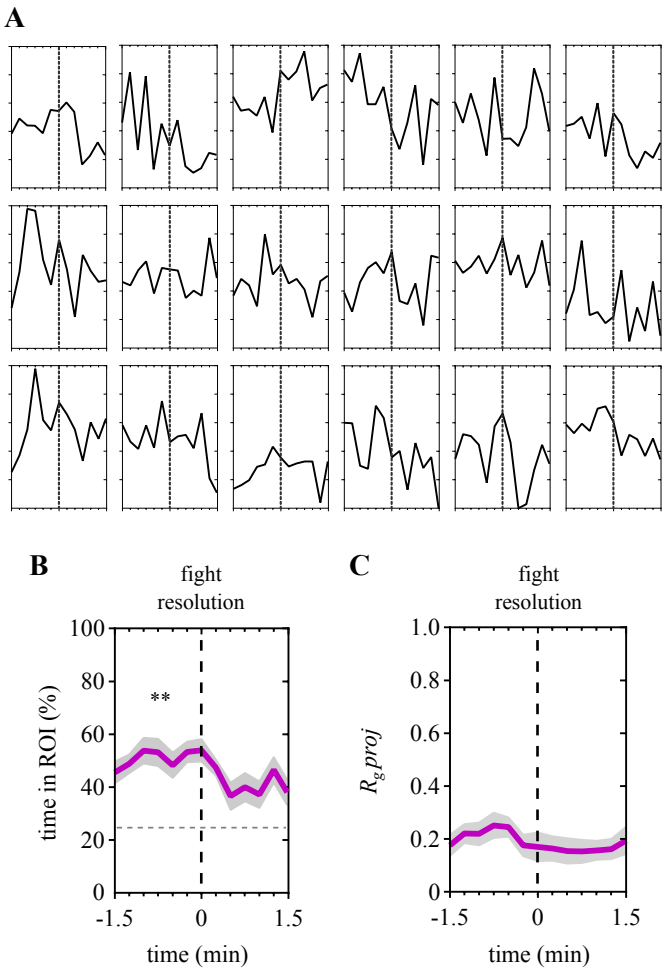

Figure 2.20 | Time in ROI and directional focus aligned by the fight resolution times. (A) Individual time series in 15 s bins of the 18 BVIC — bystanders to video of fighting conspecifics fishes' time spent in the ROI, from 1.5 min before to 1.5 min after the corresponding fight resolution time point. **(B)** Time series in 15 s bins of the BVIC (n = 18) group's mean time in the ROI aligned by the fights resolution times, from 1.5 min before to 1.5 min after the fight resolution. **(C)** Time series in 15 s bins of the BVIC mean directional focus towards the video

fights ($R_g proj$) aligned by the fights resolution times, from 1.5 min before to 1.5 min after the fight resolution. Dashed vertical lines correspond to the fight resolution time. ** $p < .01$.

Zebrafish are more attentive to an agonistic assessment interaction than to a winner-loser chasing interaction, regardless of the level of activity. After selection of a video from the 18 previously recorded video fights, the temporal dynamics of the video fight's activity levels was profiled by determining the mean speed of the fighting dyad in 30 s bins (Figure 2.21A). A 5 min sample from the pre-resolution stage and another from the post-resolution stage were selected and edited to prepare the two 30 min videos to be used as stimulus (Figure 2.21A). The activity level of the post-resolution samples was 160% higher than the pre-resolution samples (Wilcoxon test: $Z = 6.73$, $p < .001$; Figure 2.21B).

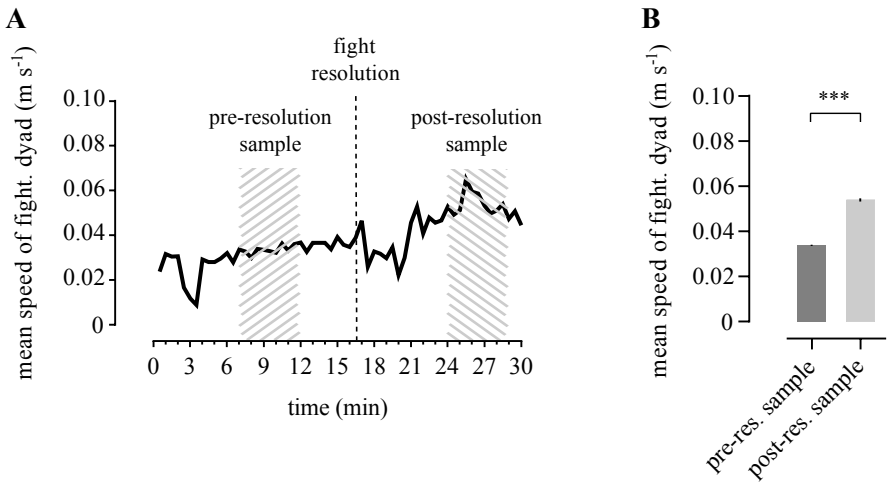

Figure 2.21 | Activity of the selected video fight and video loop samples. (A) Plot of the mean speed of the fighting dyad (black curve) in the video measured in 30 s bins, throughout 30 min. Dashed black vertical line correspond to the fight resolution time point (at min 16:33); dashed grey areas correspond to the selected 5 min pre-resolution and post-resolution samples' time periods for posterior loop editing. **(B)** Mean speed of the fighting conspecifics in the pre-resolution and post-resolution video samples.

BVICpre fish spent significantly more time in close proximity (ROI) to the stimulus than did BVICpost fish when presented with the 30 min videos [time in ROI (BVICpre) = 54.90 ± 1.96%, n = 18; time in ROI (BVICpost) = 42.26 ± 2.86%, n = 18; *t*-test: t_{34} = 3.64, p < .001, d_s = 1.21, n = 18; Figure 2.22A], even though the activity level of the post-resolution loops was 160% higher than the pre-resolution loops. Moreover, comparison of the time series for each treatment using 5 min bins, corresponding to the duration of each repeated loop, showed that the difference between the time spent in the ROI between BVICpre and BVICpost fish was sustained throughout the 30 min test [Mixed-design ANOVA, treatment: $F_{1,34}$ = 13.25, p < .001; bin: $F_{5,170}$ = 4.10, p = .001; interaction: $F_{5,170}$ = 0.50, p = .77; LSD post-hoc: (0-5 min), p' < .001; (5-10 min), p' = .002; (10-15 min), p' = .009; (15-20 min), p' < .014; (20-25 min), p' = .006; (25-30 min), p' = .02; Figure 2.22B].

Analysis of the directional focus *Rproj* revealed no significant differences between BVICpre and BVICpost treatments when considering the overall 30 min test [*Rproj* (BVICpre) = 0.16 ± 0.02, n = 18; *Rproj* (BVICpost) = 0.14 ± 0.02, n = 18; *t*-test: t_{34} = 0.75, p = .45, d_s = 0.25, n = 18; Figure 2.22C]. However, comparison of the time series

for each treatment using 5 min bins, revealed that in the first 5 min BVICpre fish were significantly more focused than BVICpost fish towards the corresponding video stimulus [Mixed-design ANOVA, treatment: $F_{1,34}$ = 0.44, p = .51; bin: $F_{5,170}$ = 8.44, p < .001; interaction: $F_{5,170}$ = 5.72, p < .001; LSD post-hoc: (0-5 min), p' = .003; remaining bins, p' > .05; Figure 2.22D].

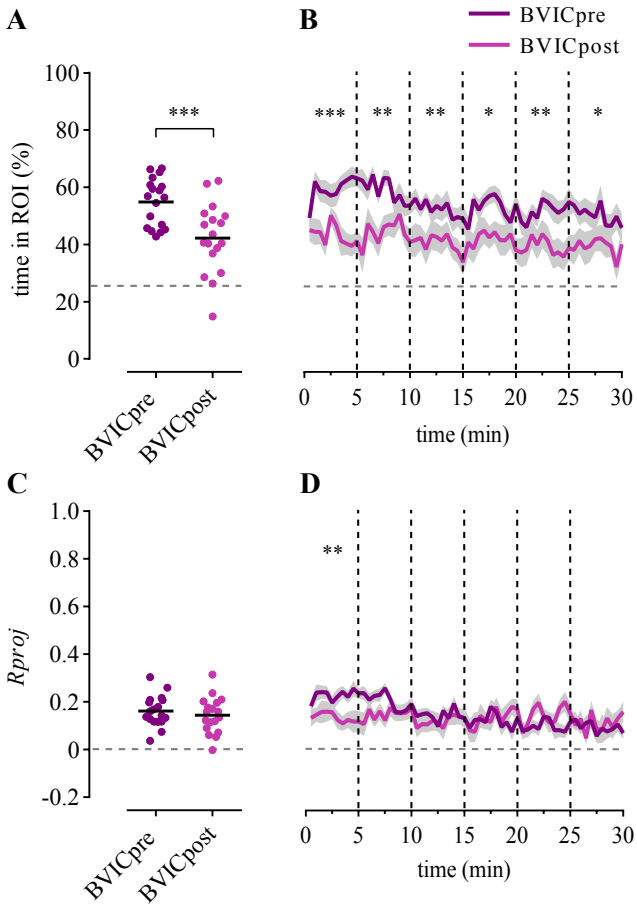

Figure 2.22 | Time in ROI and directional focus of bystanders to the pre- and post-resolution looped videos. (A) Scatter plot (n = 18) of the individual (dots) and mean (black lines) time spent in the ROI for the 30 min test.

(B) Time series of BVICpre and BVICpost mean time spent in the ROI in 30 s bins, throughout the 30 min test. **(C)** Scatter plot (n = 18) of the individual (dots) and mean (black lines) resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction (180˚) for the 30 min test. **(D)** Time series of BVICpre and BVICpost mean directional focus towards the stimulus (*Rproj*) in 30 s bins, throughout the 30 min test. BVICpre — bystanders to pre-resolution looped video of fighting conspecifics; BVICpost — bystanders to post-resolution looped video of fighting conspecifics. Dashed black vertical lines represent the start of a new loop. Dashed grey horizontal lines represented in (A,B) the value expected from a random distribution in the arena (25%) and in (C,D no directionality (*Rproj* = 0) respectively. * p' < .05; ** p' < .01; *** p' < .001.

Discussion

The results from this follow-up study strongly confirm those obtained in section 2.3 and 2.4 and show that the tuning of attention to fighting conspecific interactions is not restricted to a single zebrafish strain. Interestingly, the differences between Tübingen bystanders' responses when observing fighting or non-interacting conspecifics became significant for both proximity and directional focus towards the stimulus, with an overall increase in the response to fighting conspecifics compared to the AB strain. This is probably a consequence of the increased sample size and stimulus salience, enhanced by the smaller size of the demonstrator tanks, but can also be due to genetic differences between the two strains. In fact, differences in other cognitive abilities between zebrafish strains have been documented in the literature (e.g. social recognition; (Barba-Escobedo & Gould 2012).

The presentation of 18 different stereotypical video fights, where each fight is unique, elicited a consistent strong response in Tübingen zebrafish, further supporting the validity of using video playbacks as stimulus. Moreover, the alignment to the fight resolution's analyses also support the results previously obtained with a singular video fight in section 2.4, again showing a significant decrease in proximity to the video screen immediately after the fight resolution. This result suggests that despite the uniqueness of all fights (different levels of engagement, aggressiveness, and resolution times) the fight resolution event, which is similar in structure and common to all fights, elicits a consistent shift in attentional response from bystanders.

Furthermore, the presentation of looped videos of pre-resolution and post-resolution stages of a fight, confirmed that zebrafish are more attentive to the pre-resolution stage. Particularly in the first 5 minutes of the interaction where both attentional parameters revealed significantly higher values for the pre-resolution assessment stage. Also, results showed that this difference is not dependent on a causal relationship between the two stages (e.g. assessment coming before chasing), or the level of activity of the stimulus fish. This suggests a particularly differential attentive state both when the stimulus is novel (Wong et al. 2010) and to specific social features of the assessment interaction.

2.6 Chapter discussion

The results presented in this chapter show for the first time that the attention of a highly social species is tuned to interactions between conspecifics. This conclusion is based on the fact that zebrafish males are more attentive towards interacting than towards non-interacting conspecifics, together with the fact that this interest in interactions is not due to heightened activity levels of the interacting conspecifics making them more conspicuous to bystanders. Therefore, zebrafish bystanders' attention seems to be attracted by specific form or movement features present in the social interactions.

Interestingly, the features that drive bystanders' attention towards social interactions vary with the interaction dynamics. In the second experiment (section 2.4), at the initial phase of the agonistic interaction when opponents mutually assess each other's competitive ability (Arnott & Elwood 2009), bystanders' attention towards fighting conspecifics was higher than towards fighting dots, and with a smaller variability, indicating that form features played a key role at this stage. Such results are not surprising since the information being exchanged by the opponents at this assessment stage is mainly based on the display of species-specific stereotyped action patterns (e.g. lateral displays), which imply changes in form features rather than changes in the whole fish movement. Therefore, in order to extract relevant information on the relative competitive ability of observed conspecifics, bystanders should focus their attention on form features, during this signalling phase of the interaction.

Potentially relevant form features are the shape of a conspecific's body contour or the typical striped colouration pattern. Both are good candidates to drive attention in zebrafish, since during agonistic interactions, lateral displays imply changes in body contour (i.e. spread fins), together with changes in the intensity of body colouration also observed in aroused zebrafish (Kalueff et al. 2013). Moreover, the striped colouration and other form features are known to play a key role in the social approach response towards conspecifics in zebrafish (Engeszer et al. 2004; Rosenthal & Ryan 2005). Classic ethology studies have demonstrated the role of such simple form features of complex stimulus (aka sign stimuli or releasers) in triggering the expression of species-specific behavioural action patterns across different species (e.g. attack response of breeding male sticklebacks, *Gasterosteus aculeatus*, towards dummies with red bellies; Tinbergen 1948; Tinbergen 1951; Sevenster & Rowland 1985). Additionally it has been shown that zebrafish can integrate form and motion (aka feature binding) in a cohesive perceptual representation (Neri 2012). Therefore, the strong tendency to face the opponent in the assessment stage, which is absent in the post-resolution chasing stage, may provide specific information to eavesdroppers about the fight status, which is lost when the form features are replaced by dots. One can speculate that tuning of attention towards sign stimuli must be also part of the cognitive process that leads to an effective behavioural response. Since sign stimuli trigger the expression of adaptive behaviours in conspecifics, such cognitive processes, including selective attention, must have co-evolved with the relevant form feature. Therefore, it is expected that search images (i.e.

mental images of relevant features that enhance their detectability), which have been described in the context of foraging search behaviour (Bond 1983), may also be present in the social domain. Search images for conspecific form or movement features would be an effective way for social animals to enhance the acquisition of social information in detriment of other environmental information, similarly to the limited attention constraint that has been demonstrated for prey search images in visual predators (Dukas 2001). Like foraging search images that can be updated based on past experience of relative abundance for different food items (Langley et al. 1996), social search images may also be updated by experience or context. Future studies are needed to explore these possibilities. Finally, although form features seem to play a key role at the assessment stage, the overall level of activity of the interaction cannot be ruled out as a factor contributing partially and in an integrated way in driving bystanders' attention, as the results from the second experiment suggest.

After the fight resolution, when a clear dominant-subordinate role has been established between the interacting fish, bystanders' attentional levels towards fighting conspecifics seem to decrease. Both the second and third experiment results show that this decrease is already significantly noticeable at the fight resolution point, where there is a switch in the interaction dynamics from an assessment stage to a winner-loser chasing stage. This suggests it may be an important time point for potential eavesdroppers of fighting interactions, regarding information acquisition about the newly acquired dominance status of the fighters. Moreover, in the third experiment bystanders

independently observing a stable (looped) pre-resolution assessment or post-resolution chasing stages also showed a lower response to the latter although the movement on the screen, as measured by the average speed of the fighting conspecifics, was higher. This also supports the idea that the type of interaction dynamics and not simply the fight's sequence of events may be modulating potential eavesdroppers' level of attention. The type of interaction during the assessment stage does not yet provide information about the future status of each opponent, contrary to the post-resolution stage where a clear dominant-subordinate relationship is expressed. This uncertainty about the future social environment during the assessment stage might actually elicit higher bystanders' attentiveness, until enough information is acquired to make a decision about the social status of each opponent.

Additionally, in the second experiment attention towards fighting conspecifics and towards fighting dots became similar after the fight was resolved. This indicated that at this stage of the fight, movement features, rather than form features, were more relevant to explain attention levels. As expected, in this experiment proximity levels immediately after the resolution were also not positively correlated with movement on the screen, supporting that attention levels, although being driven by movement features, are not mainly driven by the conspicuousness of the visual stimulus.

Therefore, other movement parameters are needed to explain bystanders' attention after the fight resolution. At this stage the overall behaviour of the interacting agents (either fish or dots) is dominated by

movement components (dominants chase and attack; subordinates flee), whereas form components (e.g. lateral displays) are virtually absent. These movement features are common both to fighting fish and fighting dots, which may explain the lack of difference in the response to these two stimuli, observed at this stage of the fight. It is known that both humans and non-human animals, including fish, are tuned to attend to biological motion (Fox & McDaniel 1982; Tremoulet & Feldman 2000; Mascalzoni et al. 2010; Nakayasu & Watanabe 2014), characterized by intrinsic accelerations and changes in direction of the behavioural agent without the action of an external cause (e.g. change of direction due to hitting an obstacle). These animacy movement features are present both in the agonistic action patterns expressed during the display stage and during chasing, and can therefore play a key role in attracting the attention of bystanders. Research on pre-verbal human infants has shown that they are more attentive towards the biological motion of two behavioural agents when social contingency is present (i.e. chasing), than when they move independently from each other (Rochat et al. 1997; Frankenhuis et al. 2013). Moreover, some characteristics of chasing enhance its perceptual value, such as role reversal between the two agents (i.e. chaser and evader switching roles), "heat-seeking" chases (i.e. chaser taking the shortest path to the evader), and coherence of the orientation of the chaser according to its path of travel (Rochat et al. 2004; Gao et al. 2009; Gao et al. 2010). The tuning of attention to chasing, both in zebrafish and in humans, might represent a conserved bias in attentional processes towards a fitness relevant cue in the environment. Indeed, the outcome of a chase typically has fitness

consequences, whether these being for prey to successfully escape a predator, for a predator to successfully capture its prey, or for a subordinate individual to avoid being harmed by a dominant. Finally, it should be mentioned that despite the fact that zebrafish can perform feature binding, and therefore might integrate different features of chasing to extract meaning in terms of dominance relationships, in humans attention to chasing seems to be based on its movement features, in particular acceleration of the agents, rather than on the configuration of its features (Frankenhuis et al. 2013).

From a functional perspective the tuning of zebrafish attention to social interactions can be seen as an adaptive specialization to group living, since it allows the individual to eavesdrop on social interactions between third parties. As addressed in the general introduction (see chapter 1), social eavesdropping on aggressive interactions allows bystanders to use the collected information to infer dominance relationships and therefore to adjust their behaviour in subsequent interactions with the observed conspecifics.

In the next chapter we will follow-up on the obtained results and investigate the impact of attending to fighting interactions at the brain gene expression level.

Chapter 3
Brain transcriptomic response of attending to social interactions

3.1 Chapter summary

In this chapter we present a follow-up study of the first experiment presented in the second chapter (section 2.3), in order to start exploring the impact of attending to social interactions at the zebrafish's brain gene expression level.

- We based on the previously obtained behavioural results, showing that bystander zebrafish were more attentive towards interacting (i.e. fighting) than towards non-interacting pairs of conspecifics or social isolation, in order to select representative individuals from each of the three treatments according to distinct behavioural profiles.

- Next, we used microarray gene chips to characterize their brain transcriptome based on differential expression of single genes and gene sets. These analyses were complemented by promoter region-based techniques. Using data from both approaches we further drafted protein interaction networks.

- Overall our results suggest that attentiveness towards conspecifics, whether interacting or not, activates pathways linked to neuronal plasticity and memory formation. Furthermore, specifically observing fighting interactions further triggered pathways associated with specific genes, which suggests that observing social interactions may activate specific processes on top of those already activated just by observing conspecifics.

3.2 Introduction

As investigated in the previous chapter, in order to eavesdrop on conspecific interactions an animal must first be able to detect, approach and attend to those interactions, within a multitude of other social and non-social stimuli, in order to successfully extract relevant social information. This suggests, as discussed in the previous chapter, that tuning of attention towards social interactions should be an essential mechanism for successful eavesdropping. While social eavesdropping has been investigated at the behavioural level in several species (see chapter 1, section 1.3) to our knowledge its neural mechanisms and impact at the brain gene expression level have never been addressed. However, it is known that the input of specific social information is linked to changes in gene activation in the brain, which in turn influence subsequent behavioural outputs (Robinson et al. 2008). Moreover, different behaviours have been shown to be strongly associated with different brain gene expression profiles (Cardoso et al. 2015). For example, previous work using zebrafish has shown that a social acute agonistic event, like the experience of winning or losing a fight, is enough to elicit massive changes to the brain gene expression profiles (Oliveira et al. submitted) and functional connectivity of specific neural networks of the interacting fish (Teles et al. 2015). In the case of social eavesdropping, it should be expected that a bystander to a third party interaction will present different brain gene expression profiles, potentially reflecting its attentional state towards the

interacting conspecifics and the process of information acquisition (i.e. actively eavesdropping or not).

In this study, we selected representative individuals from each of the three treatments investigated in the first experiment of chapter 2 (section 2.3), according to their behavioural profiles, and used microarray gene chips to study their brain transcriptome. Our main goal was to characterize distinctive transcriptomic profiles and to identify candidate genes related to attentiveness to conspecifics in general, and potentially to social eavesdropping in particular. Our first approach was based on differential expression of single genes and of gene sets relative to a reference group of socially isolated individuals. We complemented this approach by considering the alignment of transcription factor (TF) motifs with the promoter region of differentially expressed (DE) genes. Finally, we used data from both approaches to draft a protein interaction network that may be used as a base to understand the mechanisms behind the obtained transcriptomes. This approach has the advantage of allowing us to analyse the social regulation of gene expression and its possible underlying biological processes in freely moving zebrafish, while in a 'naturalistic' social eavesdropping context.

3.3 A microarrays experiment

Methods

Defining behavioural profiles for transcriptomics analysis. In order to characterize different attentional profiles of the fish tested in the previous experiment (see section 2.3), we focused on the attentional behavioural parameters that revealed statistically significant differences with the socially isolated (ISOL) reference group, namely time in ROI and *Rproj*. Based on these two parameters, we clustered all samples using a partition around the medoids (PAM) method. The number of clusters was defined by maximizing the average silhouette (AS) for all possible number of clusters (between 2 and 32). The PAM clustering used Euclidean distances with normalized values (i.e. values were subtracted to the mean value and divided by the mean absolute deviation) and was performed using the R (R Development Core Team 2013) package "cluster". Based on the PAM clustering results and similarities of the focal fish's spatial and directional behavioural patterns (see Results), we selected four representative groups, each composed of 3 fish: one attentive group selected from the BIC (bystanders to fighting conspecifics) treatment and labelled sBIC; two selected groups from the BNIC (bystanders to non-interacting conspecifics) treatment — respectively one attentive group (labelled sBANIC) and one inattentive (labelled sBINIC), based on the two behavioural profiles detected in this treatment (see Results); and one selected group from the ISOL (socially isolated) treatment to act as a reference (labelled sISOL).

