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Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish

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Summary

Nonapeptides are important regulators of social behaviour across vertebrate taxa. While their role in simple grouping behaviour has been explored in estrildid finches, other taxa are understudied, prompting us to investigate nonapeptide influences on shoaling behaviour in zebrafish. Subjects received injections of isotocin, an isotocin antagonist, vasotocin, a vasotocin antagonist, or saline, followed by a test of grouping behaviour. Vasotocin decreased social interaction with the shoal. Unexpectedly, the vasotocin antagonist also reduced social interaction with the shoal, as well as general shoaling behaviour. Isotocin and its antagonist had minimal effects on grouping behaviours. These results suggest social interaction and shoaling are discrete aspects of sociality differentially influenced by vasotocin, although we cannot discount possible anxiogenic effects of vasotocin. Contrasting these results with studies in other systems demonstrates that each nonapeptide's role in social behaviour varies across taxa, and cautions against a simplistic characterisation of nonapeptides as prosocial regulators of behaviour.

Keywords:

grouping; isotocin; vasotocin; nonapeptides; fish; shoaling; zebrafish; *Danio rerio*

Introduction

Animals engage in a wide range of social behaviours which vary enormously across taxa and species. In contrast to the phenotypic variation in social behaviour, there appears to be extensive regulatory overlap between species, with the nonapeptides oxytocin and vasopressin repeatedly demonstrated to be important regulators of multiple mammalian social behaviours including parental care (Pedersen, 2013), pair bonding (Winslow et al., 1993), affiliative behaviour (Madden & Clutton-Brock, 2011), social recognition (Bielsky et al., 2004), aggression (Albers et al., 2006) and even human social interactions (Meyer-Lindenberg et al., 2011). Furthermore, differences in nonapeptide release or receptor distribution have been strongly implicated in interspecies variation in social behaviour (Insel & Shapiro, 1992; Bester-Meredith et al., 1999), as well as intra-species population differences (Beiderbeck et al., 2007) and individual differences in social behaviour (Francis et al., 2000). However, sociality is far from a uniquely mammalian attribute and accumulating evidence implicates the nonapeptides in the regulation of social behaviour in other taxa (Moore et al., 2005; Godwin & Thompson, 2012).

The influence of nonapeptides on putatively complex forms of sociality has been extensively researched, with a particular focus in recent years on nonapeptide effects on affiliative and prosocial behaviours, often ignoring one of the most fundamental forms of sociality, association with conspecifics or grouping behaviour (Goodson & Kingsbury, 2011). A notable exception to this is the extensive work of Goodson and colleagues characterising the role that nonapeptides play in grouping and sociality in estrildid finch species. For example, they have shown that variation in nonapeptide neuron number and nonapeptide receptor

density are associated with between-species variation in grouping behaviour (Goodson & Wang, 2006), and that pharmacological manipulations targeting nonapeptide receptors modulate individual grouping propensities (Goodson et al., 2009). However the influence of nonapeptides on grouping behaviour in other taxa is relatively understudied, prompting us to investigate the regulatory roles of nonapeptides on grouping behaviour in fish. Fish are the largest vertebrate class, exhibit an extensive and varied array of social behaviours (Brown et al., 2006) and express the homologous nonapeptides vasotocin (AVT) and isotocin (IT), permitting nonapeptides to be investigated in a socially rich taxon that is evolutionarily distant from mammals and birds. Fish also offer excellent opportunities for exploring grouping behaviour as many species form cohesive groups, and grouping propensities can be readily quantified.

Although much more work has been done in mammals, evidence indicates that nonapeptides influence multiple social behaviours in fish, including dominance interactions, aggression, parental behaviour, social communication and courtship (Goodson & Bass, 2000; Lema & Nevitt, 2004; Greenwood et al., 2008; O'Connell et al., 2012). Fewer studies have addressed grouping behaviour and related phenomena. Butterflyfish species (family *Chaetodontidae*) with greater territorial aggression and smaller social group sizes have larger preoptic AVT neurons and denser telencephalic AVT fibres than non-territorial, shoaling species (Dewan et al., 2008; Dewan et al., 2011). In goldfish (*Carassius auratus*), time in proximity to conspecifics ("social approach") is modulated by nonapeptide administration: IT reduces it while AVT increases it (Thompson & Walton, 2004). These effects are seen in both sexes, however they appear to be dependent on baseline levels of social approach (Thompson & Walton, 2004) and on reproductive state in this seasonally-breeding species (Walton et al., 2010).

