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1 **Behavioural responses of feral and domestic guppies (*Poecilia***
2 ***reticulata*) to predators and their cues**

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17

18

19 **Abstract**

20 Predation is an important factor during adaptation to novel environments and the feralisation
21 of introduced domestic species often involves responding appropriately to allopatric
22 predators despite a background of domestication and inbreeding. Twenty years ago domestic
23 guppies were introduced to a semi-natural environment at Burgers' Zoo in the Netherlands,
24 where they have since been exposed to avian predation. We compared predation-linked
25 behaviours in this feral population and in domestic guppies akin to the original founders. We
26 found that both populations responded to a novel predator and to conspecific alarm cues.
27 However shoaling, an important anti-predator behaviour, was higher among feral guppies
28 both at baseline and when exposed to the novel predator. We did not observe a linked suite of
29 anti-predator behaviours across shoaling, predator inspection, alarm substance sensitivity and
30 boldness, suggesting that these responses may be decoupled from one another depending on
31 local predation regimes. As we compared two populations, we cannot identify the causal
32 factors determining population differences, however, our results do suggest that shoaling is
33 either a particularly consequential anti-predator adaptation or the most labile of the
34 behaviours we tested. Finally, the behavioural adaptability of domestic guppies may help to
35 explain their success as an invasive species.

36

37

38 **Keywords**

39 predation; feralization; shoaling; grouping behaviour; domestication; guppy

40

41 **1. Introduction**

42 Predation is a major driver of variation across taxa, influencing behaviours that may lessen
43 predation risk such as social grouping (Hass and Valenzuela, 2002; Magurran, 1990a; Quinn
44 and Cresswell, 2006) and vigilance (Forsslund, 1993; Lung and Childress, 2007). However,
45 these behaviours may also carry costs. For example, grouping may increase competition for
46 resources or exposure to parasites (Krause and Ruxton, 2002). Thus anti-predator behaviours
47 are expected to vary across populations experiencing different levels of predation.

48

49 Studies of wild guppies (*Poecilia reticulata*) on the Caribbean island of Trinidad have shown
50 that populations under high levels of predation form tighter shoals (Magurran and Seghers,
51 1991; Seghers, 1974); engage in less risky predator inspection (Magurran and Seghers, 1990,
52 1994) and exhibit changes in sensitivity to conspecific alarm cues (Brown et al., 2009). While
53 extensive study of wild guppies has provided strong evidence for evolved adaptation to
54 predation in Trinidad, the guppy is also an important invasive species affecting local ecology,
55 with feral populations identified in 60 countries across six continents (Froese and Pauly,
56 2012). These feral populations are often derived from inbred ornamental guppies (Lindholm
57 et al., 2005), artificially selected for traits that may not be adaptive in the wild (Huntingford,
58 2004). Their success in novel habitats with allopatric predators suggests that domestic
59 guppies retain traits that are adaptive in new environments (Deacon et al., 2011) or have
60 sufficient genetic diversity to adapt to new environmental challenges.

61

62 We examined the influence of feralization and predation on the behaviour of guppies from
63 Burgers' Zoo in Arnhem, the Netherlands. The zoo contains a 120 m³ manatee (*Trichechus*
64 *manatus*) pool which also houses a feral population of guppies, derived from an initial

65 introduction of mixed-strain domestic guppies released by a zoo visitor in 1989, and
66 subsequently left untended. The release was observed by zoo curators, who estimate that it
67 consisted of approximately 100 individuals (Max Janse, Burgers' Zoo curator, personal
68 communication, 2015). The population has subsequently expanded considerably and is
69 estimated to number well over 100,000 guppies (Max Janse, personal communication;
70 Albers, 2000). These now-feralized guppies forage on plentiful manatee food and faeces, but
71 are subject to predation from three resident African darters (*Anhinga rufa*) which dive
72 regularly each day for guppies (personal observation) and have been reported to eat up to
73 1000 guppies each per day (Albers, 2000). The feral guppies resemble wild guppies in
74 appearance and have lost the bright colours and extravagant tails typical of domestic guppies
75 (fig. 1A). A previous study examined body colouration in these fish, and anecdotally reported
76 dense shoaling among these feral guppies (Albers, 2000). We compared this feral population
77 with mixed-strain domestic guppies akin to the ancestral founders and measured predation-
78 linked behaviours: shoaling, predator inspection behaviour, alarm substance sensitivity, and
79 boldness/exploration. We predicted the feral guppies would shoal more, inspect predators
80 more cautiously, be more sensitive to alarm substance and be less bold and exploratory as a
81 consequence of feralization and adaptation to predation.

