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Socially driven changes in neural and behavioural plasticity in zebrafish

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NOTA PRÉVIA

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Resumo

Cada espécie apresenta uma variedade de comportamentos específicos (repertório comportamental) que evoluíram de maneira adaptativa de forma a integrar o comportamento com o meio ambiente onde os animais estão inseridos. De um modo geral, a maioria dos animais vive em ambientes sociais, sendo que as suas ações afectam e são afectadas pela atividade de outros. Mesmo em ambientes sociais muito simples com um número de indivíduos reduzido, o comportamento exibido por um indivíduo vai induzir uma resposta comportamental noutro. Dependendo do número de indivíduos que constituem o grupo, graus de complexidade vão sendo acrescentados à interação entre os mesmos. Assim sendo, grupos com maiores dimensões representam tipicamente ambientes socais mais complexos, uma vez que mais interações com parceiros sociais diferentes são mais prováveis de acontecer.

À capacidade que os indivíduos têm para alterarem o seu comportamento de modo a optimizarem as suas relações sociais nestes grupos chamamos de competência social, e esta implica a capacidade de identificarem pistas sociais no ambiente e produzirem uma resposta comportamental apropriada (Plasticidade social). Assim sendo, a plasticidade comportamental assenta na flexibilidade do comportamento, que é caracterizada por uma variação da resposta comportamental ao mesmo estímulo, e encontra-se dependente de processos cognitivos como a aquisição, retenção e uso de informação pública disponível.

Vários exemplos de competência social, onde os animais extraem informação do ambiente e alteraram a sua resposta comportamental com base na informação adquirida foram descritos na natureza: (1) os animais podem observar interações entre terceiros, e recolher informação que usam posteriormente em encontros futuros (*social eavesdropping*); (2) ou servir-se da informação recolhida sobre relações conhecidas para

inferir relações desconhecidas (inferência transitiva); (3) a presença destes observadores, pode também por sua vez influenciar a interação observada num fenómeno designado por efeito de audiência; (4) a familiaridade *vs* não familiaridade entre os animais também influencia as interações sociais num fenómeno designado por "efeito querido inimigo" onde a familiaridade promove uma redução da agressão contra vizinhos, redução esta que não é observada contra estranhos; (5) por último, um efeito de experiência social, onde eventos passados influenciam acontecimentos futuros num efeito de vencedor e derrotado.

Após uma interação agonística normalmente surge um vencedor e um derrotado e estes estatutos sociais vão influenciar interações futuras de maneira que o vencedor da primeira interação tem maior probabilidade de vencer uma segunda, enquanto que o derrotado tem maior probabilidade de a perder. Curiosamente, em interações sociais onde a informação sobre o estatuto não é definida, como por exemplo, interações não resolvidas quando os animais lutam contra a sua própria imagem no espelho, a mudança de estatuto social (para vencedor ou derrotado) não ocorre, demonstrando a relevância que a informação social tem nas decisões de alteração de comportamento.

De acordo com o que foi descrito anteriormente, um único genótipo pode então ser modulado socialmente dando origem a múltiplos fenótipos comportamentais. Esta plasticidade comportamental, depende naturalmente de uma plasticidade a nível dos circuitos neurais subjacentes ao comportamento social. Os mecanismos neurais implícitos à plasticidade comportamental podem atuar de duas formas: (1) provocando alterações estruturais nos circuitos, que conduzem a mudanças comportamentais que ocorrem lentamente e são duradouras; (2) ou modulando bioquimicamente a atividade neural, provocando alterações comportamentais significativamente mais rápidas, mas contudo transientes. Recentemente foi proposto que esta modulação ocorre a nível da

social decison-making (SDM) *network* que integra um conjunto de núcleos neurais responsáveis pela regulação de comportamentos sociais (agressão, corte ou comportamento parental) com núcleos do sistema mesolímbico de recompensa que avaliam a saliência do estímulo através de uma via de sinalização dopaminérgica. Todas estas áreas estão reciprocamente ligadas, contêm receptores para hormonas esteróides e neuromoduladores, e todas elas respondem individualmente à exibição de comportamentos sociais. A hipótese subjacente a esta rede neural é que a informação é codificada de forma dinâmica, de tal forma que determinado perfil comportamental parece ser melhor explicado pelo perfil de ativação da rede na sua globalidade, do que pela atividade individual de cada nódulo. Conceptualmente, a pluralidade de combinações possíveis de ativação dos diferentes nódulos, poderá explicar a diversidade de comportamentos exibida entre espécies, entre indivíduos da mesma espécie e dentro do mesmo individuo, uma vez que esta rede é evolutivamente conservada.

Esta tese tem como principais objectivos o estudo e a possível identificação dos mecanismos proximais subjacentes à flexibilidade comportamental numa perspectiva integrativa. Para tal usámos como espécie modelo o peixe-zebra, que são animais altamente sociais, que vivem em cardumes com relações sociais bem estruturadas, tais como hierarquias de dominância e territorialidade.

No paradigma comportamental utilizado os animais foram expostos a diferentes tipos de experiências sociais: 1) interações com oponentes reais que deram origem a vencedores e derrotados, que consequentemente ajustaram o seu reportório comportamental ao novo estatuto social, e 2) lutas com espelhos, interações não resolvidas onde a expressão do comportamentos agressivos foi dissociada da experiência de vitória ou derrota. Como os peixes não reconhecem a sua própria imagem no espelho, atacam-na como se esta fosse um intruso. No entanto, como o

comportamento de submissão nunca é expresso pelo oponente, ou seja, a imagem do espelho reproduz o comportando agressivo do animal focal, o indivíduo focal nunca experiencia uma vitória ou derrota. Animais que não interagiam socialmente, i.e., que se encontravam em isolamento visual mas não químico, foram utilizados como grupo controlo.

Deste modo, o paradigma experimental utilizado para além de permitir o estudo da flexibilidade comportamental, ou seja, de diferenças específicas observadas em vencedores e derrotados aquando da comparação com o grupo controlo, permite ainda estudar o tipo de informação utilizada para esta alteração de comportamento, comparando os animais que lutam com o espelho com vencedores e derrotados, ou seja, *self- assessment*, *opponent-only assessment ou mutual assessment*, caso o mecanismo utilizado seja uma integração entre os dois modelos, num balanço entre comportamento exibido pelo próprio e pelo oponente.

No primeiro conjunto de experiências estudámos a influência dos neuromoduladores nos mecanismos de plasticidade, e para tal caraterizámos a resposta endócrina a desafios sociais (Capítulo II) e a modulação social de monoaminas (Capítulo III) e de nonapéptidos (Capítulo IV) no cérebro. Este conjunto de trabalhos identificou uma resposta dos androgénios nas lutas com oponentes reais, tanto em vencedores como em derrotados, assim como uma ativação na produção dos glucocorticóides (cortisol). No Capítulo III referente ao estudo de monoaminas, associámos o estatuto de vencedor a um aumento de atividade serotonérgica e dopaminérgica no Telencéfalo, sugerindo que o mecanismo de recompensa poderá estar envolvido na alteração de estatuto social em vencedores. No estudo do Capítulo IV, referente à variação nonapeptídica após interações sociais verificámos que a arginina-vasotocina respondeu de forma mais generalizada no grupo dos derrotados do que a

isotocina, apontando para a relevância deste péptido na regulação do comportamento agressivo em peixe-zebra, como já tinha sido descrito para outras espécies. Curiosamente, nenhum destes sistemas foi ativado em resposta à luta com o espelho, apesar deste animais apresentarem níveis de agressão semelhantes àqueles expressos pelos vencedores, apontado para o papel fundamental que a percepção social tem em transições entre diferentes estados comportamentais.

A nível neuromolecular testámos a hipótese da *social decision-making network*, contrastando alterações entre funcionalidade localizada e conectividade dentro desta rede, em resposta a alterações do estatuto social (Capítulo V) e terminámos com a caraterização de genes-chave envolvidos nos diferentes mecanismos de plasticidade (Capítulo VI).

Os dados apresentados no Capítulo V, sustentam a hipótese da *social decision-making network*, dando um suporte funcional para uma atividade em rede, ao invés da atividade específica de nodos individuais. Tendo em consideração os padrões de actividade neural conseguimos distinguir vencedores, derrotados e animais que lutaram contra a sua própria imagem no espelho.

Os resultados obtidos no Capítulo VI mostram que cada estado comportamental é caracterizado por um padrão neuromolecular de expressão de genes associados a diferentes mecanismos de plasticidade neural. Tal como ocorreu a nível fisiológico, também a nível neuromolecular os animais que lutam com o espelho apresentam padrões neurais distintos de vencedores e derrotados, indicando uma vez mais a importância a importância da percepção social na alteração de fenótipos comportamentais.

Palavras-chave: Comportamento Social, Plasticidade Social, Hormonas Esteróides, Monoaminas, Nonapéptidos, Neurogenómica, Peixe-zebra

Abstract

Social competence, the ability of individuals to regulate the expression of their social behaviour in order to optimize their social relationships in a group, is especially benefic for individuals living in complex social environments, and implies the ability to perceive social cues and produce appropriate behavioural output responses (Social Plasticity). Numerous examples of social competence can be found in nature, where individuals extract social information from the environment, and change their behavioural response based on the collected information. At the neuronal level, two major plasticity mechanisms have been proposed to underlie social plasticity, structural reorganization and biochemical switching of the neuronal networks underlying behaviour. The neural substrate for behavioural plasticity has been identified as the social decision-making (SDM) network, such that the same neural circuitry may underlie the expression of different behaviours depending on social context. The goal of this work is to study the proximate mechanism underlying behavioural flexibility in the context of experience-dependent behavioural shifts, in an integrative framework. For this purpose we exposed male zebrafish to two types of social interactions: (1) real-opponent interactions, from which a Winner and Loser emerged; and (2) Mirror-elicited interactions, that produced individuals that did not experience a change in social status, despite expressing similar levels of aggressive behaviour to those participating in real-opponent fights. In a first set of experiments, we studied the influence of neuromodulators on social plasticity mechanisms, by characterizing the endocrine response to social challenges, as well as the social modulation of brain monoamines and nonapeptides. Next we tested the SDM network hypothesis by contrasting changes in functional localization vs. connectivity across this network. Finally we characterized changes in expression of key genes for different neuroplasticity mechanisms in response

to changes in social status. Our research suggests different social plasticity mechanisms underlying Winners and Losers both at physiological and molecular levels, for Mirror-fighters, where the experience of winning or losing was decoupled for the fighting experience, few changes were detected. This, by itself suggests a pivotal role of social perception in triggering shifts between socially driven behavioural states.

Keywords: Social plasticity, Social decision-making network, steroid hormones, Monoamines, Nonapeptides, Neurogenomic, Zebrafish

Contents

Chapter I

General Introduction

1.1. The social brain

Animal species present a variety of species-specific behaviours (behavioural repertoires) that evolved in an adaptive way in order to integrate behaviour with the natural environment, and that will ultimately dictate the Darwinian fitness of individuals.

In broad terms, the vast majority of animals live in social environments, and their lives are affected by the presence and activity of others around them. In the most simplified environment, the behaviour expressed by an individual will induce a response in another, and depending on group size, layers of complexity will be added to the relationship between individuals and third parties. This way, larger groups will typically represent a more complex social environment than smaller groups, since more interactions with different social partners will be more likely to occur in the later one (Taborsky & Oliveira 2012).

The social brain hypothesis (SBH) proposed by Dunbar (1998) posits that the complexity of the social environment is one of the main driving forces for brain evolution. This hypothesis is supported by comparative data in primates and ungulates where relative brain size covaries with group size (Pérez-Barbería & Gordon 2005; Dunbar & Shultz 2007) linking brain size to high cognitive demands of more complex social interactions. There has been an attempt to generalize this hypothesis to all vertebrate taxa, however the relationship for other species was qualitative rather than quantitative, and exclusively associated with monogamy (Dunbar & Shultz 2007), one of the dimensions of sociality.

In a cooperative breeding cichlid, *Neolamprologus pulcher*, it was recently showed that the relative brain size (specific macroareas) was affected by group-size rearing, for instance, hypothalamus and cerebellum increased in fish reread in large groups, whereas

the optic tectum was bigger in fish reared in small groups, which may indicate some degree of specialization for each brain area (Fischer et al. 2015). It is also important to stress out that total brain size was unaffected, presenting the first experimental evidence for "mosaic evolution" where selective pressures act on individual brain parts (de Winter & Oxnard 2001) over "concerted evolution" where the overall brain size is selected (Finlay & Darlington 1995). At the behavioural levels, group complexity also affected the establishment of social hierarchies, where fish reared in large groups showed more submissive and less aggressive behaviour towards larger conspecifics. This behavioural response increases the chances of being tolerated in the territory of a larger dominant conspecific, which greatly enhances the survival chances under natural conditions, suggesting a better ability to cope with social challenges (Fischer et al. 2015). On the other hand, in guppies artificially selected for brain size it affected survival rate under a predation threat. Large-brained females had higher survival compared to small-brained females (Kotrschal et al. 2015), and in a predator inspection task, large-brained fish spend less time performing inspections, and group size affected the distance kept from the predator which may indicate a cognitive advantage for larger brains (van der Bijl et al. 2015).

According to this framework, the complexity of the social environment drives brain evolution and consequently cognitive abilities, which ultimately enhance fitness.

Social competence, the ability of individuals to regulate the expression of their social behaviour in order to optimize their social relationships in a group (Taborsky & Oliveira 2012) will be especially beneficial for individuals living in complex social environments, and implies the ability to perceive social cues, and produce the appropriate behaviour output response (social plasticity). Therefore, social plasticity relies on behavioural flexibility, that is variation in the behavioural response to the same

stimulus that will often depend on cognitive skills (acquisition, retention and use of information) (Taborsky & Oliveira 2012). This type of plasticity is reversible, and occurs within an individual's lifetime.

Numerous examples of social competence can be found in nature, where individuals extract social information from the environment, and change their behavioural response based on the collected information. Animals may eavesdrop interactions between third parties and collect information from the observed individuals to use in subsequent encounters ["social eavesdropping" (Oliveira et al. 1998; Earley 2010; Abril-de-Abreu et al. 2015b)], or use the collected information of known relationships to deduce unknown ones ["transitive inference" (Grosenick et al. 2007)]. The presence of bystanders may also influence the behaviour of interacting conspecifics in an "audience effect" (Doutrelant et al. 2001; Pinto et al. 2011; Cruz & Oliveira 2015)]. Familiarity with an opponent is also known to reduce aggression from a territory owner towards a neighbour rather than towards a stranger depending on the relative threat that both represent ["dear enemy effect" (Temeles 1994; Aires et al. 2015)]. Previous social experience can affect subsequent behaviour as in the case of "winner–loser effects" demonstrated across different animal taxa (Hsu et al. 2006; Rutte et al. 2006), where previous winners are more likely to win successive contests, and losers will be more likely to lose even against different opponents. Interestingly, social interactions where no information can be extracted, as is the case of unsolved fights when animals are fighting their own mirror image, behavioural flexibility (i.e. shifts between behavioural states) does not occur (Teles et al. 2013), demonstrating the relevance of social information for behavioural decisions. Thus, social competence is a key factor in the generation of different behavioural states that encompasses neural, genomic and physiological information (Cardoso et al. 2015).

1.2. Behavioural plasticity mechanisms

At the neuronal level, two major plasticity mechanisms have been proposed and both may underlie behavioural plasticity: structural reorganization and biochemical switching of the neuronal networks underlying behaviour (Zupanc & Lamprecht 2000). These mechanisms are expected to operate at different time scales. Structural reorganization is expected to underlie long-lasting behavioural changes, and may include core structural modifications through processes such as adding or removing cells from the circuit (neurogenesis and apoptosis), changes on the connectivity or *de novo* formation of synaptic connections (synaptogenesis), or by altering the responsiveness of the circuit (i.e. balance of neurotransmitter and/or neuromodulator receptors in specific neurons) (Zupanc & Lamprecht 2000; Oliveira 2009; Cardoso *et al.* 2015). On the other hand, biochemical switching, is expected to underlie short-term reversible transitions between behavioural states, and involves the modulation of synaptic transmission by changing the release of neurotransmitter molecules at the presynaptic level, changing the number, type, or properties of neurotransmitter receptors postsynaptically, or by altering the dynamic of neuromodulatory molecules (i.e. monoamines, nonapeptides or hormones) in a socially dependent fashion (Oliveira 2009).

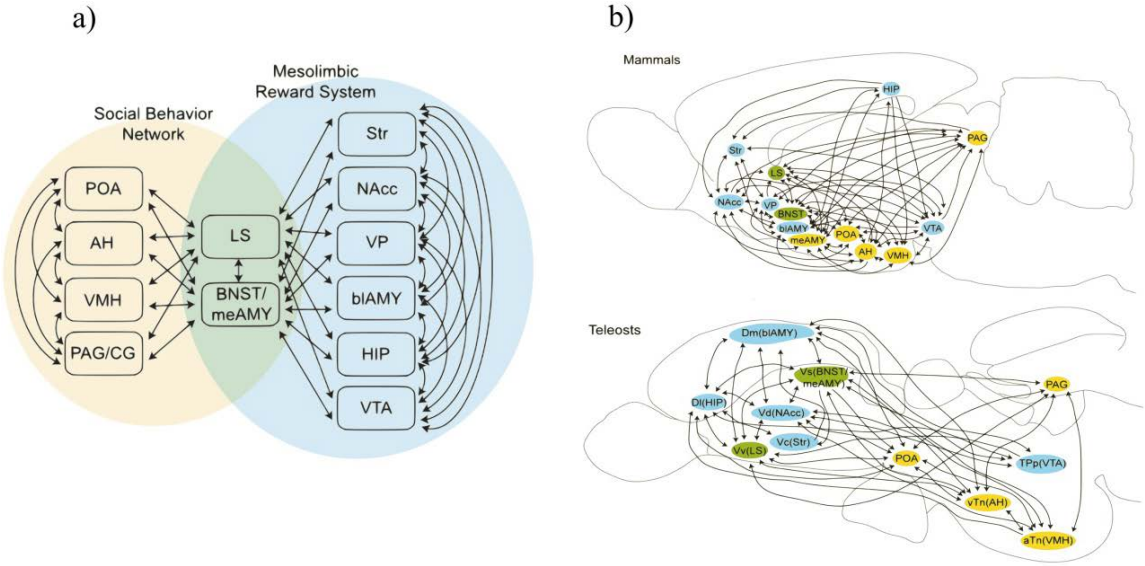
Interestingly, these two different neural plasticity mechanisms have a parallel with two different levels of endocrine regulation. Hormones can have different effects depending on their activation time, and are expected to be involved in different types of plasticity. Organizational effects occur early in development, typically within a critical or sensitive window during which the exposure to the active molecule induces a long-lasting and irreversible differentiation of a behavioural state and implies structural changes in the brain. On the other hand, activational effects typically occur later in time,

typically in adulthood, and are reversible allowing behavioural shifts (Arnold & Breedlove 1985). Thus, the integration of endocrine parameters in social decisions is expected to be mediated by activational effects in the case of behavioural flexibility (Cardoso *et al.* 2015), and by organizational effects early in developmental stages, by shaping fixed behavioural patterns that can also be influenced by environmental triggers. For instance, rats exposed to prenatal stress showed increased anxiety and depression related behaviours when tested for novelty in adulthood (Vallée *et al.* 1997). Nevertheless, at the molecular level, both mechanisms rely on the social regulation of gene expression, so that different neurogenomic states will ultimately induce different behavioural responses (Aubin-Horth & Renn 2009; Oliveira 2012).

1.3.Neuroanatomy of the social brain

According to this framework a single genotype can be socially modulated, resulting in particular phenotypes. But how does a single genome orchestrate multiple complex forms of behaviour? The neural substrate for behavioural plasticity has been identified as the social brain network (SBN) originally proposed by Newman (1999) in mammals, and confirmed to be evolutionary conserved across different vertebrate taxa by Goodson (2005). This social behaviour network is composed by six nodes located in the forebrain and midbrain areas [i.e. bed nucleus of the stria terminalis / extended medial amygdala (BNST/meAMY), lateral septum (LS), preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus, and the periaqueductal gray (PAG)], that are reciprocally connected and that together regulate several dimensions of sociality including, sexual behaviour, parental behaviour, and different forms of aggressive behaviour (Newman 1999; Goodson 2005).

As mentioned earlier, the behavioural actions expressed in response to a specific context must be adaptive to the animal, and therefore the stimuli must be properly evaluated, in terms of their valence and salience, in order to produce an appropriate response (Mendl *et al.* 2010). More recently, O'Connell and Hoffman (2012) proposed the inclusion of the mesolimbic reward system, which is generally assumed to evaluate stimulus salience via dopaminergic signalling (Wickens *et al.* 2007) and shares overlapping nodes with the SBN (lateral septum and bed nucleus of the stria terminalis), as an integrated evolutionarily ancient social decision-making (SDM) network. This network is highly conserved across vertebrates pointing to the fundamental role of the involved brain areas in vertebrate social evolution (O'Connell & Hofmann 2011, 2012), Figure 1.



(Adapted from: O'Connell & Hofmann 2011)

Figure 1 – Social decision-making (SDM) network. a) Schematic representation of the interactive nodes of the networks, brain nuclei in the social behaviour network (left) and mesolimbic reward system (right), as well as brain regions involved in both systems (centre), b) Sagittal view of a mammalian and teleost brain highlighting the connectivity between nodes of the social decision-making circuit.

According to the SDM network hypothesis the information is encoded in a dynamic fashion, and each behavioural state is better represented by the overall profile of activation across the network, rather than by the activity of single individual nodes. Different combinations of activation across nodes, and variation in the strength of the connections among them, will allow the same genotype to generate an almost infinite variation in neural states that would produce equivalent behavioural states (Goodson 2005; Cardoso *et al.* 2015). Given that most nodes of the SDM network widely express receptors for neuromodulators and steroid hormones the state of this network can be also co-regulated by these molecules (Newman 1999; Goodson 2005).

1.4.Neuromodulators contribution to plastic responses

Monoamines and neuropeptides are considered the two major classes of neuromodulators, and the action of both on social behaviour as well as their sensitivity to environmental factors, have been extensively documented (Libersat & Pflueger 2004; Goodson & Thompson 2010), which makes them major candidates to mediate changes in brain states, underlying socially driven behavioural flexibility in the SDM network.

Neuromodulators are released into broader areas than neurotransmitters, bind to receptors in the cell membrane, that are generally linked to G proteins, and subsequently activate intracellular signalling cascades with effects on the electrical activity of neurons that could last seconds, minutes, hours, days, or even weeks. At the molecular levels, neuromodulators have a wide variety of effects including: (a) modulation of ion channels and receptors, and (b) changes in protein synthesis, enzyme activity, and gene transcription (Libersat & Pflueger 2004).

a) Monoamines

Among the major representatives of the monoamines that are known to modulate well-defined behaviours are serotonin and dopamine. Serotonin (5-hydroxytryptamine, 5-HT) distribution in the CNS has been studied in different species, and found to be located exclusively in the brainstem (Takahashi *et al.* 1986; Ishimura *et al.* 1988; Johnston *et al.* 1990), where the majority of the serotonergic cell bodies reside in the dorsal and median raphe nuclei. These neurons project axons almost to the entire brain, including cortical, limbic, midbrain, and hindbrain regions (Charnay & Léger 2010), and due to this wide projection pattern, 5-HT is involved in many biological processes, such as learning and memory, mood, food intake, sleep, reproduction, circadian rhythm, thermo-regulation, pain, and social behaviour (Kiser *et al.* 2012). This molecule is catabolized from the amino acid tryptophan via 5-hydroxytryptophan and the rate-limiting factor in serotonin synthesis is the enzyme tryptophan hydroxylase (TPH1 and TPH2), whose activity depends on the availability of tryptophan (Ruddick & Evans 2006). Following release into the synapse, serotonin is either recycled back into the presynaptic neuron by the serotonin transporter (SERT), or degraded to 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme MAO. The ratio 5-HIAA/5-HT can though be used as a measurement of serotonergic activity (Shannon 1986).

Dopamine (DA) on the other hand, is a catecholamine synthesized from tyrosine via 3,4-dihydroxyphenylalanin (L-DOPA). The first step is carried out by tyrosine hydroxylase and is the rate-limiting step. In mammals, dopamine is degraded by the enzyme MAO, like in serotonin, and produce three metabolites: homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), and the 3-methoxy- tyramine (Daubner *et al.* 2011). The ratio between its metabolites and DA is also used as an index of activity.

The influence of monoamines on plastic responses, have been widely documented in the context of social status hierarchy and unpredictability, ubiquitous features in social groups.

5-HT appears to play a central role in aggressive interactions but its effects are to some extent paradoxical. While several studies have pointed out that pharmacological manipulations that increase 5-HT inhibit aggression in a wide range of vertebrates, from fish to humans (Summers *et al.* 2005), other studies, in contrast, have showed increased serotonergic activity in specific brain regions during the expression of aggressive behaviour (Winberg & Nilsson 1993; Overli *et al.* 1999; Summers *et al.* 2005). In early stages of hierarchy formation the serotonergic system appears to be activated in both dominants and subordinates, in the hippocampus, nucleus accumbens and amygdala of lizards, and in the telencephalon of fish [*Anolis carolinensis,* (Summers *et al.* 2003); rainbow trout (*Oncorhynchus mykiss,* (Overli *et al.* 1999)]. Similarly, after a chronic (5 days) agonistic interaction both dominants and subordinates showed higher levels of 5-HT activity in the telencephalon in the bicolour damselfish (*Stegastes partitus,* (Winberg *et al.* 1996), whereas in zebrafish only subordinate males increased serotonergic activity (measured as the 5-HIAA/5-HT ratios) in the hindbrain (raphe nucleus) (Dahlbom *et al.* 2012). In rats single or repeated social defeat increases serotonergic neuronal activity within the dorsal raphe nucleus in losers as evidenced by an increase in extracellular serotonin (Amat *et al.* 2010) and *c-fos* expression in serotonergic neurons (Paul *et al.* 2011). Finally, a cross-strain comparison of male mice obtained through different artificial selection breeding programs for aggression that studied the differential role of the 5-HT_{1A} receptor in aggressive and non-aggressive mice, found that highly aggressive mice had lower serotonin levels in the prefrontal cortex, and that two out of three aggressive strains had higher 5-HT_{1A} receptor

sensitivity (Caramaschi *et al.* 2007). Together, these results indicate that in addition to the activational effects on aggressive behaviour, these neuromodulator can also have organizational effects early in development.

Dopaminergic system has been classically associated with reward and motivation, with an increase in dopamine transmission leading to an increased feeling of reward. In Long-Evans rats, in a resident-intruder paradigm dopamine and serotonin levels in medial prefrontal cortex (PFC) changed in the opposite direction in dominate animals, with a sustained decrease in serotonin during and after the confrontation and an increase in dopamine after the fight in PFC and in the nucleus accumbens (NAC) (van Erp & Miczek 2000). A similar pattern has been previously observed in salmonids where dominant individuals showed higher DA activity in telencephalon than subordinate fish (Winberg *et al.* 1991).

DA is also involved in signalling unpredictability in the environment. Reversal learning is a behavioural task that requires that the animal responds to changes in reward contingencies, that is the animal has to adapt previously learned behaviours to changes in the environment. This type of learning is highly linked to both 5-HT and DA modulation. Rats treated with parachlorophenylalanine (PCPA), a drug that depletes 5-HT by the inhibition of tryptophan hydroxylase, were impaired at approaching the stimulus associated with the reward, after the reversal and this was related with lower levels of 5-HT in the ventromedial frontal cortex (Izquierdo *et al.* 2012). Recently, it was shown the pivotal role of the striatal dopamine (DA) in this process. The authors monitor the DA release in the ventromedial frontal cortex using a fast-scan cyclic voltammetry. During discrimination performance (pre-reversal), cue presentation induced phasic DA release, whereas reward delivery did not. The opposite pattern was observed in the post-reversal, that is striatal DA release occurred after reward delivery,

while cue-induced release diminished. Trial-by-trial analysis showed rapid replacement of cue-induced DA release on trials immediately following initial correct responses. This effect of positive feedback was observed in animals that learned the reversal, but not in "non-learners" (Klanker *et al.* 2015). Also in the Atlantic Salmon (*Salmo salar L.*), in an omission of the expected reward (OER) paradigm, increased DOPAC and DOPAC/DA ratios were found in the brain stem in the OER group under stress conditions (acute confinement) (Vindas *et al.* 2014).

b) Nonapeptides

The nonapeptide family includes the mammalian forms arginine vasopressin (AVP) and oxytocin (OT), as well as the nonmammalian homologues, arginine-vasotocin (AVT), and isotocin (IT) in bony fish, respectively, and mesotocin (MT) as an homologue of OT in lungfish, amphibians, birds, reptiles and some marsupials (Goodson 2008; Donaldson & Young 2008) (Thompson & Walton 2009).

All vertebrate species exhibit magnocellular and parvocellular nonapeptide cell groups in the preoptic area and hypothalamus, including the supraoptic and paraventricular nuclei in amniotes (Goodson 2008). Vasopressin and oxytocin neurons produce and pack nonapeptides into large dense-core vesicles that will be transported along the axons and can terminate in the neurohypophysis, where they are released in the bloodstream in the eminence of the anterior pituitary, and stimulate the adrenocorticotropin (ACTH) release (De Vries 2008), or project to the hindbrain, to influence autonomic functions (Thompson & Walton 2009), or other forebrain regions (Saito *et al.* 2004; Biran *et al.* 2015). In mammals these vesicles can also be released locally at the dendrites (Moos & Freund-Mercier 1984; Landgraf & Neumann 2004; Ludwig & Leng 2006) modifying their own electrical activity (Moos & Freund-Mercier

1984; Morris & Ludwig 2004). In non-mammalian species there is no direct evidence of dendritic release, although the anatomical features of both neuronal types, such as the close proximity and direct membrane appositions (Saito *et al.* 2004) may indicate a similar mechanism.

The nonapeptide system has been implicated in a variety of social behaviours, such as parental behaviour, sexual behaviour, pair-bond, mate choice, aggression and social recognition (Goodson & Bass 2001; Insel 2010), as well as in plasticity in behavioural responses in several species.

The plasticity underlying aggression, that is the ability to express aggressive behaviour under the right circumstances, may depend on previous experience and social status. In mammals this social status plasticity is linked to changes in AVP and AVP receptor (*Avpr*) distribution, with different effects being observed in dominants and subordinates. In mice, administration of lysine-vasopressin after a social defeat increases submissive behaviour in subsequent encounters compared to saline-treated animals (Roche & Leshner 1979). In the Syrian hamsters, AVP or AVP antagonist injections in the anterior hypothalamus-medial preoptic area (AH-MPOA) result in transient reversals of dominant/subordinate relationships. Subordinate animals treated with AVP display increased flank-mark behaviour (the way they communicate dominance status) while dominates treated with Avpr1 antagonist decreased its expression (Ferris *et al.* 1986). Consistently with these results, subordinate hamsters present fewer Avp-ir neurons in the magnocellular nucleus circularis (a cell group between supraoptic nucleus and paraventricular nucleus) than dominants (Ferris 1989), and following repeated agonistic encounters dominant hamsters have higher levels of *Avpr1a* binding in the ventromedial hypothalamus (VMH) when compared to their subordinate opponents (Cooper *et al.* 2005). In socially isolated hamsters, the increase

in aggression was correlated with the increase of *Avpr1a* binding in the anterior hypothalamus (AH), the paraventricular nucleus (PVN) and lateral hypothalamus, whereas socially experienced hamsters (i.e. allowed to interact with a conspecific 3 times a week) present a significant binding increase only in the amygdala (Albers *et al.* 2006). Taken together these results indicate that the AVP system, especially through the *Avpr1a* can be altered by social experience. Nevertheless, while treatment with *Avpr1a* antagonist reduced aggression in golden hamsters (Ferris *et al.* 2006), contrary to what was expected *Avpr1a* knockout (KO) mice did not show reduced aggression (Wersinger *et al.* 2008), and it was the isoform *Avpr1b* that proved to be critical for proper expression of aggression, as *Avpr1b* KO mice showed significant impairments in aggressive displays compared to wild-type controls (Wersinger & Ginns 2002).

In fish, in addition to the magnocellular and parvocellular cell groups, there is a third cellular type, the gigantocellular, and these three types of cells are distributed along the ventral portion of the preoptic area (POA) [reviewed in (Urano & Ando 2011)]. In fish the modulation of aggression is also ambiguous. In some teleost species, the expression of social dominance has been associated with higher number or size of AVT-ir cells in magnocellular (mPOA) or gigantocellular (gPOA) neuronal population, whereas social submission is associated with the number or size of pPOA AVT-ir cells [e.g. zebrafish, *Danio rerio* (Larson *et al.* 2006); African cichlid, *Astatotilapia burtoni* (Greenwood *et al.* 2008); butterfly fishes (Dewan *et al.* 2011)]. In other fish species the reversal is also true: social submission is associated with changes in the mPOA and gPOA populations (Almeida & Oliveira 2015), and aggressive behaviour with variations in size of the pPOA AVT-ir (Lema 2006). AVT manipulation can also increase or decrease aggression depending on the species (Godwin & Thompson 2012), and the expression of nonapeptide receptors can also vary depending on social status.

Evidence from zebrafish indicates arginine vasotocin-like receptor 1b (*V1b*) as one of the highest differentially expressed gene in the hypothalamus of dominant animals (Filby *et al.* 2010), whereas in the pupfish transcripts encoding the isoform *V1a1* were expressed at higher levels in the telencephalon and hypothalamus of subordinate males, and it was the variant *V1a2* that was more abundant in dominants telencephalon (Lema *et al.* 2015), similarly to what has been previously described in mice.

The oxytocin role on aggression has been scarcely investigated and, only one study in female Syrian hamsters (*Mesocricetus auratus*) clearly demonstrated that endogenous OT modulates resident-intruder aggression in adults. This experiment showed that OT infusions into the preoptic area-anterior hypothalamus (POA-AH) decreased resident-intruder aggression, and that OTR antagonist facilitated aggression (Harmon *et al.* 2002). In territorial finches (violet-eared waxbill) peripheral injections of an OTR antagonist reduced aggressive behaviour in females, and colocalization of OT-Fos found in the preoptic area and hypothalamus, was correlated negatively with aggression (Goodson *et al.* 2015), suggesting that OT may be mediating the stress response and not the aggression.

Finally the involvement of these peptides in gregariousness, a key dimension of sociality that implies behavioural flexibility, is very well described in birds. In different species of finches, mesotocin receptor distribution in the lateral septum correlates with flock size, and administration of mesotocin increases while a mesotocin antagonist reduces social behaviour, such as flock formation (Goodson *et al.* 2009).

1.5.Neurogenomics of social plasticity

Behaviour traits exhibit a great deal of plasticity and their modulation requires the integration of multiple systems as we previously stated. At the proximate level, the

direct consequence of the activation of any specific neural circuits underlying behaviour is a burst in gene expression. A neurogenomic state corresponds to distinct transcriptome profiles across the SDM network, (Robinson *et al.* 2008; Zayed & Robinson 2012) corresponding to different behavioural states. Switches between states (behavioural flexibility) are orchestrated by signalling pathways that interface the social environment and the genotype (Aubin-Horth & Renn 2009; Oliveira 2012).

At least three different neuronal activity-dependent molecular mechanisms can be proposed to translate social information into a neurogenomic state (Wolf & Linden 2012): protein phosphorylation; immediate early genes activation (IEGs), and microRNAs.

a) Activation (e.g. phosphorylation) of the intracellular signalling pathway mitogen-activated protein kinase (MAPK/ERK) is involved in the transduction of signals through a cascade of protein kinases in response to stimuli. Once this pathway is activated, ERK phosphorylates a variety of target proteins, including other protein kinases and transcription factors, for example CREB that is phosphorylated by ERK and other kinases such as protein kinase C (Roberson *et al.* 1999). This transcription factor (Brindle *et al.* 1993) binds to the CRE site present in IEG promoters, and acts as a key regulator of IEG expression activation. Several different protein kinases possess the capability of driving the phosphorylation of CREB, making it a point of potential convergence for multiple intracellular signalling cascades (Wu *et al.* 2001).

b) IEGs are the first genomic response upon cell depolarization, whose transcription can be induced without requiring *de novo* protein synthesis or

previous activation of any other responsive genes (Clayton 2000). It has been shown recently that several IEGs are poised for near-instantaneous transcription by stalling the DNA polymerase II (Pol II) in the vicinity of the promoter (Saha *et al.* 2011). In line with this, IEGs have been classified into different groups depending on the presence or absence of the DNA polymerase II (Pol II) stalling. For *rapid* IEGs, that are expressed within a few minutes after stimulation, DNA polymerase II (Pol II) stalling is in the promoter region, whereas in *delayed* IEGs, that are expressed later (ca. 1 hour post-stimulation), largely lacked this poised Pol II (Saha *et al.* 2011; Saha & Dudek 2013). This mechanism of stalling has been shown to be relevant in regulating timing and dynamics of gene responses, but not for steady-state accumulation of mRNA over time (Saha *et al.* 2011). Depending on their function, IEG proteins can act themselves as transcription factors (*e.g. c-fos* and *egr-1*), or as effector proteins (*e.g. arc* and *homer1a*), regulating synaptic function (Clayton 2000).

c) The transcription of microRNAs (miRNA), which are non-coding RNAs, function as post-transcriptional repressors of gene expression. These RNA molecules can control specific biological processes by switching off a few target genes at particular time (temporal switches) or places (during development) (Lai 2005). An example of this mechanism is the brain-expressed miR-133, recently found to play an important role in controlling behavioural shifts in migratory locusts (*Locusta migratoria*) (Yang *et al.* 2014). miR-133 controls dopamine production by targeting the genes *henna* and *pale*, which are involved dopamine synthesis and release, and related to the behavioural phase transitions (from gregarious phase to the solitary) of the migratory locust (Ma *et al.* 2011). miR-

133 sense oligonucleotides (agomir) delivery suppressed *henna* and *pale* expression, which consequently decreased dopamine production, resulting in the behavioural shift from the gregarious phase to the solitary phase, while miR-133 inhibition, promoted gregarious-like behaviour of solitary locusts. Thus, microRNA plays an important role as an activational switch in this species acting a key mediator of a transition between behavioural states.

1.6. Zebrafish as an experimental model in social neurosciences

Zebrafish (*Danio rerio*) have already proven to be a powerful model organism for the study of behavioural neuroscience including complex cognitive disorders like depression, autism spectrum disorder (ASD), drug abuse, cognitive deficits and psychoses (Kalueff *et al.* 2014). Several behavioural paradigms used in rodents to study these disorders have already been successfully adapted to zebrafish, such as exploration (open field), anxiety-like (light-dark and alarm substance), locomotion (novel tank), and social and cognitive (shoaling, social preference, predator avoidance and T-maze) tests (Kalueff *et al.* 2014). Zebrafish are also highly social animals that live in groups with structured social relationships including shoaling, dominance hierarchies, and territoriality (Spence *et al.* 2008). The utility of this species in behavioural neuroscience has grown markedly because of: its available molecular [forward and reverse genetic methods (Sivasubbu *et al.* 2007; Bill *et al.* 2009)], electrophysiological (Higashijima *et al.* 2003) and optogenetic (Douglass *et al.* 2008) tools; the variety of wild-type lines with distinct behavioural phenotypes (Kalueff *et al.* 2014), conditional transgenic lines (Kawakami *et al.* 2010); the similarity its genome presents with the human genome, where approximately 70% of the genes have human orthologues (Howe *et al.* 2013); and the conserved regulatory mechanisms with mammals, including shared modulatory

neurotransmitter systems (Panula *et al.* 2010) and homologous brain areas (Wullimann & Mueller 2004). Moreover, their small size (adults 3–4 cm long), short inter-generation time (3 months), and the large number of eggs per spawn, makes this species suitable for large-scale behavioural screens. All these features allow the study of the mechanisms underlying relevant behavioural traits.

1.7. Aims and hypothesis

The goal of this work is to study and potentially identify the proximate mechanism underlying behavioural flexibility in the context of experience dependent behavioural shifts, in an integrative framework.

Teleost fish are a group with unparalleled diversity among vertebrates in social organization. There are solitary species and species where individuals form massive schools with hundreds of others, species where no parental care is provide and species that provide parental care, either maternal, paternal or biparental (Kornfield & Smith 2000).There are also monogamous and polygynous species, species where males mimic female's behaviour (Godwin 2010), and sex change in adult animals depending on particular social conditions (Kuwamura *et al.* 2002). Thus, this group offers a unique opportunity to study how animals have adapted to social selective pressures.

