A neurobehavioural analysis of social behaviour and learning in fish and mammals

Charlotte Marijke Lindeijer

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A neurobehavioural analysis of social behaviour and learning in fish and mammals

Een neuro-gedragsanalyse van sociaal gedrag en leren in vissen en zoogdieren

(met een samenvatting in het Nederlands)

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Charlotte Marijke Lindeijer
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Promotor: Prof. dr. J. J. Bolhuis

Co-Promotor: Dr. S. M. Reader

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English Summary

In the animal kingdom, species differ strongly in their sociality. Highly social species show strong group cohesion and social affiliations, whereas more asocial species show few social interactions and weak affiliation patterns. Neuroscientists studying social behaviour have specifically linked two nonapeptide superfamilies to the regulation of social behaviour. The first includes the oxytocin family and its non-mammalian homologs isotocin (in teleost fish) and mesotocin (in birds, reptiles and amphibians). The second includes the vasopressin family and its non-mammalian homolog vasotocin. These nonapeptide lineages are highly evolutionary conserved in structure and are present in all vertebrates. The function of these nonapeptides also appears to have been highly conserved. In mammals, the regulatory role of oxytocin and vasopressin has been clearly established in mate bonding and aggression. Administration studies and mapping of brain neuro-anatomy in rodents have confirmed the regulatory role of these nonapeptides and linked different receptor distribution patterns to species and individual differences in sociality. A similar modulatory role for nonapeptides has recently been found in birds. This strongly suggests that these nonapeptide systems form part of a mechanism underlying the regulation of social behaviour, a system that is possibly conserved across all vertebrates. Taken broadly, research findings suggest that oxytocin stimulates *pro*-social behaviour (e.g. affiliation) whereas vasopressin stimulates *anti*-social behaviour (e.g. aggression). To study the neural underpinnings of social behaviour and its evolution we examined the zebrafish, *Danio rerio*. Extensive research on zebrafish has lead to an excellent understanding of its genetics, central nervous system, endocrine development and its similarities (and differences) with other vertebrates including humans. Though its behaviour is less well studied and understood, zebrafish are a tightly shoaling species and show grouping preferences early in life. Studying the zebrafish allows investigation of the multiple components of behaviour, addressing function, causation, development and evolution. Thus the zebrafish is a promising model to increase our understanding of vertebrate social behaviour, the underlying nonapeptide circuitry and its evolution. Moreover, basic knowledge on the social behaviour of zebrafish is lacking, and would be valuable to maximize the utility of this standard and increasingly used animal model.

In this thesis, three categories of social behaviour are studied to investigate sociality and its neural correlates: social learning, social memory and shoaling (social affiliation). Social learning is the acquisition of novel information from observation of, or interaction with, another animal. The propensity to use social information can be influenced by many factors including social dynamics, development and recent experience. The hypothesis that the zebrafish is a valuable model in

studying the neuroscience of social learning was first tested in a social learning paradigm and is described in Chapter 2. In a simulated predator escape test, naïve individuals were exposed to knowledgeable fish from which they could learn to escape from a moving trawl net by using a specific escape route. Here, it is demonstrated for the first time that zebrafish have the ability to socially learn escape routes. Naïve fish exposed to knowledgeable fish successfully learned to escape whereas subjects exposed to naïve fish did not.

Another realm of social behaviour of importance to social learning that has also been shown to be influenced by nonapeptides is individuals' ability to form a social memory, i.e. to remember conspecifics. The hypothesis that zebrafish could form a social memory was addressed in Chapter 3. To investigate the development of familiarization, fish were housed together for up to 20 days. At fixed time intervals, individual fish were given a preference test where they could choose to shoal with a familiar or an unfamiliar conspecific. Although subjects shoaled strongly, strong preferences for either familiar or unfamiliar conspecifics were not detected at any time point. Results thus suggest that social memory formation based on familiarity is not an important aspect of adult zebrafish social behaviour, or at least does not develop as rapidly as in other fish species.

In Chapter 4 the regulatory role of nonapeptides on sociality in zebrafish was addressed. In a preference test similar to the social memory test, I tested the predictions that vasotocin inhibits and isotocin promotes approach to a group of conspecifics. Individual subjects could join or avoid a group of conspecifics placed at either end of a tank. Prior to testing, subjects received a single peripheral injection of isotocin, vasotocin, their putative antagonists, or saline. Vasotocin-administered subjects were slower to initiate interaction with the group and spent less time interacting with it, while their shoaling tendency remained intact. These findings support the hypothesised role of vasotocin as a regulator of anti-social behaviour, and suggest its role may be dependent on behavioural context. This finding provides evidence for a conserved role of nonapeptides in the regulation of sociality in zebrafish and adds to the evidence suggesting that this is a highly conserved neural mechanism that underlies social behaviour across vertebrates.

In Chapter 5, I switched model organisms and took advantage of an extensively studied system to investigate developmental influences on social learning. The maternal care system in Norway rats, *Rattus norvegicus*, is characterized by consistent natural variations in licking and grooming behaviour of dams towards their offspring. Consequently, dams can be divided into high, mid or low maternal care licking and grooming phenotypes. These behavioural phenotypes have been shown to influence

neuro-endocrine development including oxytocin sensitivity and hippocampal development. To address the influence of early social experience and oxytocin on social learning abilities in adult rats I used the robust social transmission of food preferences paradigm. Rats that are naïve to two diets will typically reliably copy the diet that was previously eaten by a conspecific. Adult offspring of high maternal care phenotypes socially learned a food preference whereas adults that received low maternal care did not. Peripheral oxytocin administration before interaction did not show an effect on social information use. Our results show a strong correlation between maternal care received early in life and social learning performance later in life. This is the first evidence for early social experiences shaping the propensity to use social information later in life.

The findings presented in this thesis contribute to our understanding of social behaviour and its underlying neural systems. By investigating functional and developmental aspects of social behaviour and the role of the nonapeptide systems oxytocin and vasopressin, we may gain insight into the neural mechanisms underlying vertebrate sociality and the evolution of these mechanisms. Knowledge on how social experiences early in life affect sociality later in life could be used to increase our understanding of the development of human diseases related to social deficits, for instance autism, or social phobias. This information could potentially aid in the development of treatments for these problems. The results thus illustrate the value of zebrafish for research into the evolution of social behaviour.

Nederlandse Samenvatting

Er bestaan grote verschillen tussen dieren in de mate van 'socialiteit'. Enerzijds zijn er soorten die in groepsverband leven met individuen die zijn verbonden aan één partner en samen zorg dragen voor hun nakomelingen. Anderzijds zijn er soorten die voornamelijk solitair leven met individuen die zich niet verbinden aan een partner of samen nakomelingen verzorgen. Neurowetenschappelijk onderzoek naar hoe dergelijke variatie in sociale interacties tussen soorten tot stand komt of gereguleerd wordt, heeft zich voornamelijk gericht op twee verschillende hormoonsystemen, namelijk de oxytocine- en vasopressine-gerelateerde familie. Deze twee hormoonfamilies worden in alle gewervelde dieren gevonden. Het zoogdierhormoon oxytocine komt overeen met het hormoon isotocine gevonden in vissen en mesotocine gevonden in vogels, reptielen en amfibieën. Voor vasopressine is vasotocine de enige homoloog die voorkomt in alle niet-zoogdieren. De oxytocine en vasopressine families vertonen veel overeenkomsten wat betreft moleculaire structuur en functie in het lichaam. Dit betekent dat gedurende de evolutie van de vertebraten (gewervelde dieren) er relatief weinig is veranderd aan de twee hormoonsystemen. In zoogdieren is de rol van oxytocine en vasopressine in agressie en paarvorming uitgebreid bestudeerd. Als modelorganisme is in eerste instantie de woelmuis gebruikt, omdat tussen soorten grote variatie in 'socialiteit' bestaat. Er zijn 'sociale' woelmuizen die weinig agressie vertonen en monogaam zijn, maar ook 'antisociale' soorten die een hoge mate van agressie vertonen en polygaam zijn. Toediening van oxytocine en vasopressine in deze woelmuizen leidt tot afname of toename van de intensiteit van agressie en van paarvorming afhankelijk van het natuurlijke gedrag van de soort. Ook zijn de oxytocine en vasopressine neuronen (zenuwcellen) met de bijbehorende receptoren (uiteinden van zenuwcel die signalen opvangt en herkent) in de hersenen in kaart gebracht. Hieruit bleek dat de locatie en dichtheid van de receptoren tussen soorten verschilden. Deze neuro-anatomische verschillen correleerden met de verschillen gevonden in het gedrag van de 'sociale' en 'antisociale' woelmuizen. Een vergelijkbaar mechanisme is ook in andere zoogdieren en vogels aangetoond. Dit suggereert dat de oxytocine en vasopressine families een neuraal mechanisme vormen om 'socialiteit' te reguleren. Over het algemeen stimuleert oxytocine positievere interacties met soortgenoten ('prosociaal' gedrag) en vasopressine vijandigere interacties met soortgenoten ('antisociaal' gedrag).

Om meer inzicht te verkrijgen in het neurale mechanisme dat sociaal gedrag reguleert en in de evolutie van de oxytocine/vasopressine families hebben wij de zebravis bestudeerd. De zebravis is een veelvuldig gebruikt modelorganisme in de ontwikkelingsbiologie en genetica. Daarom beschikken we over uitgebreide kennis van zijn centraal

zenuwstelsel en het endocrien systeem en weten we dat er veel overeenkomsten zijn tussen deze vis en andere, later geëvolueerde vertebraten, inclusief de mens. Daartegenover is er relatief weinig bekend over het gedrag van de zebravis. Zebravissen vertonen al op vroege leeftijd sterk scholingsgedrag. Deze aspecten maken de zebravis uitermate geschikt om onderzoek naar gedrag en neurale mechanismen te combineren. Onderzoek naar gedrag in de zebravis zal de kennis van gedrag in vertebraten en het onderliggende mechanisme dat 'socialiteit' stuurt vergroten. Daarnaast zou de zebravis goed als model kunnen dienen om psychiatrische aandoeningen (bijvoorbeeld sociale fobieën) of autisme in de mens beter te begrijpen.

In dit proefschrift worden vier experimenten beschreven met de focus op verschillende gedragingen: het leren van soortgenoten ('sociaal' leren), het herkennen van soortgenoten op basis van eerdere ervaringen ('sociaal geheugen') en groepsvorming. Veel dieren kunnen nieuwe informatie leren van soortgenoten door het observeren van of omgaan met 'ervaren' individuen. Daarnaast kunnen factoren als sociale dynamiek, ontwikkeling en ervaring zorgen voor variatie in de mate van sociaal leren. In Hoofdstuk 2 staat beschreven hoe de zebravis getest is op het vermogen om te leren van soortgenoten. Testvissen werden samen met getrainde vissen in een nieuwe tank geplaatst, waarin een net was bevestigd dat, al bewegend, een roofdier moest nabootsen. De testvissen konden van de getrainde vissen, die snel en adequaat ontsnapten aan het net via een vaste route, leren om ook succesvol te ontsnappen aan de negatieve stimulus. De resultaten laten zien dat zebravissen inderdaad leerden van hun soortgenoten. Vissen die samen met getrainde vissen de taak moesten volbrengen, ontsnapten beter dan vissen die geen getrainde vissen als voorbeeld hadden.

Individuen kunnen ook selectief zijn in hun keus welk individu zij als voorbeeld gebruiken en van wie zij informatie overnemen. Het vermogen om individuen te herkennen kan dus de verspreiding van informatie beïnvloeden. Daarnaast zijn de twee hormoonsystemen oxytocine en vasopressine ook belangrijk bevonden in leer- en geheugenprocessen. In Hoofdstuk 3 is daarom de vraag of zebravissen ook een 'sociaal geheugen' kunnen vormen en een voorkeur vormen voor bekende vissen op basis van ervaring geadresseerd. Om de ontwikkeling en expressie van een sociaal geheugen te bestuderen, werden groepen vissen samen gehuisvest voor een periode van 20 dagen. Vervolgens werden individuen getest na verschillende tijdsintervallen (2, 4, 8, 12, 16 en 20 dagen). Individuen konden een keus maken tussen samenscholen met een bekende en een onbekende groep vissen. De zebravissen lieten sterk scholingsgedrag zien, maar duidelijke voorkeuren om samen te scholen met soortgenoten op basis van eerdere ervaringen, konden niet

worden vastgesteld. Dit suggereert dat in de zebravis ervaring met soortgenoten geen belangrijke rol speelt in schoolkeuzes of dat het niet ontwikkelt binnen 20 dagen.

Om de regulerende rol van het isotocine en het vasotocine hormoonsysteem op het gedrag van de zebravis te bestuderen, zijn zebravissen getest op hun scholingsgedrag na injectie van isotocine, vasotocine of hun potentiële antagonisten (deze stof blokkeert een receptor, zodat ook het signaal van isotocine of vasotocine geblokkeerd wordt). Dit experiment wordt beschreven in Hoofdstuk 4. Individuele vissen werden nadat één type hormoon was toegediend voor de keuze gesteld om samen te scholen met een grote groep soortgenoten of deze juist uit de weg te gaan. De voorspelling dat isotocine als 'prosociaal' hormoon scholingsgedrag zou stimuleren en vasotocine als 'antisociaal' hormoon groepsgedrag zou tegen gaan, werd getest. Wij vonden dat het vasotocine systeem inderdaad groepsgedrag in zebravissen beïnvloedde. Na vasotocine injecties waren testvissen langzamer om een interactie aan te gaan met de school en de totale tijd dat zij interactie met de school hadden nam af. De neiging om samen te scholen na vasotocine toediening bleef echter onveranderd vergeleken met de controle groep. Het isotocine systeem had geen invloed op het groepsgedrag. Deze bevindingen onderschrijven het idee dat vasotocine 'antisociaal' gedrag reguleert, maar suggereren ook dat de invloed van dit systeem afhankelijk kan zijn van de context. De sterke neiging van de zebravissen om in de nabijheid van een school te zijn blijft onveranderd, maar de 'interesse' in de soortgenoten kan variëren. Deze bevindingen bevestigen de algemene rol van het vasotocine systeem in de regulatie van 'socialiteit' en onderstreept het idee dat dit neurale mechanisme geconserveerd is in vertebraten.

Tot slot heb ik gebruik gemaakt van een ander modelorganisme, de rat, om te bestuderen hoe ervaringen tijdens de ontwikkeling het sociaal leren beïnvloedt. Onderzoek heeft aangetoond dat rattenmoeders variëren in de mate van verzorging en het likken van hun jongen, voornamelijk in de twee weken na de geboorte. Dit verschilt van zeer frequente, intensieve verzorging tot relatief minder intense verzorging. Deze verschillende vormen van verzorging beïnvloeden een groot aantal processen, waaronder de ontwikkeling van het centraal zenuwstelsel en het endocrien systeem (het systeem verantwoordelijk voor hormoonafscheiding in het lichaam). Er zijn bijvoorbeeld verschillen gevonden in de ontwikkeling van de hippocampus, een structuur in de hersenen die belangrijk is voor leer- en geheugenprocessen. Ook wordt de gevoeligheid voor oxytocine beïnvloed. Dit systeem hebben we gebruikt om de invloed van oxytocine en de verschillen in verzorging op sociaal leren in volwassen nakomelingen te bepalen in Hoofdstuk 5.

Nakomelingen van moeders die relatief frequente of minder frequente verzorging ontvingen, werden getest op hun informatiegebruik in een foerageertest. Ratten die als model moesten dienen voor de nakomelingen, werden vooraf getraind om één van twee nieuwe voermengsels te eten: standaard brokken met als toevoeging kaneel- of chocoladesmaak. Wanneer ratten die onbekend zijn met deze voermengsels in contact zijn geweest met een modelrat, zullen deze ratten, wanneer zij beide voermengsels aangeboden krijgen, over het algemeen kiezen om van het mengsel te eten waarvan hun model eerder heeft gegeten. Onze resultaten laten zien dat nakomelingen die intensievere zorg hebben ontvangen, de voedselkeuze van hun model kopiëren. Dit in tegenstelling tot de ratten die minder intensieve zorg hebben ontvangen: deze ratten kopieerden de voedselkeuze van hun model niet. Tegen de verwachting in, stimuleerde oxytocine injecties het sociaal leergedrag niet. De resultaten laten zien dat sociale ervaringen vroeg in het leven, op latere leeftijd kunnen leiden tot verschillen in de mate van gevoeligheid voor informatie van soortgenoten.

De bevindingen beschreven in dit proefschrift vergroten de kennis van sociale gedragingen en hoe deze worden gereguleerd door het oxytocine en vasopressine hormoonsysteem. Door te kijken naar zowel de functie van deze hormoonsystemen in sociaal gedrag als de ontwikkelingsfactoren die dit gedrag beïnvloeden, zullen we meer inzicht verkrijgen in de evolutie van zowel socialiteit als de onderliggende mechanismen. Kennis over hoe ervaringen met soortgenoten zijn weerslag kan hebben op gedragingen later in het leven, zou in de toekomst gebruikt kunnen worden om stoornissen die gepaard gaan met verminderde sociale vaardigheden, zoals autisme, maar ook angsten, zoals sociale angst of pleinvrees, beter te kunnen begrijpen en mogelijk een bijdrage kunnen leveren aan een behandeling.

Chapter 1

GENERAL INTRODUCTION

This thesis aims to contribute to a better understanding of the neural underpinnings of social behaviour and social learning. I investigated social learning in the zebrafish and the rat. To understand the role of two nonapeptide hormone families (the oxytocin- and vasopressin-related peptides) that have been linked to the regulation of 'sociality', I examined how administration of oxytocin- and vasopressin-related hormones influenced grouping behaviour in the zebrafish and social learning in rats. This Introduction provides the theoretical background for studying social behaviour and the two nonapeptide systems.

Social behaviour

Social behaviour can be broadly defined as any kind of interaction between two or more individuals, usually of the same species, such as pair bonding or social learning. Species vary dramatically in the number of interactions they have, where some live in permanent groups and others live solitarily and encounter conspecifics only when mating. Interactions between individuals can potentially offer benefits but also involve costs. Animals that live in groups, for example, reduce their predation risks, easily locate a suitable mating partner, use the behaviour of others to locate food sources more rapidly or learn about novel predators (Chapter 2) or foods (Chapter 5) from conspecifics. Larger groups, however, can also result in more competition for resources or increased risks of parasite infections (Altizer et al. 2003). Thus there is a trade off between the costs and benefits of living in groups. (Krause & Ruxton 2002)

Individuals within most animal groups do not interact with each other at random; rather, they preferentially interact with certain individuals and restrict certain social interactions to particular individuals (Krause et al. 2009). Social dynamics could therefore influence social behaviour, including the likelihood of social learning (Coussi-Korbel & Fragaszy 1995). For example, differences in affiliation between conspecifics within a group can influence attention to and salience of certain individuals that possess information over others. This implies that being able to distinguish between classes (for example age or status) or individuals and to maintain a form of social memory is important in groups (Chapter 3).

Social learning

Social learning occurs when an animal acquires information from observation of, or interaction with, another animal or its products (Heyes 1994). Alternatively, individuals can acquire their own information via asocial or individual learning (Heyes 1994). Via social learning novel behaviour patterns, also referred to as 'innovations' (Laland & Reader

1999a), can be transmitted across individuals in a population, potentially forming long-lasting traditions and spreading the behaviour across so-called 'cultural generations' (Chapter 2). Some well-known social learning examples describe the spread of novel foraging techniques. Black rats, *Rattus rattus*, living in pine forests in Israel have a special technique to extract seeds from pine cones (Zohar & Terkel 1996). Experiments have demonstrated that naïve rats learn the efficient, spiral scale removal technique via observation of conspecifics or interaction with an unfinished cone from conspecifics. This technique allows them to access the pine seeds in an energetically efficient manner, and thus occupy the novel pine forest habitat. In another field study, manipulation of reef fish populations showed that tradition determines mating site use (Warner 1988). Experimental replacement of an entire local population of bluehead wrasse, *Thalassoma bifasciatum*, led to the use of arbitrary sites which persisted long after manipulations. Sites that were used by previous populations were no more likely to be used as new sites. Additionally, control populations that were removed and replaced at their original location re-used all the previously used sites.

Both theoretical and empirical approaches have been taken to understand the evolution of social and individual learning. The use of social information as opposed to obtaining personal information via trial and error learning often may be adaptive, because an individual does not incur the costs and risks associated with the exploration of for example novel food resources or predators (Galef 1995, 1996; Laland 1996). Theoretical models (Boyd & Richerson 1985; Boyd & Richerson 1988; Rogers 1988; Laland et al. 1993; Kendal et al. 2009) have consistently found that when the environment is variable it is less likely that reliance on social cues is favoured by selection, since chances are smaller that any two individuals will experience similar environments, and thus the information will be outdated. Under stable conditions, social cues can provide reliable information on the current state of the environment and thus are more rewarding to use. At intermediate rates of change, however, a combination of social and individual learning is expected.

Experimental studies investigating the predictions from these models are scarce however. Suggestive evidence that supports a bias for social information use under more stable environmental conditions comes from a study on following behaviour of conspecifics in bats (*Chiroptera spp*.) that showed that social learning frequency is predicted by individual foraging success and resource stability (Wilkinson & Boughman 1999). In a laboratory setting, Norway rats, *Rattus norvegicus*, typically show reliable copying of a conspecific's food choice after previous interaction with it (Galef & Wigmore 1983; Galef 2002) (This paradigm is utilized in Chapter 5). With experimentally induced variation in food availability,

social influences on rats' food choices under stable conditions were more pronounced compared to variable conditions (Galef & Whiskin 2004). However, these studies considered current behaviour and did not investigate the development of social information use in an experimental setting. This issue will be further addressed in Chapter 5.

The use of social or individual information within a population has also been modelled as the frequency dependent producer-scrounger model originally describing foraging tactics (Barnard & Sibly 1981; Rogers 1988; Caraco & Giraldeau 1991). Here, individuals that obtain their own information are considered information producers and individuals that use social information are scroungers. Scroungers circumvent the costs associated with information acquisition, because they parasitize on the information provided by producers. However, the benefits they gain from scrounging depend on the frequency of information producers and scroungers in the group. The more producers there are the more it pays to scrounge. Conversely, the more scroungers there are the fewer resources are left for the producers. Eventually, an equilibrium of individuals using the producer or scrounger theory will be reached where both strategies have equal fitness. Individuals' strategy use, however, might not be fixed to either producing or scrounging information but could be deployed flexibly and depend on the situation at hand.

Species' and individuals' propensity to use social and/or asocial information can be influenced by factors such as social dynamics, the environment and (developmental) experience (Chapter 5). For example, living in groups drives general learning performance, as shown in a comparative study between a territorial and a group living columbid bird species (Lefebvre et al. 1996). Environmental conditions and social dynamics shape individual flexibility in the use of foraging tactics in birds (Giraldeau & Lefebvre 1986, 1987; Caraco et al. 1989; Morand-Ferron et al. 2011). Variation in resource availability early in life increases learning capabilities in fish (Kotrschal & Taborsky 2010). Early social environment has been shown to shape social behaviour in fish (Chapman et al. 2008) and in birds (Katsnelson et al. 2008). Guppies, *Poecilia reticulata*, that were reared in low densities increased shoaling behaviour and subsequently performed better at a social learning task compared with performances of high density rearing groups. In the house sparrow, *Passer domesticus*, social foraging tendencies of parents experienced early in life determined offspring foraging strategies later in life. Recently it has been shown that individual learning ability positively correlates with the tendency to forage as producers in house sparrows (Katsnelson et al. 2011). These findings strongly suggest that early experiences influence the employment of information acquisition

strategies in animals, which potentially leads to behaviour better suited to local environmental conditions. The influence of early life experiences and its effects on social information use in rats will be further addressed in Chapter 5.

Social learning processes

To understand how social learning works, i.e. how individuals are influenced by others and learn as a result, classification systems of underlying behavioural processes for acquisition have been described (Galef et al. 1988; Whiten & Ham 1992; Heyes 1994; Hoppitt & Laland 2008). These processes are not mutually exclusive *per se*, which has made it hard to formulate testable hypotheses. Mainly for this reason, discussions on social learning processes tend to be controversial. For the purpose of this Introduction, I will summarize a selection of the proposed social learning processes including the relevant mechanisms for this thesis (Box 1).

Hoppitt and Laland (2008; page 108) defined social learning as 'any process, both direct and indirect, through which one individual (the 'demonstrator') influences the behaviour of another individual (the 'observer') in a manner that increases the probability that the observer learns', to which it is important to note that the new behaviour is retained in the subsequent absence of the demonstrator (Nicol 1995). Learning could arise through increasing the observers' attention to certain stimuli or objects (stimulus enhancement), to specific areas in its environment (local enhancement) or when knowledgeable conspecifics expose observers to a relationship between two stimuli or between a response and a stimulus (observational conditioning), which often results in changes in the observers' behaviour towards the novel cues. Naïve individuals learn about the cues, adjust their behaviour accordingly and fine tune their behaviour due to increased interactions and individual learning experiences. Observers can also acquire a novel behavioural act by observation of a demonstrator (imitation) which is followed by individual learning experiences as well. An influential factor on performance, particularly in grouping species, is the presence of conspecifics (social facilitation).

Box 1. Social learning and related mechanisms defined

Social learning mechanisms:

a. Stimulus enhancement: occurs when observation of a demonstrator exposes the observer to a single stimulus or object and consequently changes the observer's behaviour towards similar stimuli or objects, independent of its location and in absence of the demonstrator (Spence 1937; Heyes 1994; Hoppitt & Laland 2008). This process is often followed by individual learning, where the observer further interacts with the stimulus and thus further improves its behaviour. For example, bumblebees, *Bombus impatiens*, in a new flower field preferentially foraged from the same flower type as they previously observed demonstrator bees forage from (Worden & Papaj 2005).

b. Local enhancement: occurs when observation of a demonstrator or its products attracts the observer to a particular location in its environment (Galef 1988; Hoppitt & Laland 2008). For example, guppies preferentially foraged where they had previously seen conspecifics feed (Reader et al. 2003).

c. Observational conditioning: occurs when a demonstrator exposes an observer to a relationship between two stimuli or between a response and a stimulus, respectively, which changes the observer's behaviour that is *already in their repertoire* towards this relationship in the absence of the demonstrator (Heyes 1994). Naïve zebrafish, *Danio rerio*, show anti-predatory behaviour after observing stressful behaviour in conspecifics or in response to passively released alarm substances from conspecifics' injured skin. Zebrafish that were exposed to alarm substances in combination with a novel odour subsequently learned to show alarm responses to this novel odour only (Suboski et al. 1990). Thus, subjects learned to associate a novel odour to existing anti-predatory behaviour, illustrating observational conditioning.

d. Imitation learning: occurs when the observer acquires a *novel motor act* through observation of a demonstrator. However, this process tends to be controversial and other mechanisms can not easily be ruled out (Heyes 1994). The two-action test, where observers are exposed to demonstrators that have been trained to solve the same task but using different techniques, has been suggested to distinguish between enhancement processes and imitation (Whiten & Ham 1992).

Mechanisms related to social learning:

e. Social facilitation: occurs when the presence of conspecifics, or potential demonstrators, affects observer's behaviour (Hoppitt & Laland 2008). This mechanism can indirectly affect social learning via, for example, decreases in stress levels reducing neophobia, resulting in more exploratory behaviour and thus individual learning but without actual acquisition of information from demonstrators.

f. Social support: occurs when the mere presence of conspecifics in a similar environment leads to convergent behaviour (Whiten & Ham 1992).

Social learning strategies

Evolutionary theory on the adaptive value of social learning suggests that individuals should be selective in social information use, i.e. individuals should be choosy in whom they use information from and under which circumstances, making it adaptive behaviour (Laland 2004). Laland (2004) proposed a set of learning rules, or social learning strategies, to identify under which conditions individuals would use information from others. These strategies are divided into two types: *Who* and *When* strategies.

Who strategies specify which individuals an observer should copy. For example social dynamics (i.e. association patterns) can direct observers' attention towards specific demonstrators. Examples include patterns of familiarity (Swaney et al. 2001; Galef & Whiskin 2008), affiliation (Scheid et al. 2007), relatedness (Kavaliers et al. 2005), size (Duffy et al. 2009), dominance status (Nicol & Pope 1999), and sex (Scheid et al. 2007). Observers can also show a disproportionate tendency to copy the majority of the group (also referred to as conformism) (Lachlan et al. 1998; Toelch et al. 2010). In Chapter 1 the influence of demonstrator size and number on social learning is investigated. In Chapter 3, the development of familiarity patterns (social memory) in zebrafish will be addressed.

When strategies specify under which circumstances an individual should exploit social information. For example when demonstrators always give reliable information about the environment (Leadbeater & Chittka 2009), when demonstrators' returns are higher than one's own (Pike et al. 2010), when dissatisfied or hungry (Galef et al. 2008), when asocial learning is costly or risky (Galef & Whiskin 2006; Webster & Laland 2008), or when demonstrators exhibit specific activities like feeding but not resting (Scheid et al. 2007). Thus learning seems not to be random but biased in many ways, often dependent on previous experiences that shape information use. Development of the tendency to use social information when early social cues potentially provide information about the stability of the environment is addressed in Chapter 5.

Neural underpinnings of social behaviour

Research into the neural mechanisms of social behaviour in mammals established an important role for two peptide families; the oxytocin- and vasopressin families (Donaldson & Young 2008). Therefore these peptide systems have been suggested to regulate 'sociality' in vertebrates. Another line of research has focussed on the role of nonapeptides in individual learning (e.g. active or passive avoidance paradigms) and memory, including social memory in mammals and how nonapeptides affect these behaviours (Engelmann et al. 1996). Vasopressin in particular has been shown to be important for social memory formation and social recognition (Winslow & Insel 2004). Below, I review the nonapeptide systems and research on social behaviour in vertebrates.