82

83 **2. Materials and Methods**

84 *2.1. Subjects and housing*

85 Nineteen domestic and 19 feral male guppies were used as experimental subjects, with a
86 further 14 domestic and 14 feral males used either as shoaling companions or for alarm
87 substance extraction. Domestic guppies were from a mixed strain population of
88 approximately 1400 fish, first established in 2003 within the Utrecht University Biology

89 aquarium and based on a founder population of approximately 480 domestic guppies
90 purchased from two commercial suppliers (Ruinemans, Montfoort and Ruisbroek, Maassluis,
91 both The Netherlands). Feral guppies were caught with dip nets from the manatee pool at
92 Burgers' Zoo. Feral guppies were sampled in November 2009, four months before the study,
93 and rehoused in our aquarium. Thus the feral guppies had both an evolutionary history and
94 direct experience of predation. Given an estimate of two generations per year (Magurran,
95 2005; Reznick et al., 1997), the domestic guppies are estimated to have bred for 10-15
96 generations in the Utrecht aquarium and the feral guppies for 35-45 generations at Burgers'
97 Zoo. Feral guppies were treated to remove ectoparasites after capture by 15 minute
98 immersion in 2.5% salt solution as a precautionary measure. Domestic guppies had brighter
99 and more varied colouration patterns than feral guppies, but subjects of each population were
100 similarly sized. Two male red rainbowfish (*Glossolepis incisus*) from our aquarium were
101 used as potential predators as they represented a novel, allopatric threat to both domestic and
102 feral guppies. Two weeks before the study, all experimental animals were moved to our
103 experimental laboratory.

104

105 Laboratory lights were on from 08:00 to 20:00. Fish were housed separately by
106 population/species in 80 × 50 × 40 cm tanks except for shoaling companion guppies which
107 were housed in a 90 × 50 × 25 cm tank, with domestic and feral guppies separated by an
108 opaque partition. Tanks contained gravel, plastic plants and terracotta pots, and were filtered
109 and heated to 26±1°C. Fish were fed TetraMin flake food (Tetra, Germany) daily and fresh
110 bloodworm (*Chironomidae* larvae) three times a week.

111

112 *2.2. Experimental procedures*

113 Subjects were tested for: i) boldness and exploration, ii) responses to alarm substance, and iii)
114 shoaling and predator inspection, with 3-7 days between tests. Test order was consistent so
115 any carry-over effects from each test were the same across individuals. Housing tanks were
116 divided with plastic partitions to create separate areas for subjects after testing. On test days,
117 fish were fed at the conclusion of testing. Some fish were excluded from tests due to illness
118 or for methodological reasons: 19 guppies per group were used in boldness/exploration tests,
119 17 guppies per group in alarm substance response tests, and 17 domestic and 15 feral guppies
120 in shoaling and predator inspection tests.

121

122 *2.2.1. Boldness and exploration*

123 The test tank (fig. 1B) consisted of a “sheltered” area with gravel, plastic plants and a
124 terracotta pot, and a bare, brightly lit “exposed” area, which also contained a suspended
125 opaque partition creating a novel “hidden” area not visible from the sheltered area. Notional
126 boundaries of these areas and the upper and lower halves of the tank were marked on the
127 front of the tank. At test, individual subjects were released into the sheltered area and
128 behaviour recorded for 10 minutes.

129

130 Latencies to enter exposed and hidden areas were analysed by Wilcoxon-Gehan survival test
131 (subjects that did not enter were assigned the maximum latency of 600 seconds). Time in the
132 exposed area data were log transformed and analysed by independent *t* test, time in the
133 hidden area was analysed by Mann-Whitney *U*-test. Activity was assessed by analysing
134 number of transitions between tank quadrants by independent *t* test.

135

136 2.2.2. *Alarm substance responses*

137 Mixed-population alarm substance was prepared each test day following established
138 protocols (Brown and Godin, 1999; Brown et al., 2009): a feral and a domestic male guppy
139 were euthanized in ice water and skin and skeletal muscle homogenized with 50 µl ddH₂O,
140 then filtered through glass wool with ddH₂O to a final volume of 100 ml, which was kept on
141 ice.

