

1 **Sex differences in discrimination reversal learning in the guppy**

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10

11 **ABSTRACT**

12 In several mammalian and avian species, females show higher performance than males in  
13 tasks requiring cognitive flexibility such as the discrimination reversal learning. A recent  
14 study showed that female guppies are twice as efficient as males in a reversal learning task  
15 involving yellow–red discrimination, suggesting a higher cognitive flexibility in female  
16 guppies. However, the possibility exists that the superior performance exhibited by females  
17 does not reflect a general sex difference in cognitive, but instead is confined to colour  
18 discrimination tasks. To address this issue, we compared male and female guppies in two  
19 diverse discrimination reversal learning tasks and we performed a meta-analysis of these  
20 experiments and the previous one involving colour discrimination. In the first experiment of  
21 this study, guppies were tested in a task requiring them to learn to select the correct arm of a  
22 T-maze in order to re-join a group of conspecifics. In experiment 2, guppies were observed in  
23 a numerical task requiring them to discriminate between 5 and 10 dots to obtain a food  
24 reward. In none of the two experiments did we find clear evidence of females' greater  
25 reversal learning performance, though females outperformed males in one condition of the T-  
26 maze. However, the meta-analysis of the three experiments supported the hypothesis of  
27 females' greater reversal learning ability. Our data do not completely exclude the idea that  
28 female guppies have a generally higher cognitive flexibility than males; however, they  
29 suggest that the size of this sex difference might depend on the task.

30

31 **Keywords:** Fish cognition; Numerical abilities; *Poecilia reticulata*; Reversal learning; T-  
32 maze; Sex differences

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## 36 INTRODUCTION

37           Among several polygamous mammals and birds, females show greater cognitive  
38 flexibility and reduced persistence compared to males (Guillamón et al. 1986; Ha et al. 2011;  
39 Roelofs et al. 2017; Rogers 1974). This sex difference is often studied with the discrimination  
40 reversal learning task: the animal is initially trained to choose a predetermined stimulus  
41 between two alternative options to obtain a reward; once having learned the association, the  
42 reward contingency is reversed and the speed in learning the new association is taken as  
43 measure of flexibility (Shettleworth 2010).

44           The proximate mechanisms underlying sex differences in cognitive flexibility are likely  
45 hormonal: for example, in rats, administering androgens to females and castrating males  
46 reverses the direction of the sex difference (Guillamón et al. 1986). The evolutionary  
47 explanation of this sex difference is instead less clear. A greater reversal learning ability in  
48 females is usually absent in bird species with a monogamous mating system (Brust et al.  
49 2013; Titulaer et al. 2012). Thus, this sex difference might have evolved because of the  
50 different selective pressures acting on the two sexes in polygamous species. In line with this  
51 hypothesis, greater male persistence has been supposed to evolve in order to overcome  
52 female resistance to mate (Rowe et al. 2005).

53           Recently, one study found evidence of a better performance of females in discrimination  
54 reversal learning in a polygamous fish species, the guppy *Poecilia reticulata* (Lucon-Xiccato  
55 and Bisazza 2014). Guppies were trained to dislodge small discs on the bottom of their  
56 testing tank to obtain food concealed underneath. Discs had two colours, red and yellow, but  
57 only one predetermined colour hid the food. Both males and females quickly learned to  
58 associate the presence of food with the rewarded colour, but when the reinforced colouration  
59 was reversed, females learned the new condition much faster than males that instead  
60 continued to persist on the previously reinforced colour. This result might indicate that

61 polygamy favours the evolution of greater female flexibility and greater male persistence in  
62 fish too. However, before accepting this hypothesis, an alternative explanation should be  
63 considered. In the aforementioned study, guppies were tested in a red–yellow discrimination  
64 and the possibility exists that the different performance is related to the different ecological  
65 relevance of colour discrimination for the two sexes.

66 Female guppies typically choose among available mates based on the size, number, and  
67 pattern of male carotenoid orange body spots (Houde et al. 1997). Several studies highlighted  
68 an astonishing ability of female guppies to learn the colour pattern of males after a single  
69 encounter and use this information in future mating decisions (Eakley and Houde 2004;  
70 Hughes et al. 1999). Furthermore, female guppies are known to be extremely flexible in their  
71 mating decisions. For example, the preference of a focal female for one specific male over  
72 another one can be easily reverted if the focal female could observe the non-preferred male  
73 mating with other females (Dugatkin and Godin 1992; Godin et al. 2005). Reversal of female  
74 mate preference has been observed also when the focal female is exposed to predation risk  
75 (Gong and Gibson 1996).

