Neural Mechanisms of Social Behaviour and Social Information Use in Guppies (*Poecilia reticulata*)

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Abstract

Living in groups has many advantages for individuals, such as access to social information or protection against predators. The function and evolution of sociality and social information use have received abundant attention in the past years. However, the neural mechanisms underlying these processes have not been as widely examined across taxa, which is crucial to understand their evolution in vertebrates. In this thesis, I explored the neuroendocrinal mechanisms of grouping and the neural mechanisms of social information use in the Trinidadian guppy (*Poecilia reticulata*), a species with extremely well-studied evolutionary ecology. In Chapter 2, I compared immediate early-gene (IEG) expression across brain areas of the social decision-making network when guppies were exposed to a large or small group of conspecifics, or isolated. I found higher IEG expression in the preoptic area when guppies were exposed to the large group, compared to control (isolated guppies). The preoptic area regulates social behaviour in vertebrates and is also the only region in the teleost brain producing the nonapeptides isotocin and vasotocin. Since nonapeptide homologues modulate grouping behaviour in birds and are neuromodulators of mammalian social behaviour, I hypothesized vasotocin and isotocin would have effects on grouping in fish. Thus, in Chapter 3, I developed a technique to centrally administer nonapeptides in the brain of guppies and investigated their effects on grouping. I found opposing effects of the two nonapeptides, with isotocin increasing and vasotocin reducing grouping, at 90 minutes after administration, consistent with these neuromodulators playing a role in a fundamental social behaviour in fish. An advantage of grouping behaviour is that it may facilitate the transmission of information between conspecifics. Thus, in Chapter 4, I explored the neural mechanisms of social information use by studying the brain areas activated when 'demonstrator' guppies were exposed directly to alarm substance (i.e., a reliable social cue released from damaged skin) and when they observed a conspecific reacting to alarm substance. I found that alarm substance provoked typical antipredator behaviour such as freezing and area avoidance, while visual exposure to alarmed conspecifics induced a preference to use the same area as the alarmed conspecifics. I examined patterns of IEG expression across six areas of the brain, finding that the

demonstrators had higher overall expression of *egr-1* compared to observers and control fish, and different patterns of correlated expression in demonstrators and observers, suggesting that coordinated activation across regions is involved in processing and modulating responses to social alarm cues in guppies. In summary, my thesis 1) establishes a basis for the study of the neural mechanisms of grouping and social information use in teleosts by highlighting the brain areas activated during these processes, 2) provides evidence of the conserved effects of nonapeptides on grouping in a vertebrate lineage different than birds, and 3) provides novel approaches for studies of behavioural neuroscience in small prey fish amenable to experimental studies of evolution and development.

Résumé

Vivre en groupe présente de nombreux avantages pour les individus, tels que l'accès à l'information sociale ou la protection contre les prédateurs. La fonction et l'évolution de la socialité et de l'utilisation de l'information sociale ont reçu une attention considérable au cours des dernières années. Cependant, les mécanismes neuronaux sous-jacents à ces processus n'ont pas été aussi largement examinés à travers les taxons, ce qui est crucial pour comprendre leur évolution dans les vertébrés. Dans cette thèse, j'ai exploré les mécanismes neuro-endocriniens du groupement et les mécanismes neuraux de l'utilisation de l'information sociale du guppy Trinidadien (*Poecilia reticulata*), une espèce avec une écologie évolutive extrêmement bien étudiée. Dans le chapitre 2, j'ai comparé l'expression du gène précoce immédiate (GPI) dans les zones du cerveau du réseau de prise de décision sociale lorsque les guppys étaient exposés à un grand ou un petit groupe de congénères, ou isolés. J'ai trouvé une expression du GPI plus élevée dans l'aire pré-visuelle lorsque les guppys ont été exposés au grand groupe, par rapport au contrôle (guppys isolés). L'aire pré-visuelle régule le comportement social des vertébrés et est également la seule région du cerveau téléostéen produisant les nonapeptides isotocine et vasotocine. Puisque les homologues nonapeptides modulent le comportement de groupement des oiseaux et sont des neuromodulateurs du comportement social des mammifères, j'ai émis l'hypothèse que la vasotocine et l'isotocine affecteraient le groupement des poissons. Ainsi, au chapitre 3, j'ai développé une technique pour administrer de façon centrale des nonapeptides dans le cerveau des guppys afin étudier leurs effets sur le groupement. J'ai trouvé des effets opposés des deux nonapeptides. Notamment, 90 minutes après l'administration, l'isotocine a augmenté le comportement de groupement tandis que la vasotocine l'a réduit, indiquant que ces neuromodulateurs jouent un rôle dans un comportement social fondamental des poissons. Un avantage du comportement de groupement est qu'il peut faciliter la transmission de l'information entre les congénères. Ainsi, au chapitre 4, j'ai exploré les mécanismes neuraux de l'information sociale en étudiant les zones cérébrales activées quand les guppys «démonstrateurs» étaient exposés directement à la substance d'alarme (c.-à-d., un signal social fiable libéré de la peau endommagée) et quand ils ont

observé une réaction conspécifique à la substance d'alarme. J'ai trouvé que la substance d'alarme provoquait un comportement antiprédateur typique tel que l'immobilisation et l'évitement de zone, tandis que l'exposition visuelle aux congénères alarmés induisait une préférence d'utiliser la même zone que les congénères alarmés. J'ai examiné les modes d'expression des GPI dans six régions du cerveau. Les démonstrateurs avaient une expression globale plus élevée de *egr-1* que les observateurs et les poissons témoins, et il y avait différents modèles d'expression corrélée chez les démonstrateurs et les observateurs, suggérant que l'activation coordonnée entre les régions est impliquée dans le traitement et la modulation des réponses aux signaux d'alarme sociale chez les guppys. En résumé, ma thèse 1) établit une base d'étude des mécanismes neuronaux de groupement et d'information sociale dans les téléostéens en mettant en évidence les zones cérébrales activées au cours de ces processus, 2) fournit des preuves des effets conservés des nonapeptides sur le groupement dans une lignée de vertébrés différente des oiseaux, et 3) fournit de nouvelles approches pour les études en neuroscience comportementale de petits poissons proies dans le contexte d'études expérimentales de l'évolution et du développement.

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Contributions of authors

Chapter 1 (General introduction): María J. Cabrera-Álvarez wrote the chapter with input from Simon M. Reader.

Chapter 2 (Forebrain activation during social exposure in wild-type guppies): María J. Cabrera-Álvarez, William T. Swaney and Simon M. Reader designed the study, with input from supervisory committee members Jon Sakata and Rüdiger Krahe. William T. Swaney and María J. Cabrera-Álvarez developed the immunohistochemistry protocol used in this study. María J. Cabrera-Álvarez collected and analyzed the behavioural and neural data, and wrote the manuscript with input from Simon M. Reader and William T. Swaney and anonymous reviewers from *Physiology & Behavior*. This chapter is published: Cabrera-Álvarez, M.J., Swaney, W.T. and Reader, S.M. (2017) Forebrain activation during social exposure in wild-type guppies. *Physiology & Behavior* 182, 107–113.

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Chapter 5 (General discussion): María J. Cabrera-Álvarez wrote the manuscript with input from Simon M. Reader.

Contribution to original knowledge

This thesis provides foundational work to understand the neural mechanisms underlying grouping behaviour and social information use in fish. Specifically, Chapter 2 shows that the POA is particularly important in the regulation of grouping behaviour in guppies, since there is increased expression of an immediate early gene in this region when fish are exposed to a large group of conspecifics. This finding adds knowledge to the current understanding of the neural mechanisms of grouping behaviour, mostly studied in birds. For Chapter 3, I implemented a technique to centrally administer nonapeptides in guppies and found opposing effects of isotocin and vasotocin on grouping behaviour, a result that supports a conserved role of nonapeptides on social behaviour in vertebrates. Chapter 4 is the first empirical test that studies the neural mechanisms underpinning social contagion of alarm behaviour in guppies, exploring the brain areas expressing an immediate early gene both during alarm substance exposure and during social contagion of alarm behaviour. In Chapter 5, apart from considering my results together and with previous work and their implications for a further understanding of the processes under study, I also provide several suggestions for future research.

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Chapter 1: General introduction

Living in groups has many benefits, such as increased foraging success and predator avoidance, reduced costs of movement, increased conservation of water and heat, and increased opportunities to find a mate (Krause and Ruxton 2002). Another advantage of group living is that individuals can rapidly obtain information about the environment by observing others and, consequently, make informed decisions that can improve their fitness (Seppänen *et al.* 2007). The study of group living and animal social information use has attracted the attention of many researchers in the past decades (Krause and Ruxton 2002; Seppänen *et al.* 2007; Morand-Ferron *et al.* 2010; Rieucau and Giraldeau 2011; Ward and Webster 2016). This is because understanding their function and evolution is of pivotal importance to understand complex concepts such as the evolution of culture and the emergence of cognitive traits. However, considerably less research has been carried out on the neural mechanisms underpinning sociality and animal information use. We will not have a complete understanding of these topics until we address them with an integrative approach combining Tinbergen's four questions (i.e., studying the mechanisms, development, function, and evolution; Bateson and Laland 2013; Dawkins 1989; Tinbergen 1963). To achieve this goal, and since abundant research on the function and evolution of sociality and social information use has been carried out, it is sensible to explore the underlying neuroendocrinal mechanisms that govern them. I thus decided to explore these neuroendocrinal mechanisms in an important model species for evolutionary ecology, the Trinidadian guppy (*Poecilia reticulata*), in order to set the basis for future integrative studies on this matter. In this general introduction, I introduce the term social behaviour and provide theoretical background on the neuroendocrinal mechanisms underpinning social behaviour, with a focus on the social decision-making network (i.e., a neural network involved in a wide range of social behaviours) and the nonapeptide system (i.e., neuromodulators involved in social behaviours and whose structure and function are well conserved among vertebrates). I also explain why guppies are excellent candidates for my research and describe one of their characteristics enabling the study of social information use, the alarm substance response.

Social behaviour in animals

Social behaviour, as defined by Brown (1975), is 'all behaviour directly related to potential or actual encounters between individuals'. This broad definition includes any kind of interaction with other individuals, independently of its characteristics and mechanisms. Thus, different categories of social behaviour such as aggression and parental care are classified under the term social behaviour even though they do not share the same neural mechanisms (Nelson and Trainor 2007; Kohl and Dulac 2018). Given this, drawing general conclusions about the mechanisms of social behaviour becomes difficult.

Another difficulty arises when we try to define when an animal is social. In general, animals that form groups are considered to be social. However, animals can form social groups at varying levels, from unstructured herds of mammals to sophisticated colonies of eusocial insects (i.e., species with reproductive division of labour, overlapping adult generations, and cooperative care of young). Thus, to differentiate eusocial species from other less structured groups, the term 'gregarious' has been proposed for less structured groups (Ward and Webster 2016). For the purpose of this thesis, I will use the definition suggested by Ward and Webster (2016) of a social group as 'one where two or more individuals maintain proximity in space and time through the mechanisms of social attraction'.

As previously mentioned, the obtained conclusions about the mechanisms of a specific social behaviour cannot just be extrapolated to other categories of social behaviour. Thus, to understand the underlying mechanisms of social behaviour, one can make more progress by studying a characteristic that is common to many species, such as gregariousness (henceforth, grouping behaviour). Grouping behaviour has been the focus of abundant research in the past decades. The components and characteristics of grouping behaviour, such as social recognition and social organization, as well as the

costs and benefits of living in groups, have received considerable attention (reviewed by Krause and Ruxton, 2002; Ward and Webster, 2016). However, the neuroendocrinal mechanisms underlying grouping behaviour in vertebrates, which would bring light into the internal processes regulating a widespread social behaviour, and the extent to which these are shared across taxa, have received relatively little attention (with the exception of birds, on which I elaborate below). Hence, to have a better understanding of the mechanisms of grouping behaviour in vertebrates, we need to gain knowledge on further species of vertebrates. Exploring these mechanisms in a social species of teleostean fish, the most specious group of vertebrates, will provide us with a greater scope to infer general patterns of these mechanism in vertebrates, and facilitate future comparative studies that would help us improve our understanding of the evolution of social behaviour. The following sections summarize the current knowledge on the neuroendocrinal mechanisms underlying social behaviour and introduce the study system I chose for my thesis.

The Social-Decision Making Network

The social decision making network (SDMN) is a conserved brain network involved in the modulation of a wide range of social behaviours in all vertebrates. It involves several brain areas that modulate social behaviour (the social behaviour network; Newman, 1999) as well as brain areas of the mesolimbic reward system, involved in the estimation of the salience of stimuli, with some of the areas belonging to both networks (Figs. 1.1 and 1.2; O'Connell and Hofmann, 2011; see Chapter 2 for further details). Across diverse taxa, most research effort implicating the SDMN has been targeted at 'complex' social behaviours, such as sexual and aggressive behaviour, cooperation, or nest building, while there is a noticeable paucity of research on fundamental social behaviour, such as grouping. By studying the SDMN and elucidating the brain areas involved in fundamental social behaviour in teleosts we can establish a baseline of brain activation for grouping behaviour and, due to the conservation of this network

across taxa, we can infer analogies among other gregarious vertebrates that are more difficult

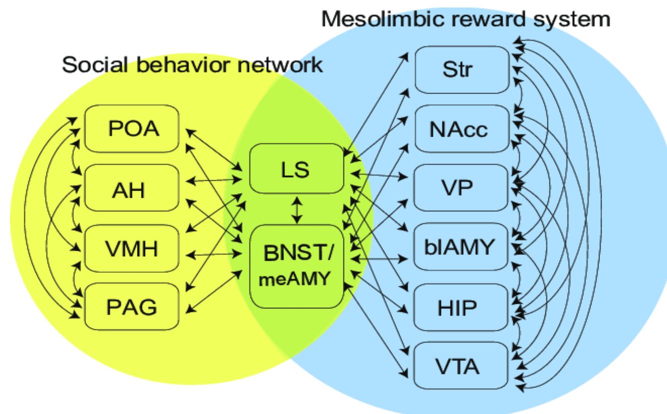

Figure 1.1. Brain areas of the social decision-making network in mammals, and their anatomical connections represented by arrows. AH, anterior hypothalamus; blAMY, basolateral amygdala; BNST/meAMY, bed nucleus of the stria terminalis/medial amygdala; HIP, hippocampus; LS, lateral septum; NAcc, nucleus accumbens; PAG/CG, periaqueductal gray/central gray; POA, preoptic area; Str, striatum; VMH, ventromedial hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area. From O'Connell and Hofmann, 2011. Reprinted with permission from John Wiley and Sons.

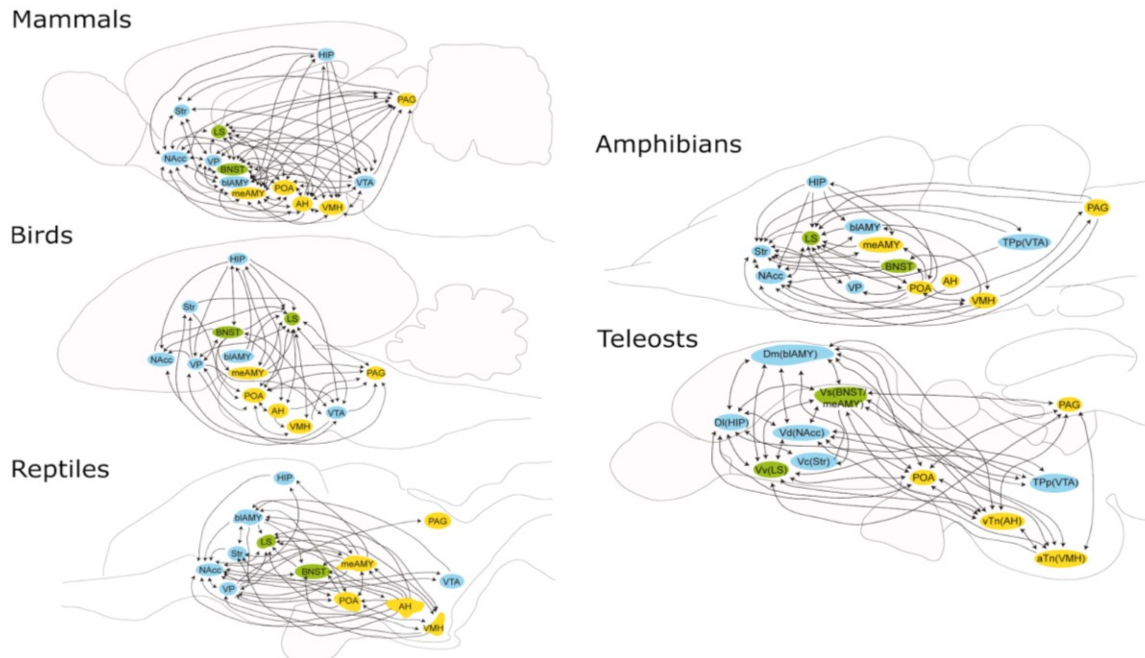

Figure 1.2. Sagittal view of the social decision-making network in each major vertebrate lineage, showing evidence for homologies by hodology (i.e., the study of connections between brain areas). From O'Connell and Hofmann, 2011. Reprinted with permission from John Wiley and Sons.

to study in a controlled environment than fish. We can also elucidate the areas involved in social information use and provide the preliminary information needed to investigate the neural mechanisms involved in more complex behaviours such as social learning. Also, by identifying the brain areas involved in grouping behaviour we open the door to future studies exploring in greater depth the characteristics of these brain regions in teleost fish and their involvement on the different categories of social behaviour.

Nonapeptides

The modulation of social behaviour is a complex neuroendocrinal process regulated by several neuropeptides (such as corticotropin-releasing factor, thyrotropin-releasing hormone, α-melanocyte-stimulating hormone, substance P, or neuropeptide Y; Crowley *et al*. 1989; Niesink and van Ree 1984; Yamada *et al*. 2000) and neurotransmitters (such as acetylcholine, GABA, serotonin, and catecholamines such as dopamine and norepinephrine; Crowley *et al*. 1989; Matthews *et al*. 2016; Watson *et al*. 2009). I decided to specifically study the role of nonapeptides in the modulation of social behaviour because their effects have been shown to be conserved among vertebrates, and in particular because of their proposed role in the modulation of grouping behaviour in birds, as I develop further below. Here, I review studies showing that the study of nonapeptides is essential for understanding the neuroendocrinal mechanisms underpinning widespread social behaviours such as grouping.

Nonapeptides are nine-amino acid peptides present in many taxa, from annelid worms to primates, and their function and structure are very well conserved. In vertebrates, nonapeptides derive from arginine vasotocin which, after an early gene duplication event, gave rise to the two evolutionary lineages of nonapeptides: the vasopressin-like nonapeptides and the oxytocin-like nonapeptides (Donaldson and Young 2008; Goodson and Kingsbury 2011). Vasopressin and oxytocin are the nonapeptide forms

present in mammals. Birds, reptiles and amphibians have vasotocin and mesotocin; and bony fish have vasotocin and isotocin (Fig. 1.3).

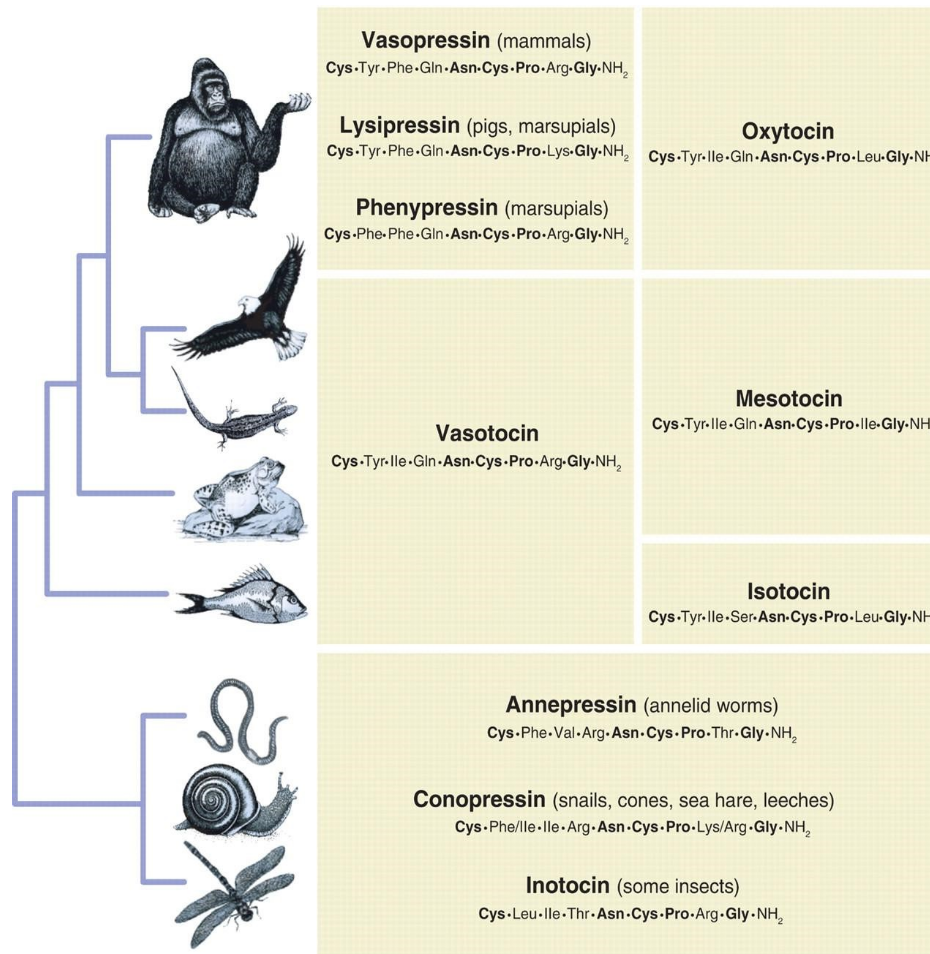

Figure 1.3. Oxytocin and vasopressin homologs, in invertebrates and vertebrates. From Donaldson and Young, 2008. Reprinted with permission from AAAS.

Nonapetides are produced in the brain. In mammals, they are predominantly synthesized in hypothalamic neurons, specifically in gigantocellular, magnocellular, and parvocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus. Small quantities of oxytocin are also produced in the bed nucleus of stria terminalis (BNST), the medial preoptic area, and the amygdala. Vasopressin-producing neurons have been found also in the preoptic area, anterior hypothalamus, BNST, medial amygdala, lateral septum, lateral habenular nucleus, and the suprachiasmatic nucleus (reviewed in Choleris, Pfaff, and Kavaliers 2013). In birds, nonapeptides are also

synthesized in the hypothalamus. Specifically, in magnocellular neurons of the supraoptic nuclei and in magnocellular and parvocellular neurons of the paraventricular nuclei. Vasotocin is also produced in the medial BNST. In teleost fish, however, nonapeptides are almost exclusively produced in the preoptic area of the hypothalamus (POA), although the anterior tuberal hypothalamus has been found to have a vasotocin cell population in several teleost species (reviewed in Choleris, Pfaff, and Kavaliers 2013), and several brain regions may be involved in vasotocin synthesis since a widespread distribution of its preprohormone has been found recently (Rodriguez-Santiago *et al*. 2017). In mammals and birds, nonapeptides are stored in large dense core vesicles distributed through the soma, dendrites and axons of nonapeptide-producing neurons, and are released centrally to different brain areas and to the third ventricle, and peripherally through the posterior pituitary (reviewed in Johnson and Young 2017). A singularity of the teleost nonapeptide system is that individual vasotocin and isotocin neurons project to both posterior pituitary and a variety of other brain regions such as the telencephalon, ventral thalamus, and various mesencephalic areas (Saito *et al.* 2004). Similar innervation of multiple targets by single vasopressin and oxytocin neurons have not been found in mammals (Urano and Ando 2011).

In mammals, there are three types of vasopressin receptors (V1a, V1b and V2) and a single type of oxytocin receptor. V2 receptors are present in the kidney (Lolait *et al.* 1992) and has not been found in the central nervous system, while V1a receptors are widely distributed through the brain (Young *et al.* 2006) and are also present in the vascular system and liver (Morel *et al.* 1992). V1b receptors are extensively expressed in the pituitary gland, and are also present in the brain (although their distribution is restricted to the hippocampus and a few cells in anterior amygdala (Young *et al.* 2006)) and in peripheral tissues such as kidney, thymus, heart, lung, spleen, uterus, and mammary glands (Sugimoto *et al.* 1994; Lolait *et al.* 1995). The oxytocin receptor is widely distributed through the brain and also present in peripheral tissues such as liver, fat cells, the adrenal and mammary glands, uterus, ovaries, and testis (Tribollet *et al.* 1992).

Teleost fish, on the other hand, have four types of vasotocin receptors (V1a1, V1a2, V2a, and V2b) and a single isotocin receptor (Konno *et al.* 2010; Lema 2010), however, their distribution differs from that of the mammalian receptors. The two types of V2 receptors have been only recently discovered (Ocampo Daza *et al.* 2012) and so the distribution of each receptor type in teleosts is still not clear. Although V2 receptors can be found in the teleostean brain, studies prior to the discovery of the two V2 receptor types located them mostly peripherally, in organs such as gills, heart, liver, kidney, testis and ovaries (Konno *et al.* 2010; Lema 2010). The V1a receptors are widely expressed in the brain, including areas implicated in social regulation, with the V1a1 receptor being very abundant in the brain, and also present in pituitary gland and testis (Lema 2010), and the V1a2 receptor in brain, heart and muscle (Lema 2010). The isotocin receptor is widely expressed in the brain, as well as present in pituitary and gonads (Lema 2010; Huffman *et al.* 2012). Mammals, birds, amphibians, and teleosts express nonapeptide receptors in all the brain areas of the social decision-making network, with the exception of the ventral pallium of birds that does not express the mesotocin receptor, and the striatum of most mammals that does not express the V1a receptor (reviewed in Huffman *et al*. 2012). The distribution of nonapeptide receptors in the social decision-making network highlights the likely importance of nonapeptides in the regulation of social behaviour and the evaluation of the salience of social stimuli.