142

143 Tests were conducted in a 40 × 25 × 25 cm tank containing 20 cm depth of water and covered
144 on three sides with white plastic. After 2 minutes for the subject to habituate, 4 ml of ddH₂O
145 was added with a pipette to start the 20 minute test. Ten minutes later, 4 ml of alarm
146 substance solution was added. Tested subjects were placed in a holding tank to prevent
147 interaction with untested subjects, then moved to the 'tested' division of their home tanks at
148 the end of each day. The test tank was cleaned and refilled before each test. Time immobile
149 ("freezing") and distance swum were analysed by repeated measures ANOVA, with
150 experimental phase (before and after addition of alarm substance) as the repeated factor and
151 population as the independent factor. Freezing data were log transformed before analysis.

152

153 2.2.3. *Shoaling and predator inspection behaviour*

154 The test tank (fig. 1C) was lined with gravel and divided with plastic partitions into left
155 (shoal), central (subject) and right (predator) sections. The transparent left partition was “one-
156 way glass” so the shoal could not see the predator or subject (Mathis et al., 1996). The right
157 partition consisted of an impermeable silicone-sealed transparent partition and a removable

158 opaque partition. The shoal section was lit with a 3W LED spotlight to ensure the
159 effectiveness of the one-way glass. Two interlocked plastic cups with matching 3 cm
160 diameter holes were suspended in the subject section, and a shoaling zone adjacent to the
161 companion shoal was marked on the front of the tank, 6 cm (2 body lengths) from the left
162 partition.

163

164 On each test day, 5 same-population companion fish were placed in the shoal section and a
165 red rainbowfish was placed in the predator section. A subject was placed in the suspended
166 cups, with the holes misaligned. After 2 minutes' habituation, the holes were aligned and
167 when the subject exited, the 20 minute test began. After 10 minutes, the opaque partition was
168 removed to reveal the red rainbowfish. We recorded time spent in the shoaling zone and
169 number of predator inspection bouts by each subject over the whole test. At the end of each
170 day, the red rainbowfish and shoaling companion fish were returned to their home tanks.
171 Populations were tested on alternate days to balance test order. Shoaling time data were
172 square root transformed and analysed by repeated measures ANOVA, with experimental
173 phase (before and after the predator was revealed) as the repeated factor and population as the
174 independent factor. Frequency of predator inspection was analysed by independent samples t
175 test.

176

177 *2.2.4. Correlations between anti-predator behaviours*

178 Relationships between responses to predation cues (the potential predator or alarm cues) were
179 analysed by correlation of behavioural difference scores. A single measure was calculated for
180 the behaviours modulated by exposure to predation cues (shoaling duration, number of
181 predator inspection bouts and freezing duration) by subtracting pre-exposure performance

182 from post-exposure performance. These three difference scores were then analysed by
183 Pearson's correlations. Shoaling data difference scores were log transformed prior to analysis.

184

185 *2.3. Analysis*

186 Tests were videoed using a Logitech Pro 9000 webcam and VirtualDub video capture
187 software. Boldness and exploration tests, and shoaling and predator inspection tests were
188 scored with JWatcher 1.0. Alarm substance tests were analysed with Ethovision XT (Noldus
189 Information Technology, Netherlands). Statistical analyses were conducted with IBM SPSS
190 Statistics 20.

191

192 *2.4. Ethics statement*

193 All procedures were carried out in accordance with Dutch law and approved by the Utrecht
194 animal experimentation commission (“Dierexperimentencommissie Utrecht”) under licence
195 2010.I.03.036.

196

197 **3. Results**

198 *3.1. Boldness and exploration*

199 Feral and domestic guppies did not significantly differ in their latency to enter the exposed
200 area (Wilcoxon-Gehan statistic = 0.10, $df = 1$, $P = 0.92$) or total time in the exposed area (t
201 test, $t_{36} = 0.70$, $P = 0.49$). There was a non-significant trend for feral guppies to enter the
202 hidden area faster (Wilcoxon-Gehan statistic = 3.30, $df = 1$, $P = 0.069$, fig. 2A) and to spend
203 more time in the hidden area (Mann-Whitney U test, $U = 240$, $N = 19$ per group, $P = 0.085$;

204 fig. 2B). Populations did not significantly differ in number of transitions between quadrants (t
205 test, $t_{36} = 0.43$, $P = 0.67$).

