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1 **Forebrain activation during social exposure in wild-type guppies**

2

3 María J Cabrera-Álvarez^a, William T Swaney^{a,b}, Simon M Reader^a

4 ^a *Department of Biology, McGill University, 1205 Dr. Penfield Ave., Montreal, Quebec, Canada*

5 ^b *School of Natural Sciences and Psychology, Liverpool John Moores University, Byrom Street,*
6 *Liverpool, United Kingdom*

7

8 Keywords: social behaviour, grouping behaviour, social decision-making network, brain
9 activation, teleost fish, guppy (*Poecilia reticulata*).

10

11 **Abstract**

12

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

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

32

33 **1. Introduction**

34

35 The social decision making network (SDMN) is a network of brain nuclei that process social
36 information and reward and which is thought to modulate social behaviour in all vertebrates
37 [1,2]. The SDMN consist of two overlapping brain networks: the social behaviour network
38 (SBN), and the mesolimbic reward system. The SBN includes six interconnected nodes (the
39 preoptic area, anterior and ventromedial hypothalamus, periaqueductal gray, lateral septum, and
40 bed nucleus of the stria terminalis/medial amygdala) that are involved in sexual, aggressive, and
41 parental behaviour across taxa [1,3]. For example, the preoptic area (POA) is involved in sexual
42 behaviour in all vertebrates, as well as aggression and parental care in mammals, birds and fish
43 (reviewed in [2]), and in mammals, the medial amygdala is involved in social recognition [4] and
44 the lateral septum is involved in social affiliation [5] and social recognition [6]. The mesolimbic
45 reward system includes eight interconnected nodes, two of them shared with the SBN (lateral
46 septum, bed nucleus of the stria terminalis/medial amygdala, striatum, nucleus accumbens,

47 ventral pallium, basolateral amygdala, hippocampus, and ventral tegmental area), and influences
48 the SBN by reinforcing adaptive social behaviours via reward [2]. For example, in mammals the
49 striatum is involved in reinforcement learning and selecting previously reinforcing actions
50 (reviewed in [7]). The SDMN is well conserved across vertebrates, albeit with differences in
51 nomenclature between taxa, and several studies in different vertebrates have linked the SDMN to
52 a wide range of social behaviours, such as mate choice [8], hierarchy formation [9], and
53 cooperative nest building [10]. While these and other studies have implicated the SDMN in
54 social behaviours across diverse taxa, it is noteworthy that most research effort has been targeted
55 at ‘complex’ social behaviours and that there has been a comparative lack of research into the
56 neural mechanisms of more fundamental social behaviour such as grouping.

57

58 Grouping is a very common phenomenon which has been the focus of extensive research in
59 behavioural, theoretical and evolutionary biology [11]. Although living in groups carries costs
60 due to potentially increased aggression, competition for resources, or transmission of parasites
61 and diseases, it can also confer benefits to the individual by reducing predation risk, increasing
62 the chances of obtaining food, increasing the opportunities of finding a mate, reducing loss of
63 heat and moisture, or reducing the cost of movement [11]. Despite the importance of this topic,
64 the neural mechanisms of grouping behaviour have received relatively little attention so far.

65 Goodson and colleagues studied the neural mechanisms involved in grouping behaviour in birds
66 and found differences between gregarious and territorial finches in the activation of brain areas
67 of the SDMN [3]. They have also shown that pharmacological manipulation of nonapeptide
68 signalling in the SDMN modulates flocking behaviour in estrildid finches [12,13]. The
69 nonapeptides are a highly conserved family of neuropeptides involved in different intra-SDMN

70 signalling pathways and studies in fish have also shown that manipulation of these nonapeptides
71 has effects on shoaling and simple social approach [14,15]. We wished to address how the
72 SDMN is involved in grouping behaviour and so investigated brain activation in teleost fish in
73 which shoaling conditions and social exposure can be readily manipulated and controlled.

74

75 For our study, we used Trinidadian guppies (*Poecilia reticulata*) as there is extensive research on
76 their shoaling tendencies, both in their natural environments and in laboratory conditions [16].

77 Trinidadian guppies vary in their shoaling tendencies across populations, with median shoal sizes
78 ranging from 1 to 21 individuals [17]. Female guppies form groups to avoid both predation and
79 sneaky mating attempts from male guppies [18]. Males, on the other hand, show a preference for
80 female rather than male shoals, and, like females and juveniles, for larger shoals rather than
81 small ones [19–21], a trait that appears to be widespread across teleost fish (e.g., banded killifish
82 [22,23], Eurasian perch [24], fathead minnows [25], three-spined sticklebacks [26,27], zebrafish
83 [28]), as well as in birds [29,30] and mammals [31,32].

84

85 We conducted two studies to investigate the neural mechanisms underlying grouping behaviour
86 in guppies. We first conducted a behavioural test to confirm subjects' preferences in the studied
87 population for large shoals over small shoals. With a second cohort of fish, we analysed brain
88 activation after a shoaling exposure test in which the subjects were exposed to one of three
89 experimental treatments: a small shoal, a large shoal, or no social exposure. After one hour, the
90 brain of each subject was dissected for immediate early gene assay of neural activation in
91 specific brain regions that are putative components of the SDMN. We expected shoals to act as a
92 social cue and a rewarding stimulus, and hence social exposure would activate areas of both the

93 SBN and the mesolimbic reward system. Thus, we selected brain areas of both networks,
94 specifically the preoptic area (POA), a node of the SBN and suggested homologue of the amniote
95 POA/paraventricular nucleus of the hypothalamus [2,33]; the dorsal part of the ventral
96 telencephalon (Vd), a node of the mesolimbic reward system homologous to the mammalian
97 striatum and nucleus accumbens [2,34]; and two nuclei belonging to both networks, the ventral
98 part of the ventral telencephalon (Vv), and the supracommissural part of the ventral pallium (Vs),
99 homologues of the mammalian lateral septum and amygdala/bed nucleus of the stria terminalis
100 respectively [2,33,34]. We did not add other brain areas of the SDMN to our study because there
101 is no consensus about teleost homologues of the mammalian areas and/or insufficient research on
102 those areas in teleost fish [2]. We hypothesized that grouping behaviour will be modulated by the
103 SDMN and so exposure to shoals would activate the selected brain areas, with greater activation
104 when the subjects were exposed to the large shoal.

105

106 **2. Materials and methods**

107

108 2.1. Experiment 1: Shoal preference study

109

110 2.1.1. Animal subjects and housing

111

112 Subjects were 30 female guppies from mixed populations of wild Trinidadian origin that had
113 been bred in captivity for at least 2 generations (henceforth ‘wild stock guppies’). Two weeks
114 before the experiment started we moved them from 110 L breeding tanks (76 x 30 x 45 cm)

115 containing both sexes to two 19 L housing tanks (40 x 20 x 25 cm) containing only the subjects.
116 We used an additional 12 wild stock female guppies to form a pool from which stimulus shoals
117 were drawn. They were unfamiliar to the subject fish and lived in the test tank (see below). All
118 tanks were kept at 26 ± 1 °C, had a filter and a heater, as well as gravel, plastic plants and a
119 shelter. Fish were fed flake food daily (TetraMin Tropical Flakes, Tetra, Germany) and
120 supplementary decapsulated brine shrimp eggs (*Artemia sp.*, Brine Shrimp Direct, Ogden UT,
121 USA) three times a week.