Nonapeptides and their receptors

One peptide superfamily has been the main focus in social neuroscience research (Insel & Young 2000). This nine-amino-acid peptide lineage (nonapeptides) has been traced far back in evolution and is present in both invertebrates and vertebrates (Fig. 1). Throughout evolution both the structure and function of nonapeptides have been well conserved. Nonapeptides differ from each other at one or two amino acid positions (Table 1), they are synthesized and released from the hypothalamus (mainly the preoptic area) in mammals or from similar neurosecretory brain regions in non-mammals, they have similar peripheral and central regulatory roles and interact with gonadal steroid hormones and neurotransmitters (e.g. dopamine and serotonin) (Gimpl & Fahrenholz 2001; Lim & Young 2006; Donaldson & Young 2008; Goodson 2008).

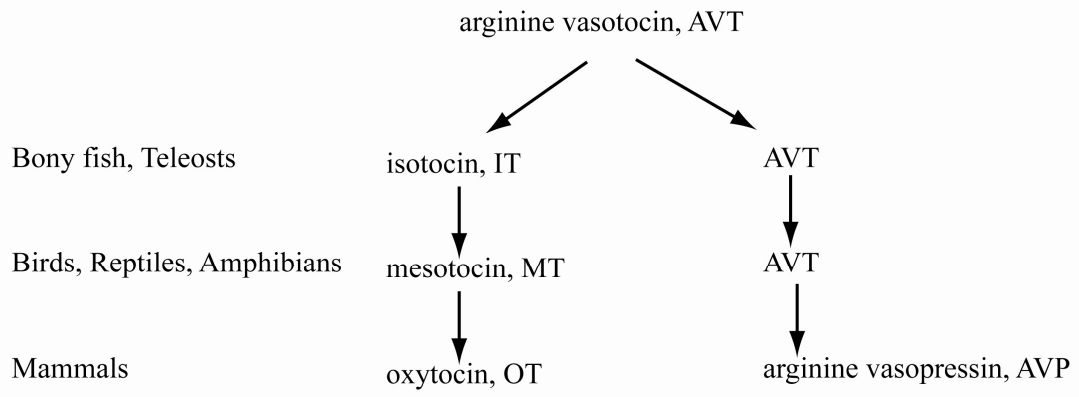

Figure 1: Overview of the nonapeptide families in vertebrates (Modified from Goodson 2008).

Table 1: Nonapeptides in vertebrates. Nine amino acid sequences of peptides compared to oxytocin. * indicates identical amino acid. (Modified from Gimpl & Fahrenholz 2001)

Peptide	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly	NH2
Isotocin	*	*	*	Ser	*	*	*	Ile	*	*
Mesotocin	*	*	*	*	*	*	*	Ile	*	*
Vasotocin	*	*	*	*	*	*	*	Arg	*	*
Vasopressin	*	*	Phe	*	*	*	*	Arg	*	*

The vertebrate nonapeptides can be divided into two groups: the oxytocin-related and the vasopressin-related family (Gimpl & Fahrenholz 2001). The non-mammalian homologs for oxytocin (OT) are isotocin (IT) in bony fish (teleosts) and mesotocin (MT) in birds, reptiles and amphibians. In mammals the main peripheral functions of oxytocin are its modulatory roles in parturition and lactation. Also, oxytocin is released during stress and after sexual stimulation. OT receptors have been found in the brain, kidney and reproductive organs (Gimpl & Fahrenholz 2001). The vertebrate homolog for arginine vasopressin (AVP) is arginine vasotocin (AVT) in all non-mammals. Vasopressin regulates homeostatic water and electrolyte balance in the liver and kidneys and is released after sexual stimulation, stress and parturition. There are three receptor types for AVP: V1a, V1b and V2. The V1a and V1b receptors are mainly found in the (fore)brain and the uterus, whereas V2 receptors in the kidney are anti-diuretic (Insel 2010). Even though OT and AVP show general regulatory roles in diverse social behaviours, species- and sex specific differences have been found in the direction of the effects of the nonapeptides (Insel & Young 2000; Goodson 2008; Insel 2010). However, OT-related peptides generally increase ‘pro-social’ behaviour (affiliation) and AVP-related peptides generally increase ‘anti-social’ behaviour (aggression) (Goodson & Bass 2001; Ross & Young 2009; MacDonald & MacDonald 2010).

Sociality and nonapeptides in mammals

Nonapeptides have been shown to influence social behaviours such as pair bonding (Young et al. 1998; Keverne & Curley 2004; Young et al. 2011), parent-offspring bonding (Insel & Young 2001), social recognition and memory (Bielsky & Young 2004; Lee et al. 2009), maternal behaviour (Keverne & Curley 2004; Campbell 2008; Neumann 2009), cooperation (Soares et al. 2010) and aggression (Greenwood et al. 2008).

The influence of OT and AVP has been extensively studied in mammalian social behaviour. One of the best described models for a regulatory role of OT and AVP in sociality is found in comparative studies in four vole species (*Microtus spp*.) that show different behaviours ranging from highly social to asocial behaviour: prairie voles (*M. ochrogaster*) and pine voles (*M. pinetorum*) are monogamous and form strong pair bonds, show biparental care and live in burrows with extensive families whereas montane voles (*M. montanus*) and meadow voles (*M. pennsylvanicus*) are promiscuous and do not pair bond, show limited maternal care and live solitarily.

Pair bond formation and aggression differences between voles can be explained by nonapeptide receptor gene expression differences that cause distinct V1a and OT receptor distribution patterns in the forebrain (Lim et al. 2004; Insel 2010). Administration studies have shown that central AVP or OT facilitate, and blocking of V1a or OT receptors inhibit, pair bonding and aggression in the monogamous males or females, respectively (Winslow et al. 1993; Williams et al. 1994; Young et al. 1997; Ross et al. 2009). Importantly, Lim and Young (2004) show that AVP release interacts with the dopaminergic system (a system important for reward-related behaviour) and that manipulations to the AVP system in one reward-related brain area particularly affect pair bonding but not other social behaviours. Similar correlations between behavioural phenotypes and receptor distribution differences in the forebrain have been found across taxa, for example in deer mice (*Peromyscus spp*.) (Bester-Meredith et al. 1999) and marmosets (*Callithrix spp*.) (Wang et al. 1997). All together, these results provide evidence for a specific neural system that underlies pair bond formation in mammals (Insel & Young 2001; Lim & Young 2004).

Apart from species typical differences in nonapeptide receptor distribution patterns and correlated behavioural differences, considerable within species differences in receptor distribution and behaviour exist (Young 1999). For example, in the promiscuous meadow vole individual differences in both receptor distribution and selective attention to a potential mate were found (Lim et al. 2004). This suggests plasticity in the nonapeptide system that would potentially allow individuals to respond to environmental or social conditions early in life that could lead to adaptive behaviour later in life. Additionally, a subpopulation of polygamous, non-social voles showed aggressive and affiliate behaviour similar to monogamous, social vole species' behaviour under specific conditions in a lab setting (Parker et al. 2001). In mammals, social interactions such as parental care and sibling interactions early in life have also shown to affect gene expression, neuro-endocrine development and influence adult behavioural patterns (Fleming et al. 1999; Cushing &

Kramer 2005). An extensively used paradigm to study such epigenetic effects is the maternal care system in rats. Dams consistently differ in the intensity of licking and grooming behaviour they provide to their offspring in the first weeks postpartum (Caldji et al. 1998; Cameron et al. 2005). These differences affect neuro-endocrine development in the pups. For example, the degree of maternal care received has been shown to shape oxytocin sensitivity, patterns of expression of oxytocin neurons and receptors, neuron projections (Shahrokh et al. 2010), social behaviours (Melo et al. 2006) and cognitive function (Champagne et al. 2008). Together, these examples show that nonapeptide receptor gene expression is highly plastic and experiences and social context could potentially alter behavioural response to nonapeptides in response to changes in the socio-ecological environment allowing animals to display adaptive behaviour which could ultimately lead to speciation (Insel & Young 2000; Lema & Nevitt 2004b; Semsar & Godwin 2004; Goodson et al. 2009a; Kabelik et al. 2009). In Chapter 5 I will take advantage of maternal care differences in rats to address developmental influences on social learning.

Sociality in birds and fish

In the last decade, research on the evolution of underlying neural mechanisms of social behaviour in non-mammals has received more attention. In birds, nonapeptidergic influences on affiliation, aggression and flocking has predominantly been studied in songbirds (Passeriformes). In the highly gregarious and non-territorial zebra finch, *Taeniopygia guttata castanotis*, infusion of AVT increases aggression in both sexes and is unaffected by MT infusion. In males only, AVT antagonist administration reduces aggression, which suggests a more important regulatory role for the AVP system in aggression in males than in females (Goodson et al. 2004). Contrary to the situation in mammals, administrations of AVT, MT or its antagonists do not influence male courtship or mate choice behaviour (Goodson et al. 2004; Kabelik et al. 2009). However, Goodson et al. (2009a) demonstrate a potential indirect role for the AVT system in mate competition by showing that AVT release specifically affected aggression levels in the context of opposite sex affiliation but not territorial aggression in zebra finches. In contrast with the enhancing effect of AVT on aggression in non-territorial songbirds, AVT administration in territorial songbirds has the opposite regulatory effects. In these bird species, AVT decreases aggression and/or agonistic song in males (Goodson 1998a, b). Thus regulatory effects of nonapeptides in birds seem to be dependent on social status and behavioural context.

Further evidence supporting the role of the AVT system in the zebra finch comes from studies on flocking behaviour. Blocking AVT

production or AVT receptors significantly decreases preferences for larger group sizes in social interactions in the zebra finch (Kelly et al. 2011). In another study, MT but not AVT infusions increased social preferences for familiar individuals and larger groups, whereas blocking MT receptors reduced both these preferences in females (Goodson et al. 2009b). Receptor distribution differences in the lateral septum in the forebrain correlate with flock size across different species of finches (Goodson et al. 2009b). These findings suggest a similar role of nonapeptides in modulating sociality to that found in mammals.

In teleost fish, the AVT system influences aggression and vocalizations depending on a species' social system and an individual's social status within its group. AVT decreases, and a V1a antagonist increases, aggressive displays in territorial males or species (Semsar et al. 2001; Santangelo & Bass 2006). In contrast, it increases aggression in non-territorial individuals or species (Semsar et al. 2001; Lema & Nevitt 2004a). Similarly, AVT increases grunt-like vocalizations during agonistic encounters in territorial male plainfin midshipman, *Porichthys notatus*, whereas IT decreases these in females and non-territorial male conspecifics (Goodson & Bass 2000b). Additionally, behavioural differences due to territoriality and social rank have also been linked to AVT expression differences in three preoptic area regions; the parvocellular, magnocellular and gigantocellular region. Higher levels of aggression correlate with smaller numbers of large AVT neurons in the magnocellular or gigantocellular preoptic area whereas lower levels of aggression correlate with higher numbers of small AVT neurons in the parvocellular preoptic area (Lema & Nevitt 2004b; Larson et al. 2006; Greenwood et al. 2008; Iwata et al. 2010; Dewan et al. 2011) (Fig. 2). These results thus correlate behavioural phenotypes to neuro-anatomical differences in the preoptic area.

While the regulatory role of AVT in male mate bonding in voles is clearly established, the involvement of AVT in fish courtship behaviour is not as evident. AVT infusions have been shown to increase or have no effect on courtship behaviour whereas V1a antagonist administrations can decrease courtship (Semsar et al. 2001; Santangelo & Bass 2010). Recently however, a study on pair bond formation in the monogamous convict cichlid, *Amatitlania nigrofasciata*, showed that the AVP/IT systems in males modulate affiliation towards females and aggression towards males (Oldfield & Hofmann 2011). Non specific blocking of AVT/IT pathways during pair formation significantly decreased both aggression and affiliation. This study thus suggests that similar neural circuits potentially underlie pair bonding in fish, birds and mammals.

Finally, social approach and withdrawal behaviour towards a conspecific and the influence of AVT and IT have been studied in the

goldfish, *Carassius auratus* (Thompson & Walton 2004). Depending on baseline sociality levels, AVT and IT affected shoaling in males. AVT infusions significantly inhibit social approach in the highly social phenotype but not in the less social fish. Whereas the AVT antagonist and the IT infusions stimulate approach in the low social phenotypes but do not affect the highly social males. Together with the finding of AVT producing cells in the preoptic area (Thompson & Walton 2009), these results show that the AVT/IT systems modulate the tendency to shoal in goldfish.

Considering the research on social behaviour and the regulatory role of the AVP/AVT and OT/IT systems in vertebrates thus far, results provide strong evidence for nonapeptides playing a role in sociality that is highly conserved across taxa. However, the direction and strength of the modulatory role of the two systems is highly dependent on species social organization and environment, but also on individual's sex and phenotype.

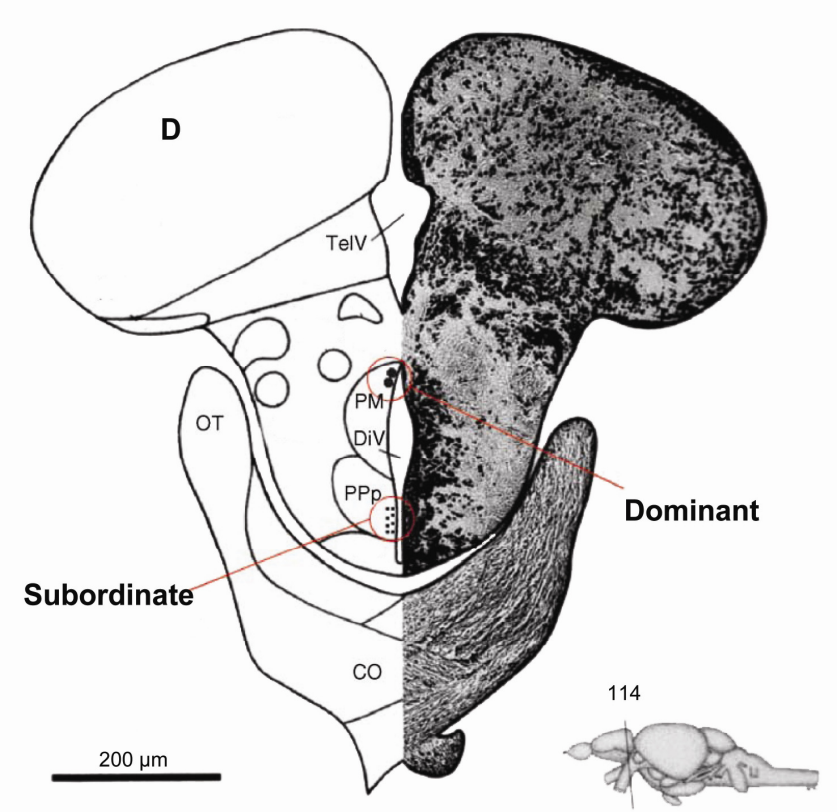

Figure 2. Cross section through the zebrafish forebrain. Inset: position of cross section. CO: optic chiasm; D: dorsal telencephalic area; DiV: diencephalic ventricle; OT: optic tract; PM: magnocellular preoptic area; PPp: parvocellular preoptic area; TelV: telencephalic ventricle. The gigantocellular preoptic area is located more caudally of the PM. The vasotocin expressing cells of dominant individuals are represented by two large black circles and the vasotocin expressing cells of subordinate individuals are represented by eight small circles (Modified from Larson et al. 2006).

The zebrafish as a model organism in behaviour

In this thesis, the main study species used was the zebrafish, *Danio rerio*. The zebrafish has been an important model organism in developmental biology, genetics and neurophysiology. Its qualities, such as a small size (2 – 3 cm standard length), easy breeding, short generation time and its relatively large and transparent eggs, made the zebrafish an excellent model for manipulation studies in the laboratory (Grunwald & Eisen 2002). Extensive research has lead to a fully sequenced zebrafish genome (www.sanger.ac.uk; The Sanger Institute, Hinxton, UK) and broad knowledge of its central nervous system function and development. Additionally, there are many genetic, neural and endocrine similarities to other vertebrates including humans. For example, comparison between the zebrafish and human genome has shown highly conserved chromosome segments (Postlethwait et al. 1998) and AVT/AVP neuro-anatomy and function has been shown to be highly conserved across vertebrate evolution (Moore & Lowry 1998). The extensive knowledge and genetic tools (e.g. knockouts, clones) available are the main reasons why the zebrafish has recently become an increasingly important and popular model organism in behavioural neuroscience (Sison et al. 2006; Spence et al. 2008) (Box 2). Importantly, it has been suggested that teleosts including the zebrafish have retained many primitive characters (Metscher & Ahlberg 1999). In particular, the neuro-anatomy of AVT in teleost fish is less complex than the AVP system in mammals, which makes it easier to study nonapeptide systems in the zebrafish than in rodents, for example (Moore & Lowry 1998). Recently, studies have shown the zebrafish's potential to replace classic rodent models in exploration and (developmental) stress research (Stewart et al. 2010; Wong et al. 2010; Steenbergen et al. 2011b).

Use of the zebrafish as a model permits us to investigate the four main questions that can be asked about behaviour, namely function, causation, development and evolution (Tinbergen 1963; Bolhuis & Verhulst 2009) (Fig. 3). It allows us to study questions that address what stimuli cause a specific behaviour and which neural mechanisms regulate it. Additionally, we can study how behaviour changes during development and into adulthood, how it potentially affects survival and allows findings to be placed into an evolutionary perspective. An evolutionary approach based on phylogenetic relations will help to identify divergent or convergent nonapeptide regulatory elements for social behaviour across vertebrates including non-mammals (Metscher & Ahlberg 1999). Even though lines leading to humans and zebrafish diverged about 450 million years ago (whereas rodents and humans diverged about 112 million years ago) (Kumar & Hedges 1998), studying simpler social behaviour (e.g. grouping) in zebrafish will allow to trace

basic, fundamental elements underlying this behaviour, which could potentially lead to the identification of some core neural mechanisms associated with more complex or phylogenetically recent social behaviour (e.g. pair bonding, attachment) in mammals. Thus zebrafish could provide additional insights on general underlying principles of vertebrate social behaviour and its evolution.

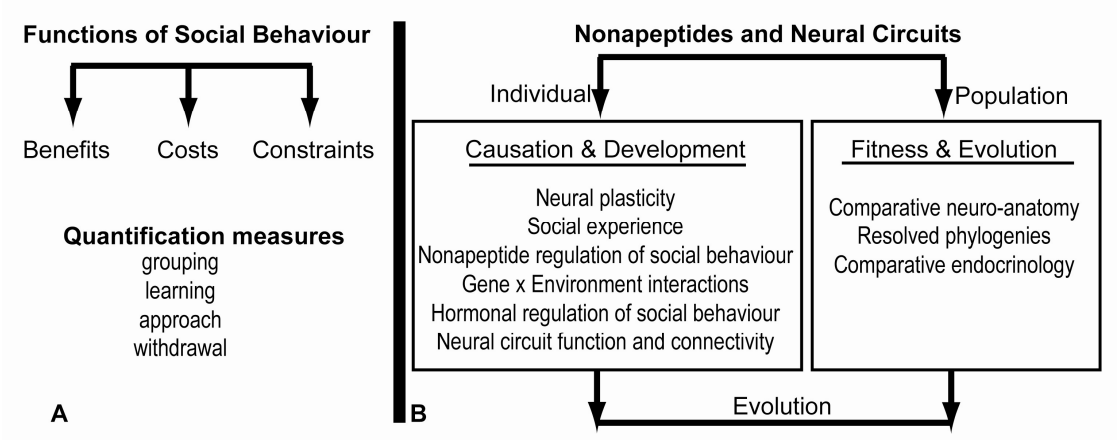

Figure 3: Conceptual areas to study the multiple components of social behaviour. Panel A: Social behaviour can result in benefits obtained, potential costs, or might be constrained. Objective quantification of sociality will aid in understanding the function of social behaviour. Panel B: To study the evolution of nonapeptides and how these potentially underlie a neural circuit to regulate sociality multiple components on the individual (left panel) and the population (right panel) level should be investigated. (Figure modified from O'Connell & Hofmann 2011)

Box 2. A short review of research on zebrafish behaviour

The zebrafish is a small shoaling diurnal freshwater teleost that naturally occurs in rice paddies or relatively shallow seasonal waters in South and Southeast Asia and mostly feeds on zooplankton and insects (Spence et al. 2006; Engeszer et al. 2007b). Knowledge on zebrafish behaviour is limited to shoaling, foraging, reproduction and individual learning (Spence et al. 2008). Zebrafish are asynchronous batch spawners and provide no parental care. Larvae start shoaling soon after hatching during which they develop a strong and robust shoaling preference based on experiences with conspecifics. When they reach the juvenile stage (approx. 1 cm standard length), they express a shoaling preference for same morph individuals as experienced during the larval stage, a preference which remains fixed into adulthood (Wright et al. 2003; Engeszer et al. 2004; Engeszer et al. 2007a). Furthermore, adult zebrafish shoaling preferences are influenced by shoal activity and shoal size (Pritchard et al. 2001), nutritional state (Krause et al. 1999), sex and shoal size (Ruhl & McRobert 2005). Zebrafish have also been used to study learning and memory processes. For example, zebrafish have been shown to associate a social reward (Al-Imari & Gerlai 2008) or food reward with a visual cue (Colwill et al. 2005) in a maze. In an active avoidance conditioning task, individuals learned to swim to a safe and dark compartment before a light switched on which predicted delivery of a mild electrical shock (Xu et al. 2007). Finally, naïve zebrafish socially learn anti-predatory behaviour via alarm substances released from conspecifics (Suboski et al. 1990). Though zebrafish are relatively new in behavioural research, there is strong experimental evidence that many fish species (e.g. guppy and stickleback, *Gasterosteus spp*.) show social learning of anti-predatory behaviour, foraging behaviour and mate choice copying (Bshary et al. 2002; Brown & Laland 2003). Further information on zebrafish behaviour will be provided in the introduction of the following Chapters.

Aim and scope of the thesis

The aim of this thesis is to elucidate the neural mechanisms of social behaviour. I address open issues such as: What neural mechanisms regulate social behaviour in bony fishes? To what extent are the nonapeptide systems the same and are they evolutionary conserved across vertebrates? How does development or experience shape social behaviour and its regulatory neural network? Specifically, I investigate social behaviour in the tightly shoaling zebrafish and how the vasotocin- and isotocin-system regulate sociality. Moreover, I examined social learning in the rat and how early development and the oxytocin-system influenced sensitivity to social information. The focus on the zebrafish as a representative model system in this thesis follows from the knowledge on its genetics and development and builds on the promising but limited studies on its behaviour. In the final Chapter, I evaluate my findings and address the further potential of this model system in the field of social neuroscience.

Thesis outline

I first investigated social behaviour in the zebrafish in order to establish a methodology for subsequent manipulation studies to gain more insight on the underlying neural mechanisms of sociality. Different categories of social behaviour are addressed in the Chapters 2 – 4. In Chapter 2, social learning of an escape response in zebrafish was studied. Having shown social learning in zebrafish successfully, I investigated the development of social memory in Chapter 3. Additionally, I transferred an established tagging procedure used in larger fish to zebrafish with success. The methodologies used to study social learning and social memory did not appear to be ideal for manipulation studies and therefore I examined shoaling behaviour. In Chapter 4, I used a simple approach - withdrawal test to investigate the influence of vasotocin and isotocin and their antagonists on grouping in the zebrafish. An administration procedure was designed to peripherally administer nonapeptides. Shoaling was affected by the vasotocin system. In Chapter 5, I took advantage of the well established developmental influences of maternal care in Norway rats to investigate both the effects of social experiences early in life and oxytocin on the propensity to use social information when adult. Here, I demonstrated that early social experiences shape the propensity to use social information later in life. By investigating different aspects of social behaviour and how these are affected by nonapeptides and development, we may gain important insights into a general conserved neural mechanism underlying vertebrate sociality. Moreover, the results illustrate the value of zebrafish for research into the evolution of social behaviour.

Chapter 2

SOCIAL LEARNING OF ESCAPE ROUTES IN ZEBRAFISH AND THE STABILITY OF BEHAVIOURAL TRADITIONS

Charlotte M. Lindeyer and Simon M. Reader

Animal Behaviour, 79, 827 – 834 (2010)

ABSTRACT

Multiple factors potentially influence the formation and longevity of behavioural traditions. In zebrafish (*Danio rerio*) we investigated whether subjects follow knowledgeable fish escaping from a novel artificial predator, learn this escape response, and maintain the demonstrated escape route and response when knowledgeable fish were removed. A moving 'trawl' net forced fish to escape, with two equidistant escape routes available. Groups of four naïve fish were placed together with demonstrator fish trained to use either one of the two routes. Observers with demonstrators were faster to escape than observers exposed to untrained fish, and were biased towards the demonstrated route, effects that persisted when demonstrators were removed. Thus zebrafish socially learned escape routes and to escape faster from the approaching trawl. To address whether escape responses were stably transmitted, we used a transmission chain with observers becoming demonstrators for further groups of observers, thus simulating three generations of social learning. Escape times remained stable along the transmission chain, but route preferences slowly collapsed. Thus while the escape response per se was reliably socially transmitted, more arbitrary choices such as route choice decayed rapidly over repeated episodes of social learning. Our results suggest pervasive species and population differences in social learning propensities.

INTRODUCTION

Social learning is widespread in animals (Galef & Giraldeau 2001), and social information can be passed repeatedly from individual to individual leading to the formation of traditions, as demonstrated in both laboratory and field studies (Corten 2001; Brown & Laland 2003; Whiten & Mesoudi 2008). Factors that influence the stability of social traditions such as group turnover, the costs and benefits of alternative actions, and the possibility of individual exploration, are all known to affect tradition dynamics (Lefebvre 1986; Galef & Whiskin 1997; Stanley et al. 2008; Whiten & Mesoudi 2008). Strikingly, traditions can maintain arbitrary behaviour patterns or inhibit the acquisition of optimal behavioural patterns (Warner 1988; Galef & Whiskin 1997; Laland & Williams 1998; Bates & Chappell 2002; Reader et al. 2008; Stanley et al. 2008; but see Galef 1995, 1996). Thus there is a need to investigate the longevity of arbitrary versus non-arbitrary socially transmitted traits (Thornton & Malapert 2009). Here, we study the social learning of predator evasion to address these issues.

Grouping provides protection against predators (Pitcher & Parrish 1993; Roberts 1996). For instance, guppies (*Poecilia reticulata)* form larger, more cohesive shoals in areas containing adept guppy predators, compared to areas of decreased predation risk (Magurran 2005). Grouping tendencies and behavioural homogeneity within groups are important to minimising predation risk: individuals that leave a group or behave differently from the group would be at increased risk (Landeau & Terborgh 1986; Theodorakis 1989), leading to the prediction that grouping individuals will follow the escape routes of others.

Individuals may gain further anti-predator benefits from group members by utilizing conspecific cues that indicate predator activity (social information use) and by learning about predators as a result of these cues (social learning). Such processes have been demonstrated in multiple taxa (Griffin 2004; Morand-Ferron et al. 2010). For example, information on predators is socially transmitted in the European blackbird *Turdus merula* by observation of mobbing (Curio et al. 1978), in minnows *Phoxinus phonixus* by observation of predator inspection behaviour (Pitcher et al. 1982; Magurran 1986), and in zebrafish *Brachydanio rerio* by visual observation of alarmed conspecifics and by alarm substances passively released from injured skin (Hall & Suboski 1995a, b). Socially learned and socially facilitated anti-predator responses to novel predator stimuli have been demonstrated in a number of shoaling fish species, as has social learning spanning several contexts, and long-lasting behavioural traditions in the wild (Brown & Warburton 1999; Corten 2001; Brown & Laland 2003; Kelley et al. 2003). This leads to the

prediction that when there is a cost to leaving the group, shoaling fish (and other grouping animals) will learn escape routes and escape responses from conspecifics, and that these escape responses will be stably transmitted to form behavioural traditions.

Studies of the social learning of escape routes provides a useful methodology to study tradition stability, but research on wild and domesticated guppies has produced apparently conflicting results (Brown & Laland 2002; Reader et al. 2003). In these studies, fish followed knowledgeable individuals. However, when these demonstrators were removed, wild but not domesticated guppies were biased towards their demonstrators' escape route. Genetic and experiential differences could account for these data, since laboratory guppies have not experienced predators within their lifetime or their recent evolutionary past. Guppies moved from a high to a low predation site show reduced shoaling tendencies (Magurran 2005), and thus it is likely that domesticated guppies show reduced anti-predator behaviour and less cohesive shoaling compared to wild guppies, both of which could limit the learning of escape routes.

Group composition and group size may be important additional influences on shoaling tendencies and social learning. Fish make active choices of who to shoal with, on the basis of characteristics such as group size, body size, familiarity, and competitive ability (Krause et al. 2000), which may direct the flow of social information (Sih et al. 2009). Although enlarged groups may compromise foraging discoveries if fish need to break visual contact with the shoal to locate food (Lachlan et al. 1998; Day et al. 2001; Stanley et al. 2008), fish in larger shoals typically perform better in foraging and escape tasks (e.g. Pitcher et al. 1982; Brown & Warburton 1999). Moreover, numerous studies show the rate of social learning increases with the number of demonstrators in a group (Laland 2004).