76 Colour discrimination has a very important role for guppies in the foraging context too.  
77 Both males and females have a strong tendency to search for small carotenoid-rich fruits that  
78 drop from the forest canopy into the rivers (Rodd et al. 2002). In the laboratory, yellow–red  
79 discrimination occurs incredibly faster than other types of discrimination (Lucon-Xiccato and  
80 Bisazza 2014; Lucon-Xiccato and Bisazza 2016). Since dietary requirements could be  
81 different in the two sexes (e.g. because carotenoids affect mating success in males but not in  
82 females; Grether 2000; Rodd et al. 2002) natural selection might have shaped discrimination  
83 learning mechanisms differently in the two sexes.

84 To clarify this issue, we need to assess whether a greater female cognitive flexibility is  
85 observed in other contexts and, in case, if the magnitude of this difference is comparable to

86 colour discrimination learning. In this study, we performed two reversal learning experiments  
87 not involving colour discrimination. In experiment 1, the task consisted in a T-maze, which is  
88 usually adopted in literature to study spatial discrimination reversal learning (Elias et al.  
89 1973; Watson and Stanton 2009). In experiment 2, the task was a discrimination between two  
90 sets of dots differing in numerosity. Both spatial and numerical abilities have been widely  
91 investigated in guppies (Agrillo et al. 2017; Kellog and Gavin 1960; Lucon-Xiccato and  
92 Bisazza 2017a). If a general sex difference in cognitive flexibility exists in guppies, females  
93 are expected to outperform males in both the discrimination reversal learning tasks. On the  
94 contrary, a lack of differences between the two sexes might imply that the previously  
95 observed sex difference in flexibility (Lucon-Xiccato and Bisazza 2014) was related to the  
96 specific context of colour discrimination.

97

## 98 MATERIALS AND METHODS

### 99 Subjects

100 We used guppies of the same strain and age and the same sample size of the previous  
101 study (Lucon-Xiccato and Bisazza 2014). These guppies derived from an outbred aquarium  
102 stock (snakeskin cobra green) bred in our laboratory since 2012. In experiment 1, we used 14  
103 males and 14 females, but one female stopped participating after the first learning phase;  
104 thus, for this experiment the final sample consisted of 13 females. In experiment 2, we used  
105 40 guppies (20 males and 20 females). A total of 12 subjects were excluded from this  
106 experiment. In detail, 2 males were excluded because they did not complete the pre-training,  
107 2 males and 3 females ceased to participate during the numerical discrimination phase and 1  
108 male and 4 females ceased to participate during the reversal learning phase (see Procedure).  
109 As a consequence, the total sample consisted of 14 males and 14 females, with a proportion  
110 of fish (70%) successfully completing the experiment similar to that reported in previous  
111 studies that used this operant conditioning procedure (Lucon-Xiccato and Bisazza 2014;  
112 Lucon-Xiccato and Bisazza 2016; Miletto Petrazzini et al. 2015b).

113 Before the experiments, guppies were maintained in 150-l tanks with natural gravel  
114 bottom, vegetation (*Hygrophila corymbosa* and *Taxiphyllum barbieri*) and water filters.  
115 Water temperature was kept at  $26 \pm 1$  °C. Fluorescent lamps illuminated the tanks with a  
116 12:12 hour light/dark cycle. We fed guppies 3 times per day using commercial food flakes  
117 and live *Artemia salina* nauplii.

118

### 119 Experiment 1 – T-maze reversal learning

120 We used a procedure previously adopted to study spatial learning abilities in guppies  
121 (Lucon-Xiccato and Bisazza 2017b). Guppies were required to choose the correct arm of a T-  
122 maze to reach their home environment in which a group of social companions was present.

123 As far as possible, we mirrored the procedure adopted in the first study on sex differences in  
124 reversal learning in the guppy (Lucon-Xiccato and Bisazza 2014).

