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Male zebrafish (*Danio rerio*) do not preferentially associate with familiar over unfamiliar conspecifics

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Abstract

Members of several shoaling species have been shown to prefer to associate with familiar individuals, enhancing the benefits of aggregation. We employed a series of social preference tasks in the laboratory to evaluate whether prior familiarity with potential partners influences preference of shoaling partner in male zebrafish (*Danio rerio*), a social species found in shallow, slow-moving waters. We found that though male zebrafish exhibited a strong preference for shoaling with a male conspecific as opposed to remaining alone, they exhibited no preference for familiar over unfamiliar conspecifics. This suggests that the benefits of familiarity for shoaling behaviour may not be as important for male zebrafish as has been shown in other social fish species.

Introduction

A wide range of fish species form social aggregations that confer various advantages to the individual members. Groups of fish often respond collectively to the threat of predation, increasing each individual's chances of

36 survival (Gerlotto *et al.*, 2006; Treherne & Foster, 1981; Webster & Laland,

37 2013). Collective decision making enables groups of fish to make better

38 decisions than lone individuals in predator avoidance (Ward *et al.*, 2008,

39 2011). In foraging contexts, for example, groups can follow noisy or faint

40 resource gradients more effectively (Grünbaum, 1998), individuals of some

41 species can learn from each other to optimize foraging (Kendal *et al.*, 2009;

42 Pike & Laland, 2010), and some piscivorous species even engage in

43 cooperative hunting (Arnegard & Carlson, 2005; Hiatt & Brock, 1948; Schmitt

44 & Strand, 1982; Strübin *et al.*, 2011; Vail *et al.*, 2013).

45   The benefits of shoaling often depend on which individuals shoal

46 together. In many species of fish, individuals prefer shoaling with

47 conspecifics of the same body size, so that no individuals stand out as

48 targets for predation (e.g. Croft *et al.*, 2005; Krause *et al.*, 2000). However,

49 individual shoaling preferences may not be beneficial to the group as a

50 whole. For example, Metcalfe and Thompson (1995) showed that European

51 minnows *Phoxinus phoxinus* (L. 1758) prefer to shoal with poor competitors

52 to enhance their own foraging success. On the other hand, guppies *Poecilia*

53 *reticulata* Peters 1859 have been shown to prefer to shoal with more

54 cooperative individuals (Dugatkin & Alfieri, 1991), presumably to reap the

55 benefits provided by cooperators, whether in predator inspection or in

56 conspicuously signaling to predators that they have been detected and have

57 nothing to gain by continuing the pursuit (Hasson, 1991; Smythe, 1970;

58 Woodland *et al.*, 1980). Preferences for associating with familiar individuals,

59 as seen in guppies (Magurran *et al.*, 1994) and three-spined sticklebacks

60 *Gasterosteus aculeatus* L. (Barber & Ruxton, 2000), can also enable

61 cooperation. For example, Chivers *et al.* (1995) showed that shoals of

62 familiar fathead minnows *Pimephales promelas* Rafinesque 1820 have a

63 more cooperative and cohesive anti-predator response than shoals of

64 unfamiliar individuals, and three-spined sticklebacks have been found to

65 exhibit less prey competition (Webster & Hart, 2007) and have increased

66 foraging success (Ward & Hart, 2005) with familiar individuals. However, that

67 is not always the case; the decrease in aggression between familiar male

68 guppies disappears when the costs of cooperation increase (Granroth-

69 Wilding & Magurran, 2013) and female cleaner fish *Labroides dimidiatus*

70 (Valenciennes 1839) are more cooperative with unfamiliar males due to

71 increased risk of punishment (Raihani *et al.*, 2012).

72   Less is known about the shoaling preferences of zebrafish *Danio rerio*

73 (Hamilton 1822), small, social fish that inhabit shallow, slow-moving waters.