Here, we studied social learning of anti-predator responses in zebrafish. Zebrafish development, genetics and neurobiology are extensively studied, making them a potentially valuable but relatively unexplored species for behavioural studies (Gerlai 2003; Miklosi & Andrew 2006; Wright et al. 2006; Spence et al. 2008). We investigated whether zebrafish follow knowledgeable conspecifics trained to escape from a novel artificial predator (a moving 'trawl' net), whether they learn from this experience, and whether this behaviour is stably transmitted across generations. Fish could escape from an approaching trawl through one of two visually distinctive holes in an opaque partition. Both escape routes were equidistant and led to the same location and were hence functionally identical.

The experiment had three phases. In phase 1, 'demonstrators' were trained to one of the escape routes. We counterbalanced demonstrator route training to account for the possibility that one route was intrinsically preferred. In phase 2, naïve fish ('observers') were placed with demonstrators while both routes were open for use. We term subjects 'observers' to follow social learning terminology, but observers could both observe and interact with demonstrators. In phase 3, we tested observers without demonstrators to examine learning. We predicted that subjects would use the same escape route as the demonstrators and escape faster than fish without trained demonstrators. Following was predicted to result in learning about the escape response and route, with these behaviours maintained in the absence of demonstrators. Moreover, we predicted improved social learning from increased numbers of knowledgeable conspecifics. We used demonstrators and subjects of two body size classes. Body mass can affect competitive ability and shoaling preferences in fish (Laland & Reader 1999b; Krause et al. 2000), potentially influencing social learning (Duffy et al. 2009).

To address the stability of socially transmitted information we used a transmission chain design. Observers in one experimental treatment became demonstrators for a further set of observers, who then acted as demonstrators for another set of observers, thus simulating three generations of social learning.

METHODS

Subjects and housing

A total of 300 female zebrafish (*Danio rerio*; age 4 – 6 months) of the AB strain were used, originally obtained from the Max-Plank Institute, Tübingen and bred and reared in the Biology aquarium at Utrecht University. We used a single sex to avoid sexual interactions during the experiment. The fish had not previously participated in any experiments. The experiment was approved by the Utrecht Ethics and Animal Care and Use Committee under protocol DEC 2008.I.03.023, and conforms to ASAB guidelines.

Fish were reared from birth in mixed sex groups. Demonstrators and observers were reared and housed separately to avoid possible familiarity effects (Swaney et al. 2001). Two weeks before the experiment fish were moved to housing tanks in all female groups. All housing tanks (80.0 x 50.0 cm) were maintained at 26 ± 1°C, and had a water depth of 30 cm. Housing was enriched with artificial plants, pot shelters and gravel floor. Fish were on a 12h light:dark schedule with lights on at 0800 hours (no natural light present). Fish were fed flake food

(TetraMin, Tetra Ltd., Germany) twice daily; one hour before and after experimental sessions.

We used fish differing in body size to allow discrimination of demonstrators and observers and to address any influence of body size, i.e. large demonstrators were tested with small observers and vice versa. Sixty fish were chosen from rearing tanks at random to act as demonstrators, with equal numbers of large and small fish. We housed these fish in three tanks, each containing 20 individuals (10 large and 10 small). One tank was assigned to yellow route training ('yellow trained demonstrators'), one to red route training ('red trained demonstrators'), and one to no training ('sham demonstrators'). Large and small demonstrators were separated from one another by a perforated transparent barrier that allowed movement of water but not fish. Demonstrators were re-used during the experiment.

Two hundred and forty fish (120 large and 120 small fish, mean mass ± SE = 0.43 ± 0.01 g, 0.34 ± 0.01 g, respectively) were subjects (observers), including 80 that participated in the transmission chain. Observers only participated once in the experiment. Fish were tested in groups. Observers were always tested in groups of four, but demonstrator number varied with experimental treatment: (1) two demonstrators (treatment 2-DEMO, N = 10 groups of observers); (2) four demonstrators (treatment 4-DEMO, N = 10 groups); or (3) six demonstrators (treatment 6-DEMO, N = 10 groups). The control treatment used four (sham) demonstrators (CONTROL, N = 10 groups). Thus 40 groups of observers (160 fish) were tested. The experiment was counterbalanced so that equal numbers of groups within each experimental treatment were exposed to red and yellow trained demonstrators (within the control treatment all demonstrators were sham demonstrators). There were also equal numbers of groups exposed to large and small demonstrators. The 4-DEMO treatment continued as a transmission chain, in which each of the 10 groups of observers became demonstrators for a group of four naïve observers, and then these observer groups became demonstrators for another 10 groups of naïve observers.

Apparatus

Testing was conducted in a large tank (150 x 50 x 30 cm; Fig. 1) divided into two equally sized compartments by a white opaque PVC partition with two square and visually distinct escape holes (1.5 x 4.0 cm each; placed at 15 cm from the bottom of the tank) 30 cm apart from one another. In the 'trawl' zone the trawl net could be moved to within 2 cm of the partition, and the tank area was otherwise empty. The other, 'escape', zone was enriched with gravel floor throughout and four randomly caught unfamiliar companion fish, plants and pot shelter were

placed behind a transparent partition (with holes to allow for olfactory cues). This enrichment was provided in an attempt to make the escape zone the preferred zone for fish, and to thus minimize any swimming back and forth between compartments. To ease visual discrimination (Spence et al. 2008; Spence & Smith 2008), escape holes were bordered by electrical insulation tape: either 3 cm of red tape surrounded by a vertical 20 x 10 cm black/white striped area, or an identically sized yellow border surrounded by a horizontal black/white striped area. The trawl device (47.0 x 42.5 cm) was made of black mesh attached to a plastic coated metal frame. Soft brushes attached to the side and base of the trawl prevented fish from escaping around the sides of the trawl. The side of the tank facing the experimenter was covered with one-way glass.

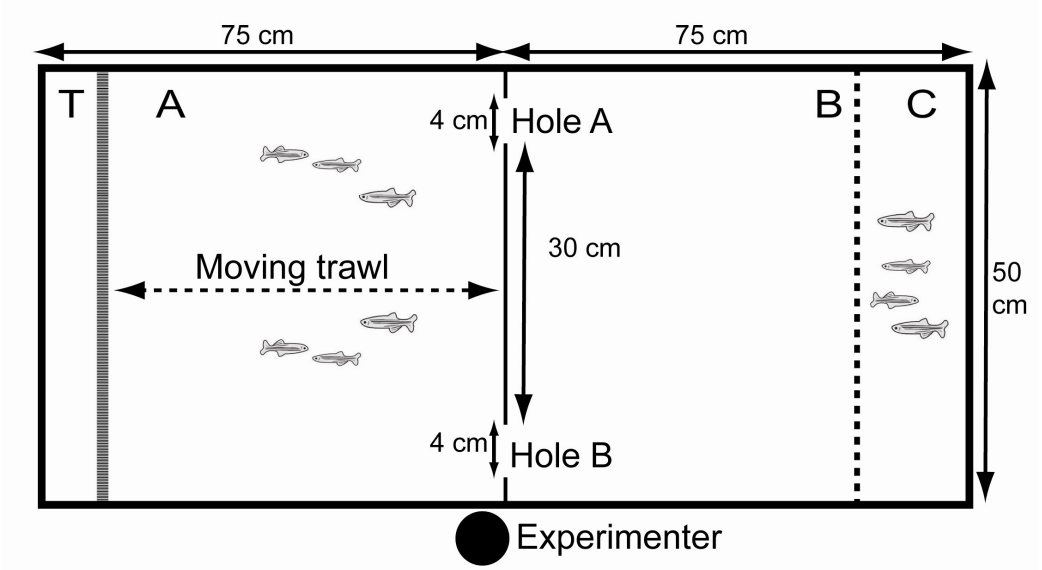

Figure 1. Schematic plan of the experimental tank. A: trawl zone, B: escape zone, C: companion fish, T: trawl device, E: experimenter. Hole A: red escape hole, Hole B: yellow escape hole. The trawl was pulled back and forth in zone A towards a central white partition with two holes that allowed subjects to escape.

Procedure

Each session consisted of four trials of 2 min each. During testing, fish were allowed 5 min to acclimatize after moving them between tanks and 1 min between trials. Fish were tested in two batches, with the schedule such that both demonstrator groups received the same number of days of training and the same interval between training and testing. Measurements were based on Sony DCR-SR55E video recordings.

Phase 1: Demonstrator training

Fish were trained in groups of 10 to use either the red- or the yellow route while the alternative route was blocked with transparent plastic. One training session consisted of four 2 min trials with 1 min rest between

trials. Demonstrators received two training sessions a day. A trial began with the trawl moving towards the partition. It was moved back and forth four times in the 2 min, pausing every 15 s at the tank end and at the partition. To begin training, enlarged escape holes extending to the bottom of the tank were used. When demonstrators did not exit through the escape hole, we used a brush to manoeuvre them to nearby the hole until they exited through it. When demonstrators were reliably swimming through the large hole in response to the trawl alone, this partition was replaced by the standard partition, with smaller escape holes. After a trial, fish that successfully escaped were gently herded back to the trawl zone by lifting the central partition. Demonstrators were considered fully trained when at least 80% escaped from the trawl within 30 sec on four consecutive trials. Training continued for 2 – 3 days after demonstrators reached this criteria and then fish were given 3 – 4 days rest. Control (sham) demonstrators were familiarized with the setup for 2 consecutive days with two sessions per day, undergoing the same procedure as the other demonstrators except that the trawl was stationary and both holes were blocked by transparent sheets to prevent fish learning the escape routes.

Phase 2: Testing demonstrators and observers

Depending on the experimental treatment, two, four or six (small or large) demonstrators were randomly selected from the trained demonstrator groups per session. Before starting the session, demonstrators repeated one 4-trial training session to confirm that they still used their trained route within 30 sec. All demonstrators met this criterion. Four randomly chosen, naïve observers of a different size class were placed with the demonstrators. Fish experienced one session composed of four trials, similar to the training session, except that both escape routes were open. Measures of escape latency and escape route were taken. If a fish did not escape from the trawl it was given a ceiling value of 2 min. Control fish underwent the same procedure but were placed with a group of sham demonstrators.

Phase 3: Testing observers

Demonstrators were removed and observers were tested after a 5-min pause for their route preference and latency in one session with four trials. The procedure was otherwise identical to phase 2.

Transmission chain

The 4-DEMO treatment continued after completion of phases 1 to 3. Former observers acted as demonstrators for a new set of four naïve observers (as in phase 2, without the training session to check

performance), and then the observers were tested alone (as in phase 3). Between sessions, new demonstrators were given a 5-min acclimatization period. These observers then acted as demonstrators for another set of four naïve observers, who were subsequently also tested alone. Thus, the 10 groups of 4-DEMO fish were further used for the transmission chain phase, resulting in 10 'chains' to which two groups of four fish were separately added. The transmission chain was counterbalanced such that there were equal numbers of groups that had experienced yellow and red route demonstrators, and equal numbers of groups of large and small body size.

Analyses

Dependent variables were escape latencies and escape route choices, calculated for demonstrators and observers separately. Latencies were individually measured, but means of the demonstrator and observer groups were used in the analyses, as zebrafish are schooling fish (Pitcher & Parrish 1993; Gerlai 2003) and thus individuals within a group could not be considered to behave independently. Route choice was measured as the relative preference for the yellow route, that is, the number of fish that used the yellow route minus the number that used the red route, divided by the total number of escapees (e.g. observers using yellow – red / all observers escaping). Values could range from -1 (all fish use the red route) to 0 (no preference) to 1 (all use the yellow route). If no fish escaped on a given trial, no route preference was calculated. We calculated route choice per group for each trial in a session.

Analyses were performed in R 2.6.2 and SPSS 16.0.2. For observer data, escape latency and escape route choice were analysed using linear mixed effects models (LMM) with repeated measures (trial) and nested random effects (formula lme of package nlme; Pinheiro et al. 2008) to estimate *P* values and degrees of freedom. Fixed effects were the number of demonstrators (2, 4, or 6), demonstrator route training (red, yellow), and demonstrator body size (large or small). Demonstrator escape latency was treated as a covariate in the transmission chain models addressing observer escape latency. Initially, all explanatory variables were entered into the models. Two-way interactions were investigated and terms were sequentially dropped until the minimal model contained only terms whose elimination would significantly reduce the explanatory power of the model. Nonsignificant ($P > 0.1$) interaction effects are not described in our results. Demonstrator size had no statistically significant effects and thus does not appear in the analyses below. We used separate analyses to make comparisons with control data. Demonstrator data were analysed using *t* tests, with latencies and route choice averaged across trials. Model assumptions were checked using box and qq-plots. Latency and route

choice measures could be estimated by normal distributions.

RESULTS

Phase 2: Demonstrator performance with observers present
Escape latency
All trained demonstrators met the criteria of escaping within 30 s when together with observers (mean escape latency ± SE = 17.2 ± 1.8 s), whereas the sham demonstrators were significantly slower than the trained demonstrators (*t* test: t_{38}= 17.42, $P < 0.0002$; mean latency ± SE = 97.5 ± 3.3 s). Yellow trained demonstrators escaped faster than red trained demonstrators, although not significantly so (*t* test: t_{28}= 2.00, P = 0.06; mean escape latencies ± SE = 13.6 ± 1.5 and 20.6 ± 2.1 s, respectively; Fig. 2). 2-DEMO demonstrators escaped significantly faster than 4-DEMO demonstrators, but there were no other significant differences in escape latency between demonstrator groups of different size (*t* test: 2-DEMO vs 4-DEMO: t_{18}= 2.59, P = 0.02; 4-DEMO vs 6-DEMO: t_{18}= 0.8, P = 0.4; 2-DEMO vs 6-DEMO: t_{18}= 1.93, P = 0.07).

Route choice
Red trained and yellow trained demonstrators escaped by their trained route on 93 % and 96 % of occasions, respectively (Fig. 3). On 75 % of trials no sham demonstrators escaped, and when escaping they took the yellow route on 65 % of occasions, suggesting a nonsignificant preference of naïve fish for the yellow route (mean route choice ± SE value of sham demonstrators = 0.25 ± 0.2; one sample *t* test: t_9= 1.39, P = 0.2). These results indicate that demonstrators performed according to their training, both in terms of their speed of escape and their route choice.

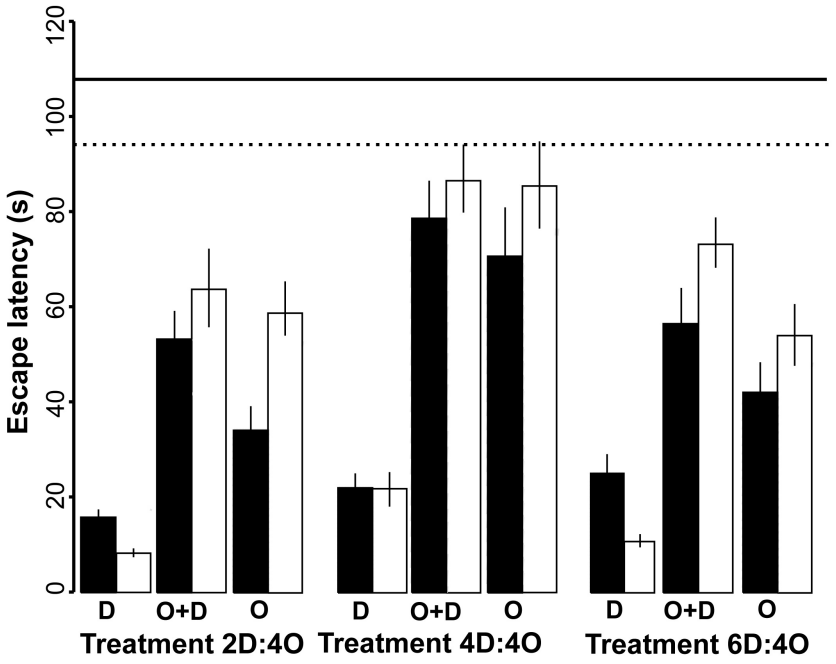

Figure 2. Mean escape latency (± SE) of groups of zebrafish demonstrators (D) and of observers in groups of four in the presence of 2, 4 or 6 demonstrators (O + D), or after these demonstrators were removed (O). Demonstrators were trained to the red route (filled bars) or yellow route (open bars). Control ('sham') demonstrators were in groups of four. Control subjects' mean escape latencies with and without sham demonstrators are shown by the solid and dashed lines, respectively.

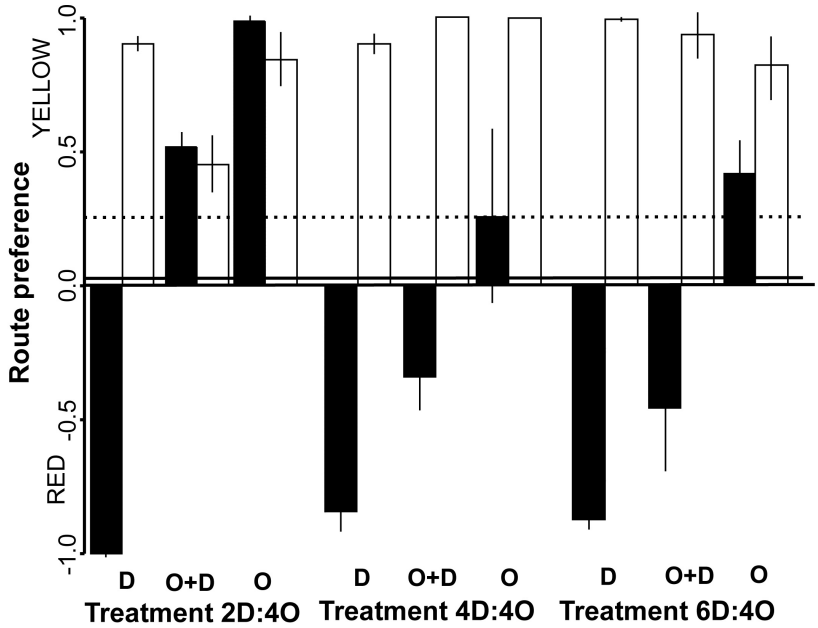

Figure 3. Mean route preferences (± SE) of demonstrators (D), of observers in groups of 4 with either 2, 4 or 6 demonstrators present (O + D) and of observers after demonstrators were removed (O). Demonstrators were trained to the red route (filled bars) or yellow route (open bars). Sham demonstrators' and control observers' route preferences are shown by the broken and solid line, respectively.

Phase 2: Observer performance in presence of demonstrators
Escape latency
Observers escaped significantly faster with trained demonstrators than with sham demonstrators (mean ± SE latencies = 69.4 ± 6.3 and 107.8 ± 3.5 s, respectively; *t* test: t_{38}= 4.06, P = 0.0002; Fig. 2).

Observers with red trained demonstrators escaped faster than those with yellow trained demonstrators (LMM: $F_{1,59}$= 4.31, P = 0.04; Fig. 2). The escape latencies of observers with trained demonstrators differed over trials (LMM: $F_{1,8}$= 5.20, P = 0.05), with observers escaping more rapidly on later trials (mean escape latency ± SE = 87.9 ± 7.8 and 70.2 ± 7.6 s for trial 1 and 4, respectively). The number of demonstrators present did not significantly influence observer escape latency (LMM: $F_{1,1}$= 0.97, P = 0.5).

Route preference
Demonstrator route training significantly influenced observer route choice (LMM: $F_{1,57}$= 21.35, P < 0.0001): observers with red trained demonstrators used the red route significantly more than those with yellow trained demonstrators (mean route choice ± SE = 0.03 ± 0.1 and 0.62 ± 0.1, respectively; Fig. 3). Thus, demonstrator route training biased observers towards the route the demonstrators took. The interaction effect between the number of trained demonstrators and demonstrator route training approached statistical significance (LMM: $F_{1,57}$= 3.40, P = 0.07), and so we investigated this further. Demonstrator route training had a significant effect on observer route in groups with 6 demonstrators present, approached statistical significance in groups with 4 demonstrators, but was not statistically significant in groups with 2 demonstrators (*t* test: 6-DEMO: t_8= 4.78, P = 0.001; 4-DEMO: t_8= 2.17, P = 0.06; 2-DEMO: t_8= 0.69, P = 0.5). Thus, observers were more likely to be biased towards the demonstrated route when more demonstrators were present. In the control condition, there was no significant difference in route choice between control observers and their sham demonstrators (paired *t* test: t_9= 1.12, P = 0.3; mean route choice ± SE observers and sham demonstrators = 0.05 ± 0.1 and 0.25 ± 0.2, respectively).

Phase 3: Observer performance in absence of demonstrators
Escape latency
When demonstrators were absent, observers that had been paired with trained demonstrators escaped more rapidly than observers that had been paired with sham demonstrators (*t* test: t_{38}= 2.96, P = 0.005; mean escape latencies = 59.2 ± 6.3 and 93.7 ± 5.0 s, respectively). Observers that had been exposed to red trained demonstrators escaped faster than those exposed to yellow trained demonstrators (LMM: $F_{1,59}$= 11.97, P = 0.001;

Fig. 2). The number of demonstrators did not significantly influence escape latency (LMM: $F_{1,1}$= 5.04, P = 0.3).

Observer groups that had experienced trained demonstrators escaped faster in the demonstrator absent phase than the demonstrator present phase (LMM: $F_{1,178}$= 6.22, P = 0.01; mean ± SE escape latencies = 59.23 ± 6.3 and 69.42 ± 5.2 s respectively).

Route preference

Observers' route choices were significantly influenced by the route training the demonstrators had received (LMM: $F_{1,59}$= 6.55, P = 0.01): former observers of red trained demonstrators used the yellow route significantly less than former observers of yellow trained demonstrators (mean route choice ± SE values = 0.53 ± 0.1 and 0.75 ± 0.1, respectively; Fig. 3). Thus, despite an apparent general bias for the yellow route, observers were biased towards the route the demonstrators had taken. The number of trained demonstrators did not have a significant effect on observer route choices (LMM: $F_{1,1}$= 0.20, P = 0.7). Control observers' route use did not significantly differ from their sham demonstrators' route use (paired t test: t_9= 0.40, P = 0.7; mean route choice ± SE values = 0.15 ± 0.2 and 0.25 ± 0.2, respectively).

Observers that experienced red route demonstrators used the red route more with than without demonstrators (t test: t_{178}= 14.35, P < 0.0002; mean route choice ± SE = 0.03 ± 0.1 and 0.53 ± 0.1, respectively). In contrast, route choice of observers of yellow route demonstrators did not significantly change when demonstrators were removed (t test: t_{28}= 0.86, P = 0.4). Similarly, route choices of observers of sham demonstrators did not significantly change when these sham demonstrators were removed (paired t test: t_9= 0.80, P = 0.5; mean observer route choice ± SE values = 0.05 ± 0.1 and 0.15 ± 0.2, with and without demonstrators, respectively).

Transmission Chain

Demonstrator performance with observers present

Escape latency

Demonstrators became slower to escape from the first to second and third generation (Fig. 4; note that second generation demonstrators were observers of first generation observers, and third generation demonstrators observed second generation demonstrators). Demonstrators escaped faster than observers, but only in generation 1 was this effect significant (t tests: generation 1: t_{18}= 5.33, P < 0.0001; generation 2: t_{18}= 0.87, P = 0.4; generation 3: t_{18}= 0.77, P = 0.5). Red or yellow trained demonstrators did not significantly differ in escape latency (t test:

generation 1: t_8= 0.09, P = 0.9; generation 2: t_8= 1.97, P = 0.09; generation 3: t_8= 0.28, P = 0.2; Fig. 4).

Route preference

Demonstrator groups in chains seeded by red or yellow trained demonstrators significantly differed in their route preferences in generation 1 but not in generations 2 and 3 (t tests: generation 1: t_8= 9.96, P < 0.0001; generation 2: t_8= 0.25, P = 0.8; generation 3: t_8= 0.82, P = 0.4). Only in generation 1 were route preferences biased towards the trained route. Red trained demonstrators showed a preference for the red route and yellow trained demonstrators a preference for the yellow route in generation 1, but in generation 2 and 3 route preferences were approximately equal (Fig. 5).

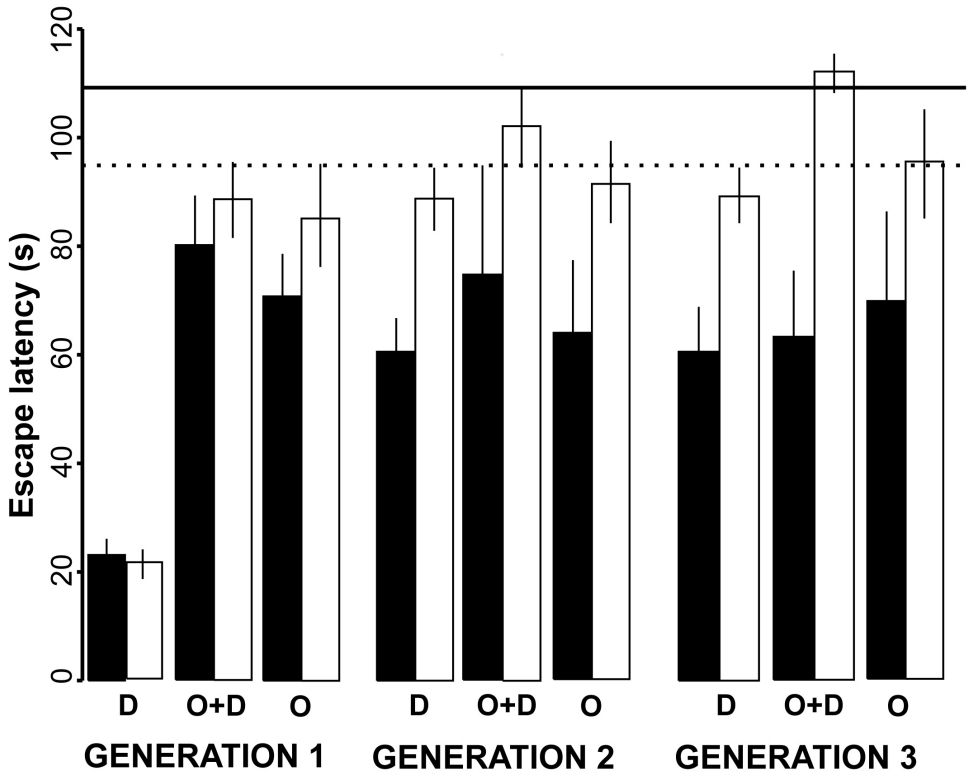

Figure 4. Mean escape latency (± SE) of demonstrators (D), observers with demonstrators present (O + D) and observers after demonstrators were removed (O) for each simulated generation in a transmission chain. Generation 1 demonstrators were trained to either the red route (filled bars) or yellow route (open bars). Observers of generation 1 demonstrators became the demonstrators of generation 2, and observers of generation 2 became the demonstrators of generation 3. Control observers' mean escape latency with and without sham demonstrators is shown by the solid and dashed line, respectively.

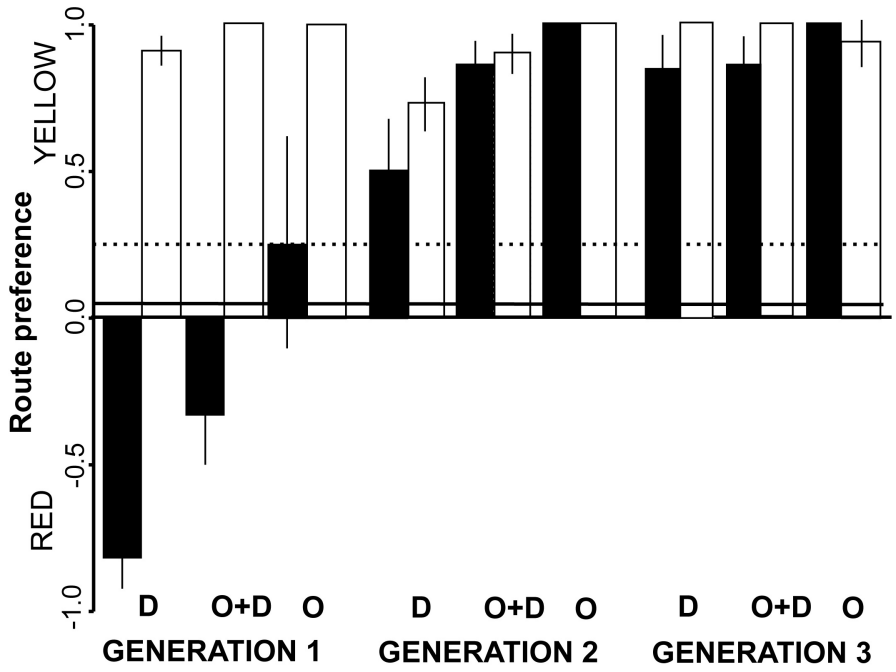

Figure 5. Mean route preferences (± SE) of demonstrators (D), observers with demonstrators present (O + D) and observers after demonstrators were removed (O) for each simulated generation of a transmission chain. Generation 1 demonstrators were trained to either the red route (filled bars) or yellow route (open bars). Observers of generation 1 demonstrators became the demonstrators of generation 2, and observers of generation 2 became the demonstrators of generation 3. Control observers' mean route preference with and without sham demonstrators is shown by the solid and dashed line, respectively.

Observer performance in presence of demonstrators

Escape latency

Demonstrator escape latency significantly influenced observer escape latency (LMM: $F_{1,56}$= 45.43, P < 0.0001): the faster demonstrators escaped the faster observers escaped. However, observer escape latency did not change significantly over generations (generation 1-2: t test: t_{18}= 0.39, P = 0.7 and generation 2-3: t test: t_{18}= 0.07, P = 0.9). There was a significant interaction effect between generation and demonstrator route training on observer escape latency (LMM: $F_{1,56}$= 3.87, P = 0.05): observers in red demonstrator seeded chains escaped faster than those in yellow demonstrator seeded chains on generation 3 ($F_{1,8}$= 12.09, P = 0.008), while their performance was not significantly different on generations 1 and 2; Fig. 4. In generation 3, observers from red but not yellow seeded chains escaped significantly faster than control observers (red vs. control $F_{1,13}$= 13.38, P = 0.003; yellow vs. control: $F_{1,13}$= 0.63, P = 0.44).