206

207 Subjects responded differently to the sheltered and exposed areas, spending significantly less
208 time in the exposed area than would be expected by chance (one-sample t test, $t_{37} = -2.63$, P
209 $= 0.012$). Similarly, subjects spent significantly less time in the hidden area after entering the
210 exposed area than would be expected by chance (one-sample Wilcoxon signed rank test, $W =$
211 180 , $N = 38$, $P = 0.005$; chance expectations were based on the relative volume of each area).
212 This avoidance of the exposed and hidden areas supports the use of time spent in these areas
213 as combined measures of boldness and exploration (sensu Réale et al., 2007).

214

215 *3.2. Alarm substance responses*

216 Alarm substance significantly increased freezing (repeated measures ANOVA, $F_{1,32} = 44.98$,
217 $P < 0.001$, fig. 2C), and decreased distance swum ($F_{1,32} = 7.79$, $P = 0.009$), but these
218 responses did not differ significantly between populations, nor were there significant
219 interactions between population and alarm substance exposure (all: $F_{1,32} \leq 0.74$, $P \geq 0.40$).

220

221 *3.3. Shoaling and predator inspection behaviour*

222 Exposure to the red rainbowfish significantly increased shoaling (repeated measures
223 ANOVA, $F_{1,30} = 14.00$, $P = 0.001$), and feral guppies spent longer shoaling than domestic
224 guppies ($F_{1,30} = 10.71$, $P = 0.003$). There was no significant interaction between population
225 and predator exposure ($F_{1,30} = 0.86$, $P = 0.36$). Both populations only engaged in predator
226 inspection once the red rainbowfish was visible (mean rate: 4.2 inspections/trial), however

227 predator inspection frequency did not significantly differ between populations (t test, $t_{30} =$
228 1.52, $P = 0.14$).

229

230 3.4. Correlations between anti-predator behaviours

231 We found no significant correlations between the difference scores for shoaling, predator
232 inspection and freezing in either domestic or feral guppies (Pearson's correlations, $-0.39 \leq r \leq$
233 0.19 , $N = 14 - 17$, all $P \geq 0.17$).

234

235 4. Discussion

236 Both feral and domestic guppies increased their shoaling behaviour and engaged in predator
237 inspection when a novel predator was revealed, and both were sensitive to alarm substance.
238 Feral guppies shoaled more than domestic guppies, both before and after exposure to a novel
239 predator, but the populations did not significantly differ in predator inspection, responses to
240 alarm substance or in exploration and boldness. There was no evidence that the anti-predator
241 responses formed a suite of coupled behaviours.

242

243 Predation levels have been strongly implicated in population differences in shoaling among
244 wild guppies (Magurran, 2005), and adaptation to predation at Burgers' Zoo may have driven
245 the shoaling phenotype in the feral guppies. Predators have been shown to target individuals
246 within a group that exhibit less cohesive grouping behaviour (Ioannou et al., 2012; Quinn and
247 Cresswell, 2006) and thus prey selection by predators would be expected to increase the
248 population-level shoaling phenotype over time. We did not find evidence for covariance of
249 the different anti-predator behaviours we measured, a result that suggests anti-predator

250 behaviours were uncoupled, and that shoaling was the most labile of the behaviours we
251 tested, perhaps because it carries the most significant costs and benefits. The different anti-
252 predator behaviours may be independently responsive, reflecting differences in the adaptive
253 value of each behaviour depending on the specific predator threat. Alternatively, the
254 uncoupling may reflect limits on the variation that can emerge, for example as a result of
255 differences in allelic diversity at loci which regulate each behaviour. Larger sample sizes may
256 have helped reveal differences in the other behaviours measured, however predation effects
257 on guppy behaviours are not always consistent (Brown et al., 2009; Seghers and Magurran,
258 1995) indicating that other factors can have an influence.