74 They shoal innately, which is believed to be primarily a defense against

75 predation (Wright *et al.*, 2006), though they also prefer to shoal with well-fed

76 conspecifics for increased foraging success (Krause *et al.*, 1999). Zebrafish

77 have been shown to prefer to shoal with larger conspecifics over smaller

78 ones (Aslanzadeh *et al.*, 2019; Fernandes *et al.*, 2015) and express

79 preferences based on species (Saverino & Gerlia, 2008; Snekser *et al.*, 2010),

80 body shape, and colour pattern (Saverino & Gerlia, 2008). In particular,

81 zebrafish exhibit a learned preference for species and colour pattern based

82 on early experience (Engeszer *et al.*, 2004; Spence *et al.*, 2008). Similarly,

83 Gerlach *et al.* (2008) found that exposure to kin early in development is

84 necessary for juvenile zebrafish to form a preference for the odor of

85 unfamiliar related individuals. Juvenile zebrafish additionally prefer the odor

86 of familiar kin over unfamiliar kin (Gerlach & Lysiak, 2006), but it is unclear

87 whether this reflects a preference for familiar individuals *per se*, or simply a

88 preference for shoaling with kin that is enhanced by familiarity. Outside of

89 the context of kinship, it has recently been shown that adult zebrafish of

90 both sexes are capable of social recognition, as they spend more time

91 inspecting novel individuals over those they have seen before (Ariyasiri *et*

92 *al.*, 2019; Norton *et al.*, 2019; Ribeiro *et al.*, 2020), and that males can still

93 distinguish familiar individuals even after 24 hours of separation (Madeira &

94 Oliveira, 2017). However, in all of the aforementioned studies on novelty,

95 familiarity was only acquired over at most 30 minutes, much less than the 12

96 days which has been found to be necessary for association preferences

97 based on familiarity to form in guppies (Griffiths & Magurran, 1997a). To our

98 knowledge, no study has evaluated whether adult zebrafish actually prefer to

99 associate with familiar partners when familiarity is able to develop over a

100 substantial time period.

101   In this study we evaluate whether male zebrafish prefer to associate

102   with familiar over entirely unfamiliar male shoalmates. A preference for

103   familiar individuals could enable cooperation in zebrafish as it does in

104   sticklebacks, minnows, and guppies (Chivers *et al.*, 1995; Croft *et al.*, 2006;

105   Milinski *et al.*, 1990) and could even promote reciprocity, as has been found

106   in guppies (Dugatkin & Alfieri, 1991).

107

108   **Materials and methods**

109

110   *Study animals*

111

112   We used adult male zebrafish purchased from Carolina Biological

113   Supply Company (Burlington, North Carolina, USA). We allocated 150

114   individual fish into seven 10 gallon home tanks that held between 15 and 20

115   individuals for familiarization (see Griffiths & Magurran, 1997b). Fish were

116   acclimated to their home tanks for 21 to 34 days prior to experiments to

117   ensure familiarity (see Griffiths & Magurran, 1997a). Tanks were separated

118   by opaque barriers so that the fish in each tank were visually and olfactorally

119   isolated from fish in neighboring tanks and as a result were only familiar with

120   the fish in their home tank. It is unlikely that fish within versus between

121   home tanks differed systematically in relatedness because they were raised

122   in ponds containing 25,000-40,000 individuals, and Carolina Biological

123   Supply comingles fish from different ponds in their holding tanks prior to

124   shipping orders (Jeff Bernia, Carolina Biological Supply Company, personal

125   communication, September 2019). The zebrafish were kept on a 14L:10D

126   cycle and were fed TetraMin Tropical Flakes (Tetra GMBH, Melle, Germany)

127   twice a day. Individual fish were used in only one trial each, after which they

128   were not returned to their home tank.

129

130   Ethical Statement

131   The care and use of experimental animals complied with United States

132   animal welfare laws, guidelines and policies as approved by the Oberlin

133   College Institutional Animal Care and Use Committee (S16TBKT-4).

134

135   *Experimental procedure*

136

137   The study consisted of two experiments, one to confirm that male

138   zebrafish prefer to shoal with male conspecifics over being alone (as is well

139   established; Ogi *et al.*, 2021), and a second to test for preferences for

140   shoaling with familiar or unfamiliar individuals.

