Shoaling preferences in female zebrafish (*Danio rerio*) and the effects of nonapeptides and environmental enrichment.

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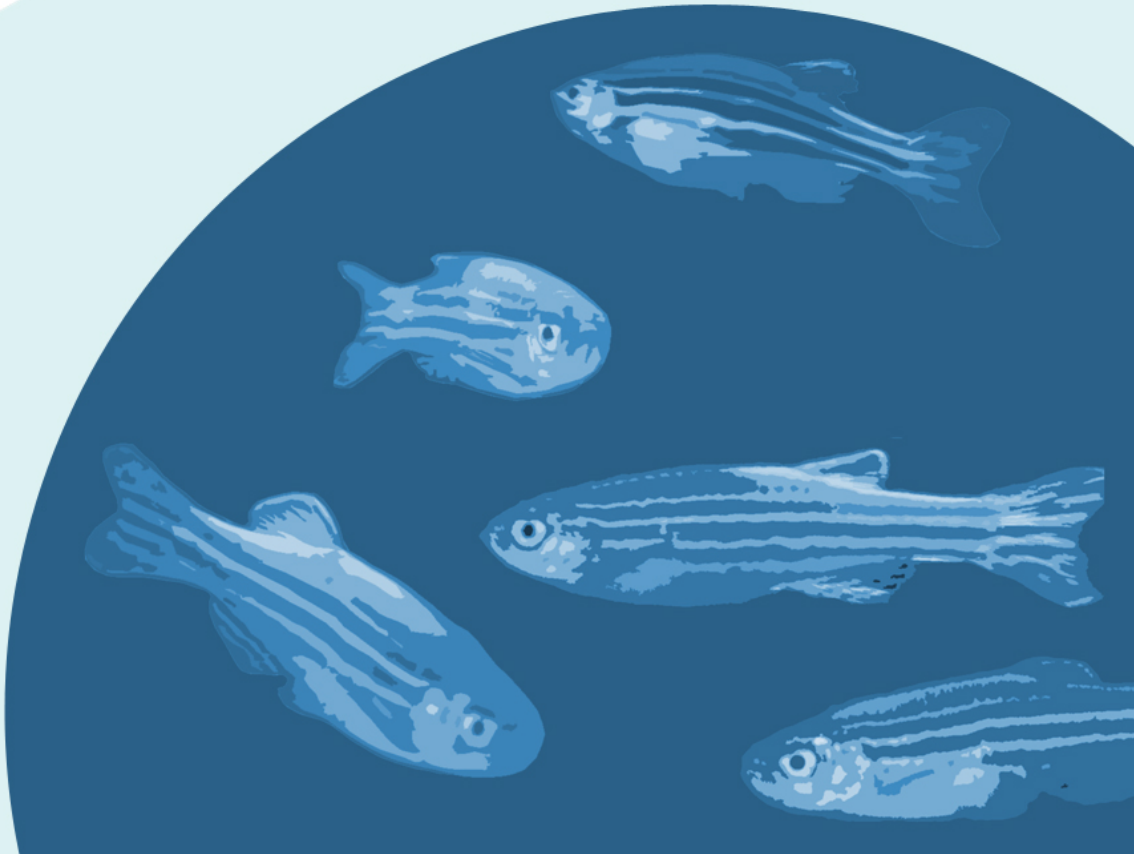

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Summary

To date, relatively few studies have addressed the mechanisms behind sociality or basic social grouping. Here, we attempted to address this empirical gap by examining the mechanisms underlying sociality in zebrafish. We investigated zebrafish shoaling preferences for groups of different sizes under different circumstances and found that zebrafish preferentially associated with larger groups of conspecifics over smaller groups. This was unaffected by social context or how stimulus shoals were presented: fixed on both tank ends, or in a way that simulated more natural shoal movement. Next, we investigated if zebrafish shoaling can be affected by nonapeptides of the isotocin/arginine vasotocin family, which have been shown to affect several social behaviour patterns in many other species. We demonstrated that vasotocin affected shoaling behaviour in zebrafish. Additionally, we investigated the effect of rearing environment – plain vs. enriched - on shoaling preferences and responses to a novel object. We found no significant effects of rearing environment on shoaling behaviour. Exploration of novel objects was also unaffected by rearing environment, though our results suggest that the novel object utilized did not induce novelty responses, compromising interpretation of this result. In conclusion, zebrafish shoaling preferences for larger groups over smaller ones appear to be relatively stable. Our results suggest a role of AVT in the regulation of zebrafish shoaling. Combined with other recent findings, this supports the hypothesis that nonapeptides may play an important role in the regulation of sociality across vertebrates.

Introduction

Social behaviour – interactions between two or more individuals where the behaviour of one individual affects the behaviour of others - is widespread throughout the animal kingdom as well as a very important component of human behaviour (Deag, 1980; Robinson et al., 2008; Ebstein et al., 2010). Studying social behaviour might reveal some of the underlying neuro-endocrine mechanisms, the evolutionary origins and how different factors can be of influence on social behaviour. This knowledge can be particularly useful in medicine, since some psychiatric disorders like autism or social phobias are strongly associated with social deficits (Bartz & Hollander, 2006).
Sociality, or the tendency to form social groups, varies among species and it can strongly influence a species' social structure (e.g. living solitary, in large groups or something in between; Krause & Ruxton, 2002; Reiczigel et al., 2008). Moreover, sociality is likely to influence social learning processes, which typically rely on social transmission of information and consequently can have important effects on numerous other behaviours important for survival, like reproduction, foraging or predator avoidance (Brown & Laland, 2003). Social aggregation inevitably poses certain challenges to individuals within a group. Living in groups means there will be competition for resources - such as food or mates - between group members (Mendl & Held, 2001; Wright et al., 2006). Also, group living increases conspicuousness to predators and susceptibility to diseases (Mendl & Held, 2001; Bolhuis & Giraldeau, 2006). The fact that group living still occurs in some species is because social aggregation also carries benefits. Group living, and the extent to which it occurs depends on cost and benefit tradeoffs. Possible benefits of group living involve anti-predatory advantages. For example, groups have a higher chance of detecting predators, according to the 'many eyes' hypothesis (Krause & Ruxton, 2002; Wright et al., 2006). Also, being in a group can reduce the chances of an individual being caught due to the dilution and the confusion effect (Krause & Ruxton, 2002; Wright et al., 2006). Living in groups can also have foraging advantages, since individuals in groups can easily use information from others to locate food resources. Also, for predator species, group hunting can increase hunting success. Similar to increasing the chances of finding food, forming social aggregations can also increase the chance of finding potential mates. (Mendl & Held, 2001; Krause & Ruxton, 2002; Wright et al., 2006)

Within groups of animals, there will be social interactions and relationships between group members (Mendl & Held, 2001). In the past decades, research has focused on the neuro-endocrine underpinnings of social behaviour and social bonding. These studies have determined a role of the nonapeptides (nine amino acid peptides) oxytocin (OT) and arginine vasopressin (AVP) and their nonmammalian homologues. Oxytocin homologues are isotocin (IT) in fish and mesotocin in birds, amphibians and reptiles. The homologue of arginine vasopressin is arginine vasotocin (AVT), found in fish, birds, amphibians and reptiles (Insel & Young, 2000; Goodson, 2008). All are known for their peripheral functions. Arginine vasopressin and its homologue are involved in osmoregulation, and oxytocin in mammals plays a role in parturition and milk let-down (Bielsky & Young, 2004). Next to these peripheral functions, nonapeptides of the oxytocin/vasopressin family function as neurotransmitters in the brain (Donaldson & Young, 2008). Here, they are known to influence various social behaviours in many vertebrate species. For example, oxytocin and vasopressin mediate pair-bonding in the monogamous prairie vole, *Microtus orchrogaster* (Young et al., 2008; Ross et al., 2009). In female prairie voles, oxytocin administrations induced pair bonding, whereas an oxytocin antagonist inhibited pair bonding (Williams et al., 1994). In males, pair bonding was inhibited by administration of a vasopressin antagonist (Winslow et al., 1993). Mesotocin has been shown to promote flocking behaviour in female zebrafinches, *Taeniopygea guttata*. In a two-choice paradigm, where subjects were able to choose between a group of 2 and a group of 10 same-sex conspecifics, centrally administered mesotocin increased the time spent near the larger group in females. A mesotocin antagonist had the opposite effect, lowering female sociality (Goodson et al., 2009).

Nonapeptide effects on social behaviour are also apparent in some fish species. In male goldfish, *Carrasius auratus*, isotocin and vasotocin affected social approach behaviour. Vasotocin inhibited social approach behaviours, whereas vasotocin receptor antagonists or isotocin stimulated social approach behaviour (Thompson & Walton., 2004). In the blue-headed wrasse, *Thalassoma bifasciatum*, vasotocin influenced courtship and aggressive behaviours (defending a spawning site against sneak-spawning males) in males, though the effects were dependent of male social and reproductive status (Semsar et al., 2001). In a recent study by Braida et al. (in press), isotocin and vasotocin were shown to affect shoaling preferences for groups of either a similar or different phenotype in the zebrafish, *Danio rerio* (in a dosage dependent manner). There have been very few experiments on nonapeptide effects in humans, but here oxytocin appears to increase trust and stimulate social interactions as well (Carter, 1998; Heinrichs et al., 2003; Kosfeld et al., 2005). Since oxytocin and arginine vasopressin and their homologues play a role in a wide range of social behaviours and often have similar effects across taxa, these nonapeptide systems might be part of a conserved underlying neural mechanism that regulates sociality across vertebrates. The studies on zebrafinches by Goodson et al. (2009) and the zebrafish experiment by Braida et al. (in press) already suggested that nonapeptides from the oxytocin/vasopressin family affect social grouping. To date, no studies have focused on how nonapeptides affect social approach behaviour in zebrafish. Here, we would like to make a start in filling this gap in research.

Zebrafish are a tightly shoaling species, shoaling being a very common social grouping phenomenon amongst many fish species. A shoal is defined as a group of individuals being within a distance of 3 - 4 body lengths apart from each other (Pitcher & Parrish, 1993; Morrell et al., 2007). Shoaling can have foraging advantages (Pitcher et al., 1982). Anti-predatory effects are also apparent, and fish that are under predation stress tend to form tight groups (Pitcher & Parrish, 1993; Krause & Godin, 1995; Wright et al., 2006). The zebrafish is a small fish and can easily be kept in large numbers, and their early development can easily be followed since zebrafish larvae are transparent, making it a popular model organism (Briggs, 2002; Key & Devine, 2003). As a result, zebrafish have been used for decades in developmental biology, neurobiology, and genetics (Larson et al., 2006). Research has shown that there are basic homologies at genetic, neural and endocrine levels between zebrafish and mammals, including humans (Larson et al., 2006; Miklosi & Andrew., 2006). Because of its social nature, the zebrafish can be used as a model for social behaviour - social grouping in particular - as well. In combination with the present knowledge on zebrafish development, neurology and genetics, studying zebrafish shoaling behaviour could give some insight on the basic mechanisms behind sociality.
Shoaling in zebrafish is unlearned and starts soon during early development, though the behaviour changes over time (Engeszer et al., 2004; 2007; Spence et al., 2008; Buske & Gerlai, 2011). Young zebrafish shoal in loose aggregations, but shoal cohesion increases with age (Buske & Gerlai, 2011). Zebrafish shoal more tightly when exposed to an alarm substance, a chemical that is released after skin damage in fish (e.g. after attack of a predator) and used to alert neighbouring fish through chemoreception (Speedie & Gerlai, 2008). This suggests that in zebrafish shoaling is an anti-predatory response and can be linked to (anti-predatory) stress. Zebrafish shoaling preferences can be influenced by various factors. One example is the effect of shoal size and activity level: when choosing between two groups of different sizes, individuals spend more time with the larger group (Pritchard et al., 2001). When able to choose between highly active stimulus groups or less active groups, subjects preferred more active groups, even when these groups were smaller than the alternative group. Zebrafish shoaling preferences are sex dependent: female zebrafish always prefer larger groups over smaller ones, whereas male zebrafish prefer to shoal with females over males, regardless of group size (Ruhl & McRobert, 2005). Group size only affected male shoaling preferences when choosing between two groups of males, subjects then preferred the larger group (Ruhl et al., 2009). Nutritional status also affects zebrafish shoaling preferences: food-deprived individuals have a preference for well fed shoals over food-deprived fish, whereas well fed individuals showed no

significant preferences (Krause et al., 1999). Zebrafish shoaling behaviour is strongly affected by an individuals' early social environment. Studies have shown that zebrafish develop visual and olfactory preferences based on early social experiences, which eventually allows them to differentiate between conspecifics, other species, kin and non-kin (Engeszer et al., 2004; Gerlach & Lysiak, 2006; Spence & Smith, 2007; Gerlach et al., 2008; Spence et al., 2008). Individuals that were reared in isolation were unable to distinguish between groups of conspecifics, groups of the closely related pearl danio, *Danio albolineatus* or groups of guppies, *Poecilia reticulata* (Spence et al., 2008). Zebrafish that were cross-reared with pearl danios had a lower preference to shoal with conspecifics and individuals that were reared with stripeless nacre/mitfa mutant conspecifics preferred to shoal with nacre mutants over wild type zebrafish (Engeszer, 2006; Spence et al., 2008). Moreover, in an odour choice experiment by Gerlach & Lysiak (2006), subjects that were reared with their own kin, showed a preference for conspecifics over other species, unfamiliar kin over unfamiliar non-kin and familiar kin over unfamiliar kin. Research showed that this olfactory preference is probably also based on early social experiences (Gerlach & Lysiak, 2006; Gerlach et al., 2008; Spence et al., 2008).

Next to the effects of early social experiences on zebrafish shoaling behaviour, it may be interesting to examine the effects of other factors. This may reveal some potential factors that affect sociality in other vertebrates. An interesting question is whether environmental complexity can affect zebrafish shoaling. There is an extensive literature on the effects of housing conditions on behaviour and physiology in captive animals. In rodents, studies have shown that environmental enrichment can affect different social behaviours, like aggression, affiliation, play behaviour and dominant – subordinate relationships (Haemisch et al., 1994; Van Loo et al., 2002; Marashi et al., 2003; Pietropaolo et al., 2004). Enrichment also affects mean activity, learning ability, exploration and stress responses (Bernstein, 1973; Boehm et al., 1996; Pham et al., 1999; Zimmermann et al., 2001; Benaroya-Milshtein et al., 2004; Görtz et al., 2008). This suggests that structural environment can affect the reliability of behavioural research, not to mention have an effect on animal welfare (Brydges & Braithwaite, 2009).

Behavioural effects of environmental enrichment are most likely a result of neural and biochemical changes occurring in the brain of animals in enriched environments (van Praag et al., 2000). In some fish species, it has been shown that the environment influences brain development. For example, in steelhead trout, *Oncorhynchus mykiss*, the relative size of the cerebellum was larger in individuals that were reared with stones as environmental enrichment than in individuals from a standard rearing tank (Kihslinger & Nevitt, 2006). In addition, individuals reared in a natural river environment had relatively larger brains and had larger relative cerebellar volumes than lab-reared fish, regardless of enrichment treatment (Kihslinger & Nevitt, 2006). Similar effects of rearing environment on brain size have been found in the guppy: guppies reared in captivity have smaller brains than wild-caught individuals, a difference that can even be seen in first generation lab-reared individuals (Burns et al., 2009).