The effect of nonapeptides on vertebrate social behaviour has been widely studied in mammals, specifically in the study of the evolution of mating systems, as well as the role of oxytocin in maternal behaviour (reviewed in Choleris *et al*. 2013). However, the involvement of nonapeptides in grouping behaviour has not been studied in mammals. In birds, the studies of Goodson and his colleagues on the evolution of social group size elucidated the roles of nonapeptides in fundamental forms of social behaviour such as grouping. First, they found evidence of differences in septal function between territorial and gregarious species after finding that vasotocin septal infusions increased aggression in a gregarious bird (zebra finches, *Taeniopygia guttata;* Goodson and

Adkins-Regan 1999) and reduced aggression to intruders in a territorial bird (field sparrows, *Spizella pusilla*; Goodson 1998). Goodson and colleagues then engaged in a series of studies exploring the role of nonapeptides in the evolution of gregariousness and the brain areas involved. They studied related species of birds with similar behaviour and ecology (mating system, parental care, food, habitat, etc.) but that differed in the number of individuals in their natural social environment. Thus, five estrildid finch species were included in their analyses, two of them territorial, two highly gregarious, and one moderately gregarious. The vasotocin circuitry of the medial BNST and lateral septal area was found to differ between gregarious and territorial finches. Specifically, vasotocin neurons in the medial BNST and vasotocin receptors in the lateral septal area were more abundant in gregarious finch species than in the territorial ones (Goodson and Wang 2006; Goodson *et al.* 2006). Goodson and colleagues also found that in gregarious species, an increased number of vasotocin neurons of the medial BNST were activated when the subjects were in the presence of same-sex individuals, while in territorial species, the number of active neurons decreased (Goodson and Wang 2006). These findings suggested that these neurons respond to the valence of the social stimulus, increasing in activation when the valence is positive, as is a social encounter for a gregarious individual, and decreasing when the valence is negative, as is a same-sex social encounter for a territorial individual. They confirmed that vasotocin is involved in the modulation of gregariousness in birds, first by blocking the vasotocin receptors in the lateral septum through infusion of vasotocin receptor antagonists, and then by blocking the production of vasotocin in the medial BNST by infusing vasotocin antisense oligonucleotides in this area (Kelly *et al.* 2011). They found that in both cases the preference for a large flock of same-sex conspecifics was reduced. All these studies strongly supported the role of vasotocin in the modulation of gregariousness in birds and the brain areas involved in vasotocin release and binding sites, BNST and lateral septum, respectively.

Goodson and colleagues also studied the effects of mesotocin (the oxytocin homologue in birds) on gregariousness. After a series of studies, they concluded that the

distribution of oxytocin-like binding sites in the lateral septum of the five studied finch species differentiates gregarious and territorial species in a clear manner (Goodson *et al.* 2009). They confirmed the involvement of the lateral septum by infusing an oxytocin antagonist in the lateral septum of zebra finches. A clear reduction of gregariousness was found, a result that was not found when the same antagonist was infused in other brain areas (Goodson *et al.* 2009). Thus, oxytocin receptors in the lateral septum are involved in modulating grouping responses in zebra finches.

In fish, relatively few studies on the role of nonapeptides on grouping and related behaviour patterns have been carried out (Table 1.1). For example, Lindeyer et al. (2015) found that vasotocin and a vasopressin receptor antagonist decreased social interactions with a shoal (i.e., swimming head first against a partition that separated them from a group of fish) in zebrafish (Danio rerio) after peripheral administration, while isotocin and an oxytocin receptor antagonist had no effect on shoaling (time swimming close to a group of fish) or social interaction. On the other hand, in the African cichlid Neolamprologus pulcher, isotocin inhibited grouping behaviour when administered peripherally, while an oxytocin receptor antagonist stimulated grouping behaviour in males (Reddon et al. 2014). N. pulcher also has higher brain gene expression of isotocin than a non-social cichlid species (Telmatochromis temporalis), and this brain gene expression of isotocin is positively correlated with social behaviours such as affiliation and submission in N. pulcher, but not in the non-social species T. temporalis (O'Connor et al. 2016). This result contrasts with a negative correlation between circulating isotocin in the brain and affiliative behaviour in N. pulcher (Reddon et al. 2015). To explain this discrepancy, O'Connor et al. (2016) suggest that more social N. pulcher not only produce higher levels of isotocin but also use higher quantities compared to less social N. pulcher. When exploring the brain gene expression of vasotocin, they found no difference between the social and non-social species, but a sex difference, with males having higher whole brain gene expression of vasotocin than females (O'Connor et al. 2016). This sexual dimorphism is in agreement

Table 1.1. Summary of nonapeptide research on grouping and related behaviour patterns in teleost fish. Note that several of these studies also addressed additional parameters, such as social status. NS: no significant effect found. NT: not tested.

Species	Type of study	Isotocin effect	Vasotocin effect	Oxytocin receptor antagonist effect	Vasopressin receptor antagonist effect	Reference
Goldfish (*Carassius auratus*)	Central administration	Increased social approach	Inhibited social approach	NT	Increased social approach	Thompson and Walton 2004
Zebrafish (*Danio rerio*)	Peripheral administration	NS effect	Decreased social interactions with a shoal	NS effect	Decreased social interactions with a shoal	Lindeyer *et al.* 2015
Feral guppy (*Poecilia reticulata*)	Peripheral administration	NS effect	Decreased shoaling	NS effect	Decreased shoaling	Swaney *et al.*, unpublished data
Guppy (*P. reticulata*)	Count of nonapeptide neurons	NT	Domestic females had fewer vasotocin neurons than the higher-grouping female feral guppies	NT	NT	Hewlett 2010
Neolamprologus pulcher	Peripheral administration	Decreased grouping behaviour	NT	Increased grouping behaviour	NT	Reddon *et al.* 2014
N. pulcher	Whole brain gene expression during pair bonding	Isotocin gene expression was positively correlated with proximity score and affiliation rate. NS effect with isotocin receptor 1 or 2 expression.	NS effect with vasotocin or V1a2 receptor.	NT	NT	O'Connor *et al.* 2016
Telmatochromis temporalis	Whole brain gene expression during pair bonding	NS effect with isotocin or isotocin receptor 1 or 2 expression.	NS effect with vasotocin or V1a2 receptor.	NT	NT	O'Connor *et al.* 2016
N. pulcher vs. *T. temporalis*	Whole brain gene expression during pair bonding	Isotocin expression higher in the social *N. pulcher* vs. the non-grouping *T. temporalis.* NS effect on isotocin receptor 1 or 2 expression.	NS effect on vasotocin or V1a2 receptor.	NT	NT	O'Connor *et al.* 2016
N. pulcher	Assay of circulating nonapeptides in the brain	Isotocin levels were negatively correlated with affiliative behaviour rate	Vasotocin levels were higher in subordinate than dominant fish	NT	NT	Reddon *et al.* 2015

with previous findings in (Jurkevich et al. 1997) and mammals (Veenema and Neumann 2008). In guppies, it was found that domestic females had fewer vasotocin neurons than females of a feral (higher grouping) strain of guppies (Hewlett 2010). Particularly relevant to my thesis is a study by Thompson and Walton (2004), given that it was the only study to date that administered nonapeptides centrally to study their effects on social behaviour in fish, particularly in goldfish (Carassius auratus). They found opposing effects of the two nonapeptides, with vasotocin inhibiting social approach and isotocin stimulating it. Inspired by these studies in fish and Goodson's studies in birds, I aimed to study the effects of nonapeptides in grouping behaviour in fish.

Study System

Studying the neural mechanisms of social behaviour is of paramount importance for understanding the basis of vertebrate social interactions, and for this purpose, it is essential to experiment with animal systems in a controlled environment. More specifically, the study system needs to have the following particular characteristics. First, it must be a social species with a wide repertoire of social behaviours that allows testing under a variety of experimental conditions. Furthermore, it should be possible for several individuals to interact with each other in the space available in a laboratory. Also, the system must be easy to handle, breed and maintain in laboratory conditions. Finally, studying a system whose behavioural repertoire, ecology, and evolutionary history are well known would have a bigger impact in the scientific community, since a wider range of additional research could be carried out in the same system and hence, a broader range of questions can be answered. In the next paragraph, I explain why I study bony fishes (teleosts), and the characteristics that make Trinidadian guppies the most suitable study system to answer my questions about the neural mechanisms of social behaviour.

Teleosts are the most specious group of vertebrates (Nelson *et al.* 2016) with approximately 30,500 species, comprising almost half of all species of vertebrates (Ravi and Venkatesh 2018). Of these, it is estimated that over 50% live in social groups as juveniles, and more than 25% of adult teleosts shoal throughout their life (Shaw 1978). By living in groups, fish have the opportunity of rapidly obtaining information about the environment via the observation of fellow shoal members (Hoare and Krause 2003). Thus, their sociality and capability of information sharing make bony fishes excellent candidates to study social behaviour and social information use. In particular, I picked Trinidadian guppies as the study system for my studies for the characteristics described below.

Trinidadian guppies are small tropical fish (Fig. 1.4) that live in Trinidad and other areas of Central and South America, and have been introduced in most parts of the world to control mosquitos or after release in the wild by aquarium owners. Guppies are social fish, and the individuals of most Trinidadian populations live their entire lives in groups. They have a wide repertoire of social behaviours, such as a variety of sexual and aggressive displays.

Figure 1.4. Laboratory reared guppies of wild Trinidadian origin. Note the considerable sexual dimorphism in body size and colouration between the larger, dull females and the smaller, orange-bodied males. Picture by Laura Chouinard-Thuly, with permission.

Trinidadian populations of guppies have been the focus of abundant research in animal behaviour, ecology, and evolution due to the differences among their natural populations, which have been shaped by environmental conditions and various levels of predation. Zebrafish, a model organism extensively studied by developmental biologist, geneticists, and neurobiologists, would be a possible alternative, but lacks extensive study of its evolution and ecology ((Spence et al. 2008), hindering the integrative approach I wished to follow.

In the wild, guppies generally live in temporary shoals that change in composition after fission and fusion events (Croft *et al.* 2003), and vary in social and intraspecific aggressive behaviour depending on their population of origin. For example, populations that experience high levels of predation form tighter shoals and are less aggressive than the populations with lower levels of predation (Magurran and Seghers 1991; Magurran 2005), and populations in rivers with prawn predation (driven mostly by odour), such as Paria river, form the loosest shoals and have the highest number of aggressive acts between conspecifics (Magurran and Seghers 1991). These differences in shoaling and aggression are maintained in the second generation of laboratory reared guppies, providing evidence for the heritability of these traits (Magurran and Seghers 1991; Huizinga *et al.* 2009). Thus, guppies are excellent candidates for the study of social behaviour in general, and grouping behaviour in particular, because their looser shoaling behaviour compared to other species of social fish that show stronger shoal cohesion (e.g. zebrafish, Lindeyer and Reader 2010; Lindeyer *et al.* 2015) allows us to prevent ceiling effects in our experimental setups, and the possibility of comparing populations that differ in their level of sociality, both in the wild and in laboratory conditions, opens a wide range of experimental opportunities for the future. However, before engaging in population comparisons, we need to establish the bases of the neural mechanisms of grouping behaviour, and for that I studied a laboratory population that derives from a mix of wild Trinidadian populations (from the Aripo, Marianne, Paria, and Quare rivers).

Social information use has also been studied in the guppy in a number of contexts, both in the wild (Reader *et al.* 2003) and laboratory (Laland and Williams 1997; Swaney *et al.* 2001; Brown and Laland 2002; Stanley *et al.* 2008). Another crucial characteristic that facilitates the study of the neural mechanisms of social information use is the behavioural response to so-called ‘alarm substance’, which I introduce in the next paragraph.

Alarm Substance

Animals use their senses to assess the presence of dangers in their surroundings. Many times, they rely on signals and cues provided by other individuals, such as the different alarm calls of Vervet monkeys (*Cercopithecus aethiops*) that alert group members to the presence of different predators (Seyfarth *et al.* 1980), or crested pigeons (*Ocyphaps lophotes)*, which recognise the sound of alarmed flight of conspecifics and take off in alarm after playback of this sound (Hingee and Magrath 2009). Similarly, many fish species react to the presence of ‘*Schreckstoff*’ or alarm substance, which is a substance created and stored in the club cells of the skin of some species of fish, and released into the water after mechanical damage of the fish’s skin, as would occur in a predation event (Pfeiffer 1967). Conspecific fish respond to the presence of alarm substance in different ways. For example, by freezing (i.e., reduced swimming), hiding, avoiding the area where alarm substance was released, swimming at the bottom or top of the water column, and increasing their vigilance for predators (Chivers and Smith 1998; Chivers *et al.* 2007). In guppies, responses to alarm substances have been confirmed both in laboratory conditions and in the wild (Brown and Godin 1999) and it has been found that guppies can differentiate between the alarm substance of different populations of conspecifics (Brown *et al.* 2010).

The reaction produced by alarm substance allows us to study not only fish behaviour, but also explore the functions of social cues, the mechanisms underlying social cues, as well as the social contagion of alarm, since it has been shown that the behavioural alarm response can be socially transmitted to conspecifics (Suboski *et al.* 1990), heterospecifics (Mathis *et al.* 1996), and even species that cannot detect alarm substance (Krause 1993). Being able to react by observing the reaction of other individuals is beneficial, since it allows a faster response towards the immediate presence of familiar or unfamiliar predators, and also stimulates the learning and recognition of unfamiliar predators. Hence, the use of alarm substance provides us with a great opportunity to investigate the neural mechanisms underlying social information use of alarm behaviour in ecologically relevant conditions.

Thesis Aims and Outline

This thesis aims to increase our understanding of the neuroendocrinal mechanisms underpinning grouping behaviour and social information use in vertebrates, with a focus on teleost fish. I first aimed to elucidate the areas of the brain that become active when guppies are exposed to a social stimulus, such as groups of conspecifics (Chapter 2). I found higher immediate early gene expression in a brain area known to be involved in social behaviour and to produce nonapeptides, i.e., neuromodulators involved in social behaviour. In a previous study, we explored the effect of nonapeptides on social behaviour when they were administered peripherally, finding no effect of isotocin and its putative receptor antagonist on shoaling, a significant reduction on shoaling when vasotocin was administered, and surprisingly, an even bigger reduction on shoaling when the vasopressin receptor antagonist was administered (W.T. Swaney, M.J. Cabrera-Álvarez, S.M. Reader; unpublished data). The probable explanation for these results is that the blood brain barrier largely blocks the entrance of nonapeptides to the central nervous system and so, the administered nonapeptides largely bind to peripheral receptors. This possibility makes it difficult to discern between whether the observed behaviour of the fish was due to the effects of nonapeptides in the brain or in

the periphery. To avoid this circumstance, I studied how the nonapeptides vasotocin and isotocin affect the social behaviour of guppies when they are administered directly into the brain (Chapter 3). Finally, I studied the brain areas activated during social information use (Chapter 4) by exposing guppies to a stressful event (exposure to alarm substance) while being observed by conspecifics. These three projects offer a broad overview of the mechanisms controlling fundamental social stimuli that many animals experience regularly through their lives. They also illustrate the value of guppies for the study of social behaviour and neuroscience.

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Chapter 2: Forebrain activation during social exposure in wild-type guppies

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Abstract

The neural mechanisms regulating social behaviour have received extensive attention in recent years, with much focus on 'complex' forms of sociality. Comparatively little research has addressed fundamental social behaviour, such as grouping, which impacts multiple determinants of fitness, such as foraging and avoiding predation. We are interested in the degree to which brain areas that regulate other forms of sociality are also involved in grouping behaviour, and so we investigated shoal-elicited activation of the brain in the guppy (*Poecilia reticulata*). Guppies are small, social fish that live in the rivers of Trinidad and, like many social fish, exhibit preferences for larger shoals. We first confirmed that our study population of wild-type guppies preferred to join a larger shoal, and then investigated the activation of four brain regions proposed to be involved in social behaviour and reward (the preoptic area, the dorsal part of the ventral

telencephalon, the ventral part of the ventral telencephalon, and the supracommissural part of the ventral pallium). Subjects were exposed to a large shoal, a small shoal, or to a tank empty of conspecifics, and we used immediate early gene expression (*egr-1*) to assess neuronal activation. We found increased activation in the preoptic area when fish were exposed to a large shoal compared to controls that had no social exposure. There were no significant differences in activation within the other brain areas examined, possibly because these brain areas are not key regulators of grouping behaviour or have only a secondary role. The higher activation of the preoptic area during social exposure suggests functional homology in this highly-conserved region across all vertebrates.

Introduction

The social decision making network (SDMN) is a network of brain nuclei that process social information and reward and which is thought to modulate social behaviour in all vertebrates (Newman 1999; O'Connell and Hofmann 2011). The SDMN consist of two overlapping brain networks: the social behaviour network (SBN), and the mesolimbic reward system. The SBN includes six interconnected nodes (the preoptic area, anterior and ventromedial hypothalamus, periaqueductal gray, lateral septum, and bed nucleus of the stria terminalis/medial amygdala) that are involved in sexual, aggressive, and parental behaviour across taxa (Newman 1999; Goodson *et al.* 2005). For example, the preoptic area (POA) is involved in sexual behaviour in all vertebrates, as well as aggression and parental care in mammals, birds and fish (reviewed in O'Connell and Hofmann 2011), and in mammals, the medial amygdala is involved in social recognition (Petrulis and Johnston 1999) and the lateral septum is involved in social affiliation (Liu *et al.* 2001) and social recognition (Landgraf *et al.* 1995). The mesolimbic reward system includes eight interconnected nodes, two of them shared with the SBN (lateral septum, bed nucleus of the stria terminalis/medial amygdala, striatum, nucleus accumbens, ventral pallium, basolateral amygdala, hippocampus, and ventral tegmental area), and influences the SBN by reinforcing adaptive social behaviours via reward

(O’Connell and Hofmann 2011). For example, in mammals the striatum is involved in reinforcement learning and selecting previously reinforcing actions (reviewed in Wickens *et al.* 2007). The SDMN is well conserved across vertebrates, albeit with differences in nomenclature between taxa, and several studies in different vertebrates have linked the SDMN to a wide range of social behaviours, such as mate choice (Cummings 2015), hierarchy formation (Maruska *et al.* 2013), and cooperative nest building (Hall *et al.* 2014). While these and other studies have implicated the SDMN in social behaviours across diverse taxa, it is noteworthy that most research effort has been targeted at ‘complex’ social behaviours and that there has been a comparative lack of research into the neural mechanisms of more fundamental social behaviour such as grouping.

Grouping is a very common phenomenon which has been the focus of extensive research in behavioural, theoretical and evolutionary biology (Krause and Ruxton 2002). Although living in groups carries costs due to potentially increased aggression, competition for resources, or transmission of parasites and diseases, it can also confer benefits to the individual by reducing predation risk, increasing the chances of obtaining food, increasing the opportunities of finding a mate, reducing loss of heat and moisture, or reducing the cost of movement (Krause and Ruxton 2002). Despite the importance of this topic, the neural mechanisms of grouping behaviour have received relatively little attention so far. Goodson and colleagues studied the neural mechanisms involved in grouping behaviour in birds and found differences between gregarious and territorial finches in the activation of brain areas of the SDMN (Goodson *et al.* 2005). They have also shown that pharmacological manipulation of nonapeptide signalling in the SDMN modulates flocking behaviour in estrildid finches (Goodson *et al.* 2009; Kelly *et al.* 2011). The nonapeptides are a highly conserved family of neuropeptides involved in different intra-SDMN signalling pathways and studies in fish have also shown that manipulation of these nonapeptides has effects on shoaling and simple social approach (Thompson and Walton 2004; Lindeyer *et al.* 2015). We wished to address how the SDMN is involved in grouping behaviour and so investigated brain activation in teleost

fish in which shoaling conditions and social exposure can be readily manipulated and controlled.

For our study, we used Trinidadian guppies (*Poecilia reticulata*) as there is extensive research on their shoaling tendencies, both in their natural environments and in laboratory conditions (Magurran 2005). Trinidadian guppies vary in their shoaling tendencies across populations, with median shoal sizes ranging from 1 to 21 individuals (Magurran and Seghers 1991). Female guppies form groups to avoid both predation and sneaky mating attempts from male guppies (Magurran and Seghers 1994). Males, on the other hand, show a preference for female rather than male shoals, and, like females and juveniles, for larger shoals rather than small ones (Lindström and Ranta 1993; Lachlan *et al.* 1998; Ledesma and McRobert 2008), a trait that appears to be widespread across teleost fish (e.g., banded killifish (Krause and Godin 1994; Hoare *et al.* 2004), Eurasian perch (Hellström *et al.* 2016), fathead minnows (Hager and Helfman 1991), three-spined sticklebacks (Krause 1993; Krause *et al.* 1998), zebrafish (Pritchard *et al.* 2001)), as well as in birds (Caraco *et al.* 1980; Elgar 1987) and mammals (Ruckstuhl and Festa-Bianchet 2001; Creel and Winnie 2005).

We conducted two studies to investigate the neural mechanisms underlying grouping behaviour in guppies. We first conducted a behavioural test to confirm subjects' preferences in the studied population for large shoals over small shoals. With a second cohort of fish, we analysed brain activation after a shoaling exposure test in which the subjects were exposed to one of three experimental treatments: a small shoal, a large shoal, or no social exposure. After one hour, the brain of each subject was dissected for immediate early gene assay of neural activation in specific brain regions that are putative components of the SDMN. We expected shoals to act as a social cue and a rewarding stimulus, and hence social exposure would activate areas of both the SBN and the mesolimbic reward system. Thus, we selected brain areas of both networks, specifically the preoptic area (POA), a node of the SBN and suggested homologue of the amniote POA/paraventricular nucleus of the hypothalamus (O'Connell and Hofmann

2011; Goodson and Kingsbury 2013); the dorsal part of the ventral telencephalon (Vd), a node of the mesolimbic reward system homologous to the mammalian striatum and nucleus accumbens (Wullimann and Mueller 2004; O'Connell and Hofmann 2011); and two nuclei belonging to both networks, the ventral part of the ventral telencephalon (Vv), and the supracommissural part of the ventral pallium (Vs), homologues of the mammalian lateral septum and amygdala/bed nucleus of the stria terminalis respectively (Wullimann and Mueller 2004; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013). We did not add other brain areas of the SDMN to our study because there is no consensus about teleost homologues of the mammalian areas and/or insufficient research on those areas in teleost fish (O'Connell and Hofmann 2011). We hypothesized that grouping behaviour will be modulated by the SDMN and so exposure to shoals would activate the selected brain areas, with greater activation when the subjects were exposed to the large shoal.

Materials and methods

Experiment 1: Shoal preference study

Animal subjects and housing

Subjects were 30 female guppies from mixed populations of wild Trinidadian origin that had been bred in captivity for at least 2 generations (henceforth 'wild stock guppies'). Two weeks before the experiment started we moved them from 110 L breeding tanks (76 x 30 x 45 cm) containing both sexes to two 19 L housing tanks (40 x 20 x 25 cm) containing only the subjects. We used an additional 12 wild stock female guppies to form a pool from which stimulus shoals were drawn. They were unfamiliar to the subject fish and lived in the test tank (see below). All tanks were kept at 26 ± 1 °C, had a filter and a heater, as well as gravel, plastic plants and a shelter. Fish were fed flake food

daily (TetraMin Tropical Flakes, Tetra, Germany) and supplementary decapsulated brine shrimp eggs (*Artemia sp*., Brine Shrimp Direct, Ogden UT, USA) three times a week.

Behavioural test

Females were tested in a 75 L tank divided into three different compartments by perforated transparent plastic partitions. Each side compartment contained a shoal of either two or 10 females (Fig. 2.1). During the testing day, we removed the plants and shelters and counterbalanced the position of the shoals and varied the member composition of each shoal at random. To measure subjects' proximity to the shoals, we drew vertical lines on the front of the tank to divide the central compartment into five zones. The subject was moved to the testing arena in a transparent plastic cup and,

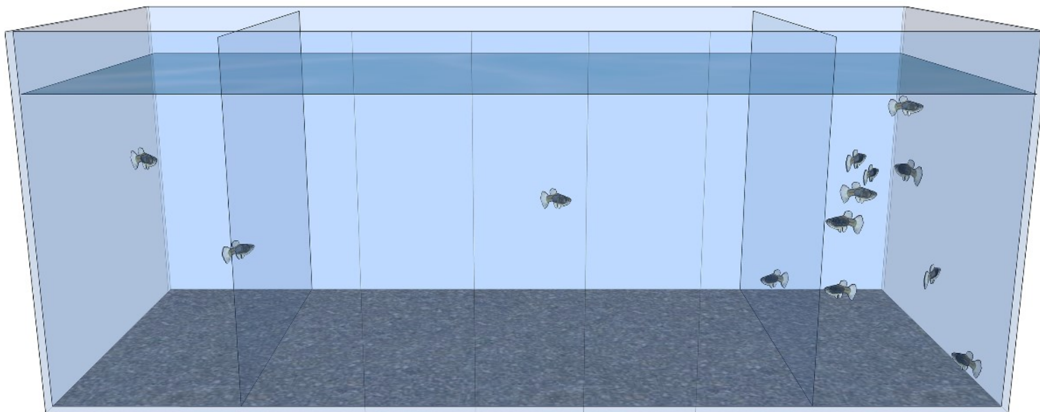

Figure 2.1. Shoal preference test. A 75 L tank (76 x 30 x 30 cm, 25 cm water depth) was divided into three compartments. Side compartments (15 x 30 x 30 cm) held either two or ten fish, and were separated from the central compartment containing the subject by perforated transparent plastic partitions. Vertical lines drawn on the front of the central compartment created 5 zones (each 9 cm wide, approximately three to four body lengths) to facilitate recording of the position of the subject. All compartments contained gravel.

after two minutes of acclimation, the cup was gently and remotely raised by the observer by pulling a string attached to the cup. The test started immediately after the subject was released. We measured the amount of time the subject spent on each of the five zones in order to calculate time shoaling with each group (i.e. time within four body lengths (Pitcher and Parrish 1986)), as well as the amount of time the subject spent interacting with the shoal (i.e. swimming head first against the transparent partitions (Lindeyer *et al.* 2015)) over 10 minutes, using the software JWatcher V1.0. We measured shoaling time and interaction time as dual estimates of grouping behaviour in fish (Lindeyer *et al.* 2015).