122

123 2.1.2. Behavioural test

124

125 Females were tested in a 75 L tank divided into three different compartments by perforated
126 transparent plastic partitions. Each side compartment contained a shoal of either two or 10
127 females (Fig. 1). During the testing day, we removed the plants and shelters and counterbalanced
128 the position of the shoals and varied the member composition of each shoal at random. To
129 measure subjects' proximity to the shoals, we drew vertical lines on the front of the tank to
130 divide the central compartment into five zones. The subject was moved to the testing arena in a
131 transparent plastic cup and, after two minutes of acclimation, the cup was gently and remotely
132 raised by the observer by pulling a string attached to the cup. The test started immediately after
133 the subject was released. We measured the amount of time the subject spent on each of the five
134 zones in order to calculate time shoaling with each group (i.e. time within four body lengths
135 [35]), as well as the amount of time the subject spent interacting with the shoal (i.e. swimming
136 head first against the transparent partitions [15]) over 10 minutes, using the software JWatcher

137 V1.0. We measured shoaling time and interaction time as dual estimates of grouping behaviour
138 in fish [15].

139

140 2.1.3. Statistical analysis

141

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

148

149 2.2. Experiment 2: Brain activation during shoal exposure test

150

151 2.2.1. Subjects and housing

152

153 Two weeks before our study started, we moved 60 females and five males to a 110 L housing
154 tank (76 x 30 x 45 cm). Of these, 36 females were used as subjects and the rest were left in the
155 housing tank as companion fish to prevent the subjects from being isolated as subjects were
156 removed from the tank as the study progressed. We also placed 24 wild stock females unfamiliar
157 to the subjects into four testing tanks (Fig. 2), two tanks had ten females forming the large shoal,
158 and the other two tanks had two females forming the small shoal. There were also two control
159 testing tanks without fish in them. Two weeks before the start of the study, we placed a

160 perforated transparent cylindrical plastic container with gravel in the middle of the testing tanks
161 to habituate the shoals to it. This container held the subject fish during the exposure test,
162 exposing them to the shoal but preventing them from interacting directly with other fish; this
163 ensured consistent exposure to stimulus shoals across subjects. A transparent plastic lid covered
164 the tank to prevent fish from jumping out. Housing conditions and feeding were the same as
165 Experiment 1. The day prior to the test, we isolated 12 subjects in separate 10 L tanks (30 x 20 x
166 15 cm) containing gravel, a plastic plant, a heater (keeping the water at 26 ± 1 °C) and an air
167 stone. The purpose of this isolation period was to set a consistent baseline of neural activity in all
168 subjects.

169

170 2.2.2. Social exposure test

171

172 On the day of the test we removed the filter and plastic plant from the experimental tank, and
173 added an air stone with a plastic plant attached to it. The air stone made the plant move, which
174 served as a control for any neural activation generated by movement, meaning that any
175 differences between treatments would be due to olfactory and/or visual exposure to the social
176 stimulus. Twenty minutes later we caught an isolated subject and placed it at random in the
177 plastic container of a testing tank containing either a large shoal, a small shoal, or no shoal
178 (control), where it was exposed to that social stimulus for an hour (Fig. 2). We monitored the
179 behaviour of the subject and companion fish and observed similarities with the behaviour
180 observed in Experiment 1: subjects appeared highly interested in the stimulus fish and spent
181 much of the exposure period attending to the stimulus fish and attempting to swim to them.
182 Although a 30 minute period has been suggested for induction of the highest expression of *egr-1*

183 in teleost fish [36], we exposed the subjects to the treatment for an hour to ensure that the brain
184 activation we observed was due to the treatment and not just due to handling and tank changing.
185 After this period, we caught the subjects and euthanized them by rapid cooling through
186 immersion in ice water [37–39]. Control tanks were emptied, rinsed and re-filled with
187 conditioned water before adding each new subject to eliminate any olfactory cues left by the
188 previous subject.

189

190 2.2.3. Immunohistochemistry (IHC) staining of egr-1

191

192 Brains were dissected out immediately after euthanasia, fixed in 4% paraformaldehyde at 4 °C
193 overnight, and then cryoprotected in 30% sucrose overnight at 4 °C before embedding in Clear
194 Frozen Section Compound (VWR International, PA, USA) and storage at -19 °C. Brains were
195 then sectioned on a cryostat at 25 µm and thaw-mounted onto Superfrost Plus slides (VWR
196 International) in two parallel series that were stored at -19 °C for less than a week before
197 processing for IHC.

198

199 One of the two series of sections was thawed and air-dried before processing for
200 immunohistochemical detection of egr-1. Sections were rinsed in 0.1M Phosphate-buffered
201 saline (PBS) for 15 minutes. After blocking for 1 hour in blocking solution (5% normal goat
202 serum and 0.3% Triton X-100 in PBS) and rinsing in PBS for 10 minutes, sections were
203 incubated in primary antibody (anti-egr-1 rabbit polyclonal, 1:1000, catalogue number sc-189;
204 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) dissolved in blocking solution at 4°C
205 overnight. Sections were then rinsed in PBS, incubated for 15 minutes in H₂O₂ solution (3.5 %

206 H₂O₂, 8.8% methanol dissolved in 0.3% Triton X-100 in PBS), and rinsed again in PBS. Sections
207 were then incubated in a biotinylated goat anti-rabbit secondary antibody solution (1:200,
208 ThermoScientific, Rockford, IL, USA) dissolved in blocking solution for 30 min at room
209 temperature, and rinsed again for 15 minutes in PBS. Sections were then washed in
210 avidin/biotinylated-horseradish peroxidase solution (1% dissolved in 0.3% Triton X-100 in PBS,
211 ABC Peroxidase staining kit, ThermoScientific) for 30 minutes and rinsed again for 15 minutes
212 in PBS. Immunoreactivity was visualized using nickel-enhanced DAB solution (0.03%
213 3,3'-diaminobenzidine, 1% cobalt chloride, 1% nickel ammonium sulphate, and 0.035% H₂O₂ in
214 PBS, all from Sigma-Aldrich, St. Louis, MO, USA). Sections were then rinsed, cleared,
215 dehydrated and coverslipped with DPX (Sigma-Aldrich). Specificity of the egr-1 antibody was
216 confirmed by western blot (see below).

217

218 2.2.4. Western blot characterization of anti-egr-1 antibody

219

220 In order to determine whether the egr-1 antibody would bind specifically to the desired antigen
221 in the guppy, the antibody was assayed using protein from four whole guppy brains by
222 radioimmunoprecipitation. Whole brains were homogenized and protein extracted in
223 radioimmunoprecipitation buffer before being diluted at 1:4 with sodium dodecyl sulphate-
224 polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and separated on a SDS-PAGE
225 gel, alongside mouse fibroblast L-cells as a control.

226

227 Whole brain extract on the gel was transferred onto a nitrocellulose membrane overnight. The
228 membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20

229 in Tris-buffered saline (TBS)), incubated in primary antibody (1:1000, anti-egr-1) for 1 hour,
230 washed three times for five minutes each in wash buffer, and then incubated in donkey-anti-
231 rabbit horseradish peroxidase (HRP)-conjugated antibody (1:1000, catalogue number AP182P,
232 EMD Millipore, Hayward, CA, USA) in blocking solution for 2 hours. After washing three times
233 for 5 minutes each with wash buffer, the blots were developed using a chemiluminescence
234 detection reagent (catalogue number WBKLS0500, EMD Millipore), and images were acquired
235 with a 16-bit CCD camera (MicroChemi DNR Bio-imaging Systems). A band was visualized
236 putatively representing egr-1 at the predicted size of 57 kDa, which is the estimated
237 unphosphorylated molecular weight of egr-1 [40–42]. We also assayed a c-fos antibody (Santa
238 Cruz Biotechnology Inc., Santa Cruz, CA, USA), and obtained two bands at 52 and 68 kDa. We
239 therefore did not use c-fos as we would have expected only a single band at 62 kDa [43] if this c-
240 fos antibody was binding specifically to the c-fos antigen in guppy.

