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

3 Charlotte M. Lindeyer¹, Esther M. A. Langen^{1,2}, William T. Swaney*^{1,3}, & Simon M.
4 Reader^{1,4}

5 ¹Department of Biology and Helmholtz Institute, Utrecht University, The Netherlands.

6 ²Department of Animal Behaviour, Universität Bielefeld, Germany.

7 ³School of Natural Sciences and Psychology, Liverpool John Moores University, UK.

8 ⁴Department of Biology, McGill University, Montreal QC, Canada.

9

10 *Corresponding author: William T. Swaney, School of Natural Sciences and Psychology,
11 Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK

12 E-mail: w.t.swaney@ljmu.ac.uk

13

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16

17

18 **Summary**

19

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

33

34 **Keywords:**

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

36

37

38

39 **Introduction**

40

41 Animals engage in a wide range of social behaviours which vary enormously across taxa and
42 species. In contrast to the phenotypic variation in social behaviour, there appears to be
43 extensive regulatory overlap between species, with the nonapeptides oxytocin and
44 vasopressin repeatedly demonstrated to be important regulators of multiple mammalian social
45 behaviours including parental care (Pedersen, 2013), pair bonding (Winslow et al., 1993),
46 affiliative behaviour (Madden & Clutton-Brock, 2011), social recognition (Bielsky et al.,
47 2004), aggression (Albers et al., 2006) and even human social interactions (Meyer-
48 Lindenberg et al., 2011). Furthermore, differences in nonapeptide release or receptor
49 distribution have been strongly implicated in interspecies variation in social behaviour (Insel
50 & Shapiro, 1992; Bester-Meredith et al., 1999), as well as intra-species population
51 differences (Beiderbeck et al., 2007) and individual differences in social behaviour (Francis
52 et al., 2000). However, sociality is far from a uniquely mammalian attribute and
53 accumulating evidence implicates the nonapeptides in the regulation of social behaviour in
54 other taxa (Moore et al., 2005; Godwin & Thompson, 2012).

55

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

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

74

75 Although much more work has been done in mammals, evidence indicates that nonapeptides
76 influence multiple social behaviours in fish, including dominance interactions, aggression,
77 parental behaviour, social communication and courtship (Goodson & Bass, 2000; Lema &
78 Nevitt, 2004; Greenwood et al., 2008; O'Connell et al., 2012). Fewer studies have addressed
79 grouping behaviour and related phenomena. Butterflyfish species (family *Chaetodontidae*)
80 with greater territorial aggression and smaller social group sizes have larger preoptic AVT
81 neurons and denser telencephalic AVT fibres than non-territorial, shoaling species (Dewan et
82 al., 2008; Dewan et al., 2011). In goldfish (*Carassius auratus*), time in proximity to
83 conspecifics (“social approach”) is modulated by nonapeptide administration: IT reduces it
84 while AVT increases it (Thompson & Walton, 2004). These effects are seen in both sexes,
85 however they appear to be dependent on baseline levels of social approach (Thompson &
86 Walton, 2004) and on reproductive state in this seasonally-breeding species (Walton et al.,
87 2010).

88

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

111

112 **Material and methods**

113

114 *Subjects and housing*

115

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

133

134 *Administration treatment groups*

135

136 Subjects were selected at random from their home tank and assigned to one of five treatment
137 groups immediately prior to testing: 1) IT (AbD Serotec Ltd, Kidlington, UK), 2) AVT
138 (Bachem AG, Bubendorf, Switzerland), 3) a putative IT receptor antagonist (IT-a), 4) a
139 putative AVT receptor antagonist (AVT-a), or 5) 0.9% saline. The IT-a was the selective
140 oxytocin receptor antagonist desGly-NH₂,d(CH₂)₅[D-Tyr²,Thr⁴]OVT (Manning et al., 1995)
141 and the AVT-a was the selective vasopressin 1a receptor antagonist
142 d(CH₂)₅[Tyr(Me)²,Dab⁵]AVP (Chan et al., 1996), both generous gifts of Professor M.
143 Manning of the University of Toledo.

144

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

153

154 *Treatment dosages and administration*

155

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

163

164 *Grouping test*

165

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

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

189

190 *Statistical analyses*

191

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

203

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 \pm 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

227 The experiment was approved by our local Animal Experimentation Committee ('Dier
228 Experimenten Commissie') under licence 2010.I.12.263, and conformed to Dutch animal
229 welfare legislation and to the ASAB/ABS Guidelines for the Use of Animals in Research.
230 Our Animal Experimentation Committee and university veterinarians discussed and observed
231 our proposed procedure before experiments began. We strived to minimise distress by
232 making the administration procedure as short as possible and not using anaesthetic: although
233 this would have led to some discomfort during the actual injection, the far shorter duration of
234 the whole procedure was judged to reduce overall distress. No adverse effects of any of the
235 tested substances were observed on behaviour or health, and as noted below, there were no
236 significant differences between groups in either individual weight or weight gain after
237 administration. Fish were euthanized at the conclusion of experiments by immersion in ice
238 water for 1 minute (following Blessing et al., 2010), as this is the fastest, most effective and
239 most humane method of euthanasia for small tropical fish such as the zebrafish (Wilson et al.,
240 2009).