Statistical analysis

We calculated the difference in time shoaling close to the large shoal minus the time shoaling close to the small shoal. This measure was not normally distributed and thus was square-root transformed to achieve normally distributed residuals. We also calculated the difference in time interacting with the large shoal minus the time interacting with the small shoal. For each measure, we ran one-sample t-tests using the software SPSS 24 to determine whether subjects preferred either shoal.

Experiment 2: Brain activation during shoal exposure test

Subjects and housing

Two weeks before our study started, we moved 60 females and five males to a 110 L housing tank (76 x 30 x 45 cm). Of these, 36 females were used as subjects and the rest were left in the housing tank as companion fish to prevent the subjects from being isolated as subjects were removed from the tank as the study progressed. We also

placed 24 wild stock females unfamiliar to the subjects into four testing tanks (Fig. 2.2), two tanks had ten females forming the large shoal, and the other two tanks had two females forming the small shoal. There were also two control testing tanks without fish in them. Two weeks before the start of the study, we placed a perforated transparent cylindrical plastic container with gravel in the middle of the testing tanks to habituate the shoals to it. This container held the subject fish during the exposure test, exposing them to the shoal but preventing them from interacting directly with other fish; this ensured consistent exposure to stimulus shoals across subjects. A transparent plastic lid covered the tank to prevent fish from jumping out. Housing conditions and feeding were the same as Experiment 1. The day prior to the test, we isolated 12 subjects in separate 10 L tanks (30 x 20 x 15 cm) containing gravel, a plastic plant, a heater (keeping the water at 26 ± 1 °C) and an air stone. The purpose of this isolation period was to set a consistent baseline of neural activity in all subjects.

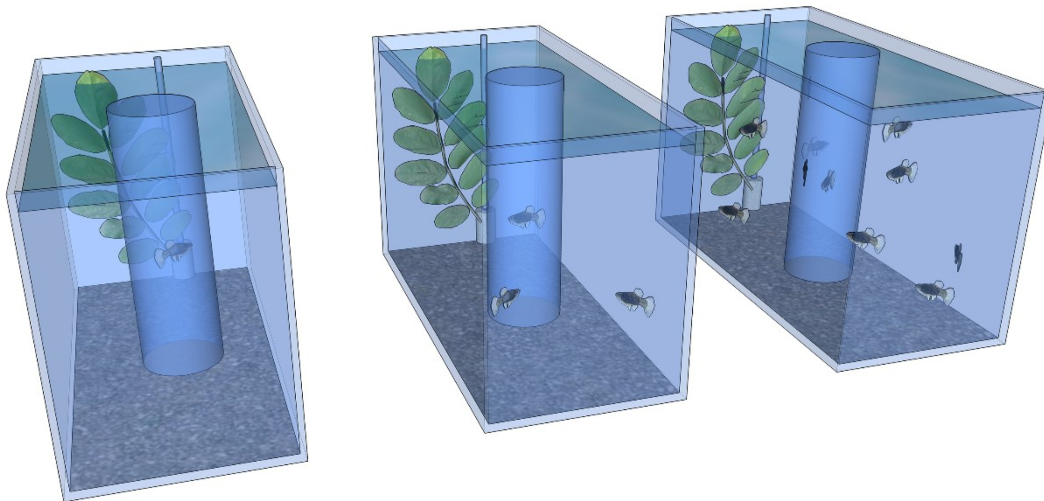

Figure 2.2. Social exposure test. Each tank (19 L, 40 x 20 x 25 cm) contained gravel, a heater, and a plant attached to an air stone, so that all subjects were exposed to visual motion. The subject fish were inside a perforated transparent plastic cylinder (diameter: 9 cm) placed in the centre of each testing tank. One testing tank was empty and served as control (left), one had two companion fish (centre), and one had 10 companion fish (right). Two sets of these three tanks were used. A transparent plastic lid covered the tanks and opaque barriers separated testing tanks so that fish in each condition could not see other fish.

Social exposure test

On the day of the test we removed the filter and plastic plant from the experimental tank, and added an air stone with a plastic plant attached to it. The air stone made the plant move, which served as a control for any neural activation generated by movement, meaning that any differences between treatments would be due to olfactory and/or visual exposure to the social stimulus. Twenty minutes later we caught an isolated subject and placed it at random in the plastic container of a testing tank containing either a large shoal, a small shoal, or no shoal (control), where it was exposed to that social stimulus for an hour (Fig. 2.2). We monitored the behaviour of the subject and companion fish and observed similarities with the behaviour observed in Experiment 1: subjects appeared highly interested in the stimulus fish and spent much of the exposure period attending to the stimulus fish and attempting to swim to them. Although a 30 minute period has been suggested for induction of the highest expression of *egr-1* in teleost fish (Burmeister and Fernald 2005), we exposed the subjects to the treatment for an hour to ensure that the brain activation we observed was due to the treatment and not just due to handling and tank changing. After this period, we caught the subjects and euthanized them by rapid cooling through immersion in ice water (Wilson *et al.* 2009; Blessing *et al.* 2010; Matthews and Varga 2012). Control tanks were emptied, rinsed and re-filled with conditioned water before adding each new subject to eliminate any olfactory cues left by the previous subject.

Immunohistochemistry (IHC) staining of *egr-1*

Brains were dissected out immediately after euthanasia, fixed in 4% paraformaldehyde at 4 °C overnight, and then cryoprotected in 30% sucrose overnight at 4 °C before embedding in Clear Frozen Section Compound (VWR International, PA, USA) and storage at -19 °C. Brains were then sectioned on a cryostat at 25 μm and thaw-mounted

onto Superfrost Plus slides (VWR International) in two parallel series that were stored at -19 °C for less than a week before processing for IHC.

One of the two series of sections was thawed and air-dried before processing for immunohistochemical detection of egr-1. Sections were rinsed in 0.1M Phosphate-buffered saline (PBS) for 15 minutes. After blocking for 1 hour in blocking solution (5% normal goat serum and 0.3% Triton X-100 in PBS) and rinsing in PBS for 10 minutes, sections were incubated in primary antibody (anti-egr-1 rabbit polyclonal, 1:1000, catalogue number sc-189; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) dissolved in blocking solution at 4°C overnight. Sections were then rinsed in PBS, incubated for 15 minutes in H_2O_2 solution (3.5 % H_2O_2, 8.8% methanol dissolved in 0.3% Triton X-100 in PBS), and rinsed again in PBS. Sections were then incubated in a biotinylated goat anti-rabbit secondary antibody solution (1:200, ThermoScientific, Rockford, IL, USA) dissolved in blocking solution for 30 min at room temperature, and rinsed again for 15 minutes in PBS. Sections were then washed in avidin/biotinylated-horseradish peroxidase solution (1% dissolved in 0.3% Triton X-100 in PBS, ABC Peroxidase staining kit, ThermoScientific) for 30 minutes and rinsed again for 15 minutes in PBS. Immunoreactivity was visualized using nickel-enhanced DAB solution (0.03% 3,3'diaminobenzidine, 1% cobalt chloride, 1% nickel ammonium sulphate, and 0.035% H_2O_2 in PBS*, all from Sigma-Aldrich*, St. Louis, MO, USA). Sections were then rinsed, cleared, dehydrated and coverslipped with DPX (Sigma-Aldrich). Specificity of the egr-1 antibody was confirmed by western blot (see below).

Western blot characterization of anti-egr-1 antibody

In order to determine whether the egr-1 antibody would bind specifically to the desired antigen in the guppy, the antibody was assayed using protein from four whole guppy brains by radioimmunoprecipitation. Whole brains were homogenized and protein extracted in radioimmunoprecipitation buffer before being diluted at 1:4 with sodium

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and separated on a SDS-PAGE gel, alongside mouse fibroblast L-cells as a control.

Whole brain extract on the gel was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20 in Tris-buffered saline (TBS)), incubated in primary antibody (1:1000, anti-egr-1)) for 1 hour, washed three times for five minutes each in wash buffer, and then incubated in donkey-anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:1000, catalogue number AP182P, *EMD Millipore*, Hayward, CA, USA) in blocking solution for 2 hours. After washing three times for 5 minutes each with wash buffer, the blots were developed using a chemiluminescence detection reagent (catalogue number WBKLS0500, EMD Millipore), and images were acquired with a 16-bit CCD camera (MicroChemi DNR Bio-imaging Systems). A band was visualized putatively representing egr-1 at the predicted size of 57 kDa, which is the estimated unphosphorylated molecular weight of egr-1 (Milbrandt 1987; Sukhatme *et al.* 1988; Cao *et al.* 1990). We also assayed a c-fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and obtained two bands at 52 and 68 kDa. We therefore did not use c-fos as we would have expected only a single band at 62 kDa (Curran *et al.* 1984) if this c-fos antibody was binding specifically to the c-fos antigen in guppy.

Quantification of neurons expressing *egr-1*

Cell nuclei containing egr-1 protein were clearly stained black and were counted using a 20× objective in a microscope (Leica DM1000LED). As no guppy brain atlas is available, we used the brain atlas of the related poeciliid, the swordtail (*Xiphophorus hellerii* (Anken and Rahmann 1994)) to distinguish the brain areas of interest (Table 2.1). We took a picture of each brain area of interest in both hemispheres using a digital camera (Leica ICC50HD with the software Leica Application Suite EZ 3.2.1). An observer blind to the experimental treatments processed all images and counted

stained nuclei. Images were converted to greyscale to sharpen images and increase contrast using ImageJ 1.50i. A defined oval sampling area that fitted centrally within each brain area of interest was applied to each image (Table 2.1) and ImageJ was used to count the number and size of stained nuclei that met minimum size and circularity criteria. The procedure was then repeated for the other hemisphere. Data on the size of each counted nucleus was then checked to account for overlapping stained nuclei. The size of each counted nucleus was divided by the size of the average nucleus. When the quotient of that division was at least 2 (i.e. two times the average size of a stained nucleus) we considered it to be an overlapping cluster of nuclei and counted it as the quotient obtained in the division. The ImageJ script used for image processing and all data will be deposited in the Dryad Digital Repository.

Table 2.1: Brain areas studied, their mammalian homologues, the brain network that they belong to (Wullimann and Mueller 2004; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013) and the mean size of the oval sampling areas used to count the number of stained nuclei in each of the four brain areas.

Fish brain area	**Mammalian homologue**	**Brain network**	**Sampling area (μm^2)**
POA: Preoptic Area	POA and VPN	Social behaviour network	6003
Vd: Ventral telencephalon – dorsal part	Nucleus accumbens and striatum	Mesolimbic reward system	4642
Vs: Ventral pallium	Amygdala/Bed nucleus of the stria terminalis	Social behaviour network & Mesolimbic reward system	4903
Vv: Ventral telencephalon – ventral part	Lateral septum	Social behaviour network & Mesolimbic reward system	5340

Statistical analysis

After counting the number of neurons in each hemisphere, we calculated the number of neurons per 100 μm^2 to standardize measurements across brain areas. The number of activated neurons per hemisphere were positively correlated across individuals (Pearson correlations; POA: $r = 0.77$, $n = 30$, $p < 0.001$; Vd: $r = 0.68$, $n = 32$, $p < 0.001$; Vv: $r = 0.58$, $n = 31$, $p=0.001$; Vs: $r = 0.69$, $n = 29$, $p < 0.001$) supporting the pooling of the counts from the two hemispheres and the reliability of our brain area identification and counts. We analysed the effect of social treatment (ten-fish shoal, two-fish shoal, social isolation) and the interaction of social treatment and brain nuclei (POA, Vs, Vd, Vv) using a linear mixed model (LMM), with brain nuclei as a repeated measure. We ran a one-way ANOVA on neuron counts for each of the brain areas and Tukey post-hoc tests to elucidate differences between treatments. We calculated the effect size for these comparisons (Cohen's d_s) and used the reference effect size values (small: $d >$ 0.2, medium: $d > 0.5$, and large: $d > 0.8$) to interpret effect sizes (Lakens 2013). All data were normally distributed and variances were homogenous. We used the software SPSS 24 for all our analyses.

Ethical note

All tests and procedures were approved by the by the Animal Care Committee of McGill University (Protocol #7133) and were carried out in accordance to the Canadian Council on Animal Care and the Association for the Study of Animal Behaviour guidelines. The subjects of behavioural tests and the fish used as shoals were placed into breeding populations at McGill University at the conclusion of the studies.

Results

Experiment 1: Shoal preference study

Guppies spent more time close to, and interacted more with the large shoal than the small shoal, with their preference scores significantly greater than 0 (One-sample t-tests; shoaling preference score: $t(29) = 9.46$, $p < 0.001$; interaction preference score: $t(29) = 3.49$, $p = 0.002$; Fig. 2.3). Fish that shoaled more also spent more time interacting with the shoal ($r = 0.76$, $n = 30$, $p < 0.001$).

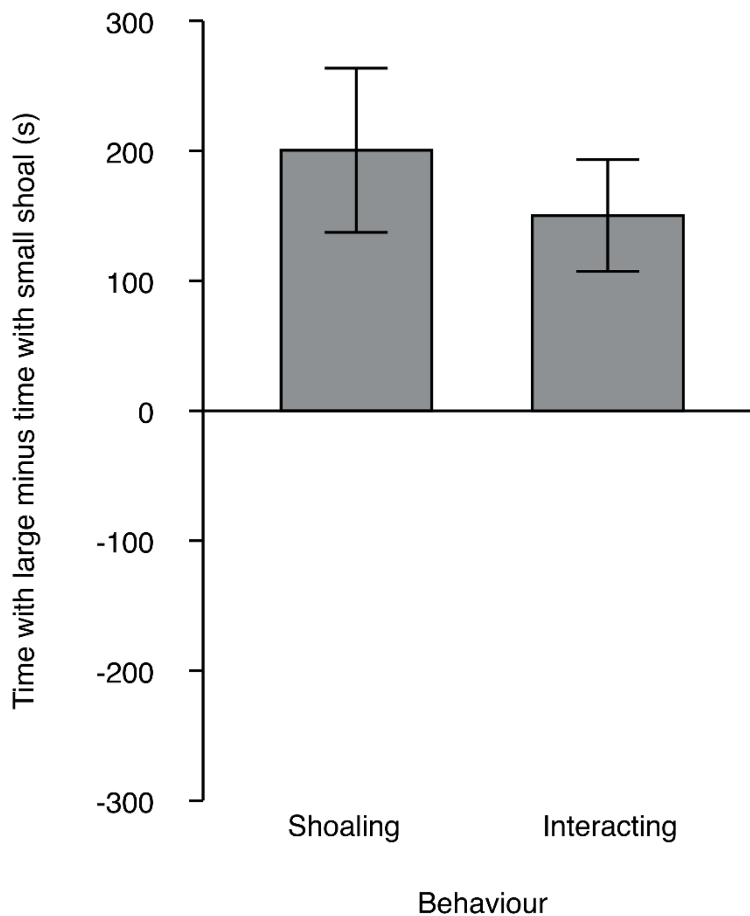

Figure 2.3. Mean ± SEM time fish spent shoaling and interacting with large shoal versus a small shoal, in a 10 minutes behavioural test. Positive values indicate a preference for the large shoal, and negative values indicate a preference for the small shoal.

Experiment 2: Brain activation during shoal exposure test

We found a significant interaction effect between treatment and brain nuclei (LMM, $F(9, 50) = 7.41$, $p < 0.001$) but no significant overall effect of treatment (LMM, $F(2, 96.18) = 1.88$, $p > 0.1$). Given the significant interaction effect, we examined each brain area individually, finding a difference among treatments in the POA (ANOVA, $F(29, 2) = 4.13$, $p = 0.027$, Fig. 2.4, Fig. A2.1), with post-hoc tests indicating that the fish exposed to a large shoal had significantly higher activation in this brain region compared to the control (Tukey, $p = 0.021$; $d = 1.18$). There were no significant differences in activation between the fish exposed to a small shoal and the control in the POA, or among treatments in the other brain areas (all $p > 0.1$).

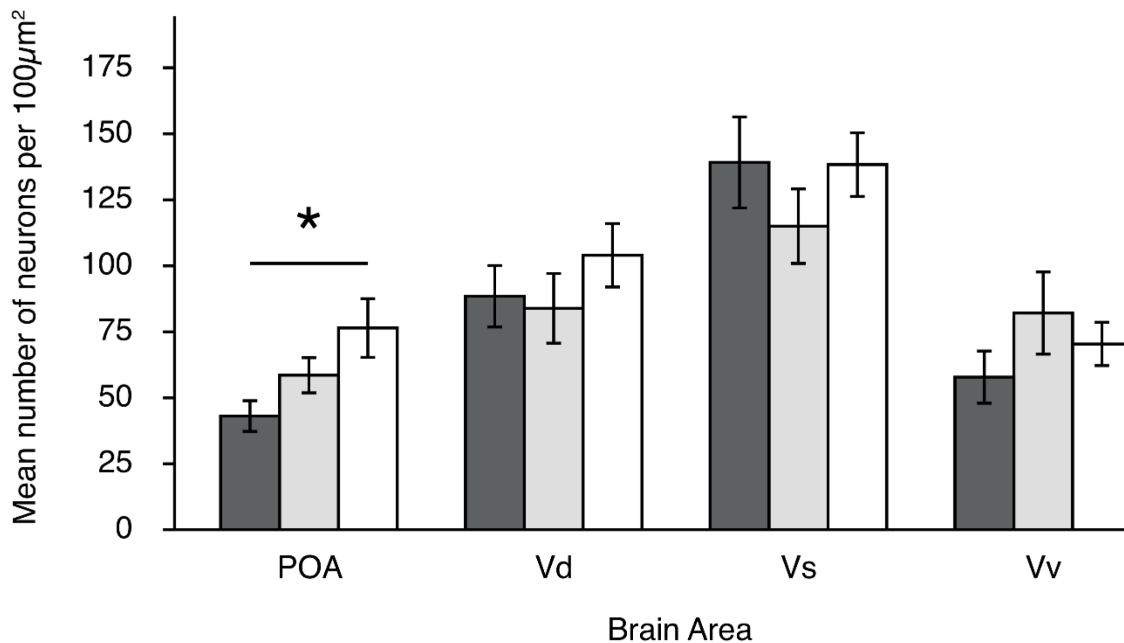

*Figure 2.4. Means ± SEM of counts of neurons per 100 μm2 in the four different nuclei (Preoptic Area (POA), Ventral telencephalon – dorsal part (Vd), Ventral pallium (Vs), Ventral telencephalon – ventral part (Vv)) in fish exposed to one of three experimental treatments (Black: control, Grey: fish exposed to a two-fish shoal, White: fish exposed to a ten-fish shoal). * p < 0.05.*

Discussion

We confirmed that our study population of guppies prefer a large over a small shoal, as has been previously demonstrated in guppies, other fish and other vertebrates (Elgar 1987; Lachlan *et al.* 1998; Krause *et al.* 1998; Creel and Winnie 2005). This preference is typically explained by anti-predator and foraging advantages for group members (Krause and Ruxton 2002). Thus, choosing a large over a small group may be a rewarding action that reinforces adaptive social behaviours. We then studied four brain areas (POA, Vs, Vd, and Vv) of the social decision making network (SDMN) involved in social behaviour in vertebrates (O'Connell and Hofmann 2011) and found that only the POA had significantly greater neuronal activation in fish exposed to a large shoal stimulus compared to isolated fish used as a control. There were no significant differences among treatments in the other brain areas examined (Vs, Vd, and Vv).

The POA is a nucleus located immediately rostral to the hypothalamus along the third ventricle and which has close functional links and connections to the hypothalamus and limbic system. As part of the hypothalamic-pituitary-gonadal axis, the POA is involved in many different reproductive behaviours in fish (Demski and Knigge 1971; Macey *et al.* 1974; Satou *et al.* 1984; Wong 2000), including social aspects such as changes in social status related to reproduction (Francis *et al.* 1993; Desjardins *et al.* 2010). The POA also mediates sexual behaviour in all vertebrate taxa, as well as parental care and aggression in mammals, birds, and teleosts (O'Connell and Hofmann 2011). Thus, its function mediating social behaviour, as well as its neurochemistry, hodology, and topography, are very well conserved among vertebrates (O'Connell and Hofmann 2011). Our finding of higher activation in the POA during grouping is similar to the results of Teles *et al.* (2015) in a more 'complex' social context, which found significantly higher *egr-1* expression in the POA when zebrafish were in a mirror test and a winner/loser context compared to isolated fish. They did not find differences between their behavioural treatments, which suggests that the POA might be processing social cues independently of the social situation experienced. Together, these results indicate

that the POA is a key component in the processing of social cues in fish, and possibly in all vertebrates. In birds, for example, there is strong evidence that the POA mediates gregariousness via the production and regulation of nonapeptides (Goodson *et al.* 2012), even though activation of the POA is not significantly different among species with different levels of gregariousness (Goodson *et al.* 2005).

The teleost POA has been suggested as the homologue to the mammalian POA and paraventricular nucleus of the hypothalamus (Goodson and Kingsbury 2013) because it includes the majority of neurons that produce vasotocin and isotocin, the teleost homologues of mammalian vasopressin and oxytocin and members of the nonapeptide family of neuropeptides that are involved in a wide range of social behaviours (Choleris *et al.* 2013). In teleost fish, vasotocin modulates aggressive behaviour (Backström and Winberg 2009; Oldfield and Hofmann 2011; Kagawa *et al.* 2013), courtship behaviour (Semsar *et al.* 2001; Salek *et al.* 2002), and behaviour related to establishing a social structure (Aubin-Horth *et al.* 2007; Greenwood *et al.* 2008; Oldfield and Hofmann 2011), while isotocin increases submissive behaviour during fights in *Neolamprologus pulcher* (Reddon *et al.* 2012) and modulates paternal care in monogamous cichlids (O'Connell *et al.* 2012). However these nonapeptides have also been implicated in simple social grouping behaviour in fish: vasotocin inhibits social approach (Thompson and Walton 2004; Thompson *et al.* 2008; Walton *et al.* 2010), and decreases social interactions with a shoal (Lindeyer *et al.* 2015), while isotocin stimulates social approach in goldfish (Thompson and Walton 2004) and inhibits it in *N. pulcher* (Reddon *et al.* 2014). Thus, the increased activation of POA neurons found in our study may reflect increased activity and signalling by nonapeptide neurons, which are located solely in this area of the teleost brain.

Our results suggest a conserved role for the POA in grouping behaviour. As this area is the key nonapeptide site in the teleost brain, this neuropeptide family may thus be involved, however, other neurochemical systems may also regulate responses to social cues. The POA has been implicated in motivation and drive (Wood *et al.* 2011) through

the high density of dopaminergic cells and dopamine receptors in the POA and local release of dopamine in response to cues from conspecifics (Hull and Dominguez 2006; O'Connell *et al.* 2011). Dopamine is a major mediator of reward and the observed higher activation in response to the large group might represent increased activation of POA dopaminergic neurons in response to the rewarding stimulus of a large group of conspecifics. In this context, the lack of activation in other areas of the SDMN in the guppy is somewhat surprising given the clear behavioural responses seen to shoaling stimuli. This is particularly true of the Vd, a putative homologue of the mammalian nucleus accumbens that mediates dopaminergic reward. Visual exposure to conspecifics has been shown to be rewarding (Al-Imari and Gerlai 2008) but despite subjects in our study showing robust preferences for large shoals, we saw no response in the Vd. This may indicate that social reward is not encoded by Vd dopamine signalling alone (Norton and Bally-Cuif 2010), but perhaps also reflects the relative paucity of information on functional teleost neuroanatomy, particularly in the guppy. Both dopamine and nonapeptides are good candidates to explain POA responses to social cues in guppies, however, our data only allow us to speculate about the nature of the active POA neurons we observed, and hence further studies are needed to elucidate this question.

Increased activity in the POA could also be explained as a neuronal response to the greater visual stimulus of multiple individuals swimming in a large shoal, however we consider this unlikely as simple visual information is processed in the optic tectum (Del Bene *et al.* 2010) and the POA is not a consensus part of this circuit. The POA is also involved in vertebrate stress responses, however we consider it unlikely that the increased POA activity is due to stress effects of social exposure. Companion fish have been shown to reduce stress-related behaviour in small shoaling fish (Barlow 1968), and simple visual exposure has been shown to be rewarding for isolated fish (Al-Imari and Gerlai 2008).