141   For both experiments, each trial took place in a 38 cm X 23 cm

142   experimental tank comprised of a translucent plastic bin divided by plastic

143   partitions into three compartments. The central 'focal fish' compartment was

144   25.4 cm wide, and was flanked on each side with a 6.3 cm wide 'partner fish'

145 compartment. The clear dividers separating focal and partner fish

146 compartments were perforated to allow water and olfactory cues to pass

147 between compartments, but the holes were too small for fish to pass

148 through. The focal fish compartment was further divided into a 12.8 cm wide

149 central 'neutral zone' flanked on each side by 6.3 cm wide 'choice zones'

150 adjacent to each of the partner fish compartments. Choice zones were

151 separated from the neutral zone by opaque barriers with openings large

152 enough to allow a fish to comfortably pass, but which were positioned such

153 that a focal fish in one choice zone could not see into the opposite choice

154 zone (as employed by Ruhl & McRobert, 2005; Snekser, *et al.*, 2006, 2010),

155 though the fish could still detect olfactory cues, which are known to be used

156 by zebrafish for distinguishing familiar from unfamiliar kin (Gerlach & Lysiak,

157 2006). Once in a choice zone, the focal fish could see and interact with the

158 occupant of the adjacent partner fish compartment, though they remained

159 separated by the clear plastic divider. Thus, the apparatus allowed us to

160 clearly identify when a focal fish was associating with one partner fish, but

161 not the other; we used the presence of fish in a choice zone as the index of

162 shoaling preference (Figure 1). Prior to each trial the experimental tank was

163 rinsed and then filled with approximately 10 L of conditioned water which

164 had never contained fish. The same experimental tank was used for both

165 experiments.

166
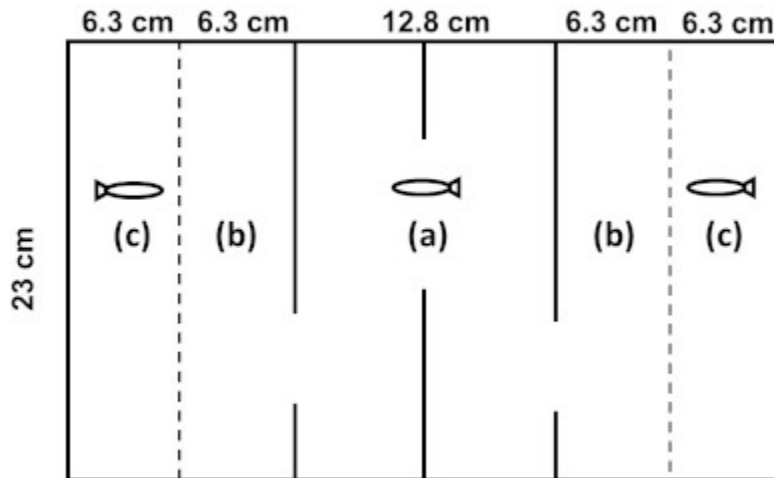
FIGURE 1. Experimental apparatus for evaluating shoaling preference in zebrafish (*Danio rerio*). Heavy lines represent opaque barriers. Dashed lines represent transparent perforated barriers. A focal fish in the neutral zone (a) could freely move into either choice compartment (b) to be near a partner fish (c) (or an empty chamber, depending on the experiment). Note that a focal fish could not see both partner fish simultaneously.

Experiment 1: Confirmation of preference for shoaling

We ran 10 trials to confirm that zebrafish preferred to shoal with a partner fish as opposed to being alone under our experimental conditions. In each trial, one focal fish was presented with a choice chamber containing one partner fish and an 'empty' choice chamber containing no fish. Each trial used two unique fish. Both the focal and the partner fish were from the same home tank in all but one trial; in the remaining trial they were from different home tanks.

182   At the onset of each trial, a focal fish was transferred directly from its

183   home tank to the middle of the neutral compartment. Then, the partner fish

184   was transferred from its home tank into a glass beaker containing

185   approximately 100 ml of water from its home tank. The contents of the

186   beaker were then poured into the designated partner compartment; which

187   side contained the partner fish (as opposed to no fish) was alternated

188   between trials. Approximately 90 seconds elapsed between the time when

189   the focal fish was transferred to the experimental tank and when the partner

190   fish was added. Fish were allowed to swim freely within their compartments

191   for the duration of the trial.