Even though environmental influences on brain development in fish are apparent, very few studies have focused on what these environmental influences mean for fish behaviour, and most work has been done on commercial species. In Atlantic cod (*Gadus morhua*), individuals from an enriched environment showed increased exploratory behaviour in new environments, recovered faster from stress and were more successful foragers when introduced to novel prey, compared to fish from standard, plain, tanks (Braithwaite & Salvanes, 2005). Enrichment also affects shoaling behaviour in this species: fish reared in an enriched environment will shoal when tested in a plain tank, but not enriched test tanks, whereas fish reared in a plain environment will always shoal, regardless of the tank they are tested in (Salvanes et al., 2007). Thus, enrichment appears to induce greater variability in shoaling behaviour in these fish. Research has also shown that rearing environment can affect aggression and dominance in fish, although the effects differ between species. In Coho salmon (*Oncorhynchus kisutch*), conventional hatchery reared individuals dominated over individuals that were reared in a natural stream environment (Rhodes & Quinn, 1998). Contrarily, in steelhead trout,

individuals reared in an enriched environment socially dominated over individuals reared in a conventional, plain, tank, even though individuals from an enriched environment were less aggressive than individuals from plain tanks (Berejikian et al., 2001). In the zebrafish, enrichment has been shown to reduce aggression (Carfagnini et al., 2009). Female zebrafish housed in enriched environments over a 13-16 week period showed reduced aggressive behaviour when compared to female individuals from bare tanks (Carfagnini et al., 2009). Since environmental enrichment influences behaviour – including shoaling behaviour - in many fish species and has already been shown to affect aggressive behaviour in the zebrafish, it might have an effect on zebrafish shoaling behaviour as well. This could in turn point to environmental influences on sociality in general.

In our studies, we investigated zebrafish shoaling preferences for groups of different sizes and some possible factors that may affect such preferences. This information is useful in future experiments, addressing the question whether zebrafish shoaling behaviour is indeed regulated by a conserved mechanism that modulates sociality across vertebrates. We first investigated zebrafish shoaling preferences for groups of different sizes. Two different versions of a two-choice paradigm were used, in which subjects were able to shoal with either a small or a large group of stimulus fish. We used a static setup, presenting the stimulus shoal on two fixed sides of the tank and a dynamic version, presenting the stimulus shoals in moving plastic cylinders. Fish shoals are naturally dynamic and simulating shoal movement may be a more natural way to present subjects with stimulus shoals. Possibly, this will yield different and perhaps more natural responses than a static version of the experiment. Results of both setups were compared after the first experiment to check for differences in shoaling response. This information was used to determine which setup would be used for following experiments. In our second shoaling experiment, we investigated whether social environment, as already being part of a small group, would affect shoaling behaviour.
After examining zebrafish shoaling preferences for small or large stimulus shoals, we performed a new shoaling experiment to test for nonapeptide effects on zebrafish sociality. We tested the effects of isotocin and vasotocin, and their respective antagonists, on zebrafish shoaling behaviour. Subjects were injected intraperitoneally with IT, AVT or their antagonists. There were 2 control groups, one receiving a saline injection, the other group non-injected. Directly after receiving the treatments, subjects were tested in a two-choice paradigm, able to choose between a group of unfamiliar conspecifics and an empty tank end.
In addition, we performed a pilot study to determine whether different rearing environments (enriched vs. standard/plain) resulted in differences in shoaling behaviour later in life, using a similar two-choice paradigm as the ones used in our first experiments, but with some environmental alterations. We also studied the effects of environmental enrichment on zebrafish exploratory behaviour through a novel object investigation experiment. Subjects were presented with a shoal of unfamiliar conspecifics and a novel object, after which their behaviour was observed. If enrichment is able to affect zebrafish shoaling and exploratory behaviour, this may open up new possibilities to investigate basic mechanisms behind such environmental influences.
Predictions are given in a small introduction for each different experiment below, after explanation of some of the general methods.

Experiments

General Methods

Subjects:
All subjects used in our experiments were F2 wild type female zebrafish, all bred in-house in the Biology aquarium at Utrecht University (Kruyt Building, Padualaan 8, 3584CH Utrecht, the Netherlands). F1 fish were purchased from a commercial supplier (Ruisbroek, Maassluis, Netherlands) and descendants of fish originally obtained from Singapore.

Housing:
In all experiments, stimulus fish and experimental fish were housed separately. Tanks were maintained at 26 +/- 1 ˚C and on a 12 hour light:dark cycle with lights on at 8:00 hours and off at 20:00 hours. Housing was enriched with artificial plants, pots to provide shelter and gravel, except for the tanks in the enrichment experiments (description of these housing tanks are given below). On regular days, fish were fed twice: in the morning between 9.00 and 10.00 (flake food; TetraMin, Tetra Ltd., Germany) and in the afternoon between 16.30 and 17.30 (*Daphnia* or bloodworms - *Chironomidae*). On test days, used subjects and stimulus fish were only fed in the afternoon (not before testing). Water quality was checked weekly by measuring nitrites, nitrates and pH levels. Also, tanks were cleaned and about 10% of the water was replaced with fresh copper and chloride-free water every two weeks.

Testing:
To avoid any effects of olfactory cues concentrating in a certain tank area, we placed a pump in the experimental tanks, with in- and outflow in the central compartment, distributing any olfactory substances equally across the tank. Additionally, after each trial, water in the tank was mixed when catching and removing fish from the tank.

For all experiments, the behaviour of the subjects was recorded using Trust Megapixel Pro (Trust international B.V., Dordrecht, The Netherlands) webcams placed in front of the test tanks and AMCAP 9.00 recording software (Microsoft Corp.) and behaviour was scored using JWatcher 1.0 (Macquarie University, Sydney, Australia and UCLA, Los Angeles, USA)

Statistical analysis:
Statistical analysis was conducted using SPSS 16.0.1 and PASW 18.0.0 (SPSS Inc., Chicago, USA). For all experiments, results were first tested for normality using a Kolmogorov–Smirnov test and Ln-transformed when necessary (see appendix). Data that met the normality criteria were analysed using ANOVA and t-tests. Non-parametric data were analysed with generalized linear models (GLMs) and Wilcoxon tests, chi square tests or Mann-Whitney U-tests.

Shoaling preference experiments and the effects of nonapeptides

1. General shoaling experiment and test of setup

Our first experiment focused on investigating basic zebrafish shoaling preferences for either a small group of 2 or a large group of 8 stimulus fish. Two different setups were used, a ‘static’ and a ‘dynamic’ version. The dynamic setup was used to simulate shoal movement, as would be typical in wild populations. Data from both setups were compared to test whether the type of setup affected fish shoaling behaviour. If so, than this information could be used to choose one of the two setups

for follow-up experiments. A setup was considered preferable if subjects responded faster and the preference measures for both stimulus groups were least ambiguous.
In general, we predicted that, as was found in similar experiments by (Pritchard et al., 2001; Ruhl & McRobert, 2005), female zebrafish would prefer to shoal with a larger over a smaller group of same-sex, non-kin conspecifics. Additionally, we expected that subjects would start to shoal faster in the dynamic setup, since stimulus shoals were moving away from them thus forcing them to make a choice faster. We also expected subjects to spend more time shoaling and show stronger preferences for large groups in the dynamic setup, because they needed to stay close to a shoal in order not to 'lose' it.

Methods

Apparatus and procedures:

Procedure A:
The first setup – 'static' setup A – was based on Engeszer et al. (2007). We used an experimental tank (100*50*40 cm, water level 25 cm) divided into three zones: two compartments (10*10 cm) on either side of the tank for stimulus shoals and one central zone in the middle where the subject was released (Fig. 1). Compartments were made with transparent non-perforated plastic barriers, thus preventing the spread of olfactory cues. Subjects were considered to be shoaling when they entered the shoaling zones, which were up to 12 cm from the stimulus shoal (about 3 - 4 body lengths, following Pitcher & Parrish, 1993). Stimulus shoals of two and eight fish were placed in the outer compartments, the side of each shoal chosen randomly, to control for any tank side biases. Subjects were caught randomly from their housing tank and placed in a transparent plastic cylinder in the central compartment, where they were allowed to acclimatize for 2 minutes. After this acclimatization period, the subjects were released by pulling up the cylinder using a string. This way, the experimenter did not have to move towards the tank and disturb the subjects. After release, shoaling behaviour was observed for 10 minutes.

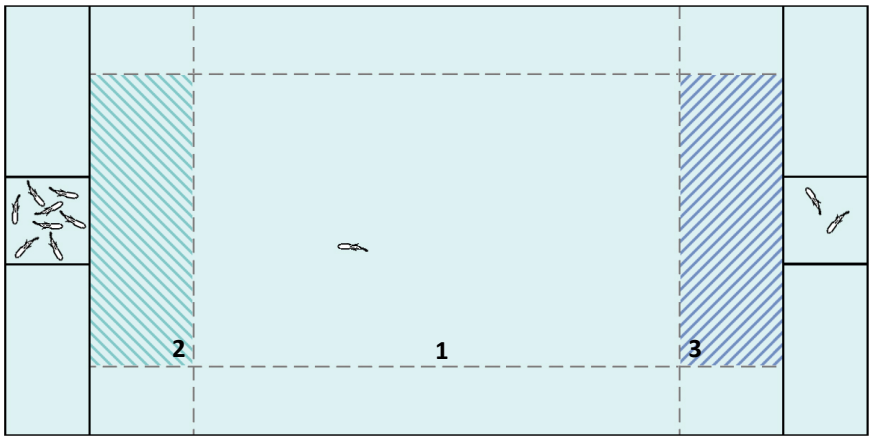

Figure 1. Schematic of the test tank with static setup, setup A, as used in experiment 1 and 4. Plan view. Stimulus shoals are presented at both tank ends behind transparent solid barriers. Dashed lines mark the boundaries of both shoaling zones (**2** and **3**), which were marked by lines on the outside and at the bottom of of the tank, at 12 cm from the stimulus shoals. **1** is the central compartment, in the centre of which the subject is released.

Procedure B:
For the second setup – a 'dynamic' setup - we used a similar setup except for the two fixed outer compartments. Here, the stimulus shoals were presented in two moveable, transparent, cylinders

(Ø12 cm, height 23 cm) close to the centre of the tank initially – and thus close to the subject – and then slowly moved towards the outer sides of the tank in approximately 5 seconds using a pulley system, similar to the setup used by (Lachlan et al. 1998) in a shoaling preference experiment with guppies (Fig. 2). Shoaling zones were again marked at 12 cm from the stimulus shoals. Borders of the shoaling zones were marked on the outside of the tank using plastic straws. These were attached to the cylinders via ropes. As the cylinders were moved towards the outer tank ends, the shoaling zone markings were pulled along (see appendix for picture). All other procedures were identical to Setup A.

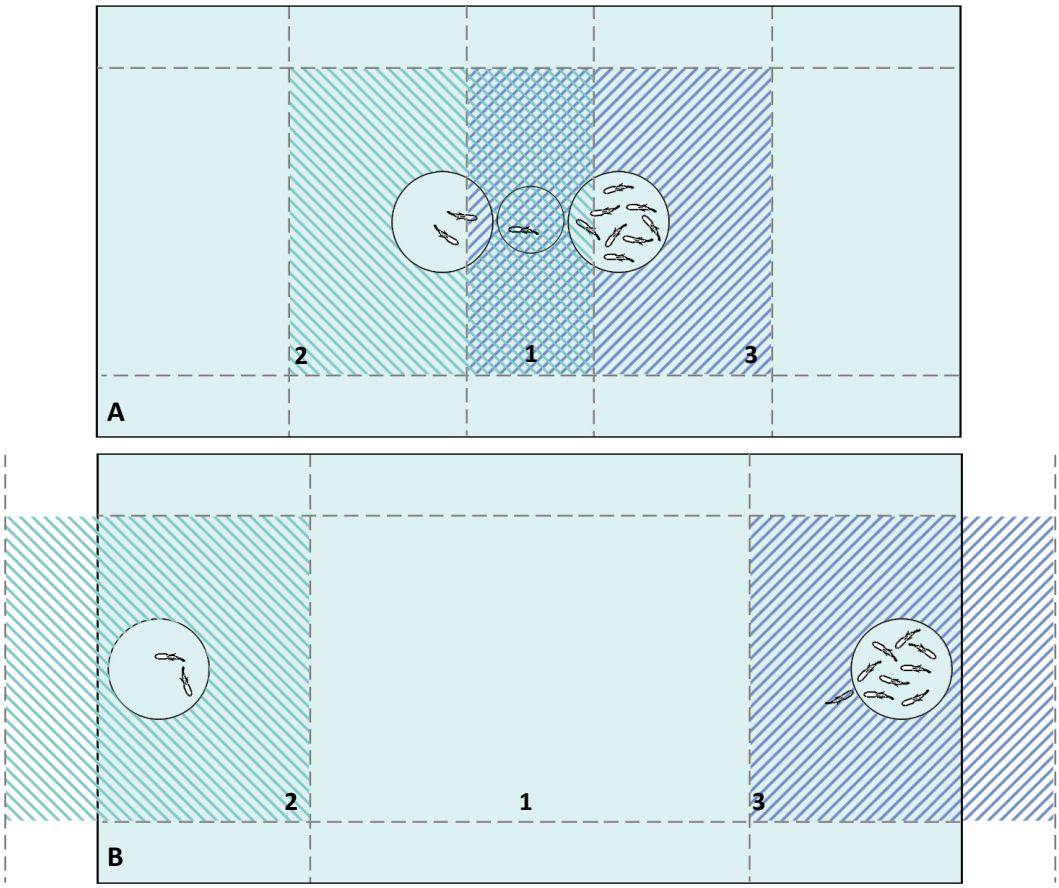

Figure 2. Schematic of the test tank with dynamic setup, setup B, as used in experiment 1. Plan view. Stimulus shoals are presented in two moveable, transparent plastic cylinders. Dashed lines mark the boundaries of both shoaling zones, which were marked by lines on the outside and at the bottom of of the tank, at 12 cm from the stimulus shoals. The diagonal lines mark both shoaling zones in the picture (**2** and **3**). **1** is the central compartment, in which the subject is released. 2A shows the situation at the start of the experiment, where the subject is still acclimatizing in a transparent plastic cylinder and both stimulus shoals are still near the center of the tank. Figure 2B shows the situation after the stimulus shoals have been moved towards the outer ends of

Subjects

A total of 70 naïve F2 wild type female zebrafish (age about 6 months) were used. We used 20 subjects and 50 stimulus fish. No males were used to avoid sexual interactions. Subjects and stimulus fish were housed separately. All subjects were tested twice – once in each setup and counterbalanced for order – with a week of rest between trials. Fish were returned to their housing tanks after trials, but as we were unable to identify individuals, used individuals were separated from naive individuals by a transparent barrier, to prevent testing the same individuals twice in the same

setup. Also, fish that were first tested in setup A were kept separate from fish tested first in setup B. Stimulus fish were caught from a pool of 50 fish. We used different stimulus groups for each trial and all stimulus fish were only used twice a day.

Measurements:

We measured a subjects' first choice (small or large shoal), the number of times a subject 'switched' in and out of the shoaling zones, the total time subjects spent shoaling and the time subjects spent shoaling with each stimulus group separately (quantifying shoaling as being within 3-4 body lengths of a stimulus shoal, i.e. within the shoaling zone).
In order to determine the subjects' shoaling preferences we used a proportional measure, calculating the difference between time spent with the large and time spent with the small shoal in relation to the total time spent shoaling:

$$\text{Shoaling preference} = \frac{\text{time spent with large shoal} - \text{time spent with small shoal}}{\text{total time spent shoaling}}$$

This results in a preference number between -1 (being a 100% preference for the small shoal) and 1 (100% preference for the large shoal).
In addition to the above measurements, subjects and stimulus fish were weighed after each trial in order to see if body mass had any effects on shoaling behaviour. We also measured subjects' latency to begin shoaling in experiment 2. This measure was not taken for experiment 1 because it did not apply for setup B, as the stimulus shoals and thus the shoaling zones were next to the subject at the beginning of the each trial.