We were somewhat surprised not to find a significant difference in activation of the POA in fish exposed to a small shoal compared to isolated fish, given shoaling preferences in the guppy, and the confirmed preference for social cues over an empty compartment (Shohet and Watt 2004; Ledesma and McRobert 2008). Our results suggest that more salient social cues than simply the presence of two other guppy females are needed to significantly activate the POA. However, it is worth noting that responses to the small shoal were intermediate to the large shoal and control conditions, consistent with POA activation increasing in step with the size of the social stimulus. We did not find a significant difference between treatments in brain activation in any of the other studied areas. This is similar to the results of Teles *et al.* (2015), who found no differences in *egr-1* expression in Vv and Vs in zebrafish during aggressive and submissive behaviour in a mirror test and a winner/loser context compared to isolated fish. However, they did find increased expression in these and other brain areas when exploring a different immediate early gene, *c-fos*, and suggested functional connectivity between several brain areas of the SDMN, supporting the SDMN hypothesis in teleosts. Similarly, Maruska *et al.* (2013) found increased activation in multiple brain regions in male cichlids (*Astratotilapia burtoni*) that had the opportunity to ascend in social rank. Our results suggest that forms of social behaviour such as grouping, which only require relatively simple social information such as recognition and approach of conspecifics, primarily activate the POA among the brain areas we examined. That said, it is an open question to what extent grouping decisions are simple, with numerous factors involving group choice. For example, guppy shoaling is influenced by cues of predation risk (Swaney *et al.* 2015), olfactory cues (Shohet and Watt 2004), early life exposure to conspecifics (Chapman *et al.* 2008), groupmates' familiarity (Griffiths and Magurran 1999), activity (Lachlan *et al.* 1998), sex (Griffiths and Magurran 1998), size (Croft *et al.* 2003), distance (Mühlhoff *et al.* 2011), and body colouration (Endler and Houde 1995).

Future studies are required to examine the neurochemical populations that the activated POA neurons belong to and whether dopamine, nonapeptides, or other neuronal signals are involved in this behaviour in fish. It is also important to consider the possibility of

activation in other brain areas that were not the focus of this study and are also involved in social behaviour in vertebrates (O'Connell and Hofmann 2011), and so, a more exhaustive study of all the brain areas of the SDMN and the use of additional immediate early genes different from *egr-1* could provide further insights into the neural modulation of grouping behaviour. While gross neuroanatomy is understood, a detailed guppy brain atlas has yet to be published, the detailed connections between nuclei have not been mapped and the functional role of much of the brain is not well understood. As the guppy is a species with an extensive, well understood and experimentally tractable suite of behaviours, addressing this lack of neuroanatomical detail would be of great assistance in exploring the neurobiology of this important species in behavioural and evolutionary biology.

In conclusion, we successfully used egr-1 immunohistochemistry to map neural activation in the four brain areas studied (POA, Vs, Vd and Vv) and showed that activation in the POA was elevated when fish were exposed to a large shoal compared to isolated fish. Our results support the idea of a conserved role of the POA in the modulation of social behaviour in vertebrates and in responses to social cues. This shows that the role of the POA in sociality extends across all forms of social behaviour, across vertebrate taxa. However, further studies are needed to clarify the neurochemical properties of the POA neurons that respond to social cues in the POA of guppies.

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Appendix to Chapter 2

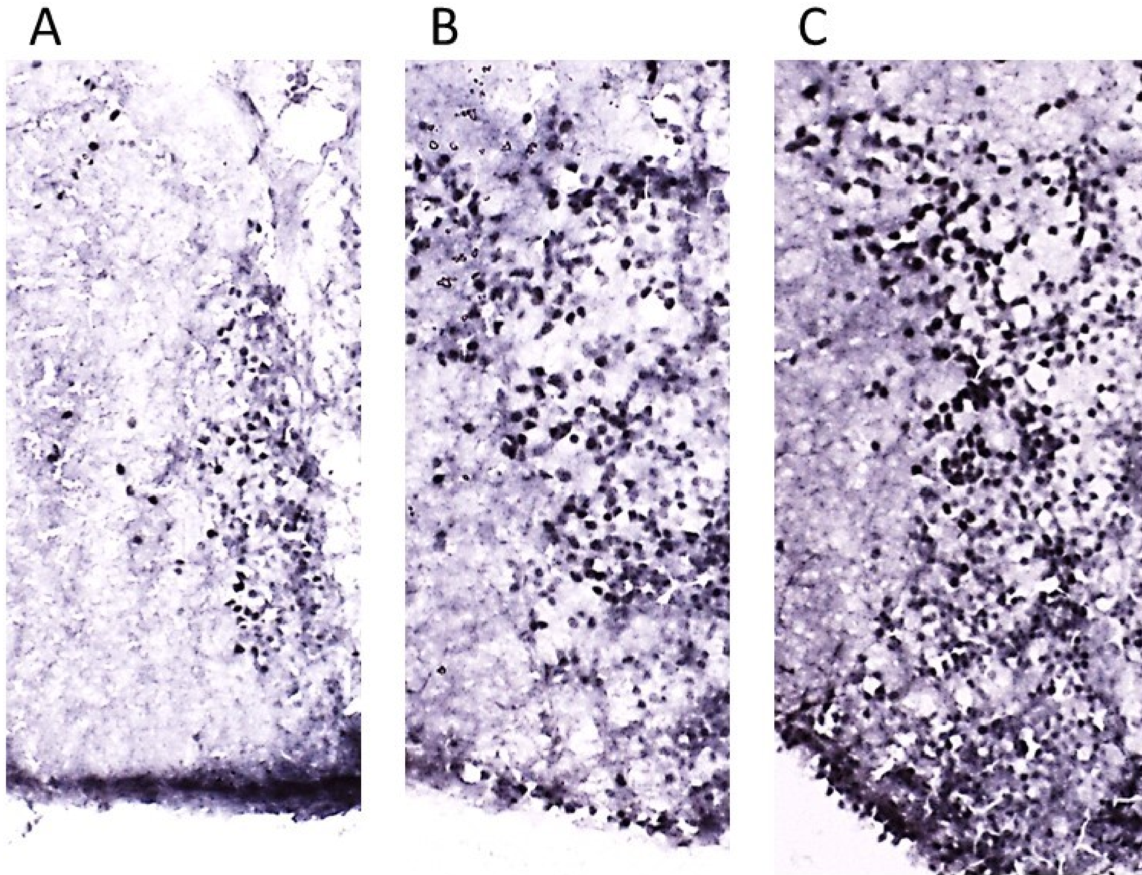

Figure A2.1. Micrographs of Preoptic Area sections showing egr-1 *activation in each treatment: A) Control; B) Fish exposed to small shoal; C) Fish exposed to large shoal. Magnification of image: 20x.*

Linking statement to Chapter 3

In Chapter 2, I studied brain areas expressing egr-1 during social exposure. I found that when guppies were exposed to a large group of conspecifics, the preoptic area (POA) had an increase in expression compared to being isolated. The POA is the only area in the teleost brain known to produce nonapeptides, which are neuromodulators involved in the regulation of a wide variety of social behaviours and are very well conserve among vertebrates. They are also involved in the modulation of grouping behaviour in birds. Thus, in Chapter 3 I studied the effect of nonapeptides on grouping behaviour in guppies.

Chapter 3: Grouping behaviour in guppies after central nonapeptide administration

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Keywords: nonapeptides, isotocin, vasotocin, guppy, social behaviour, grouping, intracerebroventricular administration, icv administration.

In revision with *Hormones and Behavior*

Abstract

The nonapeptides vasopressin, oxytocin, and their homologues are involved in the regulation of social behaviours such as mating, social recognition, and parental care across vertebrate taxa. Here, we address the role of nonapeptides in a fundamental aspect of social behaviour, grouping, in the guppy, *Poecilia reticulata*. Nonapeptides influence flocking in birds, and thus may also regulate grouping behaviour in other taxa. Guppies are a useful system for studying the evolution of social behaviour, both as a comparison to more commonly studied mammals and birds, and due to their extensively-studied evolved population differences in social behaviour. To overcome the limitations of peripheral administration in this small teleost fish, we adapted an intracerebroventricular administration technique to investigate the influence of nonapeptides on grouping behaviour in wild-type guppies. We measured grouping behaviour for 2.5 hours after intracerebroventricular administration of isotocin, vasotocin, or putative receptor antagonists, all co-administered with Evans Blue dye to confirm injection accuracy. Isotocin significantly increased shoaling behaviour and

social interaction (i.e. very close proximity to the shoal) 90 minutes after administration, while vasotocin significantly increased latency to shoal immediately after administration and reduced social interactions 90 minutes after administration. We thus found opposing effects of the nonapeptides isotocin and vasotocin on grouping but with distinct time courses for these. Our results also indicate that despite short metabolic half-lives, nonapeptides can have persistent effects after administration. Similarities across taxa support a conserved role for nonapeptides on vertebrate social behaviour, albeit with species-, taxon- and behaviour-specific variation in the precise effects.

Introduction

The nonapeptides are nine amino acid neuropeptides that are highly conserved among animal taxa and have similar molecular structures. This neuropeptide family has a shared evolutionary origin, with a gene duplication event early in vertebrate evolution giving rise to two nonapeptide lineages, one comprising mammalian vasopressin and its homologue vasotocin in all other vertebrates, and the other including mammalian oxytocin and its homologues mesotocin (birds, reptiles and amphibians) and isotocin (fish; Hoyle 1999).

Oxytocin and vasopressin were first known for their peripheral functions in mammals, such as contraction of the uterus during birth and regulation of blood pressure respectively, however in recent decades there has been extensive research into the roles of nonapeptides in the modulation of social behaviour. Oxytocin, vasopressin and their homologues have been found to influence diverse social behaviours in birds and mammals including bonding, sexual behaviour, parental care, gregariousness, social recognition, aggression, and cooperation (reviewed in Lim and Young 2006; Goodson 2013). For example, in the monogamous prairie vole, central infusion of vasopressin in males increases affiliative behaviour toward females and aggression towards males, while central infusion of oxytocin in females stimulates the formation of partner preferences, all effects which are not found in the comparatively asocial, non-

monogamous montane vole (Winslow et al. 1993; Williams et al. 1994; Young et al. 1997). The roles of nonapeptides in different vertebrate social behaviours are well supported, however, the behaviours involved, the precise direction and the importance of the effects vary greatly among species. Moreover, despite nonapeptides being well conserved among vertebrate taxa, their functional roles in behaviour are far less well understood in non-mammalian and non-avian vertebrates. Studying the behavioural effects of nonapeptides in different taxa will improve our understanding of the evolution of the underlying mechanisms regulating social behaviour in vertebrates. Goodson (2013) suggested that different forms of social behaviours may have evolved independently and thus might be differentially regulated by nonapeptides in each species, thus research into the role of nonapeptides in non-model species would help to clarify the evolution of nonapeptide regulation of diverse aspects of vertebrate social behaviour (Ondrasek 2016).

While nonapeptides have been shown to influence many different forms of social behaviour, surprisingly little is known of their role in one of the most common and fundamental social behaviours, grouping behaviour. Grouping has been the focus of extensive research in behavioural, theoretical and evolutionary biology (Krause and Ruxton 2002), however only a few studies have examined how nonapeptides influence this very common phenomenon. Goodson and colleagues (2009) found that nonapeptide administration modulates grouping behaviour in estrildid finches, with central infusions of mesotocin, but not vasotocin, increasing female preferences for a large group of conspecifics while infusion of an oxytocin receptor antagonist had the opposite effect (Goodson et al. 2009). In male finches, knockdown of vasotocin expression or blockade of vasotocin receptors decreased preferences for larger flock sizes (Kelly *et al.* 2011). Studies in fish have also shown that manipulation of nonapeptides has effects on shoaling and simple social approach, although the direction of the effect varies among species. For example, in goldfish (*Carassius auratus*), intracerebroventricular (ICV) administration of vasotocin inhibited social approach in highly social subjects, while isotocin significantly increased social approach

in less social subjects (Thompson and Walton 2004). In zebrafish (*Danio rerio*), peripheral administration of vasotocin decreased interactions with a shoal (Lindeyer *et al.* 2015), however in this same study isotocin and an oxytocin receptor antagonist had no effect on shoaling or social interaction, while a vasopressin receptor antagonist produced effects in the same direction as vasotocin, decreasing shoaling and social interactions. In a different study using considerably lower doses, both isotocin and vasotocin increased social preference in zebrafish (Braida *et al.* 2012). When isotocin was administered peripherally in the African cichlid *Neolamprologus pulcher*, it inhibited grouping behaviour, while an oxytocin receptor antagonist stimulated grouping behaviour in males (Reddon *et al.* 2014). These variable effects of nonapeptide administration suggest that nonapeptides broadly are involved in the regulation of grouping behaviour, but with interspecific differences in the modulation of grouping behaviour by nonapeptides even among related species belonging to a single class such as fish. However, the method of administration could also be involved in the differing responses seen in these studies. When administered intraperitoneally, nonapeptides may be acting on peripheral receptors and any behavioural effects may be responses to peripheral changes in physiology, with direct effects via central nonapeptide receptors blocked by the blood brain barrier. The teleost blood brain barrier consists of a single layer of membranous connective tissue, compared to the two layers of reptiles and amphibians, and the three layers of birds and mammals (Buttler and Hodos 2005). It was thought that the teleost and mammalian blood-brain barrier were physiologically different (Bernstein and Streicher 1965). However, recent studies have shown that the teleost blood-brain barrier is functionally and molecularly similar to that of other vertebrates, with the same tight junction structure conferring impermeability on the blood brain barrier endothelium in teleosts (Jeong *et al.* 2008) and similar permeability to the mammalian blood-brain barrier (Kim *et al.* 2017).

We measured shoaling behaviour in wild-type guppies (*Poecilia reticulata*) after administration of nonapeptides or putative nonapeptide antagonists with three aims: 1) to explore the effects of nonapeptides on grouping behaviour, 2) to investigate the role

that nonapeptides play in vertebrates beyond traditional mammalian models, and 3) to clarify nonapeptide effects in fish using a central method of injection in order to avoid biased responses due to peripheral receptors. The guppy is a freshwater teleost whose ecology, evolution, and behaviour, including social behaviour such as grouping, has been extensively researched (Lindström and Ranta 1993; Endler 1995; Houde 1997; Magurran 2005; Ledesma and McRobert 2008). In their natural habitat in Trinidad, waterfalls divide the downstream and upstream part of many rivers into two ecologically different areas, with and without predators, respectively. The populations of guppies in these areas exhibit different levels of social behaviour and grouping, with downstream guppies typically forming tighter and larger shoals than upstream guppies (Seghers 1974; Seghers and Magurran 1995; Song *et al.* 2011). The extensive literature on their evolutionary ecology and social behaviour make guppies an ideal subject for our study, as findings will have wider relevance and interest.

To focus on the direct central effects of nonapeptides on behaviour and to minimise secondary peripheral effects, we used ICV administration, a method successfully implemented in larger fish and more recently in zebrafish (Le Mevel *et al.* 1993; Yokogawa *et al.* 2007; Zhu 2009; Yokobori *et al.* 2011; Nishiguchi *et al.* 2012; Vijayanathan *et al.* 2017). We then observed shoaling behaviour and social interaction. We carried out two experiments, the first examining the effects of isotocin and an oxytocin receptor antagonist, and the second, vasotocin and a vasopressin receptor antagonist, observing behaviour repeatedly over 2.5 hours after administration. We used this long observation period both to find out at what point nonapeptides have their largest effect on grouping behaviour after ICV administration, and to study whether the effects of administration on grouping behaviour change over time.

Experiment 1: Isotocin

Materials and methods

Animal subjects and housing

Subjects were 76 adult female guppies selected at random from a laboratory breeding population of mixed wild Trinidadian origin housed in mixed-sex housing tanks (110 L). These fish, henceforth ‘wild stock guppies’, originated from the Aripo, Paria, Marianne, and Quare rivers, with the mixed populations reared together for several generations in captivity. We did not test males because their mating behaviour towards females and aggressive behaviour towards males would have confound the grouping behaviour we wanted to measure. We used 30 wild stock female guppies to form a pool from which the stimulus shoal was drawn. They were unfamiliar to the subject fish and lived in a 110 L tank with four male guppies. All housing tanks had gravel, plants and a shelter, as well as a filter and a heater, and were kept at 26 ± 1 °C with a 12-hour light cycle (lights on at 07:00 h with a 30-minute dawn/dusk period). Fish were fed *ad libitum* flake food during a daily feeding session (TetraMin Tropical Flakes, Germany) and supplementary decapsulated brine shrimp eggs (Artemia sp., Brine Shrimp Direct, Ogden UT, USA) three times a week.

Treatments

We performed ICV injections of either i) 200 ng/µL of isotocin dissolved in saline and 0.5% Evans Blue (EB), ii) 150 ng/µL of the selective oxytocin receptor antagonist, desGly-NH2,d(CH2)5[D-Tyr2,Thr4]OVT (Manning *et al.* 1995), dissolved in saline with 0.5% EB, iii) 0.5% EB dissolved in saline as a control, or iv) saline only as a further control to measure any effect of EB. After recovery from anaesthesia, we measured social behaviour four times over the next 160 minutes. We coadministered a dye, EB, to

allow visual confirmation of the accuracy of the injection into the ventricular system of the guppy brain. EB has been previously administered in zebrafish ICV studies to check the accuracy of the injections (Yokobori *et al.* 2011; Nishiguchi *et al.* 2012). In pilot studies, we checked the effectiveness of three dyes (Alcian blue, fluorescein, and EB) for this study, as well as different concentrations of EB, and found that 0.5% EB was the most suitable (unpublished data). The selected concentrations of nonapeptides and their receptor antagonists were based on previous studies (Thompson and Walton 2004; Thompson *et al.* 2008; Backström and Winberg 2009).

ICV nonapeptide injection

We used the technique developed by Barbosa et al. (2012) with the following modifications. We used 40 ppm eugenol (i.e. clove oil; MP Biomedicals LLC, OH, USA) to anaesthetize the subjects and, to inject the treatments, we manufactured glass capillary needles (supplementary material) and adjusted the duration of ejection according to the needle tip diameter (between 7.5 and 22.5 μm) to release 300 nL into the third ventricle of the subjects. 300 nL was sufficient to saturate the ventricles of all the subjects, based on EB diffusion (Fig. 3.1).

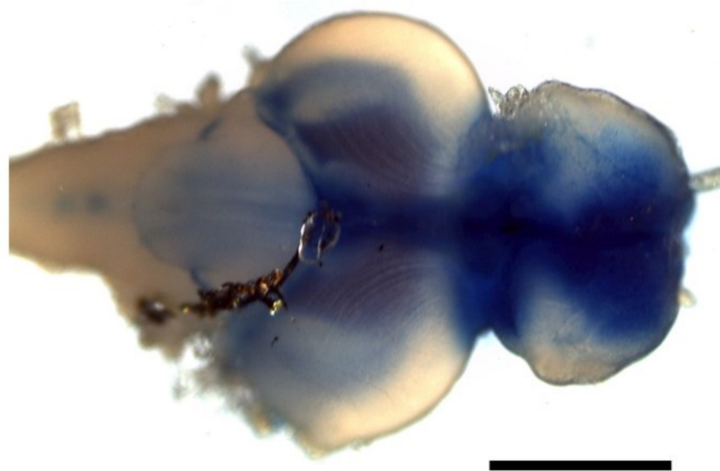

Figure 3.1. Dorsal view of guppy brain after Evans Blue administration. Rostral side on the right of the image. Scale bar = 1 mm.

We drilled a small (<200μm) hole in the skull using a microdrill (WPI, Sarasota FL, USA) and then placed the pre-loaded glass capillary needle into the third ventricle at 300 μm below the surface of the head, using a micromanipulator (Leica Leitz Micromanipulator, Germany). Using a Picospritzer III (Intracellular Microinjection Dispense System, Parker, USA) we released 300 nL of the treatment. Performing the ICV procedure (i.e. drilling the skull and administering the treatment) took a mean 116 seconds per fish. After the administration, the fish was weighed, and transferred to a transparent container (9.5 x 4.5 x 19 cm) within the testing tank where it could recover from the anaesthesia. We considered that the fish had recuperated from anaesthesia when they recovered their balance, reacted to external stimuli, were swimming like non-anaesthetized fish, and had regular opercular movements (Keene *et al.* 1998). In pilot studies, we found that all individuals recovered from anaesthesia within seven minutes. We thus began the behavioural test seven minutes after placing the subject in the recovery partition.

Behavioural test

The behavioural test was performed in a 75 L tank divided into three sealed compartments by transparent plastic partitions, with a water level of 10 cm and a sand covered bottom (Fig. 3.2). The central compartment held the subject, while one edge compartment was empty and the other held a shoal of 5 female guppies. We counterbalanced the position of the shoal and varied the member composition of the shoals at random. There were thermometers and a heater in each compartment and water temperature was maintained at 26 ± 1 °C. To measure subjects' proximity to the shoal, we drew vertical lines marked on the front of the tank to visually divide the central compartment into 10 different zones (each 6 x 31 cm). When the tank was not used for testing, it had a filter in the central compartment and an air stone in each edge

compartment. An opaque black sheet acted as a hide, so that fish could not see the experimenter. Removable white opaque partitions at each side of the central compartment prevented the subject from seeing the side compartments before the beginning of the test. The transparent container that hold the subject and opaque partitions were manually removed by the experimenter seven minutes after the subject was placed in the tank, releasing the subject, then behaviour recording started.

We measured the behaviour of the subjects for 10 minutes at four distinct recording periods during the 160 minutes of the test: (1) immediately after releasing the fish, (2) 30 min, (3) 90 min (4) or 150 min after initial release. We recorded subject location (we considered the subject as shoaling when it was in the area nearest to the shoal), the amount of time spent immobile (i.e., reduced swimming activity or total motionlessness (Millot *et al.* 2009)) and the time spent interacting with the shoal (i.e. swimming head first against the transparent partitions). Interaction time has been suggested as an additional measure for social behaviour that may indicate a greater motivation to socially interact than shoaling (Lindeyer *et al.* 2015). Eight fish were removed from the dataset: seven (5 EB, 1 IT, 1 oxytocin antagonist) displayed impaired and abnormal behaviour, while one behavioural test (oxytocin antagonist) was interrupted by a fire alarm. Fish behaviour was scored live using the software JWatcher V1.0 (Blumstein and Daniel 2007) by an observer blind to the treatments. The data will be available via the Dryad repository.

After the completion of the test, the subject fish were euthanized by immersion in ice water (Wilson *et al.* 2009; Blessing *et al.* 2010; Matthews and Varga 2012). The brain was then collected, snap frozen, and later sectioned. Visual inspection of the brains and brain sections under a microscope allowed us to ascertain the accuracy of the nonapeptide injection, which was possible due to the coinjection of EB during administration. Ten fish were removed from the dataset as the treatment did not reach the ventricle (as confirmed by three observers blind to the treatments).

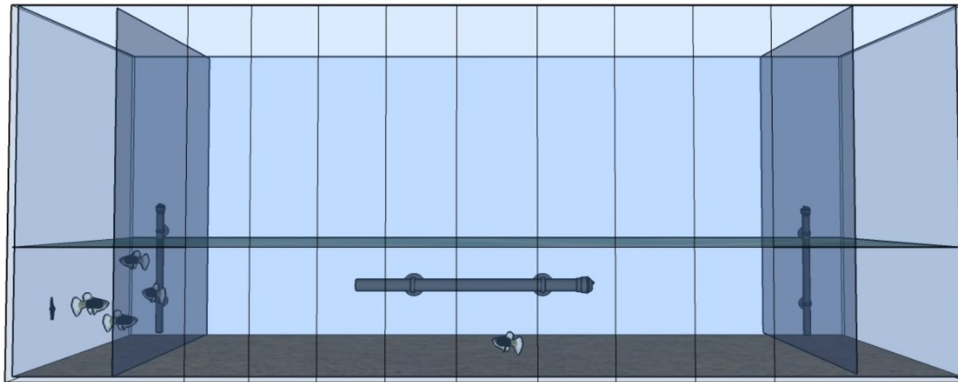

Figure 3.2. Front three-dimensional view of behavioural test tank (75 L, 76 x 31 x 31 cm) divided into three sealed compartments, the central one (60 x 31 x 31 cm) containing the subject, and the edge ones (8 x 31 x 31 cm) containing either a shoal of five female guppies or no fish. All compartments had 10 cm water level, a sand covered bottom, and a heater that maintained the water temperature at 26 ± 1 °C. The central compartment had vertical lines drawn in the glass six cm apart, creating ten zones to score the position of the subject.

Statistical analyses

Prior to the analysis, we removed the data of three fish (two isotocin treated fish, and one oxytocin receptor antagonist treated fish) that were outliers at all timepoints, as assessed via their Cook's distance and leverage values (Crawley 2013), obtaining final sample sizes of N = 12 for isotocin, N = 16 for the receptor antagonist, N = 15 for the EB control, and N = 15 for the saline group. We first ran a Pearson's correlation on each measurement for each observation period to analyse whether measurements were correlated. We then analysed the effect of EB on the behaviour of the fish by comparing it with the fish treated with saline, and then analysed the data of the fish treated with EB, isotocin and the oxytocin receptor antagonist.