Pre-processing of microarrays. RNA was extracted from the selected fishes' brains using the RNeasy Lipid Tissue Mini kit (Qiagen) with some protocol modifications. Briefly, samples were homogenized by vortex and added 20 μl of chloroform. In order to maximize RNA recovery, incubation times were increased and in the end samples were diluted in 25 μl of RNase-free water. RNA integrity was verified using Bioanalyzer prior to microarray gene array processing (Tariq et al. 2002). RNA was processed and used in Affymetrix zebrafish gene 1.1 ST array strips according to the manufacturer's protocol. Microarrays procedures were performed at the Gene Expression Unit of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). The raw data CEL files were analysed using R and Bioconductor packages (Gentleman et al. 2004). The quality of the microarrays data was assessed for high quality and the arrays were then pre-processed using the standard RMA (Robust-Multichip average) normalization (see Abril-de Abreu et. al. 2015c for further details).

Statistical analysis of microarray data. The selection of differentially expressed (DE) genes was performed considering the group sISOL as a reference and using sBIC, sBANIC and sBINIC one at a time. A linear model on $\log_2$ signal values with empirical Bayes correction to the variance (implemented in Bioconductor package 'limma') was used and the *p*-values were adjusted for multiple testing using false discovery rates (FDR). The threshold for the differentially expressed genes was set at FDR < 0.05 and fold-change > 2 or < 0.5. A

hierarchical cluster of both samples and genes was created using the pooled group of differentially expressed genes genes for the sBIC, sBANIC and sBINIC tested groups.

Genes were annotated using Entrez IDs obtained primarily from the Bioconductor, NCBI and biomart databases. A total of 21 224 genes were annotated, from which 20 944 had information on chromosome location. Over-representation analysis (ORA) was performed to assess if the differentially expressed genes of each behavioural group were enriched in some gene sets. The threshold for overrepresentation was set to $p < .10$. The gene sets considered were pathways from KEGG (Kanehisa et al. 2014) and Wikipathways (Kelder et al. 2009), terms from GO (Ashburner et al. 2000) and chromosome locations.

Because the number of obtained differentially expressed genes genes was small (see Results), we also performed gene set enrichment analysis (GSEA). Unlike ORA, GSEA uses the whole gene expression data instead of defining a list of strongly differentially expressed genes. There are many types of GSEA (Maciejewski 2014); here we applied the parametric competitive method Generally Applicable Gene-set [GAGE, (Luo et al. 2009)] which is suitable for small datasets and allows for analysis considering up-regulated genes, down-regulated genes, or both. The gene sets used were also from KEGG, Wikipathways, GO terms and chromosome locations, and the threshold was also set to $p < .10$. These analyses were performed using: Bioconductor packages 'biomaRt' and 'reutils' (annotation); 'GO.db', 'KEGG.db', 'Category' and 'GOstats' (ORA); 'gage' and 'GSEABase' (GAGE).

Promoter region analysis and transcription networks. Transcription factor (TF) binding sites (motifs) were searched in upstream regions of the zebrafish genome by calculating scores using Stubb 2.1 (Sinha et al. 2003). These scores were used to perform enrichment analysis using cis-Metalysis (Ament et al. 2012) by considering a set of differentially expressed genes identified for each behavioural group (sBIC, sBANIC, sBINIC). In brief, genomic information was obtained from UCSC Genome Browser, to which Stubb was used to score motifs every 500 bp windows with a 250 bp shift. Non-redundant motifs from Jaspar Core Vertebrate database were considered (Mathelier et al. 2014). Enrichment analyses were then performed for each motif and pair of motifs using cis-Metalysis (mode "flexible"). Using STRING 9.1 (Franceschini et al. 2013) we further constructed transcription networks considering *Homo sapiens* homologs of the list of differentially expressed genes and of enriched transcription factors for each social treatment (required confidence for edges was set to score > 0.4). These networks were then analysed regarding centralization, density, heterogeneity and structural correlation. Analyses were performed using Stubb 2.1 and cis-Metalysis within a python pipeline. Network analyses were performed using STRING 9.1 and R package "sna" (see Abril-de-Abreu et al. 2015c for further details).

Results

Clustering analysis reveals strongly attentive and weakly attentive profiles. Based on the behavioural parameters (time in ROI and *Rproj*) that revealed statistically significant differences with the socially isolated (ISOL) reference group, we performed a PAM clustering analysis for all focal fish from the three different treatments (BIC, BNIC and ISOL) tested in the previous experiment (see section 2.3). Almost all BIC fish were above chance level, while ISOL fish clustered around it (i.e. time in ROI = 25% and *Rproj* = 0). The BNIC group was composed by a majority of fish close to chance level and by some clearly above it (Figure 3.1).

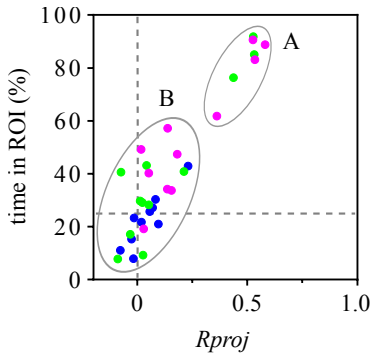

Figure 3.1 | Clustering analysis. Scatter plot of time spent in ROI vs. *Rproj* for all focal fish from the three experimental treatments: BIC (magenta), BNIC (lime), and ISOL (blue). Grey circles represent the obtained clusters. Cluster A — 'strongly' attentive profile; cluster B — 'weakly' attentive profile. Dashed grey horizontal line represents the value expected from a random distribution in the arena (25%). Dashed grey vertical line represents no directional focus (*Rproj* = 0).

The number of clusters that maximized their average silhouette AS (see Methods) was 2 with a value of 0.72, much higher than the values from all other number of clusters considered, which were consistently less than 0.50. From the cluster analysis, two distinct groups were created (Figure 3.1): cluster A, with a mean time in ROI = 82.50 ± 1.51% and mean *Rproj* = 0.50 ± 0.01, composed by four fish from BIC and three from BNIC; and cluster B, with a mean time in ROI = 28.99 ± 0.52% and mean *Rproj* = 0.05 ± 0.003, composed by the remaining BIC, BNIC and ISOL fish. This result supported the existence of a 'strongly' attentive profile (cluster A) composed by bystander fish that spent most of the time in close proximity to the stimulus and with high directional focus towards it, and a 'weakly' attentive profile (cluster B) composed by fish that did not show strong proximity and directional focus towards the stimulus.

Based on these profiles and on the matching of individual fish's spatial and directional patterns (Figure 3.2), we created four sample groups of interest for microarray analysis, each composed by 3 fish with similar time in ROI and *Rproj* values (Figure 3.3): (1) sBIC — selected bystanders attentive to fighting conspecifics (belonging to cluster A and selected from the BIC treatment); (2) sBANIC — selected bystanders attentive to non-interacting conspecifics (belonging to cluster A and selected from the BNIC treatment); (3) sBINIC — selected bystanders inattentive to non-interacting conspecifics (belonging to cluster B and also selected from the BNIC treatment); and (4) sISOL — selected inattentive socially isolated fish (belonging to cluster B and selected from the ISOL reference treatment). Interestingly, the high levels of

directional focus towards the stimulus showed by the sBIC and sBANIC fish resulted from the collapsing of a bimodal distribution peaking at an approximate 45º angle deviation from the 180º direction, which may be related to the zebrafish's eye positioning and field of view when observing the stimulus (Figure 3.2).

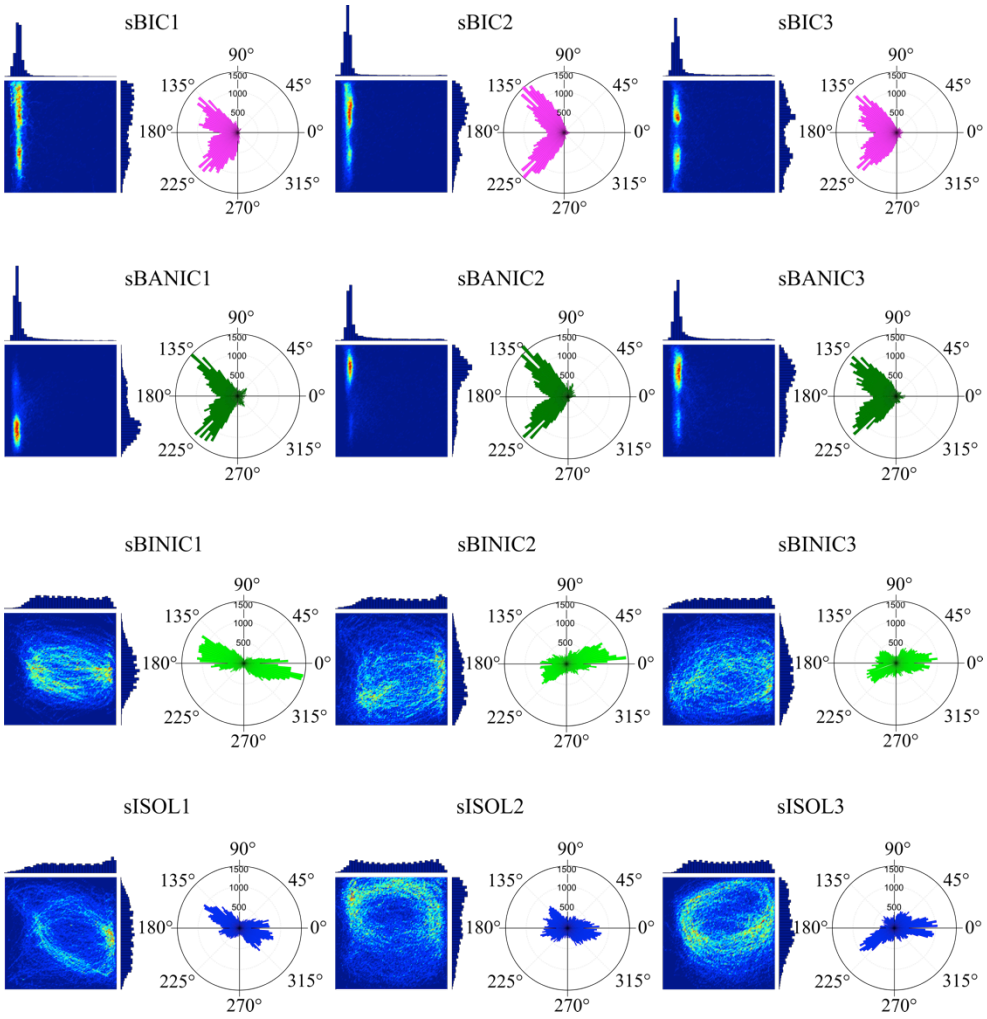

Figure 3.2 | Spatial and directional distribution patterns of the selected fish. 2D heatmaps and individual linear histograms of the time spent in each

position of the arena (left), and polar directional histograms (right) of all fish selected for transcriptomic analysis. Heatmaps are scaled from maximum relative value (red) to minimum relative value (dark blue). Linear and polar histograms represented in arbitrary scale.

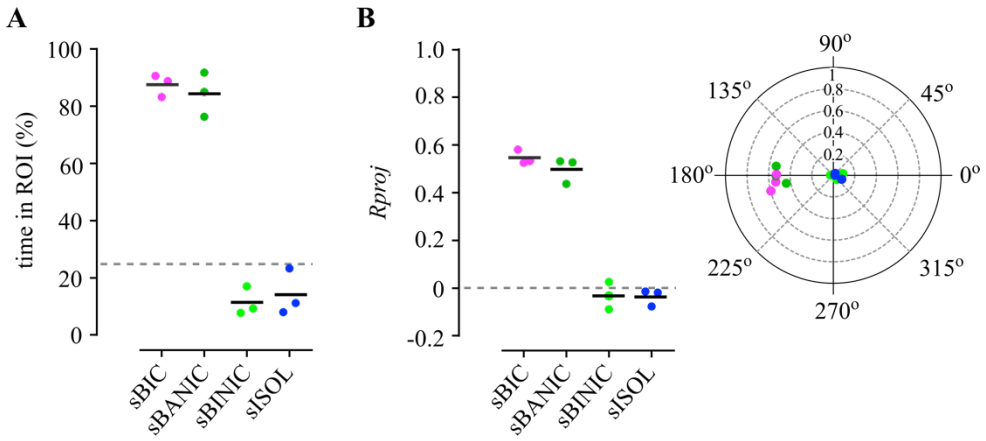

Figure 3.3 | Time in the ROI and directional focus of the selected fish. **(A)** Scatter plot of the time spent in the ROI. sBIC (magenta) — selected bystanders attentive to fighting conspecifics; sBNIC (green) — selected bystanders attentive to non-interacting conspecifics; sBINIC (lime) — selected bystanders inattentive to non-interacting conspecifics; sISOL (blue) — selected socially isolated fish. **(B)** Left — scatter plot of the individual (coloured dots) resultant vectors' lengths *R* projected (*Rproj*) onto the stimulus direction (180˚). Right — polar scatter plot of the selected fishes' individual mean resultant vectors angles α (0˚ to 360˚) combined with the corresponding vectors' lengths *R* (0 to 1), for each treatment. Dashed grey line represents in (A) the value expected from a random distribution in the arena (25%) and in (B) no directionality (*Rproj* = 0). Black lines represent mean values.

Changes in gene expression in the brain of the selected bystander fish. Comparing the whole-brain transcriptome of the reference group sISOL with the other selected behavioural groups,

revealed that four differentially expressed genes were exclusively associated to bystanders attentive to the fighting conspecifics (sBIC), five were exclusively associated to bystanders attentive to non-interacting conspecifics (sBANIC), and four differentially expressed genes were associated to both. Only one differentially expressed gene was associated to bystanders inattentive to non-interacting conspecifics (sBINIC), and two were shared by fish attentive and inattentive to non-interacting conspecifics (Figure 3.4 and Table 3.1).

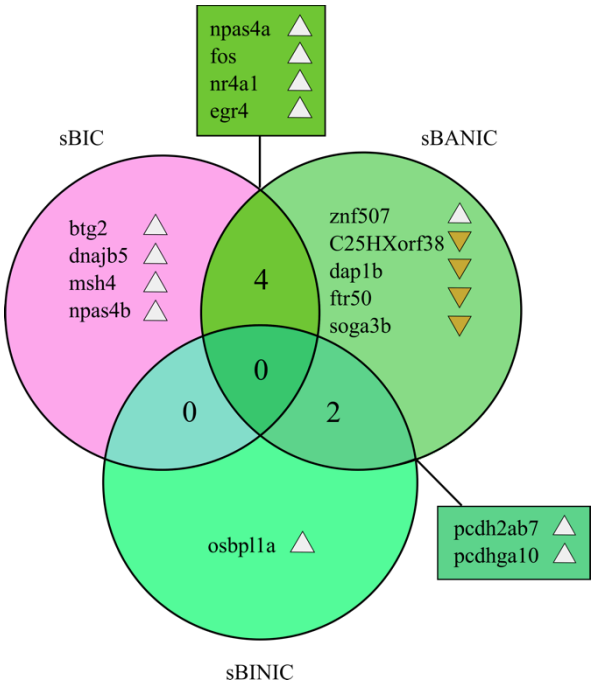

Figure 3.4 | Changes in gene expression in the brain of the selected bystander fish. Venn diagram showing the differentially expressed genes between behavioural groups sBIC (magenta), sBANIC (green) and sBINIC (lime) compared to the reference group sISOL. Listed in circles — genes exclusive to a group; listed in

squares — genes shared between groups. Up-regulated — upward triangle; down-regulated — downward triangle. Numbers of shared genes indicated at intersections.

Table 3.1 | List of differentially expressed genes

Name	FC[a]	FDR	Entrez ID	Gene Symbol	Description
sBIC					
13015447	2.24	0.001	724016	npas4a	neuronal PAS domain protein 4a
13047782	1.52	0.002	559917	msh4	mutS homolog 4 (E. coli)
13143256	1.57	0.003	795099	EGR4 (2 of 2)[a]	early growth response 4
13105945	1.96	0.003	394198	fos	v-fos FBJ murine osteosarc. viral oncogene homolog
13110394	1.41	0.003	100534657	npas4b	neuronal PAS domain protein 4
13141648	1.60	0.010	431720	nr4a1	nuclear receptor subfamily 4, group A, member 1
13124986	1.28	0.013	30079	btg2	B-cell translocation gene 2
13107726	1.78	0.016	641576	DNAJB5 (2 of 2)[b]	DnaJ (Hsp40) homolog, subfamily B, member 5-like
sBANIC					
13007436	2.56	0.000	493593	pcdh2ab7	protocadherin 2 alpha b 7
12959481	2.82	0.000	572221	ZNF507 (2 of 5)[b]	Zinc finger protein 507
13172083	-3.94	0.000	100137114	ftr50	finTRIM family, member 50
13007420	1.61	0.001	100535907	pcdhga10	protocadherin gamma-A10-like
13136272	-1.60	0.004	563485	soga3b	SOGA family member 3b
13143256	1.15	0.006	795099	EGR4 (2 of 2)[b]	early growth response 4

13015447	1.81	0.012	724016	npas4a	neuronal PAS domain protein 4a
13162324	-1.39	0.012	777611	C25HXorf 38 (1 of 2)[b]	chromosome X open reading frame 38
13105945	1.57	0.012	394198	fos	v-fos FBJ murine osteosarc. viral oncogene homolog
13141648	1.06	0.018	431720	nr4a1	nuclear receptor subfamily 4, group A, member 1
13263259	-1.04	0.031	58094	dap1b	death associated protein 1b
sBINIC					
13007436	2.55	0.000	493593	pcdh2ab7	protocadherin 2 alpha b 7
13078177	2.44	0.000	100331149	OSBPL1A (2 of 2)[b]	oxysterol-binding protein-related protein 1-like
13007420	1.38	0.019	100535907	pcdhga10	protocadherin gamma-A10-like

FC — fold change; FDR — false discovery rate; [a] $\log_2$ fold-change, negative is under-expressed, positive is over-expressed; [b] gene symbol from Ensembl; FC > $\log_2(1.1)$ and FDR < 0.05. The gene list is sorted by FDR.

All differentially expressed genes associated to both sBIC and sBANIC (*egr4*, *fos*, *npas4a* and *nr4a1*) were neuronal activity-dependent immediate early genes (IEGs) with a role in neural plasticity and brain activity. The differentially expressed genes associated only to sBIC also included neuronal activity-dependent immediate early genes associated to neuronal plasticity (*btg2* and *npas4b*; Farioli-Vecchioli et al. 2008; Ramamoorthi et al. 2011) and the late gene *dnajb5*, which has been identified in stress regulation and the circadian neuronal circuit of Drosophila (Nagoshi et al. 2010). Both differentially expressed genes associated to sBANIC and sBINIC (*pcdh2ab7* and *pcdhga10*) code for protocadherin proteins, which have been proposed to have a role in self-

recognition of individual neurons (Chen & Maniatis 2013). The differentially expressed genes *znf507* and *soga3b* associated only to sBANIC do not have a clear link to neuronal functions, however *znf507* has been implicated in human neurodevelopment disorders and *soga3b* may be related to neurogenesis (Fukushima et al. 2011; Hartl et al. 2008). The differentially expressed gene *osbpl1a*, unique to group sBINIC, also does not have a clear neural function but there has been some evidence of differential expression related to brain sterol biosynthesis (Laitinen et al. 1999). See Table 3.2 for a summary of gene functions and references.

Table 3.2 | Summary of the functions of at least one differentially expressed gene or one enriched transcription factor

Function	DE genes or enriched TF motifs[a]
cell-cell communication	*pcdh2ab7* and *pcdhga10* (Chen & Maniatis 2013)
cholesterol biosynthesis	*osbpl1a* (Laitinen et al. 1999)
circadian neuronal circuit	*dnajb5* (Nagoshi et al. 2010); *fos* (Terao et al. 2003) and JUN::FOS (Basheer & Shiromani 2001)
development of nervous system	CDX2 (Zhao et al. 2014); GATA2 (Kala et al. 2009); HNF1B (Choe et al. 2008); PDX1 (Schwartz et al. 2000); and TAL1 (Muroyama et al. 2005)
memory formation	*btg2* (Farioli-Vecchioli et al. 2008); *egr4* (Li et al. 2005); *fos* (Strekalova et al. 2003); JUN (Zearfoss et al. 2008); MEF2A (Cole et al. 2012); *npas4* (Ramamoorthi et al. 2011); SRF (Etkin et al. 2006)

neuronal cells effect	E2F1(Wang et al. 2007); JUN::FOS (Yang et al. 2008); MYC (Lee et al. 2009); and REST (Huang et al. 1999)
response to cellular stress	*dnajb5* (Nagoshi et al. 2010); JUN (Greer et al. 2011); JUN::FOS (Hess et al. 2004); MEF2A (Zhao et al. 1999); MYC (Popov et al. 2007)
sensorial system	FOXQ1 (Potter et al. 2006); RFX2 (McClintock et al. 2008)

DE — differentially expressed; TF — transcription factor; [a] DE genes are represented in italicized small caps; TF motifs are represented in all caps.

Hierarchical clustering of the samples indicated that the selected behavioural groups are well defined, although to a lesser extent between groups sBIC and sBANIC (Figure 3.5). Consistent with the behavioural profiles (Figure 3.2 and Figure 3.3), the gene expression profile of sBINIC was closer to sISOL than to the remaining groups, and results indicate a mixture between sBANIC and sBIC. Hierarchical clustering of the genes also generated a well-defined subset of genes (*btg2*, *dnajb5*, *egr4*, *fos*, *msh4*, *npas4a*, *npas4b* and *nr4a1*) with a similar profile of expression across all 12 selected fish (see Figure 3.5).

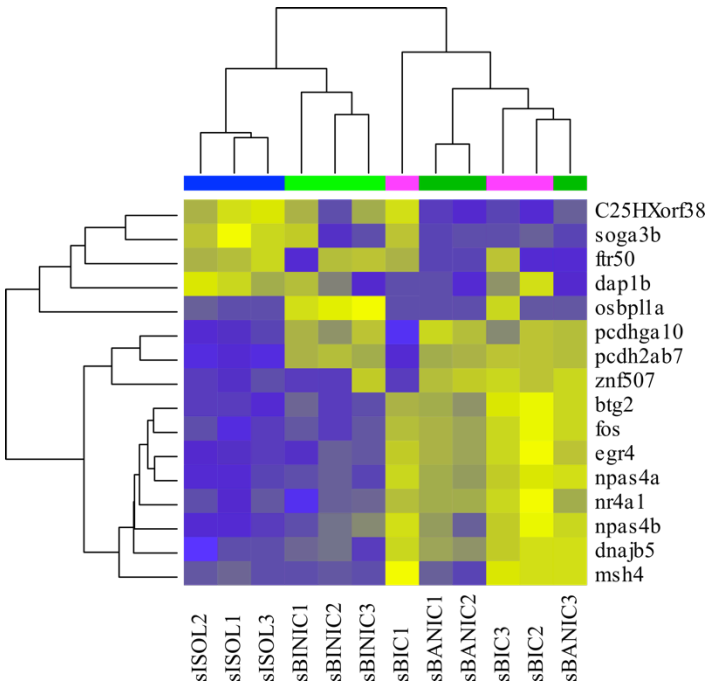

Figure 3.5 | Hierarchical clustering of the selected bystander fish's differentially expressed genes. Heatmap of the selected fish from each behavioural group sBIC, sBANIC, sBINIC (columns) and differentially expressed genes obtained (lines). Normalized gene expression levels are represented. Blue corresponds to low expression, yellow to high expression.