192   Trials were video-recorded by a camera placed above the tank.

193   Recording started before the focal fish was transferred into the tank, and

194   ended 10 minutes after the partner fish was added into the tank. The focal

195   fish was considered to be visiting a partner fish or empty compartment when

196   it was in the choice zone adjacent to that compartment. The times at which

197   the focal fish passed through the opaque barriers between neutral and

198   choice zones were recorded to calculate how often the focal fish visited each

199   choice zone and the total amount of time the focal fish spent in each choice

200   zone.

201

202   Experiment 2: Preference for familiar vs unfamiliar shoaling partners

203   We conducted 26 trials to evaluate whether zebrafish preferred to

204   associate with familiar or unfamiliar conspecifics. Experiment 2 followed the

205   same general design as Experiment 1, except that each partner

206   compartment contained a fish, one of which was familiar and the other was

207   unfamiliar to the focal fish. The focal fish and the 'familiar partner fish' were

208   taken from the same home tank, whereas the 'unfamiliar partner fish' was

209   from a different home tank. After placing the focal fish in the neutral

210   compartment, the two partner fish were transferred from their home tanks

211   into separate glass beakers containing approximately 100 ml of water from

212   their respective home tanks. Contents of the beakers were then

213   simultaneously poured into the two side compartments. Which side

214   contained the familiar partner fish and which contained the unfamiliar

215   partner fish was alternated between trials. Each trial used three unique fish.

216

217   *Statistical analyses*

218   Laboratory studies of behavioural choice in fish have used a variety of

219   metrics to assess preference. For example, studies have inferred preference

220   from the total amount of time that a focal animal spends with each stimulus

221   (e.g., Dugatkin & Alfieri, 1991; Krause *et al.*, 1999; Webster & Laland, 2013),

222   the proportion of 'active time' spent with each stimulus (i.e., disregarding

223   time spent in a neutral no-choice zone; Gerlach *et al.*, 2008; Krause *et al.*,

224   1999; TerMarsch & Ward, 2020), which stimulus was visited first (Webster &

225   Laland, 2013), and the latency to first visit (Webster & Laland, 2013).

226   Similarly, studies have begun assessment immediately after introducing the

227   focal animal into the choice arena (Dugatkin & Alfieri, 1991; Krause *et al.*,

228   1999; Webster & Laland, 2013), after an acclimation period (Gerlach *et al.*,

229   2008; Madeira & Oliveira, 2017; Metcalfe & Thomson, 1995), or after the

230   focal animal has visited or detected all available stimuli (Magurran *et al.*,

231   1994; TerMarsch & Ward, 2020).

232   Because we had no *a priori* assumption of the analytical approach that

233   would be most appropriate in our study, we used Experiment 1 to evaluate

234   the efficacy of several approaches for detecting a preference in choice trials,

235   since it is well established that zebrafish should exhibit a strong preference

236   for associating with a partner fish over an empty compartment (Ogi *et al.*,

237   2021).

238   Based on the laboratory studies that employed behavioural choice

239   tests in fish noted above, for Experiment 1 we evaluated four metrics for

240   whether zebrafish preferred the side of the choice tank that contained a

241   partner fish as opposed to the side that contained no fish, over four time

242   frames. The metrics we evaluated were the total number of visits to each

243   side, the average duration of visits to each side, the proportion of the total

244   time period spent on each side, and the proportion of 'active time' (the total

245   time the focal fish spent in either choice zone, excluding all of the time it

246   spent in the center no choice zone) spent on each side. We calculated all of

247   these metrics over four non-exclusive time frames, and we used Experiment

248   1 to determine in which time frame the focal fish actually exhibited shoaling

249   preferences. We evaluated the entire 10 minute trial, only the first five

250   minutes of each trial (to potentially avoid habituation to the apparatus), only

251   the second 5 minutes of each trial (to allow for a 5 minute acclimation period

252   before evaluating a preference), and after the focal fish had visited both

253   sides of the choice tank (ensuring that the focal fish was aware of both

254   stimuli and sufficiently acclimated to visit them). We additionally evaluated

255   the duration of the first visit to each side and which side the focal fish visited

256   first at the beginning of the trial (to potentially avoid habituation to the

257   experimental apparatus). We also used each metric to test for a bias for one

258   side of the apparatus over the other during each time frame. To evaluate

259   preference for familiar versus unfamiliar partners (Experiment 2), we used

260   the analytical approaches that were most effective at detecting the expected

261   shoaling preference in Experiment 1, and only used those approaches that

262   did not appear to exhibit a bias for the left or right side of the tank.