241

242 2.2.5. Quantification of neurons expressing egr-1

243

244 Cell nuclei containing egr-1 protein were clearly stained black and were counted using a 20×
245 objective in a microscope (Leica DM1000LED). As no guppy brain atlas is available, we used
246 the brain atlas of the related poeciliid, the swordtail (*Xiphophorus hellerii* [44]) to distinguish the
247 brain areas of interest (Table 1). We took a picture of each brain area of interest in both
248 hemispheres using a digital camera (Leica ICC50HD with the software Leica Application Suite
249 EZ 3.2.1). An observer blind to the experimental treatments processed all images and counted
250 stained nuclei. Images were converted to greyscale to sharpen images and increase contrast using
251 ImageJ 1.50i. A defined oval sampling area that fitted centrally within each brain area of interest

252 was applied to each image (Table 1) and ImageJ was used to count the number and size of
 253 stained nuclei that met minimum size and circularity criteria. The procedure was then repeated
 254 for the other hemisphere. Data on the size of each counted nucleus was then checked to account
 255 for overlapping stained nuclei. The size of each counted nucleus was divided by the size of the
 256 average nucleus. When the quotient of that division was at least 2 (i.e. two times the average size
 257 of a stained nucleus) we considered it to be an overlapping cluster of nuclei and counted it as the
 258 quotient obtained in the division. The ImageJ script used for image processing and all data will
 259 be deposited in the Dryad Digital Repository.
 260

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

261 Table 1: Brain areas studied, their mammalian homologues, the brain network that they belong to
 262 [2,33,34] and the mean size of the oval sampling areas used to count the number of stained
 263 nuclei in each of the four brain areas.
 264

265 2.2.6. Statistical analysis

266

267 After counting the number of neurons in each hemisphere, we calculated the number of neurons
268 per $100 \mu\text{m}^2$ to standardize measurements across brain areas. The number of activated neurons
269 per hemisphere were positively correlated across individuals (Pearson correlations; POA: $r =$
270 0.77 , $n = 30$, $p < 0.001$; Vd: $r = 0.68$, $n = 32$, $p < 0.001$; Vv: $r = 0.58$, $n = 31$, $p = 0.001$; Vs: $r =$
271 0.69 , $n = 29$, $p < 0.001$) supporting the pooling of the counts from the two hemispheres and the
272 reliability of our brain area identification and counts. We analysed the effect of social treatment
273 (ten-fish shoal, two-fish shoal, social isolation) and the interaction of social treatment and brain
274 nuclei (POA, Vs, Vd, Vv) using a linear mixed model (LMM), with brain nuclei as a repeated
275 measure. We ran a one-way ANOVA on neuron counts for each of the brain areas and Tukey
276 post-hoc tests to elucidate differences between treatments. We calculated the effect size for these
277 comparisons (Cohen's d_s) and used the reference effect size values (small: $d > 0.2$, medium: $d >$
278 0.5 , and large: $d > 0.8$) to interpret effect sizes [45]. All data were normally distributed and
279 variances were homogenous. We used the software SPSS 24 for all our analyses.

280

281 2.3. Ethical note

282

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

288

289 **3. Results**

290

291 3.1. Experiment 1: Shoal preference study

292

293 Guppies spent more time close to, and interacted more with the large shoal than the small shoal,
294 with their preference scores significantly greater than 0 (One-sample t-tests; shoaling preference
295 score: $t(29) = 9.46$, $p < 0.001$; interaction preference score: $t(29) = 3.49$, $p = 0.002$; Fig. 3). Fish
296 that shoaled more also spent more time interacting with the shoal ($r = 0.76$, $n = 30$, $p < 0.001$).

297

298 3.2. Experiment 2: Brain activation during shoal exposure test

299

300 We found a significant interaction effect between treatment and brain nuclei (LMM, $F(9, 50) =$
301 7.41 , $p < 0.001$) but no significant overall effect of treatment (LMM, $F(2, 96.18) = 1.88$, $p > 0.1$).
302 Given the significant interaction effect, we examined each brain area individually, finding a
303 difference among treatments in the POA (ANOVA, $F(29, 2) = 4.13$, $p = 0.027$, Fig. 4), with post-
304 hoc tests indicating that the fish exposed to a large shoal had significantly higher activation in
305 this brain region compared to the control (Tukey, $p = 0.021$; $d = 1.18$). There were no significant
306 differences in activation between the fish exposed to a small shoal and the control in the POA, or
307 among treatments in the other brain areas (all $p > 0.1$).

308

309 **4. Discussion**

310

311 We confirmed that our study population of guppies prefer a large over a small shoal, as has been
312 previously demonstrated in guppies, other fish and other vertebrates [20,26,30,31]. This
313 preference is typically explained by anti-predator and foraging advantages for group members
314 [11]. Thus, choosing a large over a small group may be a rewarding action that reinforces
315 adaptive social behaviours. We then studied four brain areas (POA, Vs, Vd, and Vv) of the social
316 decision making network (SDMN) involved in social behaviour in vertebrates [2] and found that
317 only the POA had significantly greater neuronal activation in fish exposed to a large shoal
318 stimulus compared to isolated fish used as a control. There were no significant differences
319 among treatments in the other brain areas examined (Vs, Vd, and Vv).

320

321 The POA is a nucleus located immediately rostral to the hypothalamus along the third ventricle
322 and which has close functional links and connections to the hypothalamus and limbic system. As
323 part of the hypothalamic-pituitary-gonadal axis, the POA is involved in many different
324 reproductive behaviours in fish [46–49], including social aspects such as changes in social status
325 related to reproduction [50,51]. The POA also mediates sexual behaviour in all vertebrate taxa,
326 as well as parental care and aggression in mammals, birds, and teleosts [2]. Thus, its function
327 mediating social behaviour, as well as its neurochemistry, hodology, and topography, are very
328 well conserved among vertebrates [2]. Our finding of higher activation in the POA during
329 grouping is similar to the results of Teles et al. [52] in a more ‘complex’ social context, which
330 found significantly higher *egr-1* expression in the POA when zebrafish were in a mirror test and
331 a winner/loser context compared to isolated fish. They did not find differences between their

332 behavioural treatments, which suggests that the POA might be processing social cues
333 independently of the social situation experienced. Together, these results indicate that the POA is
334 a key component in the processing of social cues in fish, and possibly in all vertebrates. In birds,
335 for example, there is strong evidence that the POA mediates gregariousness via the production
336 and regulation of nonapeptides [53], even though activation of the POA is not significantly
337 different among species with different levels of gregariousness [3].

338

339 The teleost POA has been suggested as the homologue to the mammalian POA and
340 paraventricular nucleus of the hypothalamus [33] because it includes the majority of neurons that
341 produce vasotocin and isotocin, the teleost homologues of mammalian vasopressin and oxytocin
342 and members of the nonapeptide family of neuropeptides that are involved in a wide range of
343 social behaviours [54]. In teleost fish, vasotocin modulates aggressive behaviour [55–57],
344 courtship behaviour [58,59], and behaviour related to establishing a social structure [57,60,61],
345 while isotocin increases submissive behaviour during fights in *Neolamprologus pulcher* [62] and
346 modulates paternal care in monogamous cichlids [63]. However these nonapeptides have also
347 been implicated in simple social grouping behaviour in fish: vasotocin inhibits social approach
348 [14,64,65], and decreases social interactions with a shoal [15], while isotocin stimulates social
349 approach in goldfish [14] and inhibits it in *N. pulcher* [66]. Thus, the increased activation of
350 POA neurons found in our study may reflect increased activity and signalling by nonapeptide
351 neurons, which are located solely in this area of the teleost brain.

352

353 Our results suggest a conserved role for the POA in grouping behaviour. As this area is the key
354 nonapeptide site in the teleost brain, this neuropeptide family may thus be involved, however,

355 other neurochemical systems may also regulate responses to social cues. The POA has been
356 implicated in motivation and drive [67] through the high density of dopaminergic cells and
357 dopamine receptors in the POA and local release of dopamine in response to cues from
358 conspecifics [68,69]. Dopamine is a major mediator of reward and the observed higher activation
359 in response to the large group might represent increased activation of POA dopaminergic
360 neurons in response to the rewarding stimulus of a large group of conspecifics. In this context,
361 the lack of activation in other areas of the SDM in the guppy is somewhat surprising given the
362 clear behavioural responses seen to shoaling stimuli. This is particularly true of the Vd, a
363 putative homologue of the mammalian nucleus accumbens that mediates dopaminergic reward.
364 Visual exposure to conspecifics has been shown to be rewarding [70] but despite subjects in our
365 study showing robust preferences for large shoals, we saw no response in the Vd. This may
366 indicate that social reward is not encoded by Vd dopamine signalling alone [71], but perhaps also
367 reflects the relative paucity of information on functional teleost neuroanatomy, particularly in the
368 guppy. Both dopamine and nonapeptides are good candidates to explain POA responses to social
369 cues in guppies, however, our data only allow us to speculate about the nature of the active POA
370 neurons we observed, and hence further studies are needed to elucidate this question.