Results for the over-representation analysis (ORA) should be interpreted with caution since they are based on a limited number of differentially expressed genes. Nevertheless, we note that two differentially expressed genes of sBIC and sBANIC (*fos* and *nr4a1*) were members of the "MAPK signalling pathway"; three differentially expressed genes of sBIC and sBANIC (*fos*, *nr4a1* and *npas4a*), and one unique to sBIC (*dnajb5*) had a metabolic and/or biosynthetic role; three differentially expressed genes of sBIC and sBANIC (*fos*, *nr4a1* and

npas4a) were located in the nucleus; four differentially expressed genes of sBIC and sBANIC (*egr4*, *fos*, *nr4a1* and *npas4a*), one differentially expressed gene of sBIC (*dnajb5*) and two unique to sBANIC (*pcdh2ab7* and *ftr50*) had a binding function; and finally, there was an over-enrichment of differentially expressed genes located in chromosome 23 of sBIC (*egr4* and *nr4a1*) and sBANIC (*egr4*, *nr4a1* and *soga3b*), and in chromosome 14 of sBANIC (*pcdh2ab7*, *pcdhga10* and *npas4a*) and sBINIC (*pcdh2ab7*, *pcdhga10*).

Contrary to ORA, gene set enrichment GAGE analyses are not limited by a cut-off that defines strongly differentially expressed genes, arguably making them more robust. Overall, GAGE results showed that sBIC and sBANIC had distinct profiles of differentially expressed gene sets. Pathways enriched in sBIC included 'Phototransduction', 'Exercise-induced circadian regulation', and 'Cholesterol/Steroid biosynthesis', which may be related to cortisol production and various growth-related pathways, while sBANIC and sBINIC were enriched by metabolism-based pathways and each by one pathway shared with sBIC ('FGF signalling pathway' in sBANIC and 'Cholesterol/Steroid Biosynthesis' in sBINIC). Unsurprisingly, GO analyses using biological process terms showed that all behaviour groups are enriched in transcription-related terms. However, sBIC was also enriched in the generic term 'response to stress' and in the term 'lipid metabolic process', which may be related to hormone production, whereas sBANIC was enriched in the neurogenesis-related term "notch signalling pathway" and in terms related to visual and audio sensory systems. sBINIC was also enriched in genes linked to sensory organs

development. Regarding GO terms of cellular compartments, sBIC was enriched in terms related to cell-cell communication, while sBANIC and sBINIC were enriched in the term 'peroxisome', which is related to metabolism and possibly to cholesterol biosynthesis. As for the analyses using GO terms of molecular functions, all behavioural groups were enriched in transcription-related terms. However, sBIC was also enriched in growth-related terms, the fight-or-flight term 'adrenergic receptor activity', the metabolism-related term 'cytochrome-c oxidase activity', and in terms related to cell-cell signalling; whereas sBANIC was further enriched in the term related to cell-cell signalling 'voltage-gated potassium channel activity' and in the term 'photoreceptor activity'. Finally, regarding chromosome location, we observed that genes from chromosome 14 were enriched in all behavioural groups (see Abril-de Abreu et. al. 2015c for further details).

Promoter regions and transcription networks. Promoter analyses identify transcription factors (TF) binding sites (motifs) associated to up or down-regulated genes. Since multiple transcription factors may have nearly identical motifs, the statistical findings are related to the motif itself and not to the transcription factor where it came from. Nevertheless, for simplicity, we used the transcription factor nomenclature to name the motifs. Seventeen TF motifs were enriched in at least one of the behavioural groups (Figure 3.6A; see Table 3.2 for a summary of their functions and respective references).

A

motif	*p*	FDR	sBIC	sBANIC	sBINIC
JUN::FOS	0.00002	0.00203			
MEF2A	0.00003	0.00203			
JUN	0.00003	0.00203			
CDX2	0.00046	0.02337			
NKX3.1	0.00105	0.04256			
GATA2	0.00131	0.04315			
FOXQ1	0.00180	0.04315			
E2F1	0.00180	0.04315			
SRF	0.00191	0.04315			
REST	0.00264	0.05355			
NKX3.2	0.00349	0.05459			
TAL1::GATA1	0.00362	0.05459			
PDX1	0.00366	0.05459			
RFX2	0.00376	0.05459			
HNF1B	0.00414	0.05605			
MYC	0.00580	0.07364			
FLI1	0.00835	0.09967			

B

motif1 & motif2	*p*	FDR	sBIC	sBANIC	sBINIC
GATA2 & ELK4	0.00001	0.00091			
GATA2 & FOXI1	0.00001	0.00091			
GATA2 & NR2F1	0.00002	0.00091			
GATA2 & GFI1B	0.00003	0.00103			
GATA2 & RUNX2	0.00004	0.00114			
GATA2 & T	0.00004	0.00124			
GATA2 & ELF1	0.00004	0.00129			
GATA2 & NOBOX	0.00005	0.00145			
GATA2 & REST	0.00005	0.00153			
GATA2 & SOX6	0.00005	0.00158			
GATA2 & SOX2	0.00006	0.00161			
GATA2 & TCF7L2	0.00006	0.00170			
GATA2 & CEBPA	0.00006	0.00170			
GATA2 & RORA_2	0.00007	0.00189			
GATA2 & TCF3	0.00007	0.00189			
GATA2 & SRY	0.00007	0.00198			
GATA2 & ARNT::AHR	0.00008	0.00209			
GATA2 & EN1	0.00008	0.00227			
GATA2 & STAT1	0.00009	0.00231			
GATA2 & POU5F1::SOX2	0.00009	0.00235			
GATA2 & REL	0.00010	0.00261			
GATA2 & ARID3A	0.00011	0.00283			
GATA2 & ZBTB33	0.00011	0.00290			
GATA2 & SP1	0.00012	0.00304			

C

motif1 & motif2	*p*	FDR	sBIC	sBANIC
TAL1::GATA1 & TAL1::TCF3	0.00015	0.00355		
TAL1::GATA1 & CEBPA	0.00026	0.00503		
TAL1::GATA1 & NFIC	0.00029	0.00540		
TAL1::GATA1 & ARNT::AHR	0.00029	0.00540		
TAL1::GATA1 & NOBOX	0.00039	0.00582		
TAL1::GATA1 & TBP	0.00048	0.00629		

Figure 3.6 | Transcription factor motifs enriched in differentially expressed genes for the selected behavioural groups. (A) Single motifs enriched in at least one behavioural group. **(B)** Pairs of motifs involving GATA2 enriched in sBIC and/or sBANIC. **(C)** Pairs of motifs involving TAL1::GATA1 enriched in sBIC and/or sBANIC. Associations found in each behavioural group can be strongest with up-regulated (orange) or down-regulated (purple) genes. Grey cells indicate no significance of associations to any group of differentially expressed genes. Significance was calculated using uncorrected (*p*) and corrected (FDR) *p*-values.

Focusing on the dissimilarities between sBIC (selected bystanders attentive to fighting conspecifics) and sBANIC (selected bystanders attentive to non-interacting conspecifics), we observed that only two of the transcription factors (NKX3.1, NKX3.2) were associated to differentially expressed genes in different directions (up- or down-regulated). GATA2 and TAL1::GATA1 were not associated to either up- or down-regulated genes in sBANIC. However, when considering associations between pairs of motifs (Figure 3.6B,C) two transcription factors were also associated to genes differentially expressed in different directions, when comparing sBIC and sBANIC.

Protein networks were constructed using STRING, which uses data mining to establish connections between proteins. As such, the establishment of these connections is directly related to information availability, and lack of connections between nodes can result from research biases towards more relevant pathways or any other factor that constrains data collection. Thus, the interpretation of the results should be taken with caution. The networks of sBIC and sBANIC built using differentially expressed genes and enriched transcription factors

(Figure 3.7A,B) had the same number of nodes, but sBIC's was composed by more edges (sBIC: 13 nodes and 18 edges; sBANIC: 13 nodes and 15 edges), hence having higher density (sBIC = 0.18; sBANIC = 0.16) and lower average path than sBANIC's (sBIC = 2.00; sBANIC = 2.20). The network of sBINIC (selected bystanders inattentive to non-interacting conspecifics) was composed only by 5 nodes and 2 edges (Figure 3.7C) and was excluded from the remaining network analyses. Networks of sBIC and sBANIC had very similar topologies (structural correlation coefficient = 1.00). Reassuringly, in both networks the differentially expressed up-regulated genes interacted mostly with each other and with transcription factors enriched in them, whereas differentially expressed down-regulated genes seemed to be positioned in proximity with each other and with transcription factors enriched in them (network assortativity of 0.27 and 0.17 for sBIC and sBANIC, respectively). In both networks, the gene *fos* seemed to have a central position with many connections to various genes (eigenvector centrality of 0.54 and 0.53 for sBIC and sBANIC, respectively). The gene *jun* (sBIC = 0.50; sBANIC = 0.52) also had high values of eigenvector centrality.

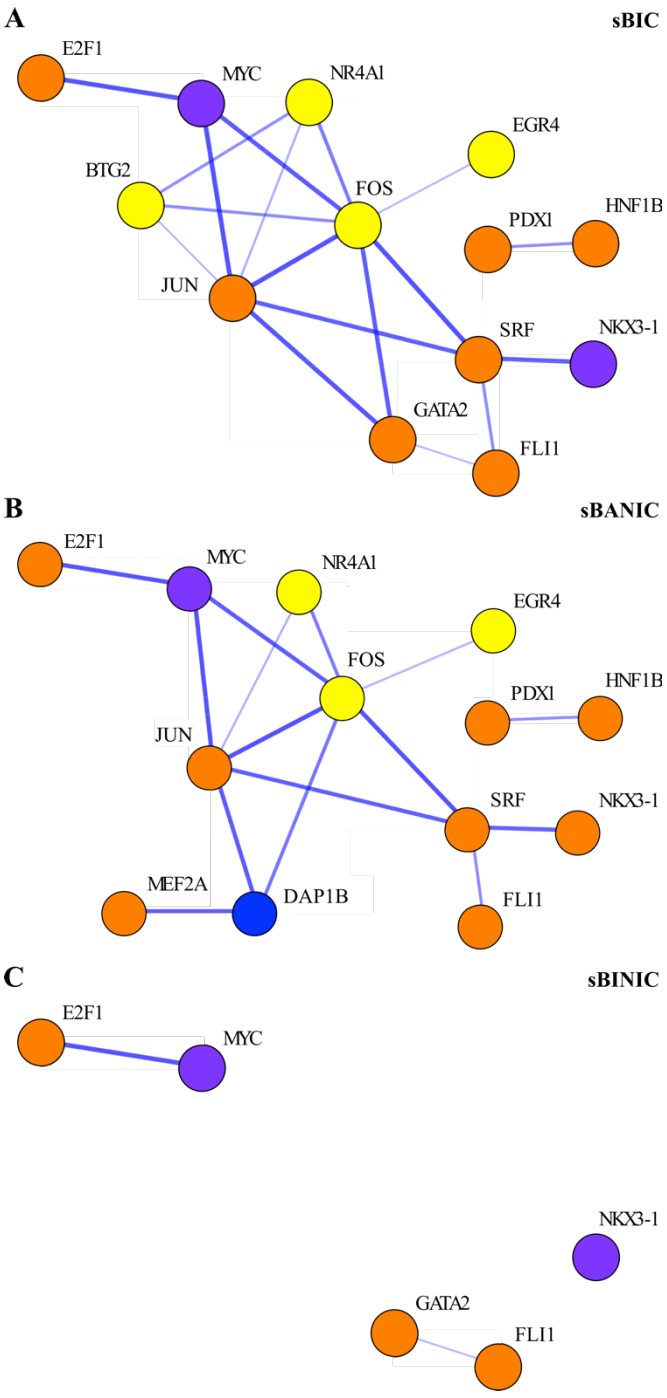

Figure 3.7 | Transcription networks of the selected behavioural groups. Networks consisting of differentially expressed genes and enriched transcription

factors for the behavioural groups: **(A)** sBIC — selected bystanders attentive to fighting conspecifics; **(B)** sBANIC — selected bystanders attentive to non-interacting conspecifics; **(C)** sBINIC — selected bystanders inattentive to non-interacting conspecifics. The thickness of the edges corresponds to the confidence score of the gene association; yellow nodes indicate up-regulated differentially expressed genes; blue nodes indicate down-regulated differentially expressed genes; orange nodes indicate transcription factors motifs mainly associated with up-regulated differentially expressed genes; and purple indicate transcription factors motifs mainly associated with down-regulated differentially expressed genes.

3.4 Chapter discussion

In the previous chapter we developed a paradigm to investigate attention to social interactions (see section 2.3) by defining an experimental task consisting of three treatments: bystander to interacting (fighting) conspecifics, bystander to non-interacting conspecifics, and socially isolated. We found that bystander zebrafish were more attentive towards fighting conspecifics, suggesting that agonistic interactions may be providing relevant social information for potential eavesdroppers. Here we followed up on these results, defining four different behavioural profiles within the tested treatments. We selected a group of bystanders attentive to fighting conspecifics (sBIC), attentive to non-interacting conspecifics (sBANIC), inattentive to non-interacting conspecifics (sBINIC) and a group of socially isolated inattentive fish (sISOL) as reference.

Transcriptomic analysis of the selected individuals from these four behavioural groups revealed differences between the socially isolated group and the remaining ones. In particular, gene expression analyses

showed that sBIC, sBANIC and sBINIC had eight, eleven and three differentially expressed genes relative to sISOL, respectively. Gene set enrichment analyses, using whole genome expression data, also showed the existence of gene sets significantly differentially expressed in all groups. These results indicate that all behaviour profiles, even when bystanders did not show attentiveness towards the stimulus, led to transcriptomic responses in the brain that differed from the isolated individuals, although in different ways. Moreover, both the behavioural results and analyses of differentially expressed genes suggest that inattentive individuals to non-interacting conspecifics had a behavioural profile and a neurogenomic state closer to the socially isolated individuals, whereas the two groups of attentive individuals, both to fighting and non-interacting conspecifics, had similar behavioural profiles and similar neurogenomic states.

Hierarchical clustering of the differentially expressed genes of all behaviour groups pooled together, showed a well-defined group of eight genes (*btg2*, *dnajb5*, *egr4*, *fos*, *msh4*, *npas4a*, *npas4b* and *nr4a1*) with a similar expression profile across the twelve analysed fish. Notably, this gene group composed all differentially expressed genes found in sBIC, suggesting that their effects may be interconnected. The interaction between four of those genes (*btg2*, *egr4*, *fos*, and *nr4a1*) was confirmed by the protein network analysis results. Together these results support the notion of a change in the neurogenomic network after exposure to conspecifics, in which some of the key players are the differentially expressed genes of sBIC.

Another noteworthy result, from the gene set enrichment analyses, was that the group of genes located in chromosome 14 was differentially expressed in all the behavioural groups. This chromosome has been previously linked to the brain transcriptome of subordinate zebrafish (Oliveira et al., submitted).

Comparing attentive bystanders to fighting conspecifics and to non-interacting conspecifics. Interestingly, *egr4*, *fos*, *npas4*, *nr4a1* found to be differentially expressed in both bystanders attentive to fighting conspecifics (sBIC) and to non-interacting conspecifics (sBANIC), together with *btg2* (only in sBIC), have also been found to be differentially expressed in a previous study in which the brain transcriptome of zebrafish was examined 30 minutes after participating in hierarchy-defining fights (Oliveira et al., submitted). In this previous study, the number of differentially expressed genes was 168 compared to the 16 from the current experiment. This was expected since interacting with conspecifics should lead to more neurogenomic changes than just observing conspecifics. The genes *egr4*, *btg2, fos*, *npas4a*, *nr4a1* are all neuronal activity-dependent immediate early genes, which could indicate that their activation merely reflects task-related brain activity. However, they are also known to have a role in neuronal plasticity (Li et al. 2005), contextual and fear memory formation (Ramamoorthi et al. 2011; Ploski et al. 2011; Strekalova et al. 2003; Hawk & Abel 2011), hence suggesting that neurogenomic changes observed in attentive bystanders are part of the changes observed in

individuals actively participating in a social interaction. These changes are likely to be related to acquisition of social information.

Nevertheless, we also found important differences between sBIC and sBANIC, which we may speculate to be associated with the acquisition of eavesdropped information by sBIC individuals. In this sense the differentially expressed genes found uniquely in sBIC may be associated with eavesdropping processes. From these, *btg2* has been shown to have a role in neuronal plasticity, contextual and fear memory formation (Farioli-Vecchioli et al. 2008). Interestingly, gene set enrichment analyses also showed a differential expression in sBIC but not in sBANIC in both the GO term "response to stress" and the Wikipathway term "exercise-induced circadian regulation". The other gene set that was uniquely differentially expressed in sBIC was the GO "adrenergic receptor activity", which is related to "fight-or-flight" response (see Abril-de Abreu et. al. 2015c for further details).

The analysis of transcription factors showed again strong similarities between sBIC and sBANIC. However, this analysis also presented important differences between the two behavioural groups: four transcription factors motifs from proteins NKX3.1, NKX3.2, GATA2 and complex TAL1::GATA1, were over-represented in genes that were differentially expressed in opposite directions in sBIC and sBANIC. These results were not unexpected since NKX3 proteins can act either as repressors or activators (Wang et al. 2009; Possner et al. 2008; Tribioli & Lufkin 1999) and have been shown to be expressed in the brain (Tanaka et al. 1999). Additionally, all of the 17 transcription factors obtained have been associated to neuronal functions or shown to

be expressed in the brain. GATA2 and TAL1 have particularly important roles in neuronal differentiation (Kala et al. 2009; Muroyama et al. 2005). Finally, the network analyses have shown that although sBIC and sBANIC networks are similar, with proteins FOS and JUN being important players, sBIC is composed by more edges and have higher density.

Together these results suggest that the neurogenomic responses in bystanders attentive to fighting conspecifics and in bystanders attentive to non-interactive conspecifics share considerable similarities, which may reflect attentional processes, but also that as a whole they possess distinct neurogenomic profiles, which may be related to the eavesdropping of social information. Pathways related to stress and flight-or-fight response and epigenetic mechanisms provided by transcriptions factors that function both as repressors and activators, for example the NKX3 proteins, are good candidates to further explore these differences.

Comparing inattentive bystanders and socially isolated fish. Regarding the inattentive bystanders to non-interacting conspecifics (sBINIC), although their behavioural profiles seem to be very close to isolated individuals, extensive transcriptomic analyses revealed important differences. Although sBINIC had only three differentially expressed genes in relation to sISOL, these genes have known important neuronal functions. Protocadherin alfa genes *pcdh2ab7* and *pcdhga10* have a role in self-recognition by individual neurons (Chen & Maniatis 2013), being important in establishing neuronal connections in

the brain (Wu & Maniatis 1999), and *osbpl1a* has been shown to be expressed at considerable high levels in cortical areas of the human brain (Laitinen et al. 1999) and to regulate cellular cholesterol metabolism in vitro (Marquer et al. 2014). Moreover, gene set enrichment analyses showed differentially expressed gene sets in areas similar to sBANIC and to sBIC, namely, cholesterol biosynthesis, metabolism, transcription and sensory organs (see Abril-de Abreu et. al. 2015c for further details). These results suggest that the mere presence of conspecifics also affects bystanders, irrespective of them being attentive or not.

Overall, we showed that the transcriptomic changes in the behavioural profiles could be divided into several areas. 'Cholesterol biosynthesis', 'Metabolism', 'Transcription' and 'Visual and audio sensory organs', are characteristic of all the behavioural groups and seem to be linked to a bystander response to the presence of conspecifics, irrespective of attentiveness. 'Cell-cell communication' and 'cell growth' are mostly characteristic of both attentive groups and we hypothesize that they may be related to neuronal plasticity and memory formation, and to the acquisition of information from conspecifics. The gene network underlying this process seems to have *fos* and *jun* as key players, while *npas4a*, *nr4a1* and *egr4* may also have an important role. "Fight-or-flight", generic "stress" responses and 'exercise-induced circadian regulation' are pathways that seem to be particularly important in attentive states to fighting interactions. The genes *btg2*, *npas4b*, *dnajb5* and *msh4* seem to be particularly important in defining this behavioural profile.

The obtained results suggest that transcriptome comparison of specific behavioural phenotypes related to conspecifics' observation tasks, can potentially allow the identification of genetic mechanisms associated with social attention processes. Both in general, as indicated by gene expression similarities between bystanders attentive to fighting conspecifics and to non-interacting conspecifics; and in particular, with the identification of genetic mechanisms associated with attention to social interactions, as indicated by the gene expression patterns exclusive to bystanders attentive to fighting conspecifics. However, further studies on the mechanisms behind these transcriptomic changes are needed. The networks drafted should be a good place to start understanding in more detail the pathways triggered by these responses. Additionally, more refined behavioural tasks need to be developed in order to better detect, understand and manipulate the acquisition and use of social eavesdropped information. With this goal in mind, in the next chapter we present a study designed to test social eavesdropping on agonistic interactions in zebrafish.

Chapter 4
Social dominance modulates eavesdropping in zebrafish

4.1 Chapter summary

In this last experimental chapter, we present a paradigm aimed at demonstrating social eavesdropping on signalling agonistic interactions in zebrafish. Moreover, we investigate its integration with private social information obtained from past social experience, specifically the eavesdroppers' own dominance status. In this study we expanded our initial focus on the bystanders' behaviour when observing the fight interactions to include analysis of their behaviour before and after observing the fights.

- We first manipulated the dominance status of bystander zebrafish by having them win or lose a fight as their latest social experience.
- Next, we either allowed or prevented bystanders from observing a fight and posteriorly assessed their behaviour towards the winners and losers of the interaction.
- We found that only dominant bystanders who had seen the fight, revealed a significant increase in directional focus (a measure of attention) towards the losers of the fights.
- Furthermore, our results indicated that information about the fighters' acquired status was collected from the signalling interaction itself and not from post-interaction status cues, which implies the existence of individual recognition in zebrafish.
- Additionally, preliminary behavioural profiling suggests that the behaviour of attentive dominant bystanders (towards the winners

and losers of the fights) was characterized by cyclic periods of sustained maximum directional focus and near immobility, alternated with higher speed circular paths around the test tank.

- Overall, we show for the first time that zebrafish, a highly social model organism, eavesdrops on conspecific agonistic interactions for subsequent use of this information and that this process is modulated by the eavesdroppers' dominance status.

4.2 Introduction

As previously discussed in chapter 1, the use of agonistic interactions for the study of social eavesdropping provides several advantages, since they are relevant for the establishment of dominance hierarchies that regulate the access to resources such as reproduction sites, mates or food. Furthermore, agonistic interactions are a salient social event, easy to manipulate experimentally and where the emergence of winners and losers provides an honest signal of competitive ability. This gives eavesdroppers the opportunity to assess the relative fighting ability of potential rivals, without directly engaging in a fight themselves (Earley 2010). Moreover, one might expect that integration of eavesdropped information with information gathered by direct past experience with others, will enable a better adaptive response to the social environment. However, little is known about this interplay between public and private social information (e.g. Lai et al. 2014).

The work presented in chapter 2 showed that zebrafish are tuned to be attentive to conspecific fighting interactions and are attracted by specific form or movement features present in those interactions, Also, previous work showed that zebrafish exhibit behavioural flexibility dependent on past social experience, as shown by the existence of winner and loser effects (Oliveira et al. 2011). Based on these results, we developed an eavesdropping paradigm, using the established proxy attentional measures of directionality and proximity towards the stimulus. We tested if bystander zebrafish, who themselves had won or lost a fight as their latest social experience, would visually extract and

differentially use information about the winners and losers of observed fighting interactions.