We wished to determine whether IT and AVT influence grouping behaviour in fish and so investigated how nonapeptides affect this fundamental component of social behaviour in zebrafish (*Danio rerio*). The zebrafish, a small freshwater fish native to South Asia (Spence et al., 2008), is a model system for genetics and developmental biology and is increasingly being used to study behaviour. Zebrafish readily shoal and nonapeptides have previously been implicated in the regulation of zebrafish social behaviours. Neuronal localization of AVT within the preoptic area is restricted to large magnocellular neurons in dominant zebrafish and to small parvocellular neurons in subordinates (Larson et al., 2006). AVT levels have also been shown to vary according to dominance status, although whether AVT expression is higher in dominant individuals (Filby et al., 2010) or in subordinates (Pavlidis et al., 2011) appears to depend on the precise makeup of the social group and the duration of such group housing. Administration of AVT has been shown to reduce aggression in zebrafish (Filby et al., 2010), while both AVT and IT have been shown to increase preferences for a same-strain shoal in zebrafish (Braida et al., 2012). We administered nonapeptides and putative nonapeptide receptor antagonists to individual zebrafish and measured shoaling and social interaction in a social behaviour test with a novel stimulus shoal. Based on Thompson & Walton's (2004) findings in goldfish, we predicted that IT would increase and AVT would decrease shoaling and social interaction. In the goldfish, a seasonal breeder, these responses are dependent on reproductive state (Walton et al., 2010). However, we did not take reproductive state into account here as reproduction in the zebrafish, also a cyprinid, is driven by food availability and so they breed year round in captivity (Spence et al., 2008).

Material and methods

Subjects and housing

A total of 125 adult female zebrafish (4 – 5 months old) were used as subjects (mean mass ± SE = 0.33 ± 0.004 g). Twenty additional adult females (mean mass ± SE = 0.35 ± 0.004 g), unfamiliar to the subjects and housed separately, served as stimulus shoals in the behavioural tests. We used females to minimise aggression and dominance effects on shoaling behaviour. All subjects were bred in-house at our departmental aquarium and were experimentally naïve F2 descendants of fish purchased from a commercial supplier ('wild type' strain, Ruijsbroek B.V., Maassluis, Netherlands). Subjects were housed in a large tank (150 × 50 cm), stimulus shoal fish in a small tank (80 × 50 cm). Once subjects had been tested, they were rehoused in separate small tanks (80 × 50 cm) by treatment group. Due to this rehousing, a further 20 adult females were included in the home tank so that the final subjects to be tested were not socially isolated. All tanks were maintained at 26 ± 1°C with 30 cm of water and were enriched with artificial plants, pot shelters and gravel. Lights were on a 12h:12h schedule with lights on at 0800 hours and no natural light. Fish were fed twice daily (at 0900 and 1700 hours) with 'TetraMin' flake food (Tetra GmbH, Melle, Germany) in the morning and bloodworm (*Chironomidae*) or *Daphnia* spp. in the afternoon. On test days, fish were given a single combined feeding after the conclusion of testing. Water quality (pH, nitrates and nitrites) was checked weekly and tanks were cleaned fortnightly.

Administration treatment groups

Subjects were selected at random from their home tank and assigned to one of five treatment groups immediately prior to testing: *1)* IT (AbD Serotec Ltd, Kidlington, UK), *2)* AVT (Bachem AG, Bubendorf, Switzerland), *3)* a putative IT receptor antagonist (IT-a), *4)* a putative AVT receptor antagonist (AVT-a), or *5)* 0.9% saline. The IT-a was the selective oxytocin receptor antagonist desGly-NH_2,d$(CH_2)_5$[D-Tyr^2,Thr^4]OVT (Manning et al., 1995) and the AVT-a was the selective vasopressin 1a receptor antagonist d$(CH_2)_5$[$Tyr(Me)^2$,Dab^5]AVP (Chan et al., 1996), both generous gifts of Professor M. Manning of the University of Toledo.