371
372 Increased activity in the POA could also be explained as a neuronal response to the greater visual
373 stimulus of multiple individuals swimming in a large shoal, however we consider this unlikely as
374 simple visual information is processed in the optic tectum [72] and the POA is not a consensus
375 part of this circuit. The POA is also involved in vertebrate stress responses, however we consider
376 it unlikely that the increased POA activity is due to stress effects of social exposure. Companion

377 fish have been shown to reduce stress-related behaviour in small shoaling fish [73], and simple
378 visual exposure has been shown to be rewarding for isolated fish [70].
379
380 We were somewhat surprised not to find a significant difference in activation of the POA in fish
381 exposed to a small shoal compared to isolated fish, given shoaling preferences in the guppy, and
382 the confirmed preference for social cues over an empty compartment [21,74]. Our results suggest
383 that more salient social cues than simply the presence of two other guppy females are needed to
384 significantly activate the POA. However, it is worth noting that responses to the small shoal were
385 intermediate to the large shoal and control conditions, consistent with POA activation increasing
386 in step with the size of the social stimulus. We did not find a significant difference between
387 treatments in brain activation in any of the other studied areas. This is similar to the results of
388 Teles et al. [52], who found no differences in *egr-1* expression in Vv and Vs in zebrafish during
389 aggressive and submissive behaviour in a mirror test and a winner/loser context compared to
390 isolated fish. However, they did find increased expression in these and other brain areas when
391 exploring a different immediate early gene, *c-fos*, and suggested functional connectivity between
392 several brain areas of the SDMN, supporting the SDMN hypothesis in teleosts. Similarly,
393 Maruska et al. [9] found increased activation in multiple brain regions in male cichlids
394 (*Astratotilapia burtoni*) that had the opportunity to ascend in social rank. Our results suggest that
395 forms of social behaviour such as grouping, which only require relatively simple social
396 information such as recognition and approach of conspecifics, primarily activate the POA among
397 the brain areas we examined. That said, it is an open question to what extent grouping decisions
398 are simple, with numerous factors involving group choice. For example, guppy shoaling is
399 influenced by cues of predation risk [75], olfactory cues [74], early life exposure to conspecifics

400 [76], groupmates' familiarity [77], activity [20], sex [78], size [79], distance [80], and body
401 colouration [81].

402

403 Future studies are required to examine the neurochemical populations that the activated POA
404 neurons belong to and whether dopamine, nonapeptides, or other neuronal signals are involved in
405 this behaviour in fish. It is also important to consider the possibility of activation in other brain
406 areas that were not the focus of this study and are also involved in social behaviour in vertebrates
407 [2], and so, a more exhaustive study of all the brain areas of the SDMN and the use of additional
408 immediate early genes different from *egr-1* could provide further insights into the neural
409 modulation of grouping behaviour. While gross neuroanatomy is understood, a detailed guppy
410 brain atlas has yet to be published, the detailed connections between nuclei have not been
411 mapped and the functional role of much of the brain is not well understood. As the guppy is a
412 species with an extensive, well understood and experimentally tractable suite of behaviours,
413 addressing this lack of neuroanatomical detail would be of great assistance in exploring the
414 neurobiology of this important species in behavioural and evolutionary biology.

415

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

422 the neurochemical properties of the POA neurons that respond to social cues in the POA of
423 guppies.

424

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439

440 **References**

441

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670

671

672 **Figure captions**

673

674 Figure 1. Shoal preference test. A 75 L tank (76 x 30 x 30 cm, 25 cm water depth) was divided
675 into three compartments. Side compartments (15 x 30 x 30 cm) held either two or ten fish, and
676 were separated from the central compartment containing the subject by perforated transparent
677 plastic partitions. Vertical lines drawn on the front of the central compartment created 5 zones
678 (each 9 cm wide, approximately three to four body lengths) to facilitate recording of the position
679 of the subject. All compartments contained gravel.

680

681 Figure 2. Social exposure test. Each tank (19 L, 40 x 20 x 25 cm) contained gravel, a heater, and
682 a plant attached to an air stone, so that all subjects were exposed to visual motion. The subject
683 fish were inside a perforated transparent plastic cylinder (diameter: 9 cm) placed in the centre of
684 each testing tank. One testing tank was empty and served as control (left), one had two
685 companion fish (centre), and one had 10 companion fish (right). Two sets of these three tanks
686 were used. A transparent plastic lid covered the tanks and opaque barriers separated testing tanks
687 so that fish in each condition could not see other fish.

688

689 Figure 3. Mean \pm SEM time fish spent shoaling and interacting with large shoal versus a small
690 shoal, in a 10 minutes behavioural test. Positive values indicate a preference for the large shoal,
691 and negative values indicate a preference for the small shoal.

692

693 Figure 4. Means \pm SEM of counts of neurons per 100 μm^2 in the four different nuclei (Preoptic
694 Area (POA), Ventral telencephalon – dorsal part (Vd), Ventral pallium (Vs), Ventral

695 telencephalon – ventral part (Vv)) in fish exposed to one of three experimental treatments
696 (Black: control, Grey: fish exposed to a two-fish shoal, White: fish exposed to a ten-fish shoal). *
697 $p < 0.05$.
698

1 **Forebrain activation during social exposure in wild-type guppies**

2

3 María J Cabrera-Álvarez^a, William T Swaney^{a,b}, Simon M Reader^a

4 ^a *Department of Biology, McGill University, 1205 Dr. Penfield Ave., Montreal, Quebec, Canada*

5 ^b *School of Natural Sciences and Psychology, Liverpool John Moores University, Byrom Street,*
6 *Liverpool, United Kingdom*

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8 Keywords: social behaviour, grouping behaviour, social decision-making network, brain
9 activation, teleost fish, guppy (*Poecilia reticulata*).

10

11 **Abstract**

12

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

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

32

33 **1. Introduction**

34

35 The social decision making network (SDMN) is a network of brain nuclei that process social
36 information and reward and which is thought to modulate social behaviour in all vertebrates
37 [1,2]. The SDMN consist of two overlapping brain networks: the social behaviour network
38 (SBN), and the mesolimbic reward system. The SBN includes six interconnected nodes (the
39 preoptic area, anterior and ventromedial hypothalamus, periaqueductal gray, lateral septum, and
40 bed nucleus of the stria terminalis/medial amygdala) that are involved in sexual, aggressive, and
41 parental behaviour across taxa [1,3]. For example, the preoptic area (POA) is involved in sexual
42 behaviour in all vertebrates, as well as aggression and parental care in mammals, birds and fish
43 (reviewed in [2]), and in mammals, the medial amygdala is involved in social recognition [4] and
44 the lateral septum is involved in social affiliation [5] and social recognition [6]. The mesolimbic
45 reward system includes eight interconnected nodes, two of them shared with the SBN (lateral
46 septum, bed nucleus of the stria terminalis/medial amygdala, striatum, nucleus accumbens,

47 ventral pallium, basolateral amygdala, hippocampus, and ventral tegmental area), and influences
48 the SBN by reinforcing adaptive social behaviours via reward [2]. For example, in mammals the
49 striatum is involved in reinforcement learning and selecting previously reinforcing actions
50 (reviewed in [7]). The SDMN is well conserved across vertebrates, albeit with differences in
51 nomenclature between taxa, and several studies in different vertebrates have linked the SDMN to
52 a wide range of social behaviours, such as mate choice [8], hierarchy formation [9], and
53 cooperative nest building [10]. While these and other studies have implicated the SDMN in
54 social behaviours across diverse taxa, it is noteworthy that most research effort has been targeted
55 at ‘complex’ social behaviours and that there has been a comparative lack of research into the
56 neural mechanisms of more fundamental social behaviour such as grouping.