Analysis

To determine whether subjects showed a preference for either the large stimulus shoal or the smaller shoal, we performed t-tests on the preference measures in both setups to see if shoaling preferences were higher than 0. To compare shoaling behaviour between the different setups, we used ANOVA's on all behavioural measures except the first choice data. First choice data was examined using a GLM. For all statistical analyses, we included location of the shoals as a random variable and subject weight and the difference between the mean weights of the stimulus shoals as covariates. Weight effects will not be discussed in the results below, since they were never significant (see appendix).

Results

Zebrafish had a significant preference for shoaling with the large shoal over the small shoal in both setups (static setup: one-sample *t* test: $t_{19} = 4.90$, $P < 0.01$; dynamic setup: one-sample *t* test: $t_{19} = 3.47$, $P < 0.01$; Fig. 3A). We did not find significant differences in shoaling preferences between both setups (ANOVA, $F_{1.0.987} = 0.25$, $P = 0.71$), nor between total time spent shoaling (ANOVA, $F_{1.35} = 0.52$, $P = 0.48$), the number of switches between zones (ANOVA, $F_{1.35} = 0.30$, $P = 0.59$). Furthermore, there were no effects of body mass or location of the shoals (see appendix). There was a marginal effect of setup on first choice, though not significant (GLM, Wald $\chi^2_1 = 3.46$, $P = 0.06$). In the static setup, subjects chose to first swim towards the small shoal significantly more often than chance (chi square test: $\chi^2_1 = 7.20$, $P < 0.01$; Fig. 3B). In the dynamic setup, there were no such effects (chi square test: $\chi^2_1 = 0.00$, $P = 1.00$; Fig. 3B).

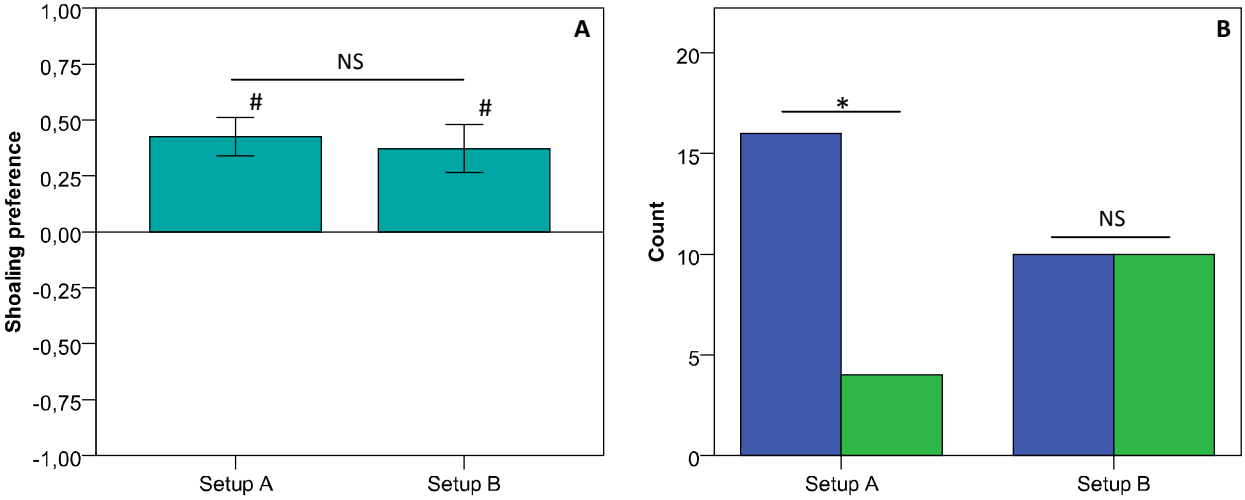

Figure 3. Experiment 1. **A**: mean shoaling preferences ± S.E. per setup. Static setup: mean ± S.E. 0.43 ± 0.09 , dynamic setup: 0.37 ± 0.11. # indicates significant shoaling preference for the large shoal over the smaller one. The difference between setup A and B was not significant (NS). **B**: first choice data per setup, blue bars represent first choice for the small shoal, green bars represent first choice for the large shoal. In setup A, the subjects significantly more often chose the small shoal first (*). In setup B, there was no difference between first choice for either the small or the large shoal (NS).

Discussion

When female zebrafish were given a choice to shoal with either a small (two fish) or a large (eight fish) group of same-sex conspecifics, they spent more time with the larger shoal, following predictions. Furthermore, we found no different results when comparing two different experimental setups; setup A, with the stimulus shoals static on the outer sides of the tank, and setup B, in which the stimulus shoals were moved from the centre of the tank towards the outer ends of the tank – which was supposed to simulate a shoals' natural way of moving. Since we found no preferable setup, we decided to use setup A for our follow up experiments because this was more practical in its use and construction.

We found that a subject's first choice was often not for the shoal that the subject in the end spent most time with, at least in setup A. Here, subjects significantly first chose the small shoal more often than the large shoal, though overall shoaling preference was for the large shoal. In setup B, first choice was random, and thus also not similar to the actual shoaling preference, which was also for the larger shoal. This finding is somewhat surprising, since one would expect a subjects' first choice to be predictive of their overall shoaling preference, but we found no indication of this in our experiments. This suggests that first choice preferences and preferences over a longer time period are not necessarily the same, a point discussed below. For our experiments, the difference in first choice and the overall shoaling preference may be explained by stress effects in the first seconds of a trial. Subjects may be slightly more stressed at the beginning of a trial, since they had just been moved into a new environment. Under the influence of stress, shoaling preferences may be affected: larger shoals may seem more threatening, resulting in an initial 'safer' choice for the smaller shoal. Differences between first choice preferences and overall preferences are smaller in the dynamic setup. This could be a result of the fact that, in this setup, subjects are close to conspecifics form the start of the experiment. Being in proximity to other fish is thought to affect stress levels (Speedie & Gerlai, 2008). If stress levels are lower at the start of our experiments using the dynamic setup, this might have resulted in less 'safe' choices for the smaller shoal.

2. Group shoaling experiment

In our second experiment, we tested whether being presented in an already existing group would change zebrafish shoaling preferences. We expected subjects to have a lower tendency to start shoaling, as they are already part of a small group. However, once they did start shoaling, we expected them to have a stronger preference for the larger shoal than subjects that were tested individually. This is because once one of the fish joins the larger shoal, it will become even larger and thus more attractive.

Methods

Apparatus and procedure:

To test for possible effects of being in an existing group on shoaling preferences, subjects were tested in the same setup as the 'static' setup A from experiment 1, but now in the company of three additional fish (Fig. 4). These three fish – all familiar with each other and the subject - were placed in the tank together with the subject, following the same procedures as described in experiment 1, static setup: in a transparent plastic cylinder, having an acclimatization period of 2 minutes. Testing was again done in trials of 10 minutes, in which the behaviour of the subject was observed. Recognition of the subject was based on its physical appearance (size, stripe pattern, colour etc.).

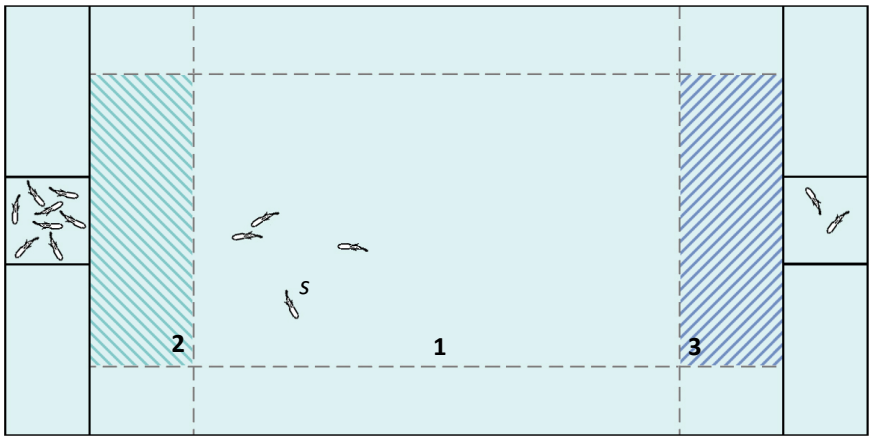

Figure 4. Schematic of the test tank as used in experiment 2. Plan view. Setup is similar to the static setup used in experiment 1, but the subject (*s*) is now accompanied by 3 additional fish. Again, stimulus shoals are presented at both tank ends behind transparent solid barriers. Dashed lines mark the boundaries of both shoaling zones (**2** and **3**), at 12 cm from the stimulus shoals. **1** is the central compartment, in which the subject and companion fish are released.

Subjects:

A total of 130 F2 wild type female (age about 7 months) zebrafish were used. We used 20 subjects and 60 companion fish, all naïve. Stimulus fish were from the same pool of 50 fish used in experiment 1 (now aged about 7 months). Fish were returned to their housing tanks after trials; used fish were kept separated from unused individuals by a transparent barrier. Stimulus fish were again only used twice a day and stimulus shoals were replaced with every trial, similar to the procedures in experiment 1.

Measurements:

We measured a subjects' first choice (small or large shoal), latency to start shoaling, the number of times a subject 'switched' in and out of the shoaling zones, the total time subjects spent shoaling and

the time subjects spent shoaling with each stimulus group separately, after which shoaling preferences were calculated. After each trial, subjects, companion fish and stimulus fish were all weighed. We compared our results with the results of the static setup from experiment 1, where we used the same setup, but with subjects tested alone.

Analysis:

Statistical analysis was similar to experiment 1. *t*-tests were used to check for shoaling preferences and to compare shoaling behaviour between the static setup from experiment 1 and the group shoaling experiment, we used ANOVA's or a GLM when appropriate. Again, weight effects were never significant and will not be discussed further (see appendix).

Results

As in experiment 1, subjects significantly preferred to shoal with the large shoal over the small shoal (one-sample *t* test: t_{19} = 8.72, P < 0.01; Fig. 5A). Being tested individually or in a group did not significantly affect shoaling preferences (ANOVA, $F_{1.35}$ = 1.78, P = 0.19). However, we found a significant overall effect of location of the shoals (location of the small shoal, ANOVA, $F_{1.35}$ = 6.86, P = 0.01). Overall, shoaling preference was higher when the small shoal was on the left, thus the larger shoal on the right (shoaling preferences, mean ± SE: small shoal on the left = 0.68 ± 0.06, n: 18; small shoal on the right: 0.38 ± 0.08, n: 22). This suggests a slight bias for the right side of the tank, though not strong enough to affect shoaling preferences.

We found no effects of subjects being tested either alone or in a group of four on total time spent shoaling (ANOVA, $F_{1.35}$ = 1.20, P = 0.28) and the number of switches in and out of the shoaling zones (ANOVA, $F_{1.35}$ = 0.22, P = 0.64). Latency to start shoaling was marginally higher when in a group (i.e. experiment 2), though the effect was not significant (ANOVA, $F_{1.35}$ = 3.54, P = 0.07; mean ± SE: experiment 2: 6.03 ± 0.74 s, experiment 1: 5.14 ± 1.08 s). We found a significant effect of experiment on first choice (GLM, Wald χ^2_1 = 6.09, P = 0.01). We already showed that there was a significant difference in first choice for the large or the small group in the static setup from experiment 1: subjects significantly more often had a first choice for the small stimulus shoal over the large (chi square test, χ^2_1 = 7.20, P = 0.01; Fig. 5B). In the group shoaling experiment, there were no differences in first choice (chi square test, χ^2_1 = 0.80, P = 0.37; Fig. 5B).

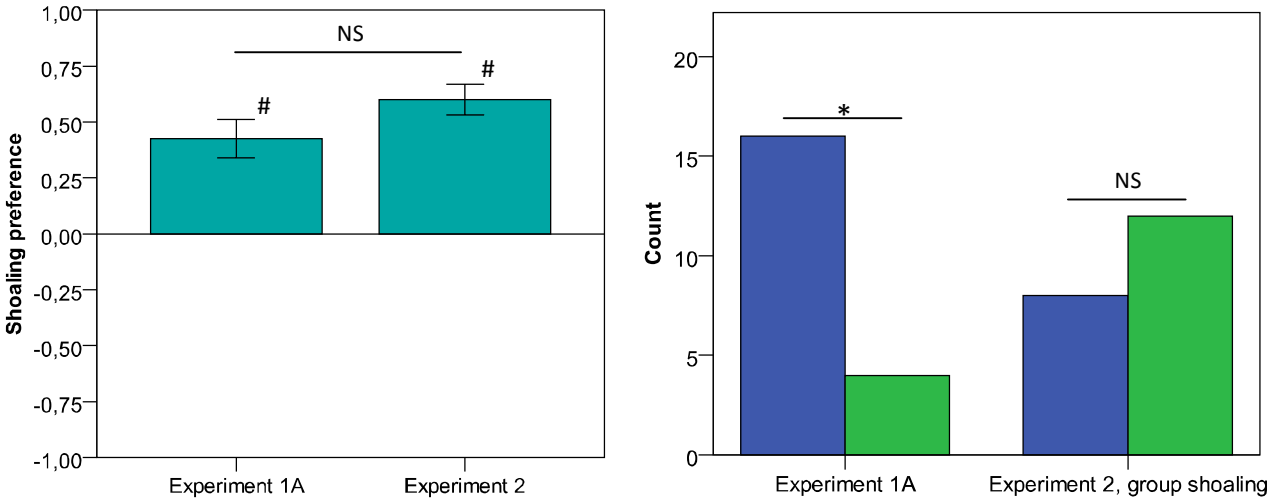

Figure 5. Experiment 2. **A**: mean shoaling preferences ± SE per experiment. Experiment 1A: 0.43 ± 0.09 experiment 2: 0.60 ± 0.07. # indicates mean shoaling preference is significantly higher than 0, meaning there is a preference for the large shoal over the smaller one. The difference between experiment 1A and experiment 2 was not significant (NS). **B**: first choice data per setup, blue bars represent first choice for the small shoal, green bars represent first choice for the large shoal. In experiment 1A, the subjects significantly more often chose the small shoal first (*). In experiment 2, the difference between first choice for either the small or the large shoal was not significant (NS).

Discussion

In our second shoaling preference experiment, subjects were tested in a group with three additional companion fish. We predicted that subjects in a group would take more time to start shoaling, since they are already in a group. Results showed that latency to start shoaling was slightly higher in experiment 2, compared to the standard shoaling preference experiment (experiment 1, static setup), but not significantly so. Our prediction that subjects tested in a group would shoal less with the stimulus shoals – since they are already in a small group – was not met. Subjects spent similar amounts of time shoaling as the subjects from the standard shoaling preference experiment. As in experiment 1, we found that subjects spent more time near the large stimulus shoal compared to the small shoal (shoaling preferences were significantly higher than 0). Comparing these results to the standard shoaling preference experiment, we found that being in a group did not affect a subject's shoaling preference. Subjects tested in groups had similar preferences to subjects tested alone. This is against our prediction that being in a group would increase shoaling preferences – we expected that, if one of the group members would start shoaling with the large shoal, the latter would become even larger and more attractive to the other fish (and to the subject), resulting in a higher preference for the large shoal compared to the standard shoaling preference test. We found no differences in first choice for the small or the large shoal in experiment 2. This is in line with our hypothesis that being close to conspecifics lowers stress and can therefore affect first choice - as discussed in experiment 1.