Route preference

Over generations, observers with red trained demonstrators increasingly used the yellow route to escape, whereas observers with yellow trained demonstrators preferred the yellow route consistently over generations (i.e. there was an interaction effect between generation and demonstrator route training on route preference; LMM: $F_{1,19}$= 10.17, P = 0.005). The effect of demonstrator route training on route preference thus differed over generations, the effect significant in generation 1 only (t test: generation 1: t_7= 3.47, P = 0.01; generation 2: t_7= 0.31, P = 0.8; generation 3: t_6= 0.75, P = 0.5; Fig. 5). Thus in generations 2 and 3 observers in red seeded chains used the yellow route similarly to those in yellow seeded chains, the opposite to their route preferences in the first generation.

Observer performance in absence of demonstrators

Escape latency

In the absence of demonstrators, former observers of red trained demonstrators escaped faster than former observers of yellow trained demonstrators (LMM: $F_{1,58}$= 13.27, P < 0.0001; Fig. 4). Observers were slower when demonstrators were present than when they were absent, but not significantly so (LMM: $F_{1,175}$= 3.34, P = 0.07). In generation 3, observers from red but not yellow seeded chains escaped significantly faster than control observers (red vs. control $F_{1,11}$= 5.15, P = 0.04; yellow vs. control: $F_{1,11}$= 0.05, P = 0.83).

Route preference

As when demonstrators were present, there was an interaction effect between generation and demonstrator route training on route preference (LMM: $F_{1,98}$= 24.57, P < 0.0001; Fig. 5): observers in chains seeded with red trained demonstrators increasingly preferred to use the yellow route over generations, while in yellow route seeded chains observers' preferences for the yellow route remained stable. The effect of demonstrator route training thus differed over generations, significant on generation 1 only (LMM: generation 1: $F_{1,7}$= 9.05, P = 0.02; generation 2: P = 1.0; generation 3: $F_{1,6}$= 2.38, P = 0.2). Observers did not show a significantly different route preference when demonstrators were present compared to absent, over all generations (LMM: $F_{1,98}$= 2.77, P = 0.09).

DISCUSSION

Naïve zebrafish with trained conspecific demonstrators escaped more rapidly from an approaching trawl than did fish with untrained demonstrators. Moreover, demonstrator route training biased observers' route choices, a bias maintained when demonstrators were removed, results consistent with the observers learning an escape route from the trained demonstrators. Contrary to recent findings in stickleback social foraging (Duffy et al. 2009), we did not find body size had a significant influence on social learning. In the transmission chain, observers at the end of the chain escaped faster than control fish, demonstrating that the escape response was transmitted across generations. However, route choice decayed rapidly over generations. Thus, arbitrary information such as route use was not stably transmitted.

Zebrafish likely learned both to escape and a specific route by following demonstrators that took only one route, increasing exposure to that route. Alternatively or in addition, demonstrators could have drawn more attention to one route (Swaney et al. 2001). Observational conditioning could also account for our results (Heyes 1994): observers could have learned to fear the trawl from visual, movement and/or olfactory demonstrator stress cues (Suboski et al. 1990), leading to more rapid escape, and, if demonstrators expose the observers to the relationship between one escape hole and the preferred 'escape' zone, to learning a particular route (Hoppitt & Laland 2008). Thus zebrafish could have learned by a number of non-mutually exclusive social learning processes (Laland & Williams 1997; Boogert et al. 2008; Hoppitt & Laland 2008). In addition, social facilitation is likely to have accelerated escape latency in the demonstrator present phase, although cannot account for continued rapid escape after demonstrators were removed. The decrease in observer latencies over trials when demonstrators were present suggests that subjects were also learning asocially how to escape, but that trained demonstrators accelerated learning and biased learning towards particular routes.

The number of demonstrators had contrary effects on observer escape latencies and routes. We found strongest route following with larger demonstrator groups present, and a similar (albeit non-significant) pattern when demonstrators were removed. In contrast, observers paired with 2 or 6 demonstrators tended to escape faster than those paired with 4 demonstrators in both demonstrator present and absent phases, although these differences were not statistically significant. This pattern contrasts with the positive relationship between the number of demonstrators and acquisition described in guppies, rainbowfish *Melanotaenia eachamensis*, rats *Rattus norvegicus* and pigeons *Columba livia* (Lefebvre & Giraldeau

1994; Brown & Warburton 1999; Laland 2004). However, in line with our findings, zebrafish learned shock avoidance faster in groups of five or one compared to two, and Artic charr *Salvelinus alpinus* learned superior predator avoidance with fewer demonstrators per observer (Gleason et al. 1977; Vilhunen et al. 2005). Opposing processes may shape the influence of group size on social learning. For example, the larger the shoal the stronger its social attractiveness is (Day et al. 2001), promoting learning, while fear may decrease with increasing shoal size, thus hindering learning of escape responses. Relations between demonstrator number and social learning may differ between behavioural contexts and be non-linear.

In common with our findings, both rainbowfish and guppies socially learn to escape faster from a moving trawl (Brown & Warburton 1999) and copy the route used by their demonstrators (Brown & Laland 2002; Reader et al. 2003). The social learning of route preferences we demonstrate in zebrafish matches findings in wild guppies tested in the field (Reader et al. 2003), but not in domesticated guppies tested in the laboratory, which escaped faster after pairing with trained demonstrators but did not show a learned preference for a particular route (Brown & Laland 2002). Minor methodological differences could account for these results, such as the fact that fish escaped through a partition in the present study and Reader et al. (2003), but through the trawl in the study of Brown & Laland (2002). Also, Brown & Laland (2002) tested observers several hours after the removal of demonstrators, another potential explanation for the loss of route preferences. Our study used a short (5 minute) gap between training and test, although demonstrators were shown to maintain their training after 3 - 4 days without reinforcement. We argue that strain and species differences provide the most likely explanation for the differences between studies. Tighter shoals are formed by wild strains of both guppies (Kelley et al. 2003) and zebrafish (Wright et al. 2006), probably reflecting relaxed selection or developmental influences in domestic environments. In wild populations, the costs of leaving the shoal and engaging in individual exploration are more likely to outweigh the possible benefits. Zebrafish also form more polarized, tighter shoals than guppies (Suboski et al. 1990), and hence social learning by following may be stronger in zebrafish than guppies. Environmental factors are tightly linked to grouping tendencies and the costs and benefits of differing from the group, potentially driving differences in social learning propensities. Our results add to findings indicating that individuals, populations and species differ in their propensity to learn from one another (Lefebvre et al. 1996; Carlier & Lefebvre 1997; Reader & Laland 2002; Bond et al. 2003; Reader 2004; Kendal et al. 2005). The mechanisms that underlie such differences in

social learning and their consequences for animal ecology and theoretical models of social learning are likely substantial but remain relatively unexplored (Reader 2004; Morand-Ferron et al. 2010).

The transmission chain demonstrated that social information on escape behaviour is transmitted over multiple generations, a finding complementing previous studies on foraging (Laland & Williams 1997; Stanley et al. 2008). Subjects escaped more rapidly than controls, even at the end of the transmission chain (generation 3). However, this finding was restricted to fish in red-seeded chains, perhaps because of unusually poor yellow demonstrators at the beginning of generation 2. Fish from red seeded chains increasingly took the yellow route over repeated episodes of transmission, resulting in equally strong preferences for yellow route use for all fish on generations 2 and 3. The two routes likely differed in attractiveness, resulting in a bias for the yellow route. At the end of the transmission chain fish had a stronger yellow route preference than control observers, an observation most likely explained by demonstration differences. Observers of sham demonstrators rarely escaped, and, when they did, escaped as individuals rather than taking one route together. In contrast, demonstrators on generation 2 and 3 provided a usable 'tip-off' as to how to escape, but not for the red route. This, combined with a slight bias for the yellow route of naïve fish, resulted in generation 2 and 3 observers escaping rapidly in coherent shoals via the yellow route.

Our finding that traditions based on what we argue to be relatively arbitrary information, such as a particular route when both routes are functionally identical, collapsed, while the more functional aspect of escaping faster was still preserved along the chain, complements results in guppies (Laland & Williams 1997) and wild meerkats *Suricata suricatta* (Thornton & Malapert 2009). However, the collapse of route preferences contrasts with the extremely stable traditions found in mate- and foraging contexts in wild fish (Helfman & Schultz 1984; Warner 1988). This apparent disparity could be explained by observers not experiencing differential feedback (or costs) (Hoppitt & Laland 2008) when they used the alternative escape route: both routes lead to the same location. In the Warner (1988) study, mating sites may be arbitrary but an individual choosing an alternative site would lose mating opportunities: there is a cost to differing from the group. Manipulation of the perceived costs and benefits of the two routes would be predicted to affect tradition stability. It is also possible that more salient landmarks would increase transmission stability. However, the fact that demonstrators remembered their route preference after a 3 - 4 day delay suggests that the opportunity for exploration of alternative equidistant routes is responsible for the collapse in the route tradition (Galef & Whiskin 1997).

Here, we attempted to mimic a realistic anti-predator situation with a simulated predator approach and a transmission chain design, i.e. creating a founder population of demonstrators representing experienced individuals, and replacing this group repeatedly, representing an influx of naïve individuals. It could be argued that the fish learned not anti-predator responses but instead routes through their habitat or to locate companions. However, subjects showed typical zebrafish alarm responses (Rehnberg & Smith 1988), and thus were learning when alarmed. Furthermore, nets and other fishing gear evoke predator escape responses in fish, and large moving objects are perceived as threat stimuli (Brown & Warburton 1997, 1999). Although zebrafish may react differently under natural circumstances with real predators, our experiment can be considered a predation test, and further investigation would indicate whether identical results would be observed in other situations or contexts. Recently, Stanley et al. (2008) argued that acquisition of tasks difficult to learn asocially (e.g. a rare innovation) provides the strongest evidence for social learning. Our task fails this criterion: control subjects did not readily escape, but would be expected to eventually learn the task individually. Acquiring rare innovations from others might provide compelling evidence for social learning. However, situations where knowledgeable conspecifics accelerate learning that could occur asocially are likely to be extremely common and relevant to fitness, and thus warrant study.

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Chapter 3

NO EVIDENCE THAT ADULT ZEBRAFISH PREFERENTIALLY SHOAL WITH FAMILIAR INDIVIDUALS

Charlotte M. Lindeyer and Simon M. Reader

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ABSTRACT

Selective interactions with conspecifics have demonstrated anti-predatory and foraging benefits. Discrimination between conspecifics on the basis of familiarity, i.e. on the basis of having previously experienced these individuals, can shape grouping decisions in many fish species. Zebrafish (*Danio rerio*) show strong shoaling behaviour and use multiple cues in choosing shoaling partners. However, the existence and development of familiarity preferences has not been clearly established in zebrafish. We investigated the development of familiarity preferences in adult female zebrafish, introducing tagged subjects to 10 unrelated and initially unfamiliar females for 20 days. At various time intervals we allowed individuals to choose between a familiar or unfamiliar pair of conspecifics, with either visual contact or visual and olfactory contact permitted between the subject and stimulus pairs. Subjects showed strong shoaling responses regardless of whether visual cues alone or also olfactory cues were available, and consistent individual differences were found in the propensity to shoal. However, examination of the time spent shoaling revealed no evidence for a preference for familiar or unfamiliar fish. Subjects in visual and olfactory contact with conspecifics made more first visits to unfamiliar fish at one time point, but this was not reflected in the time spent with familiar versus unfamiliar fish. We thus find no evidence that adult zebrafish express familiarity preferences, results in concordance with previous work. This raises the possibility that familiarity preferences are absent in adult zebrafish, or at least do not readily develop in particular populations or contexts.

INTRODUCTION

Individual grouping offers benefits such as increased safety against predators (Landeau & Terborgh 1986), increased foraging efficiency (Beyer 1976; Ioannou & Krause 2008) and increased mating opportunities (Pitcher & Parrish 1993). However, grouping also carries potential costs, such as competition for resources or increased parasite transmission. Thus there is a trade off between the costs and benefits of joining a group. Depending on the context, environment and the social dynamics of a species grouping benefits may differ (Ward et al. 2009).

Many animals do not associate randomly together. Grouping in fish is particularly well studied, partly because fish provide a practical and efficient way to investigate links between association patterns and local ecology. Fish shoaling preferences can be influenced by species, sex, morphology, size, kinship, social status, shoal distance and nutritional state, amongst other factors (Krause et al. 2000; Griffiths 2003; Mühlhoff et al. 2011). Familiarity preferences, learned preferences for individuals they have previously associated with, can also shape the decision of whom to shoal with (Griffiths 2003; Ward & Hart 2003). At least 18 fish species have been demonstrated to prefer to shoal with familiar conspecifics (Griffiths 2003; Farmer et al. 2004). For example, bluegill sunfish (*Lepomis macrochirus)* spent significantly more time with familiar rather than unfamiliar conspecifics (Brown & Colgan 1986). Familiarity preferences are learned, condition-independent, and should be distinguished from shoaling preferences that are not based on experience, such as unlearned preferences based on the appearance, kinship or current behaviour of individuals (Olsén et al. 1998; Griffiths 2003). Fish can also differentiate between conspecifics on the basis of chemical cues that are the products of shared habitat or resource use, rather than recognising individuals. This learning process thus provides an alternative social recognition mechanism to familiarity, and complicates interpretation of some familiarity studies (Ward & Hart 2003; Ward et al. 2009). Note that preferences for kin could result from both experience of kin (i.e. familiarity), or from another process, such as shared chemical cues or phenotype matching (Griffiths & Magurran 1999).

Interaction patterns affect a wide range of behaviours, including the transmission of information between individuals. Familiar fathead minnows (*Pimephales promelas*) shoaled more cohesively, showing more efficient anti-predatory behaviour than unfamiliar groups (Chivers et al. 1995). Similarly, social learning from familiar conspecifics improved foraging performance compared to learning from unfamiliar guppies (*Poecilia reticulata*) (Swaney et al. 2001). Thus selective grouping potentially offers great individual advantages. However, shoaling

preferences for familiar fish are not universal. For instance pumpkinseed (*Lepomis gibbosus)* or rock bass juveniles (*Ambloplites rupestris*) showed no familiarity discrimination (Brown & Colgan 1986). In some cases fish avoid familiar individuals, instead preferring unfamiliar fish (Kelley et al. 1999). Kelley et al. (1999) propose that this maximises mating opportunities for males.

Though the expression of shoaling preferences for familiar fish has been studied in multiple species, to our knowledge the development of familiarity preferences over time has been addressed in only two studies (Griffiths 2003; Griffiths et al. 2007). Griffiths and Magurran (1997a) demonstrated that acquisition of a preference for familiar conspecifics in guppies, a species where familiarity preferences have been extensively examined, is not expressed until after 12 days of exposure. In the second study, European minnows (*Phoxinus phoxinus*) initially shoaled with familiar rather than unfamiliar individuals (Griffiths et al. 2007). After the familiar and unfamiliar fish were housed together for two weeks they formed mixed groups, suggesting that initially unfamiliar fish were now also treated as familiar. In guppies, the preference for familiar conspecifics appears to decline with increasing shoal size, for example not developing in groups above approximately 40 individuals (Griffiths & Magurran 1997b).

Both chemical and visual cues are used in the recognition of familiar conspecifics. However, relatively few studies have examined familiarity preferences when only visual or chemical cues are present (Ward & Hart 2003). Fathead minnows required chemical cues to recognize familiars, visual cues alone being insufficient (Brown & Smith 1994), while male sticklebacks (*Gasterosteus aculeatus*) can recognise familiar rivals on the basis of visual cues only (Waas & Colgan 1994). Such results suggest potentially important species differences in cue use. Similarly, species and population differences may occur in familiarity itself (Ward et al. 2009). Guppies preferred fish they had been in visual contact with over fish they had been in solely olfactory contact with, but also preferred fish previously in olfactory contact with over fish sharing a similar chemical environment (Ward et al. 2009). In contrast, sticklebacks demonstrated no such preferences. However, like guppies, two of the three studied stickleback populations preferred fish sharing a similar chemical environment to fish from an alternative environment (Ward et al. 2009). Combined, such data suggest that habitat differences may drive within and between species differences in familiarity preferences and in the cues used to recognise others.

In our study, we used zebrafish (*Danio rerio*), a species that is increasingly used in behavioural research (Miklosi & Andrew 2006). While zebrafish development and genetics are well studied, their shoaling

and other behaviour is relatively unexplored (Miller & Gerlai 2011). Zebrafish shoal early after hatching, a behaviour that is maintained throughout life. Interactions early in life shape juvenile shoaling choices, with individuals showing visually mediated preferences for fish of a similar appearance to their rearing companions (McCann & Carlson 1982; Engeszer et al. 2004; Spence & Smith 2007). Another study established sex-specific shoaling preferences, with males preferring female shoals over larger shoals while females preferred larger shoals regardless of group composition (Ruhl & McRobert 2005). Research up to now has focussed on visual cues and suggests zebrafish shoaling is primarily visually based. Zebrafish prefer to shoal with same sized conspecifics and are specifically attracted to striped patterned conspecifics (McCann et al. 1971; Rosenthal & Ryan 2005). A recent study on sex recognition in zebrafish established that females can distinguish males from females based on visual cues alone (Hutter et al. 2011). However, zebrafish also use odour cues in conspecific discrimination. In an odour flume test, mixed-sex juvenile zebrafish (about 26 days old) preferred the area that contained odour cues from kin over non-kin and they showed familiarity biased kin preferences (Gerlach & Lysiak 2006). In adults, kin preferences changed sex-specifically: females preferred the odour of unrelated males, whereas males showed no preference for related or unrelated females (Gerlach & Lysiak 2006). Thus zebrafish have the ability to use both visual and chemical cues to base their shoaling preferences on, but it remains unclear under which conditions they use these cue types to determine shoaling choices.

Zebrafish have shown to be a useful model system to investigate shoaling and association preferences. Their shoaling increases in response to anti-predatory contexts (Speedie & Gerlai 2008). In a foraging context, nutritional status of the subject influenced the shoaling choice for either a well fed or a food deprived stimulus shoal (Krause et al. 1999). Food deprived subjects shoaled with well fed individuals and subsequently were more successful in a foraging task. Considering zebrafish show strong shoaling tendencies and association preferences under various conditions (Buske & Gerlai 2011), it is important to establish whether zebrafish, like many other fish species (Griffiths 2003), are able to distinguish between familiar and unfamiliar unrelated conspecifics.

To our knowledge, only one study has investigated familiarity discrimination in adult zebrafish (Pritchard 2001). Pritchard (2001) used the well-established method of housing mixed-sex zebrafish together for an extensive period (about 7 months) followed by a shoaling test to investigate familiarity preferences (Griffiths & Magurran 1997a; Lachlan et al. 1998; Griffiths & Magurran 1999; Barber & Ruxton 2000). Subjects could choose to shoal with a familiar or unfamiliar stimulus shoal with

either visual or both visual and olfactory cues available. Pritchard (2001) found no evidence for zebrafish expressing familiarity preferences.

In this study, we investigated the development of conspecific familiarity in adult zebrafish using an experimental design building upon Pritchard's (2001) and Griffiths' and Magurran's (1997a) studies. Only females were used to reduce variation that could be caused by sex-differences in shoaling preferences (Pyron 2003; Ruhl & McRobert 2005). Tagged subjects were introduced to 10 unrelated, unfamiliar females and housed together for a period of 20 days. At different time intervals fish were given a preference test where they could choose to shoal with either familiar or unfamiliar stimulus pairs in two cue conditions (visual and chemical cues or visual cues alone). Our aim was to determine whether zebrafish develop familiarity discrimination within 20 days and how shoaling preferences were affected by the cues available.

METHODS

Subjects and housing

A total of 260 female zebrafish (*Danio rerio*, wild type; aged 8 – 10 months at test) were used. Fish were the second generation of fish originally imported from Singapore (Ruisbroek, Maassluis, Netherlands) and bred and reared in the Biology aquarium at Utrecht University. The fish had not previously participated in any experiments. All housing tanks (80 x 50 cm) were maintained at 26 ± 1°C with a water depth of 30 cm. Housing was enriched with artificial plants, pot shelters and gravel floor. Fish were on a 12h light:dark schedule with lights on at 0800 hours (no natural light present). Fish were fed twice daily (at 0900 and 1700 hours) with flake food (TetraMin, Tetra Ltd., Germany) and bloodworm (*Chironomus*) or water fleas (*Daphnia sp*.). Otherwise, fish were left undisturbed as much as possible. The experiment was approved by the Utrecht Ethics and Animal Care and Use Committee under protocol DEC 2009.I.06.046, and conforms to ASAB guidelines. After the experiment fish were used for educational purposes.

Fish were reared in four separate large mixed sex groups (groups A, B, C and D). Fish in different rearing tanks had never thus interacted before. Three weeks before the experiment began 20 females were selected from each of the large rearing groups and were moved to experimental housing tanks while still maintaining the rearing group structure (groups A, B, C and D). Ten of the 20 fish in each tank were randomly designated as subjects and were marked according to the procedure below. The remaining fish were designated as stimulus fish and were not marked.

Tagging

Two weeks before the start of the experiment subjects were marked with an elastomer tag (Northwest Marine Technologies, Shaw Island, Washington, USA). Subjects were individually anesthetized with a eugenol (Fisher Scientific, Landsmeer, NL) solution (80 ppm; 26 °C) and injected subcutaneously with a small amount of elastomer in the abdomen region. Fish recovered within 2 - 5 min from the anaesthesia and showed no behavioural signs of discomfort of the tag. Tag combinations of two colours (red/pink/blue/green) were placed in different abdomen regions and sides to allow individual identification of fish. After one week subjects' tags were checked for visibility and re-tagged when necessary to ensure high reliability of tags during the experiment. Tags remained clearly visible for the duration of the experiment.

Procedure

Two weeks after tagging, on 'day 0' of familiarization, experimental rearing groups were mixed by moving the 10 subjects between housing tanks (moving fish between groups A and B and between groups C and D) to begin the familiarization process. In this manner we could precisely determine when subjects were first placed in contact with stimuli fish and their chemical environment, an advantage over methodologies where fish had been in prior contact before the familiarization process began. Moreover, we could identify stimulus fish without the need to tag them, which could have potentially influenced shoaling preferences. After mixing the 10 subjects with the 10 stimulus fish, fish formed one shoal (personal observation), suggesting there was no segregation between groups within tanks. Henceforth, fish in the same experimental tank are referred to as 'familiar fish' and fish in the different tanks that had never interacted before will be referred to as 'unfamiliar fish'. Subjects' preferences for individuals from the same (familiars) versus a different tank (unfamiliars) were assessed in a preference test either after 2 (N = 10), 4 (N = 30), 8 (N = 30), 12 (N = 20), 16 (N = 30) or 20 days (N = 10) of familiarization. For example, subjects in tank A were given a choice between tank A fish and tank C or D fish; they were not exposed to tank B fish since they had been in contact with these fish before familiarization began. Each individual subject was only tested on one day, and thus we used a total of 130 subjects. The experiment was conducted in four separate batches for logistical reasons. The sample size differed per testing day due to unforeseen circumstances preventing testing on some days in some batches. After testing, fish were placed back in their housing tank.

The experimental tank (Fig. 1) was divided into three compartments by transparent PVC partitions. Two stimulus fish pairs

were randomly placed in the outer compartments every two trials. Stimulus fish were presented in groups of two to increase the shoaling stimulus, to reduce stress in stimulus fish and to reduce the chance that a single fish was unusual in appearance or behaviour. Each stimulus pair was used for two consecutive trials, both as a familiar and unfamiliar pair to account for possible differences in attractiveness between stimulus pairs. Stimulus pairs were matched on size as much as possible (mean mass ± SE: stimulus pairs 0.49 ± 0.01 g; subjects 0.51 ± 0.01 g). The subject was individually placed within two PVC tubes (diameter 20 cm), one transparent and the other opaque, to acclimatise in the middle zone. After 2 minutes the opaque tube was lifted, then after 2 minutes the transparent tube was lifted, followed by a 6-minute test in which the subject's behaviour was recorded on video (Sony DCR-SR55E). A subject was considered to shoal with the stimulus fish when within 5 body lengths of the stimulus pair, i.e. 15 cm from the shoal (Pitcher & Parrish 1993). After sessions fish were weighed and returned to their experimental housing groups.

Visual versus chemical cues

To test whether the availability of both visual and chemical cues or visual cues alone would affect subjects' shoaling preferences we conducted one trial with transparent partitions with holes (diameter 0.4 cm; evenly distributed, 3.5 cm apart) and one trial with solid transparent partitions (testing order was randomised to account for any habituation). Thus subjects were tested twice, between 0930 – 1200 hours and 1330 – 1700 hours. Stimulus fish were re-used but new pairs were randomly chosen. The pump inflow (Fig. 1) was located in the middle of the tank with outflows spanning the length of the neutral zone, such that water was circulated throughout the neutral zone and was pulled through the perforated partitions. We confirmed the water circulation by adding dye to the stimuli fish zones. The dye was pulled through the perforated partitions within seconds. To control for possible odour cues left in the experimental tank from previous trials, the water was mixed after each trial.

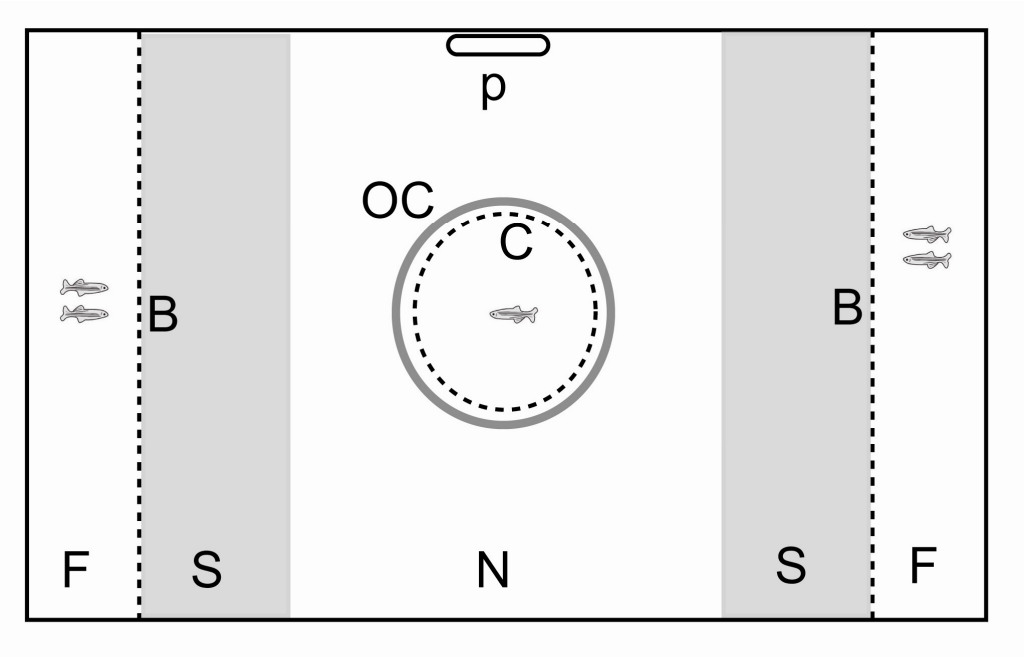

Figure 1: Schematic overview of the experimental apparatus. The experimental tank was an identical size as the housing tank, with a similar gravel floor (l x w x h: 80 x 50 x 40 cm). Subjects were placed within a transparent (C) and opaque (OC) cylinder in the neutral zone (N) to acclimatize for 4 min in total. Stimulus fish pairs were confined to the stimulus fish zones (F; 5 cm wide). The 6-min trial started when both cylinders were removed and the subject could swim freely to join either stimulus pair, familiar or unfamiliar, in the shoaling zone (S; 15 cm wide). Transparent PVC partitions (B) split zones F and S which were either perforated in the visual and chemical cues condition or non-perforated in the visual cues only condition. Lines were drawn on the outside of the tank to indicate the shoaling zones. Water circulation was provided by the pump (p) placed in the middle of the tank, which was on throughout trials.

Analyses

The dependent variables were 1) total time spent shoaling, 2) shoaling preference (the time spent shoaling on the left minus the time spent shoaling on the right), 3) first choice (left or right side), and 4) the number of switches between shoaling and neutral zones. We used generalized linear models (GLMs) with a gamma family for the total time shoaling, shoaling preference and switches measures and a binomial family for first choice. We analysed the data for the different cues available separately. In all models, the number of days after familiarization (2, 4, 8, 12, 16, 20 days) and the side the familiar stimulus pair was placed were included as fixed effects, except for analyses of total time spent shoaling and switches, where the familiar pair's location was not included. The trial order (first or second trial of the day) was included as a random effect. We initially investigated the full models including all interaction terms and sequentially dropped nonsignificant effects ($P >$

0.1). Nonsignificant trial number effects ($P > 0.05$) are not reported. Nonsignificant interactions ($P > 0.05$), with the exception of the interaction between the number of days after familiarization and the side the familiar pair was placed, are not reported. To confirm that our results were robust, we also analysed the first choice and the shoaling preferences at each time point separately, with only the familiar pair location included as a fixed effect. For exposition in Figure 2 we use the intuitive measure of time spent with the familiar pair minus time spent with the unfamiliar pair. In addition, to study individuals' preference consistency across the two trials we used Pearson's correlation for the shoal preference and the total time spent shoaling, and McNemar's X^2 test for the first choice measure. All statistical tests were two-tailed. Analyses were performed in R 2.10.1.