Each group consisted of 25 subjects. To address any possible observer bias, treatment order was pseudo-randomly determined using Microsoft Excel's RAND function, and a second researcher prepared and labelled the solutions to be administered so that the researcher conducting tests was blind to which group was being tested on each day. Tests were conducted over two weeks and to minimise order effects half of the subjects from each treatment group were tested in the first week and the remainder in the second week. Time of day of testing was counterbalanced across groups to minimise intergroup variance due to circadian effects.

Treatment dosages and administration

All substances were dissolved in 0.9 % saline and administered at a dose of 10 μg/g body weight. Doses were based on peripheral administration studies in zebrafish and other small fish (Carneiro et al., 2003; Lema & Nevitt, 2004; Filby et al., 2010). For administration, subjects were caught in the home tank with a net, weighed in water and then placed on a wet tissue for intraperitoneal injection with a 10 μl Hamilton syringe and 30G needle, with injection volumes no more than 6 μl. The administration procedure took approximately 20 seconds, after which subjects were placed in the social behaviour test tank.

Grouping test

We measured effects of administrations on zebrafish shoaling and social interaction. A large tank (150 × 50 cm) was divided into three areas by transparent plastic partitions: two side compartments each 11.5 cm wide and a central 127 cm wide compartment (Figure 1). Prior to testing, eight stimulus shoal fish were caught with a net and placed into a transparent plastic container (11.5 × 12.5 cm) filled with 26 ± 1°C water which was then placed in one of the side compartments while the opposite compartment remained empty. The stimulus shoal fish were chosen at random from the pool of 20 fish and used for 2 - 3 consecutive trials. Shoal location was randomised after every two trials. The central subject compartment was divided into three zones by boundaries drawn on the front of the tank: a central 'neutral' zone and outer 'shoaling' and 'no-shoal' zones 10 cm or 3 – 4 body lengths from each plastic partition, following Pitcher's (1983) definition of shoaling. Directly after administration, the subject was placed in a 7 cm diameter transparent plastic cylinder in the middle of the central compartment. After 5 minutes for recovery, acclimatisation and to enable administered substances to reach the brain, the cylinder was smoothly pulled upwards by rope and pulley

to release the subject and start the 10-minute trial. The post-injection recovery period was the same across treatments so that recovery from the injection procedure did not differentially influence the different treatment groups. We used a short recovery time due to the short plasma half-life of nonapeptides (Gozdowska et al., 2013). Subject behaviour was scored live with JWatcher V1.0 (http://www.jwatcher.ucla.edu) and recorded with a Megapixel Pro webcam (Trust International B.V., Dordrecht, Netherlands) and AMCap 9.20 software. After testing, subjects were moved to their post-testing housing tank. All stimulus shoal fish were weighed after being used in tests. Subjects were weighed prior to administration and again one week later to check for possible effects of administration on weight and health.

Statistical analyses

We measured shoaling behaviour and interaction with the shoal. Subjects were defined as shoaling when they were within the shoaling zone, and as interacting when they were swimming head first against the transparent partition, in a manner directed towards the stimulus shoal. This behaviour was readily distinguished from general shoaling behaviour when fish swam within the shoaling zone but did not directly approach the partition. Persistent swimming directed at the shoal may indicate greater motivation to socially interact than mere presence in the shoaling zone and so we used the shoaling and interaction measures to differentiate between grouping and more active social interest. A similar interaction measure has recently been demonstrated to give different results from grouping measures (Kelly et al., 2011), and thus could reflect a different aspect of social behaviour and motivation.

204 The dependent variables were total time in the shoaling zone, total time in the no-shoal zone,
205 total time spent interacting, latency to shoal, latency to interact and time interacting as a
206 proportion of time shoaling. We also analysed the number of transitions across zone
207 boundaries as a combined measure of activity and stress. Treatment group was a fixed effect,
208 shoal position, subject mass and mean mass of the stimulus shoal were covariates. We used
209 generalised linear models (GLMs) to investigate the effect of treatment on the behavioural
210 measures. Time and latency data were right skewed and so were analysed with a gamma
211 family of errors. To control for overdispersion, proportional data (interaction as a proportion
212 of shoaling) were analysed with a quasibinomial family of errors and count data (transitions
213 across zones) were analysed with a quasipoisson family of errors (Crawley, 2007). Treatment
214 contrasts were employed to assess the effects of each administration relative to saline, with an
215 alpha significance level of 0.05. To explore differences between treatments we defined three
216 planned comparisons of interest (AVT vs. AVT-a, IT vs. IT-a, AVT vs. IT) and ran
217 additional GLMs with a pre-defined a priori contrast matrix (package Epi) and a Bonferroni
218 adjusted critical alpha level ($\alpha = 0.0167$) for multiple comparisons. These comparisons were
219 chosen to compare effects of each nonapeptide with their putative receptor antagonist and the
220 two nonapeptides with each other. All statistical tests were two tailed and data are expressed
221 as means ± SE. Body mass of subjects, mean mass of stimulus shoals and shoal position were
222 not found to be significant predictors of shoaling behaviour ($P \geq 0.1$) and therefore are not
223 reported below. Analyses were performed in R Project 2.10.1.