3. Administration experiment

Since isotocin, vasotocin and their homologues have been shown to affect social behaviour in many different species (Young et al., 2008) we expected these nonapeptides to influence zebrafish sociality as well. Based on the results by Goodson et al. (2009) on the effects of MT and AVT on sociality in zebrafinches, we expected to see increases in sociality after IT administration in our subjects, resulting in an increase in time spent shoaling and interacting. Moreover, we expected an IT antagonist to lower zebrafish sociality. AVT had no significant effects in the zebrafinch-experiment,

but as it has been shown to inhibit social approach behaviour in goldfish (Thompson & Walton, 2004). We expected to see similar effects in our experiment: AVT decreasing shoaling tendencies, resulting in a decrease in the time a subject spent shoaling and interacting. In addition, we expected an AVT-antagonist to result in opposite effects, thus an increase in shoaling tendency.

Methods

Treatments:

To test for nonapeptide effects on zebrafish shoaling behaviour, before being tested, subjects were given intraperitoneal injections of either AVT (Bachem, Weil am Rhein, Germany), V1Ar ($d(CH_2)_5$[Tyr(Me)^2,Dab^5]AVP, a selective vasopressin antagonist and putative AVT antagonist (Gift of Prof. M. Manning), IT (AbD Serotec, Kidlington, UK), OTr (desGly-NH_2,$d(CH_2)_5$[D-Tyr^2,Thr^4]OVT, a selective oxytocin receptor antagonist and putative IT antagonist (Gift of Prof. M. Manning) or saline solution (0.9%). In addition, we included a no injection control group. Injections were given using a 10µl Hamilton syringe and 30G x ½ needles. Nonapeptides and antagonists were dissolved in 0.9% saline. Subjects received a dose of 10 µg/g body mass with injection volumes up to a maximum of 6 µl. Optimal dosages were determined in a pilot study by C.M. Lindeyer. Injection solutions were made from stock solutions every day within 10 minutes from the start of the first trial and kept on ice during experiments. The experimenter was blind to the given treatment when observing and scoring behaviour.

Apparatus and procedure:

We tested for differences in shoaling behaviour between each treatment group using a 150 x 50 x 40 cm tank, water level: 25 cm. As in previous shoaling experiments, we placed shoal compartments of 10 x 10 cm on both ends of the tank. However, we only placed one group of eight stimulus fish on one side of the tank, the other tank end was kept empty. This was done in order to 'force' a clear choice between joining a shoal or not, which is also why we used a bigger tank here than the tanks used in the previous shoaling experiments (experiments 1 and 2). These setup changes also resulted from findings in a previous pilot study by C.M. Lindeyer that had suggested that effects of nonapeptides would be more clearly distinguishable this way. Shoaling zones were marked at 10 cm from the shoal compartments, which was about 3-4 body lengths of the subjects we used (following Pitcher & Parrish, 1993; subjects were slightly smaller than subjects from the previous experiments, due to their younger age). See Fig. 6.

After receiving the treatment injections, subjects were placed in a transparent plastic cylinder in the centre of the test tank and were allowed to acclimatize for 5 minutes. The acclimatization period was slightly longer in this experiment (compared to our previous experiments) to give subjects more time to recover from the injections. After acclimatization, the subjects were again released by pulling up the cylinder using a string, to minimize disturbance. After release, the behaviour of the subjects was observed for 10 minutes.

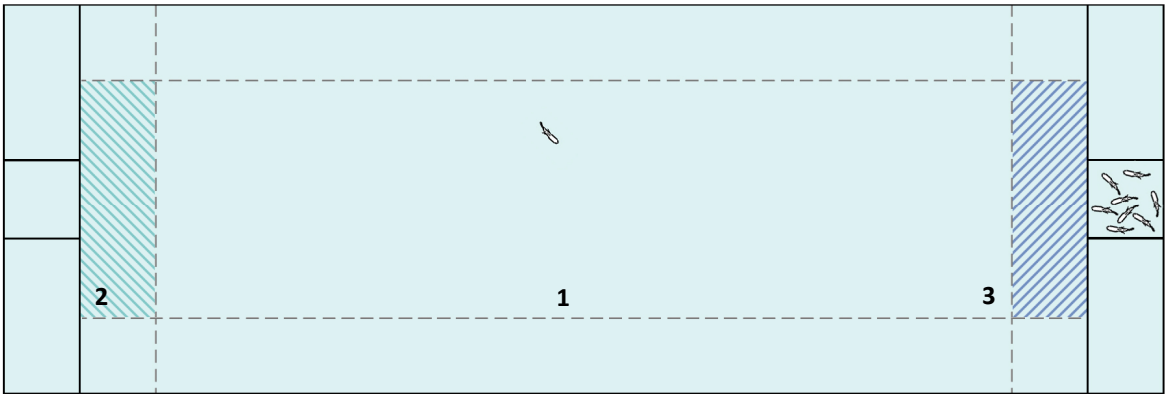

Figure 6. Schematic of the test tank as used in experiment 3. Plan view. The stimulus shoal is presented on one side of the tank behind transparent solid barriers. The other tank end, also lined with transparent solid barriers is kept empty. Dashed lines mark the boundaries of both tank end zones (2 and 3), which were marked by lines on the outside and at the bottom of of the tank, at 10 cm from the stimulus shoals. 1 is the central compartment, in which the subject is released.

Use of subjects:

A total of 170 naïve F2 wild type female zebrafish (4 – 5 months old) were used. We used a total of 150 subjects, 25 per treatment group. Stimulus fish were randomly caught from a pool of 20 fish and replaced after two to three trials. Shoal location was randomized. Testing was done over a two-week period, subjects divided over the two weeks (for each treatment group 12 subjects were tested in week 1, 13 subjects were tested in week 2). Treatment group order was randomly determined.

Measurements:

We measured the time subjects spent within 10 cm of the shoal and within 10 cm of the opposite (empty) tank end. We also measured the latency to enter the shoal zone and the latency to enter the opposite tank end zone. An additional interaction measure was added, where we measured the time subjects spent interacting with the shoal, interaction being quantified here as the subject swimming head first against the partition that separates it from the stimulus shoal. Latency to start interacting was also scored. We used the interaction measure to calculate the percentage of time within 10 cm of the shoal that subjects were actually interacting with the shoal. Stimulus fish were weighed after each trial. Subjects were already weighed before testing, in order to determine the right treatment dose, as was mentioned. We weighed the subjects again after one week to see whether the treatments had any effects on weight gain, which could point to effects on the subjects' general well-being over a longer time.

Analysis:

To check whether subjects showed a (strong) shoaling response we performed Wilcoxon tests on the whole dataset and for each different treatment group separately, comparing time spent near the stimulus and time within 10 cm of the opposite tank end. To compare treatment groups on time spent within 10 cm of the shoal, time spent on the opposite tank end, interaction times, latencies to get within 10 cm of the shoal and latencies to interact, we used GLMs to test for main treatment effects. Also, we made pairwise comparisons between all treatment groups with least significant difference correction. Location of the stimulus shoal were used as a random variable and both the weight of the subject and the mean body mass of the stimulus shoal were used as a covariate in the model, to see if these factors had any effects. These factors will not be discussed in the results below, since we did not find any weight effects, or effects of stimulus shoal location (see appendix). Data from both control groups (non-injected vs. saline injected) were compared in order to determine whether the injections alone had any effects. No significant differences were found (see appendix).

Comparisons between treatment groups and the no injection control group will thus not be discussed further.

Results

Shoaling

Overall, subjects showed a shoaling response, as they spent significantly more time near the shoal than near the empty tank end (Wilcoxon signed-ranks test: W = 24, N = 150, P < 0.01; Fig. 7). Investigating each treatment group separately, we found that only for the V1Ar group, the difference was not significant (Wilcoxon signed-ranks test*:* W = 6, N = 25, P = 0.09; mean time within 10 cm of the shoal ± SE was 111.60 ± 14.14 s; mean time within 10 cm of the opposite tank end ± SE was 70.32 ± 16.62 s. Figures and results are shown in the appendix).

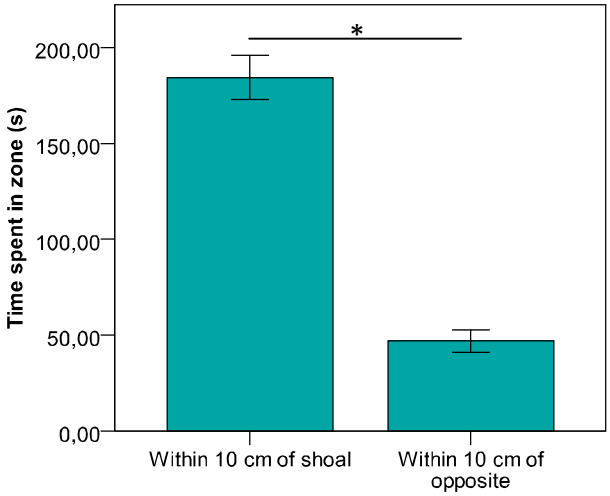

Figure 7. Experiment 3. Mean time subjects spent within 10 cm of the stimulus shoal vs. time spent within 10 cm of the opposite (empty) tank end compartment ± SE. Overall, subjects spent significantly more time near the shoal (*). Time within 10 cm of the shoal ± SE: 184.41 ± 11.37 s. Time within 10 cm of opposite end ± SE: 46.91 ± 5.86 s.

In general, treatments had no effect on time spent within 10 cm of the shoal (GLM, Wald χ^2_5 = 7.78, P = 0.17). However, there was a significant difference between the no injection group and the V1Ar group and between the AVT and V1Ar group (pairwise comparisons, respectively: P = 0.05 and P = 0.02; Fig. 8A): time spent within 10cm of the shoal was lower for V1Ar injected subjects in both cases.

Time spent near the empty tank end

A significant overall treatment effect was found on time spent within 10 cm of the opposite (empty) tank end (GLM, Wald χ^2_5 = 16.85, P = 0.01). The AVT group and the V1Ar group both showed a significant increase in time spent near the opposite tank end, compared to the no injection group (pairwise comparisons, P = 0.01 and P = 0.01, respectively) and also compared to the saline injection group (pairwise comparisons, P = 0.02 and P = 0.02). The AVT treatment group also spent more time near the empty tank end than the OTr group (pairwise comparisons, P = 0.04). See Fig. 8B.

Latency to enter shoaling zone

There was a significant overall effect of treatment on the latency to enter the 10 cm shoal zone (GLM, Wald χ^2_5 = 17.33, P < 0.01). The AVT and the V1Ar both had a significantly higher latency to

enter the 10 cm shoal zone than the no injection group (pairwise comparisons, $P < 0.01$ and $P = 0.01$, respectively). The AVT group also had a higher latency than the saline group (pairwise comparisons, $P = 0.04$). Furthermore, we found that latency to enter the 10 cm shoal zone was also significantly higher in the AVT group when compared to the IT and OTr group (pairwise comparisons, $P = 0.04$ and $P = 0.02$). See Fig. 8C.

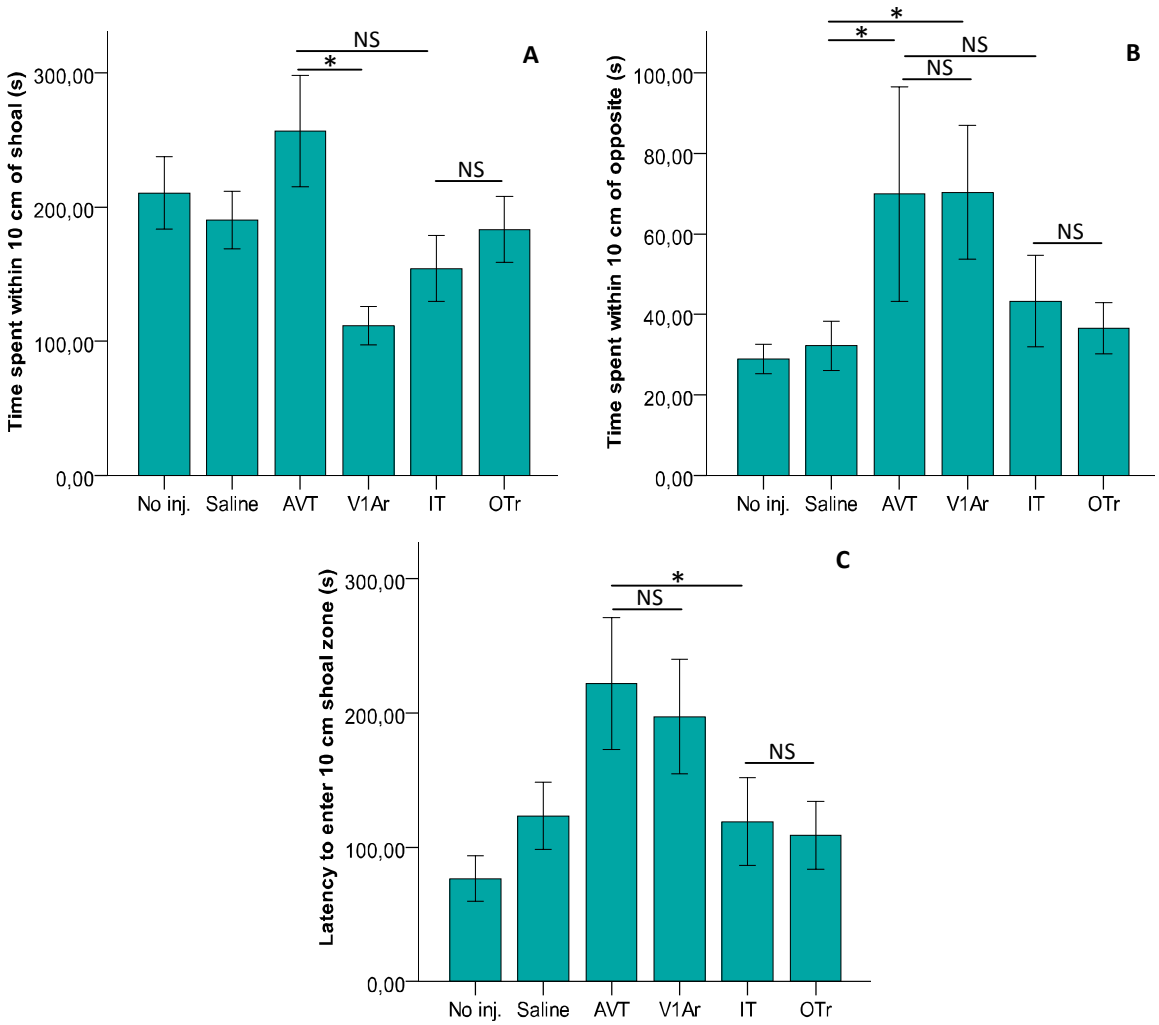

Figure 8. Experiment 3. Data per treatment group: **A**: mean time spent within 10 cm of the stimulus shoal ± S.E. No injection: 210.58 ± 27.05 s; saline: 190.16 ± 21.48 s; AVT: 256.51 ± 41.44 s; V1Ar: 111.61 ± 14.14 s; IT: 154.31 ± 24.38 s; OTr: 183.32 ± 24.61 s. **B**: mean time spent within 10 cm of the opposite tank end compartment ± S.E. no injection: 29.02 ± 3.65 s; saline: 32.25 ± 6.12 s; AVT: 69.90 ± 26.68 s; V1Ar: 70.32 ± 16.62 s; IT: 43.33 ± 11.39 s; OTr: 36.61 ± 6.34 s. **C**: mean latency to enter the shoaling zone (10 cm from the stimulus shoal) ± S.E. No injection: 76.51 ± 17.01 s; saline: 123.39 ± 25.23 s; AVT: 221.90 ± 49.32 s; V1Ar: 197.31 ± 42.56 s; IT: 118.98 ± 38.55 s; OTr: 108.87 ± 25.21 s. * indicate significant differences between treatment groups. NS indicates differences were not significant.