To accomplish our goals we chose zebrafish which are highly social animals that live in groups with structured social relationships including shoaling, dominance hierarchies, and territoriality (Spence & Simth 2005; Spence *et al.* 2008). Social behaviour in zebrafish is flexible, as recently shown by the occurrence of acute winner and loser effects (Oliveira *et al.* 2011), where short-term social interactions induce changes in social behaviour. This plasticity predicts some social cognitive skills such as social preference (Engeszer *et al.* 2007), social recognition [Kin recognition (Gerlach *et*

al. 2008) and individual recognition (N. Madeira and R.F. Oliveira, unpublished data)] social attention [(Abril-de-Abreu *et al.* 2015a)], social learning [stimulus enhancement (Lindeyer & Reader 2010), observational conditioning (Suboski *et al.* 1990)], and social inference (eavesdropping (Abril-de-Abreu *et al.* 2015b); audience effects (Cruz & Oliveira 2015), skills already described for this species.

Here we used a behavioural paradigm where animals could experience winning or losing a social interaction and consequently adjust their behaviour to a new social status (behavioural shift). The specific cues that trigger changes in social status were also investigated. There are at least two potential sources of social status information available in a social interaction: the own aggressive behaviour expressed by the individual; and the behaviour expressed by the opponent. Theoretically, animals can use just one of these two types of information (self-assessment or opponent only assessment, respectively) or combine both in mutual assessment, which assumes that contestants know their own competitive abilities, gather information about the opponent, and integrate both into a behavioural adaptive response (Elwood & Arnott 2012). Therefore, the perception that the individual has of the interaction is a key feature in the modulation of the behavioural response. In order to assess the type of assessment zebrafish uses to trigger a status-dependent behavioural shift, three social treatments were used (Figure 2):

1) Staged fights between pairs of real-opponent conspecifics, which resulted in a winner and a loser (Figure 2a);

2) Mirror-fights, which resulted in unsolved interactions and the expression of aggressiveness is decoupled from the experience of winning or losing (Figure 2b); fish do not recognize themselves on a mirror, and attack their own image as if it is an intruder (Oliveira *et al.* 2005), however since submissive behaviour is never expressed

by the opponents (i.e. the mirror image replicates the aggressive behaviour of the focal fish) focal fish never experiences a victory;

3) No agonistic interaction, which were used as a reference group (Figure 2c).

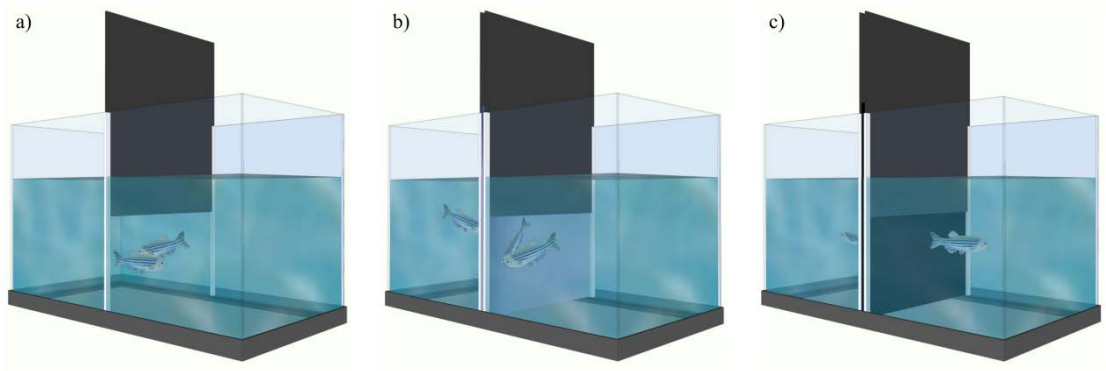


Figure 2 – Experimental apparatus: a) Real opponent interaction, fish fought with a conspecific, and a Winner and a Loser emerge; b) Mirror interaction, fish fought with their own image on the mirror but did not experience a change in social status; c) Control group, no agonistic interaction or mirror stimulation.

These social treatments generated four social behaviour states: Winners and Losers of the real opponent interaction; Mirror-fighters; and fish with no social interaction.

Our hypothesis is that winners and losers will have different neuronal states that are experience dependent, and for mirror fighters the following premises can be generated:

1) if only the individuals own behavioural expression would be relevant for the individual's assessment of fight outcome, then mirror-fighters should have a neuromolecular profile similar to that of winners;

2) if only the behavioural feedback from opponent would be relevant, then mirror-fighters should have a neuromolecular profile similar to that of losers;

3) if the comparison between perceived behaviour of the opponent and the own expressed behaviour is needed, then mirror-fighters should not activate a response because in mirror interactions they equal each other, and therefore no change in social

status would be experienced by the subject, leading to a neuromolecular profile different from those of both winners and losers.

All tests were done in pairs in order to give individuals the access to conspecific odours, which would otherwise only be present in real opponent dyads, therefore avoiding confounding effects of putative chemical cues in the comparisons between treatments.

In a first set of experiments we studied the influence of neuromodulators on social plasticity mechanisms. For this purpose we characterized the endocrine response to social challenges (Chapter II), and the social modulation of brain monoamines (Chapter III) and nonapeptides (Chapter IV). Next we tested the SDM network hypothesis by contrasting changes in functional localization vs. connectivity across this network in response to changes in social status (Chapter V). Finally, we characterized the changes in expression of key genes for different neuroplasticity mechanisms (e.g. neurogenesis, synaptogenesis, changes in synaptic strength) in response to changes social status (Chapter VI).

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Chapter II

Androgen response to social competition in a shoaling fish

Androgen response to social competition in a shoaling fish

Submitted in Hormones and Behaviour

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Abstract

Androgens respond to social challenges and this response has been interpreted as a way for males to adjust androgen-dependent behaviours to social context. However, the androgen responsiveness to social challenges varies across species and a conceptual framework has been developed to explain this variation according to differences in mating system and parental care type, which determine the regime of challenges males are exposed to, and concomitantly, the scope of response to a social challenge (e.g. care provider monogamous males are predicted to have a higher scope of response to a social challenge than polygamous males that lack parental care). However, this framework has been focused on territorial species and no clear predictions have been made to gregarious species (e.g. shoaling fish), which although tolerating same-sex individuals also exhibit some degree of intra-sexual competition. In this paper we extend the scope of this conceptual framework to shoaling fish by studying the response of zebrafish (*Danio rerio*) to social challenges. Male zebrafish exposed to real opponent agonistic interactions exhibited an increase in androgen levels (11-Ketotestosterone both in

Winners and Losers, and Testosterone in Losers). This response is absent in Mirror-fighters that expressed similar levels of aggressive behaviour to those of winners, suggesting that this response is not a mere reflex of heightened aggressive motivation. Cortisol levels were also measured, and point to an activation of the hypothalamic-pituitary-interrenal axis in real opponent fighters, but not in mirror-fighters. These results confirm that gregarious species exhibit a high scope of response to a social challenge.

Keywords: Challenge hypothesis; Androgens; 11-Ketotestosterone; Testosterone; Cortisol; Zebrafish

1. Introduction

An androgen response to social challenge has been described across vertebrates, from fish to humans (Archer, 2006; Oliveira et al., 2002), which has been interpreted as a way for individuals to adjust their expression of androgen-dependent behaviours to social context (Oliveira, 2009). However, these socially driven changes in androgen levels vary widely across species (Hirschenhauser and Oliveira, 2006). The "Challenge Hypothesis" has been proposed to explain this variation based on inter-specific differences in the regime of social challenges due to variation in mating system and/or parental care type (Wingfield et al., 1990; for reviews more recent reviews see Goymann et al., 2007; Hirschenhauser and Oliveira, 2006). For example, males from monogamous species with paternal care are expected to experience a lower regime of intra-sexual competition than males from polygamous species without paternal care, and therefore the former are expected to have lower breeding baseline androgen levels and a higher scope of response when faced with a social challenge. These predictions of the "Challenge Hypothesis" have been in general confirmed across different taxa [e.g. fish (Hirschenhauser et al., 2004) and birds (Hirschenhauser et al., 2003)]. However, most studies on the social modulation of androgen so far have concentrated on territorial species. For gregarious species, which have a high tolerance for the presence of same-sex conspecifics, the androgen responsiveness to social challenges, which occur when competing for the access to mating opportunities, can be expected to be similar to those of territorial monogamous species. In shoaling species, structured social relationships have been documented with social hierarchies and leader-follower roles [e.g. zebrafish, (Paull et al., 2010; Vital and Martins, 2013)], with dominant individuals having higher androgen levels than subordinates in some species [e.g. zebrafish (Filby et al., 2010)] but not in others [e.g. swordtail fish (Hannes, 1984)]. Moreover, both males and females

of shoaling species express aggressive behaviour when competing for resources [e.g. zebrafish (Paull et al., 2010)]. Therefore, the associations found between androgen levels and social status in male shoaling teleosts may be explained by the challenge hypothesis, reflecting a higher regime of social challenges in dominant males.

In this study we tested the hypothesis that an acute social challenge elicits an androgen response in a shoaling species, the zebrafish (*Danio rerio*), by promoting dyadic interactions between males. Furthermore, we also tested the hypothesis that it is the perception of the outcome of the interaction as a victory or a defeat that triggers the androgen response, rather than the mere expression of aggressive behaviour. For this purpose we had a treatment with mirror-elicited aggression, where males fought their own image on a mirror. In zebrafish, mirror fights elicit similar levels of aggressive behaviour to those observed in real opponent fights (Teles et al., 2013) and since the aggression expressed by the mirror-image, matches the behaviour of the focal individual, no information on fight outcome is available (Oliveira et al., 2005). Thus, despite expressing similar levels of aggressive behaviour to those of winners of real opponent fights, mirror fighters do not experience either a victory or a defeat. Thus, if the androgen response depends on the perception of fight outcome we predict androgen levels to increase in real opponent fighters but not in mirror-fighters. Since social challenges may also activate the hypothalamic-pituitary-interrenal (HPI) axis we have also sampled cortisol.

2. Material and methods

2.1. Animal housing

Adult male zebrafish from the AB strain breed and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used in this study. Fish were kept at 28 ºC under a 14L:10D photoperiod, and fed twice a day, except on the day of the experiments.

2.2. Social challenge tests

Four social treatments were used in this study (N=16 for each treatment): (1) fish that won a social interaction against a real opponent (Winners); (2) fish that lost a social interaction against a real opponent (Losers); (3) fish that interacted with its own image on a mirror and that despite fighting neither won nor lost the social interaction (Mirror-fighters); and (4) fish that did not experience a social interaction, which was used as a reference group (Control). The real opponent interactions followed a previously described behavioural paradigm (Oliveira et al., 2011). In brief, each pair was placed in the experimental arena (5cm x 8cm x6cm) where they stayed overnight isolated from each other by a removable opaque PVC partition. On the next day, the opaque divider was removed and the fish allowed to interact for 30 min. The mirror-elicited interactions followed a similar procedure but at the time of the interaction, the opaque partition separating the opponents was removed and a pair of mirrors revealed, one on each compartment. Therefore, pairs from mirror-fighters fought their own image independently but simultaneously. This procedure allowed to control for the presence of putative chemical cues during agonistic interactions, since the two compartments were not isolated chemically from each other. Pairs form the control treatment also stayed in the same conditions of the other experimental treatments but at the time of the interaction one opaque partition was lifted and second one remained to continue the

visual isolation between the pair. Fish were matched for size across treatments [body weight (mean ± SEM) = 0.32 ± 0.01 g; standard length (mean ± SEM) = 2.78 ± 0.006 cm). Behavioural tests were video-recorded for subsequent behavioural analysis.

2.3. Hormone assays

Immediately after the interaction fish were killed using an overdose of anaesthetic (MS222, Pharmaq; 500-1000 mg/L) and the spinal cord sectioned. Whole-body samples were collected and frozen at -80ºC. Steroid extraction from whole-body samples followed the protocol described in Cachat et al., 2010. Whole-body concentrations of 11-ketotestosterone (11-KT), testosterone (T) and cortisol (C) were measured using specific enzyme immunoassay kits (Cayman Chemical Company #582751, #582701, #500360, respectively) following the manufacturer's instructions. All Samples were tested in duplicate in a dilution of, 1:40 for the 11-KT, 1:20 for T and 1:4 for C. Intra-assay and inter-assay coefficients of variation were respectively: 4.2%, and 12.8% for 11-KT, 1.7% and 5.1% for T, and. 6.6% and 14.6% for C.

Behavioural analysis

Behavioural analysis was performed using a computerized multi-event recorder (Observer XT, Noldus, *Wageningen, The Netherlands*). The behaviours were divided into aggressive (bite, chase and strike) and submissive (freeze and flee) following the available zebrafish ethogram (Oliveira et al., 2011). Only the last 5 min of the interaction were analysed, when dominance relationships have already been established and winners and losers express status-specific behavioural profiles.

2.4. Data analysis

Behavioural data did not conform to parametric assumptions, hence the comparison of aggressive behaviour between winners and mirror-fighters was performed using the non-parametric Mann-Whitney U test. Hormonal data was log transformed to meet parametric criteria, which were checked by values of skewness and kurtosis for normality, and by the Levene test for the homogeneity of variances. A one-way ANOVA was used to test the main effect of social treatment on hormone levels, followed by Fisher LSD post-hoc tests to assess differences between each of the social treatments. Effect sizes were computed for all tests [partial eta-squared ($\eta^2 p$) for ANOVA and Cohen d_s for post-hoc tests]. Pearson correlations were used to assess the association between hormone levels and behaviour expression. Sample sizes varied between groups due to outlier values (i.e. mean ± 3 × standard deviation). All statistical tests were two-tailed with significance level of $p<0.05$, and were performed using the software STATISTICA v.10.

2.5. Ethics statement

All procedures used in this study followed the institutional guidelines for the use of animals in experimentation and were approved both by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 8954).

3. Results

Behavioural analysis confirmed that dominance relationships were established in real opponent fights with Winners only exhibiting aggressive behaviour, and Losers only expressing submissive behaviour (Figure 1A). Mirror fighters only expressed aggressive behaviour at a frequency that was not significantly different from that of Winners from the real opponent interaction (Mann-Whitney, Z=0.642, p=0.52, Fig. 1A).

There were significant main effects of social treatment on whole-body levels for all the measured hormones (KT: $F_{3, 53}$=4.260, p=0.009, η^2_p= 0.993; T: $F_{3, 57}$ = 2.946, p=0.040, η^2_p= 0.988; C: $F_{3, 55}$ =4.112, p=0.011, η^2_p= 0.988). Post-hoc analysis revealed that for the 11-ketotestosterone both Winners and Losers had higher levels than the Control group (p=0.0018, d_s= 1.051; and p=0.0099, d_s=0.842; respectively), and marginally non-significant (p=0.054; d_s=0.965) higher levels in Winners than in Mirror-fighters (Fig. 1B). For testosterone, the post-hoc tests detected higher levels in Losers than in either Mirror fighters (p=0.019, d_s=0.767) or Controls (p=0.010, d_s=0.834; Fig. 1C). Finally, the post-hoc analyses for cortisol revealed that both Winners and Losers had higher cortisol levels than Controls (p=0.004, d_s=1.409; and p=0.026, d_s=0.984; respectively), that Winners also had higher levels than Mirror-fighters (p=0.013, d_s=0.963), and Losers had close to significant higher levels than Mirror-fighters (p=0.064, d_s=0.703; Fig. 1D).

Correlation analysis between behaviour and hormone levels only revealed a single positive correlation between cortisol and the expression of aggressive behaviour in the mirror group (r=0.747, n=12, p= 0.005). All other correlations were non-significant.

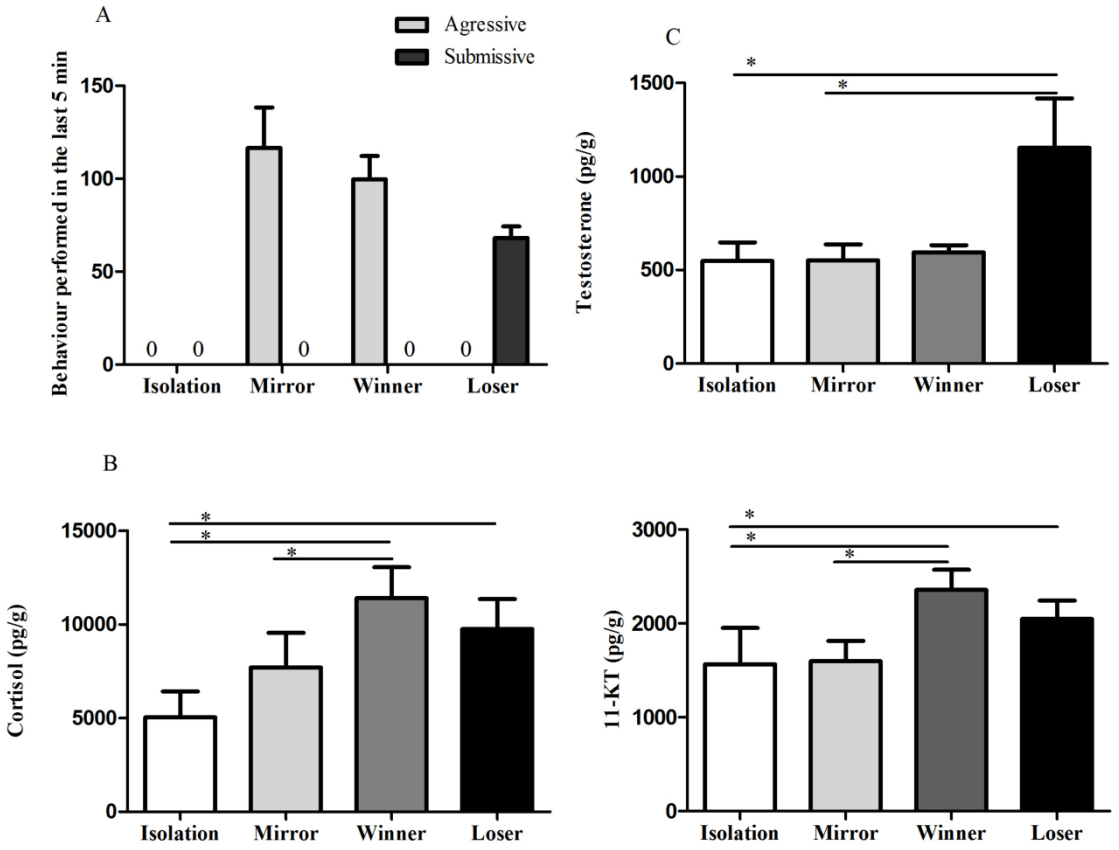

Figure 1 – Effects of social challenge in agonistic behaviour and hormonal levels: (A) Behavioural characterization of the social treatments, as the frequency of aggressive and submissive behaviours expressed at the last 5 min of the agonistic interactions; (B) whole-body 11-ketotestosterone levels; (C) whole-body testosterone levels; (D) whole-body cortisol levels. Error bars represent the standard error of the mean and asterisks indicate significant differences between treatments ($p<0.05$).

4. Discussion

Overall the results presented in this paper confirmed the occurrence of an androgen response to a social challenge in zebrafish, as predicted by the low levels of social challenges expected in a gregarious species. Indeed, 11-KT levels increased in real opponent fighters in comparison to Controls, irrespectively of the social status achieved (i.e. both in Winners and in Losers), and T levels increased in Losers, which exhibited higher levels than either Controls or Mirror-fighters. However, these results did not confirm the prediction that Winners would increase and Losers decrease their androgen

levels, as a way to adjust androgen-dependent behaviour to perceived social status (Oliveira, 2009). These results also contrast with previously reported differences in 11-KT levels between dominant and subordinate fish in long-term interactions [i.e. individuals paired for either 1 or 5 days (Filby et al., 2010)]. This difference between the two studies can be explained at least in two different ways: (1) in our study the individuals were sample immediately after the social challenge, which may have missed a divergent time course in the androgen response between Winners and Losers, which was captured when individuals were sampled after 1 or 5 days; and/or (2) the cumulative asymmetry of social status may elicit divergent androgen profiles between dominants and subordinates, which are not present immediately after a first interaction but that emerge with continuous interactions.

Interestingly, 11-KT levels of Winners were significantly higher than those of Mirror-fighters despite the similar behavioural profile expressed by these two groups. Concordantly, Mirror-fighters had similar levels of both androgens (i.e. 11-KT and T) to those of non-interacting Controls. Together these results indicate a decoupling between the expression of aggressive behaviour and the androgen response to social challenge in zebrafish, which cannot be explained merely as a reflex of a heightened aggressive motivation. This result is in line with previous studies in other species, which have also reported a dissociation between the androgen and the behavioural response in mirror-elicited aggression (Hirschenhauser et al., 2008; Oliveira et al., 2005).

Finally, the measures of cortisol taken to assess social stress during acute social challenges in zebrafish confirmed its occurrence in real opponent fights, as indicated by increased cortisol levels both in Winners and Losers. However, in Mirror-fights the HPI axis does not seem to be activated, as indicated by similar cortisol levels between Mirror-fighters and Controls, and significantly lower in the former than either in

Winners or Losers. The cortisol response to real opponent fights is in agreement with the higher cortisol levels observed in both dominant and subordinate individuals of long-term interactions (5 days), when compared to control levels in non-interacting fish (Pavlidis et al., 2011).

In summary, this study confirms the occurrence of a high magnitude [as indicated by the high effect sizes, i.e. > 0.8 (Cohen, 1988) of the reported significant results] androgen response to an acute social challenge in a shoaling species, which supports an extension of the predictions of the "Challenge Hypothesis" to gregarious species.

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Chapter III

Social modulation of brain monoamine levels in zebrafish

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Social modulation of brain monoamine levels in zebrafish

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Abstract

In social species animals tend to adjust their social behavior according to the available social information in the group, in order to optimize and improve their one social status. This changing environment requires for rapid and transient behavioral changes that relies primarily on biochemical switching of existing neural networks. Monoamines and neuropeptides are the two major candidates to mediate these changes in brain states underlying socially behavioral flexibility. In the current study we used zebrafish (*Danio rerio*) males to study the effects of acute social interactions on rapid regional changes in brain levels of monoamines (serotonin and dopamine). A behavioral paradigm under which male zebrafish consistently express fighting behavior was used to investigate the effects of different social experiences: winning the interaction, losing the interaction, or fighting an unsolved interaction (mirror image). We found that serotonergic activity is significantly higher in the telencephalon of winners and in the optic tectum of losers, and no significant changes were observed in mirror fighters suggesting that serotonergic activity is differentially regulated in different brain regions by social interactions. Dopaminergic activity it was also significantly higher in the

telencephalon of winners which may be representative of social reward. Together our data suggests that acute social interactions elicit rapid and differential changes in serotonergic and dopaminergic activity across different brain regions.

Keywords: Aggressive behavior, Behavioral plasticity, neuromodulators, Serotonin, Dopamine, zebrafish.

1. Introduction

In order to optimize the benefits of group living and to minimize its costs, social animals need to adjust the expression of their social behavior according to daily changes in their social environment. This ability of an individual to optimize its social behavior depending on available social information (aka social competence, [1]), depends primarily on mechanisms that allow for rapid and transient behavioral changes. Given the speed and liability of this type of behavioral flexibility, such mechanisms are expected to rely on socially driven biochemical switching of existing neural networks, rather than on structural rewiring of neural circuits [2]. In recent years evidence accumulated showing how neuromodulators can change the activity and even the connectivity of neural circuits in a way that each structural circuit, as represented by its connectome, may include multiple functional circuits, with some of them active and some others latent at a given moment in time [3]. Different neuromodulatory agents may interact with specific circuits and alter their functional properties, promoting either excitatory or inhibitory states. Monoamines and neuropeptides are considered the two major classes of neuromodulators, and the action of both on social behavior as well as their sensitivity to environmental factors, have been extensively documented [4, 5], which makes them major candidates to mediate changes in brain states underlying socially driven behavioral flexibility.

Monoamines have been implicated in the regulation of motivated behaviors and among them the role of the serotonergic system on the control of aggressive motivation has been demonstrated both in vertebrate and in invertebrate species [6, 7]. Interestingly the effects of serotonin (5-hydroxytryptamine, 5-HT) on aggressive behavior are to some extent paradoxical. While several studies have pointed out that pharmacological manipulations that increase 5-HT inhibit aggression in a wide range of vertebrates, from

fish to humans [8], other studies, in contrast, have showed increased serotonergic activity in specific brain regions during the expression of aggressive behavior [8-10]. Moreover, the 5-HT_{1A} and 5-HT_{1B} receptors exert functionally opposing roles in various behavioral and physiological processes such as appetite, sexual libido, motor activity, and thus it is reasonable to consider that this divergence may also be present in aggressive behavior [11-13]. Therefore, the role of 5-HT on the regulation of social behavior cannot be put simply in terms of pure inhibition or pure facilitation of aggression, but rather as a function of environmental context. The effects of dopamine (DA) on aggression are also paradoxical. For example in mammals, while D1 and D2 dopamine receptor antagonists reduce aggression [14], D2 receptors in the medial preoptic area (mPOA) and anterior hypothalamus facilitate affective defense behavior [15]. On the other hand, the mesocorticolimbic dopamine system has been shown to be involved in the preparation and execution of aggressive acts [16-20]. These neurochemical studies link elevated dopamine and its metabolites in prefrontal cortex and nucleus accumbens not only to the initiation of attacks and threats, but also to defensive and submissive responses in reaction of being attacked [19, 21]. The transition between behavioral states (e.g. inhibition or promotion of aggressive behaviors) in both monoaminergic systems appears to be sensitivity to different social contexts, which make these neuromodulators tremendously important in the regulation of social interactions.

The high diversity and plasticity of social behavior among teleost fish makes them excellent models for comparative studies on the mechanisms of social plasticity [22]. In many fish species social systems are characterized by reversible dominance hierarchies, where animals have to adjust the expression of their social behavior to their perceived

social status. In these social systems rapid changes in behavioral output occur, driven by the assessment that the animal does of the social interactions in which it is involved.

In this paper we used zebrafish (*Danio rerio*) males to study the effects of acute social interactions on rapid regional changes in brain levels of monoamines. Zebrafish were chose as a model species given their increasing use in behavioral neuroscience research and their flexible social behavior. Zebrafish is a group-living species that in nature form shoals [23] but when allowed to interact in pairs, form dominance hierarchies [24]. In this species aggression is commonly used by dominant individuals to get access to spawning sites and to protect their social status from competitors [25]. Recently, our group developed a behavioral paradigm under which male zebrafish consistently express fighting behavior and characterized the structure of these fights in male dyads [26]. Here the same paradigm is used to investigate the effects of different social experiences (i.e. individuals experiencing a victory, a defeat or fighting an unsolved interaction) on serotonin and dopamine levels in different brain regions.

2. Materials and Methods

2.1. Animals and housing

All subjects used in this experiment were adult wild-type (AB) zebrafish breed and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Fish were kept in a recirculating system (ZebraTec, 93 Tecniplast), at 28 ºC with a 14L:10D photoperiod. Water system was monitored for nitrites (<0.2 ppm), nitrates (<50 ppm) and ammonia (0.01-0.1 ppm), while pH and conductivity were maintained at 7 and 700 µSm respectively. Fish were fed twice a day with commercial food flakes in the morning and *Artemia salina* in the afternoon, except on the day of the experiments.

2.2. Experimental design

In the present study a behavioral paradigm previously developed for the study of zebrafish aggressive behavior was used [26]. Thirty-two adult males (8 in each experimental treatment) matched for standard length (mean ± SEM: 2.81 ± 0.026 cm) and body mass (mean ± SEM: 0.350 ± 0.009 g) were grouped in dyads. There were three types of dyads: 1) real opponent fight: the fish fought with a conspecific; 2) mirror fight: the fish fought with their own mirror image; 3) no fight: the fish had no agonistic interaction (Fig. 1). From these three types of dyads, came out four experimental conditions: winning the interaction, losing the interaction, fighting an unsolved interaction, or experience no interaction (control group). Subjects were always tested in pairs, in order to give them access to conspecific odours, which would otherwise only be present in real opponent dyads, therefore avoiding confounding effects of putative chemical cues.

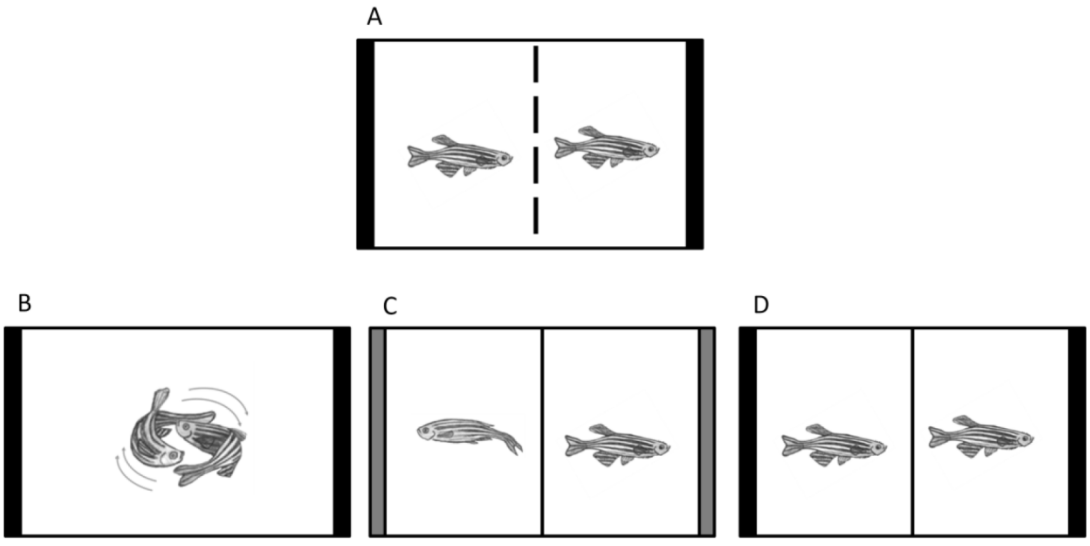


Fig. 1. Experimental procedure: A) Overnight isolation to elicit aggression. Each fish pair was placed in the experimental tank, and isolated visually, but not chemically, by a removable opaque PVC partition; B) Real opponent interaction, fish fought with a conspecific; C) Mirror interaction, fish fought with their own mirror image (grey bars); d) Control group, no agonistic interaction or mirror stimulation.

Prior to the experiment, each pair was placed in the experimental tank (20cm x 14.5cm x12.5cm) where they were kept overnight in visual isolation using a removable opaque PVC partition. Previous studies had established periods of social isolation of 5 days [24] and 24h [26] as effective to elicit aggressive behavior. However, here we established that overnight isolation was sufficient to promote the consistent expression of aggressive behavior. After the isolation period, the opaque divider was removed and the fish were allowed to interact for a period of 30 min. Behavioral interactions were videotaped (JVC-Everio S Memory camcorder-GZ-MS215) for subsequent behavioral analysis (see below).

2.3. Sampling

In order to avoid monoamine degradation during the brain macro-dissection and to keep the time of sampling after the social interactions as homogeneous as possible across dyads, only one fish from each dyad was used for monoamine quantification. These fish were sacrificed immediately after the interaction with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L) and the spinal cord sectioned. The brain was macrodissected under a stereoscope (Zeiss; Stemi 2000) into five areas: Olfactory bulb and Telencephalon (OB/TL), Optic tectum (OT), Diencephalon (DE), Cerebellum (CB), and Brain stem (BS). Immediately after collection the brain tissue was placed on dry ice and stored at -80 ºC until analysis.

2.4. Analysis of brain monoamines and metabolites

The frozen macroareas were homogenized in 4% (w/v) ice-cold perchloric acid containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA, the internal standard) using a Sonifier cell disruptor B-30 (Branson Ultrasonics, Danbury, CT, USA) and were

immediately placed on dry ice. Subsequently, the homogenized samples were thawed and centrifuged at 21000×g for 10 min at 4^{o} C. The supernatant was used for high performance liquid chromatography with electrochemical detection (HPLC-EC), analyzing the monoamines dopamine (DA) and serotonin (5-HT, 5-hydroxytryptamine) the DA metabolite DOPAC (3,4-dihydroxyphenylacetic acid) and the 5-HT metabolite 5-HIAA (5-hydroxy indole acetic acid), as described by Overli et al 1999 [10]. In brief, the HPLC-EC system consisted of a solvent delivery as system model 582 (ESA,Bedford, MA, USA), an auto injector Midas type 830 (Spark Holland, Emmen, the Netherlands), a reverse phase column (Reprosil-Pur C18-AQ 3 µm, 100 mm × 4 mm column, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) kept at 40^{o}C and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at reducing and oxidizing potentials of -40 mV and +320 mV. A guarding electrode with a potential of +450 mV was employed before the analytical electrodes to oxidize any contaminants. The mobile phase consisted of 75 mM sodium phosphate, 1.4 mM sodium octyl sulphate and 10µM EDTA in deionized water containing 7% acetonitrile brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations. To correct for recovery DHBA was used as an internal standard using HPLC software Clarity ^{TM} (DataApex Ltd, Prague, Czech Republic). The ratios of 5-HIAA/5-HT and DOPAC/DA were calculated and used as an index of serotonergic and dopaminergic activity, respectively.

For normalization of brain monoamine levels, brain protein weights were determined with Bicinchoninic acid protein determination (Sigma Aldrich, Sweden) according to the manufacturer's instructions. The assay was read on Labsystems multiskan 352 plate reader (Labsystems, Thermo Fisher Scientific) wavelength of 570 nm.

2.5. Behavioral observations

Video recordings were analysed using a computerized multi-event recorder (Observer XT, Noldus, *Wageningen, The Netherlands*). The zebrafish ethogram [26] was used as a reference and the observed behaviors were divided into aggressive (bite, chase and strike) and submissive (freeze and flee). As previously described in [26] dyadic male fights have two distinct phases: the pre-resolution phase where the fight is symmetric and both fish exhibit the same repertoire of behaviors (display, circle, and bite) and the post-resolution phase where all agonistic behaviors are initiated by the winner whereas the loser only displays submissive behaviors. Because we were only interested in the different output of the fights which generate different behavioral phenotypes (eg. winner and loser) we only analyzed the post-resolution phase (i.e. the last 5 min of the 30 min interaction). We also measured the fight resolution time (time for the social hierarchy to be established) in order to compare real opponent with mirror interactions.

2.6. Statistical analysis

Statistical analyses were performed with the software STATISTICA v.10 (StatSoft, Inc., 2011). Parametric statistic was used given that the variables match the parametric parameters. One loser and one control were removed from the analysis, one because the output of its fight was not completely clear and the second because most of the time it was trapped on the partition, resulting in a sample size of 7 for losers and control groups, and 8 for winners and mirror groups. In the behavior analyses, one animal from the winner, loser and mirror groups was removed from the analysis due to a problem with the video recordings which made the analysis impossible. A T-test was used to access differences between types of interactions (real opponent (winner) *vs* mirror) and

fight resolution time. In the monoamines analysis, four samples from the optic tectum were excluded due to problems during the sample preparation. Serotonin, dopamine levels and the respective metabolites, 5HIAA and DOPAC, as well as the activity of both neurotransmitters as measured by the ratios 5-HIAA/5-HT and DOPAC/DA, in brain macroareas were log transformed in order to meet the assumption of normal distribution. A repeated measures ANOVA (repeated factor: brain macroareas with 5 levels, independent factor: male status with 4 levels, winner, loser, mirror, control) was used to identify the main effects and the interaction between brain area and social status on the different monoamine measures, followed by a post-hoc tests and planned comparisons of least squares means between the control group (isolation) and each of the different social status. A PCA analysis was used to reduce the number of behavior variables in the real opponent paradigm. Correlations between behavior and monoamine concentrations were obtained with Pearson correlation coefficients. All tests were two-tailed and statistical significance was set at $p < 0.05$.

2.7. Ethics statement

The animal experimentation procedures used in this study followed the Association for the Study of Animal Behavior and the Animal Behavior Society guidelines for the treatment of animals in behavioral research and teaching and were approved by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 8954).

3. Results

3.1. Behavior

In the real opponent paradigm all pairs except one, develop a clear dominant / subordinate relationship. Social hierarchies were stable and the behaviors exclusive for each phenotype. During the post-resolution phase a winner never became a loser nor a loser became a winner. The behaviors are stereotyped according to social status, aggressive behaviors in winners and submissive behaviors in losers. On the other hand, in mirror interactions because the fight is symmetric along time the resulting phenotype is not apparent, they never behave like losers or winners, and aggressive levels are kept constant during the whole interaction (Fig. 2). This difference is obvious in the fight resolution time ($T=-6.39$, $p<0.0001$; Fig. 2B) where mirror fighters fight for 30 min whereas in the real opponent interaction the fight is solved in approximately 7 min, after which a post-resolution phase is established.

In order to reduce the number of behavioral variables in subsequent analyses in the real opponent paradigm, a Principal Component Analysis (PCA) was performed. Two factors, that together explain 83.1 % of the total variance (Fig. 3A), were extracted that show a clear separation between aggressive and non-aggressive behaviors: PC1 has positive loadings for aggressive behaviors and a negative load for submissive behavior (flee) and explains 65.4% of the variation; PC2 has positive loadings for submissive behavior and negative loadings for all the aggressive behaviors (bite, chase, strike) and explains 17.7 % of the variation (Fig. 3A). PC2 allows the subsequent division of submissive behavior into an active (flee, positive quadrant) and a passive (freeze, negative quadrant) style (Fig.3, B). These results support the separation of aggressive and submissive behavior in the real opponent interaction. In the mirror interaction,

because the behavioral repertoire is restricted to two behaviors (bite, strike) no PCA was performed.

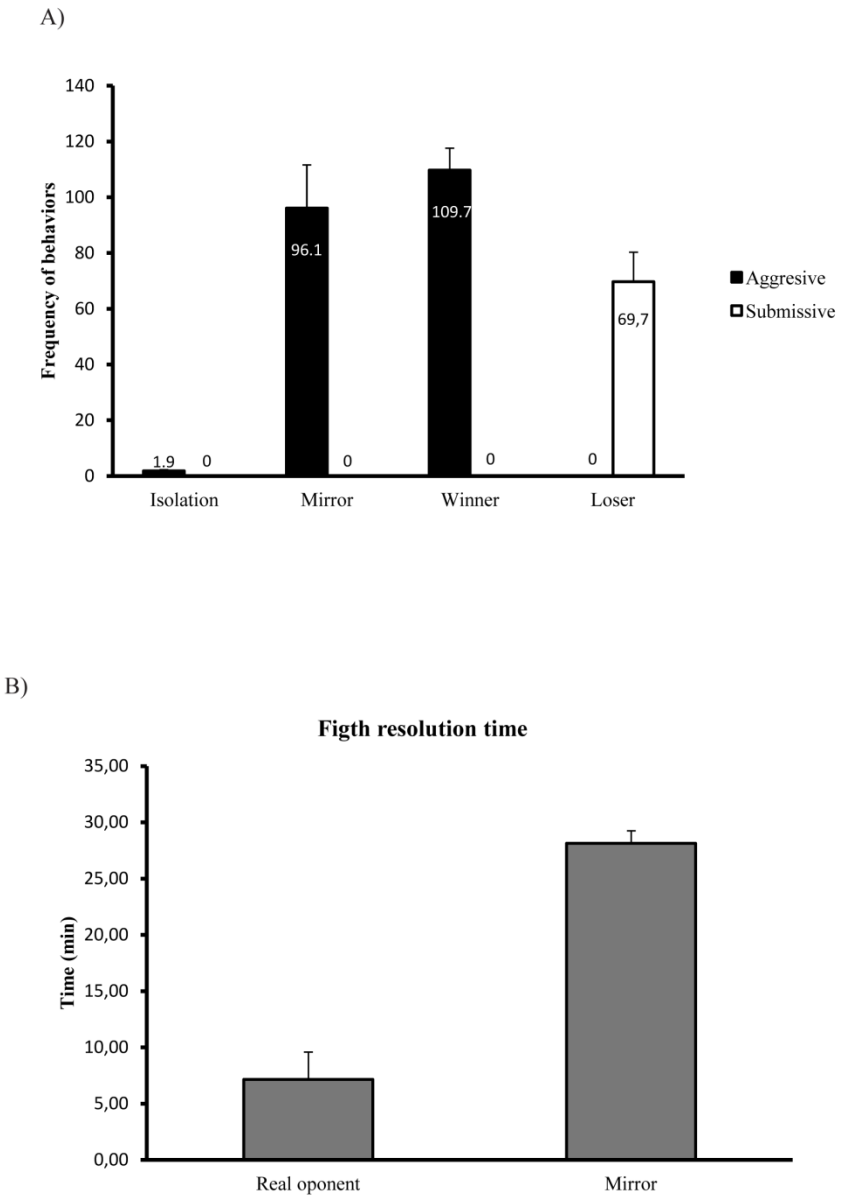


Fig. 2. Behavioral results. (A) Mean number of aggressive acts performed in the last 5 min of the 30 min agonistic interaction; error bars represent the standard error of the mean. (B) Fight resolution time, measured as the time needed for a social hierarchy to be established in the fighting male dyads (counting from the first bite to the post-resolution phase); error bars represent the standard error of the mean (t-test: $T=-6.39$, $p<0.0001$).