224

225 *Ethical note*

226

The experiment was approved by our local Animal Experimentation Committee ('Dier Experimenten Commissie') under licence 2010.I.12.263, and conformed to Dutch animal welfare legislation and to the ASAB/ABS Guidelines for the Use of Animals in Research. Our Animal Experimentation Committee and university veterinarians discussed and observed our proposed procedure before experiments began. We strived to minimise distress by making the administration procedure as short as possible and not using anaesthetic: although this would have led to some discomfort during the actual injection, the far shorter duration of the whole procedure was judged to reduce overall distress. No adverse effects of any of the tested substances were observed on behaviour or health, and as noted below, there were no significant differences between groups in either individual weight or weight gain after administration. Fish were euthanized at the conclusion of experiments by immersion in ice water for 1 minute (following Blessing et al., 2010), as this is the fastest, most effective and most humane method of euthanasia for small tropical fish such as the zebrafish (Wilson et al., 2009).

Results

Interaction with the shoal

Interaction behaviour was only observed in the shoaling zone and never at the partition in the no-shoal zone, suggesting it was directed specifically at the stimulus shoal and was an effective measure of social behaviour. Both AVT and AVT-a significantly reduced interaction time compared to saline (GLM: AVT, $t_{24} = 2.34$, $P = 0.02$; AVT-a, $t_{24} = 2.03$, $P =$

0.04, Figure 2B). Other administrations did not significantly differ from saline (GLM, $t_{24} \leq 1.60$, $P > 0.1$). IT treated fish spent less time interacting than IT-a treated fish, but not significantly so (GLM with a priori contrasts, $z_{24} = 1.94$, $P = 0.05$). AVT also significantly increased the latency to start interacting with the stimulus shoal compared to saline and IT (GLM: AVT vs. saline, $t_{24} = 2.88$, $P = 0.005$; AVT vs. IT, GLM with a priori contrasts, $z_{24} = 2.42$, $P = 0.015$, Figure 3B).

To further investigate interaction behaviour, we analysed time spent interacting as a proportion of total time spent shoaling (Figure 2C). AVT, AVT-a and IT administrations significantly decreased the proportion of shoaling time spent interacting compared to saline (GLM: AVT vs. saline, $t_{24} = 5.55$, $P < 0.001$; AVT-a vs. saline: $t_{24} = 2.46$, $P = 0.02$; IT vs. saline: $t_{24} = 2.76$, $P = 0.01$). Additionally, the proportion of shoaling time spent interacting was significantly lower after AVT administration than AVT-a or IT (GLM: AVT vs. AVT-a, $z_{24} = 3.49$, $P = 0.001$; AVT vs. IT, $z_{24} = 3.21$, P = 0.001). IT significantly decreased the proportion of shoaling time spent interacting compared to IT-a (GLM with a priori contrasts, $z_{24} = 3.09$, $P = 0.002$).

Shoaling

Time in the shoaling zone differed significantly between treatment groups (Figure 2A). AVT-a significantly reduced time shoaling compared to saline (GLM, $t_{24} = 2.58$, $P = 0.01$), however other groups did not significantly differ from the saline group (GLM, $t_{24} \leq 1.51$, $P >$ 0. 1). Shoaling was significantly higher after AVT administration than IT or AVT-a

administration (GLM with a priori contrasts, AVT vs. IT, $z_{24} = 2.48$, $P = 0.01$; AVT vs. AVT-a, $z_{24} = 3.76$, $P < 0.001$).