Interaction time

There was a significant effect of treatment on time spent interacting (GLM, Wald $\chi^2_5 = 20.90$, $P < 0.01$). There was a significant difference between the no injection group and the AVT, V1Ar and IT groups (pairwise comparisons, $P < 0.01$, $P = 0.01$ and $P < 0.01$ respectively). Subjects from the saline group spent significantly more time interacting than subjects from the AVT and V1Ar groups (pairwise comparisons, $P = 0.02$ and $P = 0.03$ respectively). The AVT, V1Ar and IT groups were all significantly lower in their interaction time when compared to the OTr group (pairwise comparisons, $P < 0.01$, $P = 0.01$ and $P = 0.03$). See Fig. 9A.

Latency to interact

Overall, treatment had a significant effect on latency to interact with the stimulus shoal (GLM, Wald $\chi^2_5 = 22.04$, $P < 0.01$). Latency to start interacting was significantly higher in the AVT group, compared to the no injection and saline groups (pairwise comparisons, $P < 0.01$ and $P = 0.01$ respectively) and compared to the IT and OTr groups (pairwise comparisons, $P = 0.02$ and $P = 0.01$). Latency to interact was also significantly higher in the V1Ar group compared to the no injection group (pairwise comparisons, $P = 0.05$). See Fig. 9B.

Percentage of time near the shoal subjects actually spent interacting

We found a significant treatment effect on the percentage of time near the shoal (within 10 cm) that subjects spent interacting (GLM, Wald $\chi^2_5 = 44.736$, $P < 0.01$). Subjects from the AVT, V1Ar and IT groups were all significantly less interactive when near the shoal than subjects from the no injection group (pairwise comparisons, respectively: $P < 0.01$, $P = 0.01$ and $P = 0.01$). The AVT and IT treatments also significantly lowered interaction within the 10 cm shoal zone compared to the saline injected group (pairwise comparisons, $P < 0.01$ and $P = 0.05$). Furthermore, the difference between the OTr and the AVT, V1Ar and IT groups was also significant: OTr injected subjects all showed significantly more interaction within 10cm of the shoal (pairwise comparisons, $P < 0.01$, $P = 0.04$ and $P = 0.02$). AVT lowered interaction within 10cm of the shoal compared to all other treatment groups (pairwise comparisons, AVT vs. no injection: $P < 0.01$, AVT vs. saline: $P < 0.01$, AVT vs. V1Ar: $P < 0.01$, AVT vs. IT: $P < 0.01$, AVT vs. OTr: $P < 0.01$). See Fig. 9C.

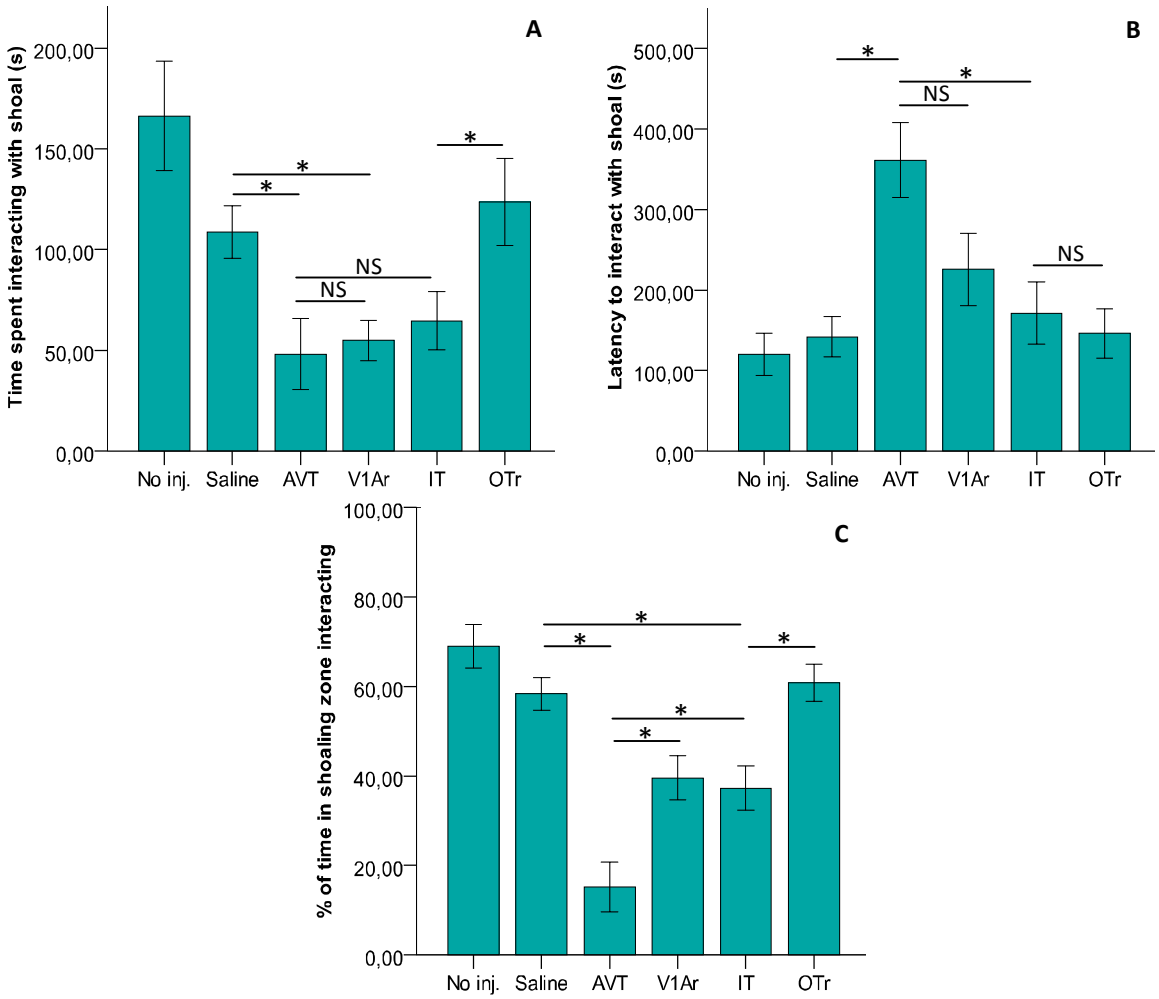

Figure 9. Experiment 3. Data per treatment group: **A**: mean time spent interacting with the stimulus shoal ± S.E. No injection: 166.37 ± 27.29 s; saline: 108.65 ± 12.96 s; AVT: 48.26 ± 17.57 s; V1Ar: 54.86 ± 9.94 s; IT: 64.65 ± 14.42 s; OTr: 123.63 ± 21.64 s. **B**: mean latency to start interacting with the stimulus shoal ± S.E. No injection: 120.17 ± 26.45 s; saline: 141.96 ± 25.16 s; AVT: 361.59 ± 46.81 s; V1Ar: 225.72 ± 44.94 s; IT: 171.49 ± 38.55 s; OTr: 146.23 ± 30.64 s **C**: mean percentage of time within 10 cm of the stimulus shoal that subjects spent interacting ± S.E. No injection: 69.00 ± 4.91 %; saline: 58.34 ± 3.69 %; AVT: 15.25 ± 5.58 %; V1Ar: 39.59 ± 4.96 %; IT: 37.29 ± 4.98 %; OTr: 60.86 ± 4.11 %. * indicate significant differences between treatment groups. NS indicates differences were not significant.

Body mass

All fish were weighed before testing and one week after. There were significant overall differences between subject weight on the day of the experiment and after one week (GLM, Wald $\chi^2_1 = 65.92$, $P < 0.01$). All subjects had significantly gained weight. Weight gain did not significantly differ between treatments (GLM, Wald $\chi^2_5 = 3.41$, $P = 0.64$). See appendix.

Discussion

Overall, we found that subjects prefer to join a group than not, since subjects spent significantly more time near the stimulus shoal than on the empty tank end. Moreover, we found that our subjects' shoaling behaviour was affected by injections with AVT, IT and V1Ar. Our initial predictions, however, were not exactly met. As predicted, we found that AVT injections decreased shoaling tendencies, as subjects showed a decrease in interaction and an increase in both their latency to enter the shoaling zone and the latency to start interacting with the stimulus group. However,

against predictions, no decrease in time spent in the shoaling zone was found. The AVT-antagonist caused a similar decrease in shoaling tendency: the time subjects spent in the shoaling zone and the time they spent interacting with the shoal both significantly decreased, compared to the control groups. In addition, the latency to enter the shoaling zone of AVT-antagonist injected subjects increased. This was against our prediction that the AVT-antagonist would have the opposite effect of AVT-injections, and thus increase shoaling. IT injections only seemed to have significant effects on interaction time, which significantly decreased, compared to the controls. No effects of IT were found on our other measures. Again, our predictions were not met here, since we expected the IT-injections to cause an increase in shoaling tendency, so an increase in time spent near the stimulus shoal and an increase in interaction time. We also expected the latency to enter the shoaling zone and the latency to start interacting to decrease, none of which were found in our experiment. The IT-antagonist never had any significant effects, though we did expect to see a decrease in shoaling here. In both the AVT and the IT-injected groups, differences in shoaling behaviour were not seen when comparing the amounts of time subjects spent near the stimulus shoal, but we did see a change in the time subjects spent interacting with the stimulus group (interaction defined as swimming head first against the partition that separated the subjects from the stimulus group). This may point to the fact that time being close to a group and the time being socially active with a group are two different things, which is important to note, since the two are often seen as one and the same measure.

Enrichment experiments

General information

Rearing:

In order to investigate the effects of environmental enrichment on shoaling preferences and exploration, all fish were reared in controlled environments in the Biology aquarium at Utrecht University. Fish were randomly distributed over four small (40*25*25 cm) rearing tanks when they were 17 days old. Two of these tanks were standard, plain tanks, the other two were ‘enriched’ with approximately 3 cm of gravel, some small rocks, a real and a plastic plant and a piece of a flower pot, to provide shelter (see Fig. 10A and C). Fish were moved into four larger (100*50*40 cm) tanks when they were approximately 1.5 months old (again, two plain tanks, two enriched, see Fig. 10B and D). During the rearing period, the groups were mixed-sex, since we were unable to distinguish males from females in 17-day old fry. However, only females were used for the actual experiments.

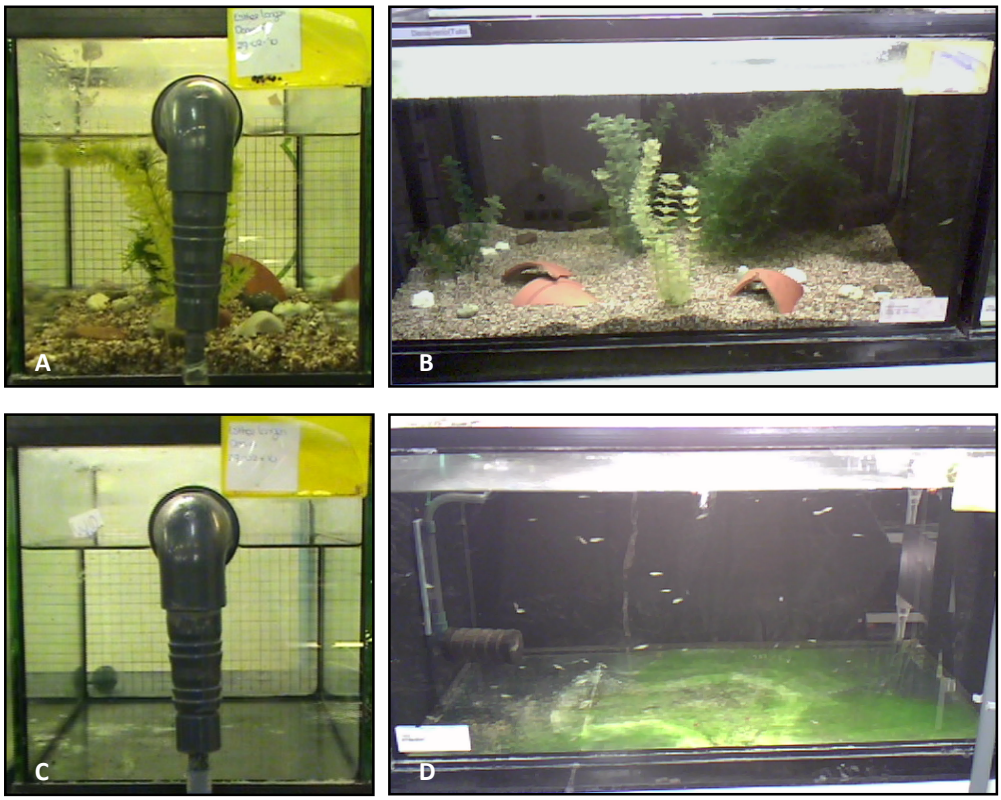

Figure 10. Pictures of the enriched and plain rearing tanks. 5A (plain) and 5C (enriched) show the smaller rearing tanks in which the subjects were housed from 17 days of age until approximately 1.5 months of age. 5B (enriched) and 5D (plain) show the larger tanks subjects were housed in from 1.5 months of age untill they were moved to the lab at approximately 4 months of age. In the lab, subjects were housed in tanks that were similar to the tanks shown in pictures 5B and D.

After a rearing period of approximately 4.5 months, female subjects were caught from their rearing tanks and moved from the Biology aquarium to new housing tanks (again, two plain, two enriched) in our laboratory. Subjects from different tanks were kept separate. After 2 weeks of acclimatization to their new housing tanks, fish were tested for their shoaling preferences (experiment 4) at 5 months of age and subjects' response to a novel object (experiment 5) was tested at approximately 9 months of age. Subjects were weighed after testing and in between both experiments at approximately 8 months of age, to see whether enrichment had any long-term effects on weight gain.

Subjects:

A total of 130 wildtype F2 female zebrafish were used, all tested in both experiment 4 and 5. We used 80 naïve wild type female subjects (age about 5 months in experiment 4 and 9 months in experiment 5), divided into four groups of 20 fish. Two groups were housed in an enriched environment, the other two groups housed in plain tanks, as described above. For stimulus fish, we re-used the same pool of 50 fish used in the experiments 1 and 2 (aged about 7 months in experiment 4 and 11-12 months in experiment 5).

4. Enrichment shoaling experiment

Enrichment has been shown to affect behaviour in many species (Haemisch et al., 1994; Van Loo et al., 2002; Marashi et al., 2003; Brydges & Braithwaite, 2009). In female zebrafish, it has been shown to reduce aggression (Carfagnini et al., 2009), a social interaction that might affect shoaling tendencies as well. Moreover, enrichment may affect stress responses (Benaroya-Milshtein et al.,

2004). Shoaling has been related to stress in zebrafish (Speedie & Gerlai, 2008). Environmental effects on stress levels may then change social behaviour and affect shoaling behaviour. Hence, we expected there to be differences in shoaling behaviour between individuals from enriched environments and subjects from plain tanks. The effects of environmental enrichment on social behaviour varied between fish species in previous experiments (Berejikian et al., 2001; Brown et al., 2003; Salvanes & Braithwaite, 2005; Salvanes et al., 2007), making it difficult to give more exact predictions.

Methods

Apparatus and procedures:

To see if environmental enrichment affected shoaling preferences, subjects were tested in the same setup as setup A from experiment 1. In addition we tested our subjects a second time after approximately one week in an enriched version of this setup, where three plastic plant models and a flower pot were placed in the test tank (Fig. 11), to see whether shoaling was affected differently when the testing environment matched or mismatched rearing and housing environment.