259

260 We used an allopatric fish predator to allow us to test the responses of both populations to a
261 novel predator, and sensitivity to predators was evident in both feral and domestic guppies'
262 shoaling, predator inspection behaviour and alarm substance responses. Many generations of
263 domestication and artificial selection has thus not eliminated predator sensitivity in domestic
264 guppies and this may help to explain the success of introduced guppies despite novel local
265 allopatric predators (Deacon et al., 2011). Predation-linked behaviours have previously been
266 implicated in the success or failure of species introductions (Holway and Suarez, 1999) and
267 other invasive poeciliid species show elevated anti-predator behaviour compared to non-
268 invasive species (Rehage et al., 2005). Our finding that anti-predator responses are
269 maintained despite domestication has implications for captive breeding and reintroduction
270 programmes for endangered fish (see e.g. Brown and Day (2002)).

271

272 We cannot discount the possibility that aspects other than predation may have shaped the
273 feral phenotype as our study compared only two populations, and additional feral populations

274 would clearly be essential to eliminate alternative explanations for our results (Dingemanse et
275 al., 2009; MacLean et al., 2012; Reader and Hrotic, 2012). While both the feral and the
276 domestic populations were originally derived from mixed domestic strains, making it
277 unlikely that phenotypic differences were due to differential levels of inbreeding, they are not
278 derived from the same source populations and so founder effects cannot be discounted. We
279 also considered other environmental influences, however differences in food availability
280 between domestic and feral guppies are unlikely to have been a factor as feral guppies are
281 able to feed ad libitum due to the manatees' feeding regime. While the manatee pool is
282 constantly filtered and tested and water quality resembles that of an aquarium, guppy
283 ectoparasites such as *Gyrodactylus* spp., are present at low levels. However, the feral guppies
284 were treated to remove parasites after capture, four months prior to the study. Moreover, any
285 direct effects of surviving parasites would be predicted to result in reduced shoaling, as
286 infected individuals are avoided within shoals (Croft et al., 2011), and *Gyrodactylus* infection
287 reduces shoal cohesion (Hockley et al., 2014). Residual developmental or evolutionary
288 effects of *Gyrodactylus* presence are also unlikely to explain the observed shoaling
289 phenotype, because these ectoparasites are transmitted socially. Consequently, a population
290 history of *Gyrodactylus* infection would be predicted to lead to reduced grouping as an
291 adaptation to avoid infection.

292

293 Caution should be taken when drawing conclusions about adaptation from two-population
294 studies such as our own, since any two populations are likely to differ on numerous
295 characteristics, not just the factor proposed to favour adaptation. However, comparisons of
296 two populations or species is a commonly used approach, and one that has made many useful
297 contributions (Dingemanse et al., 2009; Reader, 2014). Such comparisons can provide a

298 starting point, demonstrating population differences that suggest plausible hypotheses for
299 further work. They are particularly valuable when experimental manipulation of the proposed
300 causal variable presents ethical or logistical problems. The specific circumstances of the
301 Burgers' zoo population provides an independent and rare opportunity to examine 20 years of
302 guppy evolution in well-characterised and semi-controlled feral conditions. The general
303 background of the introduced fish is known, and the zoo environment affords a relatively
304 stable environment with minimal variation in factors such as water quality, temperature, or
305 food availability that can vary extensively in most feral or wild environments, often varying
306 together with predation regime (Magurran 2005). The above considerations, in combination
307 with previous work on predation and shoaling tendencies (Huizinga et al., 2009; Magurran,
308 1990b; Magurran and Seghers, 1991), suggest that 20 years of predation in a feral
309 environment is the most likely explanation for the observed increase in shoaling behaviour in
310 these guppies. Despite years of artificial selection, ornamental fish such as domestic guppies
311 retain both behavioural sensitivity to predator threats and the capacity to adapt to these.