RESULTS

VISUAL AND CHEMICAL CUES PRESENT

Shoaling preference

The location of the familiar pair did not significantly affect subjects' shoaling preferences (GLM: $t_{129} = 0.25$, $P = 0.8$). Moreover, there was no significant statistical interaction between the location of the familiar pair and the number of days of familiarization on shoaling preference (GLM: $t_{129} = 0.62$, $P = 0.5$). Thus, there was no evidence that subjects exhibited a familiarity preference or that familiarity preferences were expressed only at certain time points, a finding confirmed by analysis of shoaling preferences at each time point separately (GLMs: after 2 days, $t_9 = 1.58$, $P = 0.2$; after 4 days, $t_{29} = 0.14$, $P = 0.9$; after 8 days, $t_{29} = 1.31$, $P = 0.2$; after 12 days, $t_{19} = 1.20$, $P = 0.2$; after 16 days, $t_{29} = 1.99$, $P = 0.06$; after 20 days, $t_9 = 0.78$, $P = 0.5$; Fig. 2A).

Total time shoaling

Subjects demonstrated a strong tendency to associate with the stimulus pairs, spending more time in the shoaling zones than in the larger neutral zone (mean ± SE time shoaling = 273.12 ± 3.25 s; mean ± SE time not shoaling = 83.03 ± 3.18 s; Wilcoxon signed-ranks test: $U = 8324.5$, $N = 130$, $P < 0.0001$). The number of days of familiarization did not significantly affect total time spent shoaling (GLM: $t_{129} = 0.16$, $P = 0.9$). Trial order did not significantly affect subjects' total time spent shoaling (GLM: $t_{129} = 0.36$, $P = 0.7$).

First choice

Zebrafish had a statistically significant but marginal preference to first visit the shoaling zone where the unfamiliar pair was located (62% of first

visits to unfamiliar; GLM: z_{129}= 2.06, P = 0.04; Fig. 3A). Investigation of first choice at each time point separately showed zebrafish's choices were significantly affected by the location of the familiar pair after 8 days of familiarization (GLMs: after 2 days, z_9 = 1.75, P = 0.08; after 4 days, z_{29} = 1.45, P = 0.2; after 8 days, z_{29} = 2.13, P = 0.03; after 12 days, z_{19} = 0.45, P = 0.7; after 16 days, z_{29} = 0.37, P = 0.7; after 20 days, z_9 = 1.75, P = 0.8). Additionally, subjects' first side choice was predictive of subjects' shoaling preferences, with subjects spending more time on the side they visited first (t test: t_{128} = 5.54, P < 0.0001). We found no significant interaction between the number of days of familiarization and the location of the familiar pair on first choice (GLM: z_{129}= 0.85, P = 0.4).

Switches

Subjects' switching behaviour was not significantly influenced by the number of days of familiarization (GLM: t_{129}= 0.65, P = 0.5). Also, we did not find a statistically significant effect of trial order on switching behaviour (GLM: t_{129}= 1.01, P = 0.3).

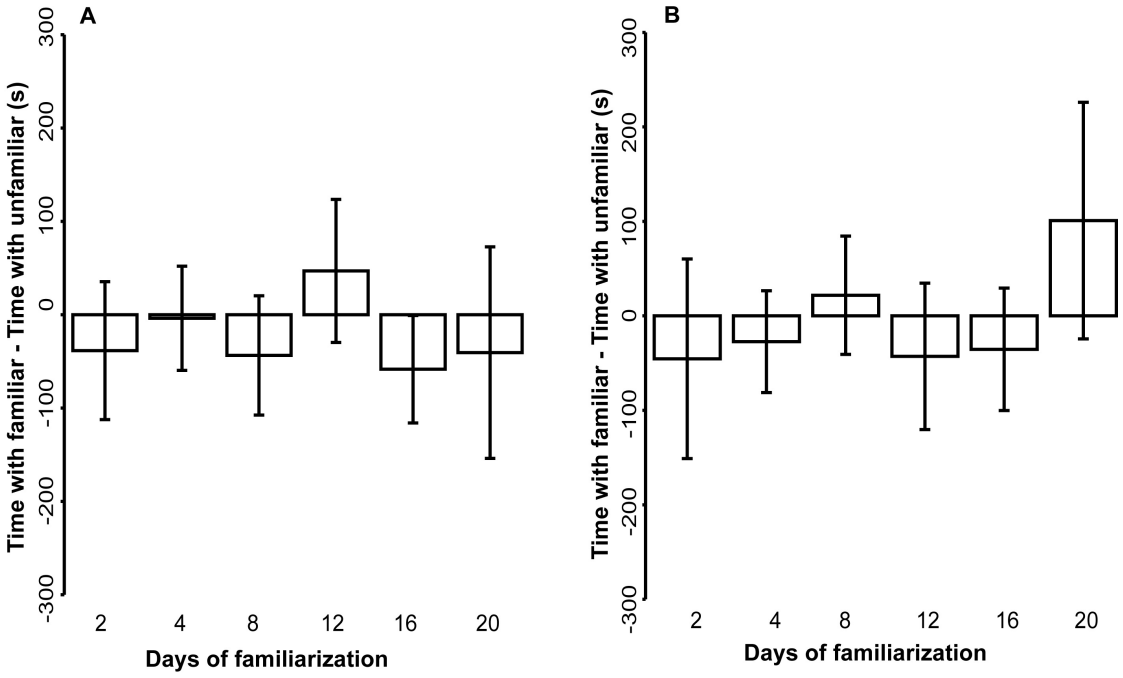

Figure 2: The change in mean (± CI) shoaling preferences over time with A) visual and chemical cues present or B) visual cues alone. Positive shoaling values indicate a preference to swim with the familiar pair and negative values represent a preference to swim with the unfamiliar pair. Zebrafish were tested at a single time point, either after 2, 4, 8, 12, 16 or 20 days. Sample sizes vary over days (Day 2: N = 10; 4: N = 30; 8: N = 30; 12: N = 20; 16: N = 30; 20: N = 10).

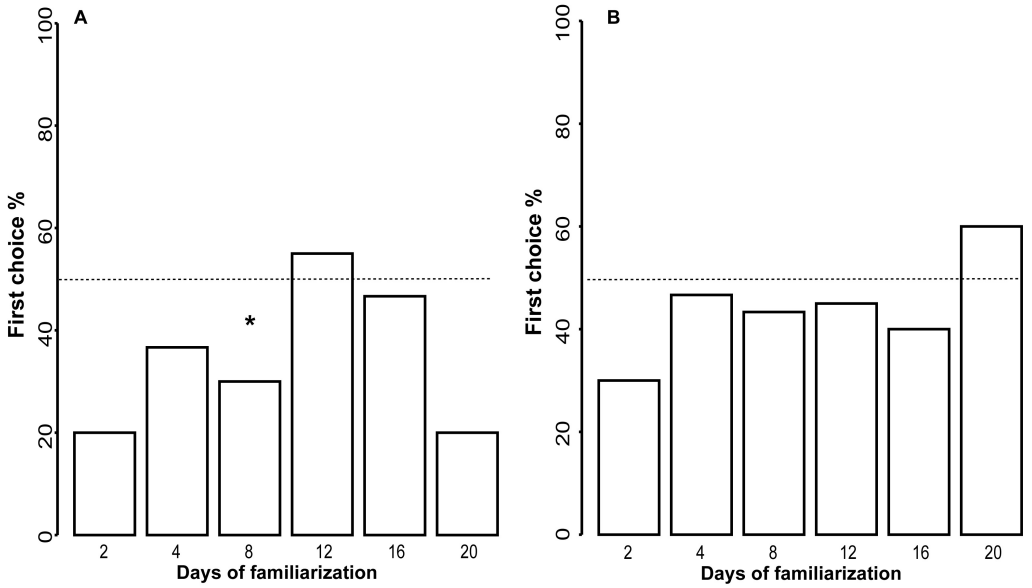

Figure 3: The change in mean first choice for familiar fish with A) visual and chemical cues present or B) when visual cues only were present. Values higher than 50% indicate a preference for the familiar pair and values lower than 50% indicate a preference for the unfamiliar pair. Sample sizes vary over days (Day 2: $N = 10$; 4: $N = 30$; 8: $N = 30$; 12: $N = 20$; 16: $N = 30$; 20: $N = 10$). Random choice is indicated by the dashed line. P value: *: $P \leq 0.05$.

VISUAL CUES PRESENT

Shoaling preference

The location of the familiar pair did not significantly affect subjects' shoaling preferences (GLM: $t_{129} = 1.15$, $P = 0.3$). Moreover, there was no significant statistical interaction between the location of the familiar pair and the number of days of familiarization on shoaling preference (GLM: $t_{129} = 0.89$, $P = 0.4$). Thus, there was no evidence that subjects exhibited a familiarity preference or that familiarity preferences were expressed only at certain time points, a finding confirmed by analysis of shoaling preferences at each time point separately (GLMs: after 2 days, $t_9 = 0.81$, $P = 0.4$; after 4 days, $t_{29} = 0.98$, $P = 0.3$; after 8 days, $t_{29} = 0.68$, $P = 0.5$; after 12 days, $t_{19} = 1.32$, $P = 0.2$; after 16 days, $t_{29} = 1.08$, $P = 0.3$; after 20 days, $t_9 = 1.90$, $P = 0.1$; Fig. 2B).

Total time shoaling

Subjects demonstrated a strong tendency to associate with the stimulus pairs, spending more time in the shoaling zones than in the neutral zone (mean ± SE time shoaling = 277.07 ± 3.05 s; mean ± SE time not shoaling = 78.72 ± 2.97 s; Wilcoxon signed-ranks test: U = 8479, $N = 130$, $P < 0.0001$). Contrary to our findings when olfactory cues were also available, when only visual cues were available we found a significant

effect of trial order on total time shoaling (GLM: $t_{129} = 3.88$, $P = 0.0002$; mean total time shoaling ± SE: trial 1 = 296.7 ± 2.6 s; trial 2 = 263.6 ± 3.1 s). Thus total time shoaling was reduced in the second compared to the first trial. The number of days of familiarization did not significantly affect total time spent shoaling (GLM: $t_{129} = 0.71$, $P = 0.5$).

First choice

Subjects' first choice of shoaling zone was not significantly influenced by the location of the familiar pairs (56 % of first visits to the unfamiliar pair; GLM: $z_{129} = 1.15$, $P = 0.3$; Fig. 3B). This finding was confirmed by analysis of shoaling preferences at each time point separately (GLMs: after 2 days, $z_9 = 1.24$, $P = 0.2$; after 4 days, $z_{29} = 0.37$, $P = 0.7$; after 8 days, $z_{29} = 0.74$, $P = 0.5$; after 12 days, $z_{19} = 0.45$, $P = 0.7$; after 16 days, $z_{29} = 1.10$, $P = 0.3$; after 20 days, $z_9 = 0.68$, $P = 0.5$). However, first choice was predictive of subjects' shoaling preferences, with subjects spending more time on the side they visited first (t test: $t_{128} = 9.79$, $P < 0.0002$). We found no significant interaction between the number of days of familiarization and the location of the familiar pair on first choice (GLM: $z_{129} = 0.54$, $P = 0.6$).

Switches

Subjects' switching behaviour was not significantly influenced by the number of days of familiarization (GLM: $t_{129} = 0.74$, $P = 0.5$). However, trial order significantly affected subjects' switching behaviour with more switching recorded on the second trial compared to the first trial (GLM: $t_{129} = 2.52$, $P = 0.01$; mean switches ± SE: trial 1 = 32 ± 1.7; trial 2 = 41 ± 1.6).

BEHAVIOURAL CONSISTENCY

We examined correlations in the behavioural measures across the two trials to address individual consistency. Individual's shoaling preferences in the first trial were not predictive of preferences in their second trial (Pearson's correlation: $r = 0.10$, $N = 130$, $P = 0.2$) nor did their first side choices in trial one predict their first choice for a side in the second trial (McNemar's Chi-square test: $X^2_1 = 1.31$, $P = 0.3$). However, there was a significant but weak positive correlation between the total time spent shoaling in the first and second trial (Pearson's correlation: $r = 0.21$, $N = 128$, $P = 0.02$).

DISCUSSION

Female zebrafish showed strong, robust shoaling tendencies regardless of the cue types available. Moreover, we found individual consistency in the propensity to shoal. However, subjects did not express association preferences for familiar compared to unfamiliar conspecifics, which is notably different from reports on the development of familiarity in similar circumstances in other species (Griffiths 2003). We thus found no evidence for familiarity preferences in adult zebrafish or evidence that fish discriminated between conspecifics on the basis of shared chemical cues. These results are concordant with Pritchard's (2001) findings, indicating that familiarity preferences are either absent in adult zebrafish or are not readily expressed in particular populations or behavioural contexts.

Although there was considerable variation in first choices for familiar versus unfamiliar fish, when olfactory and visual cues were present there was an overall tendency to first approach unfamiliar fish. This finding potentially suggests that zebrafish are able to discriminate between familiar and unfamiliar conspecifics. If this familiarity effect on first choice represents a true novelty preference for unfamiliar individuals, this means that information on conspecific familiarity has a short-lived effect and is not important in longer-term shoaling decisions. In juvenile zebrafish, familiarity information appears to be used in combination with phenotype matching to identify kin from non-kin (Gerlach & Lysiak 2006; Gerlach et al. 2008). Taken together, these findings thus suggest that familiarity discrimination is important for the development of kin recognition early in life but not in a shoaling context when adult. Thus we suggest the role of familiarity discrimination in zebrafish may be limited to kin recognition and to a sensitive period.

Though knowledge on zebrafish ecology is limited, observations suggest juveniles and adults mainly occur in slow moving to still standing water bodies such as pools, streams or rice paddies during the dry season (Pritchard 2001; Engeszer et al. 2007b; Spence et al. 2008). Zebrafish larvae show restricted mobility and are thus more likely to encounter related conspecifics early in life before they disperse as juveniles (Spence et al. 2006; Gerlach et al. 2008). Moreover, the rainy season will allow previously isolated zebrafish populations to mix (Pritchard 2001). Under these natural conditions it might be beneficial to quickly learn to recognize kin early in life to avoid potential inbreeding when adult (Gerlach & Lysiak 2006).

The behavioural context is likely to be an important determinant of the expression of familiarity preferences. In our study, fish were placed in a novel environment, which may not promote the expression of

familiarity preferences, but rather increase general shoaling tendencies, possibly as a result of increased anxiety (Speedie & Gerlai 2008; Stewart et al. 2010). That said, we did not observe stress-related behaviours such as freezing or dashing. In other behavioural contexts like foraging, mate choice or anti-predatory responses, it might be more advantageous to express a familiarity preference, as has been demonstrated in other fish species (Chivers et al. 1995; Frommen et al. 2007; Ward et al. 2009; Sievers & Magurran 2011). Alternatively, other factors that were not manipulated in our study such as social status might outweigh the benefits of shoaling with familiar conspecifics (Gomez-Laplaza & Fuente 2007). Thus zebrafish, if they can recognize familiar conspecifics, might express familiarity-biased preferences under different circumstances.

Social recognition has been shown to involve both visual and chemical signals in fish. Different types of stimuli (e.g. olfactory, visual, acoustic) can potentially signal different or more detailed information spanning across multiple behavioural contexts. For example in guppies visually attractive males were unattractive to females when olfactory cues alone were available (Shohet & Watt 2004), while males preferred larger females when a full range of stimuli was available but not when visual cues alone were presented (Herdman et al. 2004). In swordtails (*Xiphophorus pygmaeus*), females that initially showed a mate preference with visual cues alone did not show preferences when olfactory cues were added (Crapon de Caprona & Ryan 1990). A recent study on sex discrimination in female zebrafish suggested that subjects predominantly used visual cues to recognize males, but when the ambient light conditions did not allow visual discrimination they appeared to use the olfactory cues available as well (Hutter et al. 2011). These experiments illustrate that sensory mechanisms underlying social recognition are complex. Our finding that adult zebrafish made more first visits to the unfamiliar pair 8 days after familiarization provides suggestive but limited evidence that olfactory cues play a role in familiarity discrimination. To date, however, studies that compared behavioural responses in zebrafish with either visual or visual and chemical cues available did not provide evidence for differential use between these stimuli conditions (Pritchard 2001; Hutter et al. 2011).

Shoaling differences and social recognition may be shaped by both developmental and evolutionary factors (Seghers & Magurran 1995; Ward et al. 2009). For example, Kydd and Brown (2009) demonstrated that captive bred rainbowfish (*Melanotaenia duboulayi*), unlike their wild counterparts, showed no preference for familiar individuals, suggesting that captive breeding can cause the loss of familiarity preferences. In zebrafish, strain and population differences in shoaling raise the

possibility that differences in shoaling preferences for familiar individuals may also exist (Wright et al. 2003).

In this study adult zebrafish did not express familiarity discrimination in their shoaling decisions. This contrasts with the aforementioned kin-recognition studies and studies demonstrating that young zebrafish prefer to associate with fish resembling their rearing companions rather than differently patterned fish (Engeszer et al. 2004; Spence & Smith 2007). Notably, the rearing effect is lost when fish strains with more similar patterning are compared (Spence & Smith 2007). Thus learned recognition of conspecifics in zebrafish may be limited to a sensitive period, to discrimination of major phenotypic differences, to kin, or only expressed in younger individuals. Our findings, although negative, increase our knowledge on the behaviour of this important research model. Combination of existing knowledge on zebrafish development and genetics with shoaling and social recognition studies offers a valuable opportunity to study the neural mechanisms regulating social information use and grouping behaviour.

ACKNOWLEDGMENTS

We thank Henk Schriek, Ko van Rootselaar and Henk Westland for assistance with animal care, Will Swaney and Ioannis Leris for practical support or comments on a previous draft and Han de Vries for statistical advice. We acknowledge Utrecht University's 'High Potentials' programme for funding.

Chapter 4

NONAPEPTIDE INFLUENCES ON SOCIAL BEHAVIOUR: EFFECTS OF VASOTOCIN AND ISOTOCIN ON ZEBRAFISH SHOALING

Charlotte M. Lindeyer, Esther M. A. Langen,
William T. Swaney and Simon M. Reader

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ABSTRACT

Nonapeptides such as oxytocin and vasopressin have been shown to influence a range of vertebrate social behaviours and appear well conserved in function despite minor changes in molecular structure throughout evolution. To explore the role of nonapeptides in social behaviours in non-mammals, we studied grouping in zebrafish (*Danio rerio*) and investigated the influence of isotocin, vasotocin and putative antagonists on shoaling preferences. After a single peripheral injection, female subjects were given a preference test, where they could join or avoid a group of conspecifics. We predicted that vasotocin would decrease and isotocin would increase shoaling tendencies. Vasotocin administration significantly decreased the time spent interacting with the shoal and increased the time taken to begin interacting with the shoal compared to the control treatments, but had no significant effect on the time spent nearby the shoal. Contrary to expectations, a vasopressin antagonist reduced both the time subjects spent nearby the shoal and the time interacting with the shoal, relative to control subjects. Thus, as a proportion of the time spent nearby the shoal, vasotocin reduced subjects' interaction with the shoal more strongly than the vasopressin antagonist. Neither isotocin nor an oxytocin antagonist had significant effects on our behavioural measures. Our findings implicate vasotocin in the control of zebrafish shoaling and social interaction, raising the possibility that vasotocin may regulate grouping in this species. These data, combined with other recent findings, support the argument that the nonapeptides might play a general role in the regulation of sociality across vertebrates.

INTRODUCTION

A wide range of social behaviours, including pair bonding, parental care, aggression and grouping, have been shown to be influenced by the 'nonapeptides', a group of nine-amino acid proteins found in all vertebrates (Insel 2010). Two nonapeptide lineages arose from a gene duplication event early in vertebrate evolution: the first comprises arginine vasopressin (AVP) and its non-mammalian homologue arginine vasotocin (AVT), while the second is made up of oxytocin (OT) in mammals and its homologues isotocin (IT) in fish and mesotocin in birds, reptiles and amphibians. The structure and function of the nonapeptides has been highly conserved throughout evolution (Insel & Young 2000) and they have both peripheral and central effects (Young et al. 2011). Though their precise behavioural effects differ between sex and across species (Goodson 2008), the oxytocin/mesotocin/isotocin system is considered to generally play a role in 'pro-social' behaviours in vertebrates such as pair bonding whereas the vasopressin/vasotocin system regulates more 'anti-social' behaviours such as aggression (Goodson & Bass 2001; Ross & Young 2009).

The AVP system has been shown to play an important role in the control of aggression in rodents. AVP affected agonistic aggression in Syrian hamsters (*Mesocricetus auratus*) in a sex dependent manner: inter-male aggression was increased by AVP and decreased by an AVP antagonist, while inter-female aggression was decreased by AVP and increased by an AVP antagonist (Albers et al. 2006; Gutzler et al. 2010). Similarly, maternal aggression in Sprague-Dawley rats decreased after AVP antagonist administration (Nephew & Bridges 2008). Vole species (*Microtus spp.*) differ in the stability of breeding pairs (monogamous or promiscuous) and levels of aggression and these differences are associated with variation in OT and AVP systems. In monogamous male voles, AVP administration increased aggression but this was reduced by an AVP antagonist (Winslow et al. 1993). However, AVP administration also induced pair bonding in males which was blocked by an AVP antagonist (Winslow et al. 1993). Vole behavioural phenotypes have been correlated with AVP and OT receptor distribution differences (Young et al. 1998; Young et al. 2011). Similarly, extensive work has demonstrated a role for OT in social recognition. For example, OT knockout mice failed to develop social memory but this is restored after OT administration (Ferguson et al. 2000). Work in meerkats (*Suricata suricatta*) showed OT administration increased a range of cooperative behaviours and reduced aggression (Madden & Clutton-Brock 2011). Other work has demonstrated a role for OT in mother-offspring

attachment, such as the formation of a selective bond between a sheep mother and her lamb (Lim & Young 2006).

Recent work has expanded the role of nonapeptides in sociality to birds and demonstrated the different actions of mesotocin and AVT. Estrildid finches are monogamous and provide biparental care, but various species differ in terms of gregariousness and territoriality. Flocking in zebra finches (*Taeniopygia guttata*) is selectively influenced by nonapeptide administrations (Goodson et al. 2009b; Kelly et al. 2011): females spent longer with familiar than novel conspecifics after mesotocin administration, a preference reduced by administration of an OT receptor antagonist. Similarly, preferences for larger groups increased after mesotocin administration but decreased after OT antagonist administration (Goodson et al. 2009b). Moreover, AVT but not mesotocin administration increased aggression in a mate choice context in zebra finches (Goodson et al. 2004). As with the association between mating phenotype and receptor density across vole species, AVT receptor distribution differences correlated with social group size across five finch species (Goodson et al. 2009b). Together, these studies provide evidence of a regulatory role for the nonapeptides in social behaviour in birds and mammals (Donaldson & Young 2008).

While relatively few studies have investigated effects of nonapeptides on social behaviours in fish, the teleost AVT and IT systems have been shown to influence aggression, mating and dominant-subordinate behaviour (Goodson & Bass 2000a; Black et al. 2004; Lema & Nevitt 2004a; Larson et al. 2006; Greenwood et al. 2008; Filby et al. 2010; Iwata et al. 2010; Santangelo & Bass 2010). Moreover, comparative research on AVT neuro-anatomical organization across butterflyfish species (Chaetonontidae) and across pupfish populations (*Cyprinodon nevadensis* subspecies) that vary in sociality suggest a similar regulatory role for AVT to that found in birds and mammals (Lema & Nevitt 2004b; Dewan et al. 2011). Nonapeptides have also been shown to have contrasting effects on goldfish (*Carassius auratus*) social approach behaviour depending on baseline social phenotype ('highly social' or 'less social') (Thompson & Walton 2004; Walton et al. 2010). Highly social fish decreased their approach to conspecifics after AVT administration. In contrast, both AVT antagonist and IT administration increased approach in less social fish but not in highly social fish. These data suggest that AVT and IT have opposite effects and that there are opposing effects depending on the 'sociality' of the fish. These findings support the idea of a conserved neural mechanism underlying vertebrate sociality and illustrate the potential value of teleosts for comparative research into the evolution of social behaviour.

The zebrafish (*Danio rerio*) is a popular subject for developmental biology and genetics research. However, its adult behaviour is not well understood. Adult zebrafish show strong shoaling tendencies and display other social behaviours including aggression, dominant-subordinate interactions and mate choice (Spence et al. 2008; Miller & Gerlai 2011). Three studies have implicated AVT and IT in zebrafish social behaviour: neuro-anatomical differences in AVT neurons have been linked to aggression and social status (Larson et al. 2006), while variation in expression of AVT and AVT receptor genes has been linked to levels of aggression and dominance phenotypes (Filby et al. 2010). Most recently, AVT and IT were found to change the propensity to preferentially associate with fish of a similar phenotype, depending on dose (Braida et al. in press). However no study has directly addressed the influence of AVT and IT on social approach. In this study we administered nonapeptides and putative receptor antagonists to establish a functional role for AVT/IT systems in social grouping in zebrafish. We measured social approach and avoidance behaviour in a two-choice shoaling test and predicted that AVT would decrease and IT would increase social approach.

METHODS

Subjects and housing

A total of 150 adult female zebrafish (*Danio rerio*; 'wild type' strain, 4 – 5 months old) were used as subjects (mean mass ± SE = 0.33 ± 0.004 g). Twenty additional adult females (mean mass ± SE = 0.35 ± 0.004 g), unfamiliar to the subjects and housed separately, served as stimulus shoals in the behavioural tests. All subjects were bred in-house in the Biology aquarium at Utrecht University, the second generation of descendents of fish purchased from a commercial supplier (Ruisbroek, Maassluis, Netherlands). All housing tanks (small: 80.0 x 50.0 cm or large: 150.0 x 50 cm) were maintained at 26 ± 1°C with a water depth of 30 cm. Housing was enriched with artificial plants, pot shelters and gravel floor. Fish were on a 12h light:dark schedule with lights on at 0800 hours and no natural light present. Fish were fed twice daily (at 0900 and 1700 hours) with flake food (TetraMin, Tetra Ltd., Germany) in the morning and bloodworm (*Chironomidae*) or *Daphnia* sp. in the afternoon. Water pH, nitrates and nitrites were checked weekly. Tanks were cleaned fortnightly, but otherwise fish were left undisturbed as far as possible.

The subjects had not previously participated in any experiments. The experiment was approved by the Utrecht University Animal Experimentation Committee under protocol DEC 2010.I.12.263, and conforms to Dutch animal welfare standards and legislation.

Peptide treatments

Prior to each test a subject was selected at random from the pool of 150 subjects and assigned to one of six administration treatments: *1)* IT (AbD Serotec, Kidlington, UK), *2)* AVT (Bachem, Weil am Rhein, Germany), *3)* a selective OT receptor antagonist and putative IT receptor antagonist (desGly-NH_2,d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT, gift of Prof. M. Manning; Manning et al. 1995; henceforth 'OT antagonist') or *4)* a selective AVP 1a receptor antagonist and putative AVT receptor antagonist (d$(CH_2)_5$[$Tyr(Me)^2$,Dab^5]AVP, gift of Prof. M. Manning; Chan et al. 1996; henceforth 'AVP antagonist'); or a control condition with either *5)* 0.9 % saline or *6)* no administration).

Each treatment consisted of 25 replicates. Since subjects were not returned to the subject tank after testing, we added 20 additional fish to ensure that a reasonable number of fish remained. To address any possible observer bias a second researcher pseudo-randomly assigned treatments across testing days, preparing and labeling fresh solutions each testing day, such that the researcher observing the shoaling tests was blind to administration treatment. We counterbalanced the order in which treatments were administered, with half of the replicates of each administration run in the first testing week and the other half in a second testing week. We thus minimized any possible order effects.

Administration procedure

The administration protocol, doses and volumes were based on a pilot study. In the pilot study subjects had received peripheral administrations of either IT, AVT, OT antagonist or AVP antagonist solutions at three different doses (agonists: 0.1 µg/g, 1.0 µg/g and 10.0 µg/g; antagonists: 0.4 µg/g, 2.0 µg/g, and 10.0 µg/g) that were based on peripheral administration studies in other small fish (Lema & Nevitt 2004a; Santangelo & Bass 2006). Subjects were caught with a net, weighed in a cup of water and then placed on a wet tissue for intraperitoneal administration. Nonapeptides and antagonists were dissolved in 0.9 % saline and injected with a 10 µl Hamilton syringe and 30G x ½" needle at a dose of 10.0 µg/g body mass with injection volumes up to a maximum of 6 µl. No subject weighed over 0.6 g. The administration procedure took ca. 20 seconds. After administration, subjects were placed in the transparent cylinder in the middle compartment of the experimental tank (Fig. 1).