4.3 A Social eavesdropping experiment

Methods

Animals and housing. Wild-type (AB) zebrafish (*Danio rerio*), 9 to 12 months old, bred at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used. Fish were kept in mixed sex shoals of 30 individuals in environmentally enriched (gravel substrate, artificial plants and rocks) stock tanks with 50 × 25 × 30 cm (30 l) at 25 ^{o}C, under a 12L:12D photoperiod. Water was filtered and monitored for nitrites (< 0.2 ppm), nitrates (< 50 ppm) and ammonia (0.01 – 0.1 ppm). Fish were fed twice a day with commercial food flakes in the morning and with freshly hatched *Artemia salina* twice in the afternoon, except on the day of the experiment. No fish was injured as result of the expression of agonistic behaviours. Used animals were returned to stock tanks and re used in other pilot studies. All procedures were reviewed by the Instituto Gulbenkian de Ciência Ethics Committee and approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária permit 008955).

Status manipulation setup. The behavioural setup (Figure 4.1) consisted of two fight tanks (15 × 15 × 17 cm), with a 9 cm water depth, placed inside a bigger tank (50 × 25 × 30 cm) containing a mixed sex shoal of 30 individuals (to act as an audience). Each fight tank was

divided in half by an opaque removable partition. When lowered, the partition prevented visual and physical contact between two isolated fish but allowed chemical communication. When lifted, the fish could interact and fight. The audience allowed the fighting fish to assess their dominance status in a shoal-like context, similar to their 'natural' stock tank environment, while also reducing their stress levels prior to the interaction. A camera placed in front of the setup, video recorded all fights.

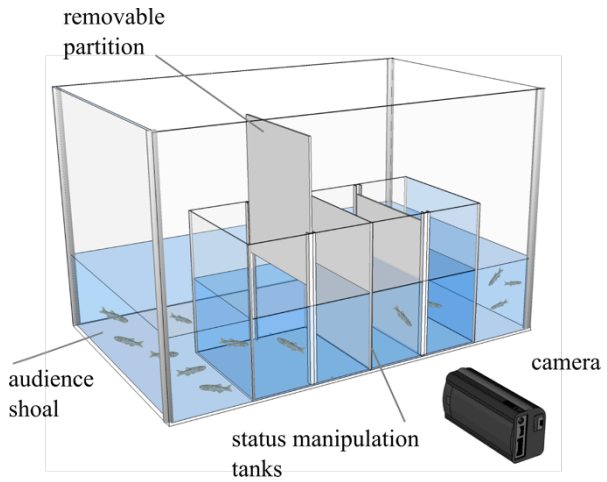

Figure 4.1 | Status manipulation setup. 3D schematic of the experimental setup.

Eavesdropping setup. The main behavioural setup (Figure 4.2) was a modified version of the experimental setup developed in chapter 2 (see Methods in section 2.3). A test tank (13 × 13 × 17 cm) was placed facing a demonstrator tank (30 × 15 × 17 cm), with a one-way mirror in-between. This allowed a bystander focal fish placed in the test tank to

see a demonstrator fish pair without itself being seen. It also prevented interactions between demonstrators and bystanders. Both tanks were filled up to a 9 cm water height. No chemical communication was possible as the tanks were self-contained. A LED light was placed over the demonstrator tank to create differential lighting required for the mirror effect. To further enhance this effect and also avoid interference of external visual cues the demonstrator tank had white opaque walls and the test tank had black walls (Figure 4.2). The demonstrator tank was divided in half by a transparent partition. The outer-half (buffer tank) buffered the fish from interference of spurious external cues and minimized stress from the experimenter's manipulations; the half adjacent to the test tank was further divided in two by an opaque removable partition and held the demonstrator fish. The removable partition was raised and lowered by a string-pulley system. When lowered, the partition prevented visual and physical contact between the two demonstrators but allowed chemical communication. A B&W mini CCTV camera (Henelec 300B, 420 TVL) with infrared sensitivity (IRs) was positioned above the test tank and connected to a laptop (HP Pavilion g6) to allow top-down view video recording of the focal fish. A second camera (SONY Handycam DCR-SR58E) was placed in front of the demonstrator tank (with the buffer tank in-between) and used to record the fighting interactions and post-interaction periods. The setup was placed over an infrared LED (850 nm) custom built lightbox to increase contrast between the background of the test tank and the focal fish (when video recording from above), without interfering with the fish's vision as IR light falls outside zebrafish's wavelength sensitivity

(Fleisch & Neuhauss 2006) . This optimized image quality for offline tracking of the focal fish's behaviour, using a custom made video-tracking system. The complete experimental setup comprised four adjacent replicas of the described setting, one for each experimental condition. A black curtain separated the setup from the rest of the behavioural room during the experiment.

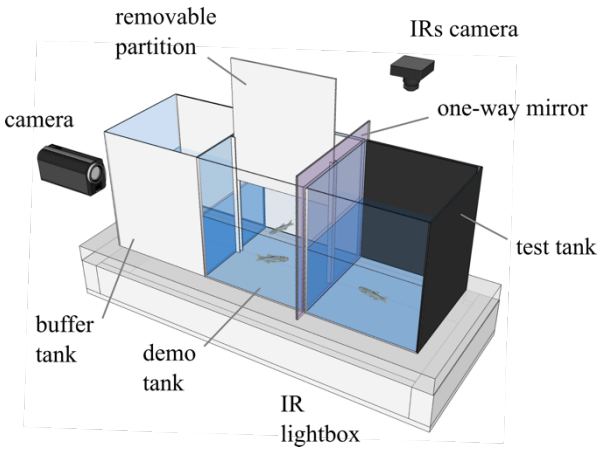

Figure 4.2 | Eavesdropping setup. 3D schematic of the experimental setup. Left wall coverings of the test and demonstrator tanks are removed for easier visualization.

Experimental procedure. On day 1 (Figure 4.3A), two pairs of unfamiliar male zebrafish matched in size were removed from their stock tanks and placed in the status manipulation setup in the two fighting tanks. Each fish from the pair was separated by an opaque partition and allowed to habituate overnight to its half of the corresponding fight tank, with full view of the audience shoal.

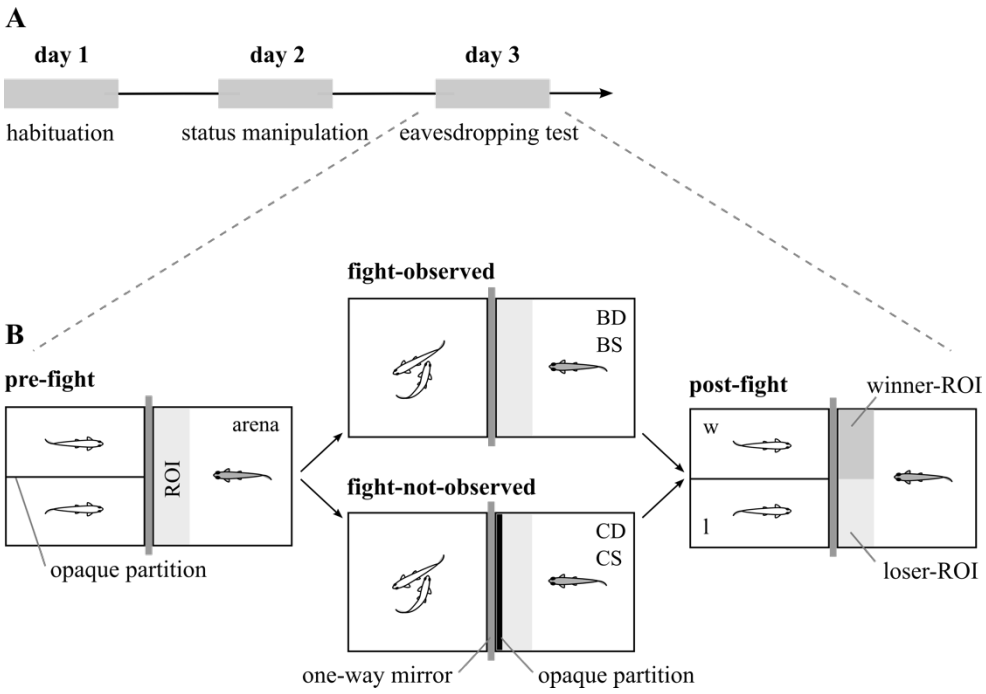

Figure 4.3 | Schematic of the experimental procedures. (A) Timeline of experimental protocol. **(B)** Schematic of eavesdropping test (day 3), composed of three 30 min stages: pre-fight, fight-observed/fight-not-observed and post-fight. Demonstrator fish represented in white and focal fish in grey, belonging to four conditions: bystander dominant (BD), bystander subordinate (BS), control dominant (CD) and control subordinate (CS). At the post-fight stage, the side of the winner (w) and loser (l) demonstrators is randomized.

On day 2 (Figure 4.3A), the opaque partitions were lifted so the fish dyads could fight while being recorded by the front camera. In these experimental conditions male zebrafish typically engage within minutes into a stereotypical structured fight for dominance, which results in a clear winner and loser of the fight (Oliveira et al. 2011). Once the fight was resolved and winners and losers emerged, they were again separated by the opaque partition. The video recordings were analysed

to identify the acquired dominance status (dominant or subordinate) of each fish. They were easily distinguishable, since the winners (labelled dominants) exhibit aggressive behaviours such as chasing, biting and striking, whereas the losers (labelled subordinates) flee and display submission and freezing postures. Two dominants and two subordinates were obtained from these two interactions to be used as focal fish. They were then individually placed in the test tanks of the eavesdropping setup and randomly assigned to bystander or control treatments. Therefore, four focal conditions were created: bystander dominant (BD), bystander subordinate (BS), control dominant (CD), control subordinate (CS). In parallel, four male pairs matched in size were removed from their stock tanks and placed in each demonstrator tank to be used as fighters, separated by an opaque partition. Each focal fish could see the corresponding demonstrator pair trough the one-way mirror to allow familiarization. All fish were left to habituate overnight.

On day 3 (Figure 4.3A,B), the eavesdropping test started with a 30 min pre fight stage (baseline), where each focal fish had full view of the separated demonstrators. It was followed by a 30 min fight-observed stage for the bystander treatment fish and a fight-not-observed stage for the control fish. Here, bystanders were allowed to observe a fight interaction between the respective demonstrator pair while controls were prevented from it by an opaque partition blocking the view. Afterwards, winners and losers were again separated by the opaque partition. The fights were video recorded with the front camera for later determination of the winner and loser's random end position in their tank (left or right), after the lowering of the partition. In the post-fight

stage, the partitions that blocked the view of the control fish were removed and all focal fish were allowed to observe for 30 min the winners and losers of the corresponding fights. During this time period no interaction occurred between the winners and losers, as they remained separated by an opaque partition. Focal fish were video recorded at all stages. On rare occasions demonstrator fish did not resolve the fight or the video recordings malfunctioned. In such cases the corresponding focal fish were discarded. One fish exhibited abnormal behaviour from the beginning in the test tank and was also discarded. A total of 71 focal fish were analysed (n = 19 for the BD condition; n = 17 for BS; n = 18 for CD; and n = 17 for CS).

Behavioural tracking and data acquisition. All focal fish were tracked at the pre-fight and post-fight stages from a top-down view, using the same custom made tracking software and methods from the experimental paradigm developed in chapter 2 (see Methods in section 2.3). For each behavioural video, a 2D region (arena) was defined for tracking (see Methods, Figure 2.3A in section 2.3). Each fish was video recorded and tracked at a 25 fps rate, which allowed determination of the position and orientation of the fish every 1/25 s.

Behavioural Analysis. All tracked data files were imported to MATLAB (MathWorks) and the behavioural parameters were determined using a custom-made script developed for this experiment. Baseline (pre-fight) and fight observation stage values of *Rproj*, time spent in a ROI closest to the demonstrator tank and speed (measure of

motor activity) were determined in the total tracked area (arena) (Figure 4.3B). The ROI had 12 × 3 cm (25 % of the tank), corresponding to the width of the arena and the mean body length of an adult zebrafish. The demonstrators' latency to fight (time to first aggressive display) and fight resolution time (from first display to winner-loser decision), were determined for all dyads. Normality and homogeneity of variances was verified and one-way ANOVAs were performed to compare all conditions.

Eavesdropping effects were investigated at the post-fight stage by comparing two defined regions of interest closest to the winner (winner-ROI) and loser (loser-ROI) demonstrator's sides (Figure 4.3B). Each region had 6 × 3 cm (12 % of the tank), corresponding to the width of a side and to the mean body length of an adult zebrafish. Directional focus towards each demonstrator (*Rproj*), time spent in each region and mean orientation (α), were determined for each focal fish and condition. A focal fish was considered in the ROI when its centroid point was inside its border. *Rproj* was defined as the projection of the fish's mean resultant directional vector's length R onto the demonstrator tank's direction (180^{o}), and ranged from 1 to -1 (see Figure 2.3C, section 2.3). Positive values indicate directionality towards the stimulus direction, negative values away from it and null values no directional focus.

Trend effects from observing or not observing a fight, were analysed by comparing pre-fight with post-fight for each condition. Mixed-design ANOVAs and planned contrasts were used. Pearson correlations were performed between the latencies to fight, resolution

times and the bystander fishes' directional focus toward the losers at the post-fight stage.

Additionally, we started a preliminary behavioural profiling of the impact of observing or not observing a fight on the focal fishes' behavioural dynamics. Pearson correlations were performed at the post-fight stage for each treatment, between the variables that revealed significant trend effects (*Rproj* and mean speed in arena), using the individual fish's mean values as sample units. The temporal dynamics of the selected behavioural variables was analysed for a representative eavesdropper, throughout the 30 min test, using 1 s bins.

Behavioural parameters were represented as mean ± SEM, except mean angles represented as mean and 95% C.I. when directionality was significant. Statistical significance was considered for $p < .05$. All analyses were performed using MATLAB R2012b (MathWorks) with the CircStat toolbox (Berens 2009), STATISTICA 12 (Statsoft, Inc.), SPSS Statistics 22 (IBM), and Oriana 4 (Kovach Computing Services).

Results

Bystanders' behaviour before the fights. Baseline (pre-fight) analysis of the focal fishes' behaviour in the total arena and ROI did not reveal any differences between conditions for the behavioural parameters analysed (*Rproj*: $F_{3,67} = 0.41$, $p = .74$; time in ROI: $F_{3,67} = 0.33$, $p = .80$; speed: $F_{3,67} = 0.38$, $p = .74$; Figure 4.4).

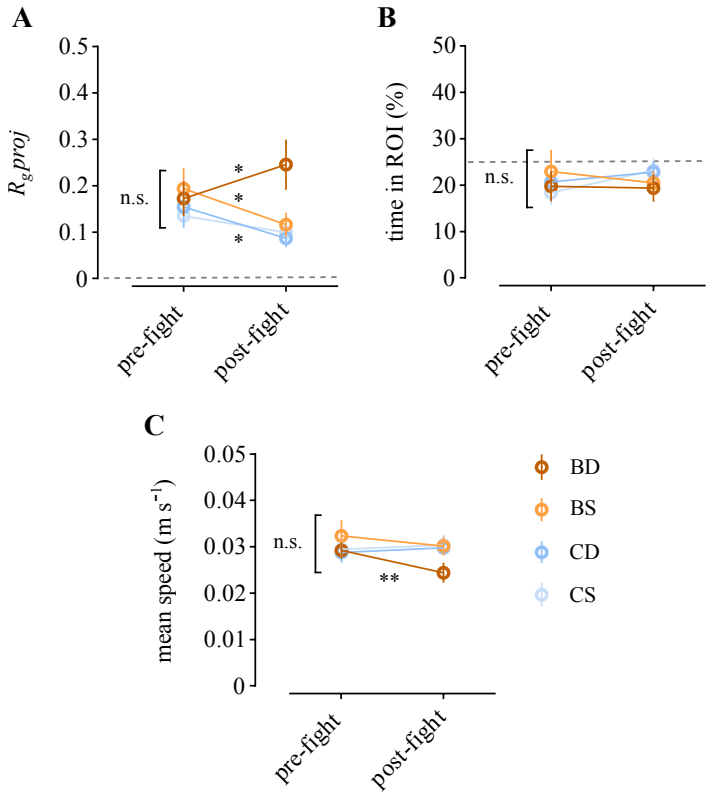

Figure 4.4 | Baseline behavioural results and trend comparisons with the post-fight stage. (A) Mean directional focus onto the stimulus direction (*Rproj*) in arena. Dashed grey line represents no directionality. **(B)** Mean time spent in the ROI. Dashed grey line represents the value expected from a random distribution in the arena (25%). **(C)** Mean speed in the arena. BD — bystander dominant; BS — bystander subordinate; CD — control dominant; CS — control subordinate. Mean ± SEM represented. n.s. — non-significant; $^{*}p < .05$; $^{**}p < .01$.

Bystanders' behaviour during the fights. During the fight interactions, the directional focus towards the stimulus and mean speed in the arena were not significantly different across conditions (*Rproj*: $F_{3,67} = 0.65$, $p = .56$; speed: $F_{3,67} = 1.28$, $p = .29$; Figure 4.5A,C). Control fish spent significantly less time in the ROI than bystander fish

irrespective of social status [$F_{3,67}$ = 5.92, p = .001; contrasts (BD–CD): t_{67} = 2.23, p = .03; d_s = 0.73; contrasts (BS–CS): t_{67} = 3.39, p = .001; d_s = 1.15; Figure 4.5B]. Analysis of the demonstrator dyads' latencies to fight (224.50 ± 39.93 s, n = 71) and fight resolution times (353.38 ± 45.98 s, n = 71) did not reveal any differences across conditions (latency to fight: $F_{3,67}$ = 0.48, p = .70; fight resolution: $F_{3,67}$ = 0.61, p = .60).

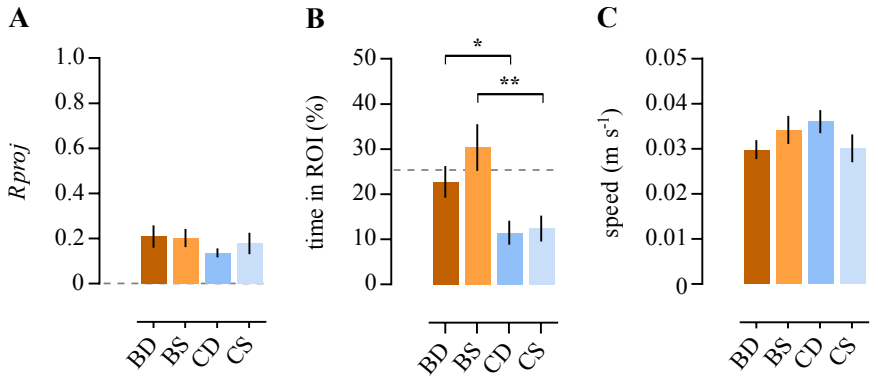

Figure 4.5 | Behavioural results at the fight-observed/not-observed stage. (A) *Rproj* in arena. Dashed grey line indicates no directionality. **(B)** Mean time spent in ROI. Dashed grey line represents the value expected from a random distribution in the arena (25%). **(C)** Mean speed in the arena. BD — bystander dominant; BS — bystander subordinate; CD — control dominant; CS — control subordinate. * p < .05; ** p < .01.

Bystanders' behaviour after the fights. In the post-fight stage, the mixed-model ANOVA revealed a main effect of treatment for the directional focus (bystanders > controls; Table 4.1). The planned comparisons showed that dominant bystanders had a significantly higher directional focus towards losers of observed fights than towards

winners (Table 4.1; Figure 4.6A). Subordinate bystanders however, showed no differences in directional focus towards winners or losers and neither did dominant and subordinate control fish. All conditions had a mean orientation around 180° (Table 4.2). There was no effect of treatment and status on the time spent in the winner and loser ROIs, with no differences detected between the two regions for any condition (Table 4.1; Figure 4.6B).

Table 4.1 | Mixed-design ANOVAs and planned comparisons of the measured behavioural parameters between winner-ROI and loser-ROI

	winner-ROI vs. loser-ROI					
	Rproj **(-1 to 1)**			**time (%)**		
	$F_{1,67}$	p		$F_{1,67}$	p	
treatment	4.67	.03		1.39	.24	
status	2.28	.14		0.08	.78	
side	0.03	.86		1.13	.29	
treatment × status	0.96	.33		0.02	.88	
treatment × side	6.39	.01		0.23	.63	
status × side	3.37	.07		0.68	.41	
treatment × status × side	0.75	.39		0.44	.51	
Planned comparisons	t_{67}	p	d_z	t_{67}	p	d_z
BD	2.80	.006	0.64	0.03	.98	0.01
BS	0.00	1.0	0.00	1.48	.14	0.36
CD	0.69	.49	0.16	0.21	.83	0.05
CS	1.62	.11	0.39	0.36	.72	0.09

treatment — bystander, control; status — dominant, subordinate; side — winner-ROI, loser-ROI; BD — bystander dominant (n=19); BS — bystander subordinate (n=17); CD — control dominant (n=18); CS — control subordinate (n=17).

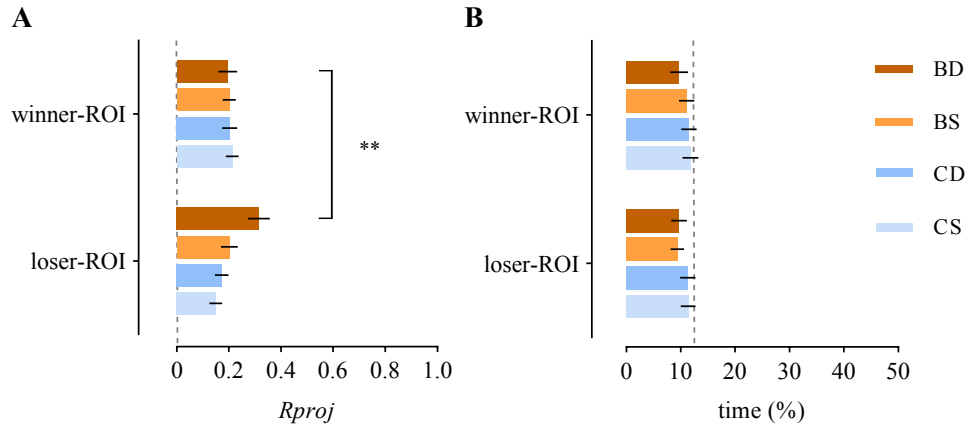

Figure 4.6 | Behavioural results at the post-fight stage. (A) Post-fight mean directional focus (*Rproj*) towards the winner and loser demonstrator fish in the winner-ROI and loser-ROI respectively, for each condition. Dashed grey line indicates no directionality. **(B)** Post-fight mean time spent in the winner-ROI and loser-ROI respectively, for each condition. Dashed grey line represents the value expected from a random distribution in the arena (12.5 %). Mean ± SEM represented. $^{**}p < .01$.

Table 4.2 | Mean orientation angles (mean, 95% C.I.)

	winner-ROI	loser-ROI	pre-fight	post-fight
		α(°)		
BD	175.87, [130.07, 233.63]	192.16, [161.04, 216.87]	184.67, [173.14, 194.40]	182.73, [170.65, 206.22]
BS	165.65, [125.77, 213.65]	193.71, [137.42, 223.93]	171.81, [161.29, 189.85]	189.07, [158.89, 212.40]
CD	199.17, [148.26, 231.22]	187.11, [142.07, 238.55]	172.39, [157.01, 227.34]	177.98, [154.28, 226.82]
CS	207.73, [163.05, 235.95]	191.65, [127.63, 242.18]	172.05, [156.01, 247.63]	183.35, [162.23, 230.32]

BD — bystander dominant (n=19); BS — bystander subordinate (n=17); CD — control dominant (n=18); CS — control subordinate (n=17).