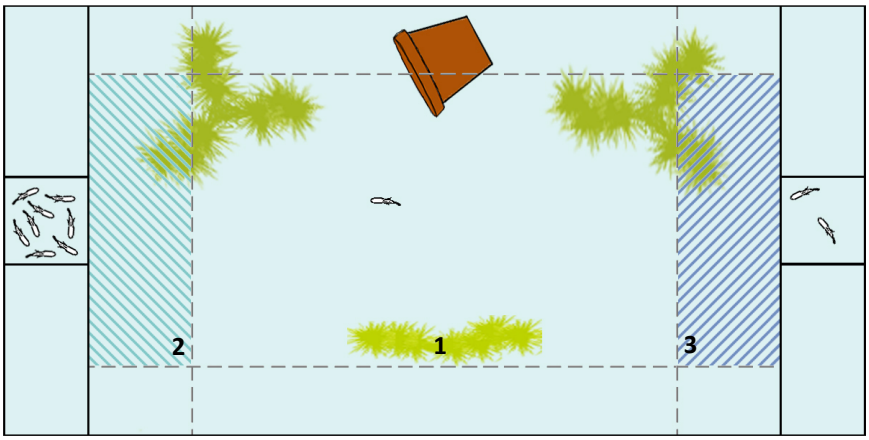

Figure 11. Schematic of the enriched test tank as used in experiment 4. Plan view. The setup is similar to the static setup of experiment 1, which was used as the plain setup in this experiment. In the enriched version, the tank is enriched with 3 plastic plant models and a piece of flower pot to provide shelter. Stimulus shoals are presented at both tank ends behind transparent solid barriers. Dashed lines mark the boundaries of both shoaling zones (**2** and **3**), at 12 cm from the stimulus shoals. **1** is the central compartment, in which the subject is released.

Measurements:

All measures were identical to experiment 1.

Analysis:

To check if subjects showed a preference for the large shoal over the small one (resulting in shoaling preferences higher than 0), *t* tests were performed, except for the data from plain reared subjects tested in an enriched environment. Shoaling preference was not normally distributed here and thus a one-sample Wilcoxon test was used, comparing the median from our data to a hypothesised median of 0. To check for effects of rearing environment on shoaling preferences and total time spent shoaling, we used a GLM similar to the model used in experiment 1 and 2. Significant effects were more thoroughly examined using posthoc *t* tests or Mann-Whitney U-tests.

Results

Shoaling preferences

All subjects preferred to shoal with the large shoal over the smaller one, both plain-reared and enriched individuals, in both testing environments (Plain reared subjects in plain test tank: *t* test,: t_{39} = 2.64, *P* = 0.01; enriched subjects in plain test tank: *t* test: t_{39} = 2.60, *P* = 0.01; plain subjects in enriched test tank: one-sample Wilcoxon test: W_{40} = 767.00, *Z* = 4.80, *P* < 0.01; enriched subjects in enriched test tank: *t* test: t_{39} = 7.46, P < 0.01). See Fig. 12A.

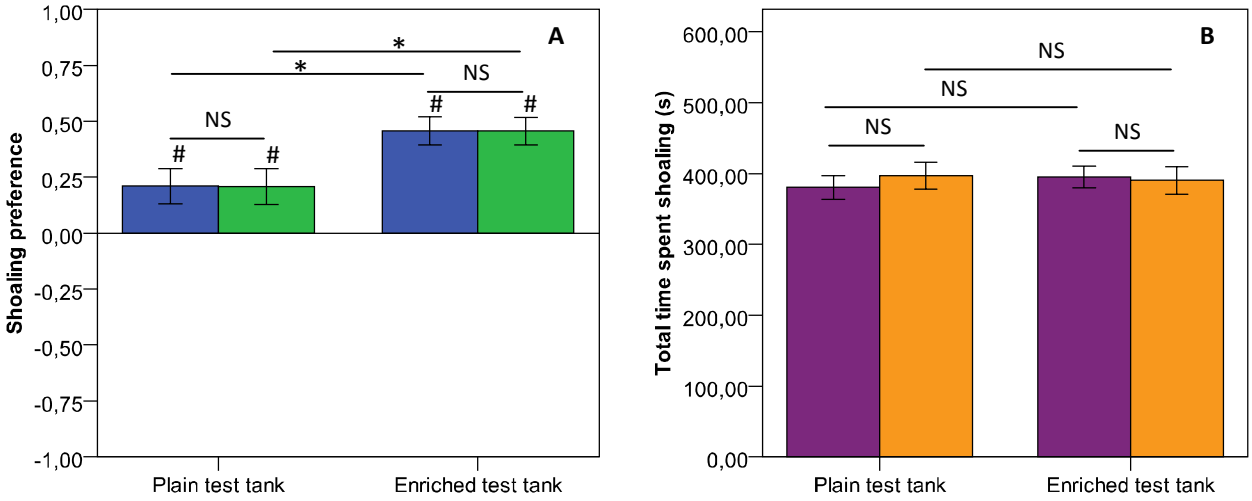

Figure 12. Experiment 4. **A**: Mean shoaling preferences ± S.E. per testing environment. Blue bars represent plain-reared subjects, green bars represent enriched subjects. Plain reared subjects in plain test tank: 0.21 ± 0.08; enriched subjects in plain test tank; plain subjects in enriched test tank: 0.46 ± 0.06; enriched subjects in enriched test tank: 0.46 ± 0.06. # indicates mean shoaling preferences were significantly higher than 0, meaning there is a preference for the large shoal over the smaller one. There was a significant difference in shoaling preferences between the two testing environments (*). There were no differences between different rearing types (NS). **B**: Mean total time subjects spent shoaling ± S.E. per testing environment. Purple bars represent plain-reared subjects, orange bars represent enriched subjects. Plain reared in plain test tank: 380.34 ± 16.65 s; enriched in plain test tank: 397.04 ± 18.90 s; plain-reared in enriched test tank: 394.98 ± 15.06 s; enriched in enriched test tank: 390.27 ± 19.35 s. There were no differences between different rearing types or testing environments (NS).

Rearing environment did not affect shoaling preferences (GLM, Wald χ^2_1 = 0.01, *P* = 0.93). However, we found significant effects of testing environment (GLM, Wald χ^2_1 = 10.44, *P* < 0.01), location of the (small) shoal (GLM, Wald χ^2_1 = 6.53, *P* = 0.01) and the difference in mean weight of the large and the small stimulus shoal (GLM, Wald χ^2_1 = 4.11, *P* = 0.04).

To determine the effect of testing environment, we compared shoaling preferences between testing environments using a Mann-Whitney *U* test. This showed that overall, shoaling preferences are higher in the enriched test tank (*U* = 2244.00, N_1 = N_2 = 80, *P* < 0.01; mean shoaling preference in plain test tank ± SE: 0.21 ± 0.06; mean shoaling preference in enriched test tank ± SE: 0.46 ± 0.04). Investigating at each rearing condition separately, we found that subjects from both rearing conditions showed a higher shoaling preference in the enriched testing environment (plain-reared subjects: Mann-Whitney *U* test: *U* = 562.00, N_1 = N_2 = 40, *P* = 0.02, enriched subjects: Mann-Whitney *U* test: *U* = 568.50, N_1 = N_2 = 40, *P* = 0.03). See Fig. 12A.

Shoaling preferences were generally higher when the small shoal was located on the left side of the tank, and the large shoal on the right (small shoal on the left vs. small shoal on the right. Mean shoaling preferences: 0.43; SE ± 0.05 and 0.24; SE ± 0.05 respectively. Mann-Whitney *U* test: *U* = 2385.50, N_1 = N_2 = 40, *P* = 0.01). This may point to a slightly higher preference of subjects for the right side of the tank over the left side. Furthermore, we found that shoaling preference was generally

higher if the mean weight of the large shoal minus the mean weight of the small shoal was higher, so the heavier fish from the large shoal were, compared to the fish from the small shoal, the more time subjects spent with the large shoal (Spearman's correlation: $r = 0.20$, $N = 160$, $P = 0.01$).

Total time spent shoaling

There were no effects of rearing environment on the total time subjects spent shoaling (GLM, Wald $\chi^2_1 = 0.46$, $P = 0.50$). There were no effects of testing environment (GLM, Wald $\chi^2_1 = 0.12$, $P = 0.73$), nor was there an interaction effect of rearing and testing environment (GLM, Wald $\chi^2_1 = 0.44$, $P = 0.51$). See Fig. 12B. We did find an effect of location of the shoals (GLM, Wald $\chi^2_1 = 10.37$, $P < 0.01$). In general, subjects spent more time shoaling when the small shoal was on the left and the large shoal on the right (mean total time spent shoaling for small shoal on the left vs. small shoal on the right: 417.46 seconds; SE ± 12.38 vs. 363.85 seconds; SE ± 11.61, *t*-test, $t_{158} = 3.16$, $P < 0.01$). So if the large shoal was on the right side of the tank, subjects not only spend a larger proportion of their total shoaling time with the large shoal they also spent more time shoaling in general. Subjects may have a bias for the right side of the tank, however, since shoaling preferences are still above 0 when the large shoal is on the left, it appears that this tank side bias is not strong enough to reverse shoaling preferences.

Switches in and out of the shoaling zones

The tank side bias was also apparent when analyzing the number of times a subject switched in and out of the shoaling zones. Location of the shoals had a significant effect on number of switches. (GLM, Wald $\chi^2_1 = 6.28$, $P = 0.01$). No effects of rearing nor testing environment were found. We found no effects of the mean weights of the shoals on both latency or nr. of switches, but we did see an effect of subject weight on the times a subject switched compartments (GLM, Wald $\chi^2_1 = 9.81$, $P < 0.01$). In general, the higher the weight of a subject, the more switches it made (Spearman's correlation: $r = 0.16$, $P = 0.05$.

Discussion

We tested the effects of environmentally enriched or deprived rearing environments on shoaling preferences. Like experiment 1 and 2, we found that shoaling preference is always for the large stimulus shoal. Rearing environment did not affect shoaling preferences, or any of our other measures (total shoaling time, latency to start shoaling, etc.) which was against our predictions. We tested subjects in the standard shoaling preference test setup, and an additional time in an enriched version of this setup, to see whether this could have any effects. In cod, effects of rearing environment were only apparent in an enriched testing environment (Braithwaite & Salvanes, 2005). Enriching our testing environment might therefore also yield different results for our subjects, compared to the standard setup. Indeed, we found differences in shoaling behaviour between the plain and enriched setup. Shoaling preferences were higher in the enriched setup. Total time spent shoaling, latency and number of switches between compartments was not affected however, and as was said before, rearing environment did not affect results: both plain and enriched subjects had higher shoaling preferences in the enriched setup. The fact that shoaling preferences were higher in the enriched setup may be explained by the fact that larger shoals can easily be seen by the subject and are therefore more attractive. When subjects were tested in the enriched setup, it was also their second time they were tested. Maybe being slightly familiar with the experiment could have had an effect on the outcome.

Subjects tended to spend more time with the large stimulus shoal if the mean weight of this stimulus shoal was higher compared to the mean weight of the small stimulus shoal. This suggests heavier fish may be more attractive shoal partners than lighter fish. Moreover, heavier subjects tended to switch in and out of the shoaling compartments more than lighter subjects, though total shoaling time and

shoaling preferences were not affected by subject weight. Heavier subjects appear to just be slightly more active than lighter subjects.

5. Enrichment novelty experiment

As shown in experiment 4, enrichment appears to have no effect on zebrafish shoaling behaviour. In this experiment, we would like to address the question of whether enrichment can affect exploratory behaviour in zebrafish, as it has been shown to do in other species (Zimmermann et al., 2001; Braithwaite & Salvanes, 2005; Görtz et al., 2008). As subjects from an enriched tank were able to habituate to a more complex environment, they were expected to be more explorative than plain-reared individuals. We expected them to be faster in showing exploration behaviour than plain reared fish and to spend more time exploring the novel object and less time shoaling.

Methods

Apparatus and procedures:

In this experiment, we tested our 80 plain and enriched subjects for differences in tendency to investigate a novel object. Subjects were tested in a large test tank, measuring 150 x 50 x 40 cm, water level 25 cm. Tanks were divided into five compartments by lines on the outside of the tank: two outer end compartments, two zones of 10 cm from the outer end compartments and the testing compartment (Fig. 8). In the centre of the tank, we placed a transparent plastic cylinder (Ø 12cm) in which a small stimulus shoal of four fish was placed. Around the stimulus shoal, we marked a shoaling zone of 12 cm (Fig. 14). In this experiment, subjects were tested in two rounds; in the first round, the tank end zones were kept empty. In the second round, however, we introduced a novel object in one of these outer compartments. We used a yellow and black striped cup as novel object, with coloured, reflective ribbons attached at the top that were moved around slightly by the water flow in the tank, to make the object look more conspicuous (see Fig. 13). The location of the object was reversed for half of the trials to control for possible tank side biases.

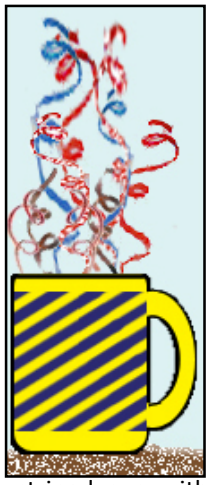

Figure 13. A striped cup with reflective coloured ribbons attached was used as novel object in experiment 5.

Before the experiment started, the subject was placed near the centre of the tank, just in front of the stimulus shoal, in a transparent plastic cylinder (Fig. 14A). It was allowed to acclimatize for 2 minutes. During this acclimatization period, both tank end zones were visually blocked from the subject by a white plastic barrier (Fig. 14A). After acclimatization, the subject was released and the barriers were moved upwards, presenting both empty tank ends simultaneously (Fig. 14B). The baseline behaviour of the subject was then observed for 10 minutes. After this first test round, the white barriers were lowered again to cover both tank ends – provided that the subject was swimming in the central compartment so that it would not be blocked or harmed in any way by the barriers. The subject was given another 2 minutes to acclimatize, during which the novel object was placed in one of the tank end compartments (Fig. 14 C). After the second acclimatization period, the white barriers were removed again, presenting the subject with the novel object (Fig. 14 D). The behaviour of the subject was then observed for another 10 minutes. Release of the subjects and moving the barriers up and down were all done by pulling strings attached to the cylinder and the barriers to minimize disturbances.