Behavioural test

We used a preference test to determine zebrafish shoaling preferences (Fig. 1). A large tank (150.0 x 50.0 cm) was divided into three areas by transparent solid plastic partitions: two outer stimulus shoal

compartments (11.5 cm wide) and a middle compartment (127 cm wide). In one of the shoal compartments a stimulus shoal of eight fish was placed in a container (11.5 x 12.5 cm), while the other shoal compartment was left empty. The stimulus shoal fish were chosen at random from the pool of 20 fish and used for 2 - 3 consecutive trials. Shoal location was randomized after every two trials. The middle compartment was divided into three zones: a 'neutral' zone in the middle, and two outer zones defined as 'shoaling' and 'non-shoaling' depending on the stimulus shoal location. Subjects were considered to be shoaling with the stimulus shoal when within 3 – 4 body lengths, i.e. 10 cm. (Pitcher 1983). The zone boundaries were marked on the outside of the tank. Fish could thus swim with a stimulus shoal on one side, with no conspecifics on the opposite side, or in the central neutral zone. At the start of the session subjects were positioned in the middle compartment within a centrally-placed transparent plastic cylinder (diameter 7 cm; Fig. 1). After a 5-minute acclimatization and recovery period the cylinder was slowly pulled upwards to release the subject, using a pulley system to minimize disturbance, and the 10-minute trial started. An observer recorded subject behaviour live using the software JWatcher V1.0 (http://www.jwatcher.ucla.edu) and all sessions were recorded with a Megapixel Pro webcam (Trust International B.V., Dordrecht, Netherlands) and AMCap 9.20 software. All stimulus fish were weighed after testing. Subjects were weighed once during testing and again one week afterwards to check for possible adverse effects of administration on weight gain. Subjects' mass did not differ significantly between the treatment conditions either before (Linear Model (LM): $t_{24} \leq 1.64$, $P > 0.1$) or after testing (LM: $t_{24} \leq 0.98$, $P > 0.3$; mean mass ± SE = 0.39 ± 0.05 g). After testing, subjects were housed separately from the naïve subjects in a tank (80 x 50 cm) assigned to their peptide treatment. During the experiment all fish were fed after testing was completed in the afternoon.

We measured the latency to enter the shoaling zone and the total time spent in the shoaling zones, as well as the latency to start interacting and total time spent interacting with the shoal. Interaction was operationally defined as when a subject swam head first against the transparent partition separating it from the shoal (i.e. the subject actively swam to join conspecifics). We used the shoaling and interaction measures to differentiate between grouping and more active social interest. A similar interaction measure has recently been demonstrated to give different results from grouping measures (Kelly et al. 2011), and thus could reflect a different aspect of shoaling.

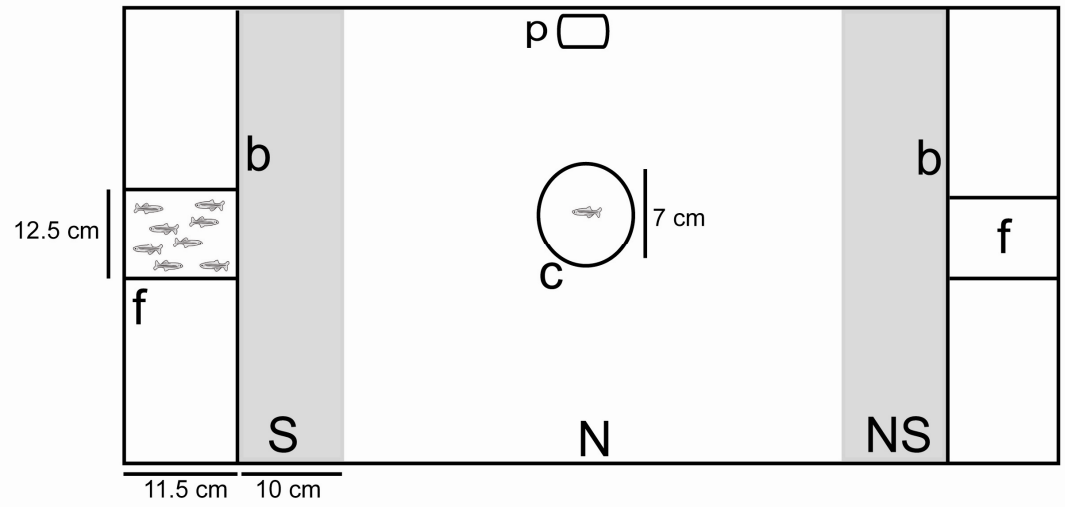

Figure 1: Schematic overview of the experimental apparatus, plan view. Lines were drawn on the outside of a large aquarium (150 x 50 cm) to mark a neutral zone (N), shoaling zone (S) and non-shoaling zone (NS), the latter two dependant on the location of a stimulus shoal. P: pump. The subject was released from a transparent cylinder (c) after acclimatization and its behaviour was recorded for 10 minutes. A large conspecific shoal was placed at random on one side of the tank behind a transparent solid partition (b) in a confined zone (f). Interaction was recorded when subjects were both in the shoaling zone (S) and swam head first against the partition (b).

Statistical analyses

The dependent variables were total time spent shoaling, total time spent interacting, latency to shoal, latency to interact and the number of switches between zones (a measure of activity). We also calculated interaction time as a proportion of the shoaling time. We analyzed peptide treatment as a fixed effect and included shoal position as a random factor and mean mass of the stimulus shoal and subject mass as covariates. We used generalized linear models (GLMs) with a gamma family (Crawley 2007), unless specified otherwise, to investigate the effect of peptide treatment on the behavioural measures. Our main analyses used the saline control as the contrast group for the peptide treatments, with an alpha significance level of 0.05. To explore differences between peptide treatments (AVT vs. IT; AVT vs. AVP antagonist; IT vs. OT antagonist) we ran additional GLMs with a pre-defined a priori contrast matrix (package Epi) and a Bonferroni adjusted critical alpha level ($\alpha = 0.0167$) for multiple comparisons. All statistical tests are two tailed. Data are expressed as means ± SE. The body mass of subjects and mean mass of stimulus shoals were not found to be significant predictors of shoaling behaviour ($P \geq 0.1$) and therefore are not reported below. Analyses were performed in R Project 2.10.1.

RESULTS

Control conditions

We compared the saline administration with the no administration control to investigate any effects of the administration procedure. We found no significant differences in any of the behavioural measures between the no administration and saline administration control groups (Fig. 2; GLMs: $t_{24} \leq 1.41$, $P > 0.2$). Similarly, saline and no administration controls did not significantly differ in mass gain (LM: $t_{24} = 0.54$, $P = 0.6$). We thus used the saline condition as the comparator group for the remaining analyses and the no administration group was not analyzed further.

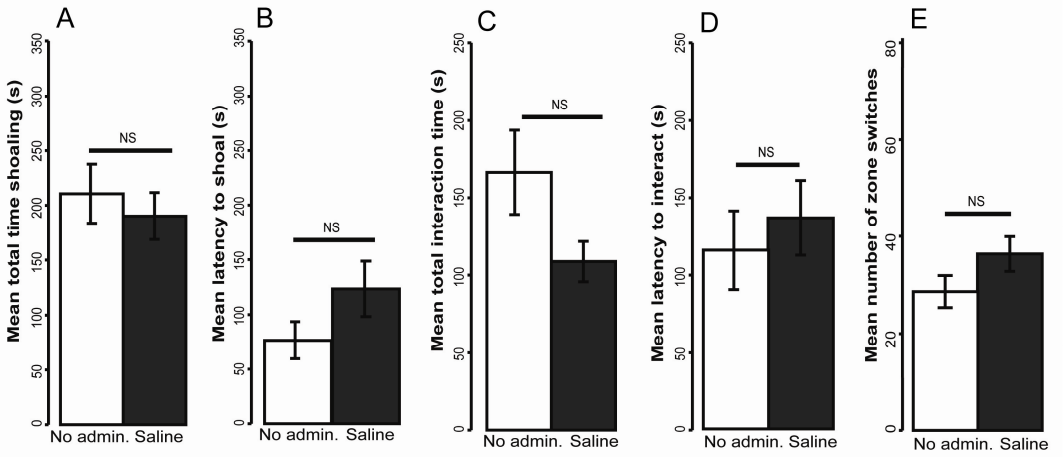

Figure 2: Zebrafish mean (± SE) values for A) total time shoaling, B) latency to shoal, C) total interaction time, D) latency to interact and E) switching frequency between the shoaling and neutral zones compared across the two control conditions (saline administration and no administration ('No admin.')). No significant differences were found on any measure (NS: $P \geq 0.1$).

Shoaling

Subjects demonstrated a strong tendency to associate with the stimulus shoal, spending more time within 10 cm of the shoal than in the non-shoaling zone (mean time with shoal ± SE = 184.4 ± 27.9 s; mean time on opposite side = 46.9 ± 14.4 s).

Treatment groups differed in the amount of time they spent with the shoal. AVP antagonist administration significantly reduced the time spent shoaling, compared to saline administration (GLM: $t_{24} = 2.58$, $P = 0.01$, Fig. 3A). Other treatments did not significantly differ from the saline control (GLM: $t_{24} \leq 1.51$, $P > 0.1$). Planned comparisons indicated that time spent shoaling was significantly higher after AVT administration than IT or AVP antagonist administration (GLM with a priori contrasts: AVT vs. IT, $z_{24} = 2.48$, $P = 0.01$; AVT vs. AVP

antagonist, z_{24} = 3.76, P = 0.0002; Fig. 3A). Subjects in all treatment groups, except for the AVP antagonist administration, demonstrated a significant preference for the shoal over the non-shoaling zone (Wilcoxon paired signed-ranks tests: AVP antagonist, U = 225, N = 25, P = 0.09; other groups, $U \geq 264$, N = 25 per group, $P < 0.005$ in all cases). AVT and AVP antagonist administration significantly increased the time spent in the non-shoaling zone, compared to saline administration (GLM: t_{24} = 2.02, P = 0.05; GLM: t_{24} = 2.03, P = 0.04; respectively). Our planned comparisons did not reveal significant differences between peptide treatments in the time spent in the non-shoaling zone (GLM with a priori contrasts: $z_{24} \leq 1.34$, $P \geq 0.2$; Fig. 3A).

Subjects typically swam away from the cylinder and back and forth in the neutral zone immediately after release, before swimming to either end of the tank. There were no statistically significant effects on latency to begin shoaling. AVT treated fish were slower to begin shoaling than both saline and IT treated fish, but not significantly so (GLMs: AVT vs. saline, t_{24} = 2.58, P = 0.09; AVT vs. IT, z_{24} = 1.78, P = 0.08; Fig. 3B).

In summary, the AVP antagonist significantly reduced time spent shoaling compared to saline and AVT treated fish; while both AVT and the AVP antagonist increased time spent at the opposite end to the tank to the shoal, compared to saline treated controls. These effects resulted in a significant preference to shoal in all but the AVP antagonist treated fish. IT administration significantly decreased shoaling compared to AVT administration.

Interaction with the shoal

Interaction was only observed nearby the shoal, never at the opposite compartment, suggesting that this was an effective measure of social interaction. Both AVT and AVP antagonist administrations significantly decreased the time interacting with the stimulus shoal compared to the saline control (GLM: AVT, t_{24} = 2.34, P = 0.02; AVP antagonist, t_{24} = 2.03, P = 0.04; Fig. 4A). Other administrations did not significantly differ from the saline control (GLM: $t_{24} \leq 1.60$, $P > 0.1$). IT treated fish spent less time interacting with the shoal than did OT antagonist treated fish, but not significantly so (GLM with a priori contrasts: z_{24} = 1.94, P = 0.05). To further investigate shoaling interactions, we examined the proportion of time interacting with the shoal out of the total time spent shoaling. This proportional measure followed a similar pattern. AVT, AVP antagonist and IT administrations significantly decreased the proportion of time interacting with the shoal compared to saline (GLM with quasibinomial family: AVT vs. saline, t_{24} = 5.55, $P < 0.0001$; AVP antagonist vs. saline, t_{24} = 2.46, P = 0.02; IT vs. saline, t_{24} = 2.76, P = 0.01; Fig. 4B). Additionally, AVT treated zebrafish spent a significantly

lower proportion of time interacting with the shoal than AVP antagonist and IT treated fish (GLM with quasibinomial family: AVT vs. AVP antagonist, $z_{24} = 3.49$, $P = 0.001$; AVT vs. IT, $z_{24} = 3.21$, $P = 0.001$; Fig. 4B). Moreover, IT administration significantly decreased the proportion of time interacting compared to OT antagonist administration (GLM with quasibinomial family: $z_{24} = 3.09$, $P = 0.002$).

AVT administration significantly increased the latency to start interacting with the stimulus shoal compared to saline and IT treated fish (GLMs: AVT vs. saline, $t_{24} = 2.88$, $P = 0.005$; AVT vs. IT, $z_{24} = 2.42$, $P = 0.015$; Fig. 4C).

We examined correlations between the total time spent shoaling and interacting, which correlated positively for all treatments except AVT (Saline, r = 0.76, $P < 0.00001$; AVT, r = 0.34, $P = 0.1$; AVP antagonist, r = 0.81, $P < 0.00001$; IT, r = 0.70, $P = 0.0001$; OT antagonist, r = 0.96, $P < 0.00001$; Fig. 5). The AVT treatment correlation coefficient was significantly lower than that of the saline treatment ($z_{44} = 2.13$, $P = 0.03$), as was the regression slope ($t_{46} = 2.68$, $P = 0.01$; Fig. 5).

To summarize, AVT strongly reduced the time spent interacting with the shoal, compared to the saline control, even when its effects on shoaling were taken into account. AVT also increased the latency to interact with the shoal. Similarly, the AVP antagonist significantly reduced interaction time with the shoal, however, once its effect on shoaling time was taken into account it did so less strongly than AVT. There was some evidence for opposing effects of IT and the OT antagonist on time spent interacting with the shoal when shoaling time was taken into account, although only the IT group differed from the saline control.

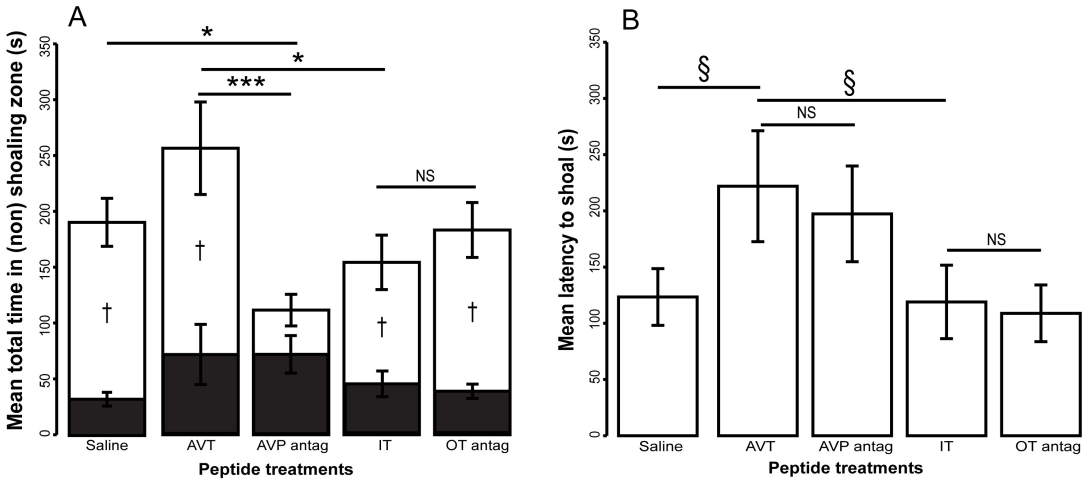

Figure 3: Zebrafish mean (± SE) values for A) the time spent in the shoaling (open bars) and the non-shoaling (filled bars) zones and for B) latency to shoal for each peptide administration ('antag': antagonist). ***: $P \leq 0.001$, *: $P \leq 0.05$, §: $P \leq 0.1$, NS: $P \geq 0.1$. NS results are only indicated for the planned comparisons (see Methods). † indicates a significant preference for the shoaling over the non-shoaling zone. Significant differences between peptide treatments in the time spent in the non-shoaling zone are not indicated here but are given in the main text.

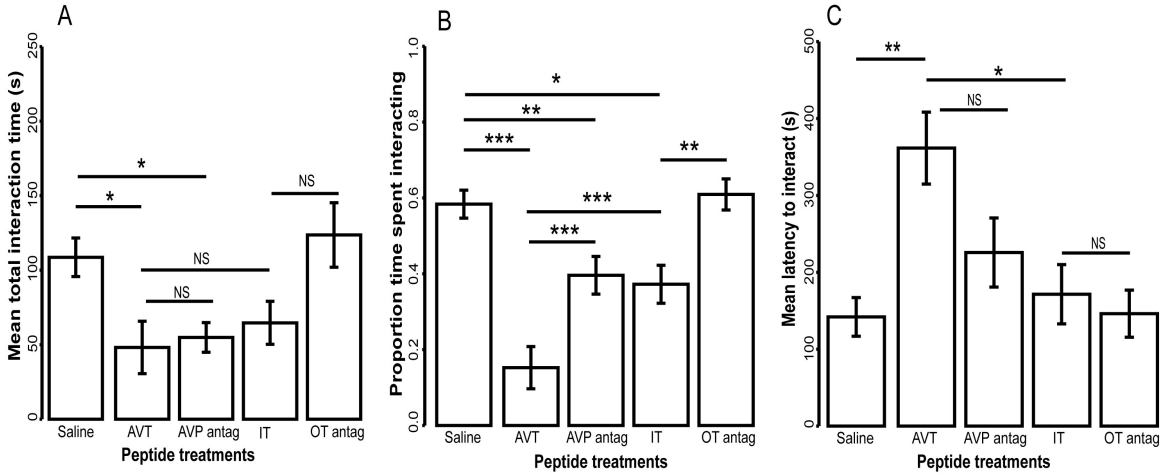

Figure 4: Zebrafish mean (± SE) values for A) the total duration of interaction with the shoal, B) the proportion of time interacting out of the time spent shoaling and C) the latency to interact with the stimulus shoal for each peptide treatment ('antag': antagonist). ***: $P \leq 0.001$, **: $P \leq 0.01$, *: $P \leq 0.05$, NS: $P \geq 0.1$. NS results are only indicated for the planned comparisons (see Methods).

Other measures

Administration of AVT, AVP antagonist and IT significantly increased the frequency that subjects switched between the shoaling, neutral and non-shoaling zones, compared to the saline group (GLM with quasipoisson family: AVT, $t_{24} = 3.42$, $P = 0.001$; AVP antagonist, $t_{24} = 2.29$, $P = 0.02$; IT, $t_{24} = 1.98$, $P = 0.05$; Fig. 6). We found no significant differences in switching between peptide treatments (GLM with a priori contrasts: $z_{24} \leq 1.90$, $P > 0.05$). Subjects significantly gained body mass in the week after testing (Wilcoxon signed rank test: $W = 5734.5$, $N = 150$, $P < 0.0001$) and their mass gain did not significantly differ between peptide treatments (LM: $t_{24} \leq 1.07$, $P > 0.3$).

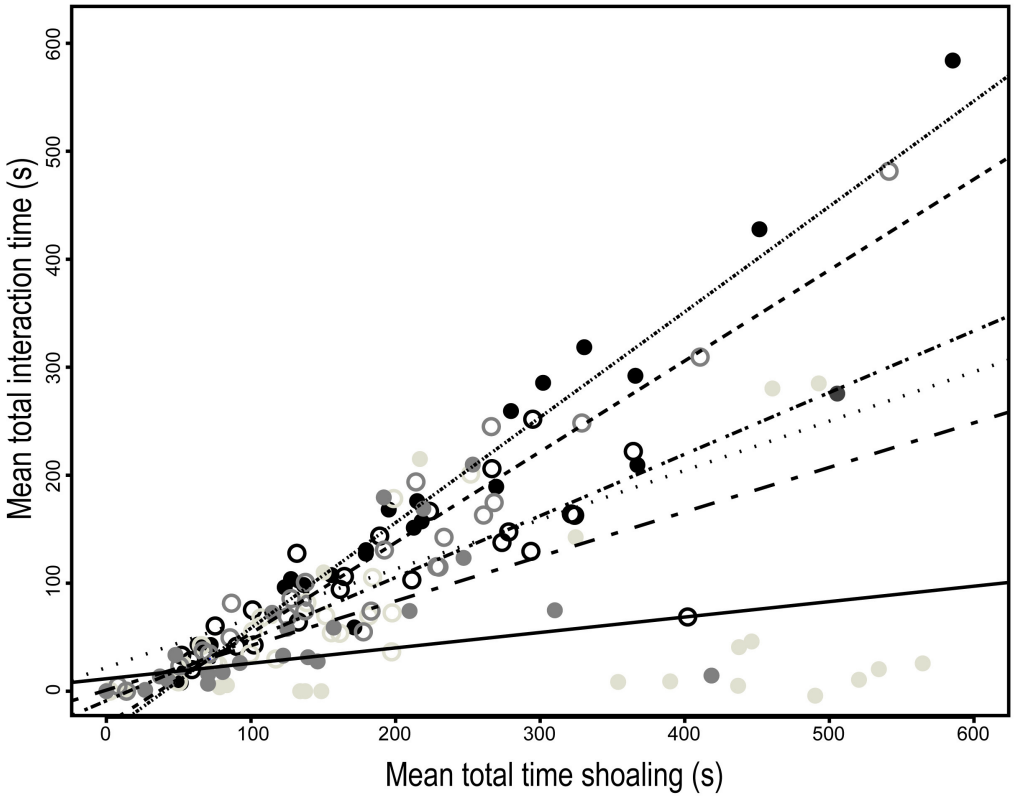

Figure 5. Mean time spent interacting with the shoal versus the mean time shoaling for the peptide treatments and the two control conditions. 1) No admin: filled black points and short-dashed line, 2) Saline: open black points and dotted line, 3) AVT: filled light grey points and solid line, 4) AVP antagonist: open light grey points and dash-with-two-dots line, 5) IT: filled dark grey points and long/short-dashed line, 6) OT antagonist: open dark grey points and medium-dashed line.

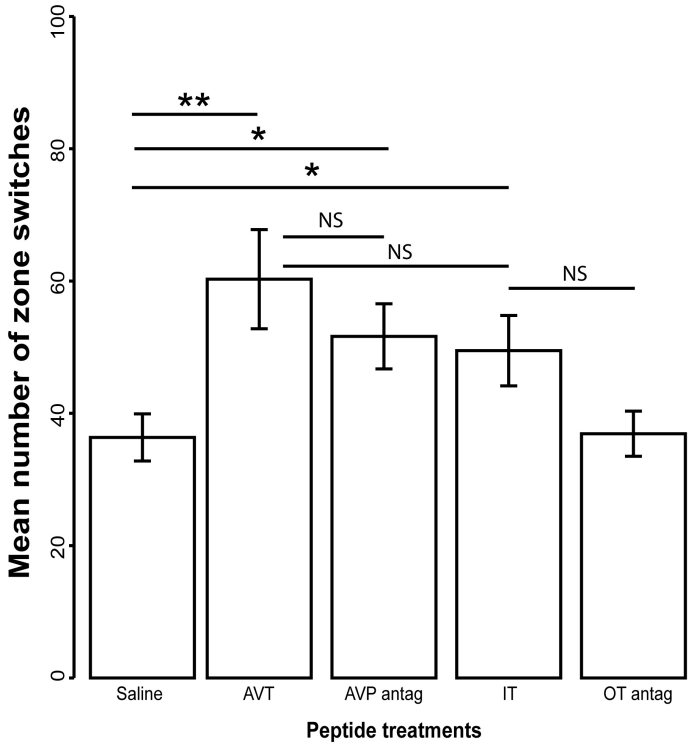

Figure 6: Mean (± SE) number of switches made between the shoaling, neutral and non-shoaling zones for each peptide treatment ('antag': antagonist). **: $P \leq 0.01$, *: $P \leq 0.05$, NS: $P \geq 0.1$. NS results are only indicated for the planned comparisons (see Methods).

DISCUSSION

Our results suggest that the AVT system modulates aspects of social behaviour in female zebrafish, namely their tendency to group and interact with conspecifics in a novel environment. Subjects administered AVT were both slower to interact with the shoal and spent less time interacting with the shoal, compared to control fish. They also spent more time away from the shoal. Contrary to predictions, the AVP antagonist significantly decreased shoaling and interaction time and increased time spent away from the shoal, compared to control fish. When effects on shoaling time were taken into account, AVT reduced interaction with the shoal more strongly than the AVP antagonist. In contrast, we found no clear evidence that the IT system strongly regulates shoaling, with few effects of IT or OT antagonist administrations, although we found some evidence that IT administration decreased interaction tendencies compared to control administrations. Thus nonapeptides influence the propensity to approach and interact with a shoal in the zebrafish.

Vasotocin

Our finding that AVT decreased the tendency to approach conspecifics in female zebrafish is matched by findings in goldfish, where AVT inhibited approach to conspecifics in both males and females (Thompson & Walton 2004; Thompson et al. 2008). Braida et al. (in press) recently reported that dose-dependent AVT intramuscular administration influenced the preference for own-strain versus different-strain conspecifics in zebrafish: doses of 1.0 $x10^{-9}$ µg/g increased the preference for same-strain individuals, doses around 5.0 $x10^{-8}$ µg/g decreased the preference for same-strain individuals, and doses around 3.0 $x10^{-6}$ µg/g had little effect. However, our study is difficult to compare to the Braida et al. study, because their doses were based on those used in intracerebroventricular administration of OT and AVP in mice and therefore considerably lower than the dose utilized in our study (10.0 µg/g) or other similar studies (Lema & Nevitt 2004a; Santangelo & Bass 2006; Filby et al. 2010). If AVT shows an inverted-U dose-response function, as Braida and colleagues suggest, an increase in shoaling might be expected at the dose utilized in our study, but we found no effect on shoaling. Moreover, in the Braida et al. study fish could choose between own-strain versus different-strain conspecifics, but in our study fish could choose to join or avoid a group of conspecifics. Thus, both studies address different aspects of social grouping, potentially explaining this discrepancy.

Contrary to our expectations and the antagonist effects Braida and colleagues reported, AVT and the AVP antagonist did not have clearly opposing effects. On some measures (time away from shoal, total interaction time, switching frequency) they had similar effects, whereas on other measures they had different effects (total time shoaling, proportion of time interacting with the shoal). Our findings suggest that the AVT system regulates, or at least affects, two aspects of social grouping: time in proximity to conspecifics and interaction with conspecifics. We found that AVT reduced interaction and the AVP antagonist reduced shoaling. These separate behavioural effects may be due to the existence of at least two AVT receptors in zebrafish (Filby et al. 2010), as well as unanticipated effects of using a mammalian AVP antagonist in zebrafish. If the two receptors mediate different components of social behaviour, then it is possible to envisage that endogenous AVT could influence social grouping and social interaction, through dose-dependent effects mediated via the different receptors. If the AVP antagonist we used has a higher affinity for one of the AVT receptors, then this might help explain the differences between responses to administration of AVT and the AVP antagonist. This potentially represents a functional difference in the role of the AVT system not seen in previous studies of the influence of nonapeptides on shoaling.

Nevertheless, it is surprising that the AVP antagonist produced behavioural responses in the same direction as AVT. It suggests that this antagonist, which is specific for the mammalian vasopressin 1a receptor (Manning et al. 2008), may interact with zebrafish AVT receptors in an unanticipated manner, possibly including triggering agonist-like responses.

Isotocin

In goldfish, IT stimulated social approach only in the subjects that showed weak shoaling behaviour under baseline conditions, and not in strong shoalers (Thompson & Walton 2004). However, zebrafish show very strong shoaling tendencies (Buske & Gerlai 2011), and our ability to detect any pro-social influence of IT administration may have been compromised by a ceiling effect, with shoaling already at a maximum. Another possibility is that IT does not have pro-social effects in zebrafish, an idea raised by recent findings that IT actually decreases preferences for same-strain zebrafish at certain doses, peaking at 1.0 $x10^{-6}$ µg/g (Braida et al. in press). While we did not see any effect on shoaling at the dose we used (10.0 µg/g), when we considered interaction behaviour while taking into account the total time spent shoaling, IT significantly decreased the proportion of time spent interacting with the shoal compared to saline administration. Though these findings should not be over interpreted, this does suggest effects of IT administration on social behaviour in our study. IT and the OT antagonist did not elicit opposing effects on any of the behavioural measures except for the proportional interaction measure. However, the putative IT antagonist we used has been shown to be effective at low doses in zebrafish (Braida et al. in press), and thus we expected to be able to observe potential behavioural differences between the IT and OT antagonist treated fish if the IT system is important for the regulation of shoaling tendency. Finally, the molecular similarity of AVT and IT raises the possibility of cross-reactivity at their respective receptors (Chini & Manning 2007; Young et al. 2011). However this may be unlikely as Mahlman and colleagues (1994) showed that fish AVT receptors respond to AVT but not IT and thus that AVT receptors are functionally insensitive to IT. This suggests that the two nonapeptide systems in fish, IT and AVT, are separate and do not interact extensively, and our results indicate that AVT seems to play the more important role in social grouping.

Specificity of response

In this study we can not rule out the possibility that the subjects' grouping behaviour was linked to exploratory behaviour and stress responses. When exposed to a novel environment, zebrafish, like many other

species, have been shown to display anxiety responses, such as freezing or increased erratic movement (Stewart et al. 2010), which influences their swimming patterns and thus potentially affects exploration of a novel area (Egan et al. 2009). These considerations are potentially relevant as the AVT system has been shown to be involved in neuro-endocrine stress responses (Balment et al. 2006). As measures of stress and exploration behaviour, we used the frequency of switching between the different zones and the time spent in the non-shoaling zone, the zone furthest away from the shoal. We observed increased switching activity in the AVT, AVP antagonist and IT administered subjects compared to the control groups, possibly indicating higher levels of anxiety. Additionally, the AVT and AVP antagonist treated subjects showed increases in the time spent away from the shoal compared to the control group suggesting greater exploration tendencies, which could be related to the AVT system's role in stress responses. However, zebrafish also show tighter shoaling in response to stress (Speedie & Gerlai 2008), and the decreased shoaling and social interaction seen in response to AVT, IT and the AVP antagonist treatment suggest that these administrations were not simply increasing stress responses but were modulating sociality specifically. This is further supported by the differences in shoaling and interaction behaviour between AVT compared to the AVP antagonist and IT, suggesting that our observed differences are not just driven by anxiety or the involvement of the AVT system in stress regulation, but due to different effects on grouping of the different nonapeptides.