Comparisons between pre-fight and post-fight, showed that observing a fight significantly increased the directional focus of bystander dominant fish towards the demonstrator fish but decreased it for bystander subordinate fish (Table 4.3; Figure 4.4A). Dominant and subordinate control fish, which did not observe the fight, also showed a decrease in directional focus, although not statistically significant for the subordinates. All conditions had a mean orientation around 180° (Table 4.2).

Table 4.3 | Mixed-design ANOVAs and planned comparisons of the measured behavioural parameters between the pre-fight and post-fight stages

	pre-fight vs. post-fight								
	Rproj (-1 to 1)			time ROI (%)			speed (m s^{-1})		
	$F_{1,67}$	p		$F_{1,67}$	p		$F_{1,67}$	p	
treatment	3.61	.06		0.06	.82		0.07	.79	
status	0.74	.39		0.05	.82		1.43	.24	
stage	2.62	.11		0.52	.47		2.12	.15	
treatment × status	0.57	.45		0.37	.55		0.82	.37	
treatment × stage	2.21	.14		2.93	.09		6.59	.01	
status × stage	3.23	.08		0.01	.92		0.50	.48	
treatment × status ×stage	7.67	.007		0.67	.42		0.60	.44	
Planned comparisons	t_{67}	p	d_z	t_{67}	p	d_z	t_{67}	p	d_z
BD	2.30	.02	0.53	0.14	.88	0.03	2.85	.006	0.65
BS	2.30	.02	0.56	0.83	.40	0.20	1.24	.22	0.30
CD	2.06	.04	0.48	0.76	.45	0.18	0.59	.55	0.14
CS	1.04	.30	0.25	1.64	.10	0.39	0.51	.61	0.12

treatment — bystander, control; status — dominant, subordinate; stage — pre-fight, post-fight; BD — bystander dominant (n=19); BS — bystander subordinate (n=17); CD — control dominant (n=18); CS — control subordinate (n=17).

No differences were detected between stages in the time spent in ROI, for any condition (Table 4.3; Figure 4.4B) with the mean values not revealing higher proximity levels towards the stimulus than what would be expected from a uniform distribution (25% of the time) in the arena. Bystander dominant fish significantly decreased their mean speed in the arena in the post-fight stage, while no differences were found for the remaining conditions (Table 4.3; Figure 4.4C).

Correlation analysis between the bystanders' directional focus towards the losers of the observed fights and the fights' latency or resolution times, revealed no significant results for bystander dominant fish (*Rproj* loser-ROI vs. latency to fight: r_p = - 0.37, p = .11; *Rproj* loser-ROI vs. fight resolution: r_p = - 0.14, p = .54; n = 19), or bystander subordinate fish (*Rproj* loser-ROI vs. latency to fight: r_p = 0.19, p = .44; *Rproj* loser-ROI vs. fight resolution: r_p = 0.20, p = .44; n = 17).

Preliminary temporal profiling of behavioural dynamics. Post-fight correlation analysis of the two behavioural parameters that showed significant differences between the pre-fight and post-fight stages (*Rproj* and mean speed in the arena), revealed a strong negative correlation between these two variables for bystander dominant fish (r_p = -0.84, p < .001, Figure 4.7A) and a moderate negative correlation for bystander subordinate fish (r_p = -0.54, p = .02, Figure 4.7B). No correlation was found for dominant (r_p = -0.35, p = .15, Figure 4.7C) and subordinate controls (r_p = -0.1, p = .71, Figure 4.7D).

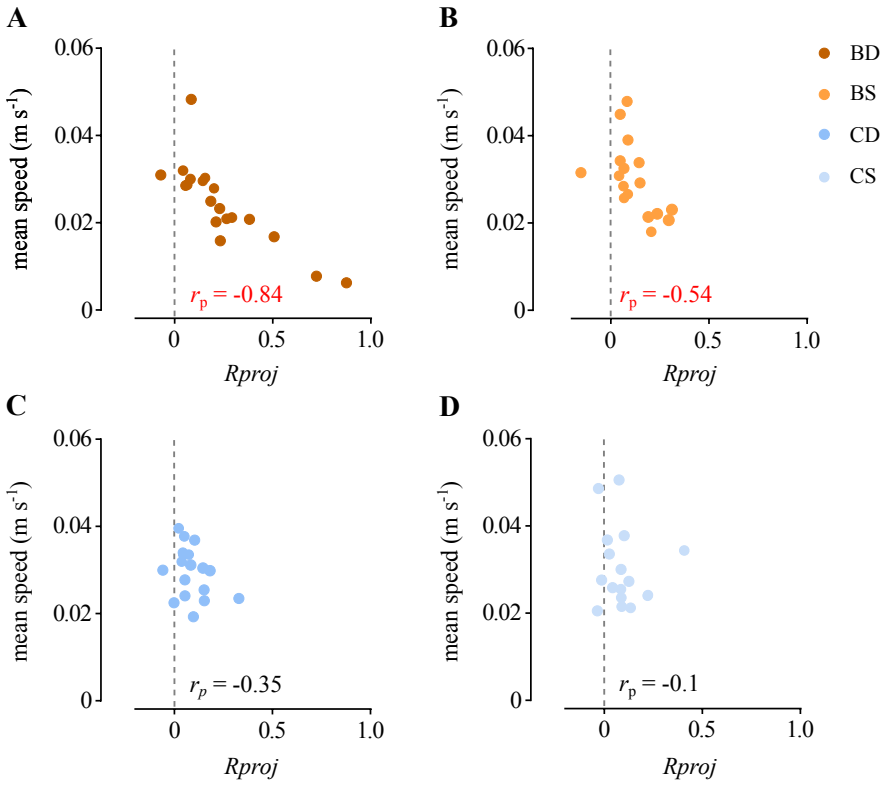

Figure 4.7 | Speed vs. directional focus at the post-fight stage. Scatter plots of the mean speed in the arena as a function of the mean directional focus towards the stimulus (*Rproj*), for the conditions: **(A)** bystander dominant (BD); **(B)** bystander subordinate (BS); **(C)** control dominant (CD); **(D)** control subordinate (CS). Coloured circles represent individual fish. Pearson's correlation coefficient r_p is shown in red when significant ($p < .05$). Dashed vertical lines represent no directional focus (*Rproj* = 0).

We further analysed the temporal dynamics of *Rproj* and speed in the arena for the bystander dominant fish that presented the highest difference in directional focus towards the loser (in the loser-ROI) compared to the winner (in the winner-ROI) (Figure 4.8A). This was the the same fish that presented the highest mean value of *Rproj* and lowest

mean speed in the arena at the post-fight stage (Figure 4.7A). Results revealed that the fish exhibited cyclic stable periods of maximum directional focus (*Rproj* = 1) towards the stimulus, interspersed with fast variation periods across all range of values (1 to -1 to 1). The mean speed values showed an alternating pattern of almost immobility with fast increases and decreases in speed in opposite phase to the *Rproj* curve (Figure 4.8B).

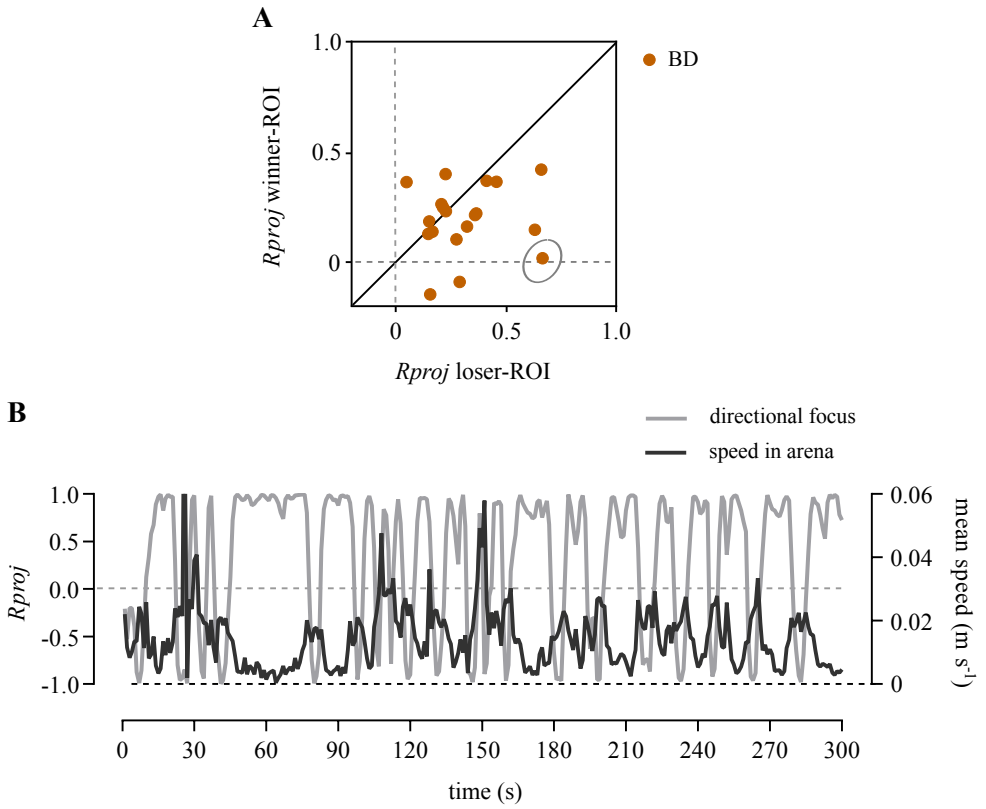

Figure 4.8 | Behavioural profile of a selected representative dominant bystander fish at the post-fight stage. (A) Scatter plot of the directional focus (*Rproj*) in the winner-ROI vs. loser-ROI for bystander dominant fish (BD). Diagonal black line represents equal *Rproj* values in the winner-ROI and loser-ROI.

The selected representative bystander is marked with a grey circle. **(B)** Temporal dynamics of *Rproj* and mean speed in the arena of the selected bystander. First 5 min are represented in 1 s bins. Dashed grey horizontal line represents no directionality (*Rproj* = 0) and dashed black horizontal line represents immobility (mean speed = 0 m s^{-1}).

4.4 Chapter discussion

In this study, we demonstrated for the first time the occurrence of social eavesdropping in zebrafish and its modulation by the bystanders' social status. After observing a fight, dominant but not subordinate bystander zebrafish became more attentive towards the losers than winners of the observed fight. Moreover, control fish that could not observe the fights did not reveal any attentional preference regardless of their dominance status. This indicates that dominant bystanders collected information about the observed fighters during the interaction and not from any post-interaction status cue, such as possible changes in colouration or body postures (Spence et al. 2008; Oliveira et al. 2011). These results also imply that zebrafish are capable of 'true' individual recognition (Tibbetts & Dale 2007) and attribution of social status to individual conspecifics as found in other fish (Grosenick et al. 2007).

No baseline differences were found between conditions for any of the parameters analysed. This showed that behaviour towards the demonstrators prior to the fight was identical and not modulated by dominance status at that stage. However, comparison between the baseline and post-fight periods confirmed that observing a fight increased the directional focus of dominant fish towards the

demonstrators, while reducing their mean speed in the arena. Conversely, the directional focus of subordinate fish decreased and activity levels were not affected, similarly to control fish, suggesting a loss of interest of these fish in the demonstrators after the fight.

During the fight observation stage no differences were found in directional focus or mean speed in the arena between conditions. Moreover, remarkably there was no increased proximity towards the demonstrators at any stage. With the exception of control fish (which avoided the opaque partition placed during the fight period), mean values remained around chance level for all conditions and stages. Thus in our study, eavesdropping was revealed by directional focus towards a conspecific rather than by proximity, a parameter which has been often used in other studies (e.g. Lai et al. 2014). This suggests that behavioural outputs of eavesdropping (and of social learning in general) can be subtle and potentially overlooked in many behavioural paradigms, emphasizing the importance of using novel behavioural parameters and automated tracking methods in the study of social interactions (e.g. Kabra et al. 2013). Particularly, in our paradigm there was no possibility of territorial intrusions or interactions after the fight and each fish controlled an adjacent territory without being able to cross it. Also, winners and losers were not aware of the bystanders' presence, thus showing no territorial or aggressive behaviours at this stage. In this context, the fact that dominant bystanders were more focused towards the losers than winners of the fights, while not preferentially approaching or avoiding either of them, may be explained as a strategy to evaluate potential territorial expansion, focused on monitoring a

weaker rival, while avoiding confrontation with a neighbouring dominant one (Ophir & Galef 2003; Amy & Leboucher 2007).

Additionally, it should be expected that the quality of the fight might provide specific information to eavesdroppers and also affect their response. For instance, the latency to start a fight might be an indicator of the level of aggressive priming, and the time it takes for a winner and a loser to emerge from the fight an indirect indicator of the differences in fighting ability of the opponents. However, we found no correlation between the dominant bystanders' increased attentiveness towards the losers of the fights and the fights' latencies or fight resolution times, which entices the use of more refined individual measures of behaviour. In our experiment we did not individually tag the demonstrators to avoid providing unintentional cues to eavesdroppers or eliciting behavioural changes during the fights. This prevented us to analyse the demonstrators' individual behaviours during the fights (Oliveira et al. 2011). Nonetheless, individual fighting performance (e.g. displays, strikes, bites, chasing) and other behavioural parameters (e.g. structure of movement) have the potential to report relevant aspects of the eavesdropped information. The recent development of new video tracking methods allowing non-invasive individual tagging of unmarked individuals (Pérez-Escudero et al. 2014), and the successful manipulation of video stimuli using fish (Abril-de-Abreu et al. 2015a; Nakayasu & Watanabe 2014) can provide the necessary tools to further develop this paradigm in future studies.

Also intriguingly, correlation analysis between the directional focus and mean speed at the post-fight stage showed that in average the more

focused were bystanders the lower was their mean speed in the arena. The coupling of these two parameters was particularly strong for dominant bystanders but it did not happen for controls (which did not see the fight), suggesting a potential post-fight behavioural pattern to eavesdroppers. This was supported by a preliminary analysis of the temporal dynamics of these variables for a representative dominant bystander, which exhibited the highest eavesdropping effect. The temporal profile revealed a cyclic behaviour between high directional focus towards the demonstrators while almost immobile at the same time, alternated with unfocused periods of activity moving around the tank. This suggests a behavioural pattern that includes sustained periods of static attentive monitoring of the stimulus. A complete profile analysis of all samples and conditions will be required to further explore the specificity of this behaviour in the future to eavesdroppers.

In conclusion, this study demonstrates the modulation of eavesdropped public information by individual past social experience, possibly a fundamental process in social learning mechanisms. Given the growing number of neurogenetic tools available for zebrafish, which allow the visualization and manipulation of neural circuits in relation to behaviour (Agetsuma et al. 2010; Ahrens et al. 2012; Okamoto et al. 2012; Muto et al. 2013; Bianco & Engert 2015); together with the development of new tracking and stimulus manipulation tools, the demonstration of social eavesdropping in zebrafish sets the stage for studying the neural mechanisms underlying social learning in a model organism.

Chapter 5
General discussion

5.1 Overview of empirical findings

In this thesis we focused on investigating the phenomenon of social eavesdropping in zebrafish, a potentially ubiquitous process in social species. We started by determining that zebrafish are tuned to attend to social interactions, a predicted requisite for social eavesdropping, and explored possible relevant features driving this attention. To achieve it, we first developed and validated an unforced-choice behavioural paradigm, using agonistic interactions between conspecifics as stimulus. We also developed an automated video tracking and combined behavioural parameters as proxies of attention, namely directional focus and proximity towards the fighting conspecifics. We found that male bystander zebrafish of different strains (AB and Tübingen), were consistently highly attentive towards unfamiliar fighting conspecifics, as measured by the selected behavioural parameters. These values significantly decreased when no interaction occurred, while activity or stress levels were not affected. Together, these results supported the hypothesis that zebrafish are tuned to attend and potentially eavesdrop on social interactions.

Subsequently, we set to explore relevant features in the fighting interactions underlying this response. We further developed our paradigm by using video playbacks of the fights as stimulus, which enabled us to manipulate both the interacting fishes' form features and also the different stages of the fight. Our results revealed that the assessment stage of the fights elicited higher attentional responses than the post-resolution chasing stage, regardless of the fighting interactions'

level of activity. Our results also suggest that the fight resolution event might be a relevant attentional switching point. Moreover, we found that during the assessment stage of a fight the shape of the fish seemed to play a key role, while after the fight's resolution, biological movement features of the dominant fish chasing the subordinate fish rather than form features, appeared to be more relevant to bystanders.

Next, based on these behavioural results we used microarray gene chips to characterize distinctive transcriptomic profiles and to identify candidate genes related to the observed attentional responses, both to conspecifics in general and to fighting conspecifics in particular. We based our approach on differential expression of single genes and gene sets. These analyses were complemented by promoter region-based techniques. Using data from both approaches, we further drafted protein interaction networks. Overall we found that all behaviour profiles, even when bystanders did not reveal attentiveness towards conspecifics, led to transcriptomic responses in the brain that differed from isolated individuals, although in different ways and with a very small number of differentially expressed genes compared to isolated fish. Attentiveness towards conspecifics whether interacting or not, activated similar neurogenomic states and pathways linked to neuronal plasticity and memory formation. However, specifically observing fighting interactions further triggered pathways associated with specific genes (*btg2*, *npas4b*, *dnajb5* and *msh4*) that seemed to be particularly important in defining this behavioural profile. For instance *btg2,* which has been shown to have a role in neuronal plasticity, contextual and fear memory formation; or for instance the "Fight-or-flight" pathway.

This suggests that observing fighting interactions activates specific processes on top of those already activated just by observing conspecifics, which might potentially be related to social eavesdropping. Overall, the obtained results suggest that transcriptome comparison of specific behavioural phenotypes using this kind of conspecifics' observation paradigms might allow the identification of genetic mechanisms associated with social attention processes in general and with social interactions in particular.

Finally, we developed a behavioural paradigm to test social eavesdropping on fighting interactions in zebrafish based on the previous developed experiments and quantified behavioural parameters. We also investigated the integration of this information with private social information obtained from past social experience, specifically the eavesdropper's own dominance status. Our results revealed for the first time that zebrafish are capable of eavesdropping on conspecific fighting interactions and subsequently use this information. Furthermore, our results showed that this process was modulated by the eavesdroppers' own dominance status. Importantly, these results also imply that zebrafish are capable of individual recognition and attribution of social status to individual conspecifics and suggest that integration of eavesdropped and private information may be ubiquitous in social learning processes.

In the following sections we will discuss specific and general aspects of these results, present future perspectives for the continuing development of this work and potential implications of studying social eavesdropping.

5.2 Measuring attention to conspecific fighting interactions

In a complex and dynamic environment, attention, i.e. the ability to select, filter and prioritize relevant information from a multitude of sensory stimuli is essential. A consensual definition of attention is still hardly achievable, as it is a construct of numerous cognitive processes. For instance, Carrasco (2011) divides visual attention using several categories: spatial attention, which can be overt (e.g. when an observer moves its eyes following a relevant location or focus of attention) or covert (e.g. when attention to a relevant location is not accompanied by overt orientation); feature-based attention, which is usually a covert form of attention and pertains to specific aspects of objects in the environment (e.g. colour, orientation, motion direction); and object-based attention, where attention can directly select discrete objects (Scholl 2001).

Accordingly, a vast amount of research combining behavioural, psychophysical, neurophysiology and neuroimaging studies in humans and other animals, specially focusing on visual attention, has been developed in recent years to investigate several attentional processes and its underlying neurobiological mechanisms (see Carrasco 2011 for a review). Most of these studies inevitably require restrained subjects. Even strictly behavioural tasks such as measuring optokinetic responses with eye-tracking systems in humans and non-human animals, require immobility of subjects or head-mounted systems. However, several

behavioural tests have also been developed using freely moving animals, similarly aiming to address several attentional processes. Bushnell (1998) for instance, reviewed and categorized from the animal behavioural literature five attentional processes, namely: orienting, expectancy, stimulus differentiation (including stimulus salience, discrimination of critical stimuli from its context, selection among stimuli), sustained attention and parallel processing. Common behavioural paradigms (mainly using rodents) encompass some of these processes. For instance, the 5-choice serial reaction time task (5-CSRTT) and the signal detection task (SDT) to assess sustained attention (i.e. when behaviour is guided by a single unpredictable stimulus in time and space); or the novel object recognition task (NOR) to assess selective attention (i.e. choosing among multiple stimuli). One of the main disadvantages of 5-CSRTT and SDT types of test are the employment of operant tasks that require extensive training procedures. Conversely, NOR types of tests have the advantage of being fast and not requiring training sessions but have the limitations of requiring independent tests to assess for instance if changes in preference, memory performance, etc. pertain to attentional variations or not (see Levin et al. 2011 for a review).

In zebrafish, there is no commonly accepted task that explicitly addresses attention as a dependent variable, although several studies exist from which attentional processes may be inferred (see Echevarria et al. 2011 for a review). A recent study by Braida et al. (2014) has addressed selective attention in zebrafish by using a modified version of the novel object recognition test, named virtual object recognition test

(VORT). Here, zebrafish were presented with a one-trial forced-choice task to discriminate between two videos of differently geometrically shaped objects (stationary or moving) displayed in opposite sites of an arena. After an initial exposure session to the same stimulus, a second test was performed where one of the stimuli was replaced with a new one and/or with a different type of movement. Results showed that zebrafish discriminated the novel stimulus or novel motion, as measured by time in proximity to each stimulus and head orientation towards it. Moreover, the memory performance of this discrimination decreased with time and disappeared after one week. These results showed that zebrafish are capable of selective attention, identifying shapes with characteristic motions. Additionally, performance increased when similar shapes were coupled with a specific motion, suggestive of the feature binding processes already discussed in chapter 2 (Neri 2012).

In our work, in order to investigate social eavesdropping in zebrafish, i.e. the ability to attend and use relevant social information from conspecific interactions, we aimed for a simple, ethologically based approach that could provide reliable and robust behavioural measures of attention to social stimuli. We started by developing a method to assess bystanders' attention to conspecific fighting interactions. Signalling in a fighting interaction is designed to 'transmit information about resource-holding power and/or intention' (Peake & McGregor 2004), thus it is expected to be a salient, ethologically relevant social stimulus in a communication network. One that we predicted could elicit eavesdropping behaviour in bystander zebrafish. Differently from Braida et al. (2014), in our paradigm we developed a

one-trial novel stimulus unforced choice paradigm, where we analysed responses to each novel stimuli (i.e. unfamiliar fighting conspecifics, non-interacting conspecifics and an empty tank as control) individually and independently. Consequently, here the 'competing' known stimulus was the baseline environment (isolation). This provided us several advantages: (1) it avoided the typical confounding effects of attraction vs. avoidance that arise in forced-choice tasks; (2) allowed analysis of the bystanders' spontaneous untrained behaviours when faced with the different conditions; and (3) enabled us to quantify at the behavioural and brain gene expression levels, specific attentional responses to each condition. Based on the previous literature, we operationally defined attention as the selective preference for a presented stimulus within the environment, and indirectly measured it by a set of overt behavioural outputs that we could easily identify and quantify as proxies of attention, namely: sustained proximity and directional focus towards the stimulus. Without neglecting the limitations inherent to assessing attentional processes by behavioural measures only, in zebrafish sustained proximity is considered a typical measure of willingness to investigate a novel object and of preference for that particular stimulus. This was particularly the case in our one-trial unforced choice task, where unfamiliar conspecifics were presented as stimulus and alternative explanations such as active avoidance of other environmental stimuli or conditioning effects, could be ruled out. Our results also showed that the significantly increased time in proximity to fighting conspecifics was not explained by differences in motor activity or stress levels, further supporting a visual selection process. Moreover,

the significantly higher directional focus towards the fighting conspecifics, compared to all other possible orientations that could have been taken by the fish (e.g. the uniform distribution pattern typical of isolated fish), allowed us to infer a reliable measure of visual attention towards that stimulus. The measure was consistent with the fish's eye positioning and field of view (Pita et al. 2015) and analogous to eye-gaze tracking attention paradigms used in other species (e.g. primates, birds, rodents; see Winters et al. 2015 for a review). Furthermore, although proximity and directional focus characterize only a subset out of a large array of attentional processes and procedures, there was a robust consistency between the two measures across the different conditions. Also, a strong positive correlation was found between these measures for bystanders observing fighting conspecifics. Together, these results supported the conclusion that each of these two behaviours are expressing an attentional process and can provide a reliable method to measure attention in the context of social eavesdropping. Additionally, in conjunction with the use of video stimuli, it provided us a reliable approach to further tease apart potentially relevant features within the fighting interactions for the acquisition of social information about the fighters, through social eavesdropping.