312

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321

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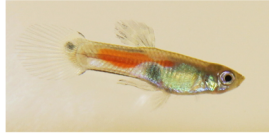
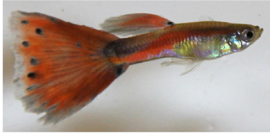
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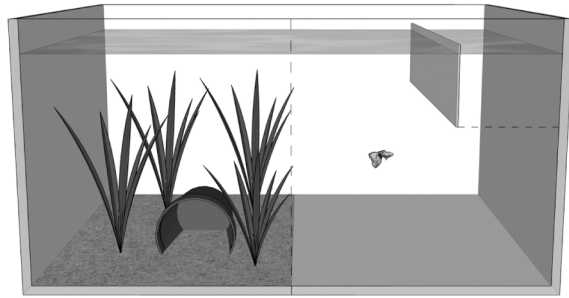
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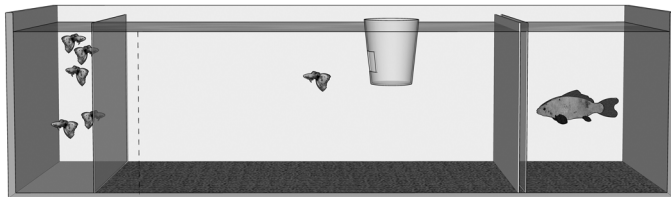
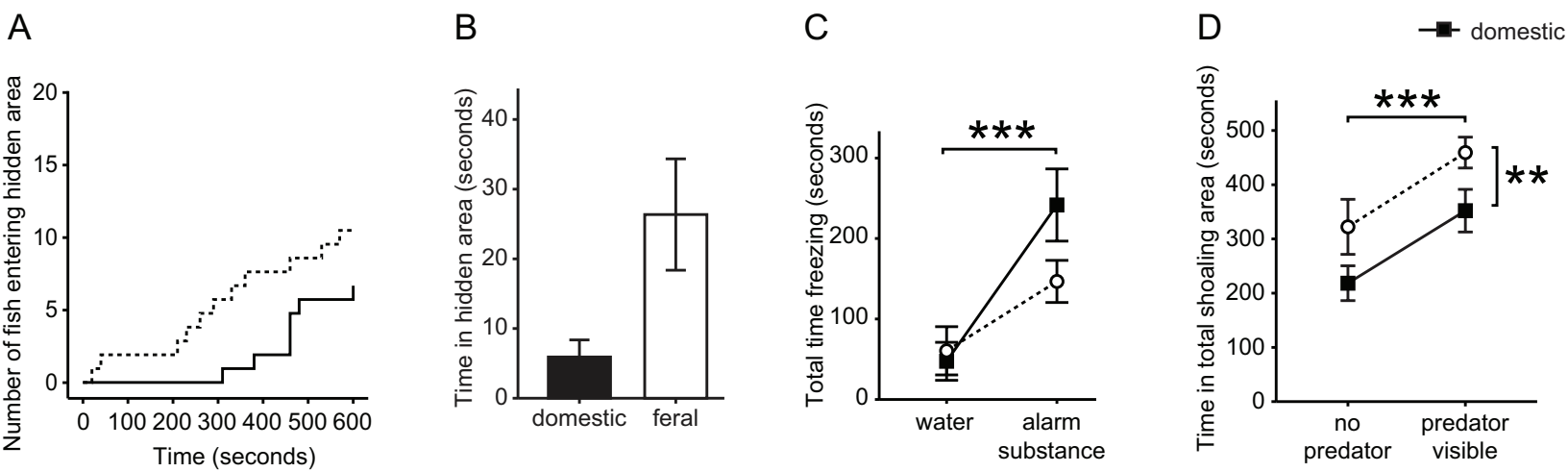


Figure 2



407 Figure 1. (A) Example photos of domestic (left) and feral (right) guppies (W.T. Swaney). (B)
408 Sketch of boldness and exploration test apparatus. The 80 cm × 50 cm × 40 cm tank
409 comprised a “sheltered” area and a brightly lit “exposed” area containing the “hidden” area
410 which subjects could not see into from their starting position in the sheltered area. (C) Sketch
411 of shoaling and predator inspection test apparatus. The 90 cm × 50 cm × 25 cm tank was
412 divided into a 10 cm wide shoal section (left), a 20 cm wide predator section (right) and a
413 subject section (centre) containing the cups from which subjects started.

414

415 Figure 2. (A) Cumulative timeline of subjects' latency to reach the hidden area and (B) total
416 time in the hidden area in exploration and boldness tests (feral $N = 19$, domestic $N = 19$). (C)
417 Total time subjects spent frozen after addition of water and alarm substance in alarm
418 substance tests (feral $N = 17$, domestic $N = 17$). (D) Total time subjects spent in proximity to
419 the shoal in “no predator” and “predator visible” parts of shoaling and predator inspection
420 tests (feral $N = 15$, domestic $N = 17$). Data are means ± SEM, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

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