To assess social grouping we used time spent in the shoaling zone (shoaling), a simple proximity measure, and the time subjects actively engaged in social approach (interaction). The interaction measure is likely to reflect the interest of the subject in the shoal, thus being a measure of social response or a measure of social motivation. This might explain why we found opposing effects in the interaction measure relative to the shoaling measure after AVT and AVP antagonist administrations and suggests that there is a behavioural distinction between shoaling tendency and social motivation in zebrafish. Our finding that administrations can selectively influence specific behavioural measures is similar to recent results on grouping in the gregarious zebra finch (Kelly et al. 2011). In this study, AVT antagonists decreased preferences for larger group sizes but increased social contact time. Thus it is important to account for the general tendency of subjects to remain in proximity of conspecifics when quantifying sociality. These findings also emphasize the importance of taking multiple measures of behaviour. Like us, Kelly et al. (2011) found different effects with a grouping versus interaction measure. Thus caution is warranted in assuming that apparently similar behaviours are linked or similarly affected by nonapeptides.

Potential peripheral effects

Due to the small size of zebrafish we administered AVT and IT peripherally. Our assumption that intraperitoneal injection of nonapeptides will reach the brain and thus increase central levels remains an important issue. Other studies in teleosts have shown clear behavioural effects after intraperitoneal or intramuscular administrations of nonapeptides (Semsar et al. 2001; Semsar & Godwin 2004; Oldfield & Hofmann 2011; Braida et al. in press) which suggest that the fish blood-brain barrier allows administered substances to reach the brain (Lema & Nevitt 2004a; Balment et al. 2006; but see Bundgaard and Abbott 2008 for an alternative view). Additionally, the behavioural effects observed could also be the result of diverse peripheral effects of both IT and AVT (Insel 2010). In fish, swimming patterns change in response to pain or stress (Clements et al. 2002; Xu et al. 2005; Backstrom et al. 2011). We used the switching measure to detect changes in swimming behaviour and quantify potential peripheral stress. Nonapeptide administrations significantly increased switching, which could be a sign of peripheral stress or discomfort. However, we did not observe stress-related behaviours such as freezing or dashing nor were longer term effects on physiology or growth found.

To further investigate AVT's role in social behaviour and to address current translational challenges in nonapeptide research, differences in distribution of AVT neurons and AVT receptors could be linked to behavioural differences across zebrafish strains, populations, individuals or between sexes (Wright et al. 2003; Larson et al. 2006; Campbell 2008; Cacioppo & Decety 2011). The zebrafish allows investigation of social and environmental factors that are known to strongly influence fish neural development (Coss & Globus 1979; Kihslinger et al. 2006; Burns et al. 2009), and thus will increase our understanding of the neural underpinnings of social behaviour in vertebrates.

CONCLUSIONS

The present experiment demonstrates that the AVT system is influential in shoaling and interaction, although our study leaves open the question of how the AVT system modulates this behaviour and why we did not find clear differences between the agonist and antagonist but instead found apparent functional differentiation. Our findings further support a general modulatory role of the AVT system in social behaviour that is similar to other fish species, other vertebrates, and invertebrates (Goodson 2008). This conserved behavioural function of AVT, combined with its conserved physiological functions and conserved structure across

taxa suggests that AVT is part of an ancestral neural mechanism underlying sociality. Comparison of relatively simple behaviours across species will increase our understanding of the neural underpinnings of social behaviour and its evolution (O'Connell & Hofmann 2011). Translating this knowledge to human social behaviour could potentially lead to a better understanding of neuropsychiatric disorders with social deficits (Meyer-Lindenberg et al. 2011).

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Chapter 5

EARLY MATERNAL CARE PREDICTS RELIANCE ON SOCIAL LEARNING ABOUT FOOD IN ADULT RATS

Charlotte M. Lindeyer, Michael J. Meaney
and Simon M. Reader

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ABSTRACT

Many vertebrates rely extensively on social information, but the value of information produced by other individuals will vary across contexts and habitats. Social learning may thus be optimized by the use of developmental or current cues to determine its likely value. Here, we show that a developmental cue, early maternal care, correlates with social learning propensities in adult rodents. The maternal behaviour of rats *Rattus norvegicus* with their litters was scored over the first six days postpartum. Rat dams show consistent individual differences in the rate they lick and groom (LG) pups, allowing them to be categorized as high, low, or mid-LG mothers. The 100-day old male offspring of high and low-LG mothers were given the opportunity to learn food preferences for novel diets from conspecifics that had previously eaten these diets ('demonstrators'). Offspring of high-LG mothers socially learned food preferences, but offspring of low-LG mothers did not. We administered oxytocin to subjects to address the hypothesis that it would increase the propensity for social learning, but there were no detectable effects. Our data raise the possibility that social learning propensities may be both relatively stable throughout life and part of a suite of traits 'adaptively programmed' by early developmental experiences.

INTRODUCTION

Individual differences in the propensity to use information provided by the activities of other individuals ('social information') and to learn from this information ('social learning') can have dramatic consequences for the spread of information through groups, for between-individual fitness differentials, and consequently for theoretical models of cultural transmission (Reader 2004; Bouchard et al. 2007). Individual differences in social learning propensities could result from constraints, such as a lack of learning opportunities, cognitive shortcomings, or perceptual deficiencies. However, individual differences in social learning propensities may also represent adaptive optimization to both local conditions and to individual characteristics. In the latter case, individual differences could be relatively fixed across individuals of a particular class. For example, individuals of one class (e.g. one sex or age) might utilize social information because this propensity consistently provides a selective advantage for these individuals and not for others. Alternatively or in addition, development and learning could flexibly shape learning propensities within a lifetime, creating individual, class, or population differences that optimize the payoffs of social learning. Recent experience has been shown to shape reliance on social information (Kendal et al. 2005; Leadbeater & Chittka 2009), but early experience may result in long-term individual differences that persist into adulthood. Such developmental influences would provide advantageous flexibility in slowly changing environments, while avoiding the costs of assessing the current value of social information.

Developmental influences resulting in differing social learning propensities are thus potentially important but have not been extensively documented. Studies of birds and fish provide suggestive findings. Populations of Zenaida doves *Zenaida aurita* differ in their social learning propensities (Carlier & Lefebvre 1997). However, it has not been established whether these differences arise from development, recent experience, or phenotypic differences between populations. Hand-reared house sparrows *Passer domesticus* raised with an artificial parent were biased towards social information use depending on the parent's previous reliability in indicating food (Katsnelson et al. 2008). Guppies *Poecilia reticulata* reared at different stocking densities from early life differed in shoaling tendencies, affecting the propensity to follow conspecifics and thus to learn from them (Chapman et al. 2008). In both the Katsnelson et al. and Chapman et al. studies, however, experimental manipulations were maintained until the time of testing so the longevity of early-life influences are unknown. To our knowledge, the only evidence for a link between experiences restricted to early life and adult social learning

propensities comes from rodents. Social learning of novel diets declines dramatically in Sprague-Dawley rats reared artificially and deprived of maternal care and littermates (Levy et al. 2003; Melo et al. 2006), raising the possibility, investigated here, that natural variation in maternal care will influence social learning tendencies when adult.

Here, we focus on maternal influences on the development of social learning propensities, by studying food preference learning in Norway rats *Rattus norvegicus*. Rats faced with a choice of novel diets prefer the food that a demonstrator conspecific has previously eaten (Galef & Wigmore 1983; Galef 2002). This social learning of food preferences combined with neophobia to novel foods should allow animals to exploit safe foods while avoiding potentially dangerous foods (Noble et al. 2001). Food preference learning thus provides an extremely well-studied social learning task relevant to the ecology of rats and several other rodents (Galef 2002; Choleris et al. 2009; Lesburgueres et al. 2011).

We took advantage of natural variation in levels of maternal care in rats, an extensively-studied influence on brain and behaviour. During the first week postpartum, rat dams show consistent individual variation in licking and grooming their pups (LG), although environmental stressors (e.g. food restriction, predator cues) influence LG rates, suggesting that maternal care provides a cue to the state of the external environment (Caldji et al. 1998; Champagne et al. 2003; McLeod et al. 2007). LG differences are associated with extensive variation in offspring gene expression, neuro-endocrine development, and behavioural development (Caldji et al. 1998; Champagne et al. 2003). Compared to high-LG offspring, low-LG offspring show enhanced stress responses and increased anxiety. Such differences in risk sensitivity have been theoretically and empirically linked to social information use, with risk sensitive individuals predicted to utilize social information more (Coolen et al. 2003; Mathot & Giraldeau 2008). However, anxiety and deficits in social interaction may also depress social information use (Choleris et al. 1998; Melo et al. 2006).

Styles of maternal care are transmitted across generations: cross-fostering studies show that genetic offspring of low-LG mothers raised by high-LG mothers show high-LG phenotypes when adult (Champagne & Meaney 2007). Pups are likely to live in a similar habitat to their mother (Levine 1994). Therefore, it has been suggested maternal care provides a route for 'adaptively programming' offspring epigenetically to their (future) environment, without the pups having to experience this habitat directly themselves (Caldji et al. 1998; Diorio & Meaney 2007).

The neuropeptide oxytocin and the closely related vasopressin are not only involved in a range of social behaviours, such as maternal care,

aggression, pair bonding, sexual behaviour, and social memory, but also in learning, anxiety related behaviour and stress coping (Neumann 2008). Oxytocin increases recall of socially acquired food preferences (Popik & Van Ree 1993), but the effects of oxytocin on acquisition of socially induced food preferences have not been explored (Choleris et al. 2009). Oxytocin could influence social acquisition through various routes, such as changes in social interactions or in approach, attention, or tolerance to conspecifics (Goodson et al. 2009b; Madden & Clutton-Brock 2011).

To investigate the joint effects of maternal care differences during development and oxytocin on social learning we utilized the well-established social food preference learning paradigm (Galef 2002), testing adult males. On the basis of maternal deprivation experiments (Melo et al. 2006), we expected high-LG offspring to show enhanced social food preference learning compared to low-LG offspring. That is, we predicted that early experience would have a lasting effect into adulthood on social learning propensities. Administration of oxytocin was predicted to enhance social learning, because oxytocin has been shown to facilitate recall for socially learned cues and to increase social approach (Popik & Van Ree 1993; Madden & Clutton-Brock 2011).

METHODS

Overview

We used adult male rats born to and reared by either high-LG or low-LG mothers. These 'observer' rats received oxytocin or saline (control) administrations prior to interaction with a single 'demonstrator' rat that had eaten one of two flavoured diets. After the interaction, the observer was separated and allowed to choose between the two novel diets. We thus determined whether interaction with a demonstrator rat influenced observers' food preferences.

Subjects and housing

Sixty-nine Long-Evans hooded male rats from a single cohort bred at the Allan Memorial Institute, Montréal, Canada participated in the experiment, 29 as demonstrators and 40 as subjects. Breeding procedures followed Champagne et al. (2003). Rats were selected as high-, mid- or low-LG phenotypes based on their mothers' maternal behaviour, defined according to Champagne et al. (2003). Maternal behaviour of individually housed dams with their litters of > 5 pups was scored daily for the first 6 days postpartum at regular times (0600, 1000, 1300, 1700 and 2100 h). Within each observation period maternal behaviour was scored every 3 minutes (for methodology see Champagne et al. (2003)). Mean LG (± SD) for the cohort was 8.16 ± 2.13. Litters with LG over 10.30 (i.e. 1 SD

above the mean) were categorized as 'high-LG', litters with LG below 6.03 (1 SD below the mean) were categorized as 'low-LG', with other litters categorized as 'mid-LG'. Twenty-nine mid-LG rats (mean LG ± SE = 8.93 % ± 0.32) acted as demonstrators, with a mean mass ± SE of 436 ± 6.12 g at 90-107 days old, the beginning of demonstrator habituation. Twenty high-LG (mean LG ± SE = 11.36 % ± 0.11) and 20 low-LG (mean LG ± SE = 4.94 % ± 0.13) naïve rats were assigned as observers. Observers were drawn from five high-LG and five low-LG litters and were 90-107 days old at the beginning of the study with a mean mass ± SE of 471 ± 6.64 g. High-LG and low-LG rats did not significantly differ in mass (*t* test; $t_{38} = 1.47$, $P = 0.15$). Observers were 107-124 days old when tested.

After weaning, rats were pair-housed with littermates in clear Plexiglas cages (20 x 23 x 44.5 cm) with sawdust bedding and rodent food pellets (Purina 5075-U.S., Charles River, Canada) and water provided ad libitum. Housing was maintained at 22 ± 4 °C and 30 ± 5 % humidity, with a 12h light-dark cycle with lights on at 0700 hours. One week prior to testing, 'experimental pairs' were formed, housing together one high-LG and one low-LG offspring per pair. Thus subjects had experienced an unfamiliar individual prior to the experimental test. In assigning experimental pairs, we matched ages within a pair as closely as possible. Demonstrators were unfamiliar to observers.

Procedures followed the guidelines of ASAB and the Canadian Council on Animal Care; protocols were approved by the McGill University Animal Care Committee (protocol #5642).

Diets

We used two diets novel in flavour to the rats: cinnamon and cocoa-flavoured. We mixed regular ground rodent food (Purina 5075-U.S., Charles River, Canada) with either 1% by weight ground cinnamon (No Name Brand, Canada) or with 2% by weight cocoa (Cadbury Fry's Premium, Canada); proportions followed Galef and Wigmore (1983). A pilot study ($N = 12$ additional low-LG rats; feeding session of 24 h) revealed diets were approximately equally matched, but with considerable individual variation and a slight (nonsignificant) preference for the cinnamon diet. We thus added granulated sugar to the cocoa diet (2% by mass) to increase its palatability relative to the cinnamon diet (Galef 2002). We utilized a counterbalanced design to account for any prior preference for one diet in the observers, with half of the demonstrators fed the cocoa diet and the other half the cinnamon diet.

Procedure

Demonstrator Habituation

Demonstrator rats were habituated to a randomly assigned diet one day prior to observer testing and re-used when necessary after one week of rest. Demonstrators were housed individually and food deprived for 23 hours. They were allowed to individually feed on 15 g of their assigned diet for one hour in a housing cage with paper tissue bedding and water ad libitum. Flavoured food was presented in a heavy (450 g) rectangular porcelain white container with 2 separate compartments (diameter 7 cm, 5 cm deep) and secured with tape to avoid overturning or excessive food spillage. Afterwards, the food was sieved to remove any foreign matter and re-weighed to measure demonstrator food intake. Observers received no habituation to diets, but food pellets were removed 1 hour before testing.

Testing

On the day of observer testing, demonstrator rats were food deprived for 22 hours, received 15 g of their assigned diet, and were allowed to feed for 2 hours with water available ad libitum. Only animals that consumed at least 3 g of their diet were used as demonstrators, following Galef and Whiskin (2001). One demonstrator did not meet the criterion and this session was not included in the diet preference analysis. Demonstrators mean consumption (± SE) was 9.53 ± 0.49 g. Demonstrators were placed with observers immediately after feeding.

We administered oxytocin or saline to observer rats 15 minutes prior to placing them with a demonstrator. Both animals within each low/high-LG experimental pair received the same administration. We injected 1 ml/kg subcutaneously of either oxytocin (OT; $C_{43}H_{66}N_{12}O_{12}S_2$; Sigma-Aldrich, Inc., Oakville, Canada) dissolved in 0.9 % saline (0.9 % sodium chloride injection USP) to a concentration of 3 ng/ml or saline as a control. We based the 3 ng/ml dosage on doses previously shown to facilitate social recognition and recall of a socially acquired taste preference when administered subcutaneously to male Wistar rats (Popik et al. 1992; Popik & Van Ree 1993). We allocated administrations so that equal numbers of high- and low-LG rats received oxytocin and saline. Similarly, age categories were matched as far as possible between the oxytocin and saline administrations. Offspring from the same litter were evenly distributed across administration and demonstrator diet groups as far as possible. Rats were left undisturbed for 15 minutes before being placed with a randomly selected demonstrator.

A single demonstrator and a single observer were placed together for 30 minutes in a clean Plexiglas cage containing fresh bedding material only. After the interaction period, demonstrators were returned to their

housing pairs. We moved single observers to a clean cage containing paper tissue bedding, water ad lib, and a weighed food cup containing 35 g of each diet (counterbalanced for location so that the cocoa diet was on the left for half the subjects and right for half the subjects). We determined individual intake of both diets after 2, 15 and 24 hours (thus at 20:00, 09:00, and 18:00 h) by weighing the remaining food. Two sieves were used to avoid cross contamination of odors between the cocoa and cinnamon diets. If less than 10 g was left of one diet, we added 10 g extra. At the end of testing rats were returned to their housing pairs and the testing cage examined for excessive diet spillage. No data had to be disregarded because of spillage.

Analyses

Statistical analyses were performed in SPSS 16.02. We measured diet preference as the mass of cinnamon diet consumed subtracted from the mass of cocoa diet consumed. We analyzed administration (OT versus saline), maternal style (high- or low-LG) and demonstrator diet (cocoa or cinnamon) as fixed effects. We investigated the effect of time period (0-2, 2-15 and 15-24 hours) as a repeated measure, and the amount eaten by demonstrators as a covariate, but neither had significant effects on diet preference, or interaction effects with independent variables ($P > 0.1$ in all cases), and thus these variables were eliminated from the model and not reported below. Hence we report diet preferences over the entire 24 h testing period. Previous studies have measured food preference as the proportion of diet cocoa consumed out of the total eaten by a subject (Galef 2002), and we use this intuitive measure for Figures 1 and 2. However, the proportional measure carries the disadvantage that preference data from rats eating very small and large amounts can be equivalent, leading us to prefer the difference measure. The proportional measure gave identical findings to the difference measure (see below).

RESULTS

Diet preference

Observers exposed to demonstrators that had eaten a cocoa diet consumed more cocoa diet than did observers exposed to cinnamon diet demonstrators (ANOVA: $F_{1,31} = 9.73$, $P = 0.004$; Fig. 1). Thus observers were biased towards their demonstrator's diet, concordant with observer social learning from demonstrators. However, not all observers were biased by demonstrator diet, which can be explained by investigating interaction effects with demonstrator diet. Maternal style (LG) had a significant statistical interaction with demonstrator diet on observer diet preference (ANOVA: $F_{1,31} = 4.81$, $P = 0.036$; Fig. 1). A similar

interaction effect was observed using the alternative diet preference measure, the proportion of diet cocoa consumed (ANOVA: $F_{1,31} = 4.24$, $P = 0.048$). Thus high-LG rats were significantly biased towards their demonstrator's diet (t test comparing high-LG observers of cocoa vs. cinnamon-demonstrators: $t_{17} = 3.52$, $P = 0.003$), in both saline and oxytocin administered rats (t tests: $t_7 = 2.70$, $P = 0.03$; $t_8 = 2.25$, $P = 0.05$; respectively; Fig. 2). However, demonstrator diet did not significantly influence low-LG rats' diet preferences (t test: $t_{18} = 0.70$, $P = 0.50$), in either saline or oxytocin administered rats (t tests: $t_8 = 1.65$, $P = 0.14$; $t_8 = 0.33$, $P = 0.75$; respectively). To partially account for the fact that observers were not all from independent litters, we took the mean diet preference for all observers from the same litter allocated to the same demonstrator diet and administration treatment combination and reanalyzed the data. Again, high-LG but not low-LG rats were significantly biased towards their demonstrator's diet (t tests: $t_{12} = 3.63$, $P = 0.03$; $t_{13} = 1.05$, $P = 0.32$).

Administration of oxytocin versus saline had no significant interaction effect with demonstrator diet (ANOVA: $F_{1,31} = 2.57$, $P = 0.12$) or with demonstrator diet and maternal style ($F_{1,31} = 0.002$, $P = 0.97$; Fig. 2). Oxytocin administration also had no significant influence on the preference for cocoa over cinnamon diet ($F_{1,31} = 0.44$, $P = 0.51$). Thus maternal style but not oxytocin administration had significant effects on social learning.

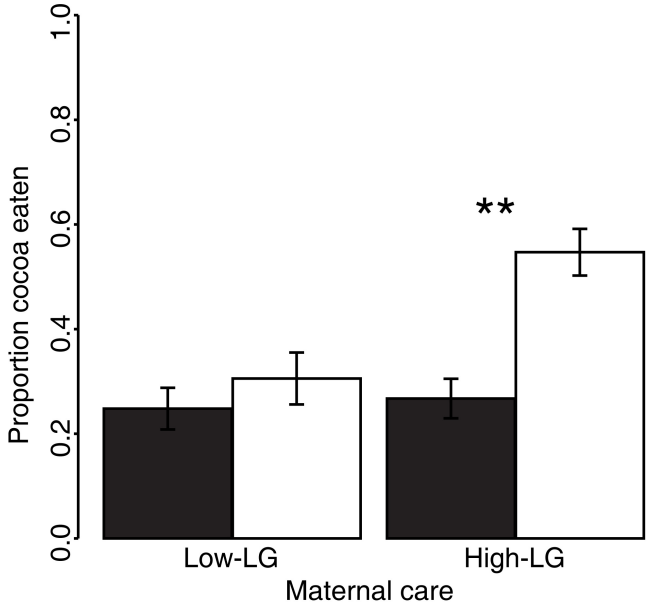

Figure 1. Mean proportion (± SE) of cocoa diet eaten by observers after exposure to demonstrators that had eaten either cinnamon (filled bars) or cocoa (open bars). Observers were raised by low-LG dams or high-LG dams. Asterisks indicate improved learning performance of the high-LG offspring ($P < 0.01$).

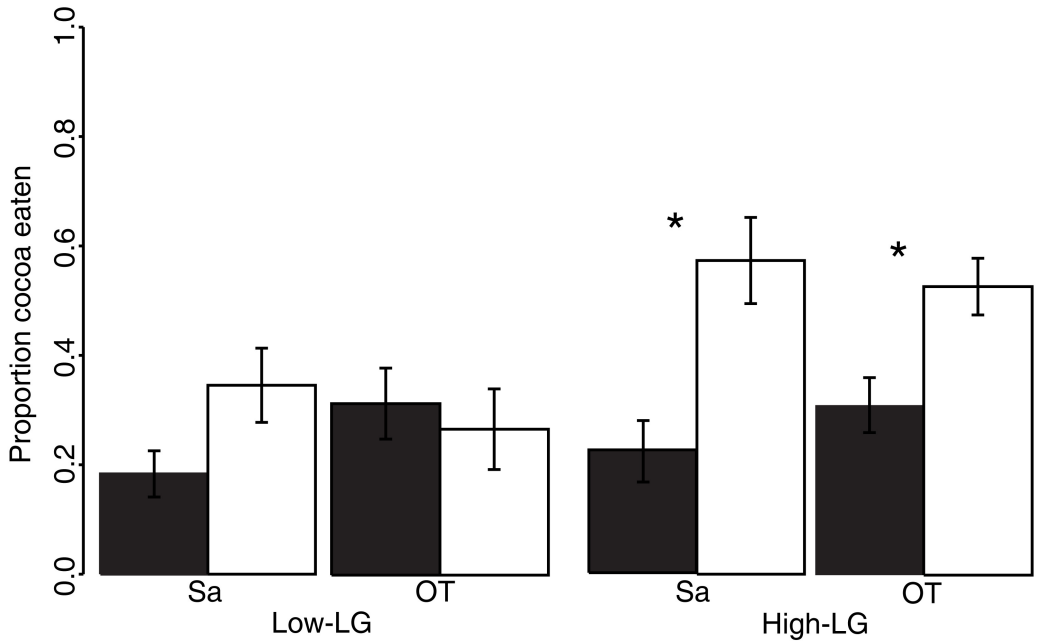

Figure 2. Mean proportion (± SE) of cocoa diet eaten by observers after exposure to demonstrators that had eaten either cinnamon (filled bars) or cocoa (open bars). Observers were administered with saline (Sa) or oxytocin (OT) 15 min. prior to interaction with demonstrators. Observers were raised by low-LG dams or high-LG dams. Asterisks indicate improved learning performance of the high-LG offspring regardless of administration ($P < 0.05$).

Total food consumption

Observers' rate of feeding decreased over time, being most rapid in the first two hours of feeding (mean ± SE = 2.01 ± 0.25 g/hr) and dropping in the second (1.13 ± 0.10 g/hr over 13 hours) and third measurement periods (0.41 ± 0.04 g/hr over 9 hours; repeated-measures ANOVA: $F_{2,76} = 30.34$, $P < 0.0001$). All observers sampled both diets within the 24-hour period, with a mean total consumption (± SE) of 22.41 ± 1.66 g. Oxytocin administration did not significantly affect total consumption (ANOVA: $F_{1,31} = 0.83$, $P = 0.37$), neither did maternal style ($F_{1,31} = 0.01$, $P = 0.91$) or demonstrator diet ($F_{1,31} = 1.84$, $P = 0.19$), nor were there significant interaction effects ($P > 0.1$).

Cocoa-demonstrators ate more than cinnamon-demonstrators (mean consumption ± SE = 11.32 ± 0.60 g, 7.84 ± 0.53 g, respectively, ANOVA: $F_{1,35} = 17.90$, $P < 0.0001$), but demonstrators assigned to high-LG observers did not eat significantly more than those assigned to low-LG observers ($F_{1,35} = 0.02$, $P = 0.89$), and there was not a significant interaction effect between observer maternal style and demonstrator diet on demonstrator food consumption ($F_{1,35} = 0.11$, $P = 0.74$). Moreover, demonstrator diet consumption was not a significant predictor of observers' total food consumption (r = 0.12, $F_{1,37} = 0.52$, $P = 0.48$). Thus

our results cannot be accounted for by differences in feeding behaviour between demonstrators.

DISCUSSION

Rats were biased towards the diet their demonstrator had previously eaten, showing social learning of food preferences for a novel diet, a well-established social learning effect (Galef 2002). However, only adult rats that had experienced one style of maternal care (high rates of licking and grooming, 'high-LG') utilized social information from demonstrators. High-LG offspring socially learned a food preference, whereas there was no evidence for such learning in low-LG offspring. This correlation between early developmental experience and social learning performance supports the idea that early maternal care could be a causal factor shaping adult social learning propensities. We found no significant effects of oxytocin on social learning.

Our finding that low-LG offspring show impaired social learning contrasts with the prevalent argument that risk-sensitive individuals are more likely to utilize social information (Coolen et al. 2003; Mathot & Giraldeau 2008). However, and in line with our findings, predation risk decreased social learning of food preferences in rats (Galef & Whiskin 2006). Our results emphasize that social learning is a multi-step process, and individual differences could arise during acquisition, retention, and/or performance. Moreover, the gathering of social information may be confounded or conjoined with other activities that show individual differences, such as vigilance. Since offspring of high and low-LG dams differ on numerous characteristics (Champagne et al. 2003; Champagne & Meaney 2007), we cannot yet identify the causal factor explaining the differences we observe.

Variation in maternal care influences a number of brain regions and neurological systems, including several implicated in rodent food preference social learning such as the hippocampus (Champagne et al. 2003; Choleris et al. 2009; Curley et al. 2011). For example, hippocampal synaptic plasticity and cholinergic innervation is reduced in adult low-LG offspring compared to high-LG offspring, characteristics that have been linked to reduced learning and memory performance (Diorio & Meaney 2007). Disrupted or low maternal care not only impairs HPA axis function, but also increases corticosterone sensitivity and thus stress sensitivity (Caldji et al. 1998). Such influences of maternal care on the brain could underlie the differences in social learning propensities we observed.

Differences in social interaction patterns between high and low-LG offspring could be an additional or alternative explanation for our results,

since interaction patterns may determine exposure to social information. For example, Norway rats learn more effectively from unfamiliar than familiar demonstrators, probably because they interact more with unfamiliar demonstrators (Galef & Whiskin 2008). In contrast, in Mongolian gerbils *Meriones unguiculatus* unfamiliar individuals evoke high aggression and anxiety and observers will not acquire food preferences from unfamiliar demonstrators unless treated with an anxiolytic (Choleris et al. 1998). Adult high-LG offspring show diminished stress responses and reduced anxiety compared to adult low-LG offspring (Champagne et al. 2003), potentially promoting learning during social interaction with the unfamiliar demonstrator. Juvenile male offspring of low-LG mothers show increased rates of aggressive play fighting compared to high-LG male offspring (Parent & Meaney 2008). Similar aggression maintained to adulthood might hinder social learning.

We speculate that high-LG offspring are developmentally adapted to more predictable environments, compared to low-LG offspring, with social learning thus providing greater payoffs (Boyd & Richerson 1985). However, evidence for such an adaptive programming hypothesis would require understanding of both the developmental mechanisms influencing social learning, and the adaptive payoffs of strategies in different environments. It has been suggested that rodent food preference learning may be an adaptive specialization (Hoppitt & Laland 2008), and theoretical simulations suggest such a system would evolve if toxic foods are generally lethal, and thus interactions with sick conspecifics are rare (Noble et al. 2001). Rats are peculiarly insensitive to the contingencies surrounding food preference learning, supporting this adaptive specialization hypothesis (Galef et al. 1999). It is possible that where the costs of assessing the value of social information are high, developmental influences provide a low-cost mechanism to adopt behaviour suited to the likely future environment.