263   In all trials, video-recording ended 10 minutes after the beaker

264   containing the partner fish was emptied into the experimental apparatus.

265   Some trials had less than five minutes remaining after the focal fish had

266   visited both choice zones; as a result, the amount of time analyzed after the

267   focal fish visited both choice zones ranged from 1:30 to 5 minutes.

268   Furthermore, when evaluating the time period after the fish visited both

269   choice zones, two trials had to be omitted from Experiment 1 and three trials

270   had to be omitted from Experiment 2 because the focal fish never visited

271   both choice zones.

272

273   All analyses were carried out in R (version 3.6.3; R Core Team, 2020).

274   We determined whether focal fish exhibited a significant preference for

275   visiting one side first using an exact binomial test in the `stats` package (R

276   Core Team, 2020). For all other analyses, we used permuted paired-samples

277   t-tests to evaluate whether focal fish exhibited a significant preference for

278   one stimulus over the other. Permutation tests relax assumptions about the

279   underlying distribution of variables, and they are less sensitive to outliers,

280   ties, and other issues that confound parametric and traditional non-

281   parametric statistical models, especially when sample sizes are small (Manly,

282   1998). We conducted permuted paired-samples tests using the

283   'independence test' procedure in the `coin` package (Hothorn *et al.*, 2008).

284

285   **Results**

286

287   In Experiment 1, zebrafish exhibited a strong preference for being in

288   the occupied choice zone over the empty choice zone based on several

289   analytical approaches (Table 1). Specifically, our ability to detect a

290   preference was strongest in the time frame after the focal fish had visited

291 both sides of the choice tank, regardless of whether we assessed preference

292 based on the proportion of total time spent on each side ($p < 0.009$), the

293 proportion of active time spent on each side ($p < 0.01$), or the mean duration

294 of visits to each side ($p = 0.008$; Table 1). We also detected a significant

295 preference in these same metrics when restricting the time frame to the final

296 5 min of each trial (all $p < 0.05$; Table 1), though this may merely reflect that

297 on average fish visited both sides at around 265 seconds (SD = 176

298 seconds), or almost 5 minutes (300 seconds) into the trial, resulting in a

299 sizable overlap between the final 5 minutes of a trial and the portion of the

300 trial that occurred after focal fish had visited both sides. We additionally

301 detected a preference ($p = 0.04$) when we evaluated the proportion of total

302 time spent on each side over the entire 10 minutes of the trial, which may

303 also have stemmed from preferences expressed late in the trial, after visiting

304 both sides. In addition, we detected a significant difference in the total

305 number of visits to each side when measured over the entire 10 minute trial

306 period ($p = 0.02$), which was only significant in that time frame (Table 1).

307 The first side visited by the focal fish was not dependent on whether the side

308 was occupied by a partner fish (4 for occupied, 6 for empty; $p = 0.754$), nor

309 was the duration of the first visit to either side (occupied: 33.5±31.2; empty:

310 31.1±37.9; $Z = -0.101$, $p = 0.912$). We detected no bias in favor of either

311 side of the apparatus based on any of the approaches (all $-1.982 < Z <$

312 0.9132, $p > 0.0502$), except for the number of visits to each side during the

313   first 5 minutes (Z = -2.335, p = 0.0166), which did not reveal any significant

314   preference for either choice zone (p = 0.952).