241

242 **Results**

243

244 *Interaction with the shoal*

245

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

250 0.04, Figure 2B). Other administrations did not significantly differ from saline (GLM, $t_{24} \leq$
251 1.60, $P > 0.1$). IT treated fish spent less time interacting than IT-a treated fish, but not
252 significantly so (GLM with a priori contrasts, $z_{24} = 1.94$, $P = 0.05$). AVT also significantly
253 increased the latency to start interacting with the stimulus shoal compared to saline and IT
254 (GLM: AVT vs. saline, $t_{24} = 2.88$, $P = 0.005$; AVT vs. IT, GLM with a priori contrasts, $z_{24} =$
255 2.42, $P = 0.015$, Figure 3B).

256

257 To further investigate interaction behaviour, we analysed time spent interacting as a
258 proportion of total time spent shoaling (Figure 2C). AVT, AVT-a and IT administrations
259 significantly decreased the proportion of shoaling time spent interacting compared to saline
260 (GLM: AVT vs. saline, $t_{24} = 5.55$, $P < 0.001$; AVT-a vs. saline: $t_{24} = 2.46$, $P = 0.02$; IT vs.
261 saline: $t_{24} = 2.76$, $P = 0.01$). Additionally, the proportion of shoaling time spent interacting
262 was significantly lower after AVT administration than AVT-a or IT (GLM: AVT vs. AVT-a,
263 $z_{24} = 3.49$, $P = 0.001$; AVT vs. IT, $z_{24} = 3.21$, $P = 0.001$). IT significantly decreased the
264 proportion of shoaling time spent interacting compared to IT-a (GLM with a priori contrasts,
265 $z_{24} = 3.09$, $P = 0.002$).

266

267 *Shoaling*

268

269 Time in the shoaling zone differed significantly between treatment groups (Figure 2A). AVT-a
270 significantly reduced time shoaling compared to saline (GLM, $t_{24} = 2.58$, $P = 0.01$),
271 however other groups did not significantly differ from the saline group (GLM, $t_{24} \leq 1.51$, $P >$
272 0.1). Shoaling was significantly higher after AVT administration than IT or AVT-a

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

275

276 Subjects demonstrated a strong tendency to associate with the stimulus shoal, spending more
277 time in the shoaling zone (mean \pm SE = 184.4 ± 27.9 s) than the no-shoal zone (mean \pm SE =
278 46.9 ± 14.4 s), however there were effects of treatment: subjects in all groups spent
279 significantly more time in the shoaling zone than the no-shoal zone, except for the AVT-a
280 group (Wilcoxon paired signed-ranks tests: AVT-a, $U = 225$, $N = 25$, $P = 0.09$; other groups,
281 $U \geq 264$, $N = 25$ per group, $P < 0.005$ in all cases). AVT and AVT-a both significantly
282 increased time in the no-shoal zone compared to saline (GLM: $t_{24} = 2.02$, $P = 0.05$; $t_{24} = 2.03$,
283 $P = 0.04$, respectively). The planned comparisons did not reveal significant differences
284 between peptide treatments in time spent in the no-shoal zone (GLM with a priori contrasts,
285 $z_{24} \leq 1.34$, $P \geq 0.2$).

286

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

292

293 *Other measures*

294

295 AVT, AVT-a and IT significantly increased the frequency of transitions across zones
296 compared to saline (GLM: AVT, $t_{24} = 3.42$, $P = 0.001$; AVT-a, $t_{24} = 2.29$, $P = 0.02$; IT, $t_{24} =$
297 1.98 , $P = 0.05$, Figure 4). The planned comparisons revealed no significant differences in
298 zone transitions between AVT vs. IT, AVT vs. AVT-a or IT vs. IT-a (GLM with a priori
299 contrasts, $z_{24} \leq 1.90$, $P > 0.05$). Subjects' body mass (mean \pm SE = 0.39 ± 0.05 g) did not
300 differ significantly between administration treatments either before (Linear Model (LM): $t_{24} \leq$
301 1.64 , $P > 0.1$) or after testing (LM: $t_{24} \leq 0.98$, $P > 0.3$). Subjects gained weight in the week
302 after testing (Wilcoxon signed rank test, $W = 5734.5$, $N = 150$, $P < 0.0001$), but weight gain
303 did not differ significantly between treatments (LM, $t_{24} \leq 1.07$, $P > 0.3$).

304

305 **Discussion**

306

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

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

321

322 *Vasotocin*

323

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

336

337 These differing effects of AVT and AVT-a administration suggest that there is a behavioural
338 distinction between shoaling tendency and social interaction in zebrafish. There are
339 similarities between these findings and recent data on grouping in the gregarious zebra finch
340 (Kelly et al., 2011). In this study, a vasopressin 1a receptor antagonist decreased preferences
341 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

367 administered nonapeptides elicit behavioural responses via central effects (Ring et al., 2006),
368 indicating that they cross the blood-brain barrier. Peripheral administration has been also
369 used to study many diverse social behaviours in fish (Carneiro et al., 2003; Lema & Nevitt,
370 2004; Santangelo & Bass, 2006; O'Connell et al., 2012; Reddon et al., 2012) and the varied
371 and complex effects reported suggest that the behavioural responses are not simply due to
372 physiological perturbation.

373

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

386

387 *Isotocin*

388

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 (Braidă 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

437 *Conclusions*

438

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

449

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451

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458

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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 \pm 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. *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$.

639

640 Figure 3: Mean \pm 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.

643

644 Figure 4: Mean \pm 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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