Oxytocin administration had no significant effects on social learning. We observed no significant interaction effects between oxytocin administration and maternal style on any measure. However, no interaction effects were predicted, since in males, unlike females, oxytocin receptor expression in three brain areas was not found to differ between high-LG and low-LG offspring (Francis et al. 2002). We used a dose established to improve retention in socially acquired flavoured tea preferences in male rats (Popik & Van Ree 1993). However, a vasopressin metabolite has been shown to increase or decrease retention of socially acquired food preferences dependent on the retention interval (Bunsey & Strupp 1990; Strupp et al. 1990), while oxytocin has a U-shaped reaction curve (Popik et al. 1992; Klenerova et al. 2009) and can attenuate or potentiate a range of processes depending on dosage. Given

that we did not vary dosage but used a single dose and retention interval, no conclusions can be drawn on the efficacy of oxytocin as a modifier of social learning.

Maternal care influences development in many species. This raises the possibility that developmental influences on social learning will be widespread in vertebrates, representing both adaptive optimizing to local conditions and byproducts of general developmental effects. An open question is the extent to which developmental influences on social information use have fixed effects throughout life, or whether recent experience or other factors can override these effects. Negative effects on social information use caused by isolation rearing were reversed by housing the artificially-reared rat pups with dams and pups for days 20-24 of life (Galef 1981; social learning was not tested). Similarly, phenotypic differences in maternal and exploratory behaviour between high and low-LG offspring can be abolished by manipulation of post-weaning environment (Champagne & Meaney 2007). However, if animals choose habitats and foraging niches partially based on their social learning propensities, this may maintain or even strengthen differences between individuals.

Given the importance of developmental influences (West-Eberhard 2003), early experience may have numerous direct and indirect effects on social information use and social learning, with interactions between genetic predispositions, development, and learning likely. Social learning plays a critical role in human development (Hermann et al. 2007), so early influences on attention to social cues and learning from these cues could be vital in shaping human social and cognitive development. Moreover, deficits in appropriate social learning are relevant to a number of mental disorders (Olsson & Phelps 2007). Thus, understanding of early influences on social learning has applied relevance to developmental and clinical psychology and psychiatry.

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Chapter 6

GENERAL DISCUSSION

The neurobehavioural studies described in this thesis concerned three categories of social behaviour: social learning, social memory and social grouping (shoaling). In a social learning experiment we demonstrated that naïve zebrafish learned from knowledgeable conspecifics to escape in a novel predator escape task (Chapter 2). The identification of social learning in zebrafish led to the investigation of whether zebrafish also have the ability to remember individual conspecifics (Chapter 3), since familiarity influences social learning processes in other species. The results suggest however that the formation of a social memory based on familiarity is not an important aspect of zebrafish social behaviour, or at least familiarity preferences are not readily expressed in our experimental setting. To investigate the regulatory role of nonapeptides on sociality, we studied zebrafish grouping behaviour after single injections of vasotocin, isotocin or their putative antagonists (Chapter 4). Vasotocin was found to decrease subjects' interaction with conspecifics, while their shoaling tendency remained unchanged. Moreover, the putative vasotocin antagonist decreased shoaling tendency and interaction with conspecifics. These findings support the hypothesized role of the vasotocin peptide family as a regulator for 'anti-social' behaviour and indicates a role of nonapeptides in zebrafish grouping. Together with the established correlation between neuro-anatomical vasotocin expression differences and social status (Larson et al. 2006) and recently established nonapeptide effects on the propensity to preferentially associate with fish of similar phenotype (Braida et al. in press), this provides further evidence for a role of nonapeptides in the regulation of sociality in zebrafish. To investigate the effects of developmental influences and oxytocin administration on social learning propensities we used the extensively studied maternal care system in Norway rats in combination with the social transmission of food preferences paradigm (Chapter 5). Here, we found that adult offspring that had received more intense maternal care socially learned a food preference but adults that had received less frequent maternal care did not. Oxytocin did not significantly affect social learning performance. Our findings link social experiences early in life with propensities to use social information in adulthood. Thus, this thesis expands knowledge on social behaviour and its underlying neural regulation and developmental influences shaping social learning in particular.

The remainder of this chapter will address the implications of the main findings presented in this thesis for *i)* The understanding of the evolution of social behaviour and its neural mechanisms, *ii)* The use of zebrafish in behavioural research and *iii)* The translational value of zebrafish findings in increasing our knowledge of human psychological disorders related to social deficits.

Evolution of nonapeptide regulation of social behaviour

Extensive research on the vasopressin/oxytocin superfamily has shown that the structure, neuronal distribution and functions of these nonapeptides are highly conserved across vertebrates. Likewise, in invertebrates, nonapeptides with amino acid sequences similar to the vertebrate vasopressin/oxytocin superfamily have been identified in hydrozoa, ringworms, insects and molluscs; and play similar roles. Research suggests that the vasopressin-related invertebrate nonapeptides regulate water balance, as in vertebrates, and the oxytocin-related invertebrate nonapeptides have been linked to socio-sexual behaviours as well, again a similarity with vertebrates (Reich 1992; Van Kesteren et al. 1992; Acher 1993; Salzet et al. 1993; Satake et al. 1999). For these reasons, the invertebrate nonapeptides have been proposed as precursor hormones of the vertebrate vasopressin/oxytocin superfamily (Insel & Young 2000).

The identification of invertebrate nonapeptides that are structurally similar to the vertebrate vasopressin/oxytocin superfamily in different taxa suggests that parts of the nonapeptide systems are highly conserved throughout evolution. Examples of vasopressin-like peptides identified in invertebrates include lysine conopressin in leeches (*Erpobdella octoculata*) and in several species of molluscs, which has 56 percent sequence homology to both vasopressin and oxytocin (Van Kesteren et al. 1992; Salzet et al. 1993), or arginine conopressin found in several other mollusc species that differs at only 2 amino acid positions from lysine conopressin and from vasotocin (Cruz et al. 1987). Finally, a diuretic hormone identified in locusts, *Locusta migratoria*, shows 78 percent homology with vasotocin (Proux et al. 1987). Examples of invertebrate oxytocin-like peptides include cephalotocin in *Octopus vulgaris* with 78 percent sequence homology to mesotocin, isotocin and vasopressin (Reich 1992) or annetocin in the earthworm (*Eisenia foetida*) that shows 42 percent sequence homology to lysine conopressin, 37 - 40 percent sequence homology to vasotocin and isotocin, and 46 percent homology to vasopressin and oxytocin (Takahama et al. 1998; Satake et al. 1999). Investigation of the freshwater hydra (*Hydra attenuate*), a Cnidariad with a structurally simple nervous system in comparison to mammals, has led to the identification of an oxytocin/vasopressin-like peptide in its nervous system (Grimmelikhuijzen et al. 1982; Acher 1993). In sum, the discovery of oxytocin/vasopressin-like peptides in invertebrates suggests that there was potentially one precursor nonapeptide system to the vertebrate oxytocin and vasopressin families present in the common ancestors of vertebrates and invertebrates.

Peripherally, invertebrate vasopressin-related nonapeptides have been shown to regulate water balance in leeches, locusts and molluscs;

similar to its vertebrate homolog vasopressin/vasotocin (Proux et al. 1987; Van Kesteren et al. 1992; Acher 1993; Salzet et al. 1993). Centrally, invertebrate oxytocin-related hormones have also been linked to social or reproductive behaviours. For example, natural variations in a neuropeptide receptor gene regulated solitary versus social foraging behaviour in the nematode *Caenorhabditis elegans* (De Bono & Bargmann 1998), the nonapeptide system in snails has been shown to project to reproductive organs (Van Kesteren et al. 1992) and annetocin induced egg-laying behaviour in earthworms and leeches (Oumi et al. 1996). In addition to these social and reproductive behaviours, the nonapeptide systems have also been linked to individual learning and memory in both invertebrates and vertebrates. Learning about the (social) environment and the formation of relevant social memories potentially influences future interactions with conspecifics. Vasopressin 1a receptor differences in the posterior cingulate cortex, a brain region that is connected to the hippocampus and thalamus that both are important for spatial memory, are associated with individual differences in space use and paternity in the monogamous male prairie vole (Phelps 2010). Males with high V1a receptor abundance showed restricted space use and partner fidelity. In contrast, males with low V1a receptor abundance showed increased space use and low partner fidelity. Increased use of space is likely to increase encounter rates with conspecifics, while improved learned associations between locations and aggressive individuals may decrease encounter rates. Also, mice lacking the oxytocin gene failed to develop a memory for familiar conspecifics (Ferguson et al. 2000). In conclusion, besides the structural conservation, peripheral and central functions of the nonapeptides also appear conserved across evolution and thus support the idea that the vasopressin/oxytocin systems are important regulators for social behaviour.

Even though research has shown that nonapeptide-related hormones are important regulators of social behaviour, considerable variation in the direction of its modulatory effects has been found. Additionally, research suggests that there are specific neuron and receptor populations that are responsible for particular types of social behaviour and not others. For example, polygamous meadow vole males with a monogamous prairie vole vasopressin receptor transgene (V1aR) in the forebrain showed increased mate bonding behaviour but paternal care was not affected (Lim et al. 2004). Also, vasotocin influenced mate competition aggression in a territorial songbird but the level of aggression related to other agonistic behaviours remained unchanged (Goodson et al. 2009a). Thus this suggests that discrete neural pathways exist across the multiple domains of sociality (Insel & Young 2000; Keverne & Curley 2004; Chapter 4).

Research has clearly established that neuro-anatomical receptor distribution differences are responsible for variation in behavioural responses between species or individuals. For example, similar correlations between receptor distributions and mate fidelity have been found in different taxa (Keverne & Curley 2004). Such differences may be influenced by social structure, and social or reproductive status (Young et al. 1997; Bester-Meredith et al. 1999; Goodson & Bass 2000a). For example, in teleosts and songbirds, vasotocin affects territoriality and aggression related to courtship dependent upon a male's dominant-subordinate status (e.g. Semsar et al. 2001; Goodson et al. 2009a). Vasotocin increased aggression in subordinate (or less aggressive) males but did not affect or reduced aggression in dominant (or highly aggressive) males. Seasonal changes in behavioural responsiveness to vasotocin have been associated with hindbrain vasotocin receptor distribution differences in the goldfish depending on reproductive status (Walton et al. 2010). Moreover, neuro-anatomical receptor distribution differences can also arise during development (for example in rats, Chapter 5) or in response to physiological fluctuations in hormone concentrations. For example, the nonapeptide systems have been shown to interact with sex steroid hormones, the dopaminergic and glucocorticoid systems (Goodson & Bass 2001). Steroid sensitivity of nonapeptide neurons has been shown in Japanese quail, *Coturnix japonica*, for instance. Eggs treated with estrogens produced male offspring with female-typical vasotocin levels, but blocking estrogen synthesis caused male-typical vasotocin levels in females (Panzica et al. 1998). Oxytocin has been shown to influence dopamine release during maternal care in female rats (Shahrokh et al. 2010) and oxytocin receptor densities in the nucleus accumbens (the brain area that mediates dopamine release) are higher in monogamous compared to promiscuous voles (Young et al. 2011). These findings suggest the dopaminergic area, already known to be important for motivation and reward related behaviours, is linked to the nonapeptide systems and involved in the maintenance and establishment of social bonds between adults and/or offspring (Young et al. 2011). Finally, the vasopressin system has been linked to stress responses as well. For example, rodents showed reduced anxiety responses in a novel environment after vasopressin administration (e.g. Appenrodt et al. 1998). Considering these findings, the nonapeptide systems, or parts of the systems, potentially regulate social behaviours via its interactions with reward/motivation related hormones and stress hormones.

Taken together, this suggests that (parts of) the nonapeptide systems are plastic which allows for flexible phenotypes depending on social context and environment, thus facilitating evolutionary responses

under certain conditions (West-Eberhard 2003). This phenotypic flexibility would allow animals to inhabit new social niches, which opens up new selection pressures. Appropriate responses to the (social) environment are likely to result in relative high fitness benefits. For instance, the formation of a pair bond will increase reproductive success or the display of reduced aggression to a highly aggressive conspecific to avoid a conflict will increase survival chances as a result. Thus, when the nonapeptide systems interact with sex/stress/reward-related hormones it allows for the modulation of particular aspects of behaviour which could drive the display of appropriate responses and therefore it is likely that interactions between these systems will be maintained or developed further. As a result, and if flexibility is costly, this will favour evolution of the nonapeptide systems. Future work should address questions that aim to understand factors contributing to receptor distribution differences and how they result in functional differentiation, but also investigate how the nonapeptide systems' interact with steroid hormones, dopamine and glucocorticoids potentially directing social behaviour. Greater understanding of the development of these interactions will allow identification of potentially one general mechanism that underlies social behaviour or rather identification of modular mechanisms that underlie different aspects of social behaviour. Also, it could provide a basis to make predictions about the direction of the effects of the nonapeptide systems. Such knowledge could offer more insight into the evolution of the vasopressin/oxytocin systems.

In the remainder of this section I propose a hypothesis for the evolution of the role of the nonapeptide systems in regulating sociality based on the literature reviewed and on the present results. Research from invertebrate species to vertebrate species strongly suggests that the nonapeptide systems are part of a conserved neural mechanism for reproductive behaviour. The invertebrate system typically consisted of a single vasopressin/oxytocin related nonapeptide. I propose that the nonapeptide system initially evolved for the modulation of primary reproductive behaviours in invertebrates through connections to reproductive organs. Possibly due to these connections to the reproductive circuit, nonapeptide interactions with the steroid hormones, testosterone and estrogens, arose. As a result, parts of the system changed and developed further. In vertebrates, a similar neural mechanism remained responsible for reproductive behaviours, however, it split into two systems, the vasotocin/vasopressin and isotocin/oxytocin family. This split could have arisen due to chance or it offered an evolutionary advantage, such as increased developmental flexibility. More importantly, particularly maternal care became crucial for the offspring's survival in many mammalian species and some species of birds and fish (Rosenblatt

2003). Therefore, it has been argued that the formation of a social bond between the mother and her offspring became important (Lim & Young 2006). Maternal care could thus also have driven the split of the two systems, with the oxytocin system modulating mother-offspring attachment. Alternatively, it cannot be ruled out that maternal care was already in place and further adapted after the split or it developed after the nonapeptide family split. However this alternative hypothesis seems unlikely as parental care in invertebrates is extremely rare (Reynolds et al. 2002). Additionally, the ability to discriminate kin from others (kin recognition) would be an important aspect in the development of social bonds, potentially evolving from or in parallel to maternal-offspring recognition. A recent finding that for the first time identified separate invertebrate oxytocin- and vasopressin-like neural systems in a cephalopod mollusc would support the idea that the two nonapeptide systems could have evolved several times (Bardou et al. 2009). Additionally, social interactions, including copulation, have been shown to release nonapeptides and these interact with the dopaminergic system (Goodson 2008). This possibly led to the reinforcement of social bonds, including the formation of partner preferences and bi-parental care, because of its link to reward and motivation on the one hand and its fitness benefits to parents and/or offspring on the other hand (Young et al. 1998; Neumann 2009; Van Anders et al. 2011). Fundamental to social bonding is the formation of a memory for conspecifics and research has shown that the vasopressin and oxytocin systems are vital in memory retrieval processes and thus important for social recognition (Ferguson et al. 2000).

A parallel line of research has focussed on the influence of steroid hormones, in particular testosterone, and how these function in the modulation of male aggression displayed during competition or courtship (Gleason et al. 2009). In a recent review, findings from the testosterone and nonapeptide literature have been considered together to establish their joint evolutionary significance in the establishment of social bonds (Van Anders et al. 2011). These authors proposed a framework to address the different contributions of testosterone and the oxytocin/vasopressin systems in the formation of social bonds. Broadly, the formation of a social bond depends on a balance between two extremes of the sociality spectrum; aggression (‘anti-social’) and affiliation (‘pro-social’) towards conspecifics. Depending on the context (e.g. reproductive or agonistic behaviour) balancing the appropriate amount of aggression and affiliation would lead to adaptive behaviour. First, higher levels of testosterone increase the amount of aggression, which is favourable for when male intruders are encountered but not when interacting with a potential female mate. Increased aggression in order to protect the mating partner or

offspring, however, will be beneficial for both parents and offspring assuming protective aggression in both contexts are linked (Sih et al. 2003). Only in the reproductive aggression context vasopressin levels would increase and therefore acts reinforcing on the maintenance of the pair bond (Van Anders et al. 2011). Second, social interactions between potential partners and offspring increase oxytocin levels and thus facilitate affiliative behaviours. Only in the courtship context testosterone levels would increase but not during father-offspring interactions (Gleason et al. 2009). Thus interactions between testosterone and the nonapeptide systems allows for context-specific modulation of pair bonding.

To summarize, the nonapeptide system could potentially have evolved from a neural circuit that initially developed to modulate fundamental reproductive behaviours and regulate water balance. This system then split into two neural mechanisms and evolved interactions with other hormones, which potentially increased behavioural flexibility. It allowed for the regulation of different types of social behaviour via distinct activation patterns and/or nonapeptides (in combination with steroid hormones) (Santangelo & Bass 2006), forming intricate neural circuits underlying social dynamics but also regulating memory processes and stress responses. For example, the hypothesized system of steroid-nonapeptide interactions would allow for dynamic regulation within the domains of affiliation and aggression (Van Anders et al. 2011). Across species, depending on an individuals' status and the behavioural context, we could make general predictions on the effect of nonapeptide administrations. For instance in monogamous and territorial male fish species, blocking of the vasotocin/isotocin system will reduce partner affiliation and initial aggression towards conspecifics (Oldfield & Hofmann 2011). Similarly, an interaction between glucocorticoids and nonapeptides could result in dynamic modulation of social behaviour via regulation of social approach or reduced aggression for instance. Further studies that address the dynamics of simple social behaviours, such as shoaling in Chapter 4, and its relation to nonapeptide brain systems' structure, ecology and life history across a wide range of taxa will increase our understanding of the evolution of this neural system and will potentially allow for broader generalizations on its behavioural influences.

Zebrafish as a model in behavioural neuroscience

Over the last decade, knowledge of zebrafish behaviour has expanded substantially increasing their importance as a model organism in human studies. In stress research, the zebrafish has been shown to show similar anxiety behaviours to rodents (Steenbergen et al. 2011b; Steenbergen et

al. 2011a). Other recent behavioural studies established that zebrafish grouping develops over time (Buske & Gerlai 2011) and that females can discriminate between sexes (Hutter et al. 2011). Because of a better understanding of zebrafish behaviour, it has been suggested that zebrafish, because of our extensive knowledge on its genetics and development and its practical advantages (discussed in Chapter 1), provide a useful vertebrate model to study neural correlates of behaviour and thus complements present research on classic rodent models (Champagne et al. 2010). For example, improved understanding of zebrafish's behaviour in the wild such as shoaling and association preferences will aid in understanding the dynamics of their social behaviour. Such knowledge could help explain why we find evidence for social learning (Chapter 2) but a lack of evidence for the formation of a social memory for familiar conspecifics (Chapter 3) in zebrafish. Additionally, it potentially provides novel and naturalistic behavioural paradigms, which could be used to study the neural mechanisms underlying social behaviours such as social learning or kin recognition. For instance, preferences for kin over non-kin conspecifics have been demonstrated in juvenile but not adult zebrafish (Gerlach & Lysiak 2006). Another study suggests that there is a sensitive period for the development of kin recognition in zebrafish (Gerlach et al. 2008). The zebrafish system would thus provide a novel system for study of the neural mechanisms of filial imprinting and sensitive periods, an important realm of study that has in the past focused on domestic chicks (*Gallus gallus domesticus*) (Bolhuis 1991; Bischof 2007; Town 2011). Our administration study on social grouping in zebrafish (Chapter 4) supports a regulatory role for the vasotocin system in their social behaviour. Additionally, our findings suggest that there are (at least) two systems rather than one system for the regulation of 'sociality' in teleosts as previously thought.

Besides the great potential to use zebrafish and their similarities to humans (discussed in Chapter 1), there are also some potential disadvantages to use this system to understand neural mechanisms of social behaviour. First, zebrafish are evolutionary more distant from humans than mammalian systems and thus translating findings might be more challenging. Second, understanding neuronal organization in vertebrates is more complicated than in invertebrates (Fetcho & Liu 1998). Third, the zebrafish genome underwent an additional duplication event about 300 million years ago, after mammal lineages diverged (Woods et al. 2000; Taylor et al. 2003). Zebrafish belong to the class of ray-finned teleosts (Actinopterygii) that diverged from the lobe-finned teleosts (Sarcopterygii) about 416 million years ago. This means that many ray-finned teleosts (including guppies, goldfish, sticklebacks and

cichlids) have polyploid genes and do not have diploid genes as in mammals (Taylor et al. 2003). The presence of more gene copies could potentially increase mutation rates and thus zebrafish research might reveal novel genes or novel gene functions. However, despite these disadvantages, the use of the zebrafish system in the study of neuronal circuits important for behaviour will provide a useful and complementary model system.

Additionally, social and ecological factors have shown to greatly influence neuro-anatomical development in fish. Teleosts show extremely high levels of neurogenesis throughout their lives in contrast to mammalian brain neurogenesis that is more restricted to early life (Birse et al. 1980; Zupanc & Sirbulescu 2011). On the one hand this could be considered a disadvantage, differentiating fish from mammals. On the other hand, it could be considered advantageous, allowing ready study of a phenomenon more challenging to study in other systems. For example, socially isolated zebrafish reared in enriched conditions for one week already showed increased forebrain cell proliferation compared to fish reared in barren conditions (Von Krogh et al. 2010). Additionally, zebrafish reared in enriched environments demonstrated increased spatial cognition (Spence et al. 2011). In another teleost species, social isolation reduced dendritic spines and branching and thus synaptic plasticity (Coss & Globus 1979). Such neural plasticity could thus relate to behavioural functions including individuals' behavioural plasticity (Kotrschal & Taborsky 2010). Thus because of the high brain plasticity observed in teleosts, studies in fish will facilitate understanding of developmental influences on brain morphology and correlated behavioural differences.

To fully unravel the neural mechanisms underlying sociality and their evolution, it is important that future research identifies nonapeptide neurons, receptors and their binding specificity in the (zebra)fish brain. For example, until recently, only one vasotocin receptor type (V1) was identified in teleosts (Mahlman et al. 1994). However, as would be predicted from the high conservation of the nonapeptide systems across taxa, other receptor subtypes (similar to V1a, V1b, V2 and OT in mammals and birds) have now been identified in a teleost species as well (Lema 2010). These findings thus provide further evidence for an evolutionary conserved neural mechanism with a vasotocin/vasopressin and isotocin/oxytocin system. Increasing knowledge on the nonapeptide systems in teleosts will facilitate the development of target-specific neural manipulations and it will help interpretation of and increase predictability of behavioural responses. Consequently, this will increase our understanding of the underlying neural mechanism of sociality.

Translational value from this research

The influence of the nonapeptide superfamily on behaviour and cognition has been studied in humans as well. Studies investigating the neural mechanisms that facilitate or inhibit social interactions may be promising in understanding human neuropsychiatric disorders, particularly those associated with social deficits or anxiety, such as autism and phobias (Olsson & Phelps 2007; Insel 2010; MacDonald & MacDonald 2010). While identification of brain regions that are active during social information processing is relatively easily done with neuro-imaging studies, investigation of more mechanistic aspects of social behaviour in humans is challenging. Thus far studies have focused on studying variations in receptor gene expression and on effects of manipulation studies via intranasal nonapeptide administration on sociality (Ebstein et al. 2009; Insel 2010). Oxytocin has been shown to influence a range of social behaviours (Striepens et al. 2011) including trust (Kosfeld et al. 2005), empathy (Bartz et al. 2010), altruism (De Dreu et al. 2010), social memory (Guastella et al. 2008b), processing of emotions (Di Simplicio et al. 2009), gaze following (Guastella et al. 2008a) and social anxiety (Scantamburlo et al. 2007). Evidence for vasopressin influencing human social behaviour is scarce however, even though people with diabetes insipidus that lack vasopressin represent an ideal natural population to study exactly this. The findings on oxytocin suggest a general 'pro-social' role, though controversial (Bartz et al. 2011), in human social behaviour as well.

Clinical studies in humans with autism spectrum disorders (e.g. impaired at social interaction and communication, limited and repetitive activities) have suggested that intranasal oxytocin treatment improves social deficits (Striepens et al. 2011). For example, patients that received oxytocin improved discrimination of and increased interaction with cooperative partners in a ball tossing game and enhanced visual screening of faces improving eye fixation in particular (Andari et al. 2010). Another study showed a reduction in the number and type of repetitive behaviours following oxytocin administrations (Hollander et al. 2003). The findings on oxytocin in human studies suggest this direction of research might be promising for clinical applications in the future.

Translating findings from non-human studies on the neural mechanism underlying sociality is a promising avenue of research that potentially could result in an effective medical treatment (e.g. pharmacological agents) that reduces social deficits or anxiety related behaviours in the future (Andari et al. 2010; Meyer-Lindenberg et al. 2011). The research reported in this thesis is a first step to uncover the neural mechanisms of sociality in the zebrafish. In particular the findings reported in Chapter 4 provide further evidence for a role for nonapeptides

in the regulation of social behaviour. This is in agreement with work in other laboratories that has demonstrated administration of nonapeptides directs social behaviours such as aggression and mate bonding in a predictable manner across species and that brain receptor distribution differences consistently correlate to differences along the 'sociality' scale between closely related species (see Chapter 1 and 4). Use of the zebrafish potentially offers new opportunities to study and insights on vertebrate social behaviour. For example, zebrafish are easier to use in testing group behaviours than mice. Thus using zebrafish to study social behaviours in particular could lead to more fundamental knowledge on social behaviour and its nonapeptide influences on behaviour in vertebrates.

However, translating findings from the zebrafish to humans needs careful consideration of species differences within their phylogenetic framework (Moore & Lowry 1998). For example, neuro-endocrine patterns that might indicate functional behavioural differentiation could be shaped by species' social behaviour or by the environment. Understanding of factors that shaped receptor differences in the brain and establishment of correlations between receptor distribution and behaviour will direct new hypotheses to investigate experimentally. For instance, it could allow prediction of species differences or prediction of the effects of early social experience on the development of social deficits in humans. This would increase our knowledge on nonapeptide influences on behaviour, which ultimately could bring us closer to developing potential treatments for human social deficits. Finally, increased understanding of species' differences in 'sociality' and its underlying nonapeptide influences could also be informative in understanding whether and how potentially different mechanisms underlie similar behaviours (Bolhuis & Macphail 2001; De Kort & Clayton 2006). Based on the nonapeptides' role in important behavioural functions (e.g. reproduction) in both invertebrates and vertebrates, apart from the high similarity of molecular structures and peripheral functions, this suggests that the oxytocin and vasopressin systems are conserved structures rather than being reinvented multiple times as a result of convergent evolution.

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Curriculum Vitae

I, Charlotte Lindeyer, was born on March 12th, 1984 in Ooststellingwerf, The Netherlands. In 1996 I attended the Menso Alting College in Hoogeveen and finished Gymnasium in 2002. My interest in animals was particularly inspired by my passion for diving, which lead me to study Biology at Leiden University in that same year. During my studies I performed undergraduate projects with the Animal Ecology group in Leiden. For my Bachelors, the project I worked on was on the evolution of female mate choice in Lake Victoria cichlids under the supervision of Dr. Inke van der Sluijs in 2004 - 2005. After obtaining my BSc. degree, I started my Masters on Evolutionary and Ecological Sciences at Leiden in 2005. After having spent six months running experiments in a basement, I was lucky enough to extend my work on my BSc. project and travel to Tanzania for field work. Afterwards, I joined Dr. Marcel Haesler from the Behavioural Ecology group at the University of Bern, Switzerland, in Zambia to help with his project on reproductive parasitism in a lekking Lake Tanganyika cichlid species. For the completion of my Master thesis I spent six months in the Azores and studied cetacean behaviour in the wild. With this data I mapped the residency patterns of bottlenose dolphins off the South Coast of Pico under the supervision of Dr. Tom van Dooren in 2006 - 2007. After my graduation I started the PhD project reported here on social behaviour and neural mechanisms in zebrafish at the Behavioural Biology group at Utrecht University in 2008, under the supervision of Dr. Simon Reader and promoted by Professor Johan Bolhuis.

List of Publications

C. M. Lindeyer, M. J. Meaney & S. M. Reader. In press. Early maternal care predicts reliance on social learning about food in adult rats. *Developmental Psychobiology*.

Haesler, M. P., **Lindeyer**, **C. M.**, Otti, O., Bonfils, D., Heg, D. & Taborsky, M. 2011. Female mouthbrooders in control of pre- and postmating sexual selection. *Behavioural Ecology*, **22**, 1033-1041.

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

Haesler, M.P., **Lindeyer**, **C. M.** & Taborsky, M. 2009. Reproductive parasitism: male and female responses to conspecific and heterospecific intrusions at spawning in a mouthbrooding cichlid. *Journal of Fish Biology*, **75**, 1845-1856.

Co-Author Affiliations

Esther M. A. Langen
Behavioural Biology, Department of Biology and Helmholtz Institute, Utrecht University, The Netherlands

Michael J. Meaney
Developmental Neuro-endocrinology Laboratory, Douglas Hospital Research Centre, McGill University, Canada

Simon M. Reader
Behavioural Biology, Department of Biology and Helmholtz Institute, Utrecht University, The Netherlands
and
Department of Biology, McGill University, Canada.

William T. Swaney
Behavioural Biology, Department of Biology and Helmholtz Institute, Utrecht University, The Netherlands.