125

## 126 Apparatus

127 The experimental apparatus consisted of a T-maze inserted in a  $68 \times 68 \times 35$  cm glass tank  
128 filled with 25 cm of water (Fig. 1a). The tank was inhabited by two subjects and by twenty-  
129 five immature guppies that served as social companions during the experiment (see  
130 Procedure). This part of the tank resembled the maintenance tanks as it was provided with  
131 natural gravel, vegetation (*H. corymbosa* and *T. barbieri*) and water filters. We turned off  
132 water filters during the trials to prevent the flow of water from affecting subjects' behaviour  
133 in the maze.

134 The maze was placed in the middle of the tank on a plastic support that kept it 2 cm below  
135 water level. The maze was made of green plastic panels and consisted of a starting chamber  
136 ( $8 \times 8$  cm), one 15-cm central arm and two identical 8-cm lateral arms. The width of the three  
137 arms was 3 cm because it was found that small corridors motivate guppies to exit a maze  
138 (Kellogg and Gavin 1960). The two lateral arms were both connected to the external tank, but  
139 the terminal part of these arms was S shaped to prevent the subjects from seeing the exit from  
140 inside the maze. To avoid latent learning of the maze (Gómez-Laplaza and Gerlai 2010), a  
141 plastic panel occluded the exit of the maze outside the trials and prevented the entrance of the  
142 subjects and the social companions. During the trials, we placed a grid net just before each  
143 exit. The grid in the correct arm presented a small hole to allow the subject to access the  
144 external tank, whereas the grid in the wrong arm did not present the hole, preventing the  
145 subject from exiting. By changing the two grids, we could modify which arm was the correct  
146 one that guppies should use to reach the external tank. The apparatus was placed in a dark  
147 room and surrounded by black curtains to prevent the fish from seeing the chamber. The

148 maze was build symmetrically with no landmarks. Thus, there were no internal or external  
149 visual cues available for the subjects to solve the task. One camera placed over the maze  
150 recorded the trials.

151

## 152 Procedure

153 We moved two subjects, one male and one female, from the maintenance tank to the  
154 external part of the experimental tank 24 hrs before the start of the experiment. We  
155 administered 6 training trials daily, divided into two 3-trial sessions (one in the morning and  
156 one in the afternoon). During each trial, we gently moved one subject into the starting  
157 chamber. We alternated the order of males and females between trials. The subject was free  
158 to swim in the maze and find the correct arm. For half of the subjects, the correct arm during  
159 the initial discrimination learning was the right arm, for the remaining subjects the correct  
160 arm was the left one. The experimenter took note of whether the subject entered first the  
161 correct or the incorrect arm. When the subject performed eight out of ten consecutive correct  
162 trials, it was considered to have learned the discrimination; thus, in the following session, we  
163 switched the correct arm and trained the fish with the reversed contingency to the same  
164 learning criterion. Since fish underwent 6 trials per day (in 2 sessions of 3 trials each) the  
165 criterion could be reached by cumulating the trials from 2 or 3 consecutive days.

166

## 167 **Experiment 2 – Numerical reversal learning**

168 In this experiment, we compared male and female guppies in a numerical task using a  
169 reversal learning paradigm similar to that previously adopted by Lucon-Xiccato and Bisazza  
170 (2014). Guppies were initially trained to discriminate between two numerosities (5 vs. 10  
171 dots). After reaching the learning criterion, the reward contingency was reversed.

172



173 Apparatus and stimuli

174 We used the apparatus previously adopted to study numerical abilities in guppies (Miletto  
175 Petrazzini et al. 2015a; Miletto Petrazzini et al. 2015b). It consisted of a glass tank (60 × 40 ×  
176 35 cm) filled with gravel and 30 cm of water maintained at a temperature of  $26 \pm 1$  °C (Fig.  
177 1b). Green opaque partitions divided the apparatus into a back compartment provided with  
178 natural vegetation and a front “experimental compartment.” A start box was inserted between  
179 the compartments and was provided with a transparent guillotine door controlled remotely by  
180 the experimenter. The apparatus was provided with two filters and each compartment was lit  
181 by a 15 W fluorescent light. A green plastic panel (20 × 15 cm) with 46 holes ( $\varnothing$  1 cm, depth  
182 0.5 cm) was placed in the experimental compartment, close to the front wall. A green net  
183 covered the bottom of the panel to allow the smell of the food reward to pervade the whole  
184 compartment in order to reduce the possibility of the subject using olfactory cues to locate the  
185 correct choice. Two yellow plastic discs ( $\varnothing$  1.2 cm, height 0.2 cm) were used during the  
186 experiment to cover the holes of the panel in front of the stimuli. In order to avoid social  
187 isolation of the subject, four social companions were kept in the apparatus and were  
188 temporarily removed before each experimental session.