We used treatment and recording period as explanatory variables, and the following four response variables: the preference for the shoal (henceforth 'shoaling preference'):

time spent close to the shoal minus time spent close to the empty container (i.e. within 6 cm or less - two to three body lengths – of the partitions at each end of the subject compartment (Pitcher and Parrish 1986; Granroth-Wilding and Magurran 2013)); the time interacting with the shoal minus time interacting with the empty container as a measure of motivation to join the shoal (henceforth 'interaction preference'); the latency to approach the shoal at the start of the test to measure the immediate effect of the treatment on social behaviour (henceforth 'shoaling latency'), and the number of transitions between the 10 different zones to estimate activity of the subjects (henceforth 'zone changes'). When the data were not normally distributed, we square-root transformed (interaction time and zone changes) or log transformed (shoaling latency) the data to achieve normality of residuals. When arithmetic transformations were not able to achieve normality (shoaling preference for the saline vs EB comparison, and interaction preference for the isotocin study), we used a rank transformation followed by analysis of the rank-transformed data (Kepner and Wackerly 1996; Baguley 2012). These rank-transformed analyses (unpublished data) gave identical results to analysis of the untransformed raw data in the case of shoaling preference, and to the logarithmically transformed data in the case of interaction preference (these being the transformations that best approached normality). The results reported for these measures are therefore the results obtained from analysis of the original data and logarithmically transformed data respectively.

We compared the treatments using a linear mixed-effects model (LMM), with recording period as a repeated measure and treatment as a between-subject effect. When there were statistically significant differences between treatments and between recording periods we ran post-hoc Bonferroni-corrected analyses to investigate the differences between each pair of treatments and each pair of recording periods. Even though there was no significant interaction between treatment and recording period for any of the explanatory variables (probably due to a low sample size and a strong effect of recording period), where the LMM revealed a statistically significant effect of treatment we also examined each recording period using one-way ANOVAs and Dunnett's tests

as post-hoc tests to explore whether the effect of a treatment observed in a specific recording period was significantly different from the other treatments, which served to verify at what point after administration the treatments had the strongest effect. Shoaling latency could only be measured in the first recording period, so we used a one-way ANOVA with treatment as the explanatory variable. We ran one-sample t-tests on shoaling preference for each treatment and recording period to confirm that subjects preferred the shoal over the empty compartment and that we were thus measuring grouping propensities (all p-values <0.05; except for EB treatment during recording period 1 ($t(14) = 1.53$, $p > 0.1$). All analyses were computed in SPSS 20.0.0.

Ethical note

All tests and procedures were approved by the by the Animal Care Committee of McGill University (Protocol #7133) and were carried out in accordance with Canadian Council on Animal Care, the Association for the Study of Animal Behaviour, and the US National Research Council 8th edition guidelines. The fish used as shoal were placed into breeding populations at McGill University at the conclusion of the studies.

Results: Experiment 1

Correlations between behavioural measures

As would be expected given the behaviour patterns were measured simultaneously, behavioural measurements generally correlated with one another (Table 3.1). However, they were not strongly collinear, and thus in subsequent analyses they were analysed independently.

Table 3.1. Pearson's correlations for each pair of behavioural measurements per recording period in Experiment 1. Shoaling latency is measured at the start of the test (Recording Period 1) only. N=58. Significant (p < 0.05) correlations are shown in bold.

	Recording period 1		*Recording period 2*		*Recording period 3*		*Recording period 4*	
	r	*P-value*	*r*	*P-value*	*r*	*P-value*	*r*	*P-value*
Shoaling preference vs. Interaction preference	***0.75***	***<0.001***	***0.73***	***<0.001***	***0.64***	***<0.001***	***0.75***	***<0.001***
Shoaling preference vs. Zone changes	***-0.29***	***0.028***	***-0.78***	***<0.001***	***-0.62***	***<0.001***	***-0.57***	***<0.001***
Interaction preference vs. Zone changes	*-0.24*	*0.066*	***-0.60***	***<0.001***	***-0.34***	***0.011***	***-0.39***	***0.003***
Shoaling latency vs. Shoaling preference	***-0.45***	***<0.001***	-	-	-	-	-	-
Shoaling latency vs. Interaction preference	*-0.25*	*0.060*	-	-	-	-	-	-
Shoaling latency vs. Zone changes	***-0.33***	***0.010***	-	-	-	-	-	-

Effect of Evans Blue on behaviour

Evans Blue had a number of impacts on behaviour compared to saline-injected fish. Evans Blue depressed shoaling preference (LMM, $F(1, 111.26) = 32.04$, $p < 0.001$), depressed interaction preference (LMM, $F(1, 102.95) = 24.41$, $p < 0.001$), and increased zone changes (LMM, $F(1, 110.77) = 32.06$, $p < 0.001$), but there was no significant effect on shoaling latency (t-test, $t(28) = 0.55$, $p > 0.1$). Shoaling preference, interaction preference and zone changes all changed across the recording periods (LMMs: $F(3, 51.72) = 6.68$, $p = 0.001$; $F(3, 50.95) = 3.19$, $p = 0.031$; $F(3, 54.05) = 5.43$, $p = 0.002$; respectively), while there were no significant interactions between recording period and treatment for these three behavioural measures (LMMs: $F(3, 51.72) = 0.38$, $p > 0.1$; $F(3, 50.95) = 0.49$, $p > 0.1$; $F(3, 54.05) = 1.43$, $p > 0.1$; respectively). EB had a clear behavioural effect compared to saline, but was essential for confirming successful ICV administration. EB was therefore the appropriate control group and we compared subsequent treatments to EB in both Experiment 1 and 2.

Effects of isotocin on behaviour

Shoaling Preference

Treatment affected shoaling preference (Fig. 3.3A; LMM, $F_{(2, 144.40)} = 4.07$, $p = 0.019$), with isotocin increasing shoaling preference relative to EB control (Bonferroni post-hoc test, $p = 0.025$). There was no significant difference in shoaling preference between fish receiving isotocin versus the antagonist (Bonferroni post-hoc test, $p > 0.1$), or between the fish receiving the antagonist versus the EB control (Bonferroni post-hoc test, $p = 0.096$). There was a significant difference in shoaling preference across recording periods (Fig. 3.3A; LMM, $F_{(3, 85.29)} = 17.88$, $p < 0.001$), with subjects shoaling less on recording period 1 compared to all other recording periods (Bonferroni post-hoc tests, $p \leq 0.001$ for all comparisons).

There was no significant interaction between treatment and recording period (LMM, $F_{(6, 85.29)} = 1.102$, $p > 0.1$), but visual inspection suggested the treatment effect might be driven by differences at recording period 3. We thus ran a one-way ANOVA for each recording period separately and found a significant treatment effect in recording period 3 only (One-way ANOVA, $F_{(2, 40)} = 4.67$, $p = 0.015$; other recording periods: Table S3.1, supplementary materials), with shoaling preference in period 3 significantly greater after isotocin than EB (Dunnett's test, $p = 0.009$) but with no significant difference between the fish treated with the oxytocin receptor antagonist and the EB control group (Dunnett's test, $p > 0.1$). Thus, the treatment effect appears to be largely driven by recording period 3.

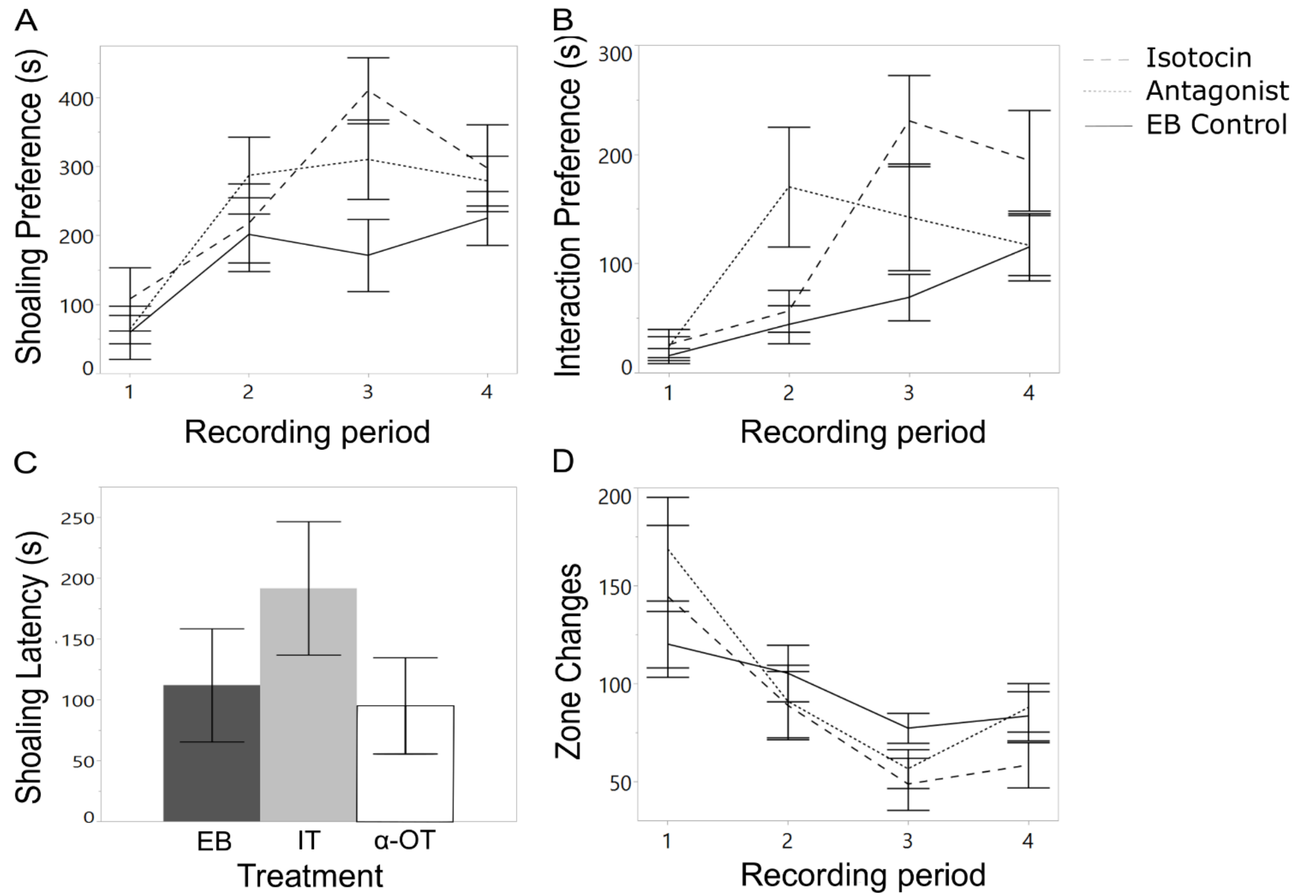

Figure 3.3. Mean ± SEM of fish treated with isotocin (IT: long-dashed line), oxytocin receptor antagonist (α-OT: dotted line), and Evans blue control (EB: solid line) on A) shoaling preference per recording period, B) interaction preference per recording period, C) shoaling latency, and D) zone changes per recording period.

Interaction preference

Treatment affected interaction preference (Fig. 3.3B; LMM, $F(2, 125.40) = 3.80$, $p = 0.025$) with the fish receiving isotocin significantly increasing their preference to interact with the shoal compared to the EB control (Bonferroni post-hoc test, $p = 0.024$). There was no significant difference in interaction preference between fish receiving isotocin versus the antagonist, or the fish receiving the receptor antagonist versus the EB control (Bonferroni post-hoc test, all p-values > 0.1).

There was a significant difference in interaction preference across recording periods (LMM, $F(3, 89.21) = 19.57$, $p < 0.001$), with subjects interacting with the shoal significantly less in recording period 1 compared to all other recording periods (Bonferroni post-hoc tests, Recording period 1 vs 2: $p = 0.015$; 1 vs 3: $p < 0.001$; 1 vs 4: $p < 0.001$). There was no significant interaction between treatment and recording period (LMM, $F(6, 89.21) = 1.92$, $p = 0.086$), but visual inspection suggested the treatment effect might be driven by recording periods 2 or 3. We thus ran a one-way ANOVA for each recording period separately, finding a significant treatment effect in recording period 3 only (One-way ANOVA, $F(2, 40) = 3.48$, $p = 0.041$; other recording periods: Table S3.1, supplementary materials), with isotocin significantly increasing time interacting with the shoal compared to the EB control (Dunnett's test, $p = 0.027$). There was no significant difference at recording period 3 between the fish treated with the receptor antagonist and the EB control group (Dunnett's test, $p > 0.1$). Thus, like shoaling preference, treatment effects on interaction preference appear to be largely driven by the third recording period.

Shoaling Latency

There was no significant difference between treatments in the latency to approach the shoal at the start of the test (Fig. 3.3C; One-way ANOVA, $F(2, 40) = 3.12$, $p = 0.055$).

Zone changes

Treatment did not significantly affect zone changes (LMM, $F(3, 147.03) = 1.70$, $p > 0.1$), and there was no significant interaction between treatment and recording period (LMM, $F(6, 84.52) = 1.00$, $p > 0.1$). There was a significant difference in zone changes across recording periods (Fig. 3.3D; LMM, $F(3, 84.52) = 9.80$, $p < 0.001$), with subjects

changing zones more in recording period 1 compared to recording periods 3 and 4 (Bonferroni post-hoc test, $p \leq 0.001$ for all comparisons).

Experiment 2: Vasotocin

Materials and Methods

Methods, statistical approach and ethical standards were identical to Experiment 1, except for the substances administered. This study involved the injection of 200 ng/µL of either i) vasotocin dissolved in saline and 0.5% EB, ii) 150 ng/µL of the selective vasopressin 1a receptor antagonist, d(CH2)5[Tyr(Me)2,Dab5]AVP (Chan *et al.* 1996), dissolved in saline and 0.5% EB, or iii) 0.5% EB dissolved in saline as a control.

We tested 51 wild stock females and removed from the dataset five subjects that displayed abnormal behaviour (2 vasotocin, 2 vasopressin antagonist, 1 EB) and 7 subjects in which the treatment did not reach the ventricle (as confirmed by 3 observers blind to the treatments), leaving the final sample size at 42 subjects (vasotocin: N = 14; antagonist: N = 15; control: N = 13). As in Experiment 1, we ran one-sample t-tests on shoaling preference to confirm that subjects preferred the shoal over the empty compartment and that we were thus measuring grouping propensities (all p-values < 0.05; except for vasotocin treatment during recording period 1 (t (13) = 1.23, $p > 0.1$)).

The data were not normally distributed. We followed the same transformation procedures as in study 1 (square root for zone changes, and logarithmic for shoaling latency) and used rank transformations when arithmetic transformations were not able to achieve normality of the residuals (shoaling preference and interaction preference) to validate the results of analysis of untransformed raw data for shoaling preference and logarithmically transformed data for interaction preference (these being the

transformations that best approached normality). We report the results obtained from the analysis of the untransformed raw data and logarithmically transformed data as these were the same as for the rank-transformed data (unpublished data).

Results: Experiment 2

Correlations between behavioural measures

As in Experiment 1, behavioural measures generally correlated with one another but were not strongly collinear (Table 3.2), and thus were analysed separately.

Table 3.2. Pearson's correlations for each pair of behavioural measurements per recording period in Experiment 2. N=42. Shoaling latency is measured at the start of the test (Recording Period 1) only. Significant ($p < 0.05$) correlations are shown in bold.

	Recording period 1		Recording period 2		Recording period 3		Recording period 4	
	r	P-value	r	P-value	r	P-value	r	P-value
Shoaling preference vs. Interaction preference	**0.86**	**<0.001**	**0.73**	**<0.001**	**0.65**	**<0.001**	**0.69**	**<0.001**
Shoaling preference vs. Zone changes	-0.16	0.300	**-0.60**	**<0.001**	**-0.51**	**0.001**	**-0.53**	**<0.001**
Interaction preference vs. Zone changes	-0.09	0.567	**-0.47**	**0.002**	-0.29	0.062	-0.30	0.054
Shoaling latency vs. Shoaling preference	**-0.42**	**0.005**	-	-	-	-	-	-
Shoaling latency vs. Interaction preference	-0.30	0.051	-	-	-	-	-	-
Shoaling latency vs. Zone changes	**-0.44**	**0.003**	-	-	-	-	-	-

Effects of vasotocin on behaviour

Shoaling Preference

Treatment did not significantly affect shoaling preference (LMM, $F(2, 140.40) = 0.34$, $p > 0.1$). There was a significant difference in shoaling preference across recording periods (Fig. 3.4A; LMM, $F(3, 82.64) = 16.98$, $p < 0.001$), with subjects shoaling less on recording period 1 compared to the other recording periods (Bonferroni post-hoc test, $p \leq 0.001$ for all comparisons). There was no significant interaction between treatment and recording period (LMM, $F(6, 82.64) = 0.92$, $p > 0.1$).

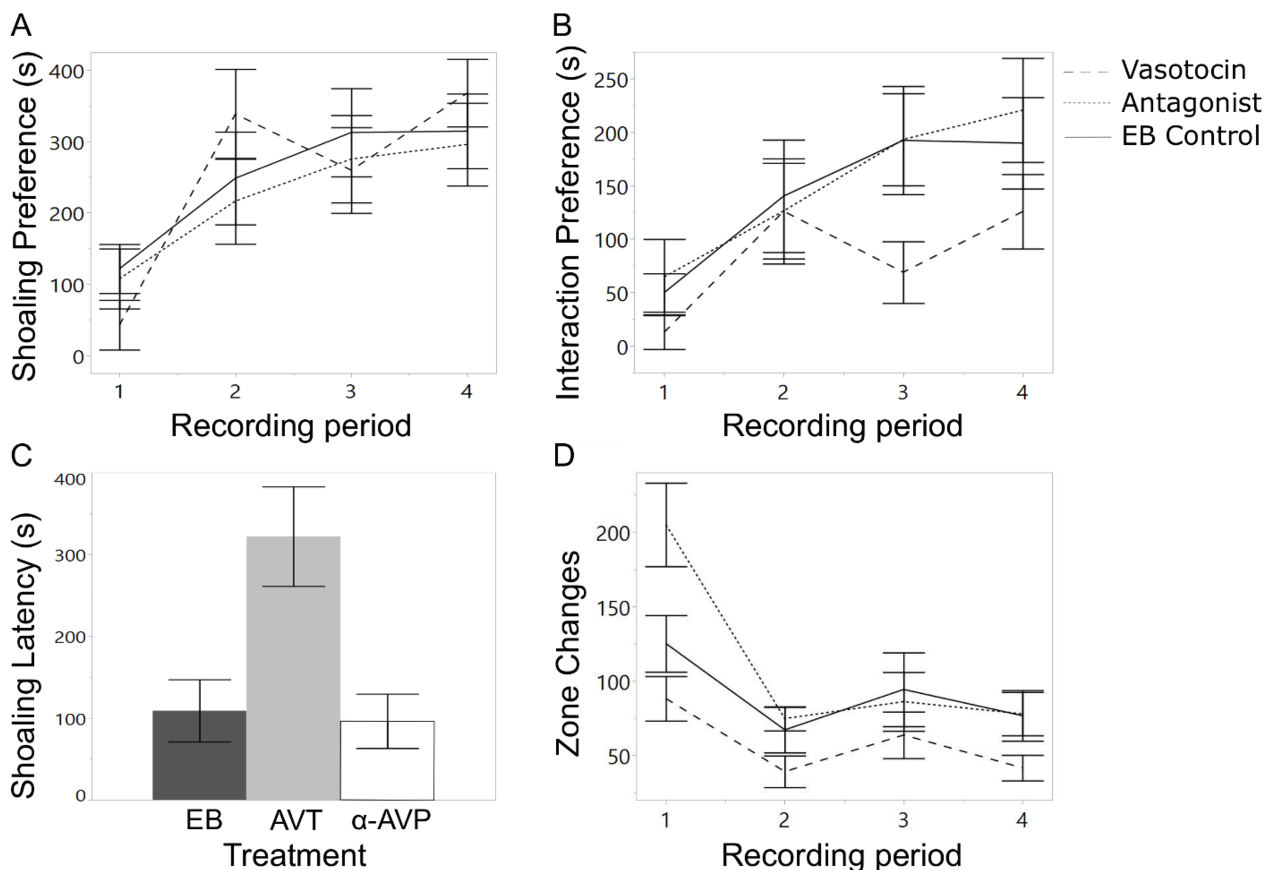

Figure 3.4. Mean ± SEM of fish treated with vasotocin (AVT: long-dashed line), vasopressin receptor antagonist (α-AVP: dotted line), and Evans blue control (EB: solid line) on A) shoaling preference per recording period, B) interaction preference per recording period, C) shoaling latency, and D) zone changes per recording period.

Interaction preference

Treatment affected interaction preference (Fig. 3.4B; LMM, $F (2, 145.77) = 4.02$, $p = 0.020$), with the fish receiving ICV vasotocin spending less time interacting than the fish that received the receptor antagonist (Bonferroni post-hoc test, $p = 0.034$), although not significantly less time than the EB control group (Bonferroni post-hoc test, $p = 0.063$). The fish treated with the receptor antagonist and the EB control group were not significantly different (Bonferroni post-hoc test, $p > 0.1$). There was a significant difference in interaction preference across recording periods (Fig. 3.4B; LMM, $F (3, 75.07) = 11.16$, $p < 0.001$), with subjects interacting with the shoal less on recording period 1 compared to recording periods 2 (Bonferroni post-hoc test, $p = 0.043$), 3 (Bonferroni post-hoc test, $p < 0.001$), and 4 (Bonferroni post-hoc test, $p < 0.001$).

There was not a significant interaction between treatment and recording period (LMM, $F (6, 75.07) = 0.50$, $p > 0.1$), but visual inspection suggested the treatment effect might be driven by differences at recording period 3. We thus ran a one-way ANOVA for each recording period separately, finding a significant treatment effect in recording period 3 only (One-way ANOVA, $F (2, 39) = 3.84$, $p = 0.03$; other recording periods: Table S3.2, supplementary material), with vasotocin significantly reducing interaction preference for the shoal compared to the EB control (Dunnett’s test, $p = 0.047$). There was no significant difference at recording period 3 between the fish treated with the receptor antagonist and the EB control group (Dunnett’s test, $p > 0.1$). Thus, treatment effects on interaction preference appear to be mainly driven by the third recording period.

Shoaling latency

Treatment affected the latency to approach the shoal (Fig. 3.4C; One-way ANOVA, $F_{(2, 39)} = 7.41$, $p = 0.002$), with the fish receiving ICV vasotocin taking more time to approach the shoal compared to the EB control group (Dunnett's test, $p = 0.003$). There was not a significant difference in shoaling latency between the EB control group and the receptor antagonist group (Dunnett's test, $p > 0.1$).

Zone changes

Treatment affected zone changes (Fig. 3.4D; LMM, $F_{(2, 147.62)} = 9.96$, $p < 0.001$), with the fish receiving ICV vasotocin changing zones less than the EB control group (Bonferroni post-hoc test, $p = 0.014$) and the receptor antagonist (Bonferroni post-hoc test, $p < 0.001$). There was not a significant difference in zone changes between the fish receiving the receptor antagonist and the EB control group (Bonferroni post-hoc test, $p > 0.1$).

There was a significant difference in zone changes across recording periods (Fig. 3.4C; LMM, $F_{(3, 77.40)} = 11.89$, $p < 0.001$), with significantly more zone changes in recording period 1 compared to all other recording periods (Bonferroni post-hoc test, Recording period 1 vs 2: $p < 0.001$; 1 vs 3: $p = 0.004$, 1 vs 4: $p < 0.001$).

There was not a significant interaction between treatment and recording period (LMM, $F_{(6, 77.40)} = 0.88$, $p > 0.1$), but visual inspection suggested the treatment effect might be driven by differences at recording period 1. We thus ran a one-way ANOVA for each recording period, finding a significant difference between treatments at recording periods 1 and 2 (Recording period 1: One-way ANOVA, $F_{(2,39)} = 7.38$, $p = 0.002$;

Recording period 2: One-way ANOVA, $F_{(2,39)} = 4.18$, $p = 0.023$; other recording periods: Table S3.2, supplementary materials), suggesting that the overall effects were mainly driven by effects at recording periods 1 and 2. However, no post-hoc tests were significant at recording period 1 or 2 (Dunnett's test, $p > 0.055$).

Discussion

We found that isotocin significantly increased the amount of time fish spent shoaling and interacting with the shoal, especially 90 minutes after administration, and that vasotocin significantly decreased the time spent interacting with the shoal, and latency to shoal. These opposing effects of isotocin and vasotocin on social behaviour resemble the direction of effects on social approach in goldfish (*Carassius auratus*) (Thompson and Walton 2004). In contrast, both mesotocin and vasotocin increased greagariousness in zebra finches (*Taeniopygia guttata*) (Goodson *et al.* 2009; Kelly *et al.* 2011), and in mammals both oxytocin and vasopressin promoted pair bonding in the monogamous praire vole (*Microtus ochrogaster*) (Winslow *et al.* 1993; Williams *et al.* 1994). Goodson (2013) reviewed the conserved role of nonapeptides in the modulation of social behaviours in vertebrates, highlighting differences in the direction of the effects between species and depending on the context and behaviour in question. Our results build on previous research into simple social behaviour in fish, such as social approach in goldfish, and indicate that isotocin and vasotocin modulate grouping in guppies, a fundamental form of social behaviour. This complements previous work showing that nonapeptides also regulate more "complex" forms of social behaviours in teleosts, such as aggression and dominance (Greenwood *et al.* 2008; Backström and Winberg 2009; Reddon *et al.* 2012, 2015; Kagawa *et al.* 2013), pair formation (Oldfield and Hofmann 2011), courtship behaviour (Semsar *et al.* 2001; Salek *et al.* 2002), paternal care (O'Connell *et al.* 2012), cooperative behaviour (Soares *et al.* 2012), and acoustic comunication (Goodson and Bass 2000). These findings indicate extensive roles for nonapeptides in a wide range of social behaviours in teleosts and other vertebrate taxa,

suggest an ancient evolutionary origin for nonapeptides in the control of social behaviour.