5.3 Using video stimuli to analyse fighting interactions

Using video playbacks as stimulus is a powerful method to manipulate the stages and social features present in the fighting interactions. It

allows exploring key features (e.g. form and structure of movement, activity, resolution times, etc.) that may drive bystander zebrafish attention. It also eliminates manipulation procedures (e.g. netting) of the demonstrator fish, which may cause stress and unwanted behavioural effects. However, a main issue usually faced when presenting a video as stimulus, is how to evaluate to which extent the observer perceives it and interprets it as a natural stimulus. One essential aspect to consider is the visual sensory system of the subject animals. The video displays used in most behavioural experiments are designed for human vision, which might differ in several aspects to other species, such as colour and luminance perception, motion detection (flicker-fusion frequency), depth perception and spatial resolution (D'eath 2007; Oliveira et al. 2000; Winters et al. 2015). In our experiments we used commercially available video cameras and displays with characteristics that took into consideration the zebrafish's visual system. We took advantage that zebrafish is a highly visual species with a similar visual system to humans (Chhetri et al. 2014), possessing an overlapping spectral wavelength sensitivity to humans (although additionally having UV-sensitive cones), similar flicker-fusion frequency (~50Hz; Branchek 1984) and visual acuity (spatial resolution) well within the range of the used video displays and camera settings (Tappeiner et al. 2012).

Another important aspect to consider is the issue of depth perception. Video displays present three-dimensional information in two dimensions, which can alter the perception of size and texture of the stimuli. Therefore, in our experiments we used real size images and

conspecifics were filmed in white, narrow tanks to avoid noisy textures and large variations in size perception[8]. Notwithstanding these caveats, the behavioural results for the different video treatments validated those obtained using real stimuli (although with lower mean levels) both for AB and Tübingen strains. This strongly suggests that the fish perceived conspecifics in the videos as real conspecifics. An even more definitive demonstration would be feasible for instance by comparing eavesdroppers' responses to real fighting interactions and to video playbacks of the same interactions, using our social eavesdropping paradigm (see chapter 4).

Moreover, our video manipulation results provided us important clues about features of the information contained in a fighting interaction that may be significant for eavesdroppers: (1) the assessment stage of the fight elicited higher attentional responses than the post-resolution chasing stage, regardless of the fights' level of activity; (2) form features of the interacting fish seemed to be particularly relevant at the assessment stage, although the dots manipulation experiment was based on one video fight only and therefore we cannot generalize this conclusion to all fighting interactions[9]; (3) The fight resolution event is a relevant attentional switching point. At first glance, when analysed individually the higher

[8] Currently we are conducting pilot tests to improve depth perception by testing different focal distances to the screen.

[9] However, we are conducting pilot experiments using within-subjects design and presenting alternating fighting fish videos and fighting dots videos to bystanders. Results are revealing strong consistent responses and fast transitions between attentive and inattentive states, correspondingly (data not shown).

interest for the assessment stage obtained in the first video experiment (section 2.4), could simply be result of an order effect and gradual loss of novelty. However the subsequent experiment (section 2.5) presenting repeated video loops from both stages independently, showed that while novelty seems to play a role, the higher levels of attention during the assessment stage are independent of a causal sequence (e.g. assessment coming before chasing) and not related to the fight's activity levels. Additionally, analysis of the bystanders' responses aligned by the fight resolution times confirmed a drop of interest around the time where transition from assessment to chasing behaviour occurred. Finally, the differences found in responses to images of conspecifics compared to dots, could simply indicate that bystanders are tuned to conspecifics and using fish's features for recognition (e.g. shape, striped colouration; see discussion in chapter 2). However, it does not explain by itself why two fish assessing each other would elicit higher attention than two fish chasing each other. Accordingly, when considered all together our results suggest that bystanders are acquiring specific social information from the assessment stage of fighting conspecifics and that this information is more relevant than information contained in the post-resolution chasing stage. This is consistent with the hypothesis that bystanders, when suddenly faced with an unexpected nearby fighting interaction between conspecifics, where the social status of each opponent is uncertain, may be immediately tuned to attend and eavesdrop on the fight; for instance in order to assess the higher future threat (i.e. winner and loser of the interaction).

If such is the case, we would expect (as our results suggest) that the assessment stage of the fight is the most relevant stage for eavesdroppers, eliciting sustained attention until a winner and loser emerges. At this stage form features of each fish should be crucial to: firstly, identify that a fighting interaction between conspecifics is occurring (e.g. proximity, physical contact, relative directionality, circling, lateral displays, bites); secondly, extract information about the conspecifics' individual identity and fighting performance (e.g. physical characteristics, absolute and relative number of bites, strikes); and thirdly, identify the behavioural shift that attributes winner or loser status to each opponent (an outcome that cannot be faked). This also includes information about the fight's duration, which can provide eventual measures of motivation and inequality between fighters (e.g. long fight reveals matched, motivated opponents).

Interestingly, in our paradigm dominance information is also unmistakably available in the post-resolution chasing interaction, where the dominant fish chases the subordinate fish that flees and freezes. While this stage also elicited strong responses by bystanders (although seemingly more dominated by movement components; see discussion in chapter 2), attentional levels at this stage were lower than in the assessment stage. Bursts of short-termed chasing events are common in zebrafish shoals, particularly between territory holders and challengers (personal observation). Whether this information is sufficient and used by eavesdroppers to determine the relative dominance status of conspecifics remains to be seen. Future experiments allying the described video manipulation methods and results in the context of our

social eavesdropping paradigm, will allow dissecting the essential aspects for successfully eavesdropping, as we will discuss next.

5.4 Social eavesdropping in zebrafish — future directions

Our social eavesdropping experiment showed that zebrafish are not only able to eavesdrop on conspecific fighting interactions but that this process is modulated by the eavesdroppers' own dominance status. Although this was still a first experiment, several important questions arise from our results that are worth considering for developing future research directions. The first one concerns what specific information are eavesdroppers acquiring in order to be able to attribute dominance status (winner and loser) to the observed conspecifics. As previously discussed in the general introduction (chapter 1), it is expected that bystanders optimize the acquisition and use of the available social information, weighting its reliability and acquisition costs (whether through social eavesdropping, non-signalling cues, or even direct experience). In order to control for other potential sources of information, in our eavesdropping paradigm subjects had no interaction experience with the fighters (whether prior, during or after the fights), ruling out any previous assessment of each fighter or possible priming effects. Also the fact that both fighters were equivalent in size and the lack of post-fight preference behaviours by control subjects (who did not see the fights), strongly suggests that conspicuous characteristic cues from each fighter were not a contributing factor either (Saverino &

Gerlai 2008). This leaves the main possibility that information about the fighters' relative status was collected during the fighting interaction itself. However, the exact source of this information, at what stage of the fight it happens, if it is dependent on aspects of the interaction dynamics only, on its outcome or both, is still unknown (see Peake & McGregor 2004 for a review). It should also be noted that absolute information about individual fighter's performance (e.g. levels of aggression, striking speed) may be additionally acquired by eavesdroppers, possibly modulating the strength of the obtained eavesdropping effects (as suggested by Earley & Dugatkin 2002).

Dominant bystanders' eavesdropping behaviour was revealed only by differences in directional focus towards winners and losers of the interactions. This monitoring-like behaviour is possibly a consequence of the used experimental design, which provided stable territories and prevented interactions between subjects and demonstrators after the fight. Selective observation without approaching might be a preferred behaviour in such circumstances, contrary for instance to a context where subsequent territorial intrusions occur after the fight, which promotes approaching and aggressive behaviours (Oliveira et al. 1998). Preliminary analysis of the eavesdroppers' post-fight behavioural dynamics is suggestive of such 'monitoring' behaviour, where periods of high directional focus seem to couple with almost immobility. Detailed analysis may reveal distinctive characteristic behavioural patterns (e.g. dependent on the eavesdroppers' dominance status).

Nevertheless, under natural circumstances eavesdropped information about the dominance status of territorial neighbours

is expected to be used in future encounters with those individuals. The addition of such a test to our current paradigm, allowing subsequent interactions between eavesdroppers and the winners and losers of the fights, can provide valuable data on how bystanders use eavesdropped information (see Oliveira et al. 1998; Earley & Dugatkin 2002). Importantly it may also reveal the use of eavesdropped information by subordinate bystanders, which in the current paradigm was not detected (e.g. Lai et al. 2014).

Another important implication from our results is that zebrafish are capable of attribution of relative social status, social memory and likely visual individual recognition of conspecifics (Tibbetts & Dale 2007; Grosenick et al. 2007). How and to what extent this happens (for instance if its context dependent, how many conspecifics can be discriminated individually and for how long) are questions that remain unanswered at this point. In our paradigm, the experimental protocol included an overnight exposure period to the demonstrators prior to the fights. This aimed to provide enough familiarization time to the future fighters in order to facilitate individual recognition in the subsequent social eavesdropping test. Surprisingly however, during the fight observation period the increased levels of both proximity and directional focus found in the previous attention experiments towards novel unfamiliar fighting conspecifics (chapter 2), were not verified. This suggests that the familiarity provided in our eavesdropping experimental context (stable neighbouring territories with familiar males) strongly reduced these overt measures of attention towards conspecifics during the fight observation period. Such was possibly

consequence of dear enemy effects (i.e. a lower perception of threat regarding familiar neighbours compared to unfamiliar ones; see Temeles 1994) and eventually even reduced the eavesdropping behavioural results obtained.

Behavioural experiments

Unravelling the topics described above, will allow us essential experimental refinement and stimulus manipulation control for future studies concerning the neural mechanisms underlying eavesdropping. A set of possible experiments follows building up on the current findings.

A first step would be to test social eavesdropping when minimizing pre-exposure to the demonstrators, similarly to the attention experiments (i.e. novel unfamiliar fighting conspecifics). While the ability to eavesdrop both on familiar and unfamiliar conspecifics might be different, the strong response exhibited by bystanders when observing unfamiliar fighting conspecifics suggests that introducing this change in the social eavesdropping paradigm could maximize eavesdropping effects. If successful, it would also allow to standardize the experimental context in both paradigms, providing us similar attentional results during the eavesdropping test's fight observation period and not only during the post-fight stage. This would enable using the simpler attention paradigm for piloting and troubleshooting of the social eavesdropping acquisition phase (fight observation) in future experiments.

Regardless of the selected protocol (familiar or unfamiliar), a second step would be to validate the social eavesdropping paradigm using video fights as stimuli, building on the results of the performed video experiments. Once validated this would allow manipulating the fights in order to pinpoint the features of the interaction that are required for eavesdropping. The obtained results from the video experiments already provide us valuable clues, namely that the assessment stage and fight resolution event might be essential. For instance three different conditions: (1) pre-resolution videos only (unresolved fight), (2) post-resolution videos only (winner-loser chasing stage), and (3) pre-resolution + fight resolution videos, could be presented to bystanders and eavesdropping tested. Once the fundamental stage for successful eavesdropping is determined, individual automated tagging and analysis (e.g. Pérez-Escudero et al. 2014; Rosenthal et al. 2015) of each fighter's individual behaviours and in relation to its opponent (e.g. relative number of bites, strikes, displays; see Oliveira et al. 2011), together with manipulation of the individual fighters' form and movement characteristics, would allow to further single out the essential aspects of the social information being acquired. After this is achieved, controlled use and manipulation of these features in the fights will become possible, while eliminating others sources of noise. One possibility would be to create realistic three-dimensional video models of the fighters (already tested in other fish) and simulate fights, where the individual features, relative movements and aggressiveness of each fish can be parameterized, manipulated and even interact with the focal bystander fish (Rosenthal

2000; Rosenthal & Ryan 2005; Butkowski et al. 2011; Woo & Rieucau 2011). This would also allow determining the characteristics that enable the predicted individual recognition of conspecifics.

Additionally, a fourth stage could be introduced to the social eavesdropping test, where bystanders would be subsequently prompted to interact with a video of the winner and loser of the fights separately (e.g. video simulated territorial intrusions). The corresponding behavioural responses and their temporal dynamics could reveal further eavesdropping effects. It would also allow future comparisons at the behavioural and neural levels between bystanders with different dominance status, providing insight on how past social experience (e.g. self-assessment) is integrated with social eavesdropping.

Neural mechanisms

Once the necessary features for successful eavesdropping on fighting interactions are identified and a video-stimuli based social eavesdropping paradigm is optimized, it will become possible to test eavesdropping at the neural level both during the fight (information acquisition, social memory formation) and at the post-fight stage (information recall and use), while minimizing variability from uncontrolled stimuli. One possible experiment would be to test different conditions where dominant and subordinate bystanders would observe simulated video fights controlled for essential eavesdropping features (e.g. two simulated fighters with a differential fixed ratio of biting behaviour). At the post-fight stage they would be presented to a single video of the winner or loser of the fights for 30 minutes. Behaviour

analysis in order to detect eavesdropped information use and its modulation by dominance status would be performed. This would allow selecting subject fish exhibiting strong eavesdropping effects and fish with little or no effects from the different conditions (such selection of the sampled population extremes could allow detecting essential aspects of the underlying mechanisms). The selected subjects would then be sacrificed, their brains extracted and single cell resolution fluorescent *in situ* hybridization (FISH) could be performed based on the mRNA expression of immediate early genes (IEGs) as transient markers of neuronal activity (e.g. *c-fos*, *egr-1*) (Lanahan & Worley 1998; Clayton 2000; Robinson et al. 2008; Okuno 2011; Kovács 2008). This in turn would provide a first map of candidate brain areas involved in the use of eavesdropped information, which would likely entail visual recognition of the presented conspecific, assessment of its dominance status (via eavesdropped information) and integration with the subject's own social status in order to appropriately adapt the subsequent behaviour (e.g. Lau et al. 2011; von Trotha et al. 2014).

It is reasonable to assume that multiple neural circuits will be involved in such processes. We hypothesize that several areas (functional homologous to mammalian areas) might potentially stand out in such mapping and be worth further analysis. We should note that care must be taken when defining homologous brain areas across species, specially in functional terms, as homologies are defined to varying degrees using a combination of (often incomplete) information from developmental and hodological studies, genetic markers, hormone receptors, neurochemical systems, anatomical connectivity and

functional lesion-stimulation studies to assess similarity of function (Goodson & Kabelik 2009; O'Connell & Hofmann 2011; O'Connell & Hofmann 2012; Goodson & Kingsbury 2013).

As we are dealing with visual stimuli, we naturally expect expression in visual processing areas onto which retinal ganglion cells project, for instance the thalamic nucleus [the ventromedial (Vm), intermediate (I), and ventrolateral (Vl) nuclei] which is a primary visual projection target; and the optic tectum (TeO), homologous to the mammalian superior colliculus, and the dominant visual centre in teleosts processing most of the visual information concerning movement, shape and colour of objects (Mueller 2012). Also, we anticipate areas that are part of the mesolimbic reward system such as the medial zone of the dorsal telencephalic area (Dm), a putative homologue of the mammalian amygdala and involved in encoding value/motivational signals and aggressive behaviour; and the lateral zone of the dorsal telencephalic area (Dl), putative homologue of the hippocampus, involved in formation of episodic memories and spatial learning (Murray 2007; Kishi et al. 2006; von Trotha et al. 2014; Portavella et al. 2002; Portavella et al. 2004). The preoptic area (POA), similar to the mammalian POA and with an important role in the regulation of sexual behaviour, aggression and parental care is also likely to be involved; additionally, the ventral nucleus of the ventral telencephalic area (Vv), putative homologue of the mammalian lateral septum, involved in the HPA (hypothalamo-pituitary-adrenocortical) axis modulation of stress activity (Singewald et al. 2011); the neurosecretory preoptic area (NPO) responsible for oxytocin producing

cells, homologue to the paraventricular nucleus (PVN) in mammals that controls hormonal release in the pituitary and homeostasis (Herget et al. 2014); and the supracommissural nucleus of the ventral telencephalic area (Vs), putative homologue of the mammalian medial extended amygdala and the bed nucleus of the stria terminalis, a key area involved in the control of social aggression and information (Fuxjager et al. 2010; Teles et al. 2015). Finally, the dorsal nucleus of the ventral telencephalon (Vd) thought to be partially a putative homologue of the mammalian nucleus accumbens (NAcc), is likely to appear in our experimental context as it is a central integrator of sensorimotor signals related to the modulation of approach/avoidance behaviour of a stimulus (Ikemoto & Panksepp 1999; Lau et al. 2011).

After candidate areas are identified and selected, sampling of those areas using micropunches or laser microdissection (O'Connell & Hofmann 2012), combined with qPCR (quantitative polymerase chain reaction) techniques, also based on transient immediate early genes markers (e.g. *c-fos*, *egr-1*), could be conducted to quantify differential changes in neural activity in the selected candidate brain areas. This would potentially allow testing functional localization and also functional connectivity between areas by comparing co-activation matrices (i.e. correlation matrices for the levels of IEG expression across the nodes within each treatment) across treatments (Hoke et al. 2005; Yang & Wilczynski 2007; Teles et al. 2015). Additionally, social information is expected to have an impact at the whole genome level and not only specific genes, with different treatments producing different neurogenomic states. Microarray analysis or mRNA

sequencing (mRNA-Seq) in conjunction with the techniques described above could be used to measure differential gene expression of candidate genes (e.g. Sneddon et al. 2011; Chandrasekaran et al. 2011; Ziv et al. 2012; Rittschof et al. 2014). For instance, *npas4* and *btg2* are candidate genes found to be relevant in the profiling of bystanders attentive to fighting interactions in our microarrays experiment (chapter 3), and which have been shown to have a role in neuronal plasticity, contextual and fear memory formation (Farioli-Vecchioli et al. 2008; Ramamoorthi et al. 2011; Ploski et al. 2011). Techniques like FISH could also be used to combine for instance the description of *c-fos* mRNA expression's spatiotemporal pattern dynamics, with quantitative analysis and co-detection with other neuronal subtype-specific markers (von Trotha et al. 2014).

The depicted approaches can provide us the first descriptive picture on specific brain areas and connectivity involved in social eavesdropping and its modulation by the dominance status of the observers. Reassuringly, previous work by Desjardins et al. (2010) using cichlid fish (*Astatotilapia burtoni*) and also measuring immediate early genes expression in several brain nuclei in a mate-choice paradigm, revealed different impacts in the neural activity of females when observing a preferred male winning or loosing a fight, indicating that specific social information acquired from observing fighting interactions can have significant effects on the brain and be correlated with neural activity.

Moreover, zebrafish adult mutant Dm lines, mutant oxytocin truncated receptor lines, and conditional (i.e. temporal) transgenics for

oxytocin producing neurons in the neurosecretory preoptic area (NPO), are currently available in our laboratory and may allow loss of function experiments in order to further advance specific hypothesis. For instance, as previously discussed, an essential aspect for eavesdropping is first to attend to conspecific interactions. Therefore it is expected that both positive valence and motivational signals may be involved in this process. In addition to studies showing that the sight of conspecifics is rewarding for zebrafish (Al-Imari & Gerlai 2008; Saif et al. 2013), the brain area Dm has been shown to be involved both in light avoidance behaviours (Lau et al. 2011) and reward-stimulated drug seeking behaviours in zebrafish (von Trotha et al. 2014). This suggests that Dm plays a role in encoding value and motivation (similarly to the mammalian amygdala) and therefore might have an important role in the tuning of attention to conspecific interactions.

Another essential aspect for successful eavesdropping is social learning and social memory. Choe et al. (2015) using male mice showed that oxytocin is selectively required both for appetitive (female — CS^+ odour pairing) and aversive (intruder aggressive male — CS^+ odour pairing) social learning. Moreover, other studies in mice have shown that mutant mice for the oxytocin gene are unable to develop social memory and show deficits in social discrimination (Winslow et al. 2000; Takayanagi et al. 2005). In zebrafish little is still known regarding the role of oxytocin. However, Nunes et al. (unpublished) using zebrafish conditional transgenics for oxytocin producing neurons demonstrated that ablation of these neurons at a critical developmental time window, significantly altered shoal preference behaviour. This suggests a role of

oxytocin in social discrimination abilities, a mechanism also expected to be essential for successful eavesdropping.

Future experiments will ideally also test and demonstrate social eavesdropping using zebrafish in juvenile stages (e.g. 21-28 days post fertilization). Recent studies point to the emergence of social behaviours at this developmental stage (Engeszer et al. 2007; Dreosti et al. 2015), and two-photon calcium imaging techniques have successfully been performed to measure and spatially localize activity of neuronal subpopulations in specific brain areas at this developmental stage (Jetti et al. 2014). At even younger ages (5-7 days post fertilization), a wide array of imaging, optogenetic and transgenic tools, allowing real time visualization and manipulation of neural circuits and its activity in relation to behaviour are already available (Agetsuma et al. 2010; Naumann et al. 2010; Ahrens et al. 2012; Okamoto et al. 2012; Muto et al. 2013; Bianco & Engert 2015). For example, Naumann et al. (2010) developed transgenic larvae expressing GFP-Aequorin in specific neural populations. This allowed monitoring neural activity with high temporal resolution and sensitivity, through the detection of the related bioluminescent emitted signals (through the fish's skull) in freely moving behaving larvae. Ahrens et al. (2012) developed a technique where brain-wide neuronal activity can be monitored at single-cell resolution and visualized using two-photon calcium imaging in live behaving zebrafish larvae. Here, fish were partially immobilized (body embedded in agarose) but could interact with a virtual environment (visual stimuli projected on a screen) and adjust their 'swimming' behaviour (tail movements) to changes in visual feedback (visual closed-

loop system). An adaptation of our proposed video-stimuli social eavesdropping paradigm would be fairly straightforward. For instance by allowing immobilized juvenile zebrafish to watch video fighting interactions and using eye gaze tracking methods for attentional measures. This could open new possibilities to explore the neuronal processes occurring during the acquisition of eavesdropped information (fight observation), even without explicit behavioural motor outputs.