57
58 Grouping is a very common phenomenon which has been the focus of extensive research in
59 behavioural, theoretical and evolutionary biology [11]. Although living in groups carries costs
60 due to potentially increased aggression, competition for resources, or transmission of parasites
61 and diseases, it can also confer benefits to the individual by reducing predation risk, increasing
62 the chances of obtaining food, increasing the opportunities of finding a mate, reducing loss of
63 heat and moisture, or reducing the cost of movement [11]. Despite the importance of this topic,
64 the neural mechanisms of grouping behaviour have received relatively little attention so far.
65 Goodson and colleagues studied the neural mechanisms involved in grouping behaviour in birds
66 and found differences between gregarious and territorial finches in the activation of brain areas
67 of the SDMN [3]. They have also shown that pharmacological manipulation of nonapeptide
68 signalling in the SDMN modulates flocking behaviour in estrildid finches [12,13]. The
69 nonapeptides are a highly conserved family of neuropeptides involved in different intra-SDMN

70 signalling pathways and studies in fish have also shown that manipulation of these nonapeptides
71 has effects on shoaling and simple social approach [14,15]. We wished to address how the
72 SDMN is involved in grouping behaviour and so investigated brain activation in teleost fish in
73 which shoaling conditions and social exposure can be readily manipulated and controlled.

74

75 For our study, we used Trinidadian guppies (*Poecilia reticulata*) as there is extensive research on
76 their shoaling tendencies, both in their natural environments and in laboratory conditions [16].
77 Trinidadian guppies vary in their shoaling tendencies across populations, with median shoal sizes
78 ranging from 1 to 21 individuals [17]. Female guppies form groups to avoid both predation and
79 sneaky mating attempts from male guppies [18]. Males, on the other hand, show a preference for
80 female rather than male shoals, and, like females and juveniles, for larger shoals rather than
81 small ones [19–21], a trait that appears to be widespread across teleost fish (e.g., banded killifish
82 [22,23], Eurasian perch [24], fathead minnows [25], three-spined sticklebacks [26,27], zebrafish
83 [28]), as well as in birds [29,30] and mammals [31,32].

84

85 We conducted two studies to investigate the neural mechanisms underlying grouping behaviour
86 in guppies. We first conducted a behavioural test to confirm subjects' preferences in the studied
87 population for large shoals over small shoals. With a second cohort of fish, we analysed brain
88 activation after a shoaling exposure test in which the subjects were exposed to one of three
89 experimental treatments: a small shoal, a large shoal, or no social exposure. After one hour, the
90 brain of each subject was dissected for immediate early gene assay of neural activation in
91 specific brain regions that are putative components of the SDMN. We expected shoals to act as a
92 social cue and a rewarding stimulus, and hence social exposure would activate areas of both the

93 SBN and the mesolimbic reward system. Thus, we selected brain areas of both networks,
94 specifically the preoptic area (POA), a node of the SBN and suggested homologue of the amniote
95 POA/paraventricular nucleus of the hypothalamus [2,33]; the dorsal part of the ventral
96 telencephalon (Vd), a node of the mesolimbic reward system homologous to the mammalian
97 striatum and nucleus accumbens [2,34]; and two nuclei belonging to both networks, the ventral
98 part of the ventral telencephalon (Vv), and the supracommissural part of the ventral pallium (Vs),
99 homologues of the mammalian lateral septum and amygdala/bed nucleus of the stria terminalis
100 respectively [2,33,34]. We did not add other brain areas of the SDMN to our study because there
101 is no consensus about teleost homologues of the mammalian areas and/or insufficient research on
102 those areas in teleost fish [2]. We hypothesized that grouping behaviour will be modulated by the
103 SDMN and so exposure to shoals would activate the selected brain areas, with greater activation
104 when the subjects were exposed to the large shoal.

105

106 **2. Materials and methods**

107

108 2.1. Experiment 1: Shoal preference study

109

110 2.1.1. Animal subjects and housing

111

112 Subjects were 30 female guppies from mixed populations of wild Trinidadian origin that had
113 been bred in captivity for at least 2 generations (henceforth ‘wild stock guppies’). Two weeks
114 before the experiment started we moved them from 110 L breeding tanks (76 x 30 x 45 cm)

115 containing both sexes to two 19 L housing tanks (40 x 20 x 25 cm) containing only the subjects.
116 We used an additional 12 wild stock female guppies to form a pool from which stimulus shoals
117 were drawn. They were unfamiliar to the subject fish and lived in the test tank (see below). All
118 tanks were kept at 26 ± 1 °C, had a filter and a heater, as well as gravel, plastic plants and a
119 shelter. Fish were fed flake food daily (TetraMin Tropical Flakes, Tetra, Germany) and
120 supplementary decapsulated brine shrimp eggs (*Artemia sp.*, Brine Shrimp Direct, Ogden UT,
121 USA) three times a week.

122

123 2.1.2. Behavioural test

124

125 Females were tested in a 75 L tank divided into three different compartments by perforated
126 transparent plastic partitions. Each side compartment contained a shoal of either two or 10
127 females (Fig. 1). During the testing day, we removed the plants and shelters and counterbalanced
128 the position of the shoals and varied the member composition of each shoal at random. To
129 measure subjects' proximity to the shoals, we drew vertical lines on the front of the tank to
130 divide the central compartment into five zones. The subject was moved to the testing arena in a
131 transparent plastic cup and, after two minutes of acclimation, the cup was gently and remotely
132 raised by the observer by pulling a string attached to the cup. The test started immediately after
133 the subject was released. We measured the amount of time the subject spent on each of the five
134 zones in order to calculate time shoaling with each group (i.e. time within four body lengths
135 [35]), as well as the amount of time the subject spent interacting with the shoal (i.e. swimming
136 head first against the transparent partitions [15]) over 10 minutes, using the software JWatcher

137 V1.0. We measured shoaling time and interaction time as dual estimates of grouping behaviour
138 in fish [15].

139

140 2.1.3. Statistical analysis

141

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

148

149 2.2. Experiment 2: Brain activation during shoal exposure test

150

151 2.2.1. Subjects and housing

152

153 Two weeks before our study started, we moved 60 females and five males to a 110 L housing
154 tank (76 x 30 x 45 cm). Of these, 36 females were used as subjects and the rest were left in the
155 housing tank as companion fish to prevent the subjects from being isolated as subjects were
156 removed from the tank as the study progressed. We also placed 24 wild stock females unfamiliar
157 to the subjects into four testing tanks (Fig. 2), two tanks had ten females forming the large shoal,
158 and the other two tanks had two females forming the small shoal. There were also two control
159 testing tanks without fish in them. Two weeks before the start of the study, we placed a

160 perforated transparent cylindrical plastic container with gravel in the middle of the testing tanks
161 to habituate the shoals to it. This container held the subject fish during the exposure test,
162 exposing them to the shoal but preventing them from interacting directly with other fish; this
163 ensured consistent exposure to stimulus shoals across subjects. A transparent plastic lid covered
164 the tank to prevent fish from jumping out. Housing conditions and feeding were the same as
165 Experiment 1. The day prior to the test, we isolated 12 subjects in separate 10 L tanks (30 x 20 x
166 15 cm) containing gravel, a plastic plant, a heater (keeping the water at 26 ± 1 °C) and an air
167 stone. The purpose of this isolation period was to set a consistent baseline of neural activity in all
168 subjects.

169

170 2.2.2. Social exposure test

171

172 On the day of the test we removed the filter and plastic plant from the experimental tank, and
173 added an air stone with a plastic plant attached to it. The air stone made the plant move, which
174 served as a control for any neural activation generated by movement, meaning that any
175 differences between treatments would be due to olfactory and/or visual exposure to the social
176 stimulus. Twenty minutes later we caught an isolated subject and placed it at random in the
177 plastic container of a testing tank containing either a large shoal, a small shoal, or no shoal
178 (control), where it was exposed to that social stimulus for an hour (Fig. 2). We monitored the
179 behaviour of the subject and companion fish and observed similarities with the behaviour
180 observed in Experiment 1: subjects appeared highly interested in the stimulus fish and spent
181 much of the exposure period attending to the stimulus fish and attempting to swim to them.
182 Although a 30 minute period has been suggested for induction of the highest expression of *egr-1*

183 in teleost fish [36], we exposed the subjects to the treatment for an hour to ensure that the brain
184 activation we observed was due to the treatment and not just due to handling and tank changing.
185 After this period, we caught the subjects and euthanized them by rapid cooling through
186 immersion in ice water [37–39]. Control tanks were emptied, rinsed and re-filled with
187 conditioned water before adding each new subject to eliminate any olfactory cues left by the
188 previous subject.