Subjects demonstrated a strong tendency to associate with the stimulus shoal, spending more time in the shoaling zone (mean ± SE = 184.4 ± 27.9 s) than the no-shoal zone (mean ± SE = 46.9 ± 14.4 s), however there were effects of treatment: subjects in all groups spent significantly more time in the shoaling zone than the no-shoal zone, except for the AVT-a group (Wilcoxon paired signed-ranks tests: AVT-a, $U = 225$, $N = 25$, $P = 0.09$; other groups, $U \geq 264$, $N = 25$ per group, $P < 0.005$ in all cases). AVT and AVT-a both significantly increased time in the no-shoal zone compared to saline (GLM: $t_{24} = 2.02$, $P = 0.05$; $t_{24} = 2.03$, $P = 0.04$, respectively). The planned comparisons did not reveal significant differences between peptide treatments in time spent in the no-shoal zone (GLM with a priori contrasts, $z_{24} \leq 1.34$, $P \geq 0.2$).

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

Other measures

AVT, AVT-a and IT significantly increased the frequency of transitions across zones compared to saline (GLM: AVT, $t_{24} = 3.42$, $P = 0.001$; AVT-a, $t_{24} = 2.29$, $P = 0.02$; IT, $t_{24} = 1.98$, $P = 0.05$, Figure 4). The planned comparisons revealed no significant differences in zone transitions between AVT vs. IT, AVT vs. AVT-a or IT vs. IT-a (GLM with a priori contrasts, $z_{24} \leq 1.90$, $P > 0.05$). Subjects' body mass (mean ± SE = 0.39 ± 0.05 g) did not differ significantly between administration treatments either before (Linear Model (LM): $t_{24} \leq 1.64$, $P > 0.1$) or after testing (LM: $t_{24} \leq 0.98$, $P > 0.3$). Subjects gained weight in the week after testing (Wilcoxon signed rank test, $W = 5734.5$, $N = 150$, $P < 0.0001$), but weight gain did not differ significantly between treatments (LM, $t_{24} \leq 1.07$, $P > 0.3$).

Discussion

Our results suggest that nonapeptides, and AVT in particular, modulate grouping behaviour in zebrafish, as administrations of both AVT and an AVT receptor antagonist had clear effects on subjects' social interaction and shoaling behaviour with a stimulus shoal. AVT-treated subjects were slower to interact and spent less time interacting with the shoal, both in absolute terms and as a proportion of time spent shoaling, than any other treatment. While AVT did not affect shoaling time, shoaling was reduced by AVT-a compared to both saline and AVT. In contrast to these diverging effects on shoaling time, the effects of AVT-a on absolute interaction time were similar to those of AVT. These differences in the responses to AVT and AVT-a across shoaling and interaction were unanticipated, and suggest differences in how AVT regulates the interaction and grouping behaviours we measured. While these social behaviours were modulated by AVT manipulations, we found little evidence of a role for IT in the regulation of social grouping in zebrafish, with no detectable responses to IT-a,

and the only significant effect of IT administration being a reduction in interaction time as a proportion of time shoaling.

Vasotocin

We saw a marked reduction in social interaction in fish that received AVT, mirroring findings in goldfish in which AVT inhibited social approach (Thompson & Walton, 2004; Thompson et al., 2008). This effect of AVT on social interaction also has parallels in the findings of Filby et al. (2010) who showed that AVT reduced aggressive behaviours, including chasing of conspecifics, in small groups of zebrafish. The observed reduction in social interaction in response to AVT may be linked to this previously reported effect of AVT on aggressive interactions: diminishing close interaction may decrease the chance of aggression, or diminished aggression may motivate less close approach of conspecifics. Intriguingly, we found that despite its effects on social interaction, AVT did not modify shoaling behaviour, as AVT-treated fish spent at least as much time as control subjects in proximity to the stimulus shoal, suggesting that these two social behaviours are differentially sensitive to AVT and that they may be decoupled.

These differing effects of AVT and AVT-a administration suggest that there is a behavioural distinction between shoaling tendency and social interaction in zebrafish. There are similarities between these findings and recent data on grouping in the gregarious zebra finch (Kelly et al., 2011). In this study, a vasopressin 1a receptor antagonist decreased preferences for larger group sizes but increased social contact time, findings that, like ours, indicate

342 regulatory separation between grouping behaviour and social interaction. These findings
343 emphasise the importance of distinguishing between general tendencies to associate with
344 conspecifics and more focused social interaction when studying social behaviour and
345 grouping. Moreover, they demonstrate that nonapeptides do not act as blanket up- or down-
346 regulators of even apparently similar social behaviours, cautioning against extrapolation of
347 the influence of nonapeptides across social behaviour more generally.