TABLE 1. Results of Experiment 1, evaluating whether male zebrafish (*Danio rerio*) prefer to shoal with a male conspecific over being alone, based on different preference metrics. Each preference metric was analyzed for the entire 10 minute trial, for the first 5 minutes of each trial, for the second 5 minutes of each trial, and after the focal fish had visited both sides of the choice apparatus. Means, 95% confidence intervals, and Z statistics and p-values from permuted independence tests are shown.

| Preference Metric | Total 10 Min (N = 10) | | | | First 5 Min (N = 10) | | | |
| | Mean (95% CI) | | | | Mean (95% CI) | | | |
	Occupied	Empty	Z	P	Occupied	Empty	Z	P
Number of Visits	5.8 (3.7, 7.9)	3.2 (1.0, 5.4)	2.182	.017*	2.7 (1.3, 4.1)	1.4 (.04, 2.8)	1.982	.064
Proportion of Total Time	.35 (.14, .56)	.11 (.02, .20)	1.924	.041*	.27 (.06, .48)	.13 (-.01, .26)	1.211	.263
Proportion of Active Time	.67 (.41, .93)	.67 (.07, .59)	1.413	.164	.50 (.19, .81)	.40 (.10, .71)	.4045	.721
Mean Visit Duration (s)	35 (10, 59)	21 (.25, 42)	.8369	.401	25 (-.49, 51)	22 (-4.6, 49)	.1728	.864

| Preference Metric | Second 5 Min (N = 10) | | | | Visit Both Sides (N = 9) | | | |
| | Mean (95% CI) | | | | Mean (95% CI) | | | |
	Occupied	Empty	Z	P	Occupied	Empty	Z	P
Number of Visits	3.1 (1.7, 4.5)	1.8 (.38, 3.2)	1.786	.095	4 (1.6, 6.4)	2.1 (.49, 3.8)	1.508	.177
Proportion of Total Time	.41 (.18, .64)	.09 (.02, .16)	2.180	.011*	.55 (.29, .81)	.12 (.03, .22)	2.230	.008**
Proportion of Active Time	.68 (.41, .95)	.22 (.004, .44)	1.961	.042*	.82 (.67, .97)	.18 (.03, .33)	2.504	.008**
Mean Visit Duration (s)	43 (9.2, 77)	9.1 (2.8, 15)	1.869	.022*	40 (1.7, 77)	10 (2.1, 18)	1.747	.007**

*Significant at α = 0.05
**Significant at α = 0.01

315

316   Given the results of Experiment 1, we analyzed data from Experiment

317   2 based on the proportion of total time spent on each side, proportion of

318   active time spent on each side, and mean visit duration, all measured after

319   the focal fish had visited both sides of the choice tank. In addition, we

320   analyzed the number of visits to each side measured across the entire 10

321   minute trial period.

322   Despite the much larger sample size in Experiment 2 (n = 26), we

323   detected no preference for familiar conspecifics based on any of the metrics

324   validated in Experiment 1. We found no significant preference for familiar or

325   unfamiliar conspecifics based on proportion of active time (familiar:

326   0.46±0.15; unfamiliar: 0.54±0.16; Z = -0.4961, p = 0.6172) or proportion of

327 total time (familiar: 0.34±0.11; unfamiliar: 0.47±0.16; Z = -1.0190, p =

328 0.3182) after the focal fish had visited both sides, or the total number of

329 visits over the entire 10 minute trial (familiar: 4.2±1.4; unfamiliar: 4.3±1.6; Z

330 = -0.2682, p = 0.8363). In fact, after the focal fish visited both sides, visits to

331 familiar fish were, on average, significantly shorter than visits to unfamiliar

332 fish (familiar: 33±14; unfamiliar: 83±42; Z = -2.1176, p = 0.0257). We

333 detected no side bias in any analysis of Experiment 2 (all -1.720 < Z <

334 1.347, p > 0.0755).

335

336   **Discussion**

337

338   We found that even though male zebrafish are clearly motivated to

339 shoal, they exhibit no preference for familiar over unfamiliar conspecifics.