189

190 2.2.3. Immunohistochemistry (IHC) staining of egr-1

191

192 Brains were dissected out immediately after euthanasia, fixed in 4% paraformaldehyde at 4 °C
193 overnight, and then cryoprotected in 30% sucrose overnight at 4 °C before embedding in Clear
194 Frozen Section Compound (VWR International, PA, USA) and storage at -19 °C. Brains were
195 then sectioned on a cryostat at 25 µm and thaw-mounted onto Superfrost Plus slides (VWR
196 International) in two parallel series that were stored at -19 °C for less than a week before
197 processing for IHC.

198

199 One of the two series of sections was thawed and air-dried before processing for
200 immunohistochemical detection of egr-1. Sections were rinsed in 0.1M Phosphate-buffered
201 saline (PBS) for 15 minutes. After blocking for 1 hour in blocking solution (5% normal goat
202 serum and 0.3% Triton X-100 in PBS) and rinsing in PBS for 10 minutes, sections were
203 incubated in primary antibody (anti-egr-1 rabbit polyclonal, 1:1000, catalogue number sc-189;
204 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) dissolved in blocking solution at 4°C
205 overnight. Sections were then rinsed in PBS, incubated for 15 minutes in H₂O₂ solution (3.5 %

206 H₂O₂, 8.8% methanol dissolved in 0.3% Triton X-100 in PBS), and rinsed again in PBS. Sections
207 were then incubated in a biotinylated goat anti-rabbit secondary antibody solution (1:200,
208 ThermoScientific, Rockford, IL, USA) dissolved in blocking solution for 30 min at room
209 temperature, and rinsed again for 15 minutes in PBS. Sections were then washed in
210 avidin/biotinylated-horseradish peroxidase solution (1% dissolved in 0.3% Triton X-100 in PBS,
211 ABC Peroxidase staining kit, ThermoScientific) for 30 minutes and rinsed again for 15 minutes
212 in PBS. Immunoreactivity was visualized using nickel-enhanced DAB solution (0.03%
213 3,3'-diaminobenzidine, 1% cobalt chloride, 1% nickel ammonium sulphate, and 0.035% H₂O₂ in
214 PBS, all from Sigma-Aldrich, St. Louis, MO, USA). Sections were then rinsed, cleared,
215 dehydrated and coverslipped with DPX (Sigma-Aldrich). Specificity of the egr-1 antibody was
216 confirmed by western blot (see below).

217

218 2.2.4. Western blot characterization of anti-egr-1 antibody

219

220 In order to determine whether the egr-1 antibody would bind specifically to the desired antigen
221 in the guppy, the antibody was assayed using protein from four whole guppy brains by
222 radioimmunoprecipitation. Whole brains were homogenized and protein extracted in
223 radioimmunoprecipitation buffer before being diluted at 1:4 with sodium dodecyl sulphate-
224 polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and separated on a SDS-PAGE
225 gel, alongside mouse fibroblast L-cells as a control.

226

227 Whole brain extract on the gel was transferred onto a nitrocellulose membrane overnight. The
228 membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20

229 in Tris-buffered saline (TBS)), incubated in primary antibody (1:1000, anti-egr-1) for 1 hour,
230 washed three times for five minutes each in wash buffer, and then incubated in donkey-anti-
231 rabbit horseradish peroxidase (HRP)-conjugated antibody (1:1000, catalogue number AP182P,
232 EMD Millipore, Hayward, CA, USA) in blocking solution for 2 hours. After washing three times
233 for 5 minutes each with wash buffer, the blots were developed using a chemiluminescence
234 detection reagent (catalogue number WBKLS0500, EMD Millipore), and images were acquired
235 with a 16-bit CCD camera (MicroChemi DNR Bio-imaging Systems). A band was visualized
236 putatively representing egr-1 at the predicted size of 57 kDa, which is the estimated
237 unphosphorylated molecular weight of egr-1 [40–42]. We also assayed a c-fos antibody (Santa
238 Cruz Biotechnology Inc., Santa Cruz, CA, USA), and obtained two bands at 52 and 68 kDa. We
239 therefore did not use c-fos as we would have expected only a single band at 62 kDa [43] if this c-
240 fos antibody was binding specifically to the c-fos antigen in guppy.

241

242 2.2.5. Quantification of neurons expressing egr-1

243

244 Cell nuclei containing egr-1 protein were clearly stained black and were counted using a 20×
245 objective in a microscope (Leica DM1000LED). As no guppy brain atlas is available, we used
246 the brain atlas of the related poeciliid, the swordtail (*Xiphophorus hellerii* [44]) to distinguish the
247 brain areas of interest (Table 1). We took a picture of each brain area of interest in both
248 hemispheres using a digital camera (Leica ICC50HD with the software Leica Application Suite
249 EZ 3.2.1). An observer blind to the experimental treatments processed all images and counted
250 stained nuclei. Images were converted to greyscale to sharpen images and increase contrast using
251 ImageJ 1.50i. A defined oval sampling area that fitted centrally within each brain area of interest

252 was applied to each image (Table 1) and ImageJ was used to count the number and size of
 253 stained nuclei that met minimum size and circularity criteria. The procedure was then repeated
 254 for the other hemisphere. Data on the size of each counted nucleus was then checked to account
 255 for overlapping stained nuclei. The size of each counted nucleus was divided by the size of the
 256 average nucleus. When the quotient of that division was at least 2 (i.e. two times the average size
 257 of a stained nucleus) we considered it to be an overlapping cluster of nuclei and counted it as the
 258 quotient obtained in the division. The ImageJ script used for image processing and all data will
 259 be deposited in the Dryad Digital Repository.

260

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

261 Table 1: Brain areas studied, their mammalian homologues, the brain network that they belong to
 262 [2,33,34] and the mean size of the oval sampling areas used to count the number of stained
 263 nuclei in each of the four brain areas.

264

265 2.2.6. Statistical analysis

266

267 After counting the number of neurons in each hemisphere, we calculated the number of neurons
268 per 100 μm^2 to standardize measurements across brain areas. The number of activated neurons
269 per hemisphere were positively correlated across individuals (Pearson correlations; POA: $r =$
270 0.77 , $n = 30$, $p < 0.001$; Vd: $r = 0.68$, $n = 32$, $p < 0.001$; Vv: $r = 0.58$, $n = 31$, $p = 0.001$; Vs: $r =$
271 0.69 , $n = 29$, $p < 0.001$) supporting the pooling of the counts from the two hemispheres and the
272 reliability of our brain area identification and counts. We analysed the effect of social treatment
273 (ten-fish shoal, two-fish shoal, social isolation) and the interaction of social treatment and brain
274 nuclei (POA, Vs, Vd, Vv) using a linear mixed model (LMM), with brain nuclei as a repeated
275 measure. We ran a one-way ANOVA on neuron counts for each of the brain areas and Tukey
276 post-hoc tests to elucidate differences between treatments. We calculated the effect size for these
277 comparisons (Cohen's d_s) and used the reference effect size values (small: $d > 0.2$, medium: $d >$
278 0.5 , and large: $d > 0.8$) to interpret effect sizes [45]. All data were normally distributed and
279 variances were homogenous. We used the software SPSS 24 for all our analyses.

280

281 2.3. Ethical note

282

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

288

289 **3. Results**

290

291 3.1. Experiment 1: Shoal preference study

292

293 Guppies spent more time close to, and interacted more with the large shoal than the small shoal,
294 with their preference scores significantly greater than 0 (One-sample t-tests; shoaling preference
295 score: $t(29) = 9.46$, $p < 0.001$; interaction preference score: $t(29) = 3.49$, $p = 0.002$; Fig. 3). Fish
296 that shoaled more also spent more time interacting with the shoal ($r = 0.76$, $n = 30$, $p < 0.001$).

297

298 3.2. Experiment 2: Brain activation during shoal exposure test

299

300 We found a significant interaction effect between treatment and brain nuclei (LMM, $F(9, 50) =$
301 7.41 , $p < 0.001$) but no significant overall effect of treatment (LMM, $F(2, 96.18) = 1.88$, $p > 0.1$).
302 Given the significant interaction effect, we examined each brain area individually, finding a
303 difference among treatments in the POA (ANOVA, $F(29, 2) = 4.13$, $p = 0.027$, Fig. 4), with post-
304 hoc tests indicating that the fish exposed to a large shoal had significantly higher activation in
305 this brain region compared to the control (Tukey, $p = 0.021$; $d = 1.18$). There were no significant
306 differences in activation between the fish exposed to a small shoal and the control in the POA, or
307 among treatments in the other brain areas (all $p > 0.1$).

308

309 **4. Discussion**

310

311 We confirmed that our study population of guppies prefer a large over a small shoal, as has been
312 previously demonstrated in guppies, other fish and other vertebrates [20,26,30,31]. This
313 preference is typically explained by anti-predator and foraging advantages for group members
314 [11]. Thus, choosing a large over a small group may be a rewarding action that reinforces
315 adaptive social behaviours. We then studied four brain areas (POA, Vs, Vd, and Vv) of the social
316 decision making network (SDMN) involved in social behaviour in vertebrates [2] and found that
317 only the POA had significantly greater neuronal activation in fish exposed to a large shoal
318 stimulus compared to isolated fish used as a control. There were no significant differences
319 among treatments in the other brain areas examined (Vs, Vd, and Vv).

320

321 The POA is a nucleus located immediately rostral to the hypothalamus along the third ventricle
322 and which has close functional links and connections to the hypothalamus and limbic system. As
323 part of the hypothalamic-pituitary-gonadal axis, the POA is involved in many different
324 reproductive behaviours in fish [46–49], including social aspects such as changes in social status
325 related to reproduction [50,51]. The POA also mediates sexual behaviour in all vertebrate taxa,
326 as well as parental care and aggression in mammals, birds, and teleosts [2]. Thus, its function
327 mediating social behaviour, as well as its neurochemistry, hodology, and topography, are very
328 well conserved among vertebrates [2]. Our finding of higher activation in the POA during
329 grouping is similar to the results of Teles et al. [52] in a more ‘complex’ social context, which
330 found significantly higher *egr-1* expression in the POA when zebrafish were in a mirror test and
331 a winner/loser context compared to isolated fish. They did not find differences between their

332 behavioural treatments, which suggests that the POA might be processing social cues
333 independently of the social situation experienced. Together, these results indicate that the POA is
334 a key component in the processing of social cues in fish, and possibly in all vertebrates. In birds,
335 for example, there is strong evidence that the POA mediates gregariousness via the production
336 and regulation of nonapeptides [53], even though activation of the POA is not significantly
337 different among species with different levels of gregariousness [3].

338
339 The teleost POA has been suggested as the homologue to the mammalian POA and
340 paraventricular nucleus of the hypothalamus [33] because it includes the majority of neurons that
341 produce vasotocin and isotocin, the teleost homologues of mammalian vasopressin and oxytocin
342 and members of the nonapeptide family of neuropeptides that are involved in a wide range of
343 social behaviours [54]. In teleost fish, vasotocin modulates aggressive behaviour [55–57],
344 courtship behaviour [58,59], and behaviour related to establishing a social structure [57,60,61],
345 while isotocin increases submissive behaviour during fights in *Neolamprologus pulcher* [62] and
346 modulates paternal care in monogamous cichlids [63]. However these nonapeptides have also
347 been implicated in simple social grouping behaviour in fish: vasotocin inhibits social approach
348 [14,64,65], and decreases social interactions with a shoal [15], while isotocin stimulates social
349 approach in goldfish [14] and inhibits it in *N. pulcher* [66]. Thus, the increased activation of
350 POA neurons found in our study may reflect increased activity and signalling by nonapeptide
351 neurons, which are located solely in this area of the teleost brain.

352
353 Our results suggest a conserved role for the POA in grouping behaviour. As this area is the key
354 nonapeptide site in the teleost brain, this neuropeptide family may thus be involved, however,

355 other neurochemical systems may also regulate responses to social cues. The POA has been
356 implicated in motivation and drive [67] through the high density of dopaminergic cells and
357 dopamine receptors in the POA and local release of dopamine in response to cues from
358 conspecifics [68,69]. Dopamine is a major mediator of reward and the observed higher activation
359 in response to the large group might represent increased activation of POA dopaminergic
360 neurons in response to the rewarding stimulus of a large group of conspecifics. In this context,
361 the lack of activation in other areas of the SDMN in the guppy is somewhat surprising given the
362 clear behavioural responses seen to shoaling stimuli. This is particularly true of the Vd, a
363 putative homologue of the mammalian nucleus accumbens that mediates dopaminergic reward.
364 Visual exposure to conspecifics has been shown to be rewarding [70] but despite subjects in our
365 study showing robust preferences for large shoals, we saw no response in the Vd. This may
366 indicate that social reward is not encoded by Vd dopamine signalling alone [71], but perhaps also
367 reflects the relative paucity of information on functional teleost neuroanatomy, particularly in the
368 guppy. Both dopamine and nonapeptides are good candidates to explain POA responses to social
369 cues in guppies, however, our data only allow us to speculate about the nature of the active POA
370 neurons we observed, and hence further studies are needed to elucidate this question.

371

372 Increased activity in the POA could also be explained as a neuronal response to the greater visual
373 stimulus of multiple individuals swimming in a large shoal, however we consider this unlikely as
374 simple visual information is processed in the optic tectum [72] and the POA is not a consensus
375 part of this circuit. The POA is also involved in vertebrate stress responses, however we consider
376 it unlikely that the increased POA activity is due to stress effects of social exposure. Companion

377 fish have been shown to reduce stress-related behaviour in small shoaling fish [73], and simple
378 visual exposure has been shown to be rewarding for isolated fish [70].
379
380 We were somewhat surprised not to find a significant difference in activation of the POA in fish
381 exposed to a small shoal compared to isolated fish, given shoaling preferences in the guppy, and
382 the confirmed preference for social cues over an empty compartment [21,74]. Our results suggest
383 that more salient social cues than simply the presence of two other guppy females are needed to
384 significantly activate the POA. However, it is worth noting that responses to the small shoal were
385 intermediate to the large shoal and control conditions, consistent with POA activation increasing
386 in step with the size of the social stimulus. We did not find a significant difference between
387 treatments in brain activation in any of the other studied areas. This is similar to the results of
388 Teles et al. [52], who found no differences in *egr-1* expression in Vv and Vs in zebrafish during
389 aggressive and submissive behaviour in a mirror test and a winner/loser context compared to
390 isolated fish. However, they did find increased expression in these and other brain areas when
391 exploring a different immediate early gene, *c-fos*, and suggested functional connectivity between
392 several brain areas of the SDMN, supporting the SDMN hypothesis in teleosts. Similarly,
393 Maruska et al. [9] found increased activation in multiple brain regions in male cichlids
394 (*Astratotilapia burtoni*) that had the opportunity to ascend in social rank. Our results suggest that
395 forms of social behaviour such as grouping, which only require relatively simple social
396 information such as recognition and approach of conspecifics, primarily activate the POA among
397 the brain areas we examined. That said, it is an open question to what extent grouping decisions
398 are simple, with numerous factors involving group choice. For example, guppy shoaling is
399 influenced by cues of predation risk [75], olfactory cues [74], early life exposure to conspecifics

400 [76], groupmates' familiarity [77], activity [20], sex [78], size [79], distance [80], and body
401 colouration [81].

402

403 Future studies are required to examine the neurochemical populations that the activated POA
404 neurons belong to and whether dopamine, nonapeptides, or other neuronal signals are involved in
405 this behaviour in fish. It is also important to consider the possibility of activation in other brain
406 areas that were not the focus of this study and are also involved in social behaviour in vertebrates
407 [2], and so, a more exhaustive study of all the brain areas of the SDMN and the use of additional
408 immediate early genes different from *egr-1* could provide further insights into the neural
409 modulation of grouping behaviour. While gross neuroanatomy is understood, a detailed guppy
410 brain atlas has yet to be published, the detailed connections between nuclei have not been
411 mapped and the functional role of much of the brain is not well understood. As the guppy is a
412 species with an extensive, well understood and experimentally tractable suite of behaviours,
413 addressing this lack of neuroanatomical detail would be of great assistance in exploring the
414 neurobiology of this important species in behavioural and evolutionary biology.

415

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

422 the neurochemical properties of the POA neurons that respond to social cues in the POA of
423 guppies.

424

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432

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439

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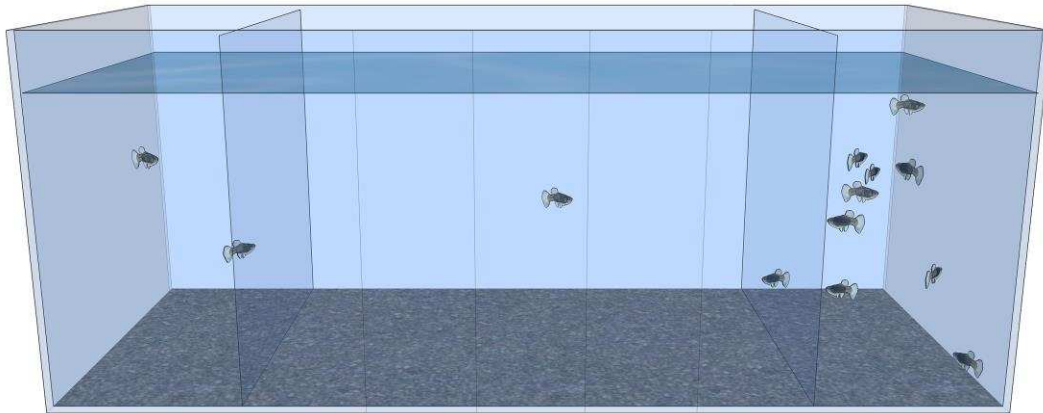
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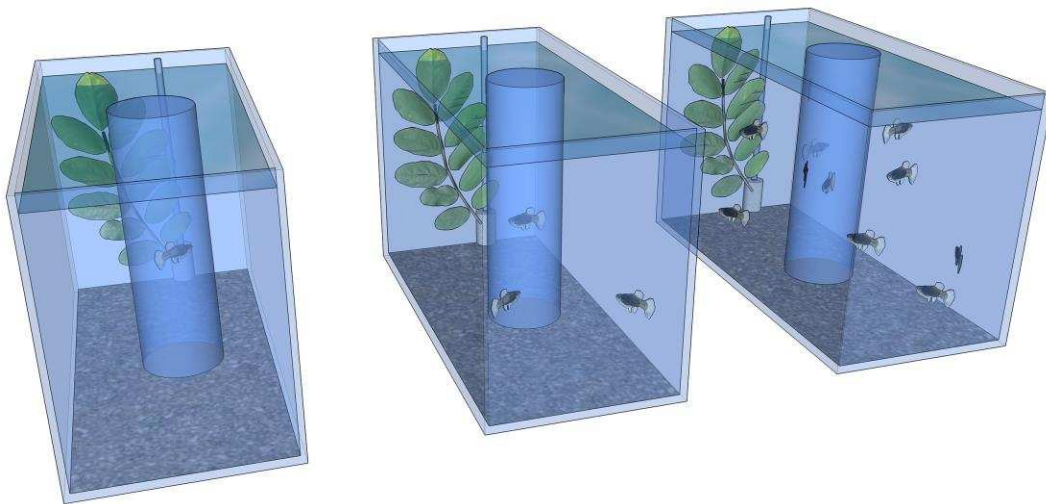
672 **Figures**

673



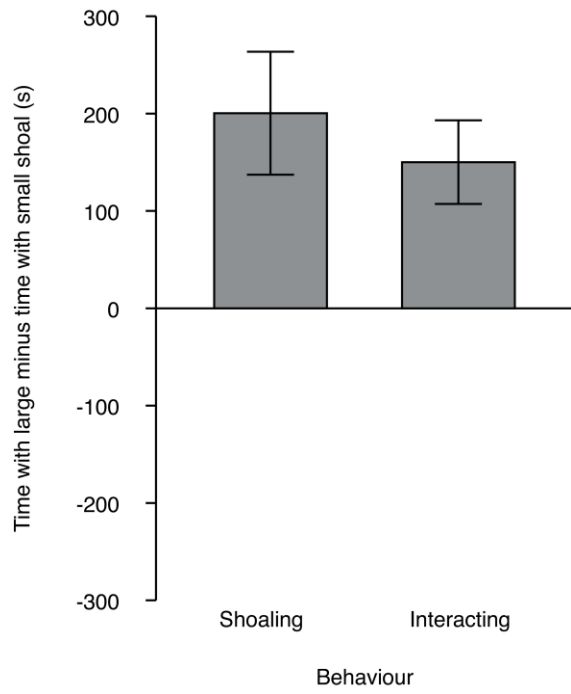
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675 Figure 1 – suggested final size: 1.5 column



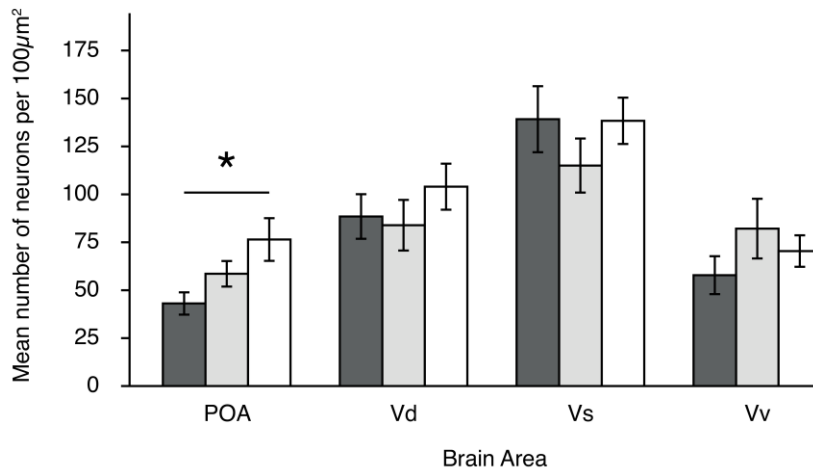
676

677 Figure 2 – suggested final size: 1.5 column



678

679 Figure 3 – suggested final size: 1 column



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681 Figure 4 – suggested final size: 1 column

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683

684 **Figure captions**

685

686 Figure 1. Shoal preference test. A 75 L tank (76 x 30 x 30 cm, 25 cm water depth) was divided
687 into three compartments. Side compartments (15 x 30 x 30 cm) held either two or ten fish, and
688 were separated from the central compartment containing the subject by perforated transparent
689 plastic partitions. Vertical lines drawn on the front of the central compartment created 5 zones
690 (each 9 cm wide, approximately three to four body lengths) to facilitate recording of the position
691 of the subject. All compartments contained gravel.

692

693 Figure 2. Social exposure test. Each tank (19 L, 40 x 20 x 25 cm) contained gravel, a heater, and
694 a plant attached to an air stone, so that all subjects were exposed to visual motion. The subject
695 fish were inside a perforated transparent plastic cylinder (diameter: 9 cm) placed in the centre of
696 each testing tank. One testing tank was empty and served as control (left), one had two
697 companion fish (centre), and one had 10 companion fish (right). Two sets of these three tanks
698 were used. A transparent plastic lid covered the tanks and opaque barriers separated testing tanks
699 so that fish in each condition could not see other fish.

700

701 Figure 3. Mean \pm SEM time fish spent shoaling and interacting with large shoal versus a small
702 shoal, in a 10 minutes behavioural test. Positive values indicate a preference for the large shoal,
703 and negative values indicate a preference for the small shoal.

704

705 Figure 4. Means \pm SEM of counts of neurons per 100 μm^2 in the four different nuclei (Preoptic
706 Area (POA), Ventral telencephalon – dorsal part (Vd), Ventral pallium (Vs), Ventral

707 telencephalon – ventral part (Vv)) in fish exposed to one of three experimental treatments
708 (Black: control, Grey: fish exposed to a two-fish shoal, White: fish exposed to a ten-fish shoal). *
709 $p < 0.05$.
710