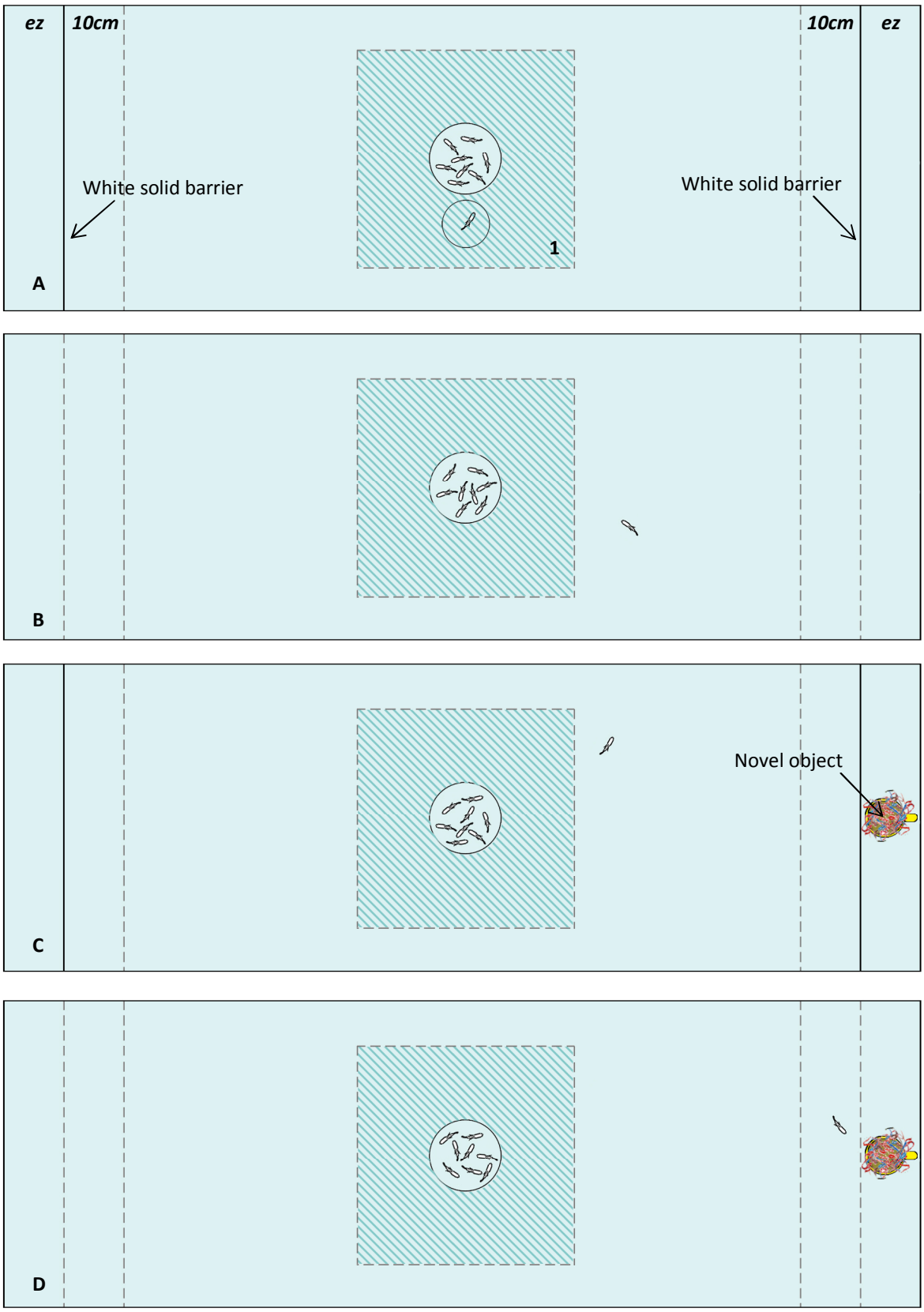

Figure 14. Schematic of the test tank with as used in experiment 5. Dashed lines mark the boundaries of both tank end zones (***ez***) and 10cm zones (***10cm***), and the shoaling zone (**1**) which is also marked by diagonal lining. The stimulus shoal is presented in transparent plastic cylinder fixed in the center of the tank.Before the first test round, the subject is placed in a transparent plastic cylinder in the center of the tank near the stimulus shoal and is allowed to acclimatize. Tank end zones are covered by white plastic barriers (14A). After acclimatization, the subject is released and the tank end zones are reveiled (14B). After 10 minutes, the tank end zones are covered again and the object will be placed on one side of the tank (14C). After another acclimatization period, the white barriers are pulled up again to reveal the object at the start of the second test round (14D).

Measurements:

We measured the time subjects spent shoaling, the time spent in each tank end compartment (object compartment or empty compartment), the time spent in proximity of the end compartments (within 10 cm of each) and the latency to swim into each of the outer end compartments. We also weighed both the subjects and the stimulus fish after each trial. Subjects were also weighed an additional time between experiment 4 and 5, when they were about 8 months old. The data of the three weighing moments were compared to each other to see if enrichment had any effects on the weight of the subjects. During the experiments, we also noted when a subject showed stress responses, such as darting or freezing.

Analysis:

Repeated measures ANOVA's were used to compare measures between the first (no object) and second (object present) test round. Rearing condition, weight of the subjects and mean weights of the shoal were all used as a factor.

Results

Total time spent shoaling

For time spent shoaling, we found no significant differences between the two testing rounds (without object vs. with object): ANOVA: $F_{1.76} = 3.83$, $P > 0.05$. There was, however, a trend towards subjects spending less time shoaling in the second 10 minutes, with the object present (mean time spent shoaling ± SE = 221.04 ± 16.11 s in round 1 and 170.62 ± 17.31 s in round 2; Wilcoxon test: $W_{80} = 18$, $Z = 4.02$, $P < 0.01$). There were no interactions with rearing condition. See Fig. 15. Weights of either the subject or the stimulus shoal did also not interact with test round. Overall, we did see a trend for an effect of the mean weight of the shoal (ANOVA: $F_{1.76} = 3.79$, $P = 0.06$; overall positive correlation (both rounds together): Spearman's correlation: $r = 0.18$, $P = 0.02$), but this effect did not interact with testing round or any other factor.

Time spent within the object zone and on opposite tank end

For the time subjects spent in the object compartment, there were no differences between both testing rounds and no effects of rearing environment. Results did suggest an effect of subject weight, though not significant (ANOVA: $F_{1.76} = 3.79$, $P = 0.06$). The overall correlation was significant: Spearman's correlation: $r = 0.26$, $P < 0.01$, so the heavier subjects were, the more time they tended to spend within the object zone.

For time spent on the opposite (empty) tank end, there were no differences between the two testing rounds or between subjects from different rearing environments. In addition, no effects of weight of the subject nor the shoal were found. The same was true for the time subjects spent within 10 cm of both end zones, the latency of subjects to enter the object zone, and the latency to enter the opposite end zone.

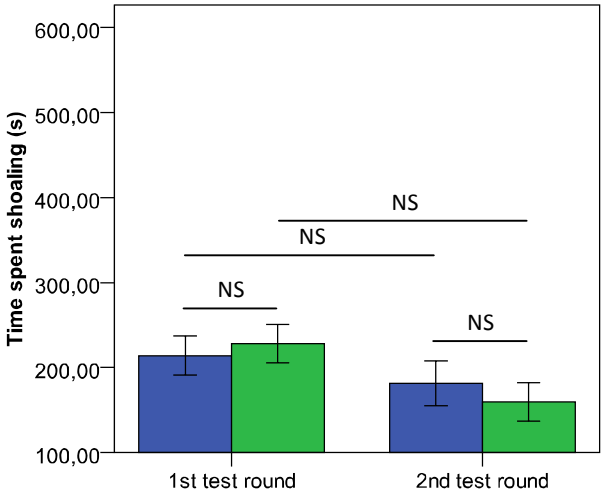

Figure 15. Experiment 5. Mean time spent shoaling ± S.E. per test round (first test round: without novel object, second test round: with novel object). Blue bars represent plain-reared subjects, green bars represent enriched subjects. Means ± SE in round 1: plain subjects: 213.94 ± 22.91 s; enriched subjects: 228.14 ± 22.89 s. Means ± SE in round 2: plain subjects: 181.54 ± 26.54 s; enriched subjects: 159.70 ± 22.45 s There were no differences between different rearing types or between test round (NS), though there appears to be a tendency for both plain and enriched subjects to shoal less in round 2.

We performed some additional tests to compare the time spent within the object zone and the time spent in the opposite tank end zone, plus the latencies to enter both zones (Table 1). These showed that subjects spent similar amounts of time on each tank end compartment (with vs. without object) for both rounds. The latencies to enter both end zones were also equal. See Fig. 16.

Selected data	Difference	Test round	Mean (± SE)	Results *t* test (test value = 0)
Overall	Ln(time in object zone) – Ln(time in opposite end zone)	1st round	0.03 (± 0.16)	t_{79} = 0.19, *P* = 0.85
		2nd round	0.07 (± 0.17)	t_{79} = 0.40, *P* = 0.69
	Ln(latency to enter object zone) – Ln(latency to enter opposite end zone)	1st round	0.15 (± 0.18)	t_{79} = 0.81, *P* = 0.42
		2nd round	-0.15 (± 0.19)	t_{79} = 0.82, *P* = 0.42
Plain subjects	Ln(time in object zone) – Ln(time in opposite end zone)	1st round	0.10 (± 0.20)	t_{39} = 0.50, *P* = 0.62
		2nd round	-0.11 (± 0.26)	t_{39} = 0.412, *P* = 0.69
	Ln(latency to enter object zone) – Ln(latency to enter opposite end zone)	1st round	-0.02 (± 0.25)	t_{39} = 0.10, *P* = 0.92
		2nd round	-0.14 (± 0.30)	t_{39} = 0.45, *P* = 0.66
Enriched subjects	Ln(time in object zone) – Ln(time in opposite end zone)	1st round	-0.04 (± 0.25)	t_{39} = 0.15, *P* = 0.88
		2nd round	0.24 (± 0.21)	t_{39} = 1.16, *P* = 0.26
	Ln(latency to enter object zone) – Ln(latency to enter opposite end zone)	1st round	0.32 (± 0.26)	t_{39} = 1.21, *P* = 0.23
		2nd round	-0.17 (± 0.23)	t_{39} = 0.76, *P* = 0.45

Table 1. Results of *t* tests on the differences between Ln transformed data of time spent in object zone and time spent in the opposite tank end zone, and between Ln transformed data of latency to enter the object zone and latency to enter the opposite tank end zone (Ln transformed data were used because the raw data were not normally distributed, see appendix). Calculations were done on the whole dataset (overall), and on the data from plain subjects and enriched subjects separately. None of the differences were statistically significant higher or lower than 0, meaning presence of the object had no effects on the time spent on each tank end or the latency to enter the end zones.

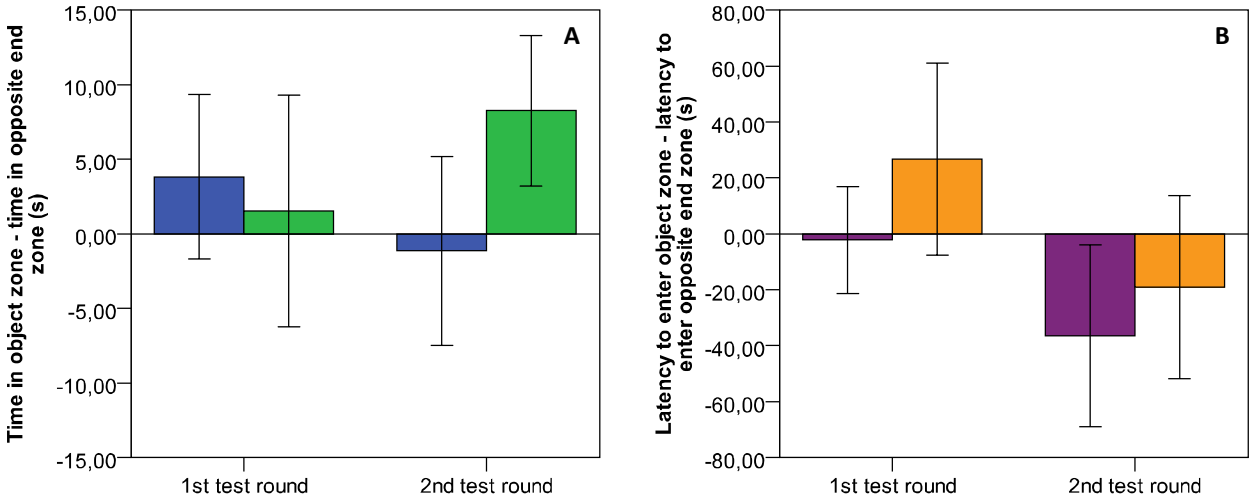

Figure 16. Experiment 5. Presence of the object has no effect on time spent in both end zones or the latency to enter the end zones. **A**: Mean difference of time spent in object zone minus time spent in opposite tank end zone ± S.E. per test round (first test round: without novel object, second test round: with novel object). Blue bars represent plain-reared subjects, green bars represent enriched subjects. Mean difference ± SE in round 1: plain subjects: 3.83 ± 5.52 s; enriched subjects: 1.52 ± 7.77 s. Mean difference ± SE in round 2: : plain subjects: -1.14 ± 6.33 s; enriched subjects: 8.26 ± 5.03 s. None are significantly different from 0. **B**: : Mean difference of latency to enter object zone minus latency to enter opposite tank end zone ± S.E. per test round. Purple bars represent plain-reared subjects, orange bars represent enriched subjects. Mean difference ± SE in round 1: plain subjects: -2.15 ± 19.11 s; enriched subjects: 26.72 ± 34.40 s. Mean difference ± SE in round 2: : plain subjects: -36.43 ± 32.55 s; enriched subjects: -19.06 ± 32.84 s. Again, none are significantly different from 0.

To see if enrichment had any influence on the weight of our subjects, all fish were weighed at three moments: at the age of 5, 8 and 9 months. Here, we found that both enriched and plain-reared subjects increased their weights significantly over time (GLM, Wald $\chi^2_2 = 207.85$, $P < 0.01$). There was a significant difference between the weights of plain and enriched subjects at the age of five months, the time of the enrichment shoaling experiment (*t* test: $t_{78} = -3.08$, $P < 0.01$). However, these differences disappeared over time, and they were gone at the moment of the second (in between experiments) and third weighing (novelty experiment). See Fig. 17.

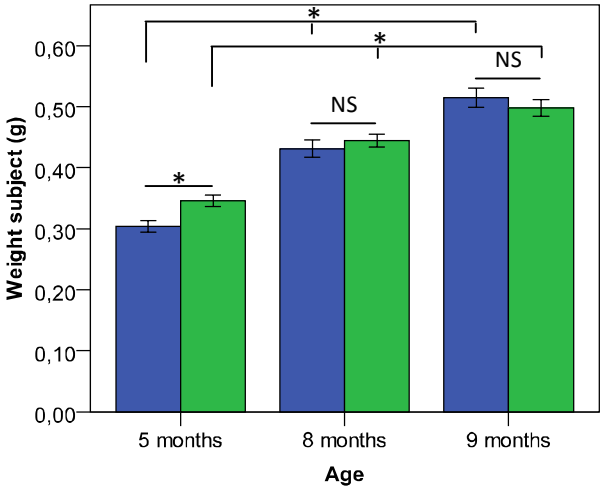

Figure 17. Experiment 5. Mean weight of subjects ± S.E. per weighing moment. Subjects were weighed at 5, 8 and 9 months of age. Blue bars represent plain-reared subjects, green bars represent enriched subjects. * indicate significant differences. Plain and enriched subjects differed significantly in mean weight at the first weighing, but over time these differences disappeared (NS). Mean weight significantly increased over time for all rearing conditions. Mean weight ± SE at 5 months: plain subjects: 0.30 ± 0.01 g; enriched subjects, mean weights ± SE = 0.35 ± 0.01 g. Mean weight ± SE at 8 months: plain subjects: 0.44 ± 0.02 g; enriched subjects: 0.44 ± 0.01 g. Mean weight ± SE at 9 months: plain subjects: 0.52 ± 0.02 g; enriched subjects: 0.50 ± 0.01 g.