348

349 Contrary to our expectations, AVT and AVT-a did not have opposing effects across our
350 different measures of social behaviour. In some cases the effects were in different directions
351 (total time shoaling), or the size of the effect was significantly different (proportion of time
352 interacting with the shoal), however on other measures the responses were similar (total
353 interaction time). These different results may be due to unanticipated effects of using a
354 putative AVT antagonist in zebrafish, which although a highly specific antagonist of the
355 mammalian vasopressin 1a receptor (Manning et al., 2008), has not been pharmacologically
356 characterised in fish. Differences between mammalian and zebrafish AVT receptor binding
357 sites may impact the affinity and efficacy of the AVT-a we used. Furthermore, nonapeptide
358 receptors in teleosts and mammals are also not uniformly equivalent: zebrafish have been
359 shown to have two receptors homologous to the mammalian vasopressin 1a receptor, both of
360 which are expressed in the brain (Iwasaki et al., 2013). Concurrent administration of AVT
361 and AVT-a would assist in determining whether AVT-a functions as a true antagonist in
362 zebrafish and so would block effects of exogenous AVT mediated via AVT receptors. It is
363 also possible that behaviour may have been influenced by peripheral, physiological responses
364 to intraperitoneal injection rather than through direct central effects. However it should be
365 noted that behavioural responses to different neuropeptides have been shown to be similar in
366 fish, whether administered centrally or peripherally (Olson et al., 1978). In mice, peripherally

administered nonapeptides elicit behavioural responses via central effects (Ring et al., 2006), indicating that they cross the blood-brain barrier. Peripheral administration has been also used to study many diverse social behaviours in fish (Carneiro et al., 2003; Lema & Nevitt, 2004; Santangelo & Bass, 2006; O'Connell et al., 2012; Reddon et al., 2012) and the varied and complex effects reported suggest that the behavioural responses are not simply due to physiological perturbation.

While we found that AVT reduced social interaction, Braida et al. (2012) recently reported that AVT administration in zebrafish increased preferences for same-strain shoals in a dose-dependent fashion. The two studies address different questions: we measured the influence of nonapeptides on shoaling tendencies, while Braida and colleagues examined specifically the effects of nonapeptides on strain preferences in choice tests, rather than on social approach directly. Moreover, Braida et al. used doses of AVT and IT based on studies of intracerebroventricular administration of oxytocin and vasopressin in mice, orders of magnitude lower than the doses we and other researchers have typically employed for intraperitoneal administration studies in fish (Carneiro et al., 2003; Lema & Nevitt, 2004; Filby et al., 2010). The inverted-U dose-response effect of AVT on same-strain shoaling preferences reported by Braida et al. (2012) might predict an increase in shoaling at the dose utilised in our study, however we did not see such an effect.

Isotocin

389 IT significantly decreased the proportion of shoaling time spent interacting compared to
390 saline, but did not have any effect on actual time spent shoaling or interacting. These findings
391 should not be over-interpreted but suggest IT influences grouping behaviour to a lesser
392 degree than AVT. While IT has been shown to stimulate social approach in goldfish, effects
393 are only seen in subjects with lower baseline levels of social approach (Thompson & Walton,
394 2004). Since zebrafish show very strong shoaling tendencies (Buske & Gerlai, 2011) our
395 ability to detect any influence of IT administration may have been limited by a ceiling effect.
396 Another possibility is that IT does not increase gregarious behaviour in zebrafish, as
397 supported by recent findings that certain doses of IT decreased preferences for same-strain
398 zebrafish (Braida et al., 2012).