A)

	PC1	PC2
Bite	**0,9709**	-0,0325
Chase	**0,9405**	-0,0077
Freeze	-0,52	**-0,8149**
Flee	**-0,7177**	0,4639
Strike	**0,8104**	-0,06406
Percent variation explained	65,40%	17,70%

B)

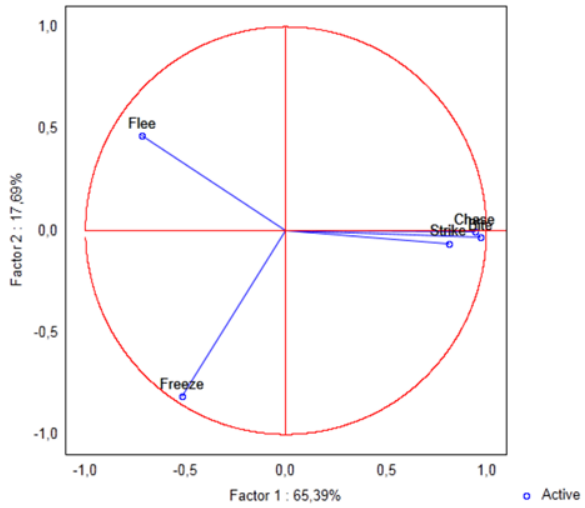


Fig. 3. Principal component (PC) analysis of aggressive and submissive behaviors in the real opponent paradigm. (A) Factor loadings of the behavioral variables and variance explained by each PC. (B) Graphic representation of the extracted PC's: PC1 represents aggressive behavior and PC2 submissive behavior, which can be further divided in active submission (flee) on the positive quadrant and passive (freeze) on the negative quadrant.

3.2. Brain monoamines

Concentrations of serotonin (5-HT), dopamine (DA) and their main metabolites (i.e. 5-HIAA and DOPAC, respectively) in the studied brain areas are given in Table 1.

There was a treatment and brain area main effect for both 5-HT (repeated measures ANOVA; social treatment: $F_{3,\ 25}$=7.86, p<0.001; brain area: $F_{4,\ 100}$=79.39, p<0.0001, respectively) and 5-HIAA (repeated measures ANOVA; social treatment: $F_{3,\ 24}$=8.55, p<0.001; brain area: $F_{4,\ 96}$=50.36, p<0.0001). The post-hoc analyses revealed that social experience increased 5-HT and 5-HIAA levels in animals that fought real opponents (W/L) and mirror image when compared to control group. For serotonin, the concentration was higher in the diencephalon, followed by olfactory bulb/telencephalon, optic tectum and brain stem and the lowest concentration was found in the cerebellum. On the other hand, for the metabolite 5-HIAA, olfactory bulb/telencephalon had the

highest concentration, followed by diencephalon, optic tectum, brain stem and finally cerebellum.

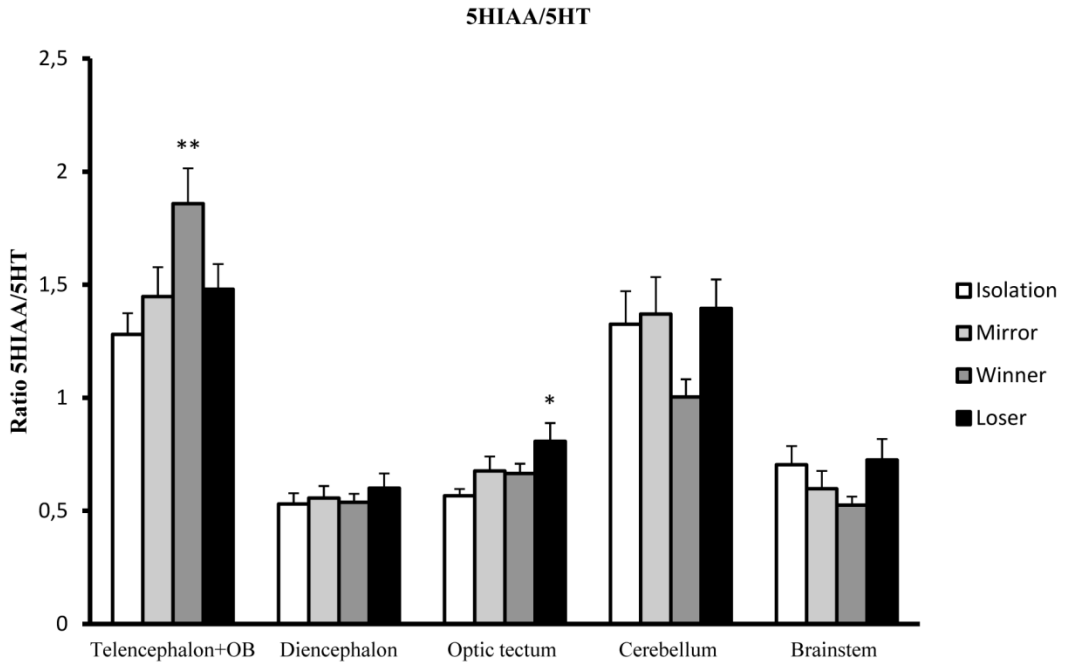
For DA and DOPAC there was also a main effect for treatment and brain area. Social experience also increased DA [$F_{3,\ 24}$=5.03, p<0.01)] and DOPAC levels [$F_{3,\ 25}$=8.31, p<0.001] in winners, losers and mirror fighters suggesting an activation of both systems in acute interactions. DA [$F_{4,96}$=85.68, p<0.0001] distribution across the brain was distinct, with elevated concentrations in the diencephalon, then olfactory bulb/ telencephalon and brain stem, and lastly optic tectum and cerebellum. For DOPAC [$F_{4,\ 100}$=39.09, p<0.0001] olfactory bulb/ telencephalon and diencephalon exhibit the highest concentration, optic tectum and brain stem were after and cerebellum showed the lowest.

There was a significant main effect of brain area but not of social status in the ratios of both 5-HIAA/5-HT (repeated measures ANOVA, brain area main effect: $F_{4,\ 88}$=83.38, p<0.0001; Social status main effect: $F_{3,\ 22}$=1.27, p=0.31) and DOPAC/DA (brain area main effect :$F_{4,\ 68}$=28.53, p<0.00001; social status main effect: $F_{3,\ 17}$=2.17, p=0.13). The post-hoc analyses revealed that 5-HIAA/5-HT ratios were significantly higher in the olfactory bulb/telencephalon, followed by the cerebellum, then optic tectum and brain stem and lastly by the diencephalon. DOPAC/DA ratios were significantly higher in the optic tectum, followed by olfactory bulb/telencephalon, then cerebellum, and diencephalon and lastly in the brain stem. Contrast analysis of 5-HIAA/5-HT and DOPAC/DA activity of an area by area basis revealed that 5-HIAA/5-HT levels were significantly higher in winners' olfactory bulb/telencephalon (F=18.43, p<0.001), and losers optic tectum (F=9.92, p<0.01; Fig 4, A). Regarding the DOPAC/DA, winners had higher activity levels in the olfactory bulb/telencephalon (F=6.32, p<0.05), and mirror and losers in the optic tectum (F=12.05, p<0.01 and F=6.67, p<0.05 respectively; Fig.4,

Table 1- Monoamine and metabolites concentrations (mean ± SEM) in different brain areas, and different treatments. Asterisk (*) in the mean indicates significative differences on specific treatments when compared to control group (Repeated measures ANOVA, *p < 0.05).

		Treatment				
Brain region	Monoamines and Metabolites	Control	Mirror fighter	Winner	Loser	Statistics
Telencephalon	5-HT	3.83± 1.02	6.40± 0.85*	6.15± 1.6	5.61±0.74	$F_{(12, 100)}$=2,48; p<0.01
	5-HIAA	5.26± 1.67	9.06± 1.66*	9,98±1.64*	9.05±1.28*	$F_{(12, 96)}$=1.17; p=0.32
	DA	2.08± 0.57	2.91±0.54	2.57± 0.53	2.28±0.31	$F_{(12, 96)}$=3.11; p<0.001
	DOPAC	0.46± 0.16	0.78±0.20	0.68±0.08	0.54±0.11	$F_{(12, 100)}$=3.55; p<0.001
Diencephalon	5-HT	8.86± 1.43	9.26± 0.75	8.19± 0.95	10.54±0.92	$F_{(12, 100)}$=2,48; p<0.01
	5-HIAA	4.44± 0.71	4.99± 0.45	4.22±0.33	5.86±0.36*	$F_{(12, 96)}$=1.17; p=0.32
	DA	6.25± 0.72	6.23±0.52	5.01± 0.68	6.93±0.65	$F_{(12, 96)}$=3.11; p<0.001
	DOPAC	0.53± 0.08	0.67±0.06	0.52±0.04	0.83±0.09*	$F_{(12, 100)}$=3.55; p<0.001
Optic tectum	5-HT	4.19±0.34	4.11± 0.36	4.04± 0.22	3.55±0.28	$F_{(12, 100)}$=2,48; p<0.01
	5-HIAA	2.37± 0.25	2.11± 0.39	2.69±0.26	2.87±0.26	$F_{(12, 96)}$=1.17; p=0.32
	DA	0.92± 0.07	1.13±0.17	1.01± 0.06	0.83±0.08	$F_{(12, 96)}$=3.11; p<0.001
	DOPAC	0.18± 0.01	0.41±0.03*	0.28±0.02*	0.33±0.05*	$F_{(12, 100)}$=3.55; p<0.001
Cerebellum	5-HT	0,32±0.06	1.72± 0.51*	1.47± 0.25*	1.08±0.26*	$F_{(12, 100)}$=2,48; p<0.01
	5-HIAA	0.92± 0.49	2.17± 0.60*	1.47±0.60*	1.75±0.42*	$F_{(12, 96)}$=1.17; p=0.32
	DA	0.20± 0.02	1.63±0.63*	1.02± 0.17*	0.72±0.17*	$F_{(12, 96)}$=3.11; p<0.001
	DOPAC	0.04± 0.01	0.30± 0.09*	0.15±0.04*	0.18±0.05*	$F_{(12, 100)}$=3.55; p<0.001
Brain stem	5-HT	3.62±0.56	3.39± 0.40	6.51± 1.18*	4.43±1.43	$F_{(12, 100)}$=2,48; p<0.01
	5-HIAA	2.37± 0.32	2.26± 0.18	3.15±0.36	3.03±0.38	$F_{(12, 96)}$=1.17; p=0.32
	DA	2.63± 0.41	2.23±0.21	4.23± 0.66*	3.21±0.86	$F_{(12, 96)}$=3.11; p<0.001
	DOPAC	0.27± 0.06	0.35± 0.03*	0.46±0.04*	0.41±0.08*	$F_{(12, 100)}$=3.55; p<0.001

A)



B)

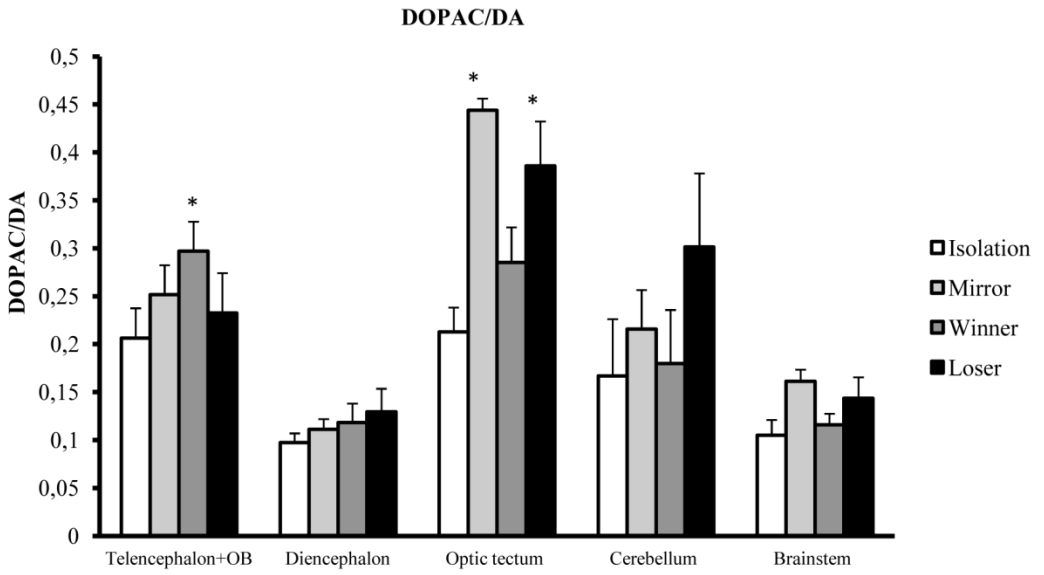


Fig. 4- Monoaminergic activity in different brain areas following an acute social interaction: (A) HIAA/5-HT ratio; (B) DOPAC/DA ratio. Error bars represent the standard error of the mean (Repeated measures ANOVA, *$p < 0.05$ and ** $p<0.01$).

B). There was also a marginally non-significant tendency for losers to have increased DOPAC/DA ratios in the cerebellum ($F=3.96$, $p=0.06$).

3.3. Relationship between monoamines and behavior

Correlations analyses between behavior and monoamine in different brain areas revealed that in the real opponent paradigm there were negative correlations between 5-HIAA levels ($r= -0.70$, $N=12$, $p<0.05$) and DOPAC levels in the diencephalon ($r= -0.58$, $N=13$, $p<0.05$) and aggressive behavior, and between 5HIAA/5HT ratio in the diencephalon and submissive behavior ($r= -0.69$, $N=12$, $p<0.05$). Positive correlations were found between DA levels in the diencephalon and submissive behaviour ($r= 0.60$, $N=13$, $p<0.05$) and DA levels in the cerebellum and aggressive behaviour ($r= 0.76$, $N=12$, $p<0.01$).

In the mirror fighting treatment there were positive correlations between bite frequency and 5-HIAA levels in the optic tectum ($r= 0.81$, $N=7$, $p<0.05$), the 5HIAA/5HT ratios in the diencephalon ($r= 0.90$, $N=7$, $p<0.01$) and optic tectum ($r= 0.83$, $N=7$, $p<0.05$) and DOPAC/DA ratio in the diencephalon ($r= 0.76$, $N=7$, $p<0.05$). Strike frequency was negatively correlated with 5-HT and DOPAC levels in the cerebellum ($r= -0.79$, $N=7$, $p<0.05$; $r=-0.79$, $N=7$, $p<0.05$) and positively correlated in the optic tectum with DA levels ($r= 0.77$, $N=7$, $p<0.05$). All other correlations were non-significant.

4. Discussion

In the current study it is shown that following an acute agonistic encounter zebrafish males express two distinct behavior profiles depending on the social status achieved: losers exhibit exclusively submissive behaviors, whereas winners express

only aggressive behaviors (Fig.2, A). After the relative fighting ability has been established, the different behavioral repertoires for each social status are stable over time (at least up to 5 days, R.F. Oliveira and co-workers, unpublished data). For animals that fought their own mirror image only aggressive behaviors were observed, with a frequency that was not significantly different from that observed in winners of real opponent fights (T-test: T=-0.84, p=0.42). However, a major difference between winners and mirror fighters is present, not on their behavioral output, but rather on the behavior observed in the opponent, since in mirror fights the opponent (i.e. own image on the mirror) never displays submissive behaviors. As a consequence mirror fights were unsolved fights, as can be demonstrated by the fact that the expression of aggressive behavior typical of the pre-resolution phase lasted for the whole duration of the trial (30 min), whereas in real opponent fights the encounter was resolved in approximately 7 min (after which post-resolution behavioral profiles were observed). Therefore, the experimental design used successfully produced four types of social phenotypes: winners, losers, individuals that expressed aggressive behavior but did not experience either a win or a loss (i.e. mirror fighters), and individuals that did not express or perceived any social behavior (control = social isolation). Therefore, the comparison of monoamine levels in regions of interest in the brain across these four social phenotypes allows the investigation of the short-term effects of acute social interactions depending on perceived outcome by the participants.

For monoamines, we found that 5-HT levels are significantly higher in the telencephalon of mirror fighters, in the brain stem of winners and in the cerebellum of all experimental groups. The increase in 5-HT brain levels in the telencephalon and brain stem suggests that mirror fighters and winners are the groups where the serotonergic system is first activated in response to a social interaction and although

they behave similarly, the brain areas activated are distinct which may indicate different perception of the context. We also found a brain area (i.e. cerebellum) that responds to acute stress independent of the interactions type (i.e. an increase in all groups was seen compared to controls).

For 5-HT metabolite (5-HIAA), significant increases were found in the telencephalon and in the cerebellum of all treatments (winner, losers, and mirror interaction), and in the diencephalon of losers. Interestingly, 5-HIAA levels in the diencephalon were negatively correlated with aggressive behavior in the real opponent paradigm supporting the diencephalon enrolment in the regulation of aggressive behaviour. On the other hand, aggressive behavior (bite frequency) was positively correlated with 5-HIAA in the optic tectum for mirror fighters. This later correlation may be primarily associated with increased visual stimulation in mirror fighters.

Our results suggest that acute interaction activated serotonergic system increasing 5-HT and 5-HIAA brain levels in response to different social conditions.

Serotonergic activity in turn, is significantly higher in the telencephalon of winners and in the optic tectum of losers, and no significant changes was observed in mirror fighters. Moreover, in real opponent fights serotonergic activity in the diencephalon was negatively correlated with submissive behavior and in mirror fights serotonergic activity both in the diencephalon and in the optic tectum is positively correlated with overt aggression (i.e. bites). Given that social interaction did not affect 5-HT levels in these brain areas, 5-HT activity was mainly determined by metabolite levels. These results suggest that serotonergic activity is differentially regulated in different brain regions by social interactions. In zebrafish three clusters of serotonergic neurons have been described: the raphe nuclei, the posterior tuberculum/hypothalamic populations and the pretectal area. The telencephalon (including the olfactory bulbs) receives projections

from the dorsal cells of the superior raphe [27,28]. Most of the 5-HT–ir fibers terminate in dorsolateral parts of the rostral telencephalon and a minor part continues ventrally into the olfactory bulb [29]. Thus, the observed increase in telencephalon and olfactory bulb serotonergic activity in winners may reflect an activation of the superior raphe projections in this social condition. Alternatively this increase in telencephalic serotonergic activity may be due to pre-synaptic stimulation of the terminal areas, which has been demonstrated, by disinhibition of GABAergic interneurons, increased glutamatergic local stimulation, and glucocorticoid infusion [30, 31].

Most of the serotonergic fibers in the optic tectum seem to originate from serotonergic neurons of the pretectal cluster [29]. Pretectal nuclei, as well as the optic tectum, have been implicated in the regulation of visual and motor behavior, multimodal sensory integration [32] and escape responses [33], which may explain the significant increased in subordinates or loser conditions, as observed in the present study. In mammals, avoidance responses are obtained from stimulations in a region of the superior colliculus that appears to represent the upper visual field [34]. Finally, serotonergic activity in the diencephalon which must represent the activation of the posterior tuberculum/hypothalamic 5-HT neuronal populations was positively correlated with overt aggression (i.e. bites) in the mirror fights and negatively correlated with submissive behavior in real opponent fights, suggesting a role for these serotonergic populations in the balance between aggressive and submissive behavior.

The activation of the serotonergic system in response to social interactions had been previously demonstrated for other species. In early stages of hierarchy formation the serotonergic system appears to be activated in both dominants and subordinates. For example, 5-HT levels were elevated after 10 min of social interaction in the limbic regions and in the locus coeruleus of dominant and subordinate fighting lizard males (in

Anolis carolinensis) [35]. In rainbow trout (*Oncorhynchus mykiss)* both dominants and subordinates increased 5-HT activity in the telencephalon and optic tectum 3h after the interaction [10]. Similarly, in the bicolor damselfish (*Stegastes partitus),* after a chronic interaction of 5d dominants as well as subordinates showed higher levels of 5-HT activity in the telencephalon [36]. Other studies have shown that serotonergic activity has similar patterns in dominants and subordinates but this pattern seems to be temporally advanced in dominants [35]. Our data does not allowed such comparison since we only collect one time point but we can speculate that the differences between social status in the brain are due to a time line that is acting at different speeds depending on social status, given that dominants and subordinates exhibit already differential patterns of 5-HT activation a short time after the resolution of the fight.

In the dopaminergic system there was a significant increase in DA levels in the cerebellum for all groups, and in the brain stem of winners. In the real opponent paradigm DA levels were positively correlated with aggressive behavior in the cerebellum and in the diencephalon with submissive behavior. For DOPAC, there was a significant increase for all groups in several brain areas; optic tectum, cerebellum and brain stem and in the diencephalon of losers. We also found a negative correlation of DOPAC in the diencephalon with aggressive behavior. These results point out the contribution of diencephalon in the regulation of submissive behavior. For mirror fighters DOPAC levels in the cerebellum were positively correlated with strikes.

On the other hand, dopaminergic activity was significantly higher in the telencephalon of winners and in the optic tectum of both losers and mirror fighters and these increases were mainly determined by the metabolite levels. Moreover, the expression of aggressive behavior was positively correlated with dopaminergic activity in the diencephalon in mirror fights. Together these results suggest an involvement of

the diencephalic monoaminergic system in the regulation of aggressive and submissive behaviors in different social conditions. This hypothesis is further supported by the known role of different diencephalic nuclei in the regulation of species-specific behaviors across vertebrates. For example, in the bluegill fish (*Lepomis macrochirus*) stimulation of the preoptic region inhibits aggressive behaviors and evoke courtship, and stimulation of a region surrounding the lateral recess elicits aggressive behavior and feeding [37]. Similarly, in golden hamsters and rats, the anterior hypothalamus [38] and the nucleus accumbens [16] respectively, have been implicated in the regulation of aggressive behaviors, and in Syrian hamsters (*Mesocricetus auratus*) the nucleus accumbens is involved in conditioned defeat [39].

Dopamine release appear to be affected also in other brain areas, as the cerebellum and brain stem, but there were no significant differences in DOPAC/DA ratios since both the neurotransmitter and the metabolite levels increased in parallel indicating an increase in monoaminergic activity.

The increased dopaminergic activity in the telencephalon when males successfully achieve dominant status (i.e. winners) may be representative of social reward. A similar pattern has been previously observed in salmonids where dominant individuals showed higher DA activity in telencephalon than subordinate fish [40]. However, in contrast to amniotes, where the dopaminergic mesolimbic reward system is located in the ventral tegmental area (VTA), that project rostrally to the nucleus accumbens, amygdala and cortical areas (e.g. prefrontal cortex in mammals), fish do not present a midbrain dopaminergic population homologous to the VTA [41]. In contrasts, in fish the DA inputs to the telencephalon originate in a local subpallial DA system and in DA neurons in the ventral diencephalon, in particular in the posterior tuberculum, that project towards the subpallium [42-44]. Therefore, although evolutionary it cannot be

considered as homologous to the mammalian VTA DA neurons, in fish this ascending DA pathway may be playing a similar role in reward behavior as the mammalian mesostriatal DA pathway. On the other hand, the increased DA activity observed in losers and mirror fighters must be a consequence of the differential activation of another DA subsystem. A pretectal DA cell group (alar plate of p1) is consistently found in bony fishes, amphibians, and most amniotes except mammals [41]. These pretectal neurons are projecting mostly on the optic tectum, in a layer-specific fashion and they may play a role in the modulation of the retino-tectal visual input [45]. In this regard it is extremely interesting to note that the similar optic tetctum DA activation in mirror fighters and losers, despite the dissimilarities of their behavioral profile (i.e. mirror fighters are as aggressive as winners, and losers in contrast, are submissive), suggests that what is driving the DA activation in this region is the perception of the interaction, which is similar in mirror fighters and losers (i.e. both are exposed to an aggressive opponent), rather that the behavioral output of the focal individual.

In summary the data presented here confirms that acute social interactions elicit rapid and differential changes in serotonergic and dopaminergic activity across different brain regions in zebrafish. Further studies are needed to elucidate the specific roles of different neuromodulatory subsystem in the regulation of social behavior. Finally, the ability of zebrafish reported here to respond to experimental manipulations of its social environment, combined with the fact that it is a species that expresses both gregarious (shoaling) and territorial behavior, makes it a promising model organism in social neuroscience. In comparison to other established models in this field, such as cichlid fish (e.g. *Astatotilapia burtoni* [46]), zebrafish has the added value of having a large genetic tool box available that can be used to genetically dissect the mechanisms involved in social decision-making.

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Chapter IV

Social modulation of nonapeptides in the zebrafish brain

Social modulation of nonapeptides in the zebrafish brain

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Abstract

The nonapeptides of the vasotocin (AVT) / oxytocin (IT) family have been implicated in the regulation of social behaviour in a wide range of taxa. In order to be efficient in modulating the expression of social behaviour according to changes in the social environment these peptides are expected to respond themselves to acute and transient changes in social context. Here we tested the hypothesis that short-term social interactions drive changes in nonapeptide (i.e. AVT and IT) levels across different brain regions. For this purpose we exposed male zebrafish to two types of social interactions: (1) real opponent interactions, from which a Winner and Loser emerged; and (2) mirror-elicited interactions, that produced individuals that did not experience a change in social status despite expressing similar levels of aggressive behaviour to those of participants in real-opponent fights. Non-interacting individuals were used as a reference group. Each social phenotypes (i.e. Winners, Losers, Mirror-fighters) presented a specific

profile of nonapeptide activity when compared to the reference group. Moreover, the comparison between the different social phenotypes allowed to disentangle the specific aspects of the interaction (e.g. assessment of opponent aggressive behaviour vs. self-assessment of expressed aggressive behaviour) that triggered the observed neuropeptide responses. Overall, AVT responded more to the agonistic interactions than isotocin, which highlights the preferential role of AVT in the regulation of aggressive behaviour, whereas IT seems to be more related to affiliative behaviours.

Keywords: arginine-vasotocin; isotocin; social dominance; aggression; stress

1. Introduction

In a wide range of vertebrate species, nonapeptides of the arginine-vasotocin (AVT) / isotocin (IT) family, the teleost homologues of the mammalian arginine vasopressin (AVP) and oxytocin (OT), respectively, have emerged as key regulators of social behaviour [1]. AVT/AVP and IT/OT are known to be associated with a variety of social behaviours [1], including the regulation of aggressive behaviour [2–4] and social status acquisition [5–10]. However, in the species studied so far there is considerable variation in the function of both circuits (direction and intensity), which appears to be species and context-dependent, making the underling mechanism relatively diverse [11]. Among fish, AVT and IT administration could either increase or decrease aggression and courtship depending on the species [4]. In general, the AVT/IT neurosecretory system in fish consists of three main cell groups distributed along the ventral portion of the preoptic area [gigantocellular (gPOA), magnocellular (mPOA), and parvocellular (pPOA), reviewed in [12]], which project fibers to multiple target areas, such as ventral telencephalon, diencephalon, and various mesencephalic structures, in addition to neurohypophysial projections [13], suggesting a diffuse neuromodulatory role for these peptides. Therefore, the nonapeptide regulation of social behaviour may occur at multiple target areas and at different levels. First, it can be influenced by the number of nonapetide producing cells and their activity (e.g. as indicated by cell body size) in the relevant cell group(s) in the POA. In some teleost species, the expression of social dominance has been associated with a higher number or size of AVT-ir cells in mPOA or gPOA, whereas social submission has been associated with the number or size of pPOA AVT-ir cells (e.g. zebrafish, *Danio rerio* [9]; African cichlid, *Astatotilapia burtoni* [8] butterfly fishes [14]); in contrast, in other species social submission has been associated with changes in the mPOA and gPOA populations (e.g. African cichlid

Oreochromis mossambicus [7]), and aggressive behaviour with variations in size of the pPOA AVT-ir instead (Pupfish, *Cyprinodon nevadensis amargosae* [15]). Secondly, the sensitivity of the target tissue to nonapeptides (e.g. as indicated by the local expression levels of their receptors) may also regulate the social behaviour. For example, in zebrafish the V1b receptor is differentially expressed in the hypothalamus according to social status [16], and in pupfish (*Cyprinodon nevadensis amargosae*) transcripts encoding V1a1 are expressed at higher levels in the telencephalon and hypothalamus of subordinate males, whereas the V1a2 variant is more abundant in the dominants telencephalon [10]. Thirdly, since these peptides are produced in the cell body of AVT/IT cells and are then transported axonally to the target areas where they are released at the synapses, the local availability of these peptides may also regulate behaviour. However, few studies have measured local peptide concentrations at regions of interest in the brain for the expression of social behaviours. In cichlids subordinate males present higher AVT levels in whole brain and pituitary than dominants, and no difference between social status was detected for IT [6,17]. In the three-spined stickleback, both AVT and IT levels are higher in whole brain of dominant individuals, whereas female levels are related to breeding and egg deposition, rather than to aggression [18,19]. Finally, among different wrasse species AVT/IT levels have been shown to vary with the degree of cleaning (mutualistic) behaviour [20]. When taken together the examples provided above suggest an association between AVT/IT systems and social status in fish, but the specific mechanism does not seem to be conserved. Since this variation can occur either at the level of the AVT/IT neuronal populations involved, or in the spatial distribution (i.e. different brain areas) or abundance of specific receptors, a good approach for the study of such diversity is the direct

measurement of these peptides in brain areas where they are hypothesized to act as neuromodulators.

In the present work we used zebrafish (*Danio rerio*) males to study the effects of changes in social status on brain nonapeptide levels. Zebrafish males establish dominance relationships through agonistic interactions, and the behaviour expressed in these interactions is well characterized. [21,22]. At the start of the interaction both opponents exhibit the same behavioural repertoire (displays, circle, and bites). After the fight is solved and a Winner and a Loser emerge, an asymmetry of expressed behaviours is observed, where all aggressive acts are initiated by the dominant and the subordinate only displays submissive behaviours[21]. In zebrafish the outcome of a fight can have a significant impact in subsequent interactions, since the Winner of an encounter is more likely to win its next interaction, whereas Losers decrease the probability of success, indicating the relevance of past experience in agonistic interactions [21]. In the behavioural paradigm used here, acute (30 min) agonistic encounters between conspecifics produced three behavioural phenotypes: fish that either won (Winners) or lost (Losers) a real-opponent interaction and that concomitantly increased or decreased its social status; and fish that fought their own image on a mirror, and therefore despite expressing aggressive behaviour and observing it in its opponent (i.e. the mirror image) do not experience either a win or a defeat and therefore do not experience a change in social status. Thus, we assessed the changes in nonapeptide levels triggered by changes in social status (increase in Winners; decrease in Losers) and by the expression/perception of aggressive behaviour independently of social status changes, as experienced by Mirror-fighters.

2. Methods

2.1. Animals

Thirty-two adult wild-type zebrafish (*Danio rerio*) males of the AB strain were used in this experiment. Animals were breed and held at the Fish Facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) in mixed sex groups under a 14L:10D photoperiod and with a water temperature of 28°C. Water quality was monitored daily for pH and conductivity (7 and 700 µSm respectively), and weekly for ammonia (0.01-0.1 ppm), nitrite (<0.2 ppm) and nitrate (<50 ppm) concentrations. Animals were fed twice a day.

2.2. Behavioural paradigm

Fish were tested in an agonistic behaviour paradigm previously described for zebrafish [21,22]. In brief, animals were grouped in size matched pairs and each pair randomly assigned to one of the following conditions: real opponent fights (mean length ± SEM: 2.78 ± 0.03 cm; mean body mass ± SEM: 0.28 ± 0.01 g); mirror elicited fights (mean length ± SEM: 2.67 ± 0.04 cm; mean body mass ± SEM: 0.27 ± 0.01 g); no social interaction (mean length ± SEM: 2.82 ± 0.05 cm; mean body mass ± SEM: 0.31 ± 0.01 g). Dyads were left overnight in the experimental tank (5 x 8 x 6 cm) that was beforehand divided with an opaque PVC partition into two visually isolated areas. After this period, the partition was removed and fish were allowed to interact with a conspecific in the real opponent interaction, or with their own mirror image on a mirror, in the mirror-elicited fight, for a period of 30 minutes. For the control group no social interaction occurred; on each side a partition was also removed (to control for putative handling stress) but the opaque PVC divider between the two animals remained in place preventing any visual contact between the two fish. Thus, four behavioural phenotypes

emerged: Winners and Losers of real opponent interactions, mirror-fighters that experience unsolved fights, and non-interacting fish that serve as a reference (control) group. Behavioural interactions were recorded with a digital camera for subsequent behavioural analysis.

2.3. Brain collection

Immediately after the encounter animals were sacrificed with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L) and the spinal cord sectioned. The brain was macrodissected under a stereoscope (Zeiss; Stemi 2000) into six areas: Olfactory bulbs (OB), Telencephalon (TL), Optic tectum (OT), Diencephalon (DE), Cerebellum (CB), and Brainstem (BS). Immediately after collection brain tissue was placed on dry ice and stored at -80 ºC until further processing. In order for the sampling time of brain tissue between individuals to be as homogeneous as possible, only one fish from each dyad was used for nonapeptide quantification.

2.4. Quantification of nonapeptides by high performance liquid chromatography with fluorescence detection (HPLC-FL)

Brain areas were weighed and sonicated in 1 mL of Milli-Q water (Microson™XL, Misonix, USA) acidified with glacial acetic acid (3 μL), and placed in a boiling water bath for 3.5 min. The homogenates were then centrifuged (12.000g, 20min, 4°C), and the supernatants loaded into solid phase extraction (SPE) columns (100 mg/1 mL, C18 Bakerbond, J.T. Baker) previously conditioned with 3 mL methanol and 3 mL Milli-Q water. To purify the samples, columns were washed sequentially with 1 mL of 5% acetic acid, 1 mL Milli-Q water and 1 mL of 5% methanol, and the peptides eluted with 2 mL mixture of ethanol: 6M HCl (2000:1, v/v). The eluate was evaporated to dryness

in a Turbo Vap LV Evaporator (Caliper Life Sciences, USA) and samples frozen, and stored at -80 °C until further processing.

For HPLC analysis, samples were dissolved in 40 µL of 0.1% TFA (trifluoroacetic acid) in 30% acetonitrile and divided into two replicates. Pre-column derivatization of AVT and IT was performed according to the procedure previously reported [23]. For derivatization reaction, 20 µL of sample and 20 µL of 0.2 M phosphate buffer (pH=9) were mixed, and 3 µL of NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg in 1 mL of acetonitrile) was added. The solution was heated at 60 °C for 3 min, cooled on ice, acidified with 4 µL of 1 M HCl and eluted in a HPLC column. Derivatized samples were measured with Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA). Chromatographic separation was done on an Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 µm particle size). The gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in acetonitrile: H_2O (3:1). A linear gradient was 40–65% of eluent B in 20 min. Flow rate was set at 1 mL/min and the column temperature set to 20 °C. Injection volume was 47 µL and fluorescence detection was carried out at 530 nm with excitation at 470 nm. The two peptides were analysed simultaneously in every sample and data expressed in pmol of peptide per tissue weight (mg).

2.5. Behavioural analysis

For the behavioural data, video recordings were analysed using a computerized multi-event recorder (Observer XT, Noldus, *Wageningen, The Netherlands*) and following the ethogram of zebrafish agonistic behaviour [21]. The following behaviour measures were taken: latency to the first interaction; fight resolution time; and

frequency of aggressive (bite, chase and strike) and submissive (freeze and flee) behaviours in the last five minutes of the interaction (post-resolution phase). The last two measures were taken at the end of the interaction because at this stage of the fights behavioural phenotypes are well differentiated: Winners only express aggressive behaviours, whereas Losers only express submissive behaviours.

2.6. Statistical analysis

To assess differences between real opponent and mirror-elicited fights, the latency for the first attack, fight resolution time, and aggression frequencies were compared with a t-test. The effects of social phenotypes (Winners, Losers, Mirror-fighters and Controls) and of the different brain areas (i.e. Olfactory bulbs, Telencephalon, Diencephalon, Optic tectum, Cerebellum and Brainstem) on neuropeptide (AVT and IT) levels were assessed using a linear mixed model (LMM) with a random effect for the subjects. Data were log transformed in order to meet the parametric assumptions of normal distribution and homoscedasticity. Post-hoc tests were used to identify which groups within each factor were responsible for significant main effects. Planned multiple comparisons analyses followed to evaluate the effect of social context (Winners vs. Losers vs. Mirror-fighters vs. Controls) on nonapeptide levels within each brain area. Pearson correlations were used to assess the association between AVT and IT levels and behavioural data. Linear discriminant function analyses (LDA) were used on AVT and IT concentrations across all brain regions, to identify the variables that contribute the most to differentiate the 4 social treatments.

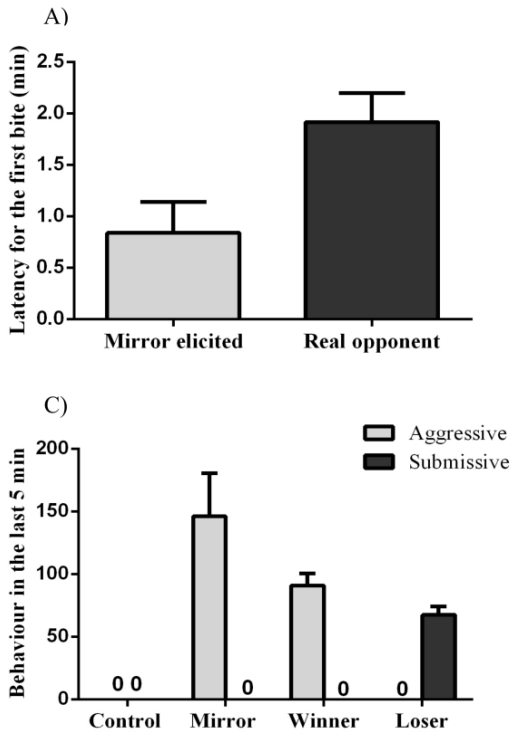
Sample sizes varied due to technical difficulties during nonapeptide quantification, or to outlier values identified for each condition using the generalized extreme studentized deviate procedure with a $p = 0.05$ and a maximum number of outliers of

20% of the sample size. Statistical analysis was performed on R [24], using the following packages: nlme (linear mixed models), multcomp (multiple comparisons), and on STATISTICA V10 and SPSSV21. For all tests the significance level used was $p < 0.05$.

3. Results

3.1. Behaviour

In real opponent fights the latency to the first attack was longer ($t=2.31$, $df=20$, $p< 0.05$; Fig. 1A) and the time to solve the interactions was shorter ($t=13.84$, $df=19$, $p<0.0001$; Fig. 1B) than in mirror-fights. There were no differences between the two types of interactions in the frequency of aggressive behaviours ($t=1.53$, $df=14$, $p> 0.05$; Fig. 1C).



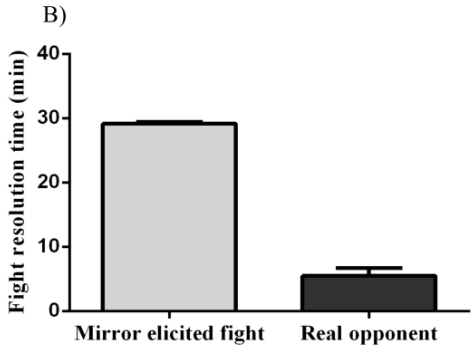


Figure 1 – Behavioural results. A) Latency for the first bite, B) Fight resolution time, measured as the time needed for a social hierarchy to be established in the real opponent dyads, C) Mean number of aggressive acts performed in the last 5 min of the 30 min agonistic interaction; error bars represent the standard error of the mean; error bars represent the standard error of the mean.