189 Stimuli consisted of pairs of laminated panels placed orthogonally to the green panel.  
190 Stimuli were groups of black dots on a white background (5 × 5 cm). Only one numerical  
191 contrast was presented during the experiment: 5 vs. 10 (0.50 ratio). Numerosity normally co-  
192 varies with several physical attributes (e.g., cumulative surface area, overall space occupied  
193 by the sets, or density of the elements), commonly called “continuous quantities”, that can be  
194 used to estimate which group is larger/smaller (Feigenson et al. 2002; Pisa and Agrillo 2009;  
195 Gebuis and Reynvoet 2012). In order to reduce the possibility of subjects using these non-  
196 numerical cues to solve the task, the stimuli were controlled for continuous quantities. In  
197 particular, in one-third of the stimuli, the cumulative surface area was matched to 100%.

198 However, a by-product of equating the cumulative surface area was that smaller-than-average  
199 items would be more frequent in the larger groups, and guppies might use this information  
200 instead of number. To reduce this possibility, the cumulative surface area was matched to  
201 75% in another third of the stimuli and in the remaining third, the cumulative surface area  
202 was not controlled (i.e., the ratio between the cumulative surface area within each pair was  
203 congruent with the numerical ratio: 0.5). Furthermore, since density and convex hull (that is,  
204 the overall surface encompassed by the most lateral dots) are negatively correlated, half of  
205 the sets was controlled for the convex hull, whereas the second half was controlled for  
206 density. Stimuli were extracted from a pool of 48 different pairs (16 pairs for each area  
207 control). The spatial distribution of the dots and their size varied across stimuli to avoid the  
208 possibility that fish could have learned the discrimination on the basis of canonical pattern  
209 recognition instead of numerical information (Mandler and Shebo 1982). Provided that no  
210 other non-numerical cue could be used to solve the task, subjects could reach the learning  
211 criterion only if they were sensitive to numerosity (reviewed in Agrillo and Bisazza 2014).

212

## 213 Procedure

214 We used a modification of the procedures recently adopted to study numerical abilities  
215 (Miletto Petrazzini et al. 2015a) and behavioural flexibility in guppies (Lucon-Xiccato and  
216 Bisazza 2014). The procedure consisted in five phases: familiarization, pre-training,  
217 numerical discrimination learning, discrimination reversal learning and olfactory cue control  
218 test.

219

### 220 *Familiarization*

221 During the 5 days preceding the beginning of the pre-training phase, fish were gradually  
222 familiarized with the apparatus and the experimental procedure. On days 1-2, two subjects

223 were introduced into the experimental apparatus and were fed with commercial food flakes  
224 released through a Pasteur pipette into the water three times a day. On days 3-5, for three  
225 times a day, fish were gently ushered into the start box using a transparent plastic panel. Once  
226 the subjects were into the start box, the guillotine door was closed and a green plastic barrier  
227 was placed in front of it to prevent the fish from seeing the experimental compartment. A pair  
228 of stimuli was placed on the far end of the green panel and dry food was delivered into four  
229 holes in the middle of the panel and equidistant from the stimuli. Subsequently, fish were  
230 allowed to enter the experimental compartment and eat the food. Each area control (non-  
231 controlled, 75% and 100%) was presented once a day. On the evening of day 5, fish were  
232 individually housed in an experimental apparatus. The training phase began the next day.