399

400 The focus of much nonapeptide research on prosocial behaviour has resulted in a widespread
401 narrative that nonapeptides, and oxytocin in particular, are the primary regulators of prosocial
402 behaviour (Barraza & Zak, 2013). However our data and those of others suggest that across
403 vertebrates this view is overly simplistic, with extensive variation in the role and importance
404 of nonapeptides across species, behaviours and contexts. For example, while IT increases
405 submissive behaviour in a cooperatively-breeding cichlid (Reddon et al., 2012), it has no
406 effect on aggressive interactions in the beaugregory damselfish *Stegastes leucostictus*
407 (Santangelo & Bass, 2006), and blockade of nonapeptide signalling disrupts neither novel nor
408 established pair bonding in a monogamous cichlid (Oldfield & Hofmann, 2011). In our
409 experiments, IT had no significant effects on grouping behaviour, suggesting at the very least
410 that this homologue of oxytocin does not function as a broadly prosocial neuropeptide in
411 zebrafish and does not mediate increased group cohesion. Furthermore, while AVT and IT
412 have sometimes been described as ‘male’ and ‘female’ nonapeptides, the responses of the

413 female subjects to AVT but not to IT, as well as the data of others (Walton et al., 2010) do
414 not support this view.

415

416 *Activity and stress responses*

417

418 Nonapeptides have been shown to be involved in neuroendocrine responses to stress in
419 teleosts, in particular AVT which stimulates cortisol release via ACTH (Balment et al.,
420 2006). We thus considered whether the reductions in shoaling and interaction after
421 nonapeptide administration might be a consequence of changes in stress responses. However
422 no specifically stress-related behaviours such as freezing or dashing (Egan et al., 2009) were
423 observed, nor did we see any effects of treatment on health or growth post-testing. The
424 increased switching rates seen in the AVT, AVT-a and IT administered subjects compared to
425 the control group could indicate increased stress or activity in these fish, as putative
426 anxiolytics have been shown to reduce swimming activity (Levin et al., 2007). However,
427 external stressors typically cause decreases in zone switching and swimming in zebrafish
428 (Bass & Gerlai, 2008; Cachat et al., 2010), suggesting that elevated activity may not always
429 be an indicator of stress. Recent work in a cichlid has shown that vasotocin administration
430 increases circulating cortisol but decreases swimming activity (Huffman et al., 2014). As
431 zebrafish show tighter shoaling in response to stress (Speedie & Gerlai, 2008), the decrease
432 in social interaction we observed in response to AVT, AVT-a and IT treatment suggests that
433 these administrations were not simply increasing stress responses but were modulating
434 sociality. However, additional research will be necessary to tease apart direct nonapeptide
435 effects on social behaviour from indirect effects mediated by HPI axis activation.

436

Conclusions

Our results demonstrate that AVT manipulations affect shoaling and social interaction, although our study also suggests that these are discrete behaviours that are differentially regulated by AVT and its receptors. Our findings offer further evidence that nonapeptides have a broad role in regulating social behaviour across vertebrates (Goodson, 2008) but also provide evidence that nonapeptides influence perhaps the most fundamental aspect of sociality, the tendency to associate with conspecifics in a group. Further comparisons of relatively simple social behaviours across species will increase our understanding of the neural underpinnings of social behaviour and its evolution (O'Connell & Hofmann, 2011), and of the degree to which nonapeptide regulation of sociality has been conserved throughout vertebrate evolution.

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627 Figure 1: Schematic overview of the experimental apparatus, plan view. Lines were drawn on
628 the outside of a large aquarium (150 × 50 cm) marking a neutral zone (N) containing a pump
629 (p), a shoaling zone (S) and a no-shoal zone (NS), the latter two dependant on the location of
630 a stimulus shoal. The subject was released from a transparent cylinder (c) after
631 acclimatisation and its behaviour was recorded for 10 minutes. A conspecific shoal was
632 placed at random on one side of the tank behind a transparent solid partition (b) in a confined
633 zone (f). Interaction was recorded when subjects were both in the shoaling zone (S) and
634 swimming head first against the partition (b).

635

636 Figure 2: Mean ± SE values for each treatment for A) time spent in the shoaling zone, B)
637 time spent interacting with the shoal, and C) time spent interacting as a proportion of time
638 spent shoaling for each treatment group. *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$.

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640 Figure 3: Mean ± SE values for each treatment for A) latency to enter the shoaling zone and
641 B) latency to interact at the partition with the stimulus shoal. §: $P \leq 0.1$, *: $P \leq 0.05$, **: $P \leq$
642 0.01.

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644 Figure 4: Mean ± SE number of switches made between the shoaling, neutral and no-shoal
645 zones for each treatment. *: $P \leq 0.05$, **: $P \leq 0.01$, NS: $P \geq 0.1$.

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