3.2. Nonapeptide levels in the brain

There were significant main effects of social status (LMM; $F_{3, 25}$ =4.49, p<0.05), and of brain area (LMM; $F_{5, 109}$ =3.56, p<0.01) on AVT levels, whereas no effect of the interaction between these two main factors was detected (i.e. social status x brain area, LMM; $F_{15, 109}$ =0.75, p> 0.05). Post-hoc analyses on the effect of social status revealed that AVT levels were higher both in Mirror-fighters and in Losers than in controls (z =3.17, p=0.008, and z =3.31, p=0.005, respectively), whereas post-hoc analyses of the main effect of the brain area revealed that the diencephalon has higher AVT levels than either the optic tectum (z = -2.46, p=0.007) or the brainstem (z =- 3.66, p=0.003). There were also close to significance higher levels of AVT in the cerebellum than in the brainstem (z =- 2.68, p=0.07). Planned comparisons of the effect of social treatment at each brain area revealed that all social phenotypes (i.e. mirror fighters, winners and losers) increased AVT levels in the telencephalon when compared to the control group (z = 2.15, p=0.03, z = 2.78, p=0.005, z = 2.54, p=0.01, respectively, Fig. 2A). In the diencephalon, only Winners (z = 2.23, p=0.02) and Losers (z = 2.25, p=0.02) heightened AVT levels, and in the optic tectum and cerebellum changes were only observed in the Losers (z = 2.17, p=0.02; z =2.02, p=0.04), always using the control group as a reference.

There were no significant main effects either of social status (LMM; $F_{3, 25}$ =0.049, p >0.05) or of brain nuclei (LMM; $F_{5, 93}$ =1.99, p=0.08) on IT brain levels. However, the interaction between these two factors was significant (LMM; $F_{15, 93}$ =2.43, p<0.01). Planned comparisons within each brain region revealed a decrease of IT levels in the olfactory bulbs of Winners in comparison with the other behavioural phenotypes (Controls: z = - 2.95, p=0.003; Mirror-fighters: z = - 3.62, p=0.0003; Losers: z = 2.82, p=0.004, Fig. 2B). In contrast, there was an increase of IT levels in the diencephalon of

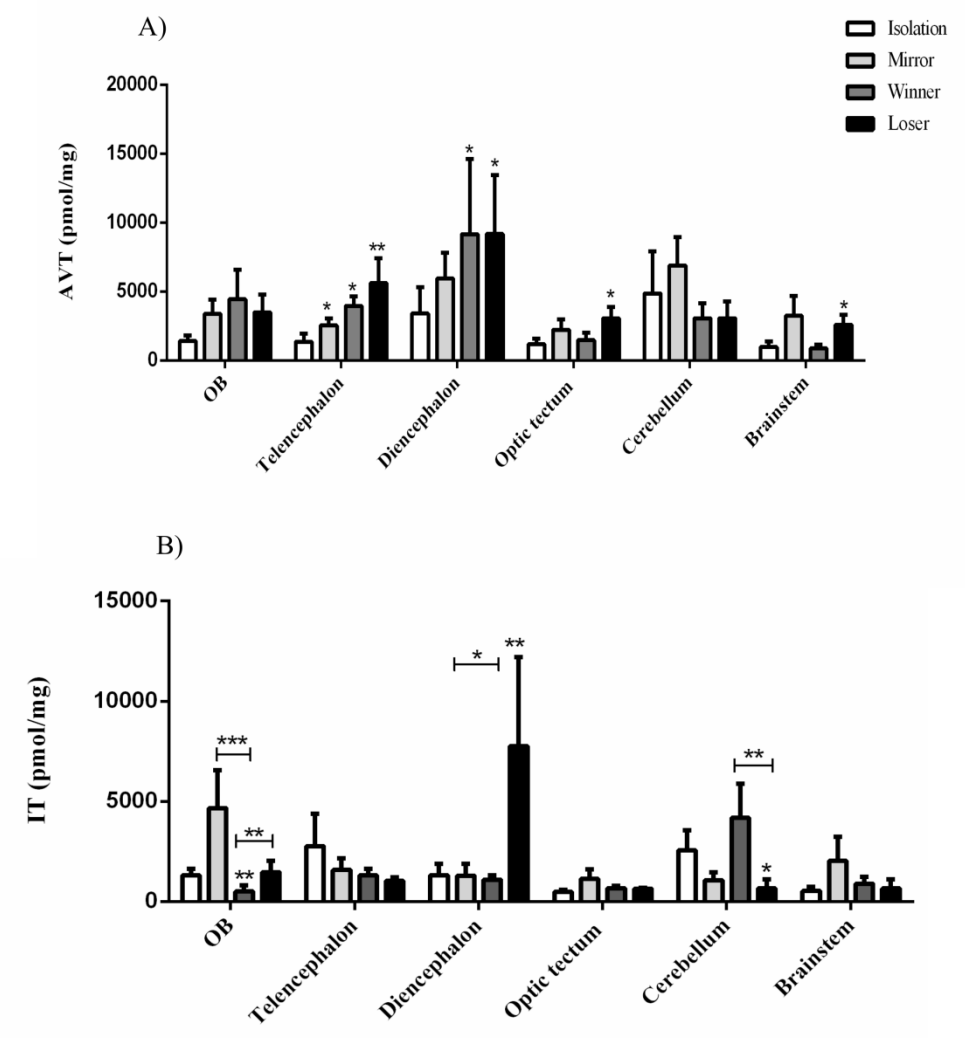


Figure 2 - Arginine-vasotocin and isotocin levels in different brain areas following an acute agonistic interaction: A) AVT; B) IT. Error bars represent the standard error of the mean (Planned comparisons, *p < 0.05, ** p<0.01, ***p> 0.001).

Losers in comparison to all other phenotypes (Controls: z = 2.74, p=0.006; Mirror-fighters: z = 2.44, p=0.014; Winners: z = 2.15, p=0.03), and a decrease in the cerebellum in comparison with either Controls (z = - 2.07, p=0.03) or Winners (z = - 2.60, p=0.009).

Linear discriminant analysis (LDA) for AVT in all regions revealed a single significant function (function 1, χ^2 = 38.89, P < 0.01) that explained 95.6% of the variance (Fig. 3A). This discriminant function was most heavily loaded by the cerebellum (-2.77), optic tectum (2.68) and telencephalon (2.17) followed by the

olfactory bulbs (1.10), suggesting that AVT levels in these three areas are the best predictor for distinguishing between the examined behavioural phenotypes: Winners (group centroid = 1.109), Losers (group centroid = 4.749), Mirror-fighters (group centroid =-4.95), and Control group (group centroid =-0.45). This LDA correctly classified 100% of the animals for all experimental groups. Regarding IT levels, LDA also revealed one significant function (function 1, χ^2 = 36.51, P < 0.01) that explained 99.8% of the variance found (Fig. 3B). This function was most heavily loaded by the telencephalon (10.52) and diencephalon (-10.46), followed by olfactory bulbs (2.45), cerebellum (2.08) and optic tectum (1.12), indicating that the areas that are the best predictors of different social phenotypes differed between AVT and IT. The discrimination between groups was not so evident for the IT LDA function: control group (group centroid = 14.26), mirror fighters (group centroid =21.62), winners (group centroid =15.70) and losers (group centroid =-127.2); and it correctly classified 100% of the animals in the Control group, 66.7% of Mirror-fighters, 80% of Winners and 33% of Losers, with an overall classification success of 75%.

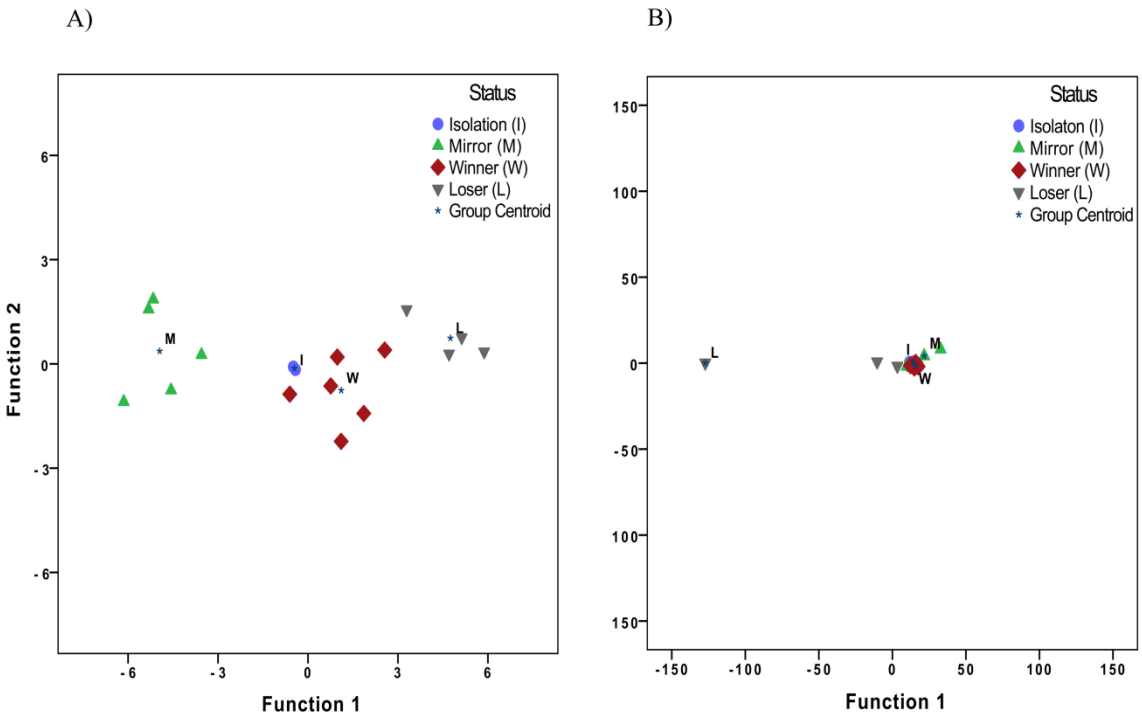


Figure 3 – Linear discriminant function analysis of nonapeptide levels. A) AVT discriminant functions, B) IT discriminant functions Discriminant scores are plotted and stars represent the centroid of each social group.

3.3. Relationship between nonapeptides and behaviour

Correlations between nonapeptide levels and behaviour were observed in all behavioural phenotypes. In Winners there were two close to significant negative correlations, between AVT levels in the telencephalon and cerebellum and aggressive displays (r= -0.80, n=6, p=0.054, r= -0.72, n=7, p=0.07, respectively). There was also a marginally non-significant positive correlation between aggression and IT levels in the telencephalon in this group (r= 0.70, n=7, p=0.08). In Losers, there was a marginally non-significant negative correlation between the AVT levels in the telencephalon and submissive behaviour (r= -0.81, n=5, p=0.09). Finally, in Mirror-fighters there were negative correlations between AVT levels in the olfactory bulb and aggressive behaviour (r= -0.93, n=5, p=0.03) and between IT levels in the brainstem and aggressive behaviour (r= -0.91, n=6, p=0.01). There was also a close to significant negative relationship between the aggressive behaviour and IT levels in the olfactory bulb (r= -0.82, n=5, p=0.08) for this group. All the other correlations were not significant (Table 1).

Table 1 – Pearson correlations between nonapeptide levels and behaviour (aggressive behaviour for Winners and Mirror-fighters, and submissive behaviour for Losers). (*) indicates $p < 0.05$; ($^{+}$) indicates $0.05 < p < 0.10$.

		Social status		
Brain region	**Peptide**	**Mirror figthers**	**Winners**	**Losers**
Olfactory bulbs	AVT	**r= - 0.93, n=5,p=0.03***	r= - 0.27, n=7,p=0.55	r= 0.31, n=6,p=0.54
	IT	**r= - 0.82, n=5,p=0.08^{+}**	**r= - 0.74, n=7,p=0.057^{+}**	r= - 0.42, n=6,p=0.40

Telencephalon	AVT	r= 0.51, n=8,p=0.20	**r= - 0.80, n=6,p=0.054**$^{+}$	r= - 0.14, n=6,p=0.79
	IT	r= 0.26, n=8,p=0.53	**r= 0.70, n=7,p=0.08**$^{+}$	r= 0.33, n=5,p=0.59
Diencephalon	AVT	r= - 0.24, n=8,p=0.57	r= - 0.62, n=7,p=0.14	**r= - 0.81, n=5,p=0.09**$^{+}$
	IT	r= - 0.41, n=7,p=0.36	r= 0.30, n=6,p=0.56	r= - 0.37, n=4,p=0.96
Optic tectum	AVT	r= 0.33, n=8,p=0.42	r= 0.32, n=7,p=0.49	r= 0.34, n=6,p=0.50
	IT	r= 0.39, n=6,p=0.45	r= 0.57, n=7,p=0.18	r= - 0.63, n=4,p=0.36
Cerebellum	AVT	r= - 0.31, n=7,p=0.49	**r= - 0.72, n=7,p=0.07**$^{+}$	r= - 0.53, n=5,p=0.36
	IT	r= - 0.60, n=5,p=0.28	r= 0.44, n=6,p=0.93	r= - 0.87, n=4,p=0.13
Brainstem	AVT	r= - 0.38, n=8,p=0.35	r= - 0.41, n=7,p=0.36	r= - 0.57, n=5,p=0.31
	IT	**r= - 0.91, n=6,p=0.01***	r= 0.26, n=6,p=0.62	r= - 0.60, n=4,p=0.40

4. Discussion

The results presented here show that an acute social interaction induces rapid changes in nonapeptide levels in the brain that depend on the specific social experience. Indeed, Winners, Losers and Mirror-fighters had different patterns of nonapeptide response to the social interaction with Losers presenting the broadest response across the brain, with increased AVT levels in the telencephalon, diencephalon, optic tectum, cerebellum and brain stem, increased IT levels in the diencephalon and decreased IT levels in the cerebellum. On the other hand, Winners exhibited increased AVT levels in the forebrain (telencephalon and diencephalon) and reduce IT levels in the olfactory bulbs. Finally, Mirror-fighters only showed increased levels of AVT in the telencephalon. Therefore, AVT seems to be more involved in the response to an acute agonistic interaction than IT, which is in line with its association with aggressive behaviour and with a more differentiated role of IT in affiliative behaviours [25]. This view is also supported by the fact that the discriminant analysis using AVT levels to

classify individuals into social groups was much more successful than that using IT levels.

The three social phenotypes (Winners, Losers and Mirror-fighters) generated by the behavioural paradigm used in this study can be contrasted among themselves and with a non-interacting reference group in order to infer the specific aspects of the social interaction that trigger the observed changes in nonapeptide changes. Differences in nonapeptide levels between either Winners or Losers and non-interacting fish, which are not present in Mirror-fighters can be interpreted as being associated with changes in social status (i.e. increase in winners; decrease in Losers). Differences in nonapeptide levels between either Winners or Losers and non-interacting fish, which are also present in Mirror-fighters must reflect aspects of fighting behaviour which are common to these three groups, and should not be associated with changes in social status since Mirror-fighters do not experience a change in status. Finally, differences in nonapeptide levels between Mirror-fighters and non-interacting fish that are not present in either Winners or Losers, should reflect specific aspects of their fighting behaviour and again should not be related to a shift in social status.

Following the rationale presented above, the increase in AVT levels observed in the telencephalon of Winners, Losers and Mirror-fighters when compared to non-interacting fish must reflect a common aspect of both interaction types (i.e. real-opponent and mirror fights) that is also shared by both Winners and Losers of the real opponent interaction. Given that Winners and Losers have distinct behavioural experiences in the post-resolution phase of the fights, and given that Mirror-fighters never solve the fight, the common factor that is eliciting the common AVT response across these three groups must reside in the pre-resolution phase of the fights. This can be the expression and/or perception of display behaviours, which are specific to the pre-resolution phase [21].

Interestingly, an evolutionary conserved social decision-making network that is mostly located in the forebrain has been recently described in vertebrates [26,27]. Most nodes of this network are known to express AVT receptors [26,28], which allow this peptide to regulate social decision-making at multiple target areas in the telencephalon. Thus, increased AVT levels in the telencephalon related to the assessment phase of the interaction may reflect AVT modulation of social decision making in a competitive context. In line with this argument a comparative study in butterfly fishes has found several associations between the density of AVT- ir varicosities in different nuclei of the telencephalon and social behaviour, particularly with aggression, and the Vv nuclei, a putative fish homolog of the lateral septum, has been identified as the best predictor of aggression, since it was able to discriminate between aggressive and non-aggressive species [29]. Interestingly, the role of the lateral septum in aggression has also been noted in birds, in which septum AVT increases aggression in non-territorial species, and decreases it in territorial species [2]. By analogy, given that zebrafish is a gregarious species one could expect AVT also to be associated with an increased aggressive response. However, in contrast to birds a negative association was found (in the olfactory bulbs) of mirror fighters and a close to significant association was also found in the telencephalon of winners, suggesting a possible negative feedback of the AVT system on aggression. In support of this idea, pharmacological administration of AVT to dominant zebrafish males inhibits aggressive behaviour [16]. However, one should keep in mind that here major brain areas were analysed and thus we cannot discriminate the contribution of each specific brain nuclei to the total AVT measured in the telencephalon.

Following the same rationale the increase of AVT in the diencephalon shared by Winners and Losers, but not observed in Mirror-fighters must reflect a component of

the agonistic interaction that is missing in a mirror fight. In this case, both phenotypes share the experience of the post-resolution phase of the fight, characterized by extended chases and attacks directed by the Winner towards the Loser. Overt-aggression is known to be more energy consuming than displaying [30,31], and therefore the post-resolution phase can arguably be considered more stressful that the assessment phase. If this is the case the observed increase in AVT may be related to social stress experienced by both social phenotypes at the post-resolution stage of the fight. This interpretation is supported by data from another study where both Winners and Losers, but not Mirror-fighters have been shown to have elevated cortisol levels (Teles et al., submitted), and by the fact that AVT in the POA is known to plays also a role in stress regulation. Alternatively, the shared increase in AVT levels between Winners and Losers may have different origins within the diencephalon. In fish two AVT cellular populations have been described in the preoptic area located in the diencephalon: the parvocellular nuclei, that appears to be more involved in the stress response, namely in the regulation of cortisol release by the action of AVT on the hypothalamic-pituitary-interrenal axis [32,33]; and the magnocellular/ gigantocellular cluster, which likely regulates aggression [8,34,35]. Thus, Winners and Losers may be activating different neuronal populations in the post-resolution phase: Winners with higher activation of the magnocellular population related to the expression of overt-aggression; and Losers with higher activation of the parvocellular population due to social stress [35]. Finally in this respect it is also worth mentioning that the results present here for peptide levels contrast with previously reported results for the gene expression, where dominant males had higher expression of the avt gene in the hypothalamus than subordinates [16]. However, the different time frames of aggression used in the two studies may explain these divergences, since in the present study a short-term (30 min) interaction was used,

whereas the gene expression profiles were performed on social stable hierarchies after 24 hours of social interaction.

The AVT increase in the optic tectum and brain stem, the IT increase in the diencephalon and the IT decrease in the cerebellum observed exclusively in Losers should thus be interpreted as being driven by the loss of social status experienced by these animals. In teleost fish the optic tectum and rhombencephalon receives AVT fibers [13], and both areas have been implicated in the regulation of visual and motor responses to sensory stimulation [36], as well as in escape behaviours [37]. Thus AVT signalling in these areas in Losers may reflect sensory-motor integration related to defensive behaviours exhibited exclusively by Losers. The high IT levels found in Losers' diencephalon might be associated with a downregulation of aggressive behaviour. This process has already been described in Syrian hamsters (*Mesocricetus auratus*), in which oxytocin administration to the preoptic area and to the anterior hypothalamus decreases aggression, whereas oxytocin receptor antagonist administration facilitates it [38].

Finally, the decrease in IT levels in the olfactory bulbs exclusively observed in Winners, can also be seen as status-acquisition driven. In fish, olfaction plays a major role in intra-specific communication [39,40] and in social recognition [41], and IT fibers are known to reach the granular layer of the olfactory bulbs [13]. In mammals, a vasopressin system has been described in the olfactory bulbs that is associated with olfactory social recognition, such that local vasopressin release will depend on previous olfactory experiences [42]. Among teleosts, although a similar system has not been described yet, very high levels of AVT have been described in the olfactory bulbs of cichlid fish [6]. Thus the lower levels of isotocin in the olfactory bulbs of winners may reflect the social regulation of olfactory memory formation.

In summary, the data presented here showed rapid nonapeptide changes across different brain areas in response to a short-term social challenge highlighting the role of these peptides in rapid modulation of social decision-making. Moreover, the pivotal roles of social perception for the triggering of shifts in social status social and of the telencephalon for fighting assessment have been unravelled.

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Chapter V

Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish

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Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish

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Abstract

According to the social decision-making (SDM) network hypothesis, SDM is encoded in a network of forebrain and midbrain structures in a distributed and dynamic fashion, such that the expression of a given social behaviour is better reflected by the overall profile of activation across the different loci rather than by the activity of a single node. This proposal has the implicit assumption that SDM relies on integration across brain regions, rather than on regional specialization. Here we tested the occurrence of functional localization and of functional connectivity in the SDM network. For this purpose we used zebrafish to map different social behaviour states into patterns of neuronal activity, as indicated by the expression of the immediate early genes *c-fos* and *egr-1*, across the SDM network. The results did not support functional localization, as some loci had similar patterns of activity associated with different social behaviour states, and showed socially driven changes in functional connectivity. Thus, this study provides functional support to the SDM network hypothesis and suggests that the neural context in which a given node of the network is operating (i.e. the state of its interconnected areas) is central to its functional relevance.

Keywords: social behaviour network, mesolimbic reward system, functional localization, functional connectivity, neural context, zebrafish

1. Introduction

Social decision-making (SDM) involves the integration of multimodal sensory information about social status and social context with previous experience in order to produce an appropriate behavioural response that is adjusted to the perceived social environment. Therefore, social decisions are expected to rely on multiple neural circuits, rather than being controlled by one specific brain region. In line with this argument, an evolutionarily conserved SDM network, composed of two interconnected neural circuits – the social behaviour network [1,2] and the mesolimbic reward circuit [3] – has been proposed to underlie the expression of social behaviour across vertebrates [4,5]. Social information would be encoded in this network of forebrain and midbrain nuclei with reciprocal connections in a distributed and dynamic fashion, such that the expression of a given social behaviour would be better reflected by the overall profile of activation across the different loci in the network rather than by the activity of a single node, and different combinations of activation across nodes and variation in the strength of the connections among them would generate an almost infinite variation in social behaviour [6]. Although the SDM network has been proposed on functional grounds, most of its current support is based on structural evidence, namely on the expression of genetic markers, hormone receptors and neurochemical/neurotransmitter systems that allow the establishment of homologies of its constitutive loci across taxa, as well as on patterns of reciprocal neuronal connections, that confirm the occurrence of structural (anatomical) connectivity among loci [4,7].

From a functional perspective, the establishment of the SDM network as a valid neurobiological construct requires the understanding of how social information is being mapped into the brain. Two hypotheses of brain function are currently available in systems neuroscience: (i) functional specialization, which proposes that different brain

regions are engaged in different cognitive functions/behaviours; [8] and (ii) functional connectivity, which postulates that specific cognitive functions/behaviours are mediated by a diffuse network of interacting brain regions [9,10]. Therefore, when two experimental conditions are compared that differ in a specific cognitive function/behaviour, the former hypothesis predicts differences in activity level between the areas relevant for that specific task, whereas the latter hypothesis predicts changes in the covariance in activity levels between different brain areas relevant for the task. Although these two hypotheses have historically been seen as antagonistic, they are not necessarily mutually exclusive as the functional relevance of a specific brain region may depend on the functional state of their connecting areas (i.e. its neural context [11,12]). In the scope of the SDM network, this hypothesis would predict that each node of the network can participate in several social behaviours through its interactions with other nodes. Therefore, both changes in activity levels in specific nodes of the SDM network and changes in its functional connectivity can be predicted in relation to relevant social stimuli. However, the key hypothesis to be tested for the functional validation of the SDM network is the occurrence of flexible functional connectivity across the network.

So far most studies that have mapped social behaviour into patterns of brain activity have only implicitly addressed the functional localization hypothesis by documenting changes in the activity or expression of molecular markers of neuronal activity (e.g. cytochrome oxidase or immediate early genes, respectively) in specific network nodes (e.g. fish [13]; birds [14,15]; mammals [16]). In fact, only a few studies have so far established links between functional connectivity and the expression of social behaviour states. For example, it has been shown that leopard geckos (*Eublepharis macularis*), a species with temperature-dependent sex determination, incubated at either male- or female-biased temperatures develop more or less aggressive behaviour, which is

paralleled by different patterns of functional connectivity across the SDM network [17]. Also, in male green anoles (*Anolis carolinensis*), exposure to video playbacks of displaying competitors elicited aggressive displays reflected in differentially connected neural networks [18]. Finally, male túngara frogs (*Physalaemus pustulosus*) exposed to the playback of relevant social calls also show changes in functional connectivity among hypothalamic nuclei [19]. However, these papers pre-date the SDM network proposal, and therefore only nodes from the social behaviour network or subsets of hypothalamic–lymbic nuclei have been considered. This means that the functional validation of the SDM network across vertebrates is still lacking.

In this study, we tested both the functional localization hypothesis and the functional connectivity hypothesis regarding the mapping of social behaviour into brain activity in the SDM network. For this purpose, we characterized the expression of two immediate early genes (*c-fos* and *egr-1*), as transient markers of neuronal activity [20,21], across selected nodes of the SDM network in male zebrafish in relation to the outcome of agonistic interactions. Zebrafish were used as a study model given their relevance as potential model organisms in social neuroscience [22]. Adult zebrafish are highly social, expressing a strong preference for shoaling with conspecifics [23–25]. However, despite this affiliative motivation, males also express aggressive behaviour when competing for resources [26,27]. Four social treatments were used: winners and losers of real-opponent interactions; mirror-fighters, which expressed agonistic behaviour towards their own image in the mirror but did not experience either a victory or a defeat; and non-interacting males, as a non-social reference group. In order to test the functional localization hypothesis, we tested for differences in immediate early gene (IEG) expression between each of the three social groups and the non-social reference group at each node of the SDM network. In order to test for the functional localization

hypothesis, co-activation matrices (i.e. correlation matrices for the levels of IEG expression across the nodes of the network within each treatment) were compared across social treatments. The use of the mirror treatment was intended to help to discriminate between perceptual and motor influences in the pattern of activity of the SDM network. Fish do not recognize themselves on a mirror, and attack their own image as if it is an intruder [28]. In zebrafish, mirror fights elicit similar levels of aggressive behaviour to those observed in real-opponent fights [29]. However, as submissive behaviour is never expressed by the mirror image, no information on fight outcome is perceived. Thus, as mirror-fighters express a behaviour output similar to that of winners but perceive different responses in the opponent (i.e. submission in the case of the winner; aggression in the case of the mirror-fighter), shared patterns of SDM network between these two groups should reflect motor output, whereas differences should be due to differences in either perception or associative processing of social information.

2. Material and methods

(a) Fish housing

All subjects used in this experiment were adult wild-type (AB) zebrafish bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Fish were kept at 28°C with a 14 L : 10 D photoperiod in a recirculating system (ZebraTec, 93 Tecniplast). Fish were fed twice a day, except on the day of the experiments.

(b) Social treatments

To create different social behaviour states we used a previously described short-term agonistic paradigm [27,29]. In brief, adult males were paired in dyads matched for

body size (mean ± s.e.m.: 0.4 ± 0.015 g) and placed, as pairs, in an experimental arena (5 × 8 × 6 cm) divided in two halves by one or more removable opaque partition(s) (see below). Therefore, there were two fish per tank, one on each side of the partition, which were kept overnight in visual isolation. At the start of the experiment one or more of the partitions were removed, and the fish were allowed to interact for a period of 30 min. Three social treatments were used: (i) fighting a real-opponent conspecific, where there was a single opaque PVC partition separating the two fish, which was removed; (ii) fighting their own image on a mirror, where there were two mirrors placed back to back, each facing one of the compartments, behind opaque partitions (the partitions were removed to uncover the mirrors); and (iii) no agonistic interaction, where there were three central opaque partitions, and only the outer two were removed (to control for putative stress effects of handling partitions in the experimental tanks). These social treatments generated four social behaviour states: winners (W, n = 12) and losers (L, n = 13) of the real-opponent interaction; fighters of unresolved interactions (i.e. mirror-fighters, M, n = 11); and fish with no social interaction (i.e. visual isolation, I, n = 12). All animals were tested in pairs in order to give them access to conspecific odours, which would otherwise only be present in real-opponent dyads, therefore avoiding confounding effects of putative chemical cues. Behavioural interactions were video-recorded for subsequent behavioural analysis.

(c) Microdissection of regions of interest in the brain

Immediately after the interaction, fish were anaesthetized with an overdose of tricaine solution (MS222, Pharmaq; 500–1000 mg l^{-1}) followed by rapid decapitation. Heads were embedded in mounting media (OCT Compound, Tissue-Tek, Sakura] and rapidly frozen on dry ice. Brains were sectioned in coronal plane at 150 µm on a

cryostat (Leica CM 3050 S) and sections were collected onto regular glass slides previously cleaned with 70% ethanol. Regions of interest, identified using the zebrafish brain atlas [30], were then microdissected under a stereoscope (Zeiss Stemi 2000; see the electronic supplementary material for details). For logistical reasons we could not sample the 12 nuclei of the SDM network [4], hence we selected the following subset of five nuclei representative of the two sub-networks: the medial zone of the dorsal telencephalic area (Dm, putative homologue of the mammalian basolateral amygdala) and the lateral zone of the dorsal telencephalic area (Dl, putative homologue of the mammalian hippocampus), from the mesolimbic reward system; the preoptic area (POA) from the social behaviour network; and the ventral nucleus of the ventral telencephalic area (Vv, putative homologue of the mammalian lateral septum) 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), common to both sub-networks [4]. Tissue was collected directly into lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen) and stored at -80°C until mRNA extraction.

(d) Gene expression analysis

Total RNA was isolated from brain nuclei using the RNeasy Lipid Tissue Mini Kit with some adjustments to the manufacturer's instructions (see the electronic supplementary material for details). RNA from each sample was then reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Biorad) in accordance with manufacturer's instructions and diluted 1 : 10 before being used as a template for quantitative polymerase chain reactions (RT-PCR) of *c-fos* and *egr-1*, using the eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*) as a reference gene (see the electronic supplementary material for details, especially table S1 for primer

sequences). Fluorescence cycle thresholds (CT) were automatically measured (Biosystems 7900HT Fast thermocycler) and the relative expression of the target genes calculated using the $2^{-\Delta C}$ method [31].

(e) Behavioural observations

Behavioural analysis was conducted using a computerized multi-event recorder (Observer XT, Noldus, Wageningen, The Netherlands), and the zebrafish aggressive behaviour ethogram [27] was used as a reference to identify both aggressive (bite, chase and strike) and submissive (freeze and flee) behaviours. As we were only interested in the behavioural outputs resultant from the different social treatments, and not in the interaction per se, we only analysed the post-resolution phase of the fight (last 5 min of the 30 min interaction), when the different social behaviour states (i.e. winners, losers, mirror-fighters and isolation) can be easily identified.

(f) Statistical analysis

The effect of the relevant social contexts (i.e. mirror-fighters versus winners) in aggressive behaviours was assessed using a t-test.

The overall effects of social behaviour state (winners, losers, mirror-fighters and isolation) and brain nuclei (Dm, Dl, Vv, Vs, POA) in *c-fos* and *egr-1* expression were assessed using linear mixed models (LMMs). As the data for winners and losers come from the same interaction, it cannot be considered independent, and a within-pair design is needed to compare these two social behaviour states [32]. On the other hand, the other two behavioural states (i.e. isolation and mirror-fighters) did not have an opponent, and thus a between-subject design is appropriate. In order to incorporate these two perspectives in the LMM analysis, two random effects were used: one for the

subjects and another for the winner–loser dyads. Parametric assumptions were checked using Shapiro–Wilk and Jarque–Bera adjusted multiplier tests to test for normality, Bartlett, Levene and Fligner–Killeen tests to test for homoscedasticity, and plots of the residuals, fitted values and estimated random effects in the LMM. Gene expression data were log-transformed before the analyses to fit parametric assumptions.

To test the functional localization hypothesis, planned comparisons were used to measure the effect of each social behaviour state (winners, losers or mirror-fighters versus isolation = reference group) on the activation (i.e. IEGs expression) of each brain nucleus. Planned comparisons among social behaviour states within each brain nucleus were also computed to test for socially driven differential activation.

To test for functional connectivity, Pearson product moment correlations were computed between the IEG expression in each pair of brain nuclei for each social behaviour state. These correlations were considered as indicative of co-activation between nuclei, in that positive correlations correspond to phasic activity and negative correlations to out-of-phase activity. Visual analyses of co-activations between nuclei were performed using heatmaps of the correlation matrices. The occurrence of different patterns of functional connectivity associated with different social behaviour states was assessed by testing the association between any two matrices using the quadratic assignment procedure (QAP) correlation test with 5000 permutations [33]. The null hypothesis of the QAP test is that there is no association between matrices. Thus, a non-significant p-value indicates that the correlation matrices are different. The occurrence of functional sub-networks within the SDM network in each social behaviour state was assessed by clustering analysis of brain areas according to correlations among them. The silhouette-based partitioning around medoids (PAM) method was used to check for clusters, and the strength of a cluster was interpreted from its average silhouette (AS)

[34]. The number of clusters to consider was calculated by maximizing the average AS for all possible number of clusters (2, 3 or 4). Finally, we have also estimated two measures of network structure (i.e. centrality and cohesion) to characterize the SDM networks underlying each social behaviour state. Eigenvector centrality, which takes into account the number of direct connections that a node has and how well connected its relations are, was used as a measure of centrality; density, the proportion of all possible connections that are present in the network, was used as a measure of cohesion [35]. In order to compare the density of connections among behavioural states (differences in the mean strengths of the relation between two nuclei), we used a bootstrap t-test approach with 5000 sub-samples.

Sample sizes varied either due to technical problems or to outlier values, identified for each condition with the generalized extreme studentized deviate procedure with $p = 0.05$ and a maximum number of outliers of 20% of the sample size (see electronic supplementary material, table S2 for detailed information on sample sizes). Statistical analyses were performed on R (www.R-project.org) using the following packages: car (Levene test), cluster (PAM), fBasics (Jarque–Bera test), Hmisc (correlations), lattice (heatmaps), multcomp (planned comparisons) and nlme (LMMs). The network analysis parameters were estimated using UCINET v. 6 [36]. Network representations were produced using Python.

3. Results

(a) Social behaviour states

As expected, the agonistic paradigm produced four social behaviour states. In real-opponent interactions, social hierarchies emerged where fish expressing two social behaviour states can be clearly identified: winners that only display aggressive

behaviours (80.2 ± 9.02 acts/5 min) and losers that only display submissive behaviours (46.9 ± 6.19 acts/5 min). Mirror-fighters only displayed aggressive behaviours, at a frequency (59 ± 12.8 acts/5 min) that was not significantly different from that displayed by the winners of the real-opponent interaction ($t_{19} = -1.39$, $p > 0.05$). However, in this treatment, the fight between the focal fish and its mirror image was symmetric and no dominance relationship was established (i.e. the focal expresses as much aggressive behaviour than it receives from the mirror image). Therefore, we considered mirror-fighters as a separate social behaviour state that did not achieve a dominant status despite expressing similar levels of aggression to winners.

(b) Effect of social behaviour state and brain region on immediate early gene expression

There were significant main effects of social behaviour state and brain nuclei both on *c-fos* and on *egr-1* expression levels, and the interaction between these two factors was not significant for either of the genes (table 1). The main effect of social behaviour state on *c-fos* expression was due to significant differences among all behaviour states (table 1). The main effect of social behaviour state on *egr-1* expression was related to a close to significant difference between non-interacting fish and winners, and between non-interacting fish and losers (table 1). For both genes the main effect of brain nuclei was due to significant differences between Vs and all other brain nuclei. For *egr-1*, there was also a significant difference between Dl and POA (table 1).

(c) Differences in functional localization among social behaviour states across the social decision-making network

Planned comparison analyses revealed a significant increase in *c-fos* expression in all nuclei for all social behaviour states (mirror-fighters, winners and losers) when compared with the reference group (i.e. isolation < mirror-fighters, winners, losers; figure 1a). The expression of *c-fos* in Dm,Vs and POA was higher in both winners and losers than in mirror-fighters, whereas in Dl and Vv mirror-fighter mRNA levels were not significantly different from those of winners, but were different from those of losers (figure 1a).

Table 1. Effect of social behaviour state and brain nuclei on *c-fos* and *egr-1* expression. Main effects, interactions and multiple comparisons were calculated using LMMs. I, isolated fish (non-social); M, mirror-fighters; W, winners; L, losers.

	c-fos		*egr-1*	
	F	p-value	F	p-value
Social behaviour state	29.52	<0.001	4.17	0.006
Brain nuclei	11.41	<0.001	19.77	<0.001
Social behaviour state * Brain nuclei	0.949	0.499	0.943	0.505
Multiple comparisons (social behaviour state)				
	z-value	p-value	z-value	p-value
I-M	4.37	<0.0001	1.44	0.14
I-W	7.26	<0.0001	1.88	0.06
I- L	8.78	<0.0001	2.58	<0.01
M-W	2.66	<0.01	0.37	0.70
M-L	4.09	<0.0001	1.05	0.29
W-L	2.35	<0.05	0.70	0.48
Multiple comparisons (Brain nuclei)				
Dm- Dl	-0.33	0.74	1.77	0.07
Dm-Vv	-0.87	0.38	0.14	0.88
Dm- Vs	4.47	<0.0001	6.01	<0.0001
Dm-POA	-0.52	0.60	-1.65	0.09
Dl-Vv	-0.53	0.59	-1.64	0.10
Dl-Vs	4.72	<0.0001	4.41	<0.0001
Dl- POA	-0.19	0.84	-3.43	<0.0001
Vv-Vs	5.24	<0.0001	5.98	<0.0001
Vv-POA	0.34	0.73	-1.81	0.07
POA-Vs	-4.92	<0.0001	-7.63	<0.0001

Regarding *egr-1*, planned comparison analyses revealed that only two brain nuclei, Dl and POA, exhibited differential activation in relation to social behaviour state (figure

1*b*). In Dl, mRNA levels of *egr-1* were not significantly different between mirror-fighters, winners and losers, but mirror-fighters and losers were significantly different from the reference group (i.e. isolation < winners, losers; figure 1*b*). The expression of *egr-1* in the POA was significantly higher in all social behaviour states than in the reference group, and there were no significant differences among the three social behaviour states (i.e. isolation < mirror = winners = losers; figure 1*b*).

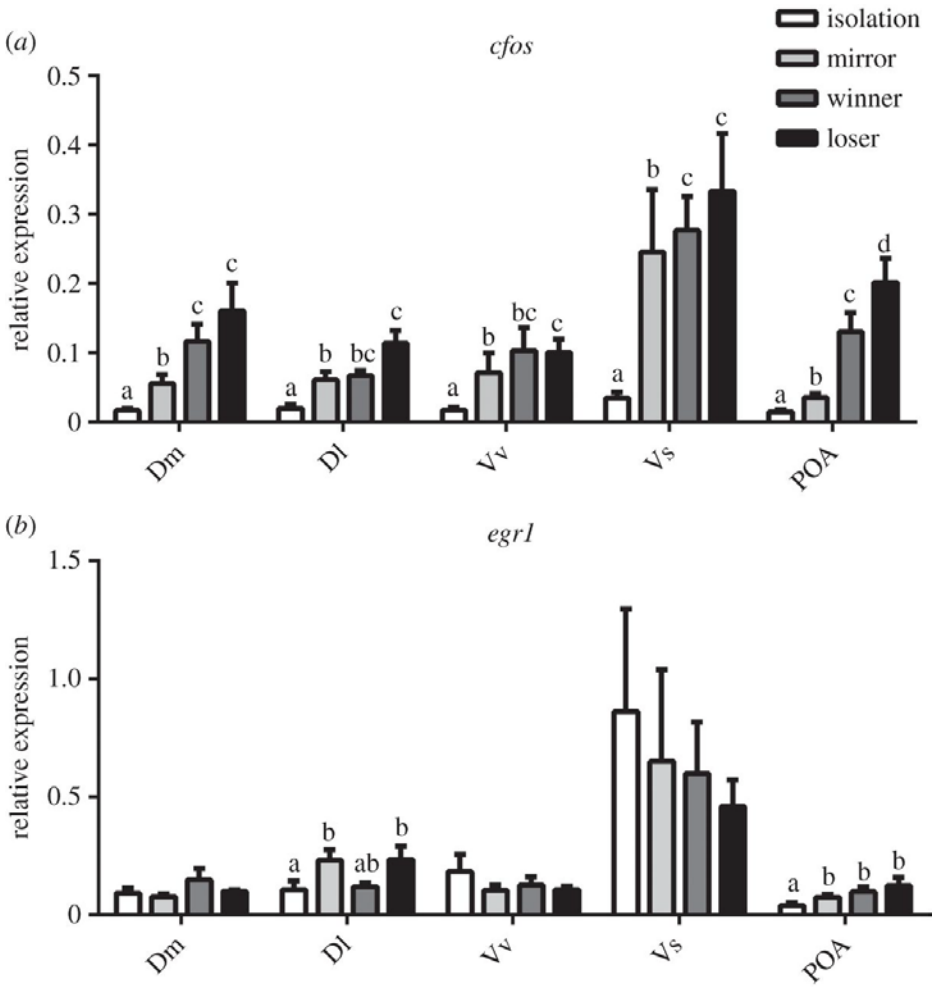

Figure 1. Immediate early gene expression in different brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area) for the different social behaviour states (i.e. isolation, mirror-fighter, winner and loser): (*a*) *c-fos* expression; (*b*) *egr-1* expression (normalized to *eef1a1l1* in both cases); the graphs represent raw data and error bars represent the standard error of the mean. Different letters indicate social

behaviour states that differ significantly from each other within a brain region, using a planned comparisons test ($p < 0.05$).