We also found differences over time, either due to changes in behaviour or changes in the effects of our treatments over time. Although, in mammals, vasopressin is metabolised in less than one minute (Stark *et al.* 1989) and oxytocin in less than two minutes (Higuchi *et al.* 1985), their behavioural and physiological effects in different vertebrate species can last for several hours (Moore and Miller 1983; Choleris *et al.* 2013) and even for days in humans (Price *et al.* 2017; Rilling *et al.* 2017). To our knowledge, this is the first study that records the effects of nonapeptides on social behaviour in teleosts for more than an hour after recovery from ICV administration. When guppies were treated with isotocin, we found an increase in shoaling and interactions 90 minutes after administration, and when guppies were treated with vasotocin we found a decrease in interactions 90 minutes after administration. Vasotocin also reduced zone changes. Nonapeptides have previously been shown to have anxiolytic effects in fish (Braida *et al.* 2012) and this may explain the reduction in zone changes over time when fish were treated with vasotocin. The opposing effects of isotocin and vasotocin on social behaviour found in our study extend well beyond the expected breakdown of administered nonapeptides, highlighting the importance of studying the effect of nonapeptides over time. The long-lasting effects we observed may be due to secondary effects of nonapeptides within the brain, such as impacts on nonapeptide receptor function or sensitivity (Gimpl and Fahrenholz 2001). It is notable that the the propensities to shoal and interact with the shoal increased over time, even in control subjects, perhaps the result of habituation to the tank or to conspecifics. This provides another possible explanation for our findings, with treatment effects only visible at particular shoaling propensities.

Despite finding overall differences between treatments, we did not find strong responses to either receptor antagonist: fish treated with an oxytocin antagonist behaved similarly to the fish treated with isotocin, and the fish treated with a

vasopressin antagonist behaved similarly to the control group. These results could be due to low affinity of the antagonists for teleost nonapeptide receptors, since the receptor antagonists we used are known to be highly specific for mammalian oxytocin and vasopressin receptors. In mammals, the oxytocin receptor antagonist is considerably more selective than widely used antagonists (Manning *et al.* 1995) and the vasopressin receptor antagonist is more selective to V1a receptors compared to both V2 and oxytocin receptors than is Manning compound (Chan *et al.* 1996). Manning compound is a receptor antagonist used in several previous fish studies (Thompson and Walton 2004; Santangelo and Bass 2006; Oldfield and Hofmann 2011), and may have a higher sensitivity to teleost receptors than the highly specific receptor antagonists that we used in this study. This would explain differences between the antagonist effects in our and previous teleost studies. Since the molecular structures of isotocin and vasotocin are so similar, it is also posible that the receptor antagonists were binding non-specifically to both isotocin and vasotocin receptors. Another posibility is that the concentration of receptor antagonist we used was insuficient to displace endogenous nonapeptides and hence impeded the observation of a behavioural effect. Future studies of nonapeptide function on social behaviour in teleosts would be greatly enhanced by the availability of antagonists that bind specifically to isotocin or vasotocin receptor antagonists.

Unexpectedly, we also found that Evans Blue significantly decreased social behaviours and increased activity relative to saline. Evans Blue is a modulator of mammlian AMPA receptors (Leßmann *et al.* 1992; Schürmann *et al.* 1997), which are glutamate receptors involved in behaviour (Kessels and Malinow 2009) including social and aggressive behaviours in rodents (Vekovischeva *et al.* 2007; Wang *et al.* 2011; Adamczyk *et al.* 2012; Gascon *et al.* 2014). In teleosts, AMPA receptors are involved in escape behaviour in goldfish (*Carassius auratus;* Patten and Ali 2007; Mirjany and Faber 2011), and glutamate receptor genes are upregulated in group-housed three-spine sticklebacks (*Gasterosteus aculeatus*; Greenwood and Peichel 2015). To our knowledge, the effect of AMPA receptor modulation on behaviour in teleosts has not

been studied and while speculative, the effects we saw of Evans Blue may have been mediated via AMPA receptors. This may have prevented a ceiling effect of endogenous isotocin, which could potentially have prevented detection of any effect of isotocin on social behaviour had we used only saline as a control.

In our study, we have also demonstrated that ICV techniques can be effectively used for central administration even in such small fish as guppies. This technique is a rapid, safe and inexpensive method that ensures the administered substance circulates throughout the brain via the ventricular system (Barbosa *et al.* 2012). Although it has technical challenges, ICV administration in small fish allows for more rigorous pharmacological manipulation of behaviour and opens up novel species, such as the intensively studied zebrafish and guppy, for behavioural neuroscience research.

In conclusion, our results show that isotocin and vasotocin modulate fundamental forms of social behaviour such as grouping, and supports the idea of an ancient evolutionary origin for the role of nonapeptides in social behaviours. We have found effects of nonapeptides on social behaviour beyond the expected time of breakdown for administered nonapeptides, highlighting the importance of measuring behavioural effects over time. We also confirmed the efficacy of ICV injection in small teleost fish and suggest this technique be more widely used when peripheral administration may not allow substances to cross the teleost blood brain barrier.

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Supplementary material

Manufacture of glass capillary needles

Glass capillary needles were manufactured using a micropipette puller (P-97, Sutter Instruments, USA) and glass capillary filaments (1 x 0.58 mm, 4”, A-M Systems, WA). We obtained a pair of identical needles for each pulled glass capillary, however, we observed variation in needles between pairs. The volume of substance released by each needle depends on the characteristics of each needle tip, the viscosity of the solution, the air pressure of ejection, and the duration of the ejection. We needed to ensure that a known and fixed volume was administered during our study, thus, we used one of each pair of identical needles to administer the experimental treatment and the other to calibrate the duration of application of constant pressure (20 psi) needed to eject 300 nL for that pair of needles. We determined the volume of substance released by ejecting the selected solution at a constant ejection pressure (20 psi) with different durations of ejections. Solutions were injected into mineral oil and the diameter of the resulting drop was measured, allowing the volume of the sphere for each ejection to be calculated, and the appropriate duration of ejection to deliver 300nl to be determined.

Supplementary tables

Table S3.1. Treatment comparisons per recording period for the isotocin experiment (One-way ANOVAs). Significant results are in bold.

	Isotocin Experiment	
	Shoaling preference	Interaction preference
Recording period 1	$F(2, 40) = 0.54$, $p = 0.59$	$F(2, 40) = 0.25$, $p = 0.77$
Recording period 2	$F(2, 40) = 0.71$, $p = 0.50$	$F(2, 40) = 2.77$, $p = 0.075$
Recording period 3	**$F(2, 40) = 4.67$, $p = 0.015$**	**$F(2, 40) = 3.48$, $p = 0.041$**
Recording period 4	$F(2, 40) = 0.69$, $p = 0.51$	$F(2, 40) = 1.20$, $p = 0.312$

Table S3.2. Treatment comparisons per recording period for the vasotocin experiment (One-way ANOVAs). Significant results are in bold.

	Vasotocin experiment	
	Interaction preference	Transitions between zones
Recording period 1	$F(2, 39) = 1.45$, $p = 0.25$	**$F(2, 39) = 7.38$, $p = 0.002$**
Recording period 2	$F(2, 39) = 0.01$, $p = 0.98$	**$F(2, 39) = 4.18$, $p = 0.023$**
Recording period 3	**$F(2, 39) = 3.84$, $p = 0.030$**	$F(2, 39) = 0.47$, $p = 0.63$
Recording period 4	$F(2, 39) = 1.26$, $p = 0.30$	$F(2, 39) = 1.88$, $p = 0.17$

Linking statement to Chapter 4

In the previous chapters, I studied the neuroendocrinal mechanisms of grouping behaviour. Being part of a group implies constant exposure to social information; and being able to properly process and respond to these stimuli is, in many cases, essential for the survival and reproductive success of individuals. An important example pertinent to my thesis is the survival benefit of responding to social cues like alarm substance in fish (a substance in the skin of fish released into the water after skin damage, acting as an honest cue of predation risk). Despite the evolutionary importance of social information use, its underlying neural mechanisms have not been widely examined across taxa. Thus, in Chapter 4, I studied the brain activation during exposure to alarm cue and during social contagion of the alarm response.

Chapter 4: Neuronal responses in the social decision-making network during social contagion of alarm in Trinidadian guppies

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Keywords: teleost, social information use, social decision-making network, alarm substance, fear, immediate early genes.

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Abstract

Alarm substances are chemical cues present in the skin of many fish that are released into the water after mechanical damage, as would occur in a predation event. Fish exposed to alarm substance respond with anti-predator behaviour, as do fish observing alarmed fish. Such alarm responses thus provide a valuable and ecologically-relevant context to study social information use and the neural mechanisms underpinning it. We exposed female guppies to alarm substance, and these fish acted as ‘demonstrators’ to ‘observers’ that were visually exposed to the demonstrators but were not directly exposed to alarm substance. Alarm substance exposure provoked a strong behavioural response in the demonstrators compared to controls, while the observers matched some elements of this response but did not engage in a full alarm response. We measured neuronal *egr-1* expression as an indicator of neural responses across six fish brain areas that have been implicated as modulating social behaviour and stress. We found broad upregulation of *egr-1* expression across the brain areas examined, and evidence for changes in functional connectivity between these areas under the different experimental treatments. Our results suggest that these areas act together in

modulating responses to alarm substance and alarmed conspecifics. Our results provide a novel insight into the study of social information use, paving the way to further understanding of the neural mechanisms underlying this process.

Introduction

Recognizing a threat and responding appropriately can be critical to individual survival. Individuals can respond with alarm to threatening situations they experience directly, but individuals can also assess threat with social cues from other individual, a phenonemon that is widely observed in many species (Olsson and Phelps 2007). Social cues provide a lower-risk method of assessing risk compared to direct experience (Danchin *et al.* 2004), although there is also the chance of acquiring inappropriate fear responses such as phobias (Debiec and Olsson 2017). Understanding the neural mechanisms underlying the social contagion and transmission of alarm information is relevant to a broad body of researchers, from ecology to psychology, and may also provide insights into the neural mechanisms of social transmission of information more broadly, an area of active controversy (e.g. Heyes 2012; Leadbeater 2015; Reader 2016).

In fish, several families of teleosts show an alarm response when exposed to a substance called *schreckstoff* or alarm substance, produced and stored in the club cells of their skin (Pfeiffer 1967) or analogous epidermal club cells (Ferrari et al. 2010). When the skin is damaged, as would happen in a predation event, alarm substance is released into the water. Alarm substance allows nearby fish to quickly detect and react to threatening events (Chivers *et al.* 2007), responding with several anti-predator behaviours (Smith 1992). For example, fish freeze (i.e., show reduced swimming activity or total motionlessness; Millot *et al.,* 2009), dash (i.e., a sudden burst of seemingly disoriented swimming; Brown *et al*., 2009), group tightly with others, and avoid the area where the alarm substance was detected (Chivers and Smith 1998). As well as allowing fish to make a rapid local response to predation threat, fish readily learn about novel

threats that are associated with alarm cue, allowing them to respond to future threats as well (Kelley and Magurran 2003; Brown 2003; Griffin 2004).

Alarm responses can be socially transmitted to conspecifics based on not only the alarm cue but also by exposure to alarmed conspecifics. This can allow individuals to learn about unfamiliar threats. For example, zebrafish (*Danio rerio*) learn to react to a novel odorant by pairing it with alarm substance, and when these fish were placed with naïve conspecifics the naïve fish acquired a similar alarm response when exposed only to the novel odorant (Suboski *et al.* 1990). Similar findings have been demonstrated between heterospecifics (Mathis *et al.* 1996) and to fish species that cannot detect alarm substance, such as naïve sticklebacks (*Gasterosteus aculeatus*) living with alarm-responsive chubs (*Leuciscus cephalus*; Krause 1993). Social transmission of alarm responses facilitates an effective anti-predator reaction towards predators that the individual has not yet encountered. Here, we focussed on social responses to alarm, comparing responses to direct exposure to alarm substance with responses to exposure to an alarmed conspecific.

Even though extensive work has investigated behavioural responses to alarm cues and the social transmission of alarm responses (Kelley and Magurran 2003; Brown 2003; Griffin 2004; Ferrari *et al.* 2010), the neural mechanisms involved in such phenomena have received relatively little attention, particularly outside of mammals (Twining *et al.* 2017). There is evidence, however, that exposure to alarm substance impacts activation of several brain regions in teleost fish. For example, in zebrafish, exposure to alarm substance causes co-activation of brain areas whose putative mammalian homologues are involved in fear and stress, such as the medial part of the dorsal telencephalon (Dm), the supracommissural nucleus of the ventral telencephalon (Vs), and the preoptic area (POA; mammalian putative homologues in Table 4.1; Faustino *et al.* 2017). Faustino *et al.* (2017) also studied the impact of the presence of unalarmed conspecifics, which 'socially buffered' behavioural alarm responses, on neural co-activation. They found the presence of unalarmed conspecifics made little difference in

the degree of activation in these regions, but did find evidence that the pattern of co-activation was changed. To our knowledge, no other work has investigated the neural mechanisms involved in processing fish alarm cues or responses to alarmed conspecifics.

Table 4.1. Brain areas studied, their mammalian homologues, the brain network that they belong to (Wullimann and Mueller 2004; O'Connell and Hofmann 2011; Goodson and Kingsbury 2013), and the mean size of the oval sampling areas used to count the number of stained nuclei in each of the six brain areas.

Fish brain area	**Mammalian putative homologue**	**Brain network**	**Sampling area (μm^2)**
POA: Preoptic Area	Preoptic Area and VPN	Social behaviour network	4854
Vd: Ventral telencephalon – dorsal part	Nucleus accumbens and striatum	Mesolimbic Reward System	7475
Vs: Ventral pallium – supracommissural part	Amygdala/Bed nucleus of the stria terminalis	Social behaviour network & Mesolimbic Reward System	7270
Vv: Ventral telencephalon – ventral part	Lateral septum	Social behaviour network & Mesolimbic Reward System	5773
Dm: Dorsal telencephalon – medial part	Basolateral amygdala	Mesolimbic Reward System (Alarm behaviour)	5648
Dl: Dorsal telencephalon – lateral part	Hippocampus	Mesolimbic Reward System (Alarm behaviour)	21807

Other teleost brain regions potentially involved in the processing of social information related to alarm are the lateral part of the dorsal telencephalon (Dl) and the dorsal and ventral parts of the ventral telencephalon (Vd and Vv). Dl is involved in fear and stress in fish (Silva *et al.* 2015), while Vd and Vv (putative homologues of the nucleus accumbens and lateral septum respectively) have not been found to be involved in fear and stress in fish, but rather in social behaviour (reviewed in O'Connell and Hofmann 2011). However, in mammals, the nucleus accumbens is involved in fear and fear learning (Levita *et al.* 2002), and the lateral septum is involved in the expression of fear, although not in acquisition nor consolidation of fear (Reis *et al.* 2010), and so the putative homologues in fish could have similar functions. Also, in sea bream (*Sparus*

aurata), immediate gene expression in Vv is associated with processing of stimulus valence (Cerqueira *et al.* 2017). All these areas are part of the social decision-making network, a brain network proposed to be involved in the processing of social behaviours in all vertebrates (O'Connell and Hofmann 2011). We hence investigated the expression of immediate early genes in these six brain areas (Dm, Dl, Vd, Vv, Vs, POA) to study the role of these regions in the modulation of alarm responses and the social contagion of alarm.

Here, we studied behavioural and neural responses to exposure to either alarm substance or an alarmed conspecific, with the aim to improve current understanding of social information use in vertebrates and underlying neural mechanisms. We conducted our study on guppies because of the abundant literature on this species on both alarm substance responses and on social information use and social learning (Laland and Williams 1997, 1998; Brown and Godin 1999; Kelly and Godin 2001; Brown 2003; Reader *et al.* 2003; Kelley and Brown 2006; Brown *et al.* 2010, 2014; Elvidge and Brown 2015; Swaney *et al.* 2015; Stephenson 2016). We hypothesized that fish exposed to alarm substance will show typical anti-predator responses, and observer fish watching these alarmed 'demonstrators' will show similar responses, but that different neuronal responses will be observed in demonstrators versus observers. We measured neuronal responses by measuring expression of an immediate early gene, *egr-1*, widely employed to map functional connectivity in the brain (Burmeister and Fernald 2005; Duclot and Kabbaj 2017).

Materials and methods

Overview

We housed fish individually, placing pairs of tanks together in a demonstrator-observer pair, or in a control-control pair, so subjects could see their paired conspecific. In the

ten-minute Phase 1, we measured the baseline behaviour of the fish when exposed to conditioned water. We then measured behaviour for the first ten minutes of the 60-minute Phase 2, in which the demonstrator fish were exposed to alarm substance, the observer fish were exposed to conditioned water, and both fish in the control pairs were exposed to conditioned water. After Phase 2, we used immediate early gene expression (*egr-1*) to assess neuronal activation in six brain areas.

Animal subjects, housing and apparatus

Subjects were 80 female guppies from mixed wild Trinidadian Northern-range populations that had been bred in captivity for at least two generations. Three days before beginning testing, we isolated 16 subject females into sixteen 9 L testing tanks to habituate them to the tanks (Fig. 4.1). Each tank was alongside another tank lengthways, allowing two fish to view one another, while opaque partitions prevented the subjects from seeing other tanks. This procedure was replicated in five batches.

Half of each testing tank was designated a ‘sheltered area’, containing three plastic plants (replicates of *Ludwigia sp.*) and a heater (keeping the water at 26 ± 1 °C), while the other half was empty and designated an ‘unsheltered area’. There was no gravel to facilitate cleaning. Two GoPro Black 3+ cameras (GoPro Inc., CA, USA) were used to record the behaviour of each subject from outside the tanks. On the day of the test a transparent plastic sheet with one vertical and three horizontal lines drawn on it was placed on the length of the tanks to facilitate behavioural scoring by dividing the tank into six visual zones (5 x 15 cm each, three on the unsheltered side and three on the sheltered side. Fig. 4.1).

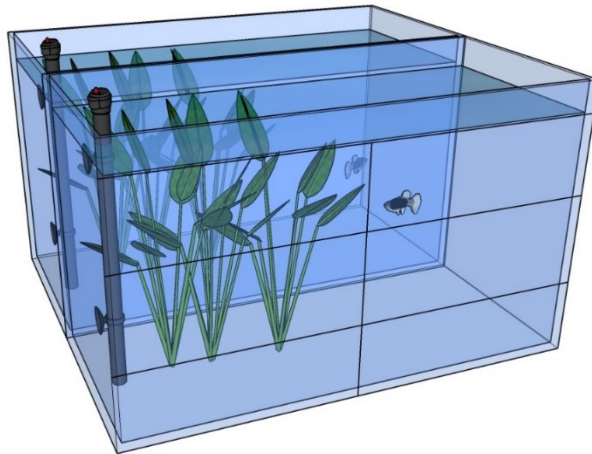

Figure 4.1. Testing aquarium (9 L, 30 x 15 x 20 cm, water depth 15 cm). One half of the tank contained shelter (plastic aquarium plants) while the other half was an unsheltered area. A transparent plastic sheet with lines drawn on it was used to differentiate between these two areas and three vertical zones.

Behavioural test

At the beginning of the day, a mix of male and female guppies were collected to extract alarm cue following previous protocols (Brown and Godin 1999; Brown *et al.* 2009). Briefly, after euthanizing them by rapid cooling through immersion in iced water (Wilson *et al.* 2009; Blessing *et al.* 2010; Matthews and Varga 2012), we removed the head, tail and viscera, leaving only skeletal muscle and skin. All carcasses were added to 10 mL of dechlorinated water, homogenized and the solution filtered through polyester filter floss. The concentration was adjusted to 0.1 cm^2 of skin/mL, again following previous protocols (Brown and Godin 1999; Brown *et al.* 2009). The alarm cue was kept on ice during the day, together with the conditioned water used as control.

The behavioural test consisted of two phases. At the beginning of Phase 1, we added 3 mL of conditioned water with a syringe to the surface of both tanks and scored the baseline behaviour of the subjects. After ten minutes, Phase 2 began and we added 3

mL of alarm cue to one tank and conditioned water to the adjacent tank (randomized and selected before baseline behaviour was scored, with a syringe, to create the alarm-cue-exposed (henceforth ‘demonstrators’) and observer groups respectively. As a control treatment, we used another pair of tanks that received 3 mL of conditioned water during Phase 2. On each phase, we scored for 10 minutes the amount of time freezing (by timing every instance when the fish became motionless for more than one second), in close ‘contact’ proximity with the fish in the adjacent tank (i.e. swimming against the shared tank glass, a measure similar to ‘interaction time’ in Cabrera-Álvarez *et al.* (2017) and Lindeyer *et al.* (2015), henceforth, ‘interaction time’), and the position of the subjects on each of the six different areas of the tank, using JWatcher 1.0 (Blumstein and Daniel 2007). Freezing is a stress response frequently observed in response to predators (Domenici 2010). The behaviour of the fish in the foremost tank was video recorded and live scored, while the behaviour of the fish in the back tank was video recorded for later behavioural scoring. Fifty minutes after we stopped scoring the behaviour (i.e. 60 min since beginning of Phase 2), one of the two control subjects and both the demonstrator and observer subjects were euthanized by rapid cooling through immersion in ice water (Wilson *et al.* 2009; Blessing *et al.* 2010; Matthews and Varga 2012), weighed, and the brains collected and put in 4% paraformaldehyde for immediate early gene expression analysis. The remaining control subject was placed at a housing tank at the conclusion of the day and kept at our facilities for breeding purposes. Between tests, tanks were emptied and rinsed with alcohol. Once the tanks were dry, they were washed, refilled, and new fish were added. For each batch of 16 fish we commenced with a different test type (demonstrator-observer or control-control) to minimize any effects of test order.

Immunohistochemistry (IHC) staining and quantification of neurons expressing *egr-1*

We followed the same protocol described in Cabrera-Álvarez *et al.* (2017) to process the brains, stain them and quantify the neurons expressing *egr-1*. Brains were dissected out immediately after euthanasia, fixed, cryoprotected, embedded in Clear Frozen

Section Compound (VWR International, PA, USA) and stored at -19 °C. Brains were then sectioned on a cryostat at 20 μm and thaw-mounted onto Superfrost Plus slides (VWR International) in two parallel series. Samples were stored at -19 °C for less than a week before processing for IHC following the aforementioned protocol.

We imaged the brain using a 20× objective in a microscope (Leica DM1000LED). We used the brain atlas of the related poeciliid, the swordtail (*Xiphophorus hellerii*; Anken and Rahmann, 1994) to distinguish the brain areas of interest (Table 4.1). We took a picture of each brain area of interest in both hemispheres using a digital camera (Leica ICC50HD with the software Leica Application Suite EZ 3.2.1). An observer blind to the experimental treatments processed all images and counted stained nuclei using ImageJ 1.50i. We processed the image and defined a single oval sampling area that fitted centrally within each brain area of interest (Table 4.1) and counted the number and size of stained nuclei that met minimum size and circularity criteria, as described in Cabrera-Álvarez *et al.*, 2017. The ImageJ script used for image processing and the data will be deposited in the Dryad Digital Repository.

Statistical analysis

Behaviour

We analyzed the effect of treatment (demonstrator fish, observer fish, control fish), phase (Phase 1: baseline behaviour, and Phase 2: post-treatment behaviour), and the interaction of treatment and phase for each of the measurements (freezing time, interaction time, and time in the two upper and two lower zones), using a linear mixed model (LMM), with phase as a repeated measure. This allowed us to examine, in a single analysis, changes over phases (including any habituation effects) and whether changes over phases differed with the experimental treatments (i.e., the interaction

effect). We excluded the middle zones from the analysis because the subjects used them as transition zones between top and lower areas (MJCA personal observation; Fig. 4.3, green lines and symbols). We included fish dyad as a random effect to account for the fact that fish could behave differently depending on the response of the fish with which they were paired. When the data were not normally distributed, we square-root transformed (time in tank areas) or logarithmically transformed (freezing and interaction time) the data to achieve normality of residuals.

When there were statistically significant differences between treatments, we ran post-hoc LSD analyses to investigate the differences between each pair of treatments. When there were significant interactions between treatment and phase for any of the explanatory variables, we calculated the difference between Phase 2 and Phase 1 of the specific measurement and ran Tukey post-hoc tests in order to examine whether there were differences between treatments.

One observer and one demonstrator from different dyads were not included in the behavioural analysis because video faults prevented data collection. One control subject died prior to testing, so its partner subject was not used for the study. The final sample size for the behavioural measurements was thus 20 subjects for the demonstrator group, 20 subjects for the observer group and 36 subjects for the control group.

Neuronal *egr-1* expression

We counted the number of neurons expressing *egr-1* in each hemisphere and calculated the number of neurons per 100 µm2 to standardize measurements across brain areas (n=60, i.e. 21 demonstrators, 21 observers, 18 control). The number of neurons expressing *egr-1* per hemisphere were positively correlated across individuals

(Pearson correlations; POA: $r = 0.74$, $n = 59$, $p < 0.001$; Vd: $r = 0.67$, $n = 57$, $p < 0.001$; Vv: $r = 0.70$, $n = 58$, $p < 0.001$; Vs: $r = 0.69$, $n = 59$, $p < 0.001$; Dm: $r = 0.80$, $n = 60$, $p < 0.001$; Dl: $r = 0.40$, $n = 59$, $p = 0.002$) supporting the pooling of the counts from the two hemispheres and the reliability of our brain area identification and counts.