340 This result is surprising because preferences for associating with familiar

341 individuals are found across many species of phylogenetically distant small,

342 shoaling fish from a wide range of habitats, including guppies, sticklebacks,

343 and minnows (Barber & Ruxton, 2000; Griffiths *et al.*, 2007; Magurran *et al.*,

344 1994). Even juvenile zebrafish have been found to prefer the odour of

345 familiar kin to unfamiliar kin (Gerlach & Lysiak, 2006) and adult male

346 zebrafish are capable of recognizing familiar individuals after 24 hours'

347 separation (Madeira & Oliveira, 2017). This combination suggests that

348 zebrafish may use familiarity primarily to support kin recognition, rather than

34   17

349   to promote cooperation among non-relatives, as may occur in other species,

350   such as guppies (Croft *et al.*, 2006). Zebrafish may instead primarily choose

351   which individuals to associate with based on factors other than familiarity,

352   such as size (Aslanzadeh *et al.*, 2019; Fernandes *et al.*, 2015), body shape

353   (Saverino & Gerlia, 2008), or colour pattern (Engeszer *et al.*, 2004; Saverino

354   & Gerlia, 2008; Spence *et al.*, 2008). Future work is needed to determine

355   what socioecological factors may be driving this surprising difference in

356   social preferences between zebrafish and many similar fish species, and in

357   doing so provide deeper insight into why many species of fish prefer to shoal

358   with familiar individuals—and why some species do not.

359   Furthermore, one of our analyses suggested male zebrafish may

360   indeed spend more time inspecting unfamiliar over familiar individuals,

361   which may reflect increased interest in a novel stimulus, as suggested by

362   studies of social cognition in zebrafish (Ariyasiri *et al.*, 2019; Madeira &

363   Oliveira, 2017; Norton *et al.*, 2019; Ribeiro *et al.*, 2020). In particular in an

364   experiment on male zebrafish by Madeira and Oliveira (2017), focal fish were

365   exposed to 'familiar' partner fish only once for a twenty minute period 24 hrs

366   prior to choice trials. Subsequently, during the choice trials, focal fish

367   explored unfamiliar fish more than familiar fish, suggesting they were

368   attracted to novel stimuli. Madeira and Oliveira (2017) showed that a brief

369   exposure to stimulus fish 24 hours prior to choice trials was sufficient time

370   for acquisition of recognition, but earlier work in female guppies has shown

371   that fish take much longer – around 12 days – to acquire shoaling

372   preferences based on familiarity (Griffiths & Magurran, 1997a). However, we

373   found a similar result to Madeira and Oliveira (2017), even though fish in our

374   study had been given 21 to 34 days to acquire familiarity, suggesting that in

375   male zebrafish, a preference for novelty and not familiarity may be at play.

376   Nonetheless, we only detected a preference for an unfamiliar partner in one

377   of the four tests we used to assess preference in Experiment 2, so this

378   pattern should be explored more thoroughly in future studies.

379   The absence of a preference for familiar individuals is unlikely to be

380   the result of a failure of the experimental procedure or small sample size

381   because the same procedure clearly detected an expected preference for

382   shoaling with a conspecific over being alone in Experiment 1, and our sample

383   size for detecting a preference for familiar over unfamiliar individuals in

384   Experiment 2 was 2.5 times larger than that in Experiment 1. We additionally

385   used Experiment 1 to ensure that we analyzed data from when focal fish

386   were acclimated to the apparatus and able to express a preference between

387   the options presented. We also confirmed that our results were not

388   confounded by side bias in the experimental apparatus. Finally, our

389   experimental apparatus was designed to give the focal fish access to visual

390   and olfactory cues, which are both known to be involved in partner choice in

391   zebrafish (Gerlach & Lysiak, 2006; Ribeiro *et al.*, 2020). Nonetheless, future

392   work should be done to evaluate whether increased access to the relevant

393 cues and motivation to choose between the partner fish reveals a more

394 subtle preference for associating with familiar individuals. For example, some

395 studies have used active water movement (e.g. Gerlach & Lysiak, 2006) or

396 close proximity to partner fish (e.g. Barber & Ruxton, 2000) to ensure that

397 olfactory cues from the stimulus fish reach the focal fish. While we separated

398 the focal fish from the partner fish with plastic partitions and used passive

399 water movement, we expect that the focal fish likely had sufficient access to

400 olfactory cues from both stimulus partners as a result of water movement

401 through perforations in the partitions due to the swimming fish (Madeira &

402 Oliveira, 2017). In our study, it is also possible that olfactory cues in the

403 water from the home tank of each partner fish that was added with that

404 partner fish could have masked some of the olfactory cues from the stimulus

405 fish themselves. Although the cues in the water from the home tanks should

406 correspond to cues of "familiar" and "unfamiliar" fish from the perspective of