5.5 Social eavesdropping as a mechanism for sociality

Growing evidence points to the ubiquitousness of eavesdropping in many social species, from fish to humans. The findings presented in this thesis provide a basic framework to investigate the mechanisms underlying this phenomenon in a model organism. Our studies focusing on the observation of dominance interactions have highlighted the relevance of attending and eavesdropping on the social relationships of others and its integration with private social information. This may prove to be essential for successful adaptation and survival in a complex social environment. At a group level, current research suggests that eavesdropping may have a role as a mechanism for distributed cognition, while also having an essential regulatory function in stabilizing conflicts, maintaining cohesion of social groups, promoting cooperation and even in the emergence of social norms.

Humans for instance are master eavesdroppers. Our decision-making processes are deeply interwoven with the behaviours of others

and therefore we are highly sensitive to social behaviour. If these mechanisms fail, such as for instance when misreading a social interaction and the information it provides (e.g. the nature of a social relationship), the consequences can range from mere awkwardness to severe conflict. While zebrafish certainly lack the complexity of human social behaviours, investigating a common fundamental social learning process such as eavesdropping, its functions and dysfunctions, can help us understand the mechanisms of our own sociality.

References

Abril-de-Abreu, R., Cruz, J. & Oliveira, R.F., 2015a. Social Eavesdropping in Zebrafish: Tuning of Attention to Social Interactions. *Scientific Reports*, 5, p.12678.

Abril-de-Abreu, R. & Oliveira, R.F., 2015b. Uncertainty about social status in agonistic interactions enhances attention in bystander zebrafish. (Submitted).

Abril-de-Abreu, R., Lopes, J.S. & Oliveira, R.F., 2015c. Brain transcriptomic response to social eavesdropping in zebrafish. (Submitted).

Abril-de-Abreu, R., Cruz, A.S. & Oliveira, R.F., 2015d. Social dominance modulates eavesdropping in zebrafish. *Royal Society Open Science*, 2(8), p.150220.

Agetsuma, M. et al., 2010. The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nature neuroscience*, 13(11), pp.1354–1356.

Ahrens, M.B. et al., 2012. Brain-wide neuronal dynamics during motor adaptation in zebrafish. *Nature*. 485. pp.471–477.

Ament, S.A. et al., 2012. New meta-analysis tools reveal common transcriptional regulatory basis for multiple determinants of behavior. *Proceedings of the National Academy of Sciences*, 109(26), pp.E1801–E1810.

Amy, M. & Leboucher, G., 2007. Male canaries can visually eavesdrop on conspecific food interactions. *Animal Behaviour*, 74(1), pp.57–62.

Aquiloni, L., Buřič, M. & Gherardi, F., 2008. Crayfish females eavesdrop on fighting males before choosing the dominant mate. *Current Biology*, 18(11), pp.R462–R463.

Aquiloni, L. & Gherardi, F., 2010. Crayfish females eavesdrop on fighting males and use smell and sight to recognize the identity of the winner. *Animal Behaviour*, 79(2), pp.265–269.

Arganda, S., Pérez-Escudero, A. & de Polavieja, G.G., 2012. A common rule for decision making in animal collectives across species. *Proceedings of the National Academy of Sciences of the United States of America*, 109(50), pp.20508–20513.

Arnott, G. & Elwood, R.W., 2009. Assessment of fighting ability in animal contests. *Animal Behaviour*, 77(5), pp.991–1004.

Ashburner, M. et al., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics*, 25(1), pp.25–29.

Barba-Escobedo, P.A. & Gould, G.G., 2012. Visual social preferences of lone zebrafish in a novel environment: strain and anxiolytic effects. *Genes, Brain and Behavior*, 11(3), pp.366–373.

Basheer, R. & Shiromani, P.J., 2001. Effects of prolonged wakefulness on c-fos and AP1 activity in young and old rats. *Molecular Brain Research*, 89(1-2), pp.153–157.

Beecher, M.D., 1989. Signalling systems for individual recognition: an information theory approach. *Animal Behaviour*, 38(2), pp.248–261.

Berens, P., 2009. CircStat: A MATLAB toolbox for circular statistics. *Journal of Statistical Software*, 31, pp.1–21.

Bianco, I.H. & Engert, F., 2015. Visuomotor Transformations Underlying Hunting Behavior in Zebrafish. *Current Biology*, 25(7), pp.831–846.

Bierbach, D. et al., 2011. Male fish use prior knowledge about rivals to adjust their mate choice. *Biology Letters*, 7(3), pp.349–351.

Bond, A.B., 1983. Visual search and selection of natural stimuli in the pigeon: The attention threshold hypothesis. *Journal of Experimental Psychology: Animal Behavior Processes*, 9.3, p.292.

Bonnie, K.E. & Earley, R.L., 2007. Expanding the scope for social information use. *Animal Behaviour*, 74(2), pp.171–181.

Boyd, R. & Richerson, P.J., 1988. An evolutionary model of social learning: the effects of spatial and temporal variation. In T. R. Zentall & T. B. Galef Jr., eds. *Social learning: psychological and biological perspectives*. Lawrence Erlbaum Associates, Hillsdale, NJ., pp. 29–48.

Braida, D. et al., 2014. A new model to study visual attention in zebrafish. *Progress in neuro-psychopharmacology & biological psychiatry*, 55, pp.80–86.

Braida, D. et al., 2012. Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. *Psychopharmacology*, 220(2), pp.319–330.

Branchek, T., 1984. The development of photoreceptors in the zebrafish, brachydanio rerio. II. Function. *The Journal of comparative neurology*, 224(1), pp.116–22.

Bshary, R. & Grutter, A.S., 2006. Image scoring and cooperation in a cleaner fish mutualism. *Nature*, 441(7096), pp.975–978.

Burke, C.J. et al., 2010. Neural mechanisms of observational learning. *Proceedings of the National Academy of Sciences of the United States of America*, 107(32), pp.14431–14436.

Bushnell, P.J., 1998. Behavioral approaches to the assessment of attention in animals. *Psychopharmacology*, 138(3-4), pp.231–259.

Buske, C. & Gerlai, R., 2011a. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. *Neurotoxicology and Teratology*, 33(6), pp.698–707.

Buske, C. & Gerlai, R., 2011b. Shoaling develops with age in Zebrafish (Danio rerio). *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 35.6, pp.1409–1415.

Butkowski, T. et al., 2011. Automated interactive video playback for studies of animal communication. *Journal of visualized experiments : JoVE*, (48), pp.3–7.

Byrne, R.W. & Whiten, A., 1989. Machiavellian Intelligence: Social Expertise and the Evolution of Intellect in Monkeys, Apes, and Humans. *American Scientist*, 77, p.599.

Cachat, J. et al., 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. *PLoS ONE*, 6(3).

Cardoso, S.D., Teles, M.C. & Oliveira, R.F., 2015. Neurogenomic mechanisms of social plasticity. *The Journal of experimental biology*, 218(1), pp.140–149.

Carrasco, M., 2011. Visual attention: The past 25 years. *Vision Research*, 51(13), pp.1484–1525.

Chan, J.P.Y. et al., 2008. The effects of male-male contests and female eavesdropping on female mate choice and male mating success in the jumping spider, Thiania bhamoensis (Araneae: Salticidae). *Behavioral Ecology and Sociobiology*, 62(5), pp.639–646.

Chandrasekaran, S. et al., 2011. Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. *Proceedings of the National Academy of Sciences of the United States of America*, 108(44), pp.18020–18025.

Chen, W. V & Maniatis, T., 2013. Clustered protocadherins. *Development*, 140(16), pp.3297–302.

Chhetri, J., Jacobson, G. & Gueven, N., 2014. Zebrafish—on the move towards ophthalmological research. *Eye*, 28(4), pp.367–380.

Choe, H.K. et al., 2015. Oxytocin Mediates Entrainment of Sensory Stimuli to Social Cues of Opposing Valence. *Neuron*, 87(1), pp.152–163.

Choe, S.-K. et al., 2008. hnf1b genes in zebrafish hindbrain development. *Zebrafish*, 5(3), pp.179–187.

Clayton, D.F., 2000. The genomic action potential. *Neurobiology of Learning and Memory*, 74(3), pp.185–216.

Cole, C.J. et al., 2012. MEF2 negatively regulates learning-induced structural plasticity and memory formation. *Nature Neuroscience*, 15(9), pp.1255–1264.

Colwill, R.M. et al., 2005. Visual discrimination learning in zebrafish (Danio rerio). *Behavioural Processes*, 70(1), pp.19–31.

Connor, R.C., 2010. Cooperation beyond the dyad: on simple models and a complex society. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 365(1553), pp.2687–2697.

Coolen, I. et al., 2003. Species difference in adaptive use of public information in sticklebacks. *Proceedings of the Royal Society of London B: Biological Sciences*, 270(1531), pp.2413–2419.

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, pp.184–190.

Crockford, C. et al., 2007. Baboons eavesdrop to deduce mating opportunities. *Animal Behaviour*, 73, pp.885–890.

Cruz, A.S. & Oliveira, R.F., 2015. Audience effects and aggressive priming in agonistic behaviour of male zebrafish, Danio rerio. *Animal Behaviour*, 107, pp.269–276.

D'eath, R., 2007. Can video images imitate real stimuli in animal behaviour experiments? *Biological Reviews*, 73(3), pp.267–292.

Dabelsteen, T. et al., 1996. Is the signal value of overlapping different from that of alternating during matched singing in great tits? *Journal of Avian Biology*, 27(3), pp.189–194.

Danchin, E. et al., 2004. Public information: from nosy neighbors to cultural evolution. *Science (New York, N.Y.)*, 305(2004), pp.487–491.

Desjardins, J.K., Klausner, J.Q. & Fernald, R.D., 2010. Female genomic response to mate information. *Proceedings of the National Academy of Sciences of the United States of America*, 107(49), pp.21176–21180.

Doutrelant, C., Mcgregor, P.K. & Oliveira, R.F., 2001. The effect of an audience on intrasexual communication in male Siamese fighting fish, Betta splendens. *Behavioral Ecology*, 12, pp.283–286.

Dreosti, E. et al., 2015. Development of social behavior in young zebrafish. *Frontiers in Neural Circuits*, 9, pp.1–9.

Dugatkin, L.A. & Earley, R.L., 2004. Individual recognition, dominance hierarchies and winner and loser effects. *Proceedings of the Royal Society of London B: Biological Sciences*, 271(1547), pp.1537–1540.

Dukas, R., 2001. Limited attention: the constraint underlying search image. *Behavioral Ecology*, 12(2), pp.192–199.

Dunbar, R.I.M. & Shultz, S., 2007. Evolution in the social brain. *Science*, 317(5843), pp.1344–1347.

Dzieweczynski, T.L., Gill, C.E. & Perazio, C.E., 2012. Opponent familiarity influences the audience effect in male-male interactions in Siamese fighting fish. *Animal Behaviour*, 83(5), pp.1219–1224.

Earley, R.L., 2010. Social eavesdropping and the evolution of conditional cooperation and cheating strategies. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 365(1553), pp.2675–2686.

Earley, R.L. & Dugatkin, L.A., 2002. Eavesdropping on visual cues in green swordtail (Xiphophorus helleri) fights: a case for networking. *Proceedings of the Royal Society of London B: Biological Sciences*, 269(1494), pp.943–952.

Echevarria, D.J., Jouandot, D.J. & Toms, C.N., 2011. Assessing attention in the zebrafish: Are we there yet? *Progress in neuro-psychopharmacology & biological psychiatry*, 35(6), pp.1416–20.

Engeszer, R.E. et al., 2008. Sex-specific perceptual spaces for a vertebrate basal social aggregative behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 105(3), pp.929–933.

Engeszer, R.E. et al., 2007. Timing and plasticity of shoaling behaviour in the zebrafish, Danio rerio. *Animal Behaviour*, 74, pp.1269–1275.

Engeszer, R.E., Ryan, M.J. & Parichy, D.M., 2004. Learned social preference in zebrafish. *Current Biology*, 14(1946), pp.881–884.

Etkin, A. et al., 2006. A Role in Learning for SRF: Deletion in the Adult Forebrain Disrupts LTD and the Formation of an Immediate Memory of a Novel Context. *Neuron*, 50(1), pp.127–143.

Farioli-Vecchioli, S. et al., 2008. The timing of differentiation of adult hippocampal neurons is crucial for spatial memory. *PLoS Biology*, 6(10), pp.2188–2204.

Fawcett, T.W. & Mowles, S.L., 2013. Assessments of fighting ability need not be cognitively complex. *Animal Behaviour*, 86(5), pp.e1–e7.

Félix, A.S. et al., 2013. Noninvasive measurement of steroid hormones in zebrafish holding-water. *Zebrafish*, 10(1), pp.110–5.

Fiorito, G. & Scotto, P., 1992. Observational Learning in Octopus vulgaris. *Science*, 256(5056), pp.545–7.

Fitzsimmons, L.P. & Bertram, S.M., 2013. Playing to an audience: the social environment influences aggression and victory displays. *Biology letters*, 9(4), p.20130449.

Fleisch, V.C. & Neuhauss, S.C.F., 2006. Visual behavior in zebrafish. *Zebrafish*, 3(2), pp.191–201.

Fox, R. & McDaniel, C., 1982. The perception of biological motion by human infants. *Science*, 218, pp.486–487.

Franceschini, A. et al., 2013. STRING v9.1: Protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Research*, 41(D1), pp.D808–D815.

Frankenhuis, W.E. et al., 2013. Infants' perception of chasing. *Cognition*, 126(2), pp.224–33.

Fukushima, Y. et al., 2011. Sema3E-PlexinD1 signaling selectively suppresses disoriented angiogenesis in ischemic retinopathy in mice. *Journal of Clinical Investigation*, 121(5), pp.1974–1985.

Fuxjager, M. et al., 2010. Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. *Proceedings of the National Academy of Sciences*, 107(27), pp.12393–12398.

Galef jr, B.G. & White, D.J., 1998. Mate-choice copying in Japanese quail, Coturnix coturnix japonica. *Animal behaviour*, 55(3), pp.545–52.

Gao, T., McCarthy, G. & Scholl, B.J., 2010. The wolfpack effect. Perception of animacy irresistibly influences interactive behavior. *Psychological science*, 21(12), pp.1845–53.

Gao, T., Newman, G.E. & Scholl, B.J., 2009. The psychophysics of chasing: A case study in the perception of animacy. *Cognitive psychology*, 59(2), pp.154–79.

Gentleman, R.C. et al., 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome biology*, 5(10), p.R80.

Gerlach, G. et al., 2008. Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings of the Royal Society of London B: Biological Sciences*, 275, pp.2165–2170.

Gerlach, G., 2006. Pheromonal regulation of reproductive success in female zebrafish: female suppression and male enhancement. *Animal Behaviour*, 72(5), pp.1119–1124.

Gerlach, G. & Lysiak, N., 2006. Kin recognition and inbreeding avoidance in zebrafish, Danio rerio, is based on phenotype matching. *Animal Behaviour*, 71, pp.1371–1377.

Gigerenzer, G., 1997. The modularity of social intelligence. In A. Whiten & R. W. Byrne, eds. *Machiavellian intelligence II: Extensions and evaluations*. Cambridge University Press, p. 264.

Godin, J.-G.J. & Smith, S.A., 1988. A fitness cost of foraging in the guppy. *Nature*, 333(6168), pp.69–71.

Goodson, J.L. & Kabelik, D., 2009. Dynamic limbic networks and social diversity in vertebrates: from neural context to neuromodulatory patterning. *Frontiers in Neuroendocrinology*, 30(4), pp.429–441.

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), pp.103–12.

Gori, S. et al., 2014. Do fish perceive illusory motion? *Scientific reports*, 4, p.6443.

Goto-kazeto, R. et al., 2004. Localization and expression of aromatase mRNA in adult zebrafish. General and comparative endocrinology, 139(1), pp.72–84.

Grafen, A., 1987. The logic of divisively asymmetric contests: respect for ownership and the desperado effect. *Animal Behaviour*, 35(2), pp.462–467.

Grant, J.W.A. & Kramer, D.L., 1992. Temporal clumping of food arrival reduces its monopolization and defence by zebrafish, Brachydanio rerio. *Animal Behaviour*, 44(1), pp.101–110.

Green, J. et al., 2012. Automated high-throughput neurophenotyping of zebrafish social behavior. *Journal of Neuroscience Methods*, 210(2), pp.266–271.

Greer, J.E., McGinn, M.J. & Povlishock, J.T., 2011. Diffuse traumatic axonal injury in the mouse induces atrophy, c-Jun activation, and axonal outgrowth in the axotomized neuronal population. *The Journal of neuroscience*, 31(13), pp.5089–5105.

Grosenick, L., Clement, T.S. & Fernald, R.D., 2007. Fish can infer social rank by observation alone. *Nature*, 445(7126), pp.429–32.

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*, 63(3), pp.229–240.

Hartl, D. et al., 2008. Transcriptome and proteome analysis of early embryonic mouse brain development. *Proteomics*, 8(6), pp.1257–1265.

Hawk, J.D. & Abel, T., 2011. The role of NR4A transcription factors in memory formation. *Brain Research Bulletin*, 85(1-2), pp.21–29.

Herget, U. et al., 2014. Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. *J Comp Neurol*, 522(7), pp.1542–1564.

Hess, J., Angel, P. & Schorpp-Kistner, M., 2004. AP-1 subunits: quarrel and harmony among siblings. *Journal of cell science*, 117(25), pp.5965–5973.

Heyes, C., 2012. What's social about social learning? *Journal of comparative psychology*, 126(2), pp.193–202.

Heyes, C.M., 1994. Social Learning in Animals: Categories and Mechanisms. *Biological Reviews*, 69(2), pp.207–231.

Hitti, F.L. & Siegelbaum, S. a, 2014. The hippocampal CA2 region is essential for social memory. *Nature*, 508(7494), pp.88–92.

Hoke, K.L., Ryan, M.J. & Wilczynski, W., 2005. Social cues shift functional connectivity in the hypothalamus. *Proceedings of the National Academy of Sciences of the United States of America*, 102(30), pp.10712–10717.

Hoppitt, W. & Laland, K.N., 2013. *Social learning: An introduction to mechanisms, methods, and models*, Princeton University Press.

Howe, K. et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, 496(7446), pp.498–503.

Huang, Y., Myers, S.J. & Dingledine, R., 1999. Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nature neuroscience*, 2(10), pp.867–872.

Humphrey, N.K., 1976. The Social Function of Intellect. In P. Bateson & R. Hinde, eds. *Growing points in ethology*. Cambridge: Cambridge University Press, pp. 303–317.

Ikemoto, S. & Panksepp, J., 1999. The role of nucleus accumbens dopamine in motivated behavior: A unifying interpretation with special reference to reward-seeking. *Brain Research Reviews*, 31(1), pp.6–41.

Jetti, S.K., Vendrell-Llopis, N. & Yaksi, E., 2014. Spontaneous Activity Governs Olfactory Representations in Spatially Organized Habenular Microcircuits. *Current Biology*, 24(4), pp.434–439.

Jia, J., Fernandes, Y. & Gerlai, R., 2014. Short-term memory in zebrafish (Danio rerio). *Behavioural brain research*, 270, pp.29–36.

Johnstone, R.A. & Bshary, R., 2004. Evolution of spite through indirect reciprocity. *Proceedings of the Royal Society of London B: Biological Sciences*, 271(1551), pp.1917–1922.

Johnstone, R.A., 2001. Eavesdropping and animal conflict. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16), pp.9177–80.

Kabra, M. et al., 2013. JAABA: interactive machine learning for automatic annotation of animal behavior. *Nature methods*, 10, pp.64–67.

Kala, K. et al., 2009. Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. *Development*, 136(2), pp.253–262.

Kalueff, A. V et al., 2013. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish*, 10(1), pp.70–86.

Kanehisa, M. et al., 2014. Data, information, knowledge and principle: Back to metabolism in KEGG. *Nucleic Acids Research*, 42(D1).

Kanwisher, N., McDermott, J. & Chun, M.M., 1997. The fusiform face area: a cortical region specialized for the perception of faces. *The Journal of neuroscience*, 17(11), pp.4302–4311.

Karnik, I. & Gerlai, R., 2012. Can zebrafish learn spatial tasks? An empirical analysis of place and single CS-US associative learning. *Behavioural Brain Research*, 233(2), pp.415–421.

Kelder, T. et al., 2009. Mining biological pathways using WikiPathways web services. *PLoS ONE*, 4(7).

Kendal, R.L. et al., 2005. Trade-Offs in the Adaptive Use of Social and Asocial Learning. *Advances in the Study of Behavior*, 35(05), pp.333–379.

Kendal, R.L., Coolen, I. & Laland, K.N., 2004. The role of conformity in foraging when personal and social information conflict. *Behavioral Ecology*, 15(2), pp.269–277.

Kishi, T. et al., 2006. Topographical projection from the hippocampal formation to the amygdala: A combined anterograde and retrograde tracing study in the rat. *Journal of Comparative Neurology*, 496(3), pp.349–368.

Kovács, K.J., 2008. Measurement of Immediate-Early Gene Activation-*c-fos* and Beyond. *Journal of Neuroendocrinology*, 20(6), pp.665–672.

Kurvers, R.H.J.M., Wolf, M. & Krause, J., 2014. Humans use social information to adjust their quorum thresholds adaptively in a simulated predator detection experiment. *Behavioral Ecology and Sociobiology*, 68(3), pp.449–456.

Lai, W.-S. et al., 2014. A new method for studying social eavesdropping using male golden hamsters. *Physiology & behavior*, 128, pp.202–211.

Laitinen, S. et al., 1999. Family of human oxysterol binding protein (OSBP) homologues. A novel member implicated in brain sterol metabolism. *Journal of lipid research*, 40(12), pp.2204–2211.

Lanahan, a & Worley, P., 1998. Immediate-early genes and synaptic function. *Neurobiology of learning and memory*, 70(1-2), pp.37–43.

Langley, C.M. et al., 1996. Visual search for natural grains in pigeons (Columba livia): Search images and selective attention. *Journal of Experimental Psychology: Animal Behavior Processes*, 22, p.139.

Lau, B.Y.B. et al., 2011. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proceedings of the National Academy of Sciences*, 108(6), pp.2581–2586.

Leadbeater, E. & Chittka, L., 2007. Social Learning in Insects - From Miniature Brains to Consensus Building. *Current Biology*, 17(16), pp.703–713.

Lee, H. et al., 2009. The neuronal expression of MYC causes a neurodegenerative phenotype in a novel transgenic mouse. *The American journal of pathology*, 174(3), pp.891–897.

Lefebvre, L. & Giraldeau, L.-A., 1996. Is Social Learning an Adaptive Specialization? In C. M. Heyes & B. G. Galef, eds. *Social Learning in Animals: The Roots of Culture*. Academic Press, pp. 107–128.

Levin, E.D., Bushnell, P.J. & Rezvani, A.H., 2011. Attention-modulating effects of cognitive enhancers. *Pharmacology Biochemistry and Behavior*, 99(2), pp.146–154.

Li, L. et al., 2005. The neuroplasticity-associated arc gene is a direct transcriptional target of early growth response (Egr) transcription factors. *Molecular and cellular biology*, 25(23), pp.10286–10300.

Lindeyer, C.M. & Reader, S.M., 2010. Social learning of escape routes in zebrafish and the stability of behavioural traditions. *Animal Behaviour*, 79(4), pp.827–834.

Lucon-Xiccato, T. & Dadda, M., 2014. Assessing memory in zebrafish using the one-trial test. *Behavioural processes*, 106, pp.1–4.

Luo, W. et al., 2009. GAGE: generally applicable gene set enrichment for pathway analysis. *BMC bioinformatics*, 10, p.161.