We analysed the effect of treatment (demonstrators, observers, control) and the interaction between social treatment and brain area (Dl, Dm, POA, Vs, Vd, Vv) using a linear mixed model (LMM), with brain area as a repeated measure, and dyad (i.e., each pair of subjects) as a random effect to account for our paired observations not being independent from each other. We square-root transformed the data to achieve normally distributed residuals. We ran one-way ANOVAs on each brain area to inspect which brain areas were driving the overall effect of treatment. Here, when the residuals were not normally distributed we square-root or logarithm transformed the data to achieve normally distributed residuals. We also ran this analysis with olfactory bulb *egr-1* expression as a covariate to account for any difference in general *egr-1* expression between individuals. The olfactory bulb was chosen as an area exterior to the brain networks under study. We confirmed that there were no significant differences between treatments in olfactory bulb *egr-1* expression (logarithmically transformed data, one-way ANOVA, $F(2,57) = 1.42$, $p = 0.25$). However, as a robustness check we present these results with and without the olfactory bulb covariate given that the choice of covariate brain area could impact our findings.

Since the SDMN functions as a network (O'Connell and Hofmann 2011; Teles *et al.* 2015), we examined co-activation between areas by running social network analyses (see Wong *et al.* 2012; Teles *et al.* 2015; and Faustino *et al.* 2017 for similar approaches). Network analyses are pertinent in our case because our observations are not independent of each other (i.e., the data of the POA in an individual brain is not independent for the data on the Vv, Vd, Vs, Dl, and Dm of that same brain). *Egr-1* expression was not normally distributed and is a non-linear response variable, so we computed Spearman's correlations between *egr-1* expression in each pair of brain

areas for each treatment, as well as partial Spearman's correlations using the olfactory bulb as a covariate as a method of examining correlations between *egr-1* expression after accounting for brain-wide changes in *egr-1* expression, producing heatmaps of the two types of correlation matrices for visual analysis. These matrices represent *egr-1* co-activation between brain areas, with positive correlations showing that *egr-1* expression in two areas increases together, and negative correlations showing that *egr-1* expression in one area increases while decreasing in the other.

To study whether the co-activation of brain nuclei was different from a baseline pattern of co-activation, we constructed a null network for each treatment by generating a random correlation matrix by swapping rows at random across treatments, keeping the number of fish in each treatment constant, and using 5000 permutations. We then compared our observed correlation matrices for each treatment with this null network. We used the IBM software SPSS 24 for all our analyses, except for the network analyses, which we ran using R version 3.4.3 (R Core Team 2018).

Ethical note

All tests and procedures were approved by the by the Animal Care Committee of McGill University (Protocol # 2012-7133 and 2015-7708) and were carried out in accordance to the Canadian Council on Animal Care and Association for the Study of Animal Behaviour guidelines. The remaining control subjects were placed into breeding populations at McGill University at the conclusion of the studies.

Results

Behaviour

We found a significant interaction between treatment and phase in the time guppies were freezing (Fig. 4.2, LMM, $F(2, 59.9) = 6.16$, $p = 0.004$). That is, although guppies froze more on Phase 2 than Phase 1 (LMM, $F(1, 59.9) = 19.86$, $p < 0.001$), this effect differed between treatments. Tukey post-hoc tests on differences between Phase 1 and 2 revealed that demonstrators increased their freezing behaviour in Phase 2 compared to the control ($p = 0.047$), but this effect was not significant when demonstrators were compared to the observers ($p = 0.089$), and there was no significant difference between the observers and the control group ($p > 0.1$). As expected, given that the Phase 1 baseline is the same for all treatments, we did not find an overall effect of treatment (Fig. 4.2, LMM, $F(2, 134.4) = 1.39$, $p > 0.1$).

We found a significant effect of treatment on the time spent in the lower unsheltered area (Fig. 4.3, Table 4.2), with both the demonstrator and observer groups spending more time there compared to the control group (LSD test, demonstrator vs. control: $p = 0.001$, observer vs. control: $p < 0.001$, demonstrator vs. observer: $p > 0.1$). There was also a significant interaction of treatment by phase on time spent in the lower unsheltered area (Fig. 4.3, Table 4.2). That is, guppies spent more time in the lower unsheltered area on Phase 2 than Phase 1 (Table 4.2), but this effect differed between treatments. Tukey post-hoc tests on differences between Phase 1 and 2 revealed that demonstrators significantly increased their time in the lower unsheltered area in Phase 2 compared to the control group ($p = 0.004$), with no significant differences between the observer group and the demonstrators ($p > 0.1$) or the observer and the control group ($p > 0.1$). The interaction effect suggests that the overall treatment effect was driven by behavioural differences on Phase 2.

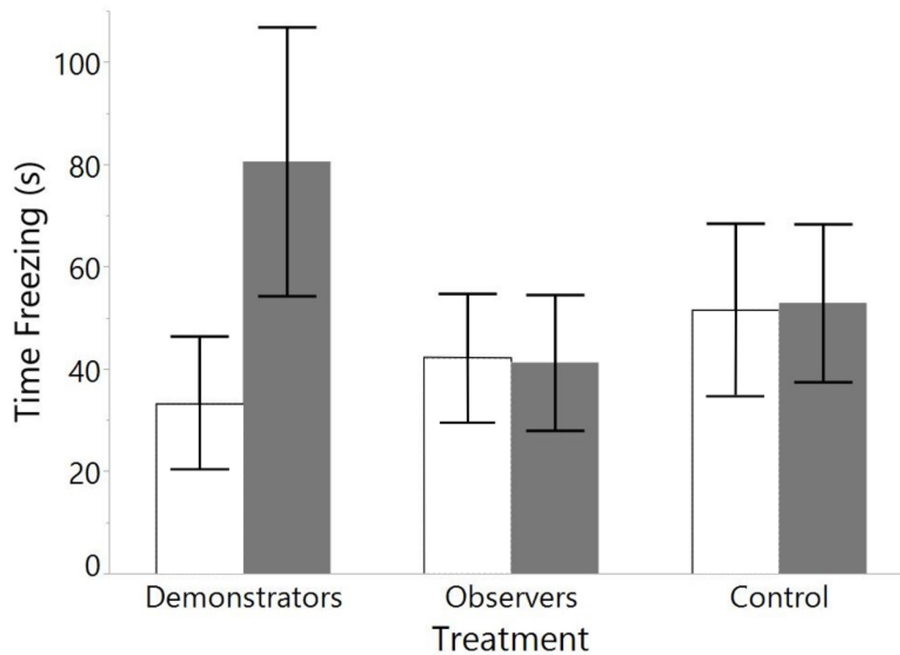

Figure 4.2. Mean ± SEM time freezing (s) per treatment, in Phase 1 (open bars) and Phase 2 (filled bars). Each Phase was 10 minutes (600 s).

Examining time spent in the other three tank areas, we did not find a significant interaction between treatment and phase (Table 2, Fig. 4.3), nor significant differences between treatments (Table 4.2, Fig. 4.3). Time in the top sheltered and top unsheltered areas were significantly reduced in Phase 2 (Table 4.2, Fig. 4.3). We also examined interaction time and did not find a significant interaction between treatment and phase (LMM, $F(2, 59.1) = 0.49$, $p > 0.1$), a significant difference between treatments ($F(2, 131.5) = 1.53$, $p > 0.1$), or a significant difference between phases ($F(1, 59.1) = 0.00$, $p > 0.1$).

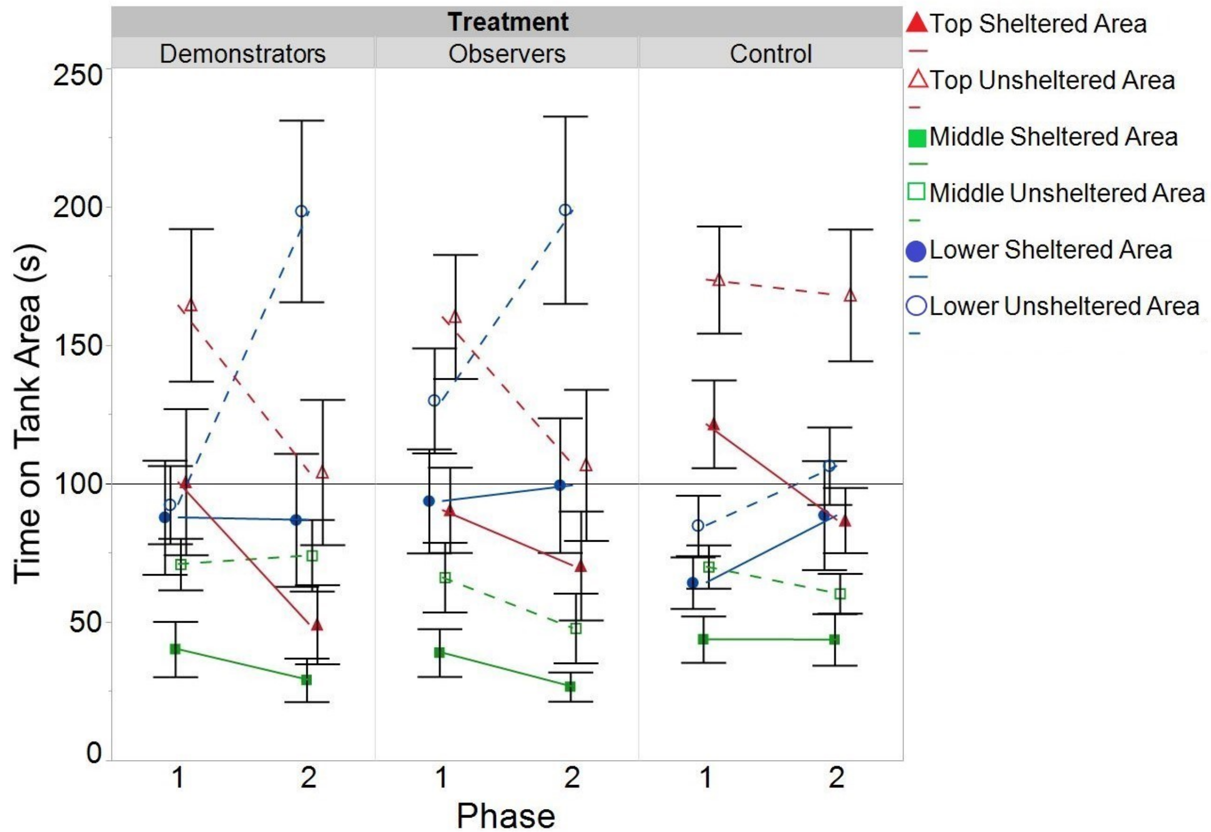

Figure 4.3. Mean ± SEM time spent in each tank area (filled symbols and continuous line: sheltered area; open symbols and dashed lines: unsheltered area; blue circles: lower area; green squares: middle area; red triangles: top area) per treatment per phase. Each phase was 10 minutes (600 s). The horizontal line at 100 seconds represents the expected time per area if fish were swimming at random.

*Table 4.2. LMM analyses of time subjects spent in each of four tank areas, for Treatments: Demonstrators, Observers, Control; Phase: 1 or 2; and Interaction of Treatment * Phase. Significant values are in bold.*

Area	Treatment	Phase	Treatment*Phase
Lower Sheltered	F (2, 127.1) = 2.25 P = 0.11	F (1, 47.4) = 0.18 P = 0.67	F (2, 47.4) = 0.05 P = 0.95
Lower Unsheltered	**F (2, 136.1) = 11.63** **P < 0.001**	**F (1, 62.7) = 22.29** **P < 0.001**	**F (2, 62.7) = 3.25** **P = 0.050**
Top Sheltered	F (2, 117.4) = 2.13 P = 0.12	**F (1, 63.3) = 11.14** **P = 0.001**	F (2, 63.3) = 0.73 P = 0.49
Top Unsheltered	F (2, 141.4) = 1.16 P = 0.32	**F (1, 141.4) = 5.72** **P = 0.018**	F (2, 141.4) = 0.85 P = 0.43

Neuronal *egr-1* expression

Treatment had a significant effect on *egr-1* expression across the brain areas examined (Fig. 4.4; LMM, F (2, 57.2) = 4.98, p = 0.010), with significantly more *egr-1* expression in demonstrators than the observers (LSD, p = 0.003) and the control fish (LSD, p = 0.035), but there was no significant difference between the observers and the control (LSD, p > 0.1). Although the treatment by brain area interaction was not statistically significant (LMM, F (10, 78.2) = 1.0, p > 0.1), the treatment effect appeared to be mainly driven by increases in *egr-1* expression in area Vs (Fig. 4.5; One-way ANOVA, F(2,56) = 4.40, p = 0.017; demonstrators had significantly higher expression than the observers (Tukey, p = 0.026) and higher, but not statistically significant expression compared to the control (Tukey, p = 0.052)) and area Dl (Fig. 4.5; One-way ANOVA, F(2,56) = 3.96, p = 0.025; demonstrators had significantly higher expression than the observers (Tukey, p = 0.034) and higher, but not statistically significant expression compared to the control (Tukey, p = 0.074)). Expression did not significantly differ across treatment in other brain areas (all p-values > 0.05, Supplementary material Table S4.1). Brain areas differed from one another in *egr-1* expression (Fig. 4.5, LMM, F (5, 78.2) = 93.5, p < 0.001).

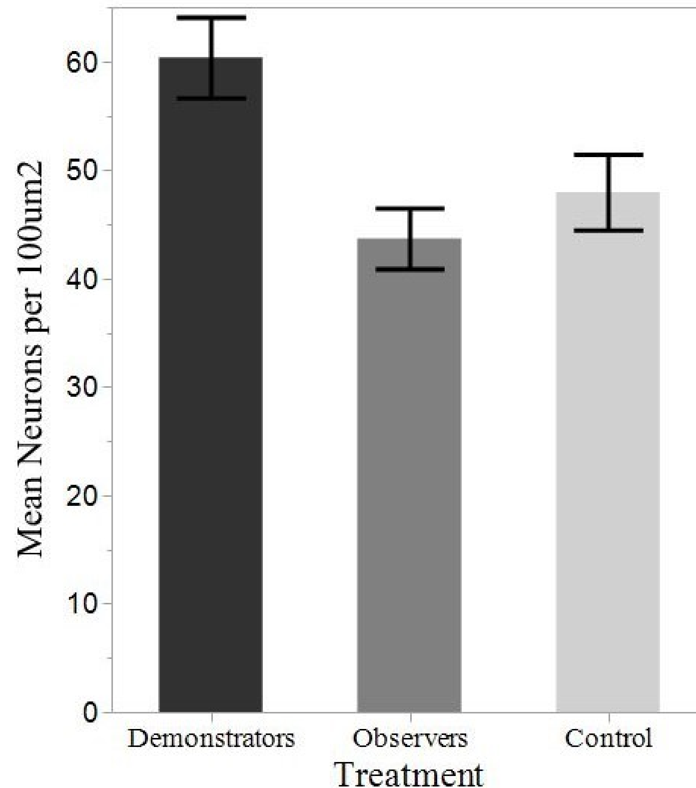

Figure 4.4. Mean ± SEM number of total number of neurons expressing egr-1 per 100 µm2 per treatment in six brain areas (Dm, Dl, Vd, Vv, Vs, POA). Demonstrators: directly experienced alarm cue; Observers: visually exposed to demonstrators while exposed to control cue (water); Control: exposed to control cue (water) and another control fish.

We found a similar pattern of results when we redid the analyses with olfactory bulb as a covariate in the model to account for any differences in brain-wide *egr-1* expression. The effect of treatment approached statistical significance (LMM, $F(2, 66.7) = 5.14$, $p = 0.08$), a pattern driven by a significant difference between demonstrators and observers (LSD, $p = 0.03$) and demonstrators and control (LSD, $p = 0.023$), but with no significant difference between observers and control (LSD, $p > 0.1$). Again, brain areas differed in *egr-1* expression ($F(5,85.7) = 83.4$, $p < 0.001$) but there was no interaction of treatment by brain area ($F(10, 86.1) = 1.50$, $p > 0.1$).

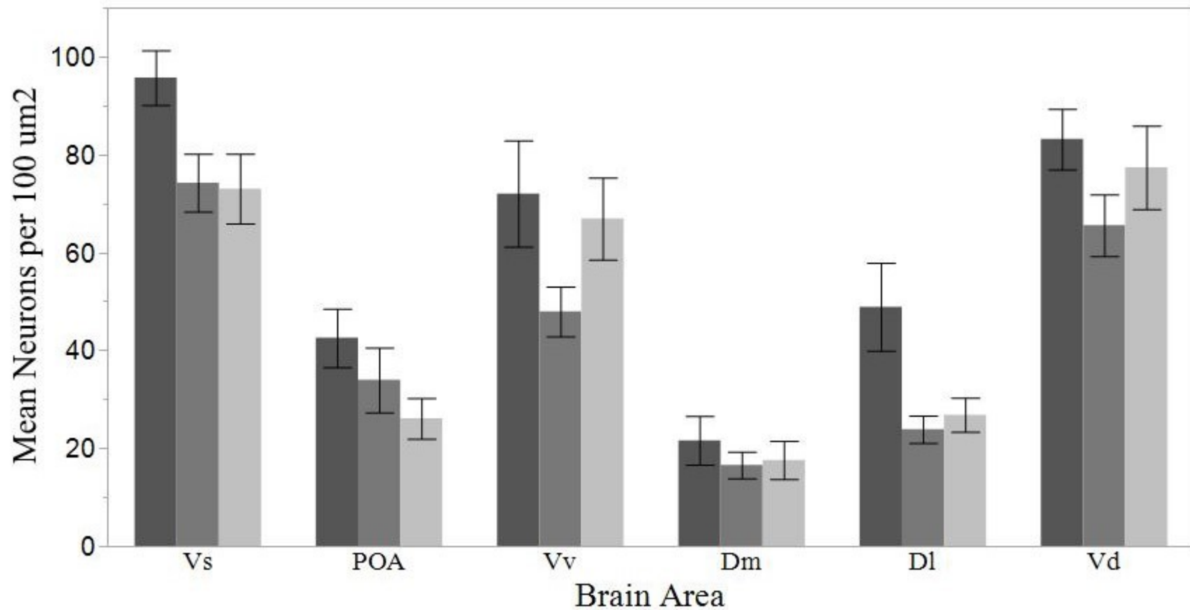

Figure 4.5. Mean ± SEM of egr-1 expression in different brain areas for demonstrators (dark grey) observers (medium grey) and control groups (light grey). Brain area abbreviations are in Table 4.1.

The partial correlation matrices (Fig. 4.6) indicate that in the demonstrator group, Vs and Dl expression was significantly and positively correlated (Spearman's partial correlation, r_s (16) = 0.71, p = 0.001), as were Vd and Vv expression (Spearman's partial correlation, r_s (16) = 0.53, p = 0.024) and Vv and Dm expression (Spearman's partial correlation, r_s (16) = 0.48, p = 0.044). Vd and Vs expression were significantly and negatively correlated (Spearman's partial correlation, r_s (16) = -0.56, p = 0.017). In contrast, in the observer group, Vd and Dm were significantly and positively correlated (Spearman's partial correlation, r_s (15) = 0.50, p = 0.039), as was Vs and the POA (Spearman's partial correlation, r_s (16) = 0.50, p = 0.042). There were no significant correlations in the control group (Fig. 4.6).

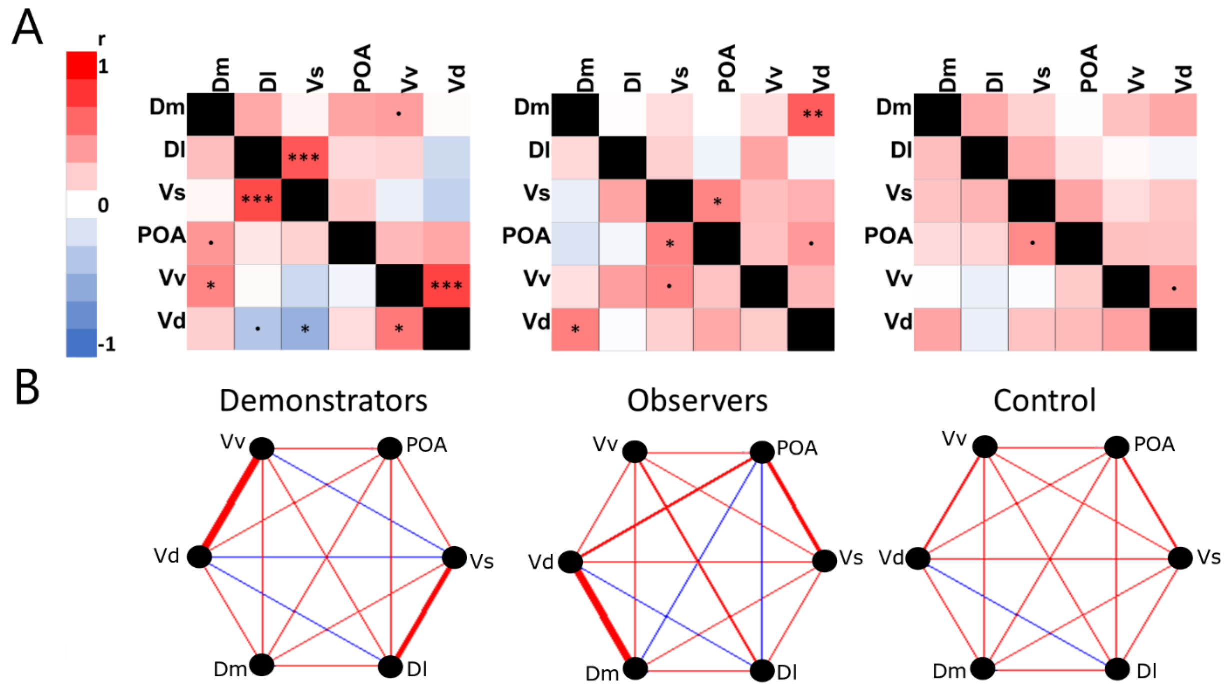

*Figure 4.6. A) Heatmaps of Spearman's correlations matrices (above diagonal) and partial Spearman's correlation matrices (below diagonal) of egr-1 expression between pairs of brain areas (abbreviations in Table 4.1) for each treatment. Demonstrators: directly experienced alarm cue; Observers: visually exposed to demonstrators while exposed to control cue (water); Control: exposed to control cue (water) and another control fish. Colour scheme represents rs values from -1 (blue) to 1 (red). Asterisks indicate significant correlations: * = p < 0.05, ** = p < 0.01, *** = p < 0.001; dot (.) indicates p < 0.1. B) Network diagrams based on egr-1 expression for each treatment, obtained from Spearman's correlation coefficients (rs) Lines linking brain areas have a thickness proportional to rs values, positive rs values in red and negative rs values in blue.*

Using social network analysis, we found significant correlations between brain areas when we compared each matrix to a null network that represented the baseline pattern of *egr-1* expression. Specifically, in demonstrators, Vd and Vs ($p = 0.006$) and Vd and Dm ($p = 0.042$) were more negatively correlated than expected by chance, while in observers a positive correlation between Vd and Dm approached significance ($p = 0.068$). In the control fish, no correlation differed significantly from that expected by chance.

Discussion

We studied the behavioural responses of guppies to alarm substance and the social contagion of the alarm response between conspecifics, as well as underlying neural mechanisms. Fish exposed to alarm substance showed a behavioural alarm response, freezing and avoiding the area where alarm substance was released by spending more time in the unsheltered lower area of the tank. The area avoidance behaviour was transmitted to the observer fish, which spent a similar amount of time as the demonstrators in the unsheltered lower area compared to the control group. Demonstrators had significantly more neurons expressing the immediate early gene *egr-1* across the six brain areas we studied, compared to the other two treatment groups, a difference mainly driven by increases in expression in two areas, Vs and Dl. When examining patterns of *egr-1* expression across the brain areas, we found evidence for different networks of expression between demonstrators, observers, and control, consistent with changes in functional neuronal connectivity depending on exposure to different indicators of risk.

The fact that demonstrators increased the amount of time in the lower unsheltered area after exposure to alarm substance is likely to represent an avoidance response to the water surface, where the alarm substance was released and an area that may present higher predation risk. For example, the river bed is safer in case of aerial attack (Templeton and Shriner 2004) and provides camouflage (Endler 1980). The observers matched this area use, indicating social contagion of the alarm response. We expected that the sheltered area would be used as a predator refuge by alarmed guppies, but perhaps the open area was preferred since it would allow increased visibility of any predator and also of conspecifics. It is noteworthy that we did not observe increased freezing in the observers, while this antipredator behaviour was displayed by the demonstrators. Although freezing is a useful antipredator behaviour, it can also be costly, since it reduces the amount of time the individual performs other useful behaviours such as foraging (Chelini *et al.* 2009). For a female guppy, the costs of

reduced foraging are high (discussed in Griffiths 1996), and visual exposure to a single alarmed demonstrator may be a less salient or reliable cue of current risk than exposure to alarm substance. Thus, observers may show only a partial anti-predator alarm response in response to a single alarmed conspecific.