407 the focal fish, it would be useful in future studies to better isolate the

408 olfactory cues of the stimulus fish. In addition, giving the focal fish the

409 opportunity to visually compare the partner fish simultaneously might

410 increase its motivation to choose one individual over the other (but see Ruhl

411 & McRobert, 2005; Snekser, *et al.*, 2006, 2010). Motivation to shoal may also

412 be increased by the presence of potential threats, such as predator cues; the

413 focal fish in our study may have had low motivation to shoal if the benefits of

414 shoaling in our experimental context were perceived to be low. Notably, all

415 fish used our study experienced the same diet and identical habitats to

416 ensure that any preferences were based on familiarity with individuals, as

417 opposed to preferences for familiar diet and habitat cues, as have been

418 found in three-spined sticklebacks (Ward *et al.*, 2004). Future work should be

419 done to evaluate whether zebrafish likewise exhibit preferences based on

420 familiar diet and habitat cues, even though they do not prefer familiar

421 individuals *per se*, as those cues may be more available or more relevant to

422 fish seeking shoaling partners.

423   Our study only used male zebrafish to avoid any possible confounds of

424 mate choice or mating behaviour. It is possible that these results may extend

425 to females, as preferences for familiar individuals are typically found in both

426 sexes (Griffiths & Ward, 2011). However in guppies, which are highly

427 sexually dimorphic, females have been found to be more likely to associate

428 repeatedly with the same individuals (Croft *et al.*, 2003), whereas the

429 evidence of preferences for familiar individuals in males is more equivocal

430 (Croft *et al.*, 2004; Griffiths & Magurran, 1998). Zebrafish are not highly

431 sexually dimorphic, but they exhibit some sex-based differences in

432 behaviour, cognition and neurophysiology (Genario *et al.*, 2020), and males

433 and females differ in shoaling preferences based on strain (Snekser *et al.*,

434 2010), sex, and shoal size (Ruhl & McRobert, 2005). Both males and females

435 are capable of recognizing familiar individuals (Ariyasiri *et al.*, 2019; Madeira

436 & Oliveira, 2017; Norton *et al.*, 2019; Ribeiro *et al.*, 2020) and future work

437   should be done to explore whether, as may be the case in guppies, only

438   females prefer to shoal with familiar individuals, or whether both males and

439   females have no preference or only exhibit a novelty effect.

440   Our study used domestic zebrafish from a biological supply company,

441   as is typical of this very well established laboratory species (Ogi *et al.*, 2021).

442   The fish that we used descended from source populations that likely spent

443   dozens of generations in an environment with decreased exposure to

444   predation and increased access to essential resources. Predator avoidance

445   and increased foraging efficiency are some of the key functions of shoaling in

446   fish such as zebrafish. In captivity, where these pressures may be

447   diminished, the cost of competition may outweigh the advantages of

448   shoaling and lead to decreased shoal cohesion and selectivity (Wright *et al.*,

449   2006). In zebrafish, it has been found that older domesticated and more

450   recently established strains learn foraging tasks at different rates and

451   respond differently to social perturbations (Vital & Martins, 2011).

452   Furthermore, if shoaling is less important in captivity, there may be less

453   pressure selecting for the maintenance of shoaling preferences in general.

454   There may also be some costs to increased shoal cohesion, which is one of

455   the known benefits of associating with familiar individuals in the wild

456   (Chivers *et al.*, 1995). Such costs may even select against a shoaling

457   preference in captivity. Experiments comparing shoaling behaviour of

458   domestic and wild zebrafish could reveal whether domesticity can account

459   for a decrease in shoaling preferences overall, as well as whether wild male

460   zebrafish similarly do not exhibit shoaling preferences based on familiarity.

461

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470

471   **Contributions**

472   A. F. B. generated all the data, K. A. T. acquired funding, and both A. F. B.

473   and K. A. T. contributed ideas, analyzed the data, and prepared the

474   manuscript.

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