(d) Differences in functional connectivity among social behaviour states across the social decision-making network

Expression of *c-fos* revealed distinct co-activation patterns in all social behaviour states (figure 2). The QAP correlations detected a close-to-significant negative relationship between the isolation group and the losers' matrices ($r = -0.724$, $p = 0.054$), and all other QAP correlation tests were not significant (isolation versus mirror: $r = -0.097$, $p = 0.369$; isolation versus winner: $r = -0.119$, $p = 0.397$; loser versus mirror: $r = -0.091$, $p = 0.421$; loser versus winner: $r = -0.201$, $p = 0.351$; and mirror versus winner: $r = 0.048$, $p = 0.451$). From the correlation matrices it can also be seen that each social behaviour state has different sets of significant correlations between different network nodes, which are indicative of behaviour state-specific co-activation patterns (figure 2). Cluster analysis confirmed these different co-activation patterns, as different clusters were found for each social behaviour state (electronic supplementary material, figure S1).

The structural characterization of the *c-fos* SDM networks revealed that in the isolation group there was no evident central nucleus as the values for all areas were very similar, except for POA, which was the most peripheral nucleus. This network appears to have very similar connections (number of relations between the nodes) given no extra weight to any specific area in the expression of this neutral behaviour state (table 2). For mirror-fighters, Vs was the most central nucleus and the Dm was the most marginal one. For winners, POA and Vs were the most connected ones and Dl the less associated nucleus (table 2). Finally, in losers, POA was the most central area (table 2). Regarding cohesion, the density of the *c-fos* SDM network was significantly higher in the isolation

group than in any of the other social behaviour states (versus mirror-fighters: $t = 2.831$, $p = 0.0002$; versus winners: $t = 2.947$, $p = 0.0018$; versus losers: $t = 1.929$, $p = 0.0184$; table 2), and all the other comparisons were not statistically significant.

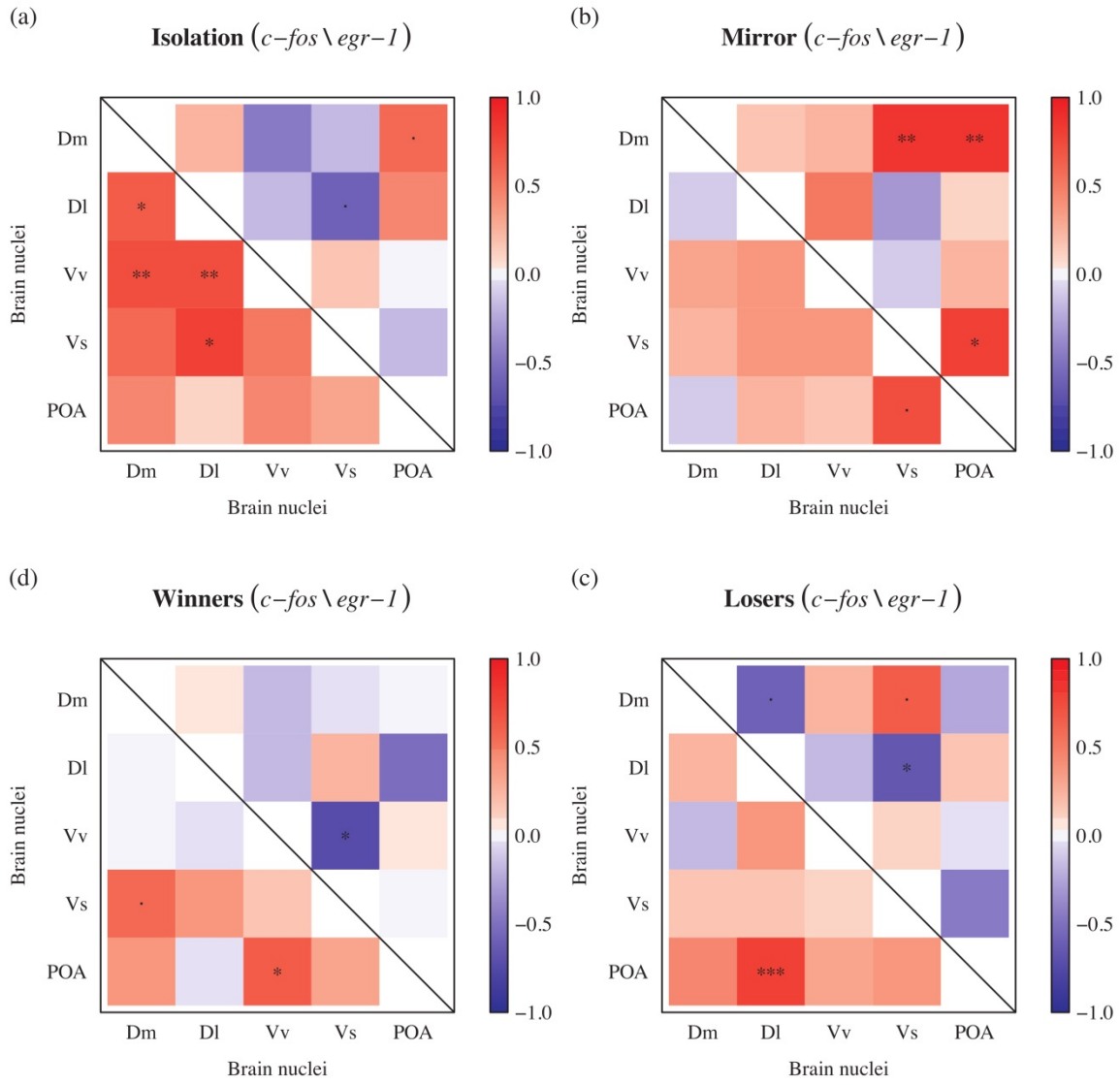

Figure 2. Functional connectivity in the SDM network as measured by Pearson correlations (*r*) of *c-fos* (below the diagonal) and *egr-1* (above the diagonal) expression between pairs of brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area) for each social behaviour state: (*a*) isolated fish (non-social); (*b*) mirror-fighters; (*c*) winners; (*d*) losers; colour scheme represents *r* values from −1 (blue) to 1 (red); asterisks indicate significant correlations after *p*-value adjustment: dot (.) $p < 0.1$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2. Quantitative characterization of the SDM network for each social behaviour state, using *c-fos* or *egr-1* as reporters of neuronal activity. Values correspond to centrality measures (eigenvalues) for each network node: Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area and cohesion (density) for each behaviour state.

		c-fos				*egr-1*			
Brain Nuclei		Isolation	Mirror-fighter	Winner	Loser	Isolation	Mirror-fighter	Winner	Loser
Eigenvalues	Dm	0.481	0.321	0.452	0.372	0.498	0.552	0.201	0.514
	Dl	0.489	0.402	0.248	0.541	0.50	0.251	0.445	0.512
	Vv	0.484	0.427	0.402	0.353	0.326	0.265	0.581	0.208
	Vs	0.463	0.566	0.534	0.313	0.414	0.536	0.577	0.563
	POA	0.284	0.483	0.536	0.589	0.474	0.524	0.302	0.336
Density		0.529	0.319	0.269	0.332	0.318	0.414	0.205	0.338

Expression of *egr-1* also showed distinct co-activation patterns for each social behaviour state (figure 2), as indicated by the lack of significant QAP correlations between any two matrices (isolation versus loser: $r = 0.211$, $p = 0.250$; isolation versus mirror: $r = 0.013$, $p = 0.497$; isolation versus winner: $r = 0.083$, $p = 0.383$; loser versus mirror: $r = 0.307$, $p = 0.158$; loser versus winner: $r = -0.343$, $p = 0.155$; and mirror versus winner: $r = -0.653$, $p = 0.009$). The correlation matrices for egr-1 expression also show different sets of significant correlations between different network nodes for each social behaviour state, which is suggestive of behaviour state-specific co-activation patterns (figure 2). Cluster analysis also supports the occurrence of different functional connectivity patterns in different social behaviour states, as different clusters were found for each social behaviour state (electronic supplementary material, figure S2).

The structural characterization of the *egr-1* SDM networks showed variation in the most central areas across social behaviour states. In the isolation group they were the Dl and Dm, whereas in mirror-fighters, winners and losers the most well-connected areas were the Dm, Vv and Vs, and Vs, respectively (table 2). Concerning network cohesion, the more densely connected *egr-1* network was observed in mirror-fighters (0.414),

which was significantly more connected than that of winners (t = 2.055, p = 0.0280), and that of winners was also more densely connected than that of isolated fish (t = 1.6311, p = 0.0428).

Egr-1 and *c-fos* expression patterns and clusters for the same social behaviour states also showed clear distinctions (figure 2; electronic supplementary material, S1 and S2). Correlation analyses between *c-fos* and *egr-1* expression for the same brain nuclei and social behaviour state showed a general lack of association between the expression of these two IEGs (electronic supplementary material, figure S3). Notable exceptions were the expression of *c-fos* and *egr-1* in Vs in the mirror group and in Vv in the isolation group (with r = 0.94 and r = 0.72, respectively).

(e) Association between immediate early gene expression and behaviour

Correlation analyses between aggressive and submissive behaviour and IEG expression in different brain nuclei for different social contexts showed no significant results either for *c-fos* or *egr-1* (electronic supplementary material, figure S4). However, for *c-fos* there was a tendency for negative correlations between aggressive behaviour in winners and expression levels in Dl, Vv and Vs (r = −0.54, p = 0.072; r = −0.51, p = 0.088; and r = −0.59, p = 0.071, respectively). For *egr-1*, we found a single close-to-significant positive correlation between submissive behaviour in losers and expression in Vv (r = 0.61, p = 0.063).

4. Discussion

Here we provide functional evidence that supports the SDM network hypothesis in zebrafish by confirming its implicit assumption that SDM relies on integration across different regions of the network, rather than on regional specialization of specific

network nodes. Specifically, we showed that there were no specific patterns of localized activity in a given node associated with specific social behaviour states, whereas the expression of socially driven behavioural behaviour states was associated with specific patterns of functional connectivity across the SDM network. These results suggest that the neural context in which a given node of the network is operating (that is, the state of its interconnected areas) is central to its functional relevance. Interestingly, IEG expression for *c-fos* and *egr-1* showed distinct neuronal activation patterns for all the considered social contexts (mirror, winners, losers; electronic supplementary material, figure S5), which also suggests that these genes are not working in unity but their activity rather reflects different behaviour state-related processes; *c-fos* appears to be a good neuronal marker for general brain activity, as all brain nuclei in all conditions responded to social interactions with an increase of *c-fos* mRNA levels in comparison to the reference non-social group, whereas *egr-1* expression seems to be more region- and process-specific.

(a) Functional localization

Although there were main effects of both social behaviour state and brain nuclei on the expression levels of both immediate early genes, in both cases, the interaction between social behaviour state and brain region was not significant, indicating independence between social behaviour state and regional differences in gene expression. The subsequent planned comparisons of neuronal activity, as indicated by IEG expression, confirmed the lack of functional localization of social behaviour states in any of the tested nodes of the SDM network. When comparing each of the three social behaviour states against the non-social reference behaviour state (i.e. isolation) the *c-fos* data indicated an activation of all brain regions in all behaviour states, whereas

egr-1 data only revealed activation of POA for all behaviour states and of Dl for mirror-fighters and losers. Moreover, when comparing the three social behaviour states among themselves (i.e. winners versus losers versus mirror-fighters), different behaviour states shared the same patterns of localized activity. For example, despite the contrasting behaviour states winners and losers had similar levels of *c-fos* expression in all studied brain regions, and the three social behaviour states (i.e. winners, losers and mirror-fighters) shared similar *egr-1* expression levels in the two brain nuclei where this gene responded to social experience (i.e. Dl and POA). Furthermore, winners and mirror-fighters also had similar levels of *c-fos* expression in Dl and Vv. These results are coincident with those reported for another fish species, the African cichlid *Astatotilapia burtoni*, where stable dominant and stable subordinate males express different status-specific behavioural profiles, which are also not paralleled by differences in either *c-fos* or *egr-1* expression in any of the studied nodes of the SDM network—which in this case also included, the anterior (ATn) and the ventral tuberal nuclei (VTn) [37]. However, in another study with the same species winners and losers of an acute agonistic interaction show different expression profiles across the network, with localized higher expression of *c-fos* in the POA and the ATn, and of *egr-1* in Dm, Dl, Vv, Vs and VTn of losers [38]. Together these results suggest that socially driven changes in neuronal activation in the SDM network are transient, and that stable social behaviour states do not rely on localized differences in brain activity. Accordingly, the observed behavioural states of winners and losers in this experiment should be seen as stable status-dependent states. This view is supported by the fact that winner and loser effects are observed in zebrafish at least 1 h after a single status establishing fight [27].

Although lacking a behaviour state-specific pattern of activation, from the brain regions studied here, Vs was the one that responded the most to social interactions. This

region has been proposed as a teleost putative homologue of the mammalian medial amygdala based on hodological, genomic and functional evidence [4]. However, this view has been recently questioned by a study of molecular markers in the adult zebrafish brain, which suggests that the dorsal and medial Vs are homologous to the central amygdala and its ventral part to the bed nucleus of the stria terminalis [39]. Independent of one-to-one homologies between Vs and specific components of the mammalian amygdala, our study supports the central role of this region in the processing of social information.

Finally, the comparison between winners and mirror-fighters, which share similar behavioural outputs (i.e. both are aggressive) but perceive different behaviours on their opponents (i.e. winners have submissive opponents and mirror-fighters face an aggressive opponent), potentially allows the identification of areas whose activity is better explained either by motor (i.e. when activity is similar between winners and mirror-fighters) or by perceptual information processing (i.e. when activity is different between them). According to this rationale perceptual processes were associated with amygdala (i.e. Dm and Vs) and preoptic *c-fos* expression, whereas behavioural output was associated with *c-fos* expression in Dl and Vv and with *egr-1* expression in Dl and POA. Thus, this approach revealed a functional differentiation between the two IEGs used in this study, with *c-fos* more associated with perceptual processes and *egr-1* exclusively associated with behavioural output.

(b) Functional connectivity

Our results showed that different social behaviour states exhibited different patterns of functional connectivity, as evidenced by: (i) lack of association between any two correlation matrices that capture the patterns of co-activation of SDM nodes for each

social behaviour state (there was only one close to significant association, between isolation and losers for *c-fos*, and it had a negative sign, indicating opposite and not coincident co-activation patterns); (ii) different clusters (i.e. sub-networks) present in each social behaviour state; (iii) different nodes occupying the central position in the network in each social behaviour state; and (iv) significantly different densities of connections in each social behaviour state. For *c-fos*, the non-social reference treatment was the one that presented the lowest activity in each node and the most connected SDM network, which breaks apart into different functional networks in the other three social behaviour states without significant differences in connectivity among them (electronic supplementary material, figure S5). This result resembles resting-state functional connectivity networks observed in fMRI human cognition studies, which have been interpreted as intrinsic neural activity reflecting the underlying structural connectivity architecture of the network [40,41]. Similarly, for the SDM network this high functional connectivity in the non-social state may reflect the known reciprocal anatomical connections among the different nodes of the network. For *egr-1*, mirror-fighters were the behaviour state that presented the most densely connected SDM network, with winners presenting the lowest connectivity.

The fact that *c-fos* and *egr-1* expression depict different functional networks (i.e. for the same social behaviour state, the two IEGs show different patterns of co-activation across the SDM network; electronic supplementary material, figure S5) suggests that different socially driven neuromolecular processes are operating in parallel and that different connectivity layers, corresponding to each of these processes, can be simultaneously present in the SDM network. This possibility contradicts the classic view of a single functional connectivity pattern associated with a specific behavioural behaviour state. Indeed different information-processing processes (e.g. attention,

memory, decision-making) may contribute to the same social behaviour state, and each of these processes may be differentially represented in the network by different signalling pathways. For example, in the case of *egr-1*, its expression has been classically associated with the induction of LTP and the expression of long-term memories in mammals [42]. Similarly in the electric fish *Apteronotus leptorhynchus*, *egr-1* expression in the dorsal telencephalon has been associated with the memory of individual conspecifics based on their electric organ discharge frequency, and this memory can last for several days [43]. Thus, the observed expression of *egr-1* in Dl and its associated sub-networks may reflect social memory formation in some of the social behaviour states.

5. Conclusion

The results presented here provide functional support to the SDM network hypothesis [4,7], as we have identified functionally connected networks that integrate nodes from both the mesolimbic system and the social behaviour network. Our results also show that the functional relevance of each network node to the social behaviour state depends on the activity in the network nodes to which they are connected, thus highlighting the relevance of neural context for social behaviour states.

Ethics

The animal experimentation procedures used in this study followed the institutional guidelines for the use of animals in experimentation and were approved by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 8954).

Data accessibility

Supporting qPCR and behavioural data can be accessed at Dryad: http://dx.doi.org/10.5061/dryad.826h4.

Authors' contributions

M.C.T. and R.F.O. designed the experiment; M.C.T. and O.A. performed the experiment; M.C.T. and J.S.L. analysed the data; M.C.T. and R.F.O. wrote the paper with contributions from all authors.

Competing interests

We declare we have no competing interests.

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Supplementary Material

Microdissection of the regions of interest in the SDM network

To identify and microdissect specific brain nuclei, slides were placed on a glass petri dish, filled with dry ice to maintain low temperatures, and viewed under a stereoscope (Zeiss; Stemi 2000). Tissue was collected with a modified 27G needle (inner diameter = 210µm) attached to a syringe. To prevent cross contamination between brain nuclei, one needle per nuclei was used and the needles were cleaned sequentially with distilled water and ethanol 70% between individuals. The nuclei in the vicinity of the ventricular area (i.e. Vv, Vs, and POA) were collected from both hemispheres at a single sampling point, due to their small size when compared to the diameter of the microdissection tool and due to their closeness to other nuclei. The remaining nuclei (i.e. Dm and Dl) were sampled from both hemispheres separately, and tissue from the two hemispheres were then pooled directly into lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen) and stored at -80 until mRNA extraction.

RNA extraction

Tissue was homogenised in qiazol lysis reagent and incubated for 7 min at room temperature (RT). Chloroform (1:2) was added, and the sample incubated at RT for 5

min. Samples were subsequently centrifuged at 13000 *g* for 20 min at 4ºC, after which the upper aqueous phase was transferred to a new tube where 1 volume of 70% ethanol was added. This mixture was then transferred to an RNEasy column, remained 5 min at RT, and was centrifuged for 1 min at 9000 *g*. A sequence of buffers (provided by the RNeasy Lipid Tissue Mini Kit) was added to the Rneasy column: 700 µl of Buffer RW1, 500 µl of Buffer RPE and an additional 500 µl Buffer RPE. After each buffer, samples were centrifuged for 1 min at 9000 *g* and the flow-through was discarded. The RNeasy column was then placed in a new 2 ml tube and centrifuged for 3 min at 14000g. The column was transferred to a new 1.5 ml tube, RNA eluted with 25 µl of RNAse-free water, and centrifuged for 2 min at 9000 *g*. The elution step was repeated with the same 25 µl of RNAse-free water in order to increase RNA recovery efficiency. RNA concentration and purity of all samples was estimated by spectophotometric absorbance (260 nm and 280nm) in the Nanodrop (Thermo Scientific NanoDrop 2000), and the RNA integrity of a random group of samples was checked using Bionalyzer (Agilent 2100 Bioanalyzer).

Quantitative RT-PCR (qRT-PCR)

Primer sequences for qRT-PCR were designed on Primer 3 (Premier Biosoft International, Palo Alto, CA, USA), tested for quality in the FastPCR 5.4., and the PCR products were sequenced to confirm the amplicon (table S1). qRT-PCR reactions were performed in an Applied Biosystems 7900HT Fast thermocycler in 8 µl triplicate reactions with SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and primers at 50 µM. Thermocycling conditions were 5 min at 95º C, followed by 40 cycles of: 95ºC for 30 s, specific annealing temperature for each primer for 30 s (Table S1), and 72 ºC for 30 s. After PCR, a melting curve program from 55 to 95ºC with 0.5 º

C changes was applied and the presence of a single reaction product in each well was confirmed. All reactions were performed in triplicate and technical replicates were run on the same plate. Before the analysis, the threshold value was adjusted manually for each plate at the inflection point of the amplification curve, and the same threshold was used in all assays of the same gene.

Table S1- Primer sequences and qRT-PCR parameters.

Gene	Accession No.	Primer sequence (5' → 3')	Annealing temperature (C°)	Amplicon length (pb)
eef1a1l1	NM_131263	F-CAAGGAAGTCAGCGCATACA R-TCTTCCATCCCTTGAACCAG	60	134
c-fos	NM_205569	F-CCGATACACTGCAAGCTGAA R- CGGCGAGGATGAACTCTAAC	59	111
egr-1	NM_131248	F- GTGAGCCCAACCCCATCTAT R- CCAGGCTGATCTCACTTTGC	58	216

F - primer forward; R - primer reverse

Table S2- Final sample sizes (n) after outliers removal. Brain nuclei: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA).

		Social behaviour state			
Brain Nuclei	**Gene**	**Isolation**	**Mirror–fighters**	**Winners**	**Losers**
Dm	*cfos*	12	11	12	13
	egr-1	11	11	12	11
Dl	*cfos*	12	10	12	13
	egr-1	12	10	12	13
Vv	*cfos*	12	11	12	12
	egr-1	12	11	12	11
Vs	*cfos*	7	7	10	12
	egr-1	9	8	10	12
POA	*cfos*	11	11	12	13
	egr-1	11	11	12	12

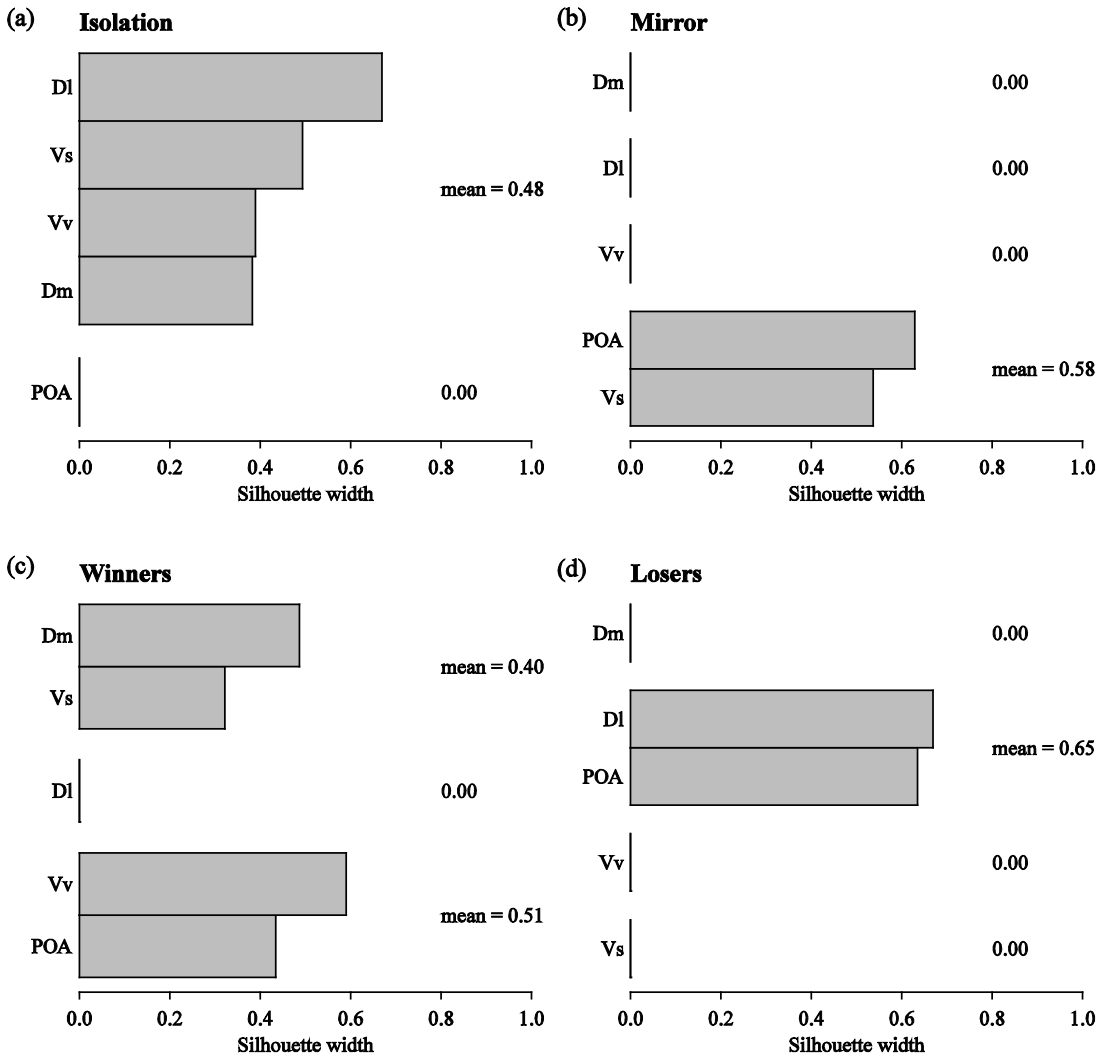

Figure S1 – Clustering of different brain regions and their silhouette width according to correlation values of *c-fos* expression for various social contexts: (a) no interaction (Isolation); (b) unsolved mirror interaction (Mirror); winning conspecific interaction (Winners); and losing conspecific interaction (Losers). Considered brain regions were: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Average silhouette width per cluster is presented right to the silhouettes cluster (AS < 0.25 = no structure; 0.25 < AS < 0.5 = weak structure; 0.5 < AS < 0.7 = reasonable structure; AS > 0.7 = strong structure).

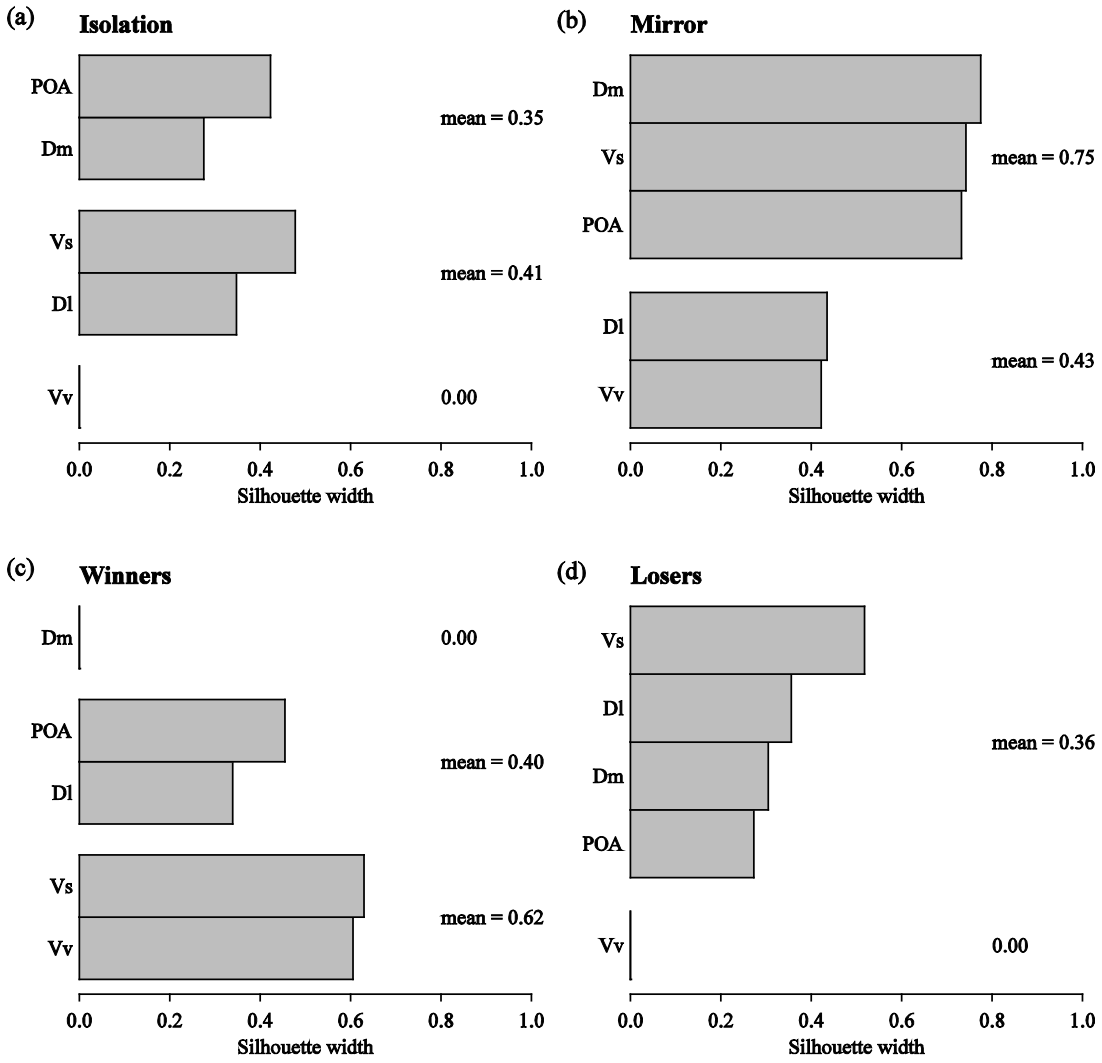

Figure S2 – Clustering of different brain regions and their silhouette width according to *egr-1* expression for various social contexts: (a) no interaction (Isolation); (b) unsolved mirror interaction (Mirror); winning conspecific interaction (Winners); and losing conspecific interaction (Losers). Considered brain regions were: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Average silhouette width per cluster is presented right to the silhouettes cluster (AS < 0.25 = no structure; 0.25 < AS < 0.5 = weak structure; 0.5 < AS < 0.7 = reasonable structure; AS > 0.7 = strong structure).

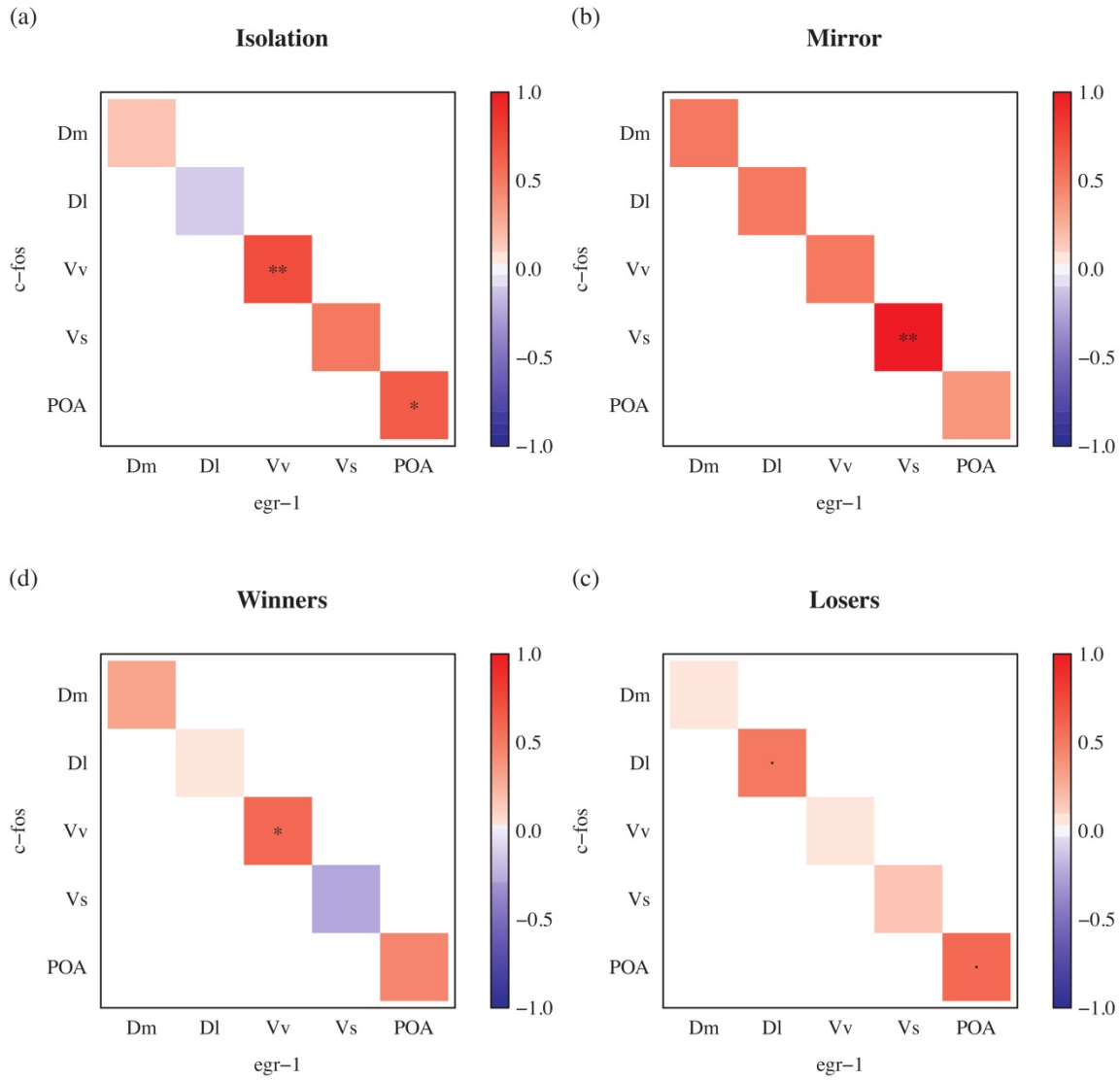

Figure S3 – Pearson correlations between *c-fos* and *egr-1* expressions for various social contexts: (a) no interaction (Isolation); (b) unsolved mirror interaction (Mirror); winning conspecific interaction (Winners); and losing conspecific interaction (Losers). Considered brain regions were: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Colour scheme represents correlation values from -1 (blue) to 1 (red). Asterisks indicate significant correlations after p-value adjustment (dot (.), $p < 0.1$; *, $p < 0.05$; **, $p<0.01$; ***, $p< 0.001$).

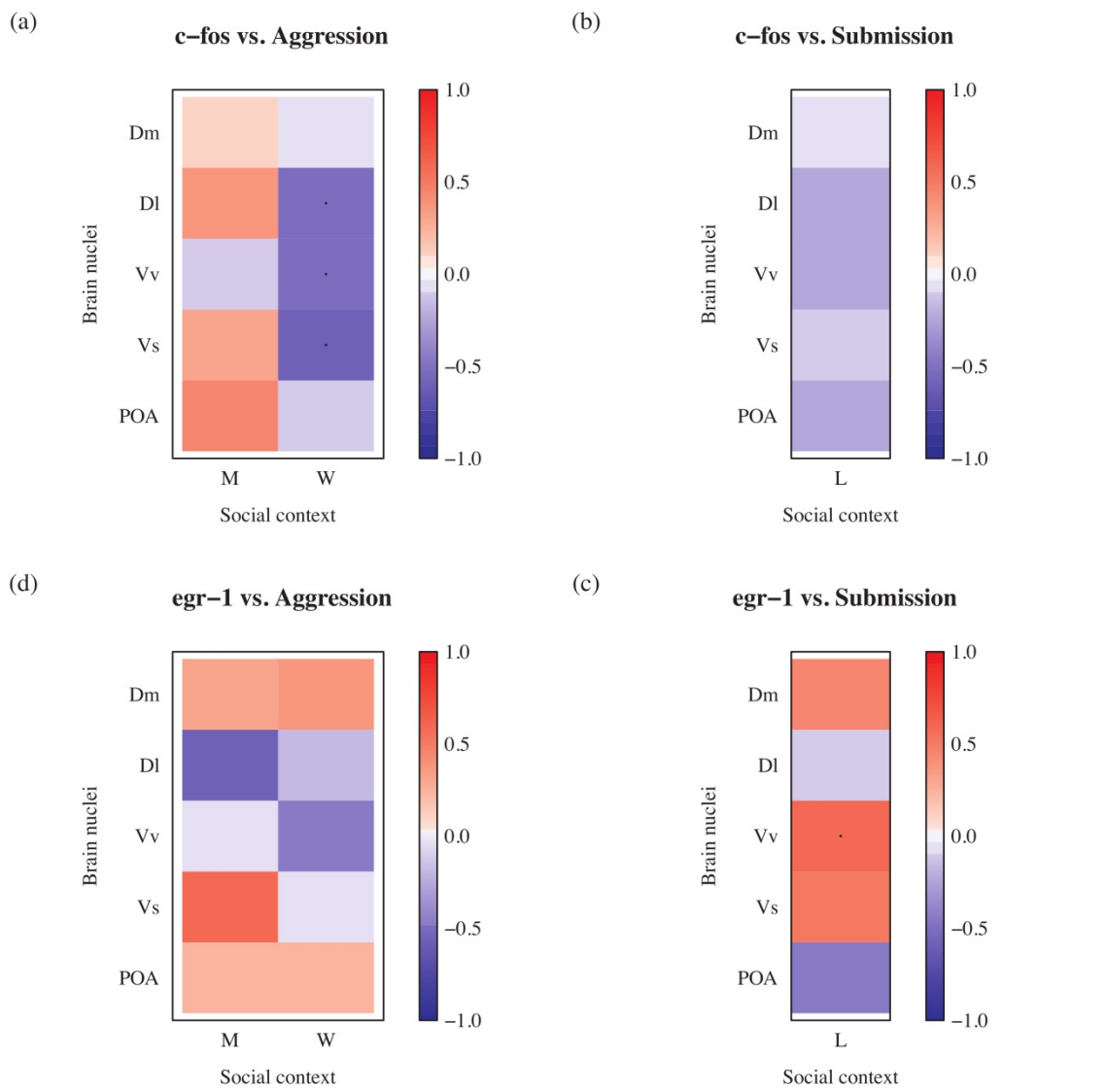

Figure S4 – Pearson correlations between immediate early genes expression and behaviour towards conspecifics for various social contexts: (a) association between *c-fos* expression in various brain regions and aggressive behaviour in relevant social contexts; (b) association between *c-fos* expression in various brain regions and submissive behaviour in relevant social contexts; (c) association between *egr-1* expression in various brain regions and aggressive behaviour in relevant social contexts; and (d) association between *egr-1* expression in various brain regions and submissive behaviour in relevant social contexts. Considered brain regions were: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Considered social contexts were: winning conspecific interaction (W); losing conspecific interaction (L), unsolved mirror interaction (M); and no interaction (I). Colour scheme represents correlation values from -1 (blue) to 1 (red). Asterisks indicate significant correlations after p-value adjustment (dot (.), $p < 0.1$; *, $p < 0.05$; **, $p<0.01$; ***, $p< 0.001$).