The demonstrators had higher neural *egr-1* expression across the brain areas of the social-decision making network that we studied, compared to the control and the observers, while *egr-1* expression in an area outside the social-decision making network, the olfactory bulb, did not significantly differ. Thus alarm substance increased the number of neurons expressing *egr-1* in guppies, particularly in the Vs and Dl, two areas involved in alarm and stress responses in fish (Silva *et al.* 2015; Faustino *et al.* 2017). Vs has been implicated in the processing of social information during intraspecific agonistic interactions (Teles *et al.* 2015), and its mammalian homologue, the subpallial amygdala, is also known for its role in fear and stress (Li *et al.* 2004; Shackman and Fox 2016). However, we find surprising that Dm, a third brain area implicated in stress responses in fish (Silva *et al.* 2015; Faustino *et al.* 2017) and homologous to the mammalian pallial amygdala (O'Connell and Hofmann 2011; Goodson and Kingsbury 2013), did not show higher *egr-1* expression in the demonstrator group. Nevertheless, Dm *egr-1* expression was correlated with expression in other areas in a different manner for the demonstrators and the observers.

In demonstrators we found positive partial correlations between three brain areas pairs (Vs-Dl, Vd-Vv, and Vv-Dm) and a negative partial correlation between Vd and Vs. In observers, positive partial correlations were found in the POA-Vs and Vd-Dm brain area pairs. A social network analysis comparing the networks to a null, baseline network found that in demonstrators the Vd-Vs and Vd-Dm pairs were more negatively correlated than expected by chance, while in observers only the positive Vd-Dm correlation approached statistical significance. These results suggest differences between demonstrators and observers, and support the ideas that these brain areas act in a functional network during the processing of alarm, and that different sources of

alarm have different effects on how the network nodes interact. Here, we utilized two social cues of alarm from conspecifics: alarm substance and alarmed behaviour. It would be valuable to compare our findings with other cues of alarm, such as exposure to predators, in order to determine the specificity of the responses we observed to social alarm cues.

Dl, Dm and Vs are involved in stress responses in fish, while Vv and Vd are the teleostean homologues to the mammalian lateral septum and striatum/nucleus accumbens, respectively. These two mammalian areas are also involved in responses to fear and stress (Yadin *et al.* 1993; Levita *et al.* 2002; Singewald *et al.* 2011; Rodriguez-Romaguera *et al.* 2012), while, interestingly, to our knowledge, these two areas have not previously been implicated in stress responses in teleost fish. In mammals, the striatum/nucleus accumbens is also involved in social reward and evaluation of actions, having an important role in goal directed movements and decision making (Báez-Mendoza and Schultz 2013). The lateral septum modulates social behaviour in mammals and birds via nonapeptides binding to this area (Liu *et al.* 2001; Goodson *et al.* 2009), and in fish the Vv processes stimulus valence (Cerqueira *et al.* 2017) and odour information (Kermen *et al.* 2013). Our findings in demonstrators may thus be due to a role of Dl, Dm and Vs in the processing of stress-induced cues, of Vd and Vv in the modulation of stress responses, and of Vv in the processing of odour cues. Future studies of these six areas are needed to elucidate the specific neural mechanisms by which these areas are involved in responses to threat and to social information in fish.

The POA is active in fish during social exposure (Cabrera-Álvarez *et al.* 2017) and is involved in many types of social behaviours in fish, including reproduction and changes in social status (Demski and Knigge 1971; Macey *et al.* 1974; Satou *et al.* 1984; Francis *et al.* 1993; Wong 2000; Desjardins *et al.* 2010; Wong *et al.* 2012), and the POA, Vs and Dm have recently been found to be involved in social buffering (i.e., reduced stress responses when conspecifics are present; Faustino *et al.* 2017). Finding activation in

brain areas in observers that are related to stress and social behaviour is consistent with our behavioural data supporting social information use of the alarm information by the observers. Our partial correlation and network analyses implicate the POA, Vs, Vd, and Dm as acting together in the processing of social information cues, specifically cues related to alarm. Future studies are needed to elucidate the generality of these findings to other stressful cues.

Our results suggest that examination of both the nodes (i.e. brain regions) and the links between the brain areas of the SDMN is essential. Furthermore, our results emphasize the importance of examining areas outside the SDMN in order to establish changes specific to the SDMN. Previous studies in fish have shown that the SDMN works indeed as a network, by activating all nodes of the network and showing different activation patterns for different stable social behaviour states, rather than a localized functionality of a specific brain regions for each state (Maruska *et al.* 2013b; Teles *et al.* 2015; Faustino *et al.* 2017). However, other studies in fish have shown localized functionality in specific brain regions of the SDMN depending on the social treatment (Maruska *et al.* 2013a; Cabrera-Álvarez *et al.* 2017). Therefore, future work would usefully examine the characteristics of a social situation necessary to trigger a localized activation of specific nodes of the network. Also, studies comparing the effect of the same stimulus condition on the expression of different immediate early genes in neurons uncovered different (Maruska *et al.* 2013a; Teles *et al.* 2015) or similar patterns (Fernald and Maruska 2012; Maruska *et al.* 2013b). Furthermore, immediate early gene expression is not a simple proxy for neuronal electrical activity (Mello *et al.* 1992; Clayton 2000). Thus, our results should be considered as specific to *egr-1* expression, and we do not discard the possibility of uncovering different brain activation patterns with different immediate early genes or other markers of neuronal response. We also note that our subjects lived in a benign laboratory setting, and different patterns of *egr-1* expression may be observed in fish that had extensive experience with alarm cues. That said, our study illustrates neural and behavioural responses during socially contagion of alarm, in a species amenable to experimental manipulations of development and evolution (Reznick and

Bryga 1987; Magurran 2005; Leris and Reader 2016), thus providing a valuable route to investigate the neural underpinnings of adaptive social information use and social learning.

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Supplementary material

Supplementary table

Table S4.1. Analysis of egr-1 *expression under the experimental treatments for different brain areas. Transformation indicates the data transformation employed to meet the assumptions of parametric analysis. Other columns provide the results of One-way ANOVA and Tukey post-hoc analyses for each brain area. Abbreviations: D: Demonstrators; O: Observers; C: Control.*

Brain area	Transformation	F	p	Tukey
Vs	None	F (2,56) =4.40	0.017	D vs O = 0.026
				D vs C = 0.052
				O vs C = 0.96
Dl	Square Root	F (2,56) =3.96	0.025	D vs O = 0.034
				D vs C = 0.074
				O vs C = 0.94
POA	Square Root	F (2,56) =1.63	0.205	
Vv	Square Root	F (2,55) =1.59	0.213	
Vd	Square Root	F (2,55) =2.57	0.086	D vs O = 0.077
				D vs C = 0.78
				O vs C = 0.28
Dm	Logarithmic	F (2,57) =0.03	0.97	

Chapter 5: General discussion

This thesis examined the neural mechanisms underlying social behaviour and social responses to alarm in a small prey fish, the guppy. In this general discussion, I consider my results together and with previous work, implications for a further understanding of the processes under study, as well as avenues for future research. Particularly, I discuss the social role of the preoptic area, measures of social interaction, interpretations of observers' responses to alarmed conspecifics, the possibility that nonapeptides also modulate non-social behaviours, statistical approaches to study neural activation, and considerations regarding the use of immediate early genes in social behaviour studies.

The social role of the preoptic area

My thesis provided several lines of evidence consistent with the preoptic area (POA) playing an important role underpinning social behaviour. In Chapter 2, *egr-1* expression in the POA increased in fish exposed to a large group of ten conspecifics, compared to isolated fish. In Chapter 4, in fish observing alarmed conspecifics, *egr-1* expression in the POA correlated with that in the Vs, the homologue of the mammalian amygdala and BNST, a pattern not observed in the other experimental groups. And in Chapter 3, nonapeptides produced only in the POA, isotocin and vasotocin, were found to change grouping behaviours after central administration. Previous studies in fish have shown that the POA is involved in modulating parental care, sexual behaviour and aggression (O'Connell and Hofmann 2011). These results, together with my findings, suggest that the POA has a critical role processing social information in a range of contexts (aggression, mating, alarm, grouping) to generate an appropriate behavioural response.

Examining the results of Chapter 2 and 3 together, it is possible that the increased *egr-1* expression observed in response to large conspecific groups was due to upregulation of isotocin and downregulation of vasotocin release. Since vasotocin reduced social interactions in guppies after central administration, this suggests that vasotocin prompts social avoidance. The lack of a social cue in the control of Chapter 2 can explain why there was not increased *egr-1* expression of vasotocin neurons in the POA when fish were isolated. However, changes in nonapeptide neuronal activity in the POA in response to grouping is only a speculative idea, since this was not directly measured in my studies, and it is possible that other neurochemical systems may have been involved, such as dopamine (see discussion, Chapter 2). Further studies using techniques such as triple-labelling *egr-1* expressing neurons, vasotocin-expressing neurons, and isotocin-expressing neurons in fish under various social conditions would be vital in resolving these questions. Interestingly, in Chapter 4, I found no evidence for changes in functional connectivity between the POA and other brain areas when fish were directly exposed to conspecific alarm cue, which implicates the POA in processing relevant visual social cues from conspecific behaviour.

Social interactions across situations

As a measure of motivation to interact with conspecifics, I measured the propensity of fish to be in extremely close proximity to the barrier separating them from conspecifics in Chapter 2-4, thus measuring this in three situations: choosing between a large and small group, choosing to group after nonapeptide administration, and choosing to group during alarm substance exposure or observation of alarmed conspecifics. Guppies spent more time socially interacting with a large shoal than with a small shoal, and, 90 minutes after central administration, isotocin increased social interactions while vasotocin reduced them. Similar results were found in a study with zebrafish, in which vasotocin decreased social interactions with a group of conspecifics (Lindeyer *et al.* 2015). However, in Chapter 4 we found exposure to alarm substance or alarmed conspecifics had little impact on my social interaction measure. This was unexpected,

given that predator threat is known to impact grouping behaviour (Caraco *et al.* 1980; Hoare *et al.* 2004; Botham *et al.* 2006).

If the observed lack of increase of the social interaction measure in response to alarm cues is replicated, one possible explanation is that contextual differences between Chapter 2-3 and Chapter 4 are responsible. In the Chapter 4 study, subjects were exposed to a stressful event, while in the Chapter 2 and 3 studies subjects were not exposed to a highly stressful event but to a group of conspecifics, which has been shown to reduce stress in fish (Barlow 1968; Al-Imari and Gerlai 2008). Hence, the results of Chapter 4 may suggest that social interactions are too costly to engage in during a stressful event, probably because this behaviour would prevent the fish from engaging in anti-predator behaviours such as freezing. Another explanation is that a single conspecific, as was the available potential groupmate in the Chapter 4 study, was not a strong enough stimulus to generate a robust social interaction response in the subjects. This explanation is in accordance with the behavioural results found in Chapter 2, since a large stimulus shoal generated a stronger behavioural response than a smaller one. Also, the subjects in Chapters 2 and 3 were unfamiliar to the stimulus shoals, while in Chapter 4, each fish had been exposed to that same individual for three days. Several studies have shown that female guppies prefer to shoal with familiar conspecifics (Magurran *et al.* 1994; Griffiths and Magurran 1997, 1999; Lachlan *et al.* 1998). However, in the absence of familiar fish, such as in Chapters 2 and 3, I suggest the novelty of the stimulus shoal might induce social interactions in guppies. Thus, my results show the importance of the environmental context, as well as the novelty and salience of the stimulus when exploring behavioural responses to social stimuli.

Since many studies explore the behaviour of fish in captivity, and social interactions are a common social behaviour that we have observed in guppies, and is modulated by nonapeptides both in guppies and zebrafish (Lindeyer *et al.* 2015), we need further studies exploring social interactions to better understand what this behaviour pattern represents. For example, it will be informative to know if fish would display social

interactions with fish in a separate compartment if there are other individuals in their immediate proximity with which they can interact. It will also be informative to study whether social interactions represent behaviours involved in social dominance interactions with newly encountered conspecifics: several dominant and subordinate displays and behaviours can only be performed in close proximity (Gorlick 1976). We do not know whether the social interaction measure used represents affiliative or aggressive behaviour, or different motivations in different individuals or contexts. Thus, we could explore whether fish with established hierarchies would still engage in social interactions. My work, and that of Lindeyer *et al.* (2015) in zebrafish and Kelly *et al.* (2011) in birds find different patterns for grouping and the close-proximity social interaction measure, suggest that different motivations underlie these phenomena.

Observers' responses to alarmed conspecifics

In Chapter 4, 'demonstrator' fish exposed to alarm substance spent more time in a lower unsheltered area of the tank, and observer fish spent similar amount of time in this region. The neural results confirmed that the alarm information was transmitted to the observers because brain areas involved in social behaviour and stress in fish were co-activated in observers. Although the observers could have reacted to alarm information differently, for example, hiding in the sheltered area, they used the same area as the demonstrators. This behavioural response can have several interpretations. For example, the observers may approach the other fish as an anti-predator response, for example diluting the risk of being predated upon when they are close to other conspecifics (Krause and Ruxton 2002). This could potentially be driven by observers staying in the same area as the demonstrators because visualizing a conspecific is both rewarding (Al-Imari and Gerlai 2008) and stress-relieving (Faustino *et al.* 2017). A speculative explanation is that the observers, unexposed to any other alarm cue than the visualization of the alarmed conspecific, stayed in the same area as the demonstrators to reduce the stress of the companion fish. This line of thought opens the discussion of whether fish can show empathy for other conspecifics as it has been

shown in recent studies in mammals (Fraser *et al.* 2008; Bartal *et al.* 2012; Sato *et al.* 2015; Smith *et al.* 2016) and birds (Fraser and Bugnyar 2010; Perez *et al.* 2015). For example, Bartal *et al.* (2012) exposed a trapped rat (*Rattus norvegicus*) showing signs of distress (i.e., emitting alarm calls) to a free rat and found that the free rats showed increased activity and opened the door of the cage significantly more than rats exposed to empty cages or to cages containing a toy rat, even if the free rats were not rewarded with the social presence of the rat after opening. The authors concluded that this pro-social behaviour was driven by an 'emotional motivation, arguably the rodent homologue of empathy' (Bartal *et al.* 2012).

Empathy is the ability to understand and share the feelings of another. In psychology, the term empathy is only used when it involves both emotional engagement and adopting the other's point of view (de Waal 2008), however, adopting the other's point of view is difficult to measure in animals and does not necessarily match the classical definition of empathy. In fact, whether an animal is adopting the other's point of view and showing emotional engagement are both difficult to prove, especially since there can be alternative explanations for what are considered empathic behaviours. For example, in the Bartal *et al.* (2012) study, the authors confirmed emotional engagement of the free rats by showing increased activity when exposed to the trapped cagemates, and discarded the alternative explanation that the free rats liberated their cagemates to stop hearing their alarm calls, because alarm calls occurred too infrequently. However, rats increase their activity when exposed to the odour of stressed conspecifics (Mackay-Sim and Laing 1981). Thus, the fact that free rats increased their activity and opened the cages may not be due to empathy, but rather to personal distress (de Waal 2008). Thus, eliminating alternative explanations is challenging. Given that there is increasing behavioural and neuroanatomical evidence that shows that it is possible that fish can experience fear (reviewed in Braithwaite and Boulcott, 2007; Branson, 2008; Chandroo *et al*., 2004), and that they are capable of a wide variety of complex cognitive processes (Brown *et al.* 2006) despite lacking a neocortex (a mammalian brain area involved in higher-order brain functions), it is not unreasonable to consider that empathic

behaviours are a possibility in fish, since it is our responsibility to consider all possible explanations to a behaviour. However, given the difficulty of measuring emotions in animals, it is in our hands to parsimoniously interpret behavioural results and to conduct the appropriate studies to answer important questions about the evolution, function and mechanisms of social cognition such as whether fish can be empathic.

Nonapeptides and social behaviour

In Chapter 3, I found that isotocin and vasotocin modulate grouping behaviour in guppies by influencing shoaling and social interactions. Although oxytocin has been popularly designated as the 'love' hormone, given its influence in pair bonding in monogamous rodents and its effects on prosocial behaviour in many species, there is more and more evidence that it is also involved in the regulation of 'anti-social' behaviours, such as aggression, social selectivity, and fear (Beery 2015), as well as mediating negative emotions in humans, such as envy and gloating (Shamay-Tsoory *et al.* 2009). In fact, a new theory suggests that oxytocin is not specifically a modulator of social behaviour, but instead it enhances the salience of personally relevant and emotionally evocative stimuli (whether social or non-social; Harari-Dahan and Bernstein 2014). Harari-Dahan and Bernstein (2014) reviewed the human and rodent literature and suggested that oxytocin modulates motivation to approach positive stimuli by upregulating the neural circuits linked to reward and modulates motivation to withdraw from/avoid negative stimuli by downregulating the neural circuits involved in fear and threat. They named this new approach of the mechanisms of action and function of oxytocin as the general approach-avoidance hypothesis of oxytocin (GAAO). A recent study of intranasal administration of oxytocin in men and women provides evidence that oxytocin reduced behavioural avoidance when they were exposed to negative-valenced stimuli (both social and non-social), and not when they were exposed to neutral-valence stimuli (Harari-Dahan and Bernstein 2017). However, they found no significant results for the effect of oxytocin on the behavioural approach when exposed to positively-valenced information, a result they explain by methodological factors such as lower

valence of the positive stimuli compared to the negative stimuli, and to their mixed-block design (Harari-Dahan and Bernstein 2017). This new interpretation of the results found in oxytocin studies helps clarify some conflicting results. For example, oxytocin increases aggressivity of female mother rats towards intruders, and reduces it towards pups (Campbell 2008). If oxytocin was simply modulating aggressive behaviour, the 'social hormone' explanation cannot justify this selectivity, in contrast to the GAAO, which suggests that oxytocin facilitates information processing salience of cues in the environment, in this example enhancing maternal behaviours towards the positive-valence stimuli (pups) and promoting aggressive acts towards the negative-valence stimuli (intruders).

Despite the importance of this new approach to understand the functions and mechanisms of action of oxytocin, to my knowledge, it has not been directly verified in animals yet. This may be due to the difficulty of evaluating the salience of stimuli and the emotional response to a stimulus in animal studies, as opposed to human studies in which the subjects can rate their emotions. However, an effort in the field should be made to confirm whether the GAAO is also valid to explain the mechanisms of action of oxytocin and its homologues in animals, and so, whenever possible, non-social stimuli control should be included in the experimental design of nonapeptide studies. For example, positive and negative smells, such as food smells and naphthenic acid (Reichert *et al.* 2017) respectively, could be used to test whether nonapeptides modulate approach and withdrawal to non-social stimuli in fish.

Statistical analysis of neural data

In this thesis I have used two approaches to analyse neural data. The first approach was to use a linear mixed model (LMM) and a second, complementary approach was to use a social network analysis. Both types of analyses have benefits and disadvantages. For example, a benefit of using the LMM is that it allowed comparison of experimental

treatments and find whether there are specific brain areas that differ in activation between treatments. However, the LMM has the disadvantage of not treating the neural data as a network, and hence, when the differences between treatments are not high, or statistical power is limited, it is not possible to observe different patterns of network activation. The social network analysis, on the other hand, allows us to have a more realistic view of our data by studying it as a combination of brain areas that are simultaneously activated. However, there is no consensus on how best to approach the data and there is variation between studies. For example, recent studies have used the quadratic assignment procedure (QAP; Faustino *et al*., 2017; Teles *et al.*, 2016, 2015) to compare the networks generated by different treatments. The QAP is a social network analysis, equivalent to a Mantel test, that correlates whole networks, with a significant p-value indicating that the networks are correlated (in contrast with most statistical analysis, in which significant p-values indicate a difference between treatments). Thus, this analysis is useful if our question of interest is to explore whether two networks are similar; however, it cannot be used if what we want to explore is whether there are significant differences between networks. Thus, although social network analyses are useful to interpret neural network data, we should be cautious in their use and interpretation. In conclusion, when analysing neural data, it is appropriate to use both standard statistical analysis and social network analyses, as long as we are aware of the advantages and disadvantages of using one or another.

Immediate early gene (IEG) expression as a measure of neuronal activity

IEG expression in the brain has been used as a tool to visualize neuronal activity and there is a variety of IEGs that can be used (Okuno 2011). However, IEG expression is not a simple proxy for neuronal electrical activity (Clayton 2000) and the expression of different IEG may vary between regions depending on contexts. For example, when the African cichlid *Astatotilapia burtoni* has the opportunity to rise in social rank, there is increased activation of *egr-1* and *c-fos* in all the studied brain areas of the social decision-making network (Maruska *et al.* 2013b), whereas when they descend in social

rank there is distinct expression of these two IEGs across the SDMN (Maruska *et al.* 2013a). Similarly, when zebrafish win or lose a contest, *egr-1* and *c-fos* show distinct patterns of expression, suggesting that the expression of these IEGs reflect different behaviour state-related processes (Teles *et al.* 2015). Teles *et al.* (2015) suggested that *c-fos* might be a better neural marker for general brain activity during social interactions because all brain nuclei increased their expression of this IEG compared to the non-social control, while *egr-1* expression might be more region- and process-specific. In a similar experiment, instead of studying IEGs during winner-looser contexts, they explored the expression in the SDMN of several genes involved in neural plasticity and found that each social treatment showed a specific neuromolecular pattern across the SDMN, suggesting that there are several neuroplasticity mechanisms modulating different social behaviour changes (Teles *et al.* 2016). In summary, these studies show the complexity of the neuromechanisms influencing social behaviour in fish and provide evidence of differences in IEGs expression patterns. Thus, the results I show in this thesis should be considered as specific to *egr-1* expression, since I cannot discard the possibility that different methods of measuring neuronal responses could reveal different patterns of brain activation.

Implications and further studies

This thesis furthers our understanding of the neural mechanisms of grouping behaviour and social information use in guppies. The results of Chapter 2 showed that the brains of wild-type guppies have a different pattern of *egr-1* expression when they are exposed to a large group compared to when they are isolated. Thus, this result shows that for guppies, the consequences of being in a large group does not only have the potential to alter their fitness (Krause and Ruxton 2002), but it also affects their neural physiology, by inducing responses in an area of the brain known for its implications in social behaviour. This result opens the door to further investigations of this matter. For example, wild guppies of different populations show different levels of sociality (specifcally, grouping and intraspecific agression; Seghers 1974; Magurran and

Seghers 1991) and so, the study of the neural responses to sociality in each population could help us understand the evolution of gregariousness. For example, if we expose different populations to large shoals, will the neural response be the same on different populations, showing a conserved pattern of brain regulation of sociality in this species, or on the contrary, will each population show a different neural response, showing the adaptability of the brain to the ecologically relevant circumstances of each population? If that is the case, will raising individuals of different populations in social contexts that differ from their original populations (i.e., asocial populations raised in highly social environment, and vice versa) affect the neural response to sociality? In other words, can development affect the neural response to sociality, and how does this interact with evolved differences? All these questions would help us achieve a better understanding of the neural processes underpinning sociality.

Likewise, the central administration technique used in Chapter 3 and the obtained results allow us to further explore the modulation of sociality by nonapeptides in fish, as well as their effect on other social paradigms. For example, by using the previous knowledge of differential group sizes in wild guppy populations (Seghers 1974; Magurran and Seghers 1991) and following previous protocols in teleost fish (Huffman *et al.* 2012; Reddon *et al.* 2015, 2017; O'Connor *et al.* 2016), we can explore whether there are different levels of circulating nonapeptides in different populations, different number of nonapeptide neurons, as well as different number of nonapeptide receptors in their brains. These studies can help us understand the role of nonapeptides in the modulation of sociality in a single species with multiple independent replicates, which has the benefit of narrowing down the specific ecological factors that influenced the evolution of grouping behaviour. The role of nonapeptides on other relevant social paradigms can also be studied, for example, we could study the role of nonapeptides in the processing of social versus asocial cues, testing the general approach-avoidance hypothesis of an oxytocin homologue in fish, which provide insights on the recently suggested mechanism of action of nonapeptides in animals other than humans (Bartal *et al.* 2012). Lastly, the results of Chapter 3 highlight the importance of measuring the

behavioural effect of nonapeptides over time, which, apart from suggesting the use of longer behavioural tests in future studies of nonapeptides, also leads questions on the mechanisms of action of nonapeptides in fish grouping behaviour. For example, we could study whether the observed long-term effects were due to activation of complex cascades of neural activation, and whether it also involved interactions with other neurotransmitters such as dopamine, as observed in mammals (Love 2014).

As previously mentioned, knowing how the brain processes social information help us towards resolving complex questions currently under debate, such as whether social and individual (asocial) learning involve the same or different processes (Heyes 2012; Leadbeater 2015; Reader 2016). In Chapter 4, I showed that guppies had a different pattern of *egr-1* expression in the brain when exposed to relevant visual social cues compared to fish exposed to chemical social cues and to control fish, which had social exposure but to a non-alarmed conspecific. This result shows that the salience and nature of the social cue is key to generate a specific pattern of *egr-1* expression in the brain, showing neural processing of this cue, which in turn is key to generating an appropriate behavioural response. A similar approach could be used to address questions in the social versus asocial learning debate by exposing individuals to either relevant asocial or social cues and exploring whether the neural activation is different, as well as exploring the neural responses during social and asocial learning. Thus, the results of Chapter 4 open the door to a new set of neurobehavioural studies that can advance our understanding of processes that have a large impact in the ecology and evolution of many species, such as social learning (Hoppitt and Laland 2013).

In conclusion, the approaches and findings in this thesis provide foundational work to understand the neural mechanisms underlying grouping behaviour and social information use in fish, and open the door to future research in fish that can explore hotly debated ideas, such as the controversy about whether social and asocial learning are the same process (Heyes 2012), or whether oxytocin is not necessarily modulating only social behaviours (Harari-Dahan and Bernstein 2014).

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