Maaswinkel, H., Zhu, L. & Weng, W., 2013. Assessing Social Engagement in Heterogeneous Groups of Zebrafish: A New Paradigm for Autism-Like Behavioral Responses. *PLoS ONE*, 8(10).

Maciejewski, H., 2014. Gene set analysis methods: Statistical models and methodological differences. *Briefings in Bioinformatics*, 15(4), pp.504–518.

Marler, P., Dufty, A. & Pickert, R., 1986. Vocal communication in the domestic chicken: II. Is a sender sensitive to the presence and nature of a receiver? *Animal Behaviour*, 34, pp.194–198.

Marquer, C. et al., 2014. Increasing membrane cholesterol of neurons in culture recapitulates Alzheimer ' s disease early phenotypes. *Molecular neurodegeneration*, 9(1), pp.1–13.

Mascalzoni, E., Regolin, L. & Vallortigara, G., 2010. Innate sensitivity for self-propelled causal agency in newly hatched chicks. *Proceedings of the National Academy of Sciences of the United States of America*, 107(9), pp.4483–4485.

Mathelier, A. et al., 2014. JASPAR 2014: An extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic Acids Research*, 42(D1).

Matos, R.J., Peake, T.M. & McGregor, P.K., 2003. Timing of presentation of an audience: Aggressive priming and audience effects in male displays of Siamese fighting fish (Betta splendens). *Behavioural Processes*, 63(1), pp.53–61.

Matzel, L.D. & Kolata, S., 2010. Selective attention, working memory, and animal intelligence. *Neuroscience and biobehavioral reviews*, 34(1), pp.23–30.

McClintock, T.S. et al., 2008. Tissue expression patterns identify mouse cilia genes. *Physiological genomics*, 32(2), pp.198–206.

McElreath, R., 2003. Reputation and the evolution of conflict. *Journal of theoretical biology*, 220(3), pp.345–357.

McGregor, P.K., 2005. *Animal Communication Networks.* P. K. McGregor, ed., Cambridge University Press, Cambridge.

Mcgregor, P.K., 2000. Playback experiments : design and analysis. *Acta ethologica*, 3(1), pp.3–8.

McGregor, P.K., 1993. Signalling in Territorial Systems: A Context for Individual Identification, Ranging and Eavesdropping. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 340(1292), pp.237–244.

McGregor, P.K. & Dabelsteen, T., 1996. Communication networks. In D.E. Kroodsma & E.H. Miller, eds. *Ecology and Evolution of Acoustic Communication in Birds*. New York, NY, US: Cornell University Press, pp. 409–425.

McGregor, P.K., Dabelsteen, T. & Holland, J., 1997. Eavesdropping in a territorial songbird communication network: preliminary results. *Bioacoustics*, 8, pp.253–254.

McGregor, P.K. & Peake, T.M., 2000. Communication networks: social environments for receiving and signalling behaviour. *Acta ethologica*, 2(2), pp.71–81.

McGregor, P.K., Peake, T.M. & Lampe, H.M., 2001. Fighting fish Betta splendens extract relative information from apparent interactions: what happens when what you see is not what you get. *Animal Behaviour*, 62, pp.1059–1065.

Mennill, D.J., Ratcliffe, L.M. & Boag, P.T., 2002. Female eavesdropping on male song contests in songbirds. *Science*, 296, p.873.

Mesterton-Gibbons, M. & Sherratt, T., 2007. Social eavesdropping: a game-theoretic analysis. *Bulletin of Mathematical Biology*, 69(4), pp.1255–1276.

Milinski, M., 1984. A predator's costs of overcoming the confusion-effect of swarming prey. *Animal Behaviour*, 32(4), pp.1157–1162.

Miller, N. & Gerlai, R., 2007. Quantification of shoaling behaviour in zebrafish (Danio rerio). *Behavioural brain research*, 184(2), pp.157–166.

Mosher, C.P., Zimmerman, P.E. & Gothard, K.M., 2014. Neurons in the Monkey Amygdala Detect Eye Contact during Naturalistic Social Interactions. *Current Biology*, 24(20), pp.2459–2464.

Mueller, T., 2012. What is the Thalamus in Zebrafish? *Frontiers in Neuroscience*, 6, pp.1–14.

Mueller, T. & Wullimann, M.F., 2009. An evolutionary interpretation of teleostean forebrain anatomy. *Brain, Behavior and Evolution*, 74(1), pp.30–42.

Munger, S.D. et al., 2010. An olfactory subsystem that detects carbon disulfide and mediates food-related social learning. *Current biology*, 20(16), pp.1438–44.

Muroyama, Y. et al., 2005. Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature*, 438(7066), pp.360–363.

Murray, E.A., 2007. The amygdala, reward and emotion. *Trends in Cognitive Sciences*, 11(11), pp.489–497.

Muto, A. et al., 2013. Real-Time Visualization of Neuronal Activity during Perception. *Current Biology*, 23(4), pp.307–311.

Nagoshi, E. et al., 2010. Dissecting differential gene expression within the circadian neuronal circuit of Drosophila. *Nature neuroscience*, 13(1), pp.60–68.

Naguib, M., Fichtel, C. & Todt, D., 1999. Nightingales respond more strongly to vocal leaders of simulated dyadic interactions. *Proceedings of the Royal Society B: Biological Sciences*, 266(1419), p.537.

Naguib, M. & Todt, D., 1997. Effects of dyadic vocal interactions on other conspecific receivers in nightingales. *Animal behaviour*, 54(6), pp.1535–1543.

Nakayasu, T. & Watanabe, E., 2014. Biological motion stimuli are attractive to medaka fish. *Animal cognition*, 17(3), pp.559–75.

Naumann, E.A. et al., 2010. Monitoring neural activity with bioluminescence during natural behavior. *Nature neuroscience*, 13(4), pp.513–520.

Neri, P., 2012. Feature binding in zebrafish. *Animal Behaviour*, 84, pp.485–493.

Norton, W. & Bally-Cuif, L., 2010. Adult zebrafish as a model organism for behavioural genetics. *BMC neuroscience*, 11(1), p.90.

Nowak, M.A. & Sigmund, K., 1998. Evolution of indirect reciprocity by image scoring. *Nature*, 393(6685), pp.573–577.

Nowak, M.A. & Sigmund, K., 2005. Evolution of indirect reciprocity. *Nature*, 437(7063), pp.1291–1298.

O'Connell, L.A. & Hofmann, H.A., 2012. Evolution of a Vertebrate Social Decision-Making Network. *Science*, 336(6085), pp.1154–1157.

O'Connell, L.A. & Hofmann, H.A., 2012. Social Status Predicts How Sex Steroid Receptors Regulate Complex Behavior across Levels of Biological Organization. *Endocrinology*, 153(3), pp.1341–1351.

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), pp.3599–3639.

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), pp.386–394.

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

Oliveira, R. et al., 2001. Watching fights raises fish hormone levels. *Nature*, 409(6819), p.475.

Oliveira, R.F. et al., 2000. Considerations on the use of video playbacks as visual stimuli : the Lisbon workshop consensus. *Acta ethologica*, 3(1), pp.61–65.

Oliveira, R.F., 2013. Mind the fish: zebrafish as a model in cognitive social neuroscience. *Frontiers in neural circuits*, 7, pp.1-15.

Oliveira, R.F., McGregor, P.K. & Latruffe, C., 1998. Know thine enemy: fighting fish gather information from observing conspecific interactions. *Proceedings of the Royal Society B: Biological Sciences*, 265(1401), pp.1045–1049.

Oliveira, R.F., Silva, J.F. & Simões, J.M., 2011. Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. *Zebrafish*, 8(2), pp.73–81.

Olsson, A. & Phelps, E.A., 2007. Social learning of fear. *Nature neuroscience*, 10(9), pp.1095–1102.

Ophir, A.G. & Galef, B.G., 2003. Female Japanese quail that "eavesdrop" on fighting males prefer losers to winners. *Animal Behaviour*, 66, pp.399–407.

Otter, K. et al., 1999. Do female great tits (Parus major) assess males by eavesdropping? A field study using interactive song playback. *Proceedings of the Royal Society B: Biological Sciences*, 266(1426), p.1305.

Parker, M.O. et al., 2012. Discrimination reversal and attentional sets in zebrafish (Danio rerio). *Behavioural Brain Research*, 232(1), pp.264–268.

Paull, G.C. et al., 2010. Dominance hierarchies in zebrafish (Danio rerio) and their relationship with reproductive success. *Zebrafish*, 7(1), pp.109–117.

Pavlidis, M. et al., 2011. Adaptive changes in zebrafish brain in dominant-subordinate behavioral context. *Behavioural Brain Research*, 225, pp.529–537.

Pavlidis, M. et al., 2013. Husbandry of zebrafish, Danio rerio, and the cortisol stress response. *Zebrafish*, 10(4), pp.524–31.

Peake, T. et al., 2002. Do great tits assess rivals by combining direct experience with information gathered by eavesdropping? *Proceedings of the Royal Society B: Biological Sciences*, 269(1503), pp.1925–1929.

Peake, T.M., 2005. Eavesdropping in communication networks. In P. K. Mcgregor, ed. *Animal communication networks*. Cambridge: Cambridge University Press, Cambridge, pp. 13–37.

Peake, T.M. et al., 2001. Male great tits eavesdrop on simulated male-to-male vocal interactions. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1472), pp.1183–1187.

Peake, T.M. et al., 2005. Song type matching, song type switching and eavesdropping in male great tits. *Animal Behaviour*, 69(5), pp.1063–1068.

Peake, T.M., Matos, R.J. & McGregor, P.K., 2006. Effects of manipulated aggressive "interactions" on bystanding male fighting fish, Betta splendens. *Animal Behaviour*, 72(5), pp.1013–1020.

Peake, T.M. & McGregor, P.K., 2004. Information and aggression in fishes. *Animal Learning & Behavior*, 32(1), pp.114–121.

Pereira, A.G. et al., 2012. Silence resulting from the cessation of movement signals danger. *Current biology*, 22(16), pp.R627–R628.

Pérez-Escudero, A. et al., 2014. idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature methods*, 11(7), pp.743–748.

Pinto, A. et al., 2011. Cleaner wrasses labroides dimidiatus are more cooperative in the presence of an audience. *Current Biology*, 21(13), pp.1140–1144.

Pita, D. et al., 2015. Vision in two cyprinid fish: implications for collective behavior. *PeerJ*, 3, p.e1113.

Plath, M. et al., 2008. Male Fish Deceive Competitors about Mating Preferences. *Current Biology*, 18(15), pp.1138–1141.

Ploski, J.E. et al., 2011. The neuronal PAS domain protein 4 (Npas4) is required for new and reactivated fear memories. *PLoS ONE*, 6(8).

Popov, N. et al., 2007. Fbw7 and Usp28 regulate Myc protein stability in response to DNA damage. *Cell Cycle*, 6(19), pp.2327–2331.

Portavella, M., Torres, B. & Salas, C., 2004. Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. *Journal of Neuroscience*, 24(9), pp.2335–2342.

Portavella, M., et al., 2002. The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. *Brain research bulletin*, 57(3), 397-399.

Possner, M. et al., 2008. Functional analysis of NKX3.1 in LNCaP prostate cancer cells by RNA interference. *International journal of oncology*, 32(4), pp.877–884.

Potter, C.S. et al., 2006. Evidence that the satin hair mutant gene Foxq1 is among multiple and functionally diverse regulatory targets for Hoxc13 during hair follicle differentiation. *Journal of Biological Chemistry*, 281(39), pp.29245–29255.

Pritchard, V.L. et al., 2001. Shoal choice in zebrafish, Danio rerio: the influence of shoal size and activity. *Animal Behaviour*, 62(6), pp.1085-1088.

Qin, M. et al., 2014. Induction of social behavior in zebrafish: live versus computer animated fish as stimuli. *Zebrafish*, 11(3), pp.185–97.

R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramamoorthi, K. et al., 2011. Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science*, 334(6063), pp.1669–75.

Rittschof, C.C. et al., 2014. Neuromolecular responses to social challenge: Common mechanisms across mouse, stickleback fish, and honey bee. *Proceedings of the National Academy of Sciences*, 111(50), pp.17929–17934.

Roberts, A.C., Bill, B.R. & Glanzman, D.L., 2013. Learning and memory in zebrafish larvae. *Frontiers in neural circuits*, 7, p.126.

Robinson, G.E., Fernald, R.D. & Clayton, D.F., 2008. Genes and social behavior. *Science*, 322(5903), pp.896–900.

Rochat, P., Morgan, R. & Carpenter, M., 1997. Young infants' sensitivity to movement information specifying social causality. *Cognitive Development*, 12(4), pp.537–561.

Rochat, P., Striano, T. & Morgan, R., 2004. Who is doing what to whom? Young infants' developing sense of social causality in animated displays. *Perception*, 33(3), pp.355–369.

Rosa Salva, O., Sovrano, V.A. & Vallortigara, G., 2014. What can fish brains tell us about visual perception? *Frontiers in Neural Circuits*, 8, pp.1–15.

Rosenthal, G.G., 2000. Design considerations and techniques for constructing video stimuli. *Acta ethologica*, 3(1), pp.49–54.

Rosenthal, G.G. & Ryan, M.J., 2005. Assortative preferences for stripes in danios. *Animal Behaviour*, 70, pp.1063–1066.4

Rosenthal, S.B. et al., 2015. Revealing the hidden networks of interaction in mobile animal groups allows prediction of complex behavioral contagion. *Proceedings of the National Academy of Sciences*, 112(15), pp.4690–4695.

Rutte, C., Taborsky, M. & Brinkhof, M.W.G., 2006. What sets the odds of winning and losing? *Trends in Ecology and Evolution*, 21(1), pp.16–21.

Saif, M. et al., 2013. Sight of conspecific images induces changes in neurochemistry in zebrafish. *Behavioural brain research*, 243, pp.294-299.

Saverino, C. & Gerlai, R., 2008. The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural brain research*, 191(1), pp.77–87.

Scholl, B.J., 2001. Objects and attention: the state of the art. *Cognition*, 80(1), pp.1–46.

Schwartz, P.T. et al., 2000. Pancreatic homeodomain transcription factor IDX1/IPF1 expressed in developing brain regulates somatostatin gene transcription in embryonic neural cells. *Journal of Biological Chemistry*, 275(25), pp.19106–19114.

Sevenster, P. & Rowland, W.J., 1985. Sign Stimuli in the Threespine Stickleback (Gasterosteus Aculeatus): a Re-Examination and Extension of Some Classic Experiments. *Behaviour*, 93(1), pp.241–257.

Shettleworth, S.J., 1999. Perception and Attention. In *Cognition, Evolution and Behaviour*. Oxford University Press, pp. 57–95.

Shettleworth, S.J., 1993. Varieties of learning and memory in animals. *Journal of Experimental Psychology: Animal Behavior Processes*, 19, pp.5–14.

Singewald, G.M. et al., 2011. The Modulatory Role of the Lateral Septum on Neuroendocrine and Behavioral Stress Responses. *Neuropsychopharmacology*, 36(4), pp.793–804.

Sinha, S., van Nimwegen, E. & Siggia, E.D., 2003. A probabilistic method to detect regulatory modules. *Bioinformatics*, 19(suppl 1), pp.i292–i301.

Sison, M. & Gerlai, R., 2010. Associative learning in zebrafish (Danio rerio) in the plus maze. *Behavioural brain research*, 207(1), pp.99–104.

Slocombe, K.E. & Zuberbu, K., 2007. Chimpanzees modify recruitment screams as a function of audience composition. *Proceedings of the National Academy of Sciences*, 104(43), pp.17228-17233.

Sneddon, L. et al., 2011. Molecular Correlates of Social Dominance: A Novel Role for Ependymin in Aggression. *PLoS ONE*, 6(4), p.e18181.

Spence, R., 2006. Mating preference of female zebrafish, Danio rerio, in relation to male dominance. *Behavioral Ecology*, 17(5), pp.779–783.

Spence, R. et al., 2008. The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews*, 83(1), pp.13–34.

Spence, R., 2011. Zebrafish ecology and behaviour. In A.V. Kalueff & J.M. Cachat, eds. *Zebrafish Models in Neurobehavioral Research*. Humana Press, pp. 1–46.

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, p.5.

Stewart, A.M. et al., 2014. Zebrafish models for translational neuroscience research: From tank to bedside. *Trends in Neurosciences*, 37, pp.264–278.

Strekalova, T. et al., 2003. Memory retrieval after contextual fear conditioning induces c-Fos and JunB expression in CA1 hippocampus. *Genes, brain, and behavior*, 2(1), pp.3–10.

Suboski, M.D. 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), pp.101–112.

Sumbre, G. & De Polavieja, G.G., 2014. The world according to zebrafish : How neural circuits generate behaviour. *Frontiers in Neural Circuits*, 8, p.91.

Takayanagi, Y. et al., 2005. Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, 102(44), pp.16096–16101.

Tanaka, M., Lyons, G.E. & Izumo, S., 1999. Expression of the Nkx3.1 homobox gene during pre and postnatal development. *Mechanisms of Development*, 85(1), pp.179–182.

Tappeiner, C. et al., 2012. Visual acuity and contrast sensitivity of adult zebrafish. *Frontiers in Zoology*, 9(1), p.10.

Tariq, H., Niaz, T. & Nadeem, S., 2002. *Bioanalyzer: A Software Package for Sequence Analysis and Manipulation an Application Software*, LAP Lambert Academic Publishing.

Teles, M.C. et al., 2013. Social modulation of brain monoamine levels in zebrafish. *Behavioural brain research*, 253, pp.17–24.

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. *Proc. R. Soc. B*, 282, p.20151099.

Temeles, E.J., 1994. The role of neighbours in territorial systems: When are they “dear enemies”? *Animal Behaviour*, 47, pp.339–350.

Terao, A. et al., 2003. Region-specific changes in immediate early gene expression in response to sleep deprivation and recovery sleep in the mouse brain. *Neuroscience*, 120(4), pp.1115–1124.

Tibbetts, E.A. & Dale, J., 2007. Individual recognition: it is good to be different. *Trends in Ecology and Evolution*, 22, pp.529–537.

Tinbergen, N., 1948. Social releasers and the experimental method required for their study. *The Wilson Bulletin*, pp.6–51.

Tinbergen, N., 1951. *The study of instinct.*, New York, NY, US: Clarendon Press/Oxford University Press.

Tremoulet, P.D. & Feldman, J., 2000. Perception of animacy from the motion of a single object. *Perception*, 29, pp.943–951.

Tribioli, C. & Lufkin, T., 1999. The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development*, 126(24), pp.5699–5711.

Trivers, R.L., 1971. The Evolution of Reciprocal Altruism. *The Quarterly Review of Biology*, 46(1), pp.35–57.

von Trotha, J.W., Vernier, P. & Bally-Cuif, L., 2014. Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. *The European Journal of Neuroscience*, 40(9), pp.3302–3315.

Tsao, D.Y., Moeller, S. & Freiwald, W. a, 2008. Comparing face patch systems in macaques and humans. *Proceedings of the National Academy of Sciences of the United States of America*, 105(49), pp.19514–19519.

von Uexküll, J., 1934. *Streifzüge durch die Umwelten von Tieren und Menschen*, Verlag von Julius Springer, Berlin.

Valone, T.J., 2007. From eavesdropping on performance to copying the behavior of others: A review of public information use. *Behavioral Ecology and Sociobiology*, 62(1), pp.1–14.

Vignet, C. et al., 2013. Systematic screening of behavioral responses in two zebrafish strains. *Zebrafish*, 10(3), pp.365–75.

Vital, C. & Martins, E., 2011. Strain differences in zebrafish (Danio rerio) social roles and their impact on group task performance. *Journal of Comparative Psychology*, 125(3), p.278.

Vital, C. & Martins, E.P., 2013. Socially-Central Zebrafish Influence Group Behavior More than Those on the Social Periphery. *PLoS ONE*, 8(1).

Wang, L., Wang, R. & Herrup, K., 2007. E2F1 works as a cell cycle suppressor in mature neurons. *The Journal of neuroscience*, 27(46), pp.12555–12564.

Wang, P. et al., 2009. Nkx3.1 and p27(KIP1) cooperate in proliferation inhibition and apoptosis induction in human androgen-independent prostate cancer cells. *Cancer investigation*, 27(4), pp.369–375.

Wedekind, C. & Milinski, M., 2000. Cooperation through image scoring in humans. *Science*, 288(5467), pp.850–852.4

White, D. & Galef jr BG, 1999. Mate choice copying and conspecific cueing in Japanese quail, Coturnix coturnix japonica. *Animal behaviour*, 57(2), pp.465–473.

White, D.J., 2004. Influences of social learning on mate-choice decisions. *Learning & behavior*, 32(1), pp.105–113.

Wiley, R.H., 2013. Specificity and multiplicity in the recognition of individuals: Implications for the evolution of social behaviour. *Biological Reviews*, 88(1), pp.179–195.

Wilkinson, A. et al., 2010. Social learning in a non-social reptile (Geochelone carbonaria). *Biology letters*, 6(5), pp.614–6.

Winslow, J.T. et al., 2000. Social amnesia in mice lacking the oxytocin gene. *Nature Genetics*, 25(3), pp.284–288.

Winters, S., Dubuc, C. & Higham, J.P., 2015. Perspectives: The Looking Time Experimental Paradigm in Studies of Animal Visual Perception and Cognition. *Ethology*, 121(7), pp.625–640.

Wong, K. et al., 2010. Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural brain research*, 208(2), pp.450–7.

Woo, K.L. & Rieucau, G., 2011. From dummies to animations: A review of computer-animated stimuli used in animal behavior studies. *Behavioral Ecology and Sociobiology*, 65(9), pp.1671–1685.

Wu, Q. & Maniatis, T., 1999. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell*, 97(6), pp.779–790.

Wulliman, M.F., Rupp, B. & Reichert, H., 2012. *Mario F., Barbara Rupp, and Heinrich Reichert. Neuroanatomy of the zebrafish brain: a topological atlas.*, Birkhäuser.

Wullimann, M.F. & Mueller, T., 2004. Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. *Journal of Comparative Neurology*, 478(4), pp.427–428.

Yang, E.-J. & Wilczynski, W., 2007. Social experience organizes parallel networks in sensory and limbic forebrain. *Developmental Neurobiology*, 67(3), pp.285–303.

Yang, H. et al., 2008. Basic fibroblast growth factor-induced neuronal differentiation of mouse bone marrow stromal cells requires FGFR-1, MAPK/ERK, and transcription factor AP-1. *The Journal of biological chemistry*, 283(9), pp.5287–5295.

Zearfoss, N.R. et al., 2008. A molecular circuit composed of CPEB-1 and c-Jun controls growth hormone-mediated synaptic plasticity in the mouse hippocampus. *The Journal of neuroscience*, 28(34), pp.8502–8509.

Zhao, M. et al., 1999. Regulation of the MEF2 family of transcription factors by p38. *Molecular and cellular biology*, 19(1), pp.21–30.

Zhao, T. et al., 2014. β-catenin regulates Pax3 and Cdx2 for caudal neural tube closure and elongation. *Development*, 141(1), pp.148–157.

Ziege, M. et al., 2009. Audience effects in the Atlantic molly (Poecilia mexicana)-prudent male mate choice in response to perceived sperm competition risk? *Frontiers in zoology*, 6, p.17.

Ziv, L. et al., 2012. An affective disorder in zebrafish with mutation of the glucocorticoid receptor. *Molecular Psychiatry*, 18(6), pp.1–11.

Zuberbühler, K., 2008. Audience effects. *Current Biology*, 18(5), pp.189–190.

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