233

#### 234 *Pre-training*

235 During this phase, fish were trained to dislodge the discs. For this aim, fish underwent a  
236 total of 9 trials with partially covered holes. In trials 1-3, discs covered 25% of the holes; in  
237 trials 4-6, they covered 50%; and in trials 7-9, they covered 75% of the holes. The food  
238 reward was placed only under the disc of the positive stimulus. All subjects were presented  
239 with the same numerical contrast: 5 vs. 10. However, the positive stimulus was the larger  
240 numerosity (10) for half of the fish whereas the positive stimulus was the smaller numerosity  
241 (5) for the other half. These 9 trials were excluded from the analysis. Only subjects that  
242 learned to dislodge the discs were admitted to the numerical discrimination learning phase.

243

#### 244 *Numerical discrimination learning*

245 During this phase, the fish were subjected to 6 trials per day, subdivided into two sessions  
246 of 3 trials each with a 4-hour inter-session interval. Each trial started with the subject in the  
247 start box, the guillotine door closed and the green barrier in front of it to block the view of the

248 experimental compartment. The experimenter placed a pair of stimuli on the green plate, hid  
249 the food reward in the hole in front of the positive stimulus and used the yellow discs to  
250 entirely cover the holes in correspondence with the stimuli. Subsequently, the barrier was  
251 removed and the fish was allowed to look at the stimuli for 30 s. After that, the door was  
252 opened and the subject could enter the experimental compartment to make its choice. The  
253 choice was defined as the first disc dislodged by the fish. We used a correction procedure to  
254 keep the subjects motivated, and if the fish opened the disc associated with the wrong  
255 stimulus, it was allowed to open the disc associated with the correct one and eat the food. If  
256 no discs were dislodged within 5 min, the trial was considered invalid and repeated later. The  
257 position of the stimuli on the plate and their distance from the corridor were determined with  
258 a pseudorandom rule. To avoid any side bias, the left–right position of the stimuli was  
259 counterbalanced over trials and the positive stimulus was never presented more than twice in  
260 a row on the same side. Each cumulative surface area control (100%, 75% and non-  
261 controlled) was presented two times a day. The training continued until the subject reached a  
262 learning criterion of 8 correct responses out of 10 consecutive trials as in experiment 1. Once  
263 the criterion was reached, the fish entered the discrimination reversal learning phase.

264

#### 265 *Discrimination reversal learning*

266 The procedure was the same one used in the numerical discrimination learning phase but  
267 the reward contingency was reversed. The positive stimulus was 10 dots for the subjects  
268 previously trained to select 5 dots and vice versa. The trials always started in the session  
269 following the one in which the subjects had reached the criterion in the numerical  
270 discrimination task. The learning criterion was the same used in the numerical discrimination  
271 learning phase.

272

273 *Olfactory control test*

274 Subjects were given 15 trials using the same procedure of the reversal learning except that  
275 they were presented with pairs of stimuli of identical numerosity. The subjects trained to  
276 select 10 dots in the reversal learning phase were presented with 10 vs. 10, whereas the  
277 subjects trained to select 5 dots in the reversal learning phase were presented with 5 vs. 5.  
278 The food reward was hidden only under one disc with the assumption that, if the subjects  
279 used olfactory cues to locate the correct choice in the previous phases, they were expected to  
280 select the rewarded stimulus more than chance.

281

282 **Statistical analysis**

283 Analyses were performed in R version 3.2.2 (The R Foundation for Statistical Computing,  
284 Vienna, Austria, <http://www.r-project.org>). In the text, we provided mean  $\pm$  SD. All the  
285 statistical tests were two-tailed and the significance threshold was set at  $P = 0.05$ . The  
286 number of errors in experiment 2 was log transformed before the analysis because of a right-  
287 skewed distribution. For both experiments, we compared the performance in the initial  
288 discrimination learning and in the reversal learning phase. To do this, we performed a  
289 repeated measures ANOVA on the pooled data of the initial and the reversal learning phase,  
290 fitted with phase (initial/reversal) as within-subjects factor. To compare the performance of  
291 the two sexes in the two experimental phases, we fitted ANOVAs on the number of errors to  
292 criterion with sex as factor. We also fitted the side initially associated with the reward (right  
293 or left) and the initially rewarded numerosity (5 or 10) as factor in experiment 1 and  
294 experiment 2, respectively. We used one-sample  $t$  test to compare the choice for the baited  
295 disc in the control test of experiment 2 against chance level (50 %). In case of absence of  
296 significant effect of the sex in the previous analysis, the use of the null hypothesis  
297 significance testing does not allow to conclude that the performance of males and females