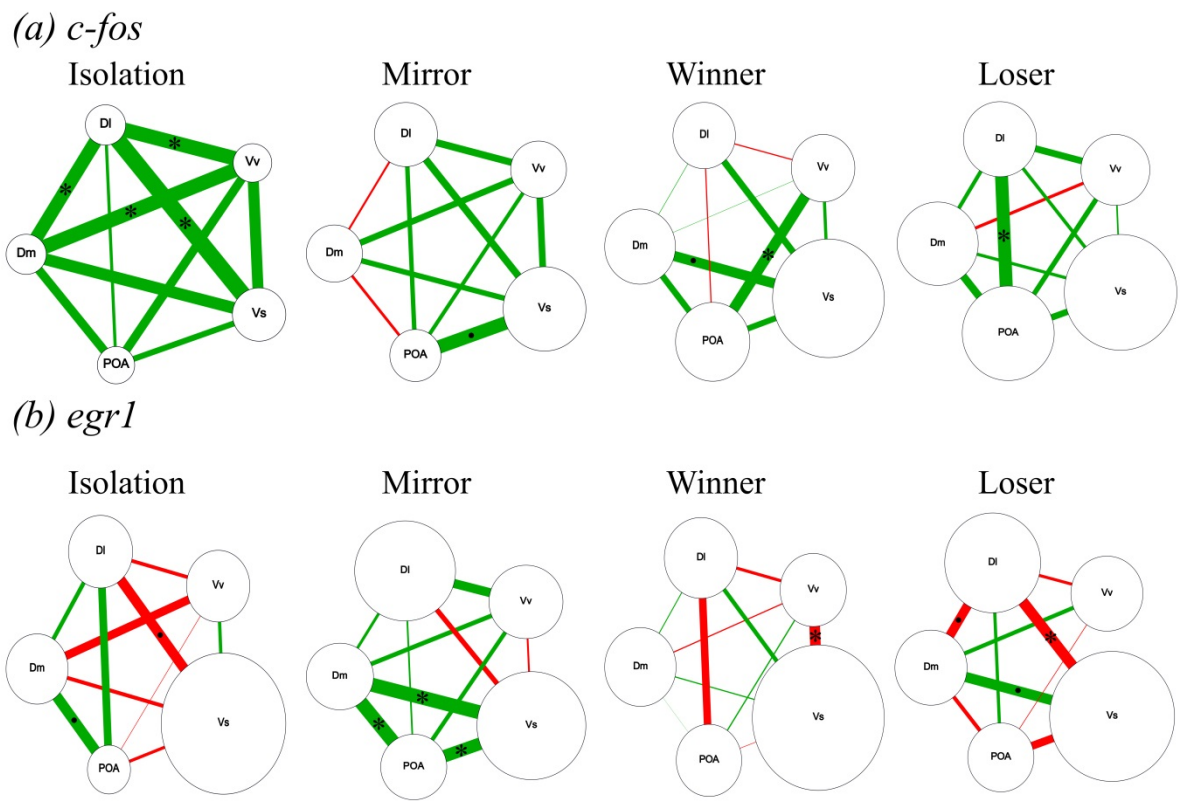

Figure S5 – Representation of the state of the social decision-making network for each social behavioural state using: *(a) c-fos* expression and *(b)* egr-1 expression as reporters of neuronal activity. Circle diameter represents the activity level at each network node: medial zone of the dorsal telencephalic area (Dm); lateral zone of the dorsal telencephalic area (Dl); ventral nucleus of the ventral telencephalic area (Vv); supracommissural nucleus of the ventral telencephalic area (Vs); and preoptic area (POA). Lines linking pairs of nodes represent the functional connectivity between them as measured by Pearson correlation coefficients of IEG expression, such that: the thickness of the line is proportional to the R value and the colour scheme represents positive (green) or negative (red) correlations. Asterisks indicate significant correlations after p-value adjustment: dot (.) $p < 0.1$; * $p < 0.05$; ** $p<0.01$; *** $p< 0.001$.

Chapter VI

Social plasticity relies on different neuroplasticity mechanisms across the brain social decision-making network in zebrafish

Social plasticity relies on different neuroplasticity mechanisms across the brain social decision-making network in zebrafish

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Abstract

Social living animals need to adjust the expression of their behaviour to their status within the group and to changes in social context and this ability (social plasticity) has an impact on their Darwinian fitness. At the proximate level social plasticity must rely on neuroplasticity in the brain social decision-making network (SDMN) that underlies the expression of social behaviour, such that the same neural circuit may underlie the expression of different behaviours depending on social context. Here we tested this hypothesis in zebrafish by characterizing the gene expression response in the SDMN to changes in social status of a set of genes involved in different types of neural plasticity: *bdnf*, involved in changes in synaptic strength; *npas4*, involved in contextual learning dependent establishment of GABAergic synapses; neuroligins (*nlgn1* and *nlgn2*) as synaptogenesis markers; and genes involved in adult neurogenesis (*wnt3* and *neurod*). Four social phenotypes were experimentally induced: Winners and Losers of a real-opponent interaction; Mirror-fighters, that fight their own image in a mirror and thus do not experience a change in social status despite the expression of aggressive behaviour; and non-interacting fish, which were used as a reference group. Our results show that

each social phenotype (i.e. Winners, Losers and Mirror-fighters) present specific patterns of gene expression across the SDMN, and that different neuroplasticity genes are differentially expressed in different nodes of the network (e.g. BDNF in the dorsolateral telencephalon, which is a putative teleost homologue of the mammalian hippocampus). Moreover, the role of cortisol on the gene expression response to social plasticity depends on social status achieved with Winners showing overall positive associations between gene expression and cortisol levels, Losers negative associations and Mirror-fighters a mosaic of positive and negative associations depending on brain region. These results indicate that social plasticity relies on multiple neuroplasticity mechanisms across the SDMN, and that there is not a single neuromolecular module underlying this type of behavioural flexibility.

Keywords: behavioural flexibility, social competence, social behaviour, neuroplasticity, synaptic plasticity, neurogenesis

1. Introduction

Social plasticity (aka 'social competence', (Taborsky and Oliveira, 2012)), defined as the ability to adaptively change the expression of social behaviour according to previous experience and to social context, is ubiquitous among group-living animals. The effect of previous social experience on subsequent behaviour has been described in a wide range of animals both in competitive and cooperative contexts, as illustrated by experience-dependent winner-loser effects (Hsu et al., 2006; Rutte et al., 2006) and reciprocity of cooperative behaviour (Bshary and Grutter, 2006; Rutte and Taborsky, 2007) , respectively. Similarly, the effect of social context on social behaviour can be illustrated by different social phenomena present in many different species, such as 'dear enemy'/'nasty neighbours' effects (Müller and Manser, 2007; Temeles, 1994), audience effects (Doutrelant et al., 2001; Pinto et al., 2011), social eavesdropping (Earley, 2010; Oliveira et al., 1998) and mate choice copying (Witte and Ryan, 2002). All these examples illustrate how social plasticity allows animals to optimize their social relationships in relation to the complexities of their social environment, and therefore it should be seen as a key determinant of their Darwinian fitness (Oliveira, 2009; Taborsky and Oliveira, 2012). Given its biological relevance there are important implications of social plasticity both for the study of behaviour and evolution. First, given the prominent role of the nervous system in orchestrating flexible responses to cues that signal environmental change, the understanding of the mechanisms underlying social plasticity is crucial for understanding behaviour and brain evolution (e.g. (Dunbar and Shultz, 2007). Secondly, social plasticity can be seen either as a constraint or as a motor of evolution depending on environmental heterogeneity, availability of cues that signal environmental changes and the costs and limits of plasticity (DeWitt et al., 1998; Pigliucci, 2005; Price et al., 2003). Knowledge of the genetic architecture and the

proximate mechanisms underlying social plasticity is crucial to understanding its costs, limits and evolutionary consequences. Therefore, the study of the neuromolecular mechanisms of social plasticity should be seen as a central topic in current behavioural research.

In terms of proximate mechanisms, social plasticity can be conceptualized as reversible shifts between behavioural states (i.e. the consistent expression of a set of behaviours) in response to relevant social information, which are paralleled by shifts between neurogenomic states [i.e. the expression of co-regulated gene sets,(Cardoso et al., 2015)]. Thus, at the molecular level socially-driven behavioural flexibility must rely on neuronal activity-dependent mechanisms that change the neurogenomic state of the brain in response to perceived social stimuli (Cardoso et al., 2015). For example, the activation (e.g. phosphorylation) of relevant proteins (e.g. cAMP response element-binding, CREB), which then act as transcription factors (e.g. pCREB), may lead to the expression of immediate early genes (IEG). These IEGs can encode other transcription factors (e.g. c-fos, egr-1) or synaptic proteins (e.g. Arc, Homer1a), hence acting as neuromolecular switches that change the neurogenomic state of the brain (Aubin-Horth and Renn, 2009; Cardoso et al., 2015; Wolf and Linden, 2012).

The neuromolecular mechanisms potentially involved in social plasticity discussed above must be in action at brain regions relevant for the expression of social behaviour. Recently it has been proposed the occurrence of an evolutionary conserved social decision making network (SDMN) in vertebrate brains, that regulates a variety of social behaviours, from aggression, to mating and parental care (O'Connell and Hofmann, 2011, 2012). According to this proposal the SDMN is composed by two interconnected neural circuits, the social behaviour network (Goodson, 2005; Newman, 1999) and the mesolimbic reward system (O'Connell and Hofmann, 2011). Together these two

circuits include a core collection of nuclei that are reciprocally connected and that encode information in a distributed fashion, such that the expression of a specific social behaviour is better explained by the overall pattern of activation of the network rather than by the activity of a single node (Goodson and Kabelik, 2009). Thus, not only temporal but also spatial changes in gene expression across the SDMN may contribute for the differential activation of the network and concomitantly to the generation of different behavioural states. Given that, at the molecular level, different neural plasticity mechanisms may be in action it is important for the understanding of the genetic architecture of social plasticity to assess if they occur independently at each of the nodes of the SDMN. Previous studies have already established that behavioural transitions are associated with changes in the pattern of IEGs expression across the SDMN. In the African cichlid fish *Astatotilapia burtoni*, the opportunity to rise in social rank increased the expression of IEGs in all studied SDMN nuclei, whereas descend in social rank showed a distinct activation across the SDMN for the IEGs *c-fos* and *egr-1* (Maruska et al., 2013a, 2013b). In zebrafish winners and losers of a single social interaction also exhibit acute changes in the pattern of expression of *c-fos* and *egr-1* across the SDMN suggestive of socially-driven changes in functional connectivity among the nodes of these network (Teles et al., 2015). However, these studies have only focused on the expression of IEGs, and the hypothesis that different neuromolecular mechanisms involved in neuroplasticity may act independently at each of the nodes of the SDMN remains to be tested.

In this paper we used zebrafish (*Danio rerio*) to study socially-driven changes in behavioural state as a model to study social plasticity. Specifically, we assessed how induced changes in male zebrafish social status impact the expression of a set of genes known to be involved in different types of neuroplasticity across different nodes of the

SDMN. Male zebrafish (*Danio rerio*) express experience-dependent dominance behaviour, such that dominant and subordinate individuals express different behavioural profiles (Paull et al., 2010), and the outcome of a single agonistic interaction in socially isolated individuals is enough to induce experience-dependent shifts in status-dependent behavioural state (Oliveira et al., 2011). We used an established agonistic paradigm under which male zebrafish socially isolated overnight consistently express aggressive behaviour and a dominance relationship is established with a clear winner and a clear loser (Teles et al., 2013). We consider that winners and losers experience a change in social status in opposite directions (gain and loss, respectively), given their different perceived ratio of the aggressive acts given and received during the interaction. Two control treatments were also included in the experiment: (1) non-interacting fish that were kept in social isolation for the same amount of time; and (2) fish that fought their own image on a mirror, and therefore despite expressing aggressive behaviour did not experience a change in social status, since the number of aggressive acts performed equals those perceived in the opponent (mirror-image). The non-interacting control treatment provides a reference group, whereas a comparison of real-opponent fighters (i.e.Winners and Losers) with the mirror-fighters will allow us to distinguish gene responses associated with a behavioural shift (present in winners and losers) from those related to the expression of fighting behaviour (also present in mirror fighters, but where no status shift occurred). In summary our interpretation of possible results is the following:

(1) changes in gene expression between Winners/Losers and non-interacting fish that are not present in Mirror-fighters are associated with changes in social status (i.e. social plasticity);

(2) changes in gene expression between Winners/Losers and non-interacting fish also present in Mirror-fighters reflect aspects of fighting behaviour and are not associated with changes in social status;

(3) changes in gene expression between Mirror-fighters and non-interacting fish that are not present in Winners/Losers reflect their fighting behavioural state and are not associated with a shift in social status.

The following genes were used as markers of different types of neuroplasticity: brain-derived neurotrophic factor (*bdnf*), involved in changes in synaptic plasticity by increasing synaptic strength in response to excitatory transmission (Leal et al., 2014); neuronal PAS domain protein 4a (*npas4*), involved in homeostatic plasticity, by enhancing inhibitory synapses in response to excitatory transmission (Lin et al., 2008); neuroligin 1 (*nlgn1*) and neuroligin 2 a/b (*nlgn2*), as synaptogenesis markers (Krueger et al., 2012); and neuronal differentiation 1(*neurod*) and wingless-type MMTV integration site family, member 3 (*wnt3*) as indicators of neurogenesis (Aimone et al., 2014). Plasma cortisol levels were also measured to detect rapid physiological changes.

2. Material and methods

2.1. Animals

Our study subjects consisted of forty-five adult wild-type (AB) zebrafish males breed and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Fish were kept in a recirculating system (ZebraTec, 93 Tecniplast), at 28 ºC with a photoperiod of 14L:10D in mixed tanks. Water system was 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 7 respectively. Fish were fed twice a day with *Artemia salina* in the morning and commercial food flakes in the afternoon, except on the day of the experiments.

2.2. Experimental procedure

A behavioural paradigm previously used to study agonistic interactions (Oliveira et al., 2011; Teles et al., 2013) was followed. In brief, males were paired in size-matched dyads [standard length (mean ± SEM)= 3.78 ± 0.03 cm; body mass (mean ± SEM): 0.4 ± 0.00 g], and placed in a experimental arena (5 x 8 x 6 cm), which was divided in two compartments by one or more removable opaque partition(s) (see below). Members of each dyad were kept overnight in visual isolation, each one on each compartment of the experimental arena. After this period, one or more of the partitions were removed and the fish were allowed to interact for 30 min. Three social treatments were used: 1) fighting a real-opponent conspecific, where there was a single opaque PVC partition separating the two fish, which was removed; 2) fighting their own image on a mirror, where there were two mirrors, each facing one of the compartments, behind opaque partitions; the partitions were removed to uncover the mirrors but a central partition separating the two compartments remained in place; and 3) no agonistic interaction, where there were three central opaque partitions, and only the outer two were removed (to control for putative stress effects of handling partitions in the experimental tanks). These social treatments generated 4 social behaviour states: winners (W, n=12) and losers (L, n=11) of the real opponent interaction; mirror-fighters (M, n=12); and non-interacting fish (i.e. visual isolation, I, n=10). All animals were tested in pairs in order to give them access to conspecific odours, which would otherwise only be present in real opponent dyads, therefore avoiding confounding effects of putative chemical cues in the comparisons between treatments. Behavioural interactions were video-recorded for subsequent behavioural analysis. Two hours after the end of the interaction, animals were killed with an overdose of tricaine solution (MS222, Pharmaq; 500-1000 mg/L), and blood collect for hormonal analysis.

2.3. Blood collection and hormone analysis

Blood samples were collected from the caudal vein using a 300 µl syringe with a 30G needle. Blood was subsequently centrifuged at 10 g for 10 min, and the plasma collected into a new tube, diluted in EIA buffer (1:50) and stored at - 20°C until further processing. Cortisol levels were quantified using a commercially available enzyme immunoassay kit (Cayman Chemical Company, ref. 500360) following the manufacturer's instructions. Plasma samples were used directly into the kit without extraction, since it has been previously shown that there are no interferences of other putative immunoreactive substances with this kit in non-extracted plasma (Félix et al., 2013).

2.4. Brain microdissection

After euthanasia, fish were quickly decapitated by cervical transection, the head removed, embedded in mounting media (OCT, Tissue teck) and rapidly frozen on dry ice. Brains were subsequently sectioned in coronal plane at 150 µm on a cryostat (Leica, CM 3050 S), and sections collected onto regular glass slides previously cleaned with 70% ethanol. The following brain nuclei of interest were selected for microdissection based on proposed homologies between the fish and the mammalian brain (O'Connell and Hofmann, 2011), which are indicated between brackets below, and identified in the zebrafish brain according to the available brain atlas (Wullimann et al., 1996): Dm, medial zone of the dorsal telencephalic area (basolateral amygdala); Dl, lateral zone of the dorsal telencephalic area (hippocampus); Vv, ventral nucleus of the ventral telencephalic area (lateral septum); Vs, supracommissural nucleus of the ventral telencephalic area (extended amygdala/bed nucleus stria terminalis); and POA, preoptic area. Microdissection was performed with a modified 27G needle attached to a syringe

under a stereoscope (Zeiss; Stemi 2000). Tissue was collected directly into lysis buffer (RNeasy Lipid Tissue Mini Kit-Qiagen) and stored at -80ºC until mRNA extraction.

2.5. Gene expression

Total RNA extraction was carried out immediately after thawing using the RNeasy Lipid Tissue Mini Kit (Qiagen) with some adjustments to the manufacturer's instructions (see the electronic supplementary material for details). RNA quality and concentration were estimated using NanoDrop 1000 spectrophotometer and cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Quantitative real-time PCR (qPCR) primers for the target genes (*bdnf, npas4, nlgn1, nlgn2, wnt3, neurod)* were designed at specific gene regions, therefore when necessary, homologous regions underlying gene family functions were excluded from primer design. However, for *nlgn2,* which is duplicated, both gene forms (*i.e. nlgn2a* and *nlgn2b*) where targeted by designing primers in homologous regions between the two sequences. The eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*) was used as a reference gene. For each sample, transcript levels of candidate and reference gene were measured in 25 µl reactions on an Mx3000P qPCR system (Stratagene) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta BioSciences). No-template controls for each primer mix were also included in each run. (see the electronic supplementary material for further details). For the analysis, raw fluorescence data was submitted to PCR Miner (Zhao and Fernald, 2005) to calculate reaction efficiencies and cycle thresholds (CT) for each sample, and parameters subsequently used to determine the relative initial template concentration from 1/1(1+E)^CT. Relative amount of mRNA in each sample was then normalized to the reference gene.

2.6. Behavioural analysis

Behavioural analysis was performed using a computerized multi-event recorder (Observer XT, Noldus, *Wageningen, The Netherlands*). The behaviours were divided into aggressive (bite, chase and strike) and submissive (freeze and flee), following the ethogram for zebrafish agonistic behaviour (Oliveira et al., 2011). The following behaviour variables were quantified: (1) latency for the first attack (i.e. time between the beginning of the recording period and the first bite); (2) fight resolution time (i.e. time needed for a social hierarchy to be established); (3) frequency of aggressive displays and (4) submissive behaviours, expressed in the last 5 min of the interaction, when winners and losers were easily distinguished allowing the recording of individual behaviour.

2.7. Statistical analysis

T-tests were used to compare the behavioural variables (i.e. latency for the first attack, fight resolution time, and overt aggression) between real opponent and mirror elicited fights. The effects of social treatment (Mirror-fight, Winner, Loser, Non-interacting) and brain nuclei (Dm, Dl, Vv, Vs, POA) in the expression of different genes (*bdnf, npas4, nlgn1, nlgn2, neurod, wnt3*) were evaluated using linear mixed models (LMM) with two random effects, one for the subjects and another for the dyads involved in real opponent interactions. The inclusion of the random effect for the dyadic real opponent interactions aims to address the fact that the data for Winners and Losers can not be considered independent from each other since the behaviour of each of them influences the other, and hence a matched-dyad analysis is more appropriate (Briffa and Elwood, 2010). To assess the assumptions of the mixed-effects models plots of the residuals, fitted values, and estimated random effects were used. Planned multiple comparisons analyses were then used to evaluate the effect of social treatment (Mirror-

fight vs. Winners vs. Losers vs. Control) on gene expression at each brain nuclei. Effect sizes (Cohen's d_s for independent samples, and Cohen's d_z for dependent samples) for these comparisons were reported and reference effect size values (small: d>0.2, medium: d>0.5, and large: d>0.8) used to interpret the mean difference of the effect (Cohen, 1988). A one-way ANOVA with Welch correction for violation of homoscedasticity, followed by post-hoc tests (Tukey HSD) were used to compare cortisol levels among social treatments. Pearson correlations were also computed to test for associations between: (1) expressed behaviour and gene expression; (2) expressed behaviour and cortisol levels; and (3) cortisol levels and gene expression.

Sample sizes varied either due to technical problems (i.e. problems with blood collection or video recordings) or to outlier values, identified for each condition with the generalized extreme studentized deviate procedure with a $p = 0.05$ and a maximum number of outliers of 20% of sample size. Statistical analyses were performed on R [(R CoreTeam, 2015);, nlme (linear mixed models), multcomp (multiple comparisons) and Hmisc (correlations)] and on SPSS v. 21 (one-way ANOVAs with Welch correction). For all tests the significance level used was $p < 0.05$.

2.8. Ethics Statement

The animal experimentation procedures used in this study followed the institutional guidelines for the use of animals in experimentation and were approved by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 8954).

3. Results

3.1. Behaviour

The influence of social treatment in aggressive behaviour was measured in the pre-resolution phase by the latency to the first attack, and by the fight resolution time. During the assessment phase, the latency for the first bite was significantly lower in mirror fighters ($t=4.15$, $df=20$, $p< 0.001$; Figure 1A), whereas the fight resolution time was significantly higher ($t=28.73$, $df=20$, $p<0.000$; Figure 1B) when compared to real opponent interactions. Real opponent fights were solved in approximately 7 min, after which a dominance relationship was established. In contrast, mirror fighters fought for the entire interaction period. In the post-resolution phase of real-opponent fights, aggressive behaviour was only performed by winners, whereas losers only expressed submissive behaviour (Figure 1C). In mirror-fighters aggressive behaviour was exhibited during the entire interaction and submissive behaviour was never observed.

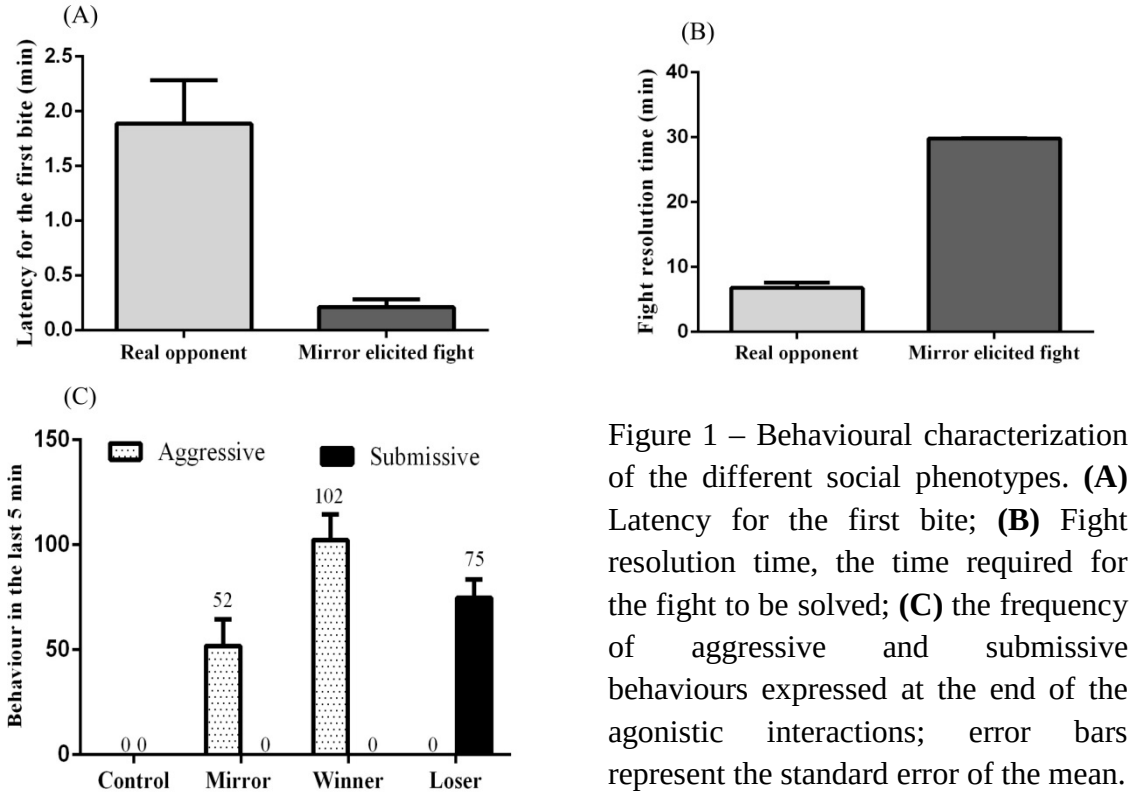

Figure 1 – Behavioural characterization of the different social phenotypes. **(A)** Latency for the first bite; **(B)** Fight resolution time, the time required for the fight to be solved; **(C)** the frequency of aggressive and submissive behaviours expressed at the end of the agonistic interactions; error bars represent the standard error of the mean.

3.2. Expression of *bdnf* across the SDMN

There was a significant main effect of brain nuclei on the expression of *bdnf* ($F_{(4, 152)}$ =80.75, p<0.0001; Dl > Dm > Vv > POA > Vs, Table 1). In contrast, no effects were found either for social treatment ($F_{(3, 7)}$=0.83, p=0.52), or for the interaction between social treatment and brain nuclei ($F_{(12, 152)}$ = 0.89, p= 0.55). Planned comparison analysis to evaluate the effect of social treatment on *bdnf* expression at each brain nuclei revealed that mirror fighters and losers increased mRNA levels in the Dl when compared to the control group (Isolation) (z =2.41, p=0.015, d_s =0.90; z =2.80, p=0.005, d_s =0.77, respectively), and that Losers also increased in comparison to Winners (z =1.99, p=0.04, d_z =0.48) (Figure 2 A).

3.3. Expression of *npas4* across the SDMN

There was a significant main effect of brain nuclei ($F_{(4, 145)}$ = 10.13, p< 0.0001), with Dm, Dl and Vv presenting higher mRNA levels than Vs and POA, and Vs was also significantly different from POA (Table 1),. There were no significant effects of either social status or the interaction between the two factors ($F_{(3, 7)}$ = 0.51, p= 0.69; $F_{(12, 145)}$ = 1.02, p= 0.43) on the expression of *npas4*. Planned comparisons revealed an increase in *npas4* expression in the Dm of the Winners compared to Losers (z = - 1.99, p=0.046, d_z =0.51) and a decrease in the mRNA levels of the Mirror fighters in the Dl when compared to the control group (z = -2.21, p=0.026, d_s =0.80, Figure 2 B).

Table 1 – Multiple comparisons analysis calculated using linear mixed models on the brain nuclei.

	bdnf		***npas4***		***nlgn1***		***nlgn2***		***wnt3***		***neurod***	
Brain nuclei	z-value	p-value	z-value	p-value	z-value	p-value	z-value	p-value	z-value	p-value	z-value	p-value
Dm-Dl	-3.86	<0.001	1.31	0.19	-0.67	0.50	-1.14	0.25	0.42	0.67	-6.75	<0.0001
Dm-Vv	6.01	<0.0001	1.61	0.10	-0.60	0.54	-3.82	<0.001	0.24	0.80	10.73	<0.0001
Dm-Vs	9.67	<0.0001	3.86	<0.001	1.97	<0.05	-7.63	<0.0001	2.36	<0.05	11.37	<0.0001
Dm-POA	9.14	<0.0001	5.98	<0.0001	3.09	<0.01	-1.68	0.09	2.08	<0.05	11.45	<0.0001
Dl-Vv	9.79	<0.0001	0.30	0.76	0.08	0.93	-2.74	<0.01	-0.18	0.85	17.69	<0.0001
Dl-Vs	13.31	<0.0001	2.60	<0.01	2.50	<0.05	-6.67	<0.0001	1.89	0.06	18.13	<0.0001
Dl-POA	12.77	<0.0001	4.71	<0.0001	3.62	<0.001	-0.55	0.57	1.60	0.10	18.47	<0.0001
Vv-Vs	3.80	<0.0001	2.35	<0.05	2.48	<0.05	-4.01	<0.0001	2.09	<0.05	0.89	0.37
Vv-POA	3.30	<0.0001	4.44	<0.0001	3.61	<0.001	2.15	<0.05	1.81	0.07	0.55	0.58
Vs-POA	0.47	<0.0001	1.99	<0.05	0.92	0.36	6.11	<0.0001	0.35	0.72	-0.3	0.71

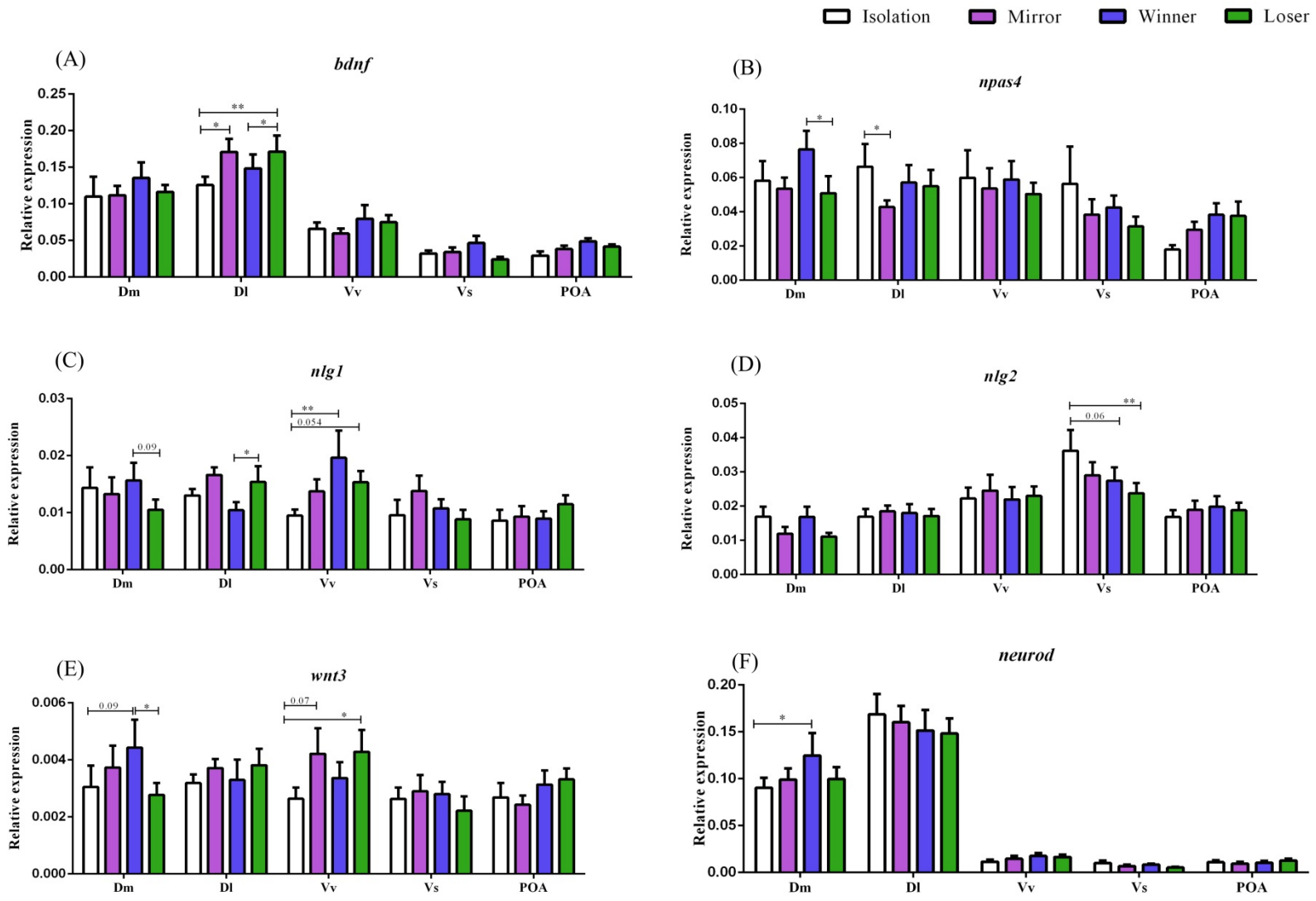
Isolation
Mirror
Winner
Loser
(A)
bdnf
(B)
npas4
(C)
nlg1
(D)
nlg2
(E)
wnt3
(F)
neurod
Relative expression
Dm
Dl
Vv
Vs
POA
0.09
0.054
0.06
0.07

Figure 2- Gene expression for the analysed genes (*bdnf*, *npas4*, *nlgn1*, *nlgn2*, *wnt3* and *neurod*) in different brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area;Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area) for the different social phenotypes. Control group is represented by the white bars, Mirror-fighter by the grey bars, Winner by the blue bars, and Loser by the green bars: **(A)** *bdnf* expression; **(B)** *npas4* expression; **(C)** *nlgn1* expression; **(D)** *nlgn2* expression; **(E)** *wnt3* expression; **(F)** *neurod* expression (normalized to *eef1a1l1* in); error bars represent the standard error of the mean. Asterisks indicate significant differences: * $p < 0.05$; ** $p<0.01$; using a planned comparisons test.

3.4. Expression of neuroligin genes across the SDMN

For *nlgn1* there was a main effect of brain nuclei ($F_{(4, 141)} = 6.49$, $p< 0.001$), with higher expression levels in Dm, Dl, and Vv compared to Vs and POA (Table 1). There was also an effect of the interaction between social treatment x brain nuclei ($F_{(12, 141)} = 1.84$, $p= 0.04$). No effects were detected for social treatment ($F_{(3, 7)} = 0.37$, $p= 0.77$). Planned comparison analysis revealed an increased in the Dl of Losers when compared to Winners ($z = 2.06$, $p=0.04$, $d_z =0.57$) and in the Vv of both Winners and Losers relative to controls ($z = 2.74$, $p=0.006$, $d_s =0.84$, $z = 1.92$, $p=0.054$, $d_z =0.27$). A close to significance response was also found in the Dm, where Losers decreased *nlgn1* expression in comparison to Winners ($z = - 1.66$, $p=0.09$, $d_s =0.56$, Figure 2 C).

For *nlgn2*, a main effect of brain nuclei was also detected ($F_{(4, 139)} = 16.42$, $p< 0.0001$), with major expression levels in the Vv and Vs compared to the other nuclei (Table 1), and no effects were found either for social treatment ($F_{(3, 7)} = 0.06$, $p= 0.97$) or for the interaction between social status and brain nuclei ($F_{(12, 139)} = 1.10$, $p= 0.35$). Planned comparisons identified a response in Vs with a significant decrease in the mRNA levels of Losers, and a close to significance decrease of Winners when compared with the control group ($z = - 2.59$, $p=0.009$, $d_s =0.98$; $z = - 1.84$, $p=0.06$, $d_s =0.65$, Figure 2 D).

3.5. Expression of neurogenesis genes across the SDMN

There was a main effect of brain nuclei on the expression of both *wnt3* and *neurod* ($F_{(4,\ 144)}$ = 3.11, p= 0.017; $F_{(4,\ 143)}$ = 149.2, p< 0.001; respectively). For *wnt3* higher abundance of transcripts was detected on Dm compared to Vs and POA, and also on Vv compared to Vs, for *neurod*, Dm and Dl were the areas with higher expression levels (Table 1). There was no main effect for social treatment nor for the interaction between social treatment and brain nuclei for either of these two genes (*wnt3*, social treatment: $F_{(3,\ 7)}$ = 0.58, p= 0.64; social status x brain nuclei: $F_{(12,\ 144)}$ = 0.94, p= 0.51; *neurod*, social treatment: $F_{(3,\ 7)}$ = 0.27, p= 0.84; social status x brain nuclei: $F_{(12,\ 143)}$ = 0.57, p= 0.86). Planned comparison analysis identified a significant increase in *wnt3* expression in the Dm of Winners in contrast with Losers (z = -2.07, p=0.04, d_s =0.44), and also a close to significance increase in the expression levels of Winners when compared to the control group (z = 1.68, p=0.09, d_s =0.38). There was also a marginally non-significant increase in the expression of *wnt3* in Vv both in Mirror fighters (z = 1.78, p=0.07, d_s =0.43) and in Losers (z = 2.16, p=0.03, d_s =0.43, Figure 2 E) when compared to the control group. An increase in *neurod* expression was observed in the Dm of Winners when compared to the control group (z = 2.10, p=0.03, d_s =0.56, Figure 2 F).

3.6. Circulating cortisol levels

Circulating levels of plasma cortisol sampled 2h after the social interaction showed overall differences across groups ($F_{(3,\ 10.68)}$ =5.83, p= 0.013). A post-hoc analysis revealed that Mirror-fighters and Winners had significantly higher cortisol levels than either Losers or individuals from the control group (Tukey HSD, p<0.05, Figure 3).

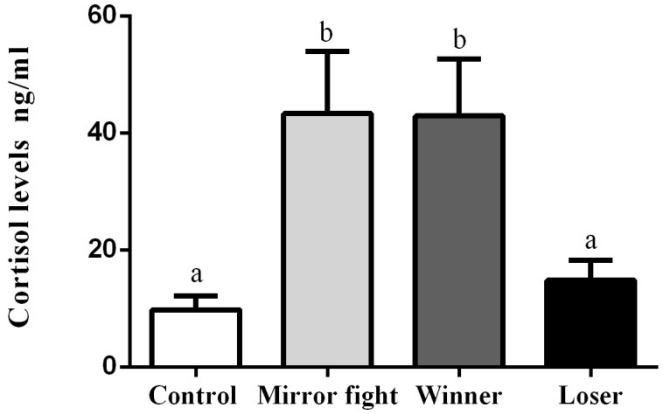

Figure 3- Plasma cortisol concentrations (ng/ml) in the different social phenotypes 2h post-interaction. Error bars represent the standard error of the mean, and different letters indicate significant differences between the groups ($p<0.05$).

3.7. Association patterns between behaviour, gene expression, and cortisol levels

For Winners positive correlations were found between aggressive behaviour and the expression of the genes *neurod* and *wnt3* in the POA (r=0.73, n=10, p=0.016; r=0.71, n=10, p=0.021, respectively), as well as a marginally non-significant correlation between aggressive behaviour and *bdnf* in the Dm (r=0.59, n=10, p=0.07). Both for Mirror-fighters and for losers no significant correlations were found, except for a close to significance association between submissive behaviour and the expression of *wnt3* in the Vs (r=0.58, n=9, p=0.09, Figure S1).

Regarding the relationship between behaviour and cortisol levels on the different social treatments, only a negative correlation was found in Mirror-fighters (r=-0.84, n=6, p=0.036; correlations were not significant in all other social treatments, Figure S2).

For gene expression and cortisol levels on the different social treatments: Mirror-fighters had a positive correlation for the expression of *nlgn2* in the Vs and a negative correlation in the POA. A close to significant correlation between *bdnf* expression and cortisol was also found in the Vs (Figure 4). In Winners the expression of *bdnf*, *npas4*, *wnt3* and *neurod* in the Dm was positively correlated with cortisol, and a close to

significant correlation was observed for the *nlgn1*. In the Dl *npas4 and nlgn2* were positively correlated with the hormonal levels, and a marginally non-significant correlation for *bdnf* was also detected. Finally, in the Vs a marginally non-significant correlation was observed for *nlgn1* (Figure 4). Losers in the Dl correlate negatively with the expression of *bdnf* and *neurod*, as well as a positively with *neurod* expression in the Vs. Close to significance negative correlations between cortisol levels and the expression of *wnt3* and *neurod* were also found in the Vv. All other correlations between cortisol levels and gene expression were not significant (Figure 4).