Discussion

We tested whether rearing environment affected our subjects' responses to a novel object. Our predictions were that enriched individuals would show a 'bolder' response than plain reared individuals when presented with an unfamiliar object: we expected enriched individuals to shoal less than individuals from plain tanks, and to spend more time in proximity of the object, to investigate it. We also expected enriched individuals to approach the novel object faster than plain reared fish. However, our results showed that in our experiment, there were no differences between enriched and plain individuals. Rearing environment did not affect shoaling time, nor did it have an effect on how fast subjects approached the novel object or how much time they spent in its proximity. We did find that all subjects spent less time shoaling in the second test round. We also found that that there were no differences between the amounts of time subjects spent in the object zone and in the empty tank end zone, or between the latencies to enter both zones. This suggests that the end compartment containing the object was not more or less attractive to the subjects than the opposite end. The decrease in time spent shoaling in the second round was thus not likely caused by the presence of the object. Possibly, the differences in shoaling response between the two testing rounds simply reflect a decrease in shoaling behaviour over time, as the subjects get used to the testing environment. Stress levels will then decrease, and since shoaling is often also a response to stress, it may occur less in the second round. This also suggests that stress levels did not increase in the second test round, when the object was presented. It appears that the object used was unable to induce fear or exert exploratory behaviour. This might explain why we found no differences in response between enriched or plain reared subjects. In the future, it would be interesting to perform

a similar experiment, but with an object that evokes stronger responses. Overall in this experiment, we found a trend for mean weight of the stimulus shoal to affect shoaling time, subjects appear to shoal more when the stimulus fish are heavier. This is in line with our findings in experiment 3, where subjects spent more time with the large shoal if the mean weight of that shoal was higher, in comparison with the mean weight of the small shoal. Our experiment also showed that the body weight of subjects is positively correlated with the time a subject spent with the novel object. This suggests that heavier subjects are more investigative than lighter fish, especially since this weight effect was not seen in the empty tank compartment.

General discussion

Basic shoaling preference experiments and the effects of nonapeptides

Our shoaling preference experiments showed that – following predictions - female zebrafish have a preference to aggregate with larger groups of eight fish over smaller groups of two. These results are in line with the findings of Pritchard et al. (2001) and Ruhl & McRobert (2005). New here is that our results suggest that first choice shoaling preferences and shoaling preferences over a longer time period are different from each other. This is remarkable, as one would expect a subjects first choice to be predictive of their overall preferences. To date, no researches have focused on investigating differences between first choice and preferences over longer time in choice experiments.

Our nonapeptide administration experiment suggests that the AVT and possibly the IT system play a role in regulating zebrafish grouping behaviour. Though AVT injections did not decrease the time spent in close proximity of the stimulus shoal as we predicted, it did decrease interaction time and both the latency to enter the shoaling zone and to start interacting. Contrary to our predictions and out of line with the results from Braida et al. (in press), an AVT antagonist did not have opposing effects compared to AVT. The AVT antagonist decreased time spent near the shoal, time spent interacting and the latency to enter the shoaling zone and to start interaction. The difference with AVT injections were that AVT did not affect time spent near the shoal and that the effects of AVT on interaction were stronger. For IT, we found that besides reducing effects on interaction, there appear to be no effects on any other of our behavioural measures. As with AVT, we found no contrasting effects between IT and its antagonist, the latter not affecting any of our measures. It is surprising that our antagonists were unable to induce effects opposing those of the agonists. Possibly, this is because the antagonists used are both mammalian (OT and AVP). Perhaps this resulted in unexpected interactions with zebrafish receptors. Also, zebrafish have at least two different AVT receptors in the brain (V1a and V1b, see Lema, 2010). AVT and the AVP antagonist may differ in the affinity for both receptor types, which could result in behavioural effects through different pathways. However, these possible explanations are not in line with the results of Braida et al. (in press), where the agonists and antagonists (which were also the mammalian type) did have opposing effects.

We expected IT and AVT to have opposing effects on sociality since they are often linked to seemingly opposing social interactions (IT/OT often linked to social bonding and affiliation and AVT /AVP often linked to aggression and social avoidance, see Insel & Young, 2000 and Donaldson & Young, 2008). However, in our experiments we did not find any results supporting this. This could suggest that the AVT and IT systems may affect behaviour through different pathways and they do not necessarily have to work against each other (Goodson & Bass, 2001; Donaldson & Young, 2008; Lema, 2010). Possibly, in the zebrafish, both IT and AVT have similar decreasing effects on sociality, though our results suggest a stronger role of the AVT system in this respect.

Next to their behavioural effects, IT and AVT injections may have had physiological effects in our experiments. The IT and AVT systems are both very complex, which is especially true for the AVT system. There are multiple kinds of AVT receptors and zebrafish have at least three different types. As mentioned earlier, two of these types are found in the brain, where they can affect behaviour. The other receptor type is found in the body, where AVT has peripheral effects, for example on osmoregulation. In addition, in other fish species AVT receptors have been found on the gills, suggesting that AVT can affect gill functioning as well (Balment et al., 1993; Lema, 2010). The IT system has not yet been studied well to date, but here there may also be peripheral effects. In our experiments, treatment injections were given intraperitoneally. This means that treatment substances were all able to reach any peripheral receptors, possibly resulting in some physiological effects and affecting the outcome of our experiments. We found no indications of physiological effects in the long term, as all subjects showed a similar increase in body mass after one week. Still, the possibility of peripheral effects should be kept in mind. In addition to the fact that our treatments might have resulted in different physiological effects, we can also not be sure that our treatments were able to have any central effects here. It might be that the treatments did not reach

the brains of our subjects sufficiently to affect behavioural pathways. In other experiments intraperitoneal injections of nonapeptides were sufficient to result in behavioural effects (Semsar et al., 2001; Lema & Nevitt, 2004; Thompson & Walton, 2004; Braida et al., in press). This suggests the fish blood – brain barrier is at least partially permeable to the administered substances (Balment et al., 2006). Nonetheless, at this point we cannot be sure the administrations were able to reach the brain in our experiment. In order to ensure treatments are able to reach the brain sufficiently and to lower the chances of possible physiological effects in the future, research should focus on refining techniques in order to be able to inject treatments directly into the zebrafish brain. It would also be a good idea to look at additional measures that can reveal if any physiological effects are occurring, for example by measuring activity levels, through swimming speed and the distances travelled.
Though our exact predictions were not met in our experiments, we did see some behavioural effects of the nonapeptides AVT and (to lesser extend) IT on social grouping in the zebrafish. Peptide effects on sociality are also reported in the research done by Braida et al. (in press) and the experiment on flocking behaviour of zebrafinches by Goodson et al. (2009). In combination with the knowledge from research on social behaviours in other species, this could point to the fact that there is indeed a conserved neural mechanism behind sociality in which AVT, IT and their homologues play an important role (Insel & Young, 2000; Thompson & Walton, 2004; Donaldson & Young, 2008).

Enrichment experiments

Our results suggest that, contrary to predictions, shoaling behaviour of adult female zebrafish is not affected by environmental enrichment in their rearing period. Additionally, we found no influences of different testing environments matching or mismatching our subjects' rearing environments. This suggests that enrichment has no effects on basic grouping behaviour in the zebrafish, unlike in other species (Pietropaolo et al., 2004; Braithwaite & Salvanes, 2005; Salvanes & Braithwaite, 2005; Salvanes et al., 2007). In our novel object experiment, we found no differences in the tendency to investigate a novel object between plain reared individuals and subjects from enriched housing tanks, against predictions (following Braithwaite & Salvanes, 2005). As was discussed earlier, the novel object used here was probably unable to exert exploratory behaviour, which would explain why we found no differences in exploratory behaviour.
We did find some weight effects on shoaling preferences and exploratory behaviour in our experiments. In the shoaling preference experiment, we found that preferences for the larger shoal increased when the mean weight of this group was higher compared to the mean weight of the small shoal. In the second enrichment experiment, on responses to a novel object, we found similar increasing effects of mean shoal weight on shoaling response. This suggests heavier fish may be more attractive shoal partners than lighter fish. Previous research by (Krause et al., 1999) showed that, to food-deprived zebrafish, well fed individuals are more attractive to shoal with than other food-deprived fish. This could correlate with the present findings, since heavier fish are most likely better fed individuals. Additionally we found a positive correlation between a subjects' body weight and the time spent with the novel object, an effect that was not seen on the empty tank end. This suggests that heavier subjects are more investigative than lighter fish. In other fish species, boldness has been linked to increased foraging success and growth (Ward et al., 2004; Brown et al., 2005; Mas-Muñoz et al., 2011). Our results suggest that this may also be the case in zebrafish. This would imply that zebrafish fitness is affected by the boldness of an individual. Effects of different personality traits on reproductive success and growth have been examined in a range of different species in the past decades (Laland & Reader, 1999; Réale et al., 2007; Smith & Blumstein, 2008). Within populations, there can be great variability in personality traits between individuals and selection for different traits will vary with environment (Smith & Blumstein, 2008). Personality is thought to affect the ecology and evolution of species, which makes it an interesting field of research (Réale et al., 2007; Smith & Blumstein, 2008). If boldness as a personality trait indeed affects fitness in zebrafish, as our results may suggest, it may be interesting to be further investigated. Thereby, it might be possible to investigate other personality traits and their fitness consequences in this species. Since the species'

development and genetics are so well known (Larson et al., 2006; Miklosi & Andrew, 2006), this may give some insight on the genetics behind personality and how this can affect survival.
To conclude, it should be kept in mind that our enrichment experiments were only pilot studies. Our results were obtained using only four different groups of fish, divided over two rearing conditions. Data on individuals within the groups are not independent of each other. The results mentioned here should therefore not be over interpreted. To address the question of enrichment effects on shoaling and exploratory behaviour in the zebrafish more thoroughly it may be useful to perform a similar but larger scale experiment in the future.

Overall, our experiments have shown that zebrafish preferences to shoal with larger groups over smaller ones are relatively stable. It appears to be unaffected by how stimulus shoals are presented (static or dynamic), whether the subject is on its own or part of a group, or environmental enrichment. Our results suggest a role of AVT in the regulation of zebrafish shoaling, though the exact mechanism behind this modulation of shoaling remains unclear. Also, as we found no differences between agonists and antagonists in our study, this poses some questions on how the different AVT and IT receptors may respond to the given treatments and subsequently affect behaviour. As studies in other species have reported a possible role of AVT, IT and their homologues in the regulation of social behaviour, our results suggest that there is indeed a conserved neural mechanism behind sociality (Insel & Young, 2000; Thompson & Walton, 2004; Donaldson & Young, 2008). Unravelling the secrets of these mechanisms in the future can be particularly useful in medicine, for example in studying specific psychiatric disorders that are strongly associated with social deficits, like autism or social phobias (Bartz & Hollander, 2006). Moreover, since sociality inevitably affects social learning, it can also affect on numerous other behaviours, like reproduction, foraging or predator avoidance (Brown & Laland, 2003). Understanding the basic mechanisms behind sociality could therefore also help understanding these other kinds of behaviour.

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Appendix

Raw data

Raw data (excel sheets) are all on the CD 'Shoaling preferences in female zebrafish (*Danio rerio*) and the effects of nonapeptides and environmental enrichment - Esther Langen' located in separate maps per experiment. Summaries of the data per experiment - which is the data used for analysis - are located in the sub maps 'Summary data and SPSS files'. These also include the used SPSS files. References to the appendix in the main text all refer to the SPSS output files located here, containing the results of all statistical tests used and some additional graphs.

Each raw data file (excel) contains the following worksheets:

- **General info and weight data (may be divided over different sheets per week/setup type etc.):** contains date, time, trial number, references to video files and info such as treatment, location of the stimulus shoals (as location of the small shoal) etc. plus additional comments (if there were any).
- **Rough time data (may be divided over different worksheets per week/setup type etc.):** contains the scored behavioural data from all experiments, per trial.
- **Explanation codes:** explains the codes used in the 'Rough time data' worksheets (under the 'code' column)

Raw data from experiment 3, the admin experiment contains two additional worksheets:

- **Weights stimulus shoal:** the weights of the stimulus shoals used for each trial.
- **Weights, 1 week after testing:** the weights of the used subjects, one week after the experiment.

The summary data excel files contain two worksheets:

- **Data:** contains all data that was retrieved from the raw data files – this was the data used for statistical analysis.
- **Coding:** explanation of each column in the 'Data' worksheet.

Data on weight effects of enrichment are in the map Appendix CD Esther Langen/Experiment 5 – Enrichment novelty experiment/Summary data and SPSS files/Weight data enrichment experiments. Here you can find the raw excel data on the weights of the subjects from both enrichment experiments over time. The worksheet 'Coding' again explaining the columns of the 'Data' worksheet. SPSS files are also included.

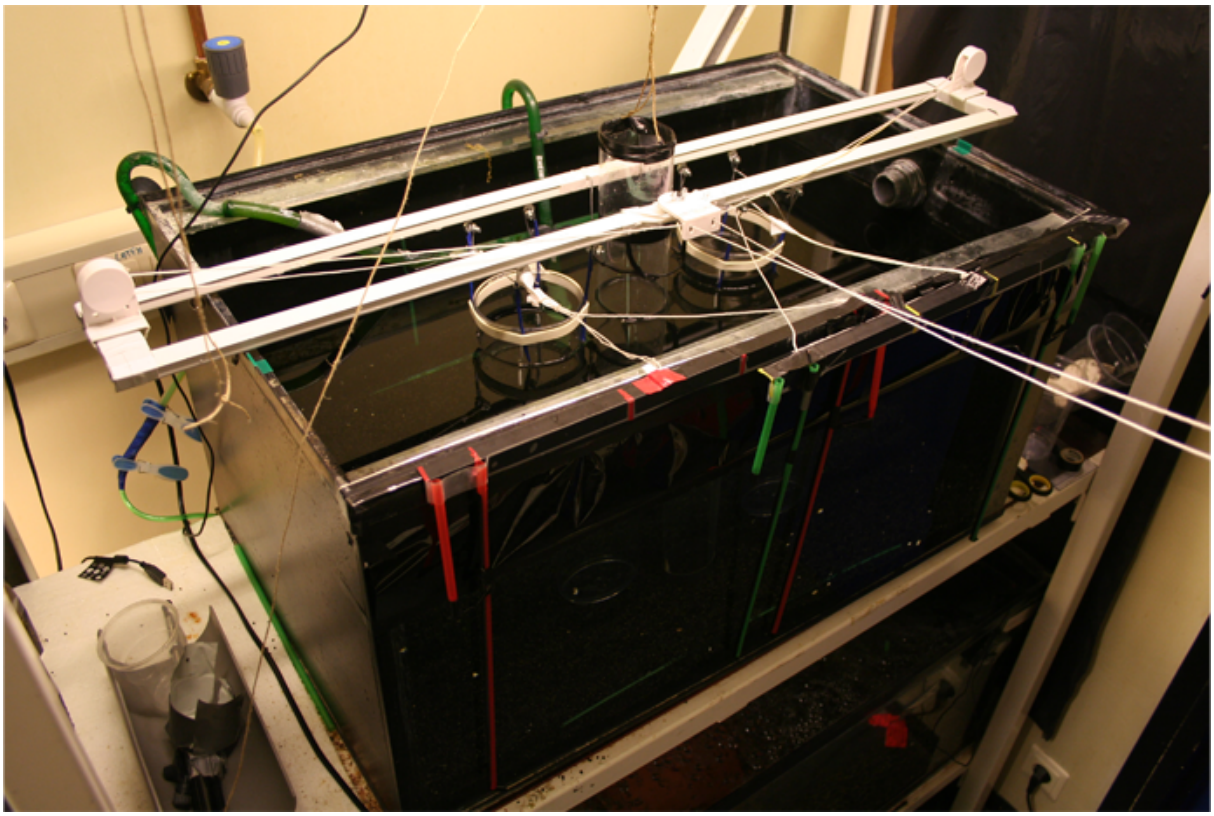
HD's

The external hard drives 'Student Data', 'Students 3' and 'Esther data admin study' contain video files of all experiments, which are also divided over different maps per experiment. Which video file can be found where is further specified in the raw data files.

Student Data: video files of experiments 1, 2 and 4
Students 3: video files of experiment 5
Esther data admin study: video files of experiment 3

Pictures dynamic setup experiment 1:

Positions of stimulus shoal cylinders and shoaling zone markings at start of trial:



Moved towards the outer tank ends: