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Forebrain activation during social exposure in wild-type guppies

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

Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Experiment 1: Shoal preference study

2.1.1. Animal subjects and housing

Subjects were 30 female guppies from mixed populations of wild Trinidadian origin that had been bred in captivity for at least 2 generations (henceforth ‘wild stock guppies’). Two weeks 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

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

2.1.3. Statistical analysis

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

2.2. Experiment 2: Brain activation during shoal exposure test

2.2.1. Subjects and housing

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

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

2.2.2. Social exposure test

On the day of the test we removed the filter and plastic plant from the experimental tank, and added an air stone with a plastic plant attached to it. The air stone made the plant move, which served as a control for any neural activation generated by movement, meaning that any differences between treatments would be due to olfactory and/or visual exposure to the social stimulus. Twenty minutes later we caught an isolated subject and placed it at random in the plastic container of a testing tank containing either a large shoal, a small shoal, or no shoal (control), where it was exposed to that social stimulus for an hour (Fig. 2). We monitored the behaviour of the subject and companion fish and observed similarities with the behaviour observed in Experiment 1: subjects appeared highly interested in the stimulus fish and spent much of the exposure period attending to the stimulus fish and attempting to swim to them. 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 H_2O_2 solution (3.5 %

206 H_2O_2, 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% H_2O_2 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

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

2.2.5. Quantification of neurons expressing egr-1

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

3. Results

3.1. Experiment 1: Shoal preference study

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

3.2. Experiment 2: Brain activation during shoal exposure test

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

4. Discussion

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

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

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

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

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

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

Increased activity in the POA could also be explained as a neuronal response to the greater visual stimulus of multiple individuals swimming in a large shoal, however we consider this unlikely as simple visual information is processed in the optic tectum [72] and the POA is not a consensus part of this circuit. The POA is also involved in vertebrate stress responses, however we consider 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

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

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

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

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

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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 ± 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 ± 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

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

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Forebrain activation during social exposure in wild-type guppies

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

Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Experiment 1: Shoal preference study

2.1.1. Animal subjects and housing

Subjects were 30 female guppies from mixed populations of wild Trinidadian origin that had been bred in captivity for at least 2 generations (henceforth ‘wild stock guppies’). Two weeks 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

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

2.1.3. Statistical analysis

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

2.2. Experiment 2: Brain activation during shoal exposure test

2.2.1. Subjects and housing

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

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

2.2.2. Social exposure test

On the day of the test we removed the filter and plastic plant from the experimental tank, and added an air stone with a plastic plant attached to it. The air stone made the plant move, which served as a control for any neural activation generated by movement, meaning that any differences between treatments would be due to olfactory and/or visual exposure to the social stimulus. Twenty minutes later we caught an isolated subject and placed it at random in the plastic container of a testing tank containing either a large shoal, a small shoal, or no shoal (control), where it was exposed to that social stimulus for an hour (Fig. 2). We monitored the behaviour of the subject and companion fish and observed similarities with the behaviour observed in Experiment 1: subjects appeared highly interested in the stimulus fish and spent much of the exposure period attending to the stimulus fish and attempting to swim to them. 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 H_2O_2 solution (3.5 %

206 H_2O_2, 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% H_2O_2 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

2.2.6. Statistical analysis

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

2.3. Ethical note

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

3. Results

3.1. Experiment 1: Shoal preference study

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

3.2. Experiment 2: Brain activation during shoal exposure test

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

4. Discussion

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

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

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

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

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

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

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

fish have been shown to reduce stress-related behaviour in small shoaling fish [73], and simple visual exposure has been shown to be rewarding for isolated fish [70].

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

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

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

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

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

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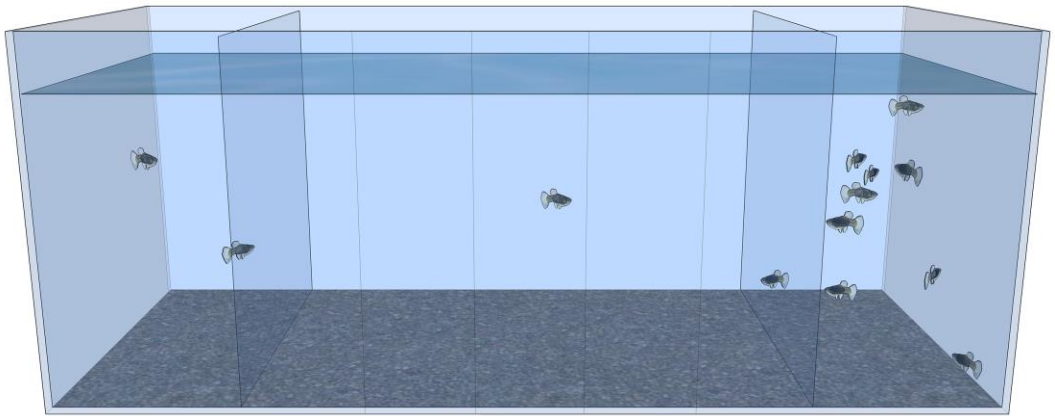
671

672 **Figures**

673



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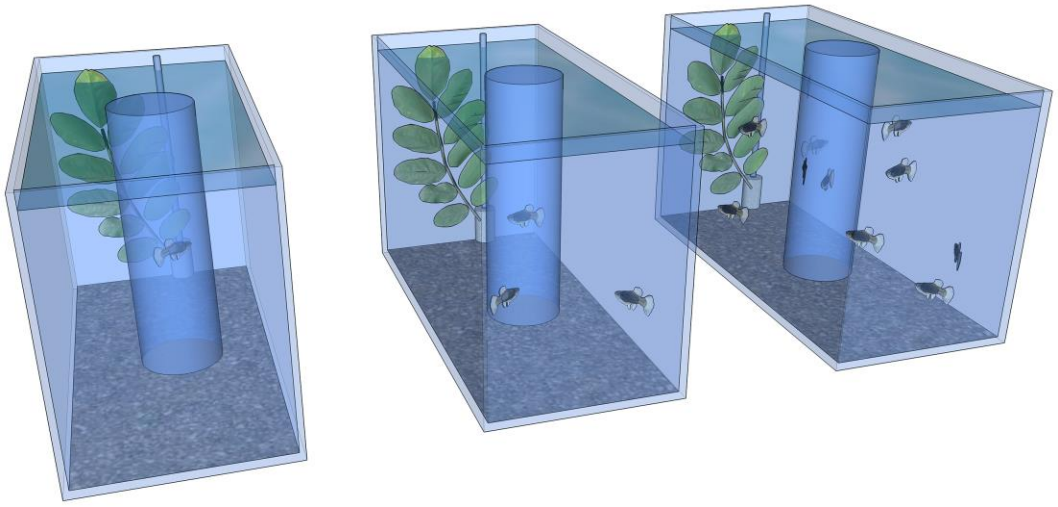
675 Figure 1 – suggested final size: 1.5 column



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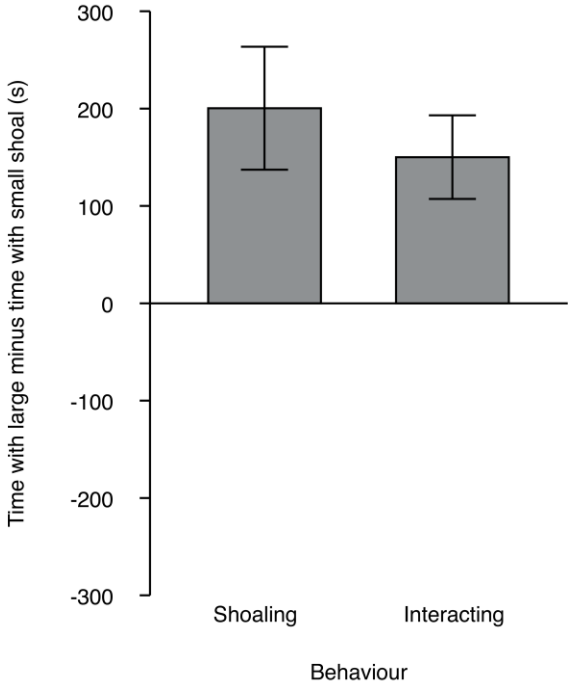

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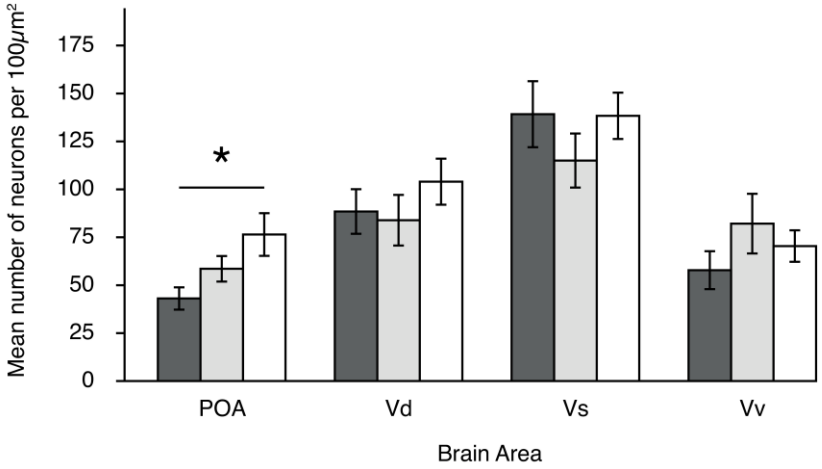

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681 Figure 4 – suggested final size: 1 column

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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 ± 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 ± 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

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