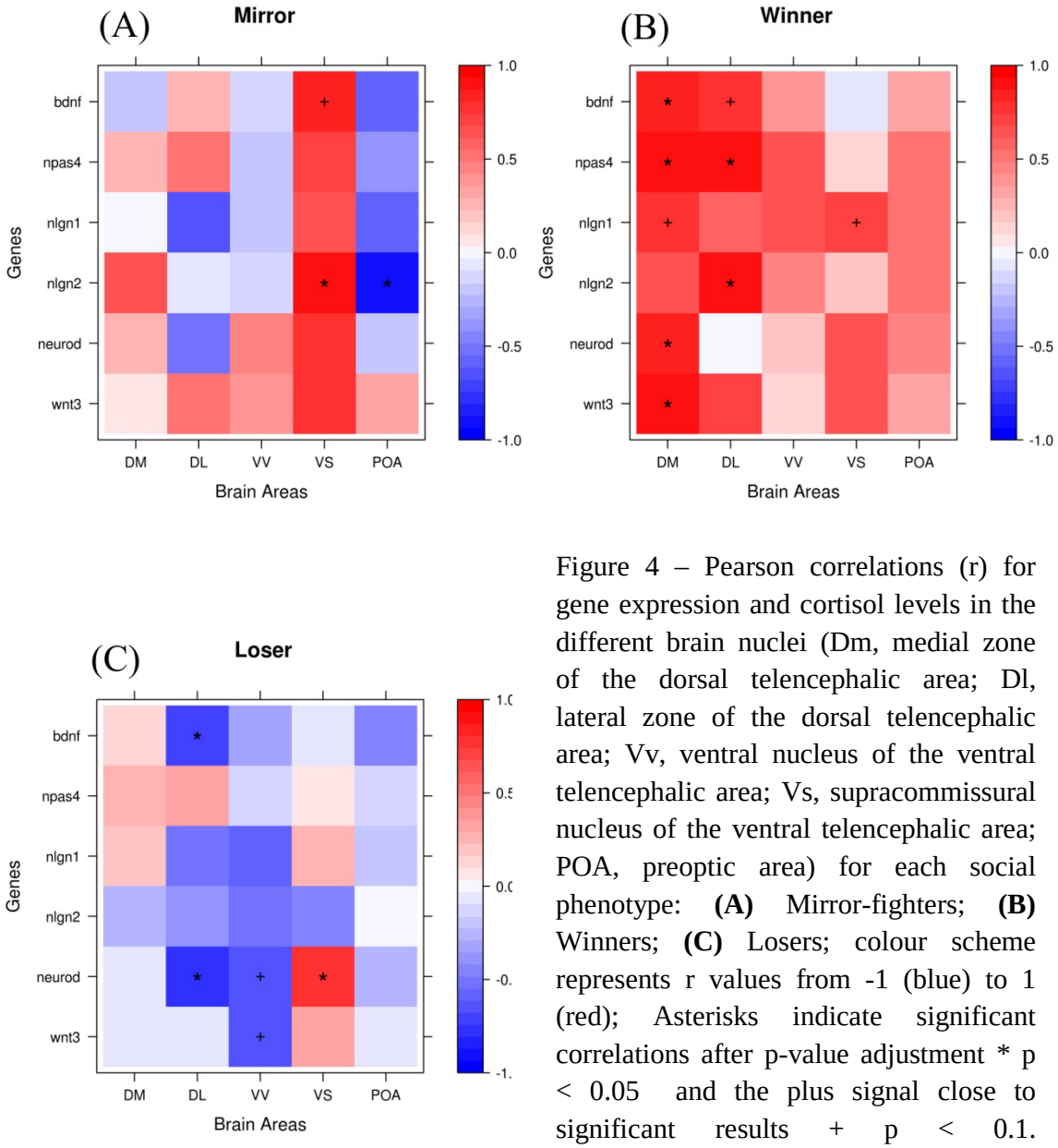

Figure 4 – Pearson correlations (r) for gene expression and cortisol levels in the different brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area) for each social phenotype: **(A)** Mirror-fighters; **(B)** Winners; **(C)** Losers; colour scheme represents r values from -1 (blue) to 1 (red); Asterisks indicate significant correlations after p-value adjustment * $p < 0.05$ and the plus signal close to significant results + $p < 0.1$.

4. Discussion

The results of this study are summarized in Table 2 and can be interpreted at two different levels: (1) the comparisons between each social treatment (i.e. Winners, Losers and Mirror-fighters) and the reference group (i.e. controls: non-interacting individuals) allow the characterization of the neuromolecular response specific to each social treatment; (2) the comparisons of the different social treatments among themselves allow the interpretation of the source of the observed neuromolecular responses, according to the predictions presented at the end of the Introduction. In the Discussion of the results below we will address each of these two levels of analysis sequentially.

Table 2 – Differential expressed genes in the SDM network in comparison with the control group and between Winners and Losers. Red arrows (↑) indicates a significant increase in the expression, blue arrows (↓) a significant decrease in the expression with $p < 0.05$, and the plus (+) indicates close to significant results, $p < 0.1$. Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area;Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area.

	Brain nuclei				
Social phenotype	Dm	Dl	Vv	Vs	POA
Mirror - Control		*bdnf* ↑ *npas4* ↓	*wnt3* ↑+		
Winner - Control	*neurod* ↑ *wnt3* ↑+		*nlgn1* ↑	*nlgn2* ↓+	
Loser - Control		*bdnf* ↑	*wnt3* ↑+	*nlgn2* ↓	
Winner - Loser	*npas4* ↑ *nlgn1* ↑+ *wnt3* ↑	*bdnf* ↓ *nlgn1* ↓			

4.1. Socially triggered neuroplasticity profiles for each social phenotype

Each social treatment was characterized by a specific neuromolecular pattern across the SDMN (Table 2). The Winner phenotype was characterized by an increase of the expression of neurogenesis genes (*wnt3* and *neurod*) in Dm (putative basolateral

amygdala homologue), and of neuroligin genes (*nlgn1* and *nlgn2*) in Vv and Vs (putative homologues of the lateral septum and extended amygdala, respectively) (O'Connell and Hofmann, 2011). *Wnt* signaling is one of the main regulators of adult neurogenesis (Lie et al., 2005), and its expression has been shown to be activity-dependent and to be associated with LTP and synaptic plasticity (Chen et al., 2006). Moreover, *wnt3* mediates *neurod* activation via the canonical Wnt/β-catenin pathway (Kuwabara et al., 2009), which in turn is important for adult neurogenesis and survival of progenitor cells. Thus, the up-regulation of these two genes suggests a remodelling of Dm (amygdala) circuits in Winners. In mammals, the amygdala together with the dorsal hippocampus (putatively Dl in teleosts) are critically involved in the formation of contextual fear memory, with the former tracking emotional valence, and the latter forming a representation of the context (Zelikowsky et al., 2014). Thus our results, that suggest remodelling of Dm in the absence of regulation of the Dl, may indicate changes at the level of valence encoding without context modulation in Winners. The differential expression of neuroligin genes in Winners is also interesting. These are post-synaptic cell adhesion molecules involved in synaptogenesis and synapse maturation in an activity-dependent fashion, by binding to pre-synaptic neurexins (Scheiffele et al., 2000). Neuroligins also affect synaptic function by recruiting and stabilizing key synaptic components, such as neurotransmitter receptors and channels. Both mammals and fish express 4 neuroligin genes (in zebrafish *nlgn* 2–4 are duplicated) with *nlgn1* and *nlgn2* exclusively expressed in excitatory and inhibitory synapses respectively, whereas *nlgn3* and *nlgn4* may be present in both (Südhof, 2008; Rissone et al., 2010). The region-specific effects of social phenotype on *nlgn1* in Vv and of *nlgn2* in Vs overall match previously described distribution of these genes in the CNS of zebrafish (Davey et al., 2010). In Winners the increase of *nlgn1* mRNA levels in the lateral

septum homologue (Vv) may be associated with the storage of associative memories related to status-acquisition, given the role *nlgn1* in excitatory glutamatergic synapses in associative learning (Kim et al., 2008). The decrease in *nlgn2* expression in the extended amygdala (Vs) maybe associated with behavioural disinhibition in winners related to a down-regulation of GABAergic synapses. Indeed, the manipulation of *nlgn2* has been shown to increase anxiety-like behaviours in mice (Hines et al., 2008).

Losers were characterized by an increase of the expression of *bdnf* in Dl and of the neurogenesis gene *wnt3* in Vv and by a decrease in the expression of the synaptic gene *nlgn2* in Vs. Interestingly, Mirror-fighters showed a neuromolecular pattern that partially overlapped with that of Losers: an increased expression of *bdnf* in Dl and of wnt3 in Vv. Additionally Mirror-fighters also exhibited a decrease of *npas4* in Dl. BDNF is a key molecule involved in the control of neuronal differentiation and survival, synapse formation, and in the regulation of activity-dependent changes in synapse structure and function (Park and Poo, 2013). In particular BDNF signalling in the mammalian hippocampus has been implicated in learning and memory through its effect on long-term potentiation and depression ((Egan et al., 2003); (Kovalchuk et al., 2002); (Park and Poo, 2013)). Thus, the region-specific effect of social treatment on *bdnf* expression localized in the fish homologue of the tetrapod hippocampus [Dl, (O'Connell and Hofmann, 2011)] is not surprising, and may suggest an involvement of Dl in social memory in both Losers and Mirror-fighters, that would thus recognize dominant individuals. In this respect it is worth noting that these two social phenotypes are the ones that have an opponent that expresses high levels of aggressive behaviour, and this might be a key feature to trigger social recognition mechanisms in an aggressive context. This hypothesis is also supported by the known role of hippocampus-dependent memory in social recognition in mice (Kogan et al., 2000).

Wnt3 was also up-regulated in the Vv of both Losers and Mirror-fighters, pointing to the occurrence of structural reorganization processes in this area in both social phenotypes. In mammals the lateral septum is involved in fear suppression (Thomas et al., 2013) as well as in and extinction of social fear conditioning (Zoicas et al., 2014), and a variety of anxiolytic or antidepressant drugs activate it (Rodrìguez-Landa et al., 2007; Thomas et al., 2005). Thus, for both phenotypes, *wnt3* mediated changes in this region may be related with anxiety and fear control: in Losers due to the defeat, and in Mirror-fighters due to the anxiety of an unsolved fight. The distinction between these two social phenotypes comes from a down-regulation of *npas4* in the Dl of Mirror-fighters, and of *nlgn2* in the Vs of Losers. *Npas4* is an activity-dependent transcription factor expressed in inhibitory and excitatory synapses that modulates the excitatory-inhibitory balance within neural circuits that are being activated (Lin et al., 2008). It has a cell-type-specific transcription gene program that induces inhibitory outputs on both cell types decreasing circuit activity; in excitatory neurons by the expression of synaptic connectivity regulators (e.g. *bdnf*), and in inhibitory neurons by a different gene set (Spiegel et al., 2014). *Npas4* has recently been implicated in the formation of contextual memories in the hippocampus (Ramamoorthi et al., 2011). Thus, the decreased expression of *npas4* in Mirror-fighters may indicate a decline in synaptic inhibition, as well as a lack of contextual memory formation. On the other hand, the decrease in the expression of *nlgn2* in the Vs of Losers, similar to what happens in Winners, may be related with anxiety behaviours as well. Despite the fact that Winners and Losers express different behavioural phenotypes, anxiety–like behaviours are expected to occur in both phenotypes after an agonistic interaction.

4.2. Status-specific and fighting triggered neuromolecular states of the SDMN

According to the rational described above (see Introduction) changes in gene expression in Winners/Losers shared by Mirror-fighters in relation to the reference group should reflect neuromolecular changes triggered by fighting behaviour, whereas the same changes not shared by Mirror-fighters should reflect status-specific neurogenomic states. Accordingly, the increase in neurogenesis genes in Dm and the changes in synaptic genes in Vv and Vs observed in Winners should be seen as status-specific. Similarly, the decrease in the expression of *npas4* in Mirror-fighters' Dl should be seen as specific of this phenotype. In contrast, there were no status-specific neuromolecular changes in Losers. Neuromolecular changes shared by different social treatments that hence reflect fighting behaviour, rather than status-specific neurogenomic states included: (1) the decrease of *nlgn2* in Vs both in Winners and Losers; and (2) the increase in *bdnf* in Dl and the increase of *wnt3* in Vv observed both in Losers and Mirror-fighters. Because the shared experiences between Winners and Losers and between Losers and Mirror-fighters are different, one may infer what aspect of the agonistic behaviour is driving these changes. In the former case, both social phenotypes (i.e. Winners-Losers) share the expression of displaying behaviour during the initial phase of the fights when individuals assess each other (Oliveira et al., 2011). Only after this assessment phase an asymmetry in agonistic behaviour emerges and Winners chase and attack Losers that only express submissive behaviour in this post-resolution phase of the fight (Oliveira et al., 2011). Therefore, the shared pattern of gene expression between Winners and Losers (i.e. decreased expression of *nlgn2* in Vs) most probably reflects the similar behavioural display patterns expressed by both phenotypes in the initial phases of the fights. In the latter case, the behavioural experience shared by

both Losers and Mirror-fighters is not the behaviour expressed, which is aggressive in Mirror-fighters and Submissive in Losers, but rather the behaviour observed in the opponent, which is the aggressive behaviour displayed either by the real opponent in the case of Losers or by the mirror-image in the case of Mirror-fighters. Therefore, the shared neuromolecular pattern observed in Losers and Mirror-fighters (i.e. increase in *bdnf* in Dl and the increase of *wnt3* in Vv) most probably is triggered by the perception of aggressive behaviour in a fighting opponent.

As a result of the increases/decreases of expression of the different genes in relation to the reference group discussed above, significant differences between social treatments may also emerge. In this study such differences were only observed between Winners and Losers in Dm for *npas4*, *nlgn1* and *wnt3*, and in Dl for *bdnf* and for *nlgn1*. These differences between social phenotypes are difficult to interpret since they may results from variations in each of the two phenotypes that are being compared in relation to the reference group.

4.2. Brain region specific neuroplasticity

This study also allowed to test if there are region specific neuroplasticity mechanisms across the SDMN in relation to the expression of social behaviour. Such regional variation was indeed observed with some neuroplasticity mechanisms being associated with social behaviour only at certain regions of the SDMN. In the amygdala homologue (Dm) only neurogenesis genes (*wnt3* and *neurod*) were associated with one of the social phenotypes (Winners). In the hippocampus homologue (Dl), only genes involved in molecular processes related to memory (*bdnf, npas4*) were associated with social phenotypes (Losers, Mirrror-fighters). In the lateral septum homologue (Vv), genes related to cell proliferation (*wnt3*) and to synaptic plasticity (*nlgn1*) were

associated with social phenotypes (Losers and Mirror fighters, and Winners, respectively). In the extended amygdala homologue (Vs) only genes involved in synaptic plasticity (*nlgn1, nlgn2*) were associated with social phenotypes (Winners and Losers). Finally, no neuroplasticity changes were observed in the POA.

4.3. Role of cortisol on socially-driven neuroplasticity

We have also investigated the cortisol response to each social treatment, given the potential role of social stress in shaping the neuromolecular responses described above. Our data showed that 2h post-interaction both Winners and Mirror-fighters had elevated cortisol levels whereas Losers' cortisol levels were similar to control levels. It has been previously shown that in zebrafish cortisol levels sharply increase after an acute stressor reaching a peak at 30 min, and then return to basal levels after 2h (Pavlidis et al., 2015). Thus, both Winners and Mirror-fighters seem to maintain elevated cortisol levels beyond the acute stress response. Differential regulation of the cortisol response across social treatments was also evident by the different correlations between cortisol and behaviour across the different phenotypes: a negative correlation between cortisol and aggressive behaviours was observed in the Mirror-fighters, but not in Winners, which also expresses aggressive behaviour; and so correlation between cortisol and submissive behaviour was observed in Losers. Correlations between cortisol and gene expression were also divergent depending on the social treatment and the brain nuclei analysed, indicating a social and region specific pattern of response. Overall, Winners presented positive correlation between the expression of the different neuroplasticity genes and cortisol in most brain nuclei, whereas losers mainly exhibited negative correlations (Figure 4). Thus, cortisol is associated with either an up- or down-regulation of neuroplasticity genes depending on gain or loss of social status by the individual.

Interestingly, Mirror-fighters, who despite the expression of aggressive behaviour do not experience a shift in social status, show a mosaic of positive and negative correlations between cortisol and gene expression across the SDMN.

BDNF interaction with cortisol is an example of these contrasting effects. In Winners there was a significant positive association between cortisol and *bdnf* expression both in Dm and Dl, while in Losers this association was only significant in Dl and was negative. This context-dependent interplay between BDNF and glucocorticoids has already been described previously and has been shown to vary with brain region (Gray et al., 2013), suggesting that either protective or detrimental effects of the interaction between neurotrophins and corticosteroids may occur in a social-dependent way. In the same line, the direction of the association between cortisol and *neurod* expression also varied with social treatment (i.e. positive in Winners and negative in Losers) again suggesting that the effect of cortisol on adult neurogenesis is dependent on social context. For instance in mice it has been shown that an increase in cortisol associated with a rewarding experience did not compromise adult neurogenesis (Leuner et al., 2010), whereas in stressful conditions elevated stress hormones impaired structural plasticity (Gould and Tanapat, 1998; Tanapat et al., 2001). Thus, one can speculate that cortisol in winners may be promoting neurogenesis, whereas in losers may be decreasing cell proliferation.

In summary our study presents the first experimental evidence that after an acute agonistic interaction different neuroplasticity mechanisms are activated in a brain-region specific fashion, which parallel the social-status specific changes in social behaviour observed. This indicates that social plasticity relies on multiple neuroplasticity mechanisms across the SDMN, and that there is not a single neuromolecular module underlying this type of behavioural flexibility.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution

MCT and RFO designed the experiment; MCT and SC performed the experiment; MCT analysed the data; MCT and RFO wrote the paper with contributions from all authors.

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Supplementary Material

RNA extraction

Tissue was homogenised in qiazol lysis reagent by vortex followed by an incubation of 7 min at room temperature (RT). Chloroform was added in a proportion of 1:2 and the sample incubated at RT for 5 min. Samples were subsequently centrifuge at 13000 x *g* for 20 min at 4ºC, and the upper aqueous phase transferred to new tube where 1 volume of 70% ethanol was added. This mixture was transferred to an RNEasy column, remained 5 min at RT, and was centrifuged for 1 min at 9000 x *g*. A sequence of buffers was added to the Rneasy column according to the manufacturer's instructions, and RNA eluted with 25 µl of RNAse-free water

Primers design and quantitative RT-PCR (qRT-PCR)

Primers were designed using Primer3web (Untergasser et al., 2012), with parameters adjusted to avoid dimer and hairpin formation, and their specificity confirmed by Primer-BLAST search (NCBI). Primers were commercially synthetized by Eurofins MWG Operon. Thermocycling conditions were 5 min at 95°C followed by 40 cycles of: 95°C, primer specific annealing temperature (see Table S1 for detailed information), and 72°C for 30 s each. A melting curve program from 55°C to 95°C with 0.5°C change in 10s intervals concluded the cycling protocol. The presence of single peaks in melting curves and gel electrophoresis performed on the PCR products confirmed the specificity of each primer pair. The identity of PCR products for each gene was also verified by DNA sequencing.

For each sample, transcript levels of candidate and reference gene were measured in 25 µl reactions and primers used at a concentration of 0.4 µM.

Table S1 – List of genes and corresponding primer sequences and parameters for qPCR.

Gene name	Abbreviation	Accession No.	Primer Sequences (5' → 3')	Annealing temperature (°C)	Amplicon length (bp)
eukaryotic translation elongation factor 1 alpha 1, like 1	*eef1a1l1*	NM_131263	F-CAAGGAAGTCAGCGCATACA R-TCTTCCATCCCTTGAACCAG	60	134
brain-derived neurotrophic factor	*bdnf*	NM_131595	F- GCTGCCGAGGAATAGACAAG R- CTGCCCCTCTTAATGGTCAA	58	157
neuronal PAS domain protein 4a	*npas4a*	NM_001045321	F- GACACGGGTTGAGAATGGTT R- GCACCAAGCACCCTGTAAAT	59	165
wingless-type MMTV integration site family, member 3	*wnt3*	NM_001114552	F- CTGTTGGGGGACTACCTGAA R- GGCGTATTTGGCTCGTAGTG	57	108
neuronal differentiation 1	*neurod1*	NM_130978	F- AAGTCAGATCCCTGCGTCAT R- GGGAATTGTGCAACTCTGC	63	185
neuroligin 1	*nlgn1*	NM_001142265	F- TCAACGAGGTCAGCCAGATA R- TGAAGCACCGACAGCAATAG	59	221
neuroligin 2 a/b	*nlgn2a* *nlgn2b*	NM_001166336 NM_001166329	F- GTCTGCCAAAGGGAACTATG R- ATGGTGGGACAGGATGAGTA	59	157

F - primer forward; R - primer reverse

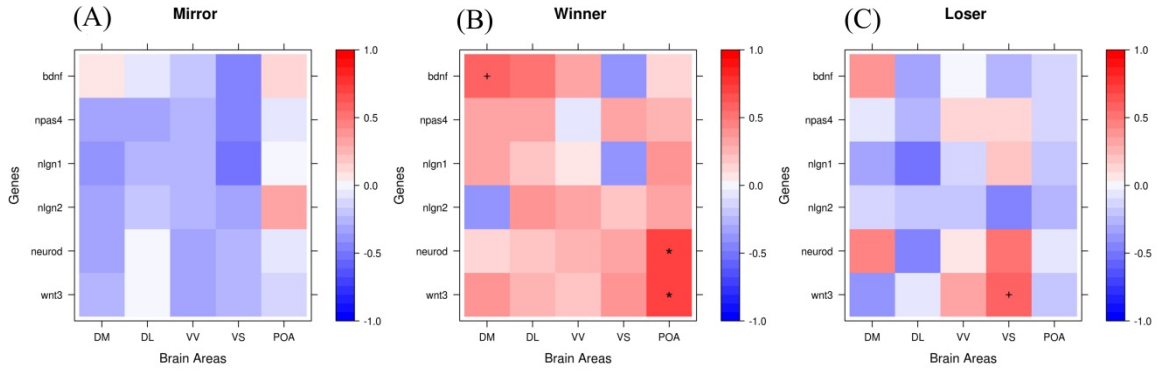

Figure S1 – Pearson correlations (r) between behaviour (aggressive and submissive) and gene expression in the different brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area; POA, preoptic area) for each social phenotype: **(A)** Mirror-fighters; **(B)** Winners; **(C)** Losers; colour scheme represents r values from -1 (blue) to 1 (red); Asterisks indicate significant correlations after p-value adjustment: + $p < 0.1$; * $p < 0.05$

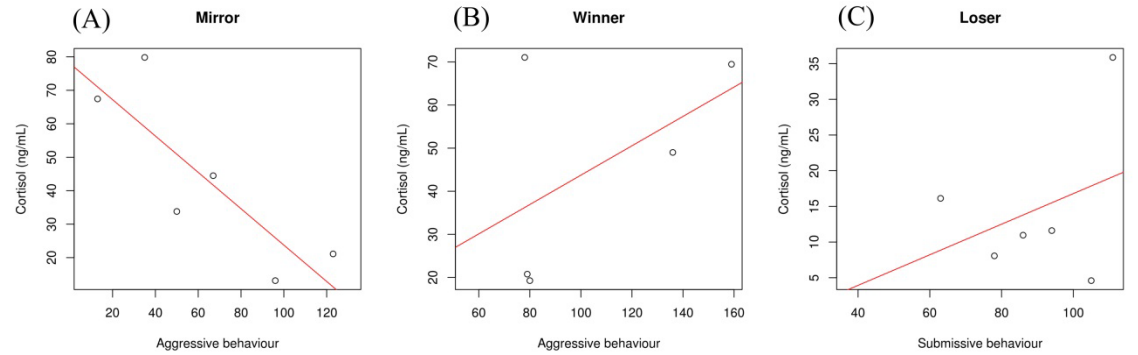

Figure S2 – Pearson correlations (r) plots for behaviour (aggressive and submissive) and cortisol levels. **(A)** Mirror-fighters, **(B)** Winner**, (C)** Loser.

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Chapter VII

General Discussion

7.1. Overview of results

The aim of the present thesis was to identify the neuronal mechanisms underlying socially driven changes in zebrafish behaviour. In order to do that, we used an agonistic paradigm that produce three different behavioural states: Winners, Losers and Mirror-fighters, and investigated the differences among these states in an integrative way by looking at the different neuromodulators molecules involved in behavioural shifts, and ultimately at changes in genes expression levels in the nodes of the social decision-making network.

In Chapter II to IV the neuromodulation processes were evaluated. Immediately after the acute interaction (30 min) animals were sacrificed, and levels of steroid hormones (i.e. androgens and cortisol) in the whole-body, and monoamines and nonapeptides concentrations in different brain areas were quantified. It was found that:

In Chapter II zebrafish males exposed to real opponent agonistic interactions exhibited an increase in androgen levels (11-Ketotestosterone increased both in Winners and Losers, while testosterone only increased in Losers). This androgen response was absent in Mirror-fighters, despite them expressing similar levels of aggressive behaviour to those of Winners. Cortisol levels were higher in real opponent fighters (Winners, Losers), but not in mirror fighters, when compared to the control group.

In Chapter IV serotonergic activity, measured by the ratio 5HIAA/5HT was significantly higher in the telencephalon of Winners and in the optic tectum of Losers, and no significant changes were observed in Mirror-fighters. Dopamine activity measured by the ratio DOPAC/DA was also significantly higher in the telencephalon of Winners and in the optic tectum of Losers and Mirror-fighters, when compared to non-interacting fish.

Chapter IV highlighted that AVT concentration in the telencephalon increased in the three social behavioural states (i.e. Winners, Losers and Mirror-fighters) and in the diencephalon of Winners and Losers. Isotocin on the other hand, decreased in the olfactory bulbs of Winners, and in the cerebellum of Losers, and increased in the diencephalon of the latter group.

In Chapter V and VI neurogenomic changes underlying behavioural flexibility were characterized.

In Chapter V after the agonistic interaction specific brain nuclei that compose the social decision-making network were microdissected and the expression of immediate early genes (IEGs) and candidate plasticity genes was analysed.

The neuronal activity patterns mapped by the IEG's *c-fos* and *egr-1* across the SDM network allowed to distinguish between the three behavioural states, since the co-activation patterns across the nuclei were behaviour state-specific (i.e. Winners ≠ Loser ≠ Mirror-fighters). Additionally, these results also supported the SDM network hypothesis since it was shown that social information was processed in a distributed fashion rather than locally.

In Chapter VI different neuroplasticity mechanisms were examined through the expression of a set of candidate genes (*bdnf, npas4, nlgn1, nlgn2, wnt3* and *neurod*) in the nodes of the SDM network. Each social treatment was characterized by a specific neuromolecular pattern across the SDMN. Winners were characterized by an increase of the expression of neurogenesis genes (*wnt3* and *neurod*) in Dm, and of neuroligin genes (*nlgn1* and *nlgn2*) in Vv and Vs. Losers were characterized by an increase of the expression of the memory gene *bdnf* in Dl and of the neurogenesis gene *wnt3* in Vv and by a decrease in the expression of the synaptic gene *nlgn2* in Vs. Interestingly, Mirror-fighters showed a neuromolecular pattern partially overlapped with that of Losers: an

increased expression of *bdnf* in Dl and of wnt3 in Vv, plus a decrease of *npas4* in Dl. Together these results indicate the occurrence of region specific neuroplasticity mechanisms across the SDMN in the different behavioural states.

7.2. Behavioural and physiological modulators of social plasticity

7.2.1. When behavioural repertoire speaks about social status

Aggressive behaviours are a pivotal component of the behavioural repertoire of animals. They serve numerous adaptive functions, including the establishment of dominance hierarchies and the competition for basic resources such as food, shelter, mates, or territories. Similarly to other species, the behavioural repertoire of zebrafish agonistic behaviour consists of a series of stereotyped body postures and movements that have been previously characterized (Oliveira *et al.* 2011). In dyadic male fights two distinct phases have been described: (1) a pre-resolution phase, where both fish exhibit the same repertoire of behaviours [display (lateral and frontal), circle, and bite]; this phase lasts until the first chase or flee is observed, which marks the establishment of a behavioural asymmetry between the contestants (i.e. fight resolution); and (2) a post-resolution phase, characterized by an asymmetry of expressed behaviours, where all agonistic behaviours are initiated by the dominant fish whereas the subordinate only displays submissive behaviours.

Overall our behavioural results (Chapters II to VI) have shown that following an acute agonistic encounter zebrafish males express two distinct behaviour profiles depending on the social status achieved: Losers exhibit exclusively submissive behaviours, whereas Winners express only aggressive behaviours. For animals that fought their own mirror image only aggressive behaviours were observed, with a frequency that was not significantly different from that observed in Winners of real

opponent fights. However, a major difference between Winners and Mirror-fighters is present, not on their behavioural output, but rather on the behaviour observed in the opponent, since in mirror fights the opponent (i.e. own image on the mirror) never displays submissive behaviours. As a consequence Mirror-fighters represent a group kept in the pre-resolution phase of the fight were the behaviours are symmetric between the contestants (i.e. focal fish and his own mirror image), and no behavioural shift occur, as can be demonstrated by the fact that the expression of aggressive behaviour typical of the pre-resolution phase lasted for the whole duration of the trial (30 min), whereas in real opponent fights the encounter was resolved in approximately 7 min (after which post-resolution behavioural profiles were observed). Other differences were also found between real opponent interaction and mirror elicited fights. For instance mirror fighters have lower latencies for the first bite, and this may be related to the absence of an assessment phase, in particular with the antiparallel displays impossible to perform with the mirror.

Conceptually, there are also several differences between the two behavioural protocols, which one should take in consideration depending on the specific goals of the study. Nevertheless, this is not in the scope of the present discussion, and a more detailed clarification is provided in Supplement I.

In summary our experimental design successfully produced four types of social phenotypes: Winners, Losers, individuals that expressed aggressive behaviour but did not experience either a win or a loss (i.e. Mirror-fighters), and individuals that did not express or perceived any social behaviour (control = social isolation). Therefore, our data can be interpreted in the perspective of behavioural shifts, social perception, or fighting mechanisms depending on the following comparisons:

Behavioural shifts	Social perception	Fighting behaviour
Differences between Winners or Losers against control group that are not present in Mirror-fighters suggest specific plasticity related processes.	Similarities between Mirror-fighters and Winners not present in Losers point to self-assessment evaluation; Similarities between Mirror-fighters and Losers, not present in Winners point to opponent-only assessment.	Similarities between Winners, Losers and Mirror-fighters, not present in the control group point to fighting related mechanisms.

7.2.2. Physiological modulation of social plasticity

In fish, like in other vertebrates the neuroendocrine system is organized in a hierarchical fashion with the hypothalamus controlling the activity of the anterior pituitary gland that in turn controls peripheral endocrine glands (e.g. gonads, anterior kidney, etc.) (Oliveira & Gonçalves 2008). As in other vertebrates, the fish pituitary gland consists of two types of tissue, the adenohypophysis and neurohypophysis. The secretion of the adenohypophysial hormones is under the direct control of releasing factors produced by hypothalamic neurons (e.g. hypothalamic releasing hormones, such as gonadotropin-releasing hormone GnRH, corticotropin-releasing hormone CRH), which in turn control other releasing factors [e.g. adrenocorticotropic hormone (ACTH)]. On the other hand, the neurohypophysis receives neural projections from the magnocellular neurons of the preoptic area, which may end in a capillary network, where the neurohormones are released into the bloodstream or project to other brain regions (Oliveira & Gonçalves 2008).

7.2.2.1. Steroid hormones

It has long been described that androgens respond to social challenges and this response has been interpreted as a way for animals to adjust androgen-dependent behaviour to social context (Oliveira 2009). Androgens have been involved in the

modulation of several social phenomena that require flexible behavioural responses. The role of androgens in the dear enemy effect, according to which the territorial individuals adjust their aggressive behaviour according to the threat imposed by intruders (familiar vs unfamiliar) (Temeles 1994), has been recently tested in a territorial species, the cichlid fish *Oreochromis mossambicus*. The authors found that familiar intruders elicited lower levels of aggression and a weaker androgen (11-Ketotestosterone, the androgen with higher biological activity in fish) response than strangers. Following repetitive intrusions the androgen response was significantly reduced and the difference between the two types of intruders no longer existed, suggesting the involvement of the androgen in the modulation of the response (Aires *et al.* 2015). The role of androgens in the audience effect, that is changes in the behavioural response due to the presence of bystanders, has also been tested in Siamese fighting fish (*Betta splendens*) and 11-KT was higher in males trying to establish a territory in the presence of bystander male (Dzieweczynski *et al.* 2006). Watching a territorial contest may also induce changes in the behaviour of the observer (bystander effect), and in *O. mossambicus* bystander males increased both 11-KT and testosterone (Oliveira *et al.* 2001). Androgens are also involved in tuning the expression of social behaviour in future interactions. The winner and loser effect associates previous experience with future interactions, for instance, prior winning increases the likelihood of winning future fights, whereas losing decreases the change of being a winner in subsequent encounters. 11-KT was found to mediate part of this phenomenon, since Winners treated with the anti-androgen cyproterone acetate lacked the winner effect, whereas 11-KT administration failed to reverse the loser effect, suggesting different underlying mechanisms for these two antagonic effects (Oliveira *et al.* 2009). However,

in another species (Japanese quail, *Coturnix japonica*) post-contest T administration reversed the loser effect (Hirschenhauser *et al.* 2013).

As we previously described most of these behavioural effects have already been described in zebrafish (Oliveira *et al.* 2011; Cruz & Oliveira 2015; Abril-de-Abreu *et al.* 2015a; b). However little is known about their hormonal modulation. Our results from Chapter II indicate an androgen response to an acute social challenge. 11-KT levels increased in real opponent fighters (Winner/Loser) in comparison to Controls. Nevertheless, these results did not confirm the prediction that Winners would increase and Losers decrease their androgen levels, as a way to adjust androgen-dependent behaviour to perceived social status (Oliveira 2009), and actually they contrast with previously reported differences in overall levels of 11-KT between dominant and subordinate zebrafish (Filby *et al.* 2010a). Nonetheless there are numerous dissimilarities between the two studies: (1) in our study animals were tested in pairs (dyadic interaction) whereas in the other study animals were tested in shoals of four individuals (2 males and 2 females); (2) in our study we used a short-term interaction (30 min) whereas Filby and co-workers used a long term interaction (1 or 5 days); and (3) the hormonal levels reported by Filby and co-workers refer to final levels (1 and 5 days for both social status), and individual differences within the same day were not reported, as we are showing here. All these methodological differences may explain the divergences observed between the two studies. Thus, to the best of our knowledge, this is the first study reporting androgen levels in response to acute social challenges in zebrafish.

The fact that there were no differences between the two social status (Winners and Losers), suggests an androgen response to social challenges that is independent of the social status achieved. Mirror-fighters had similar levels of both androgens (i.e. KT and

T) to those of non-interacting Controls, indicating a lack of activation of the hypothalamus-pituitary-gonads (HPG) axis, even when aggressive behaviour was expressed, suggesting a decoupling between the behaviour expression and the hormonal response to social challenges. This result is in line with previous studies for other species, which have also reported a dissociation between the androgen and the behavioural response in Mirror-elicited aggression (Oliveira *et al.* 2005; Hirschenhauser *et al.* 2008).

Corticosteroids, such as cortisol, are a measure of stress activation because the production of this hormone measures the activity of the hypothalamus-pituitary-interrenal axis (HPA). Here we have also investigated the cortisol response to each social treatment (Winner/Loser/Mirror-fighter), given the potential role of social stress in shaping the behaviour response. Social stress was confirmed in real opponent fights, as indicated by increased cortisol levels both in Winners and Losers. In Mirror-fights the HPI axis does not seem to be activated, as indicated by similar cortisol levels between Mirror-fighters and Controls. The cortisol response to real opponent fights observed in our study is in agreement with the higher cortisol levels observed in both dominant and subordinate individuals of long-term interactions (5 days), when compared to control levels in non-interacting fish (Pavlidis *et al.* 2011). Nevertheless, the cortisol stress response has a temporal dynamic. Zebrafish cortisol levels sharply increase after an acute stressor reaching a peak at 30 min, and then return to basal levels after 2h (Pavlidis *et al.* 2015). In Chapter VI we measured plasma cortisol levels 2h after the social interactions, and we found that Winners maintained elevated cortisol levels, Losers decreased to basal levels, and Mirror-fighters seemed to elevated cortisol levels beyond the acute stress response. These data are somehow puzzling, and can be a consequence of our behavioural paradigm. For the 2h sampling, at the end of the

interaction the respective partitions were placed back (i.e. in real opponent fights the central partition, preventing the interaction between the 2 conspecifics; and in the mirror elicited fights the partitions covering the mirrors), and although animals could not physically interact, odour cues were still available within each pair. Thus, two alternative explanations, which are not mutually exclusive, can be proposed. First, for the Winner, the chemical cues circulating in the tank signal the presence of the Loser, but it is no longer accessible, hence maintaining the anxiety levels; for the Loser, the Winner is still present but it can no longer attack, leading to a decrease in the anxiety state; for Mirror-fighters, the interaction was not solved and anxiety states may reflect frustration. Secondly, it can be related to the degree of activity (swimming) and glucose metabolism which is higher in Winners and Mirror-fighters (Mommsen *et al.* 1999).

Differential regulation of the cortisol response across social treatments was also evident by the different correlations between cortisol and behaviour across the different social states in Chapter VI. A negative correlation between cortisol and aggressive behaviours was observed in the Mirror-fighters, but not in Winners, which also expressed aggressive behaviour, and a correlation between cortisol and submissive behaviour was observed in Losers.

In summary zebrafish fighting a real-opponent showed an androgen, and a glucocorticoid activation, whereas both axis (HPG and HPI) failed to respond in Mirror-fighters. According to our initial hypothesis this can be explained by the absence of information on the interaction outcome in the latter group.

7.2.2.2. 5HT and DA

Similar to other behaviours, aggression is most likely influenced by the interplay of multiple neurotransmitters. The activation of the serotonergic system in response to

social challenges has been previously reported for several fish species. Interestingly the effects of serotonin 5-HT on aggressive behaviour are to some extent paradoxical. While several studies have pointed out that pharmacological manipulations that increase 5-HT inhibit aggression in a wide range of vertebrates, from fish to humans (Summers *et al.* 2005), other studies, in contrast, have shown increased serotonergic activity in specific brain regions during the expression of aggressive behaviour (Winberg & Nilsson 1993; Overli *et al.* 1999; Summers *et al.* 2005).

In Chapter III we showed that serotonergic activity (given by the ratio 5HIAA/5HT) was significantly higher in the telencephalon and olfactory bulbs of Winners and in the optic tectum of Losers, whereas no significant changes were observed in Mirror-fighters. According to our predictions these results point to social status specific responses of the serotonergic system in Winners and Losers, and the absence of activation in the Mirror group, points out the key role of the interaction outcome in social plasticity.

In zebrafish the dorsal cells of the superior raphe nuclei project to the telencephalon (Lillesaar *et al.* 2007, 2009), and telencephalon activity, may thus reflect the activation of this nucleus after an increase in social rank. The specific activation of the optic tectum in Losers appears to be a result of serotonergic neurons of the pretectal cluster (Kaslin & Panula 2001), which together with the optic tectum, have been implicated in the regulation of visual and motor behaviour, multimodal sensory integration [32] and escape responses (Herrero *et al.* 1998), which may explain the observed increase in Losers.

The effects of dopamine on aggression have been related with initiation and execution of aggressive acts (Louilot *et al.* 1986; Puglisi-Allegra & Cabib 1990; van Erp & Miczek 2000; Ferrari & Erp 2003). These neurochemical studies link elevated

dopamine and its metabolites in prefrontal cortex and nucleus accumbens not only to the initiation of attacks and threats, but also to defensive and submissive responses in reaction of being attacked (Puglisi-Allegra & Cabib 1990; Tidey & Miczek 1996) suggesting that dopamine may modulate motivational aspects of aggressive behaviour.

The DA system has also been linked with aggression in fishes. In juvenile Arctic charr (*Salvelinus alpinus*), L-dopa (the immediate precursor of dopamine) administration induces dominant status and caused a dose-dependent increase in dopamine brain levels (Winberg & Nilsson 1992). In rainbow trout dominate fish exhibited overall higher levels of dopamine than the subordinates (McIntyre *et al.* 1979), and in salmonids dominant individuals showed higher levels of homovanillic acid (HVA), a major DA metabolite, in the telencephalon when compared to subordinate fish (Winberg *et al.* 1991).

Our results point to status specific and area dependent modulation of this system. Dopaminergic activity was significantly higher in the telencephalon of Winners and in the optic tectum of both Losers and Mirror-fighters, and these increases were mainly determined by the metabolite levels.

For the Winners our results confirmed previous reports, where dominant fish increase DA activity after raise in social rank, and this increased dopaminergic activity in the telencephalon may reflect social reward. In amniotes, the mesolimbic dopaminergic system consists of the ventral tegmental area (VTA) projecting to many forebrain nuclei in what has been described as the reward system and is important for reinforcing learned behaviours (Young & Wang 2004). However, fish do not present a midbrain dopaminergic neuronal population homologous to the VTA (Smeets & González 2000). In contrast, DA inputs to the telencephalon originate in a local subpallial DA system and in DA neurons in the ventral diencephalon (i.e. posterior

tuberculum) that project towards the subpallium (Rink & Wullimann 2001, 2002; Tay *et al.* 2011). Therefore, in fish this ascending DA pathway may be playing a similar role in reward behaviour as the mammalian mesostriatal DA pathway.