298 was similar (Barchard 2015; Dienes 2014) nor to exclude the presence of a sex difference  
299 smaller compared to that observed in the previous reversal learning study (Lakens and Evers  
300 2014). For this reason, we calculated Cohen's  $d$  effect sizes and their 95% CIs using the  
301 'effsize' R package. We also calculated the approximate Bayes factor ( $BF$ ) in favour of the  
302 null hypothesis (i.e., the two sexes have similar performance) by comparing the Bayes  
303 information criterion of the linear model fitted with and without the term sex (Wagenmakers  
304 2007).  $BF$  provides relative support to the null hypothesis that is robust even with reduced  
305 sample size (Kass and Raftery 1995). For example, a  $BF > 20$  would conventionally be  
306 considered strong evidence for the absence of sex difference (Kass and Raftery 1995).

307 To analyse all data available on sex differences in reversal learning in guppies, including  
308 the previous study (Lucon-Xiccato and Bisazza 2014), we adopted a meta-analysis approach  
309 using the 'rma' function of the 'metafor' R package on the log transformed data of the  
310 experiments (Viechtbauer 2010). One last analysis was aimed to understand whether possible  
311 differences between the present and the previous reversal learning experiment might be due  
312 to stochastic resampling of the same population. Using bootstrap, we simulated 10000 data  
313 sets of 14 males and 14 females based on the data of the colour discrimination reversal  
314 learning (Lucon-Xiccato and Bisazza 2014) and we calculated the mean performance  
315 difference between males and females (Crawley 2007). From the distribution of the simulated  
316 mean differences and the mean differences observed in the two reversal learning experiments  
317 of this study, we calculated  $P$  values that describe the likelihood of observing the data of the  
318 present experiments due to stochastic resampling from the data of the previous experiment.

319

## 320 RESULTS

### 321 Experiment 1 – T-maze reversal learning

322 In the initial learning phase, guppies made  $13.81 \pm 11.96$  errors before learning the correct  
323 arm of the maze. In the reversal learning phase, guppies made  $14.49 \pm 14.85$  errors before  
324 learning the new reward contingency. The repeated measures ANOVA did not find a  
325 significant difference between the number of errors made in the two phases ( $F_{1,26} < 0.001$ ,  $P$   
326  $= 0.987$ ).

327 The ANOVA analysing the number of errors in the initial learning phase did not find  
328 differences between males and females (males:  $15.93 \pm 11.94$ ; females:  $11.54 \pm 12.03$ ;  $F_{1,23} =$   
329  $2.335$ ,  $P = 0.140$ ; Cohen's  $d = 0.31$ , 95% CI [-0.49, 1.11]; Fig. 2a). Guppies made fewer  
330 errors when the correct arm was the right one (right arm:  $8.43 \pm 5.81$ ; left arm:  $19.62 \pm 14.25$ ;  
331  $F_{1,23} = 6.959$ ,  $P = 0.015$ ). There was not significant sex  $\times$  side interaction ( $F_{1,23} = 0.212$ ,  $P =$   
332  $0.650$ ).

333 The ANOVA analysing the number of errors in the reversal learning phase did not find  
334 differences between males and females (males:  $17.79 \pm 17.78$ ; females:  $11.69 \pm 9.59$ ;  $F_{1,23} =$   
335  $0.643$ ,  $P = 0.431$ ; Cohen's  $d = 0.27$ , 95% CI [-0.53, 1.07]; Fig. 2a). There was no difference  
336 in the number of errors between guppies initially trained with the right or the left arm  
337 associated with the reward (right arm:  $15.71 \pm 11.25$ ; left arm:  $13.92 \pm 17.78$ ;  $F_{1,23} = 1.038$ ,  $P$   
338  $= 0.319$ ). The sex  $\times$  side interaction was significant in the model ( $F_{1,23} = 4.492$ ,  $P = 0.045$ ),  
339 indicating that females performed better than males when the initial rewarded arm was the  
340 left one (Fig. 2b). The Bayesian analysis provided limited support to the absence of sex  
341 difference ( $BF = 3.98$ ).

342

### 343 Experiment 2 – Numerical reversal learning

344 In the initial