The increased DA activity found in the optic tectum of both Losers and Mirror-fighters, suggests that what is driving this activity is what they see (both observe aggressive behaviour in the opponent) rather than the behaviour they express.

In summary, our results characterized the monoaminergic systems response to social challenges, across major brain regions. Furthermore, with our behavioural paradigm we could detect that serotonin and dopaminergic systems are associated with different motivational states.

7.2.2.3. AVT and IT

In the species studied so far there are considerable variation in the function of both AVT and IT, which appear to be species and context-dependent (Goodson 2008). For instance AVT and IT administration could either increase or decrease aggression and courtship depending on the species (Godwin & Thompson 2012), and the expression of nonapeptide receptors can also vary depending on social status (Filby *et al.* 2010b; Lema *et al.* 2015). Therefore, there is clear neuromodulation of social status by AVT/IT systems. In zebrafish different neuronal population have been associated with different social status, (i.e. Winner with higher activation of the magnocelular population, and losers in the parvocelular neuronal population) (Larson *et al.* 2006) supporting a segregation of the Winner/ Loser within the same neuronal system.

In Chapter IV we show that AVT is related with:

a) Loser social status transition, by the expression of higher levels of this peptide in the optic tectum and brain stem;

b) General fighting mechanisms, by an overall increase in the telencephalon of all behavioural phenotypes (Winner/Loser/ Mirror-fighters);

c) Real opponent fighting characteristics, by the raise of AVT in the diencephalon of only Winners and Losers.

For Losers our data are in line with previous reports in zebrafish, which show that pharmacological administration of AVT induce a decrease in agonistic behaviour, whereas an antagonist of V1a receptors (Manning compound) is able to restore aggression to sham-treated levels (Filby *et al.* 2010b). Interestingly, the two brain regions involved (optic tectum and rhombencephalon) have been implicated in the regulation of visual and motor responses to sensory stimulation (Iwasaki *et al.* 2013), as well as escape behaviours (Herrero *et al.* 1998). Thus, the observed AVT changes in these areas in Losers can be modulating submissive behaviour by coordinating sensory inputs to motor circuits.

The telencephalon receives AVT projection from the preoptic area (Saito *et al.* 2004), and in butterfly fishes an association between the density of AVT- ir varicosities and aggressive behaviour in the Vv (homolog of the lateral septum) has been identified (Dewan *et al.* 2011). Our findings also support a key role of the telencephalon in the regulation of aggressive behaviour. However, since we analysed major brain areas rather than specific brain nuclei, we cannot unravel to role of individual areas.

Isotocin was associated with Winners' social ascension, who exhibited a decrease of IT levels in the olfactory bulb. The plasticity of the olfactory system may be related with memory formation. In mice this peptide has been shown to induce LTD in synapses involved in long-term olfactory memory formation (Gur *et al.* 2014). Thus, in zebrafish IT may be also modulating synaptic plasticity underlying learning and memory formation in relation to status acquisition.

In Losers we detected a significant decrease of this peptide in the cerebellum and increase of IT levels in the diencephalon. Cerebellar LTD has also been described in fish Purkinje-like cells (Han *et al.* 2000) and it has been hypothesized to be important for motor learning. Thus the observed changes of IT levels in the cerebellum may be related to motor learning in Losers. On the other hand, the diencephalon levels may be related to decrease aggression, as already describe in Syrian hamsters (*Mesocricetus auratus*) (Harmon *et al.* 2002).

It is relevant to highlight that also for the AVT/ IT system Mirror-fighters did not show any response suggesting once again the pivotal role of perception in triggering transitions between behavioural states.

7.3.Neurogenomic shifts: the first line of response

So far we have discussed the neuromodulation of social plasticity, which can be conceptualized as a reversible process between different behavioural states (i.e. the consistent expression of a set of behaviours) in response to relevant social information. These behavioural states are paralleled by shifts between neuronal states, which, from the molecular perspective, rely on the social regulation of gene expression (Aubin-Horth & Renn 2009; Oliveira 2012).

In the African cichlid fish, *Astatotilapia burtoni*, neuronal activity-dependent gene expression has been reported to occur in the social decision-making (SDM) network, after a shift in social status. Socially ascending males increased mRNA levels of the IEGs *c-fos* and *egr-1* in all nuclei of the SDM network, and this increased expression was not found either in stable dominant or stable subordinate males (Burmeister *et al.* 2005; Maruska *et al.* 2013a). In contrast, in socially descending males changes in IEGs expression levels were nuclei specific both for *c-fos* and for *egr-1*, but never

simultaneously for both (Maruska *et al.* 2013b), suggesting distinct activation patterns in the SDM network depending on social experience. Nevertheless, the theoretical hypothesis underlying the SDM network is that the information is processed in a dynamic fashion across the nodes of the network, and that the neuronal state that parallels the behavioural changes is best represented by its overall pattern of activity. Our data from Chapter V) tested the SDM network hypothesis in zebrafish by contrasting changes in functional localization vs. functional connectivity across the SDM network in response to changes in social status. The analysis of individual nuclei (functional localization) showed an mRNA increase of *c-fos* levels in all brain regions for all behavioural states (i.e. Winners, Losers, Mirror-fighters) relative to Controls, whereas *egr-1* data only revealed activation of Dl (putative homologue of the mammalian hippocampus) for Mirror-fighters and Losers, and of the POA for all behaviour states. Despite the contrasting behaviour profiles, Winners and Losers had similar levels of expression in all studied brain regions, suggesting an absence of localized social status specific activation in the SDM network. However, the analysis of functional connectivity, given by the co-activation pattern between the nuclei, showed that different social behaviour states exhibited different patterns of functional connectivity as evidenced by: 1) the lack of association between any two correlation matrices that capture the patterns of co-activation of SDM nodes for each social behaviour state; (2) different clusters (i.e. sub-networks) present in each social behaviour state; (3) different nodes occupying the central position in the network in each social behaviour state; and (4) significantly different densities of connections in each social behaviour state. Thus, our results show for the first time that social behaviour state depends on the activity of the interconnected nodes of the SDM network, rather than on the localized activity of individual nuclei. Based on network

activity patterns, we found different neuronal states for Winners and Losers, suggesting social-status related changes. Regarding Mirror-fighters, one can conclude that during the fights the assessment is performed by an integration between perceived behaviour of the opponent and the own expressed behaviour, since the neuronal state of Mirror-fighters was different from Winners and Losers, showing the relevance of social perception on the modulation of gene expression.

In line with the same rational in Chapter VI we characterized neural plasticity in the different behavioural states across the SDM network. In order to do that, we selected a set of genes involved in different types of neural plasticity: *bdnf*, involved in changes in synaptic strength; *npas4*, involved in contextual learning dependent establishment of GABAergic synapses; neuroligins (*nlgn1* and *nlgn2*) as synaptogenesis markers; and genes involved in adult neurogenesis (*wnt3* and *neurod*) to look for differences across the SDM network and across the different social states.

Our results illustrate that each behavioural state (i.e. Winners, Losers, Mirror-fighters) was characterized by a specific neuromolecular pattern across the SDM network (results are summarized in table 2). Relatively to non-interacting fish, Winners presented the most distinct phenotype with increased expression of neurogenesis genes (*wnt3* and *neurod*) in Dm (putative basolateral amygdala homologue), and neuroligin genes (*nlgn1* and *nlgn2*) in Vv and Vs (putative homologues of the lateral septum and extended amygdala, respectively). Losers were characterized by a decrease in the expression of the synaptic gene *nlgn2* in Vs (putative homologues of extended amygdala, respectively) and shared with Mirror-fighters a neuromolecular pattern consisting of an increased expression of *bdnf* in Dl (putative hippocampus homologue) and *wnt3* in Vv (putative basolateral amygdala homologue). Additionally, Mirror-fighters also exhibited a decrease of *npas4* in Dl (putative hippocampus homologue).

Thus, this study presents the first experimental evidence that after an acute agonistic interaction different neuroplasticity mechanisms are activated in a brain-nuclei specific fashion, indicating that social plasticity relies on multiple neuroplasticity mechanisms across the SDM network. Winners and Losers proved to be different also in the neuroplasticity mechanisms that are activated, and Mirror-fighters were different from either of them despite sharing some similarities with Losers. This latter result may indicate that perceived behaviour in the fighting opponent is the relevant cue to activate some brain nuclei, as already suggested in Chapter III for dopamine activity in the optic tectum of Losers and Mirror-fighters.

7.4. Concluding remarks and future perspectives

In the present work we have shown how highly responsive the brain is to social plasticity. The neural plasticity revealed in these studies, in response to an acute agonistic interactions is remarkable. Influenced by social stimuli, there are massive changes in the brain both at the physiological (i.e. hormonal, monoamines, nonapeptides) and genomic (activity-dependent genes expression, and plasticity genes expression) levels. Based in our data a physiological and a neuronal profile (Table 3) for each social status can be established.

In general, the differences found between Winners and Losers strongly suggest different social plasticity mechanisms underlying the different behavioural states both at physiological and molecular levels, given us an integrative view of the process. The specific cues that trigger this shift in social status remain elusive, however, since in Mirror-fighters, where we were able to decouple the experience of winning or losing for the fighting experience, few changes were detected. This, by itself suggests a pivotal role of social perception in triggering shifts between socially driven behavioural states.

Table 3- Physiological levels (hormones, monoamines and nonapeptides) and genomic patterns found in Winners, Losers, and Mirror-fighters in comparison with the control group. A Red arrow (↑) indicates a significant increase, a blue arrow (↓) a significant decrease, with $p < 0.05$.

	Winner	**Loser**	**Mirror-fighter**
Testosterone		↑	
11-Ketotestosterone	↑	↑	
Cortisol	↑	↑	
Serotonin activity	↑ (Telencephalon)	↑ (Optic tectum)	
Dopamine activity	↑ (Telencephalon)	↑ (Optic tectum)	↑ (Optic tectum)
Arginine-Vasotocin	↑ (Telencephalon) ↑ (Diencephalon)	↑ (Telencephalon) ↑ (Diencephalon) ↑ (Optic tectum) ↑ (Brain stem)	↑ (Telencephalon)
Isotocin	↓ (Olfactory bulbs)	↓ (Cerebellum)	
IEGs	*c-fos* ↑ (all SDMN) *egr-1* ↑ (POA)	*c-fos* ↑ (all SDMN *egr-1* ↑ (Dl) *egr-1* ↑ (POA)	*c-fos* ↑ (all SDMN) *egr-1* ↑ (Dl) *egr-1* ↑ (POA)
Memory-related genes		*bdnf* ↑ (Dl)	*bdnf* ↑ (Dl) *npas4* ↓ (Dl)
Synaptogenesis genes	*nlgn1* ↑ (Vv) *nlgn2* ↑ (Vs)	*nlgn2* ↓ (Vs)	
Neurogenesis genes	*neurod* ↑ (Dm) *Wnt3* ↑ (Dm)	*wnt3* ↑ (Vv)	*wnt3* ↑ (Vv)

Although this work was manly focused in one of the two major plasticity mechanism, biochemical switching, our data on the expression of neurogenesis and synaptogenesis related genes suggest that both types of neuroplasticity may occur in parallel and not at different time scales as initially proposed (Zupanc & Lamprecht 2000). To test this hypothesis further studies will be needed that would contrast chronic

and acute socially induced changes in behavioural states to see their impact in the rearrangement of neuronal circuits.

7.5. References

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Supplement I

Teles, M. C. & Oliveira, R. F. 2013 Quantifying aggressive behaviour in Zebrafish.

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Quantifying aggressive behaviour in Zebrafish

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Summary

Aggression is a complex behaviour that influences social relationships and can be seen as adaptive or maladaptive depending on the context and intensity of expression. A model organism suitable for genetic dissection of the underlying neural mechanisms of aggressive behavior is still needed. Zebrafish has already proven to be a powerful vertebrate model organism for the study of normal and pathological brain function. Despite the fact that zebrafish is a gregarious species that forms shoals, when allowed to interact in pairs, both males and females express aggressive behaviour and establish dominance hierarchies. Here we describe two protocols that can be used to quantify aggressive behaviour in zebrafish, using two different paradigms: (1) staged fights between real opponents; and (2) mirror elicited fights. We also discuss the methodology for the behaviour analysis, the expected results for both paradigms, and the advantages and disadvantages of each paradigm in face of the specific goals of the study.

Keywords: aggression; social dominance; behaviour; ethogram; event recorder; zebrafish.

1. Introduction

Aggression can be defined as any behaviour directed towards another individual with the intention to cause harm (1). It is usually seen as an adaptive behaviour expressed throughout most animals' lives, which has evolved in the context of intraspecific competition for resources, such as food, shelter, mating opportunities or social status. However, heightened aggression levels may become maladaptive, and in humans they are often associated with psychiatric disorders (2). Therefore, the study of aggression has been prompted both by fundamental and by applied questions. Despite significant progress in the identification of the neurobiological factors associated with aggression, there is still a need to understand in more detail the neural circuits and the active molecules that control this behaviour. Similar to other complex behaviours, aggression is induced by the interplay of genes, neurotransmitters and hormones, in the building and regulation of neural circuits, that appear to be conserved across vertebrate species (3, 4). Thus, progress in this area needs a model organism with a genetic toolbox available that allows for real-time visualization of brain activity and for the precise manipulation of specific neural circuits, in order to enable the mapping of behavior into neural circuits (5).

Zebrafish have already proven to be a powerful animal model for the study of complex cognitive disorders like depression, autism spectrum disorder (ASD), drug abuse, cognitive deficits and psychoses (6). Several behavioural paradigms used in rodents to study these disorders have already been successfully developed in zebrafish, such as exploration (open field), anxiety-like (light –dark and alarm substance), locomotion (novel tank), and social and cognitive (shoaling, social preference, predator avoidance and T-maze) tests (6). The utility of this species in behavioural neuroscience has grown markedly because of its available molecular [forward and reverse genetic

methods (7, 8)], electrophysiological (9) and optogenetic (10) tools, the variety of wild-type lines with distinct behavioural phenotypes (6), conditional transgenic lines (11), and the similarity its genome presents with the human genome, where approximately 70% of the genes have human orthologs (12). All these features make zebrafish an ideal model for translational neuroscience.

Although zebrafish is a gregarious species that in nature form shoals (13), when allowed to interact in pairs, both males and females express aggressive behaviour and establish dominance hierarchies (14–16). In this species aggression is commonly used by dominant individuals to get access to spawning sites and protect their social status from competitors (16). Similarly to other species, the repertoire (i.e. ethogram) of zebrafish agonistic behaviour consists of a series of stereotype body postures and movements that have been previously characterized (Table 1) (15). In dyadic male fights two distinct phases have been described: (1) a pre-resolution phase, where both fish exhibit the same repertoire of behaviors (display, circle, and bite); this phase lasts until the first chase or flee is observed, which marks the establishment of a behavioural asymmetry between the contestants (i.e. fight resolution); and (2) a post-resolution phase, characterized by an asymmetry of expressed behaviours, where all agonistic behaviours are initiated by the dominant fish whereas the subordinate only displays submissive behaviours. Therefore, the expression of the different aggressive behaviour action patterns has a specific temporal structure (Figure 1). An agonistic interaction usually starts with both opponents exhibiting lateral displays in an anti-parallel position, and circling each other. Then, it progresses to mutual bites, still in the pre-resolution phase. Finally, in the post-resolution phase, dominant individuals bite, chase and strike towards subordinates, whereas the latter flee, freeze and retreat.

Table 1- Ethogram of zebrafish aggressive behaviour [adapted from Oliveira el al, 2011].

Behavioural patterns	Description
Displays	In short distance of the opponent, usually less than one body length, fish erects its dorsal and anal fins, and flares its body flank towards the opponent.
Circle	Two fish approach each other in antiparallel positions with their fins erected and circle one another ascending in the water column. It can last from a few seconds to minutes.
Strike	The fish swims rapidly towards the opponent, but no physical contact occurs.
Bite	Fish opens and closes its mouth in contact with the body surface of its opponent, usually directed towards the ventral or the posterior parts of the body of the target fish.
Chase	Similar to strike but with an active pursuit by the aggressor. This behaviour stops when one fish stops chasing, and/or the other fish adopts a Freeze behaviour.
Retreat	Fish swims rapidly away from the opponent in response to a strike or a bite.
Flee	Continued escape reaction in response to a Chase. Fish swims rapidly away from the aggressor.
Freeze	Fish stays immobile with all fins retracted and the caudal region downwards near the bottom or the surface of the aquaria.

Given that fish lack visual self-recognition, when exposed to a mirror they usually display aggressive behaviour towards their mirror-image (17). Therefore, aggressive behaviour in fish has been quantified using either their response towards real opponents (14, 15), or towards their own mirror images (17–20, 26, 27). However, recent studies have questioned whether these two tests of aggression are measuring the same aspects of behaviour, since they elicit different hormonal responses in cichlid fish (17, 27). In zebrafish, mirror elicited fights also failed to arouse the same brain responses as real

opponents in gene expression (R.F. Oliveira lab, unpublished data) and in the monoaminergic activity (19). Despite these physiological differences elicited by the two protocols, there are no significant differences between the level of overt aggression exhibited towards a mirror image or a real opponent (19, 20). Thus, both protocols seem suitable for quantifying overt aggression measures, but the decision to use one or the other should take into consideration known differences between the two (Table 2), which may be advantageous or disadvantageous, depending on the specific goals of the study. Here we describe two protocols that can be used to quantify aggressive behaviour in zebrafish, using each of these two paradigms: (1) staged fight test, between real opponents; and (2) mirror elicited aggression test.

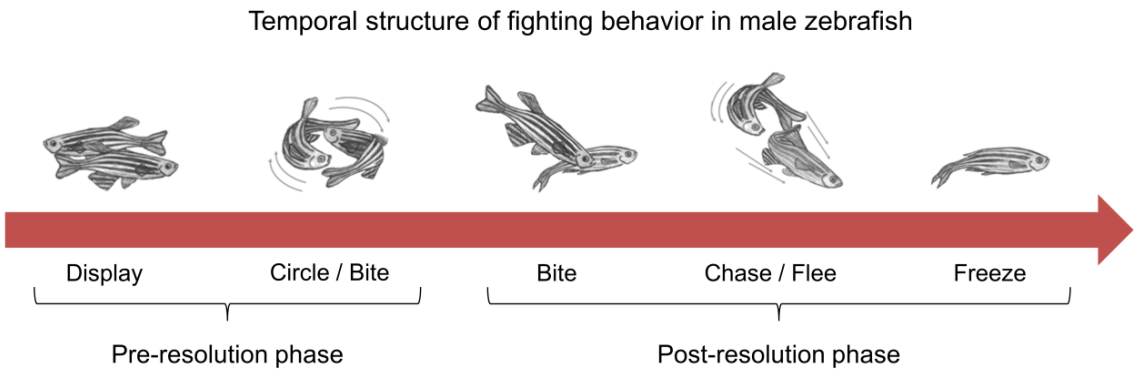

Figure 1- Zebrafish male fights exhibit a typical temporal structure. Fights can be divided into a pre-resolution phase and a post-resolution phase. The pre-resolution phase is defined by the expression of symmetric behaviours by both contestants, and behaviours such as displays, circles, and mutual bites occur. The post-resolution phase is characterized by a transition to asymmetric expression of behaviours between the opponents, where bites, chases and strikes are performed by the dominant individual, whereas, retreat, flee and freeze are expressed by the subordinate. The arrow represents the temporal occurrence of each type of behaviour in the respective phase [Adapted from Oliveira el al, 2011].

Table 2- Advantages and disadvantages between real-opponent and mirror-elicited fights as tests of aggression in zebrafish.

	Real opponent fight	Mirror elicited fight
Advantages	- Provide the most natural social stimulus. -Promote the establishment of social dominance with the emergence of dominant and subordinate phenotypes.	- The opponent's behaviour is standardized to that of the focal fish (i.e. it is the same). - Fighting individuals are not exposed to physical injuries, which makes it ethically more acceptable.
Disadvantages	- The researcher has no, or limited control of the stimulus fish, and the behaviour of the focal fish depends to a great extent on the behaviour of the opponent. - Fighting individuals can be physically injured, and thus it is less acceptable from an ethical perspective.	- The fights are unsolved and therefore the focal fish never experiences either a victory or a defeat (26). - Prevents the expression of lateral display in an antiparallel position, which is a common action pattern in real opponent fights**.** - The dynamics of the fight are atypical, since the opponent never initiates behavior and never displays submissive behaviour.

2. Materials

1. Electronic balance
2. Ruler/ Calliper
3. Buffered tricaine methane sulfonate (MS222, **See note** 1)
4. Spring scissor
5. Forceps

6. Fish holding support (**See note** 2)
7. 27G needle (internal diameter 0.210 mm)
8. Nylon monofilament 0.14 mm
9. Povidone-iodine (Betadine®) or any other microbicide like *chlorhexidine* to disinfect the material)
10. Nail polish
11. Zebrafish maternity tanks (18x10 x9cm)
12. Video camera
13. Multievent recorder software for behaviour recording and analysis (Observer XT)

3. Methods

3.1. Animal housing

The protocols described here were developed using adult wild-type zebrafish of the AB strain (**See note 3)**. Fish are kept in a recirculating housing system (ZebTec Multilinking System, TECNIPLAST, Italy), at 28 ºC with a 14L:10D photoperiod. The water is monitored for nitrites (<0.2 ppm), nitrates (<50 ppm) and ammonia (0.01-0.1 ppm), and pH and conductivity are maintained at 7 and 700 µSm, respectively. Fish are fed twice a day, except on the day of the experiments.

3.2. Individual tagging

In staged fights it is important to identify each individual during the whole interaction, such that the behavior of each opponent can be quantified separately. For this purpose, individuals need to be individually tagged. There are three commonly used procedures to tag zebrafish: fin clipping (15), colour tagging with nylon monofilament

(21, 22), and colour tagging with implanted elastomers (23) (**See note 4**). Here we describe the two methods that are currently used in our lab.

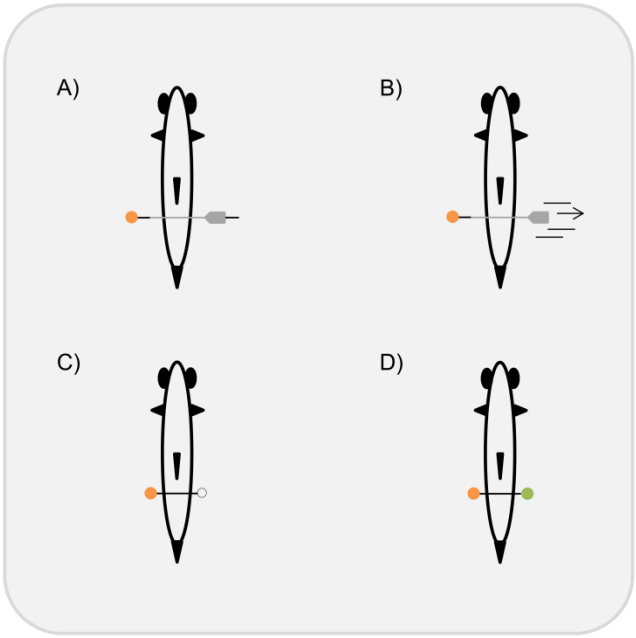

Figure 2- Color tagging with nylon monofilament. The fish is represented in a top view: A) Insertion of the hypodermic needle through the dorsal musculature of the fish and guiding the nylon monofilament already tagged through the needle hole. B) Removal of the needle leaving the monofilament in place. C) Giving knots on one side of the monofilament. D) Painting it with nail polish [Reproduced with permission from Patzner, R.A, 1984].

3.2.1. Fin clips:

1) Anesthetize the fish by immersion in Tricaine solution (160 mg/L) in a petri dish (**See note 5**).

2) Use the spring scissor to clip the extremities of the caudal, dorsal or anal fins in different combinations between pairs of opponents.

3.2.2. Colour tagging with nylon monofilament:

1) Prepare the nylon monofilament by cutting approximately 5 cm; give three or four knots with the help of the forceps in one tip and paint the knots with nail polish. (**See note 6**).

2) Cut the other tip of the nylon monofilament in diagonal, in order to be pointed.

3) Place all material, including the painted nylon monofilament previously prepared, in Povidone-iodine (Betadine®) or any other microbicide solution.

4) Anesthetize the fish by immersion in Tricaine solution (320 mg/L) in a petri dish.

5) Place the fish in an appropriate bedding (**See note 2**).

6) Insert the hypodermic needle (27G) through the dorsal musculature immediately below the posterior insertion of the dorsal fin.

7) Insert the pointed nylon monofilament already tagged through the needle hole (**Figure 2A**).

8) Remove the needle out of the fish body leaving the monofilament behind **(Figure2 B)**.

9) Give three or four knots, with the help of the forceps, on this tip and paint with nail polish **(Figure 2 C-D)** (**See note 7**).

3.2.3. Recovery from anaesthesia

1) Fill a zebrafish maternity tank with water (approximately 800 ml) and place the fish to recover after any of the tagging procedures described above. Do not use more than 5 animals per tank to mitigate stress (24).

2) Animals will recover very fast from the anesthesia (in minutes); however in order maximize the anesthetic withdrawal, keep animals in the recovery tank for 1h before moving them back to the home tank (**See note8**) (25).

3.3. Behavioural recording

1) We typically use an experimental tank of 12.5 cm x 8 cm x 6 cm divided into two parts: (1) the posterior part (7.5 cm x 8 cm x 6 cm) containing a mechanical filter and a heater (water temperature is kept at 28ºC also during the tests); and (2) the anterior part (5cm x 8cm x 6 cm), hereafter designated as arena, where the tests take place (**See note** 9).
2) Cover the back wall of the arena with white PVC, in order to improve contrast between fish and the background in video recordings.
3) Divide the arena into 2 parts of the same size by a removable PVC partition **(Figure 3)**: (a) for staged fights the PVC partition separates the two fish in the right and left sides of the tank (**Figure 3A**); (b) in mirror elicited fights the PVC partition contains one mirror on each side, and is perforated on the sides to allow water flow between the 2 parts; a second removable partition should be placed in front of it to hide the mirrors from the focal fish before the start of the interaction (**Figure 3B**).

3.3.1 Staged fights

1) Pair the animals according to their weight and standard length. **(See note 10)**

2) Prior to the experiment, place each pair in the experimental tank, one fish on each side of the arena divided by the opaque partition, where they stay overnight in visual isolation (**See note 11, Figure 3A-B**). Before the experiment, set up a standard video camera (**See note 12**) in front of the tank to record the interaction.

3) Gently remove the opaque partition and allow the two fish to interact for a period of 30 min (**See note13, Figure 3A'**).

4) At the end of the test period (30 min), a dominant and a subordinate fish should be easily identified by the different behaviours they express (i.e. winners only express aggressive behaviours, and losers only express submissive behaviours); place the partition back into the observation tank to separate the two fish again, and note the identity of the dominant and of the subordinate fish.

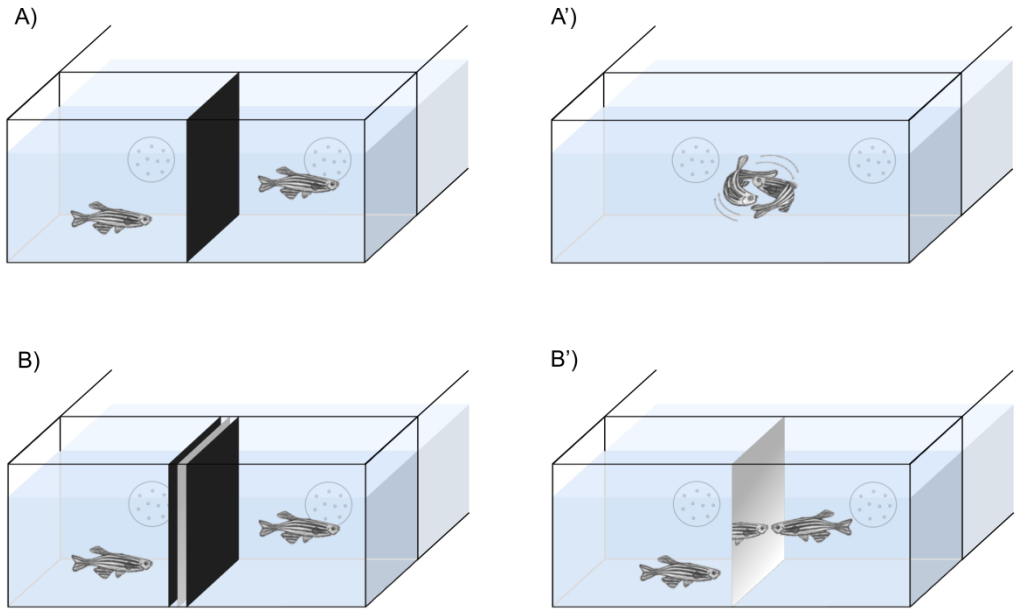

Figure 3- Observation tanks are divided into a posterior part, which contains a mechanical filter and a heater, and an anterior part where the test takes place (the arena). Perforated plastic circles along the glass dividing the two compartments, allow water exchange between the arena and the filter compartments. The arena is divided into 2 same-size parts by an opaque PVC partition; depending on the test (real-opponent or

mirror fight), this partition can be removed or not. A) For real-opponent fights, animals are separated by a removable opaque PVC partition. A') The opaque divider is removed, and the fish are allowed to interact for 30 min. B) For mirror elicited fights, the arena is divided by a PVC partition containing one mirror on each side, and a second removable partition is placed in front of each mirror to cover it. B') The two outer partitions are removed and the fish are allowed to interact with their own mirror image throughout the test period (30 min).

3.3.2. Mirror elicited fights

a) Repeat steps a and b from the staged fights protocol in 3.3.1.

b) Gently remove the two opaque partitions that are covering the mirrors, and allow the two fish to interact with each mirror simultaneously. (**See note 14, Figure 3 B'**)

c) After the 30 min period, place the two opaque partitions back in place, in order to end the interaction of each fish with its own mirror image.

3.3.3. Quantitative behavioural analysis

1) Analyse the video recordings using a computerized multi-event recorder (Observer XT, Noldus, *Wageningen, The Netherlands*).

2) Use the ethogram of zebrafish agonistic behaviour to identify the relevant action patterns (15), which are divided into aggressive for dominants (bite, chase and strike), and submissive for subordinates (freeze and flee).

3) Identify the selected behaviours as states or events, and quantify the frequency or the duration of the respective behaviours (**See note 15**).

3.3.4. Typical results

For staged fights, a typical encounter starts with mutual displays (lateral displays, circling) characteristic of the pre-resolution phase. In the post-resolution phase when the dominant-subordinate status has already been established, chase and bites are the most frequent action patterns **(Figure 4, A)**.

When comparing staged fights with mirror-elicited fights several differences can be observed **(Figure 4, B-D)**:

(1) The latency for the first attack (i.e. bite) is significantly lower in mirror fights when compared to staged fights, which may be a result of mirror "opponents" providing ambiguous information leading mirror fighters to escalate their aggressive behavior faster than individuals fighting a real opponent (**See note 16**).

(2) The opposite pattern is observed for the fight resolution time, with staged fights being solved more rapidly (in approximately 7 minutes) than mirror fights (usually still ongoing at the end of the 30 min observation period). This may result from the fact that during the pre-resolution phase, fish mutually assess their relative fighting ability and adjust their behavior accordingly.

(3) Since there is no fight resolution in mirror fights, mirror fighters do not either win or loose the fight; therefore, they do not adopt the respective dominant or subordinate phenotype, observed in real opponent fights, despite the expression of significant amounts of aggressive behaviour.

(4) Indeed, there are no significant differences in the levels of overt aggression between mirror fighters and dominants of real opponent fights. Thus, one can conclude that a major difference between the two protocols is not so much in the behaviour expressed by the focal fish, but rather in the behaviour expressed by the opponent.

As a final recommendation we suggest that researchers intending to use the mirror test to phenotype aggression should first validate it by comparing individual responses between real opponent and mirror tests. This has been done recently for a set different cichlid species and the results appear to be species specific, since in some species (i.e. *Neolamprologus pulcher* and *Astotilapia burtoni*) the results of the two tests are correlated (18, 28), whereas for other species (i.e. *Telmatochromis vittatus*, *Lepidiolamprologus elongates* and *Amatitlania nigrofasciata*) no relationship was found between mirror and real opponent aggression (28).

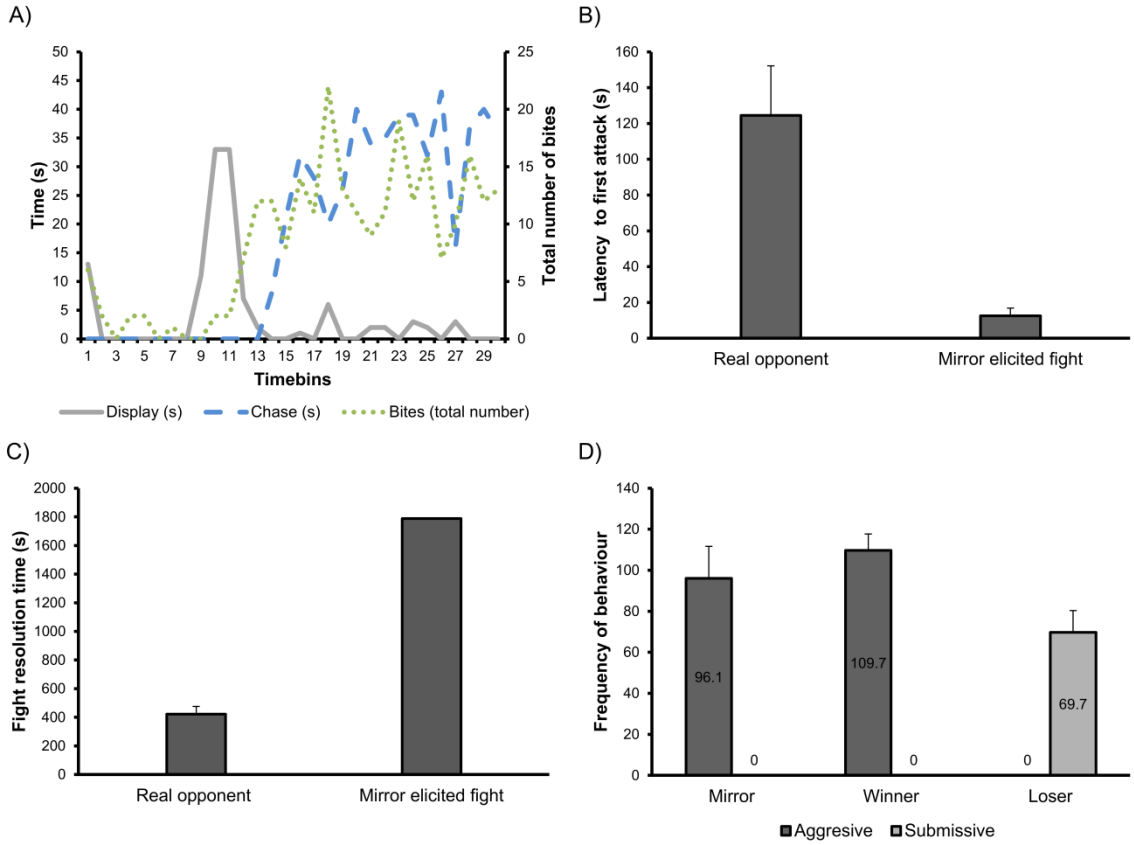

Figure 4 – Typical results for the two protocols used to quantify aggressive behaviour. A) Temporal dynamics of a real opponent fight analysed in 1 minute time bins for the 30 min interaction (unpublished data). The full line represents the time in display, a typical behaviour of the pre-resolution phase, and the dashed and dotted lines represent the time in chase and number of bites, respectively, behaviours typically expressed in the post-resolution phase. B) Mean latencies to the first attack in real opponent and in mirror elicited fights (unpublished data). C) Fight resolution time, measured as the time

needed for a social hierarchy to be established, in real opponent and in mirror elicited fights (unpublished data). D) Mean number of aggressive acts performed in the last 5 min of the 30 min interaction test for winners and losers of real opponent fights and for mirror fighters; Error bars represent the standard error of the mean [Reproduced with permission from [Teles el al, 2013].

4. Notes

1- Buffered tricaine methane sulfonate (stock solution): 4000 mg/L tricaine methane sulfonate (MS222), buffered with tris-base 1M, pH=9 to a final pH=7 solution.

2- The bedding can be a small petri dish filled with aquarium graded silicone, with a small depression in the middle to hold the fish in a dorso-ventral position.

3- One should keep in mind that aggressive behavior might differ between different wild-type strains, as it has been described for other behaviours (29).

4- The choice of the tagging method depends on the experimental procedure to be used. For example, fin clips are normally used for short-term experiments since fin regeneration occurs rapidly, whereas colour tagging is more appropriate for long term experiments, despite being a more intrusive technique. Finally, visible implant elastomers are more suitable for experiments that do not require video analysis because visible implant elastomer tags may be difficult to distinguish in video-images (e.g. yellow vs. orange or pink vs. red can be easily confused). Furthermore, colour identification may depend on ambient light which becomes a constraint when video recordings are used (23).

5- With this dose of anesthetic a deeper anesthesia will be induced, which promotes a total loss of equilibrium and muscle tone and a very slow ventilation rate (almost absent)(30). This will occur very fast. As soon as these signs are present remove the fish from the anesthetic solution.

6- Beforehand prepare a sheet with the colour combinations that you intend to use to tag the fish, to avoid repetitions of colour codes.

7- Leave some clearance between the knots and the fish body to avoid skin infections and interference with body growth.

8- After tagging the animals, there must be a quarantine period before starting the behavioural tests. For fin clips one should wait at least 24 hours, and for colour tagging 10 days to guarantee wound healing. Animals should be monitored during this period for tag loss and health status.

9- The perforated plastic circles along the glass dividing these two parts of the tank, allow water exchange between the two compartments (Figure 3).

10- Since body size is highly correlated with dominance, size differences (length or weight) between opponents should not exceed 10% of total body size, in order to avoid an a priori advantage of the larger individual. Take the opportunity of having fish anesthesized for the tagging procedures to take body measurements (weight, standard length) of all individuals.

11- Previous studies had established different periods of social isolation of 5 days (14, 31) and 24h (15) as effective to elicit aggressive behavior in zebrafish. However, overnight isolation proved sufficient to induce consistent expression of aggressive behaviour for the duration of the tests (30 min) (19).

12- The camera we used had a resolution of 720x576 and frame rate of 25 frames per second; however, higher resolution cameras with higher frame rates are also appropriated

13- In order to minimize the interaction between the observer and the focal fish, the partitions can be pulled up from a distance with the help of pulleys.

14- Subjects were also tested in pairs in the mirror elicited test, in order to provide them with conspecific odours, which would otherwise only be present in real opponent dyads, therefore avoiding confounding effects of putative chemical cues used in agonistic interactions.

15- For behaviour quantification it is important to distinguish between two fundamental types of action patterns, based on the time expression, because this will influence the type of measures that one should take: (1) events, are action patterns that are discrete in time (i.e. have very short duration) such that it is difficult to establish their start and finish time (e.g. bites, strikes); the relevant measure of events is their frequency (number of occurrences per unit of time); (2) states, are action patterns that have a significant time duration which allows to easily define their start and their end (e.g. display, chase, freeze and flee); states can be quantified both in terms of their frequency and their duration (e.g. percentage of time displaying). Latency, defined as the time from some specified time point (e.g. start of the test) to the first occurrence of the relevant action pattern, can also be measured, both for events and for states. Latency to initiate a fight is usually interpreted as a measure of aggressive motivation, whereas frequency and duration of events and states, respectively, reflect the engagement in the interaction. Since the engagement in the fight depends not only on the

motivation of the focal fish but also on the response of the opponent, measures of latency are expected to better measure the intrinsic aggressive motivation of individuals. In our protocols we typically analyse the latency to the first interaction and the frequency and duration of aggressive and submissive behaviours.

16- When laterally displaying to each other, as a way of assessing each others competitive ability (32), fish can align either in a parallel (head to head) or anti-parallel (head to tail) position (33). However, since there is a left-eye bias in zebrafish for social stimuli, they prefer to display the left side of the body, making the head to tail alignment, which is not present in mirror interactions, more common during mutual displays (20). Thus, mirror fights also change the structure of the fight making mirror fighters escalate faster than real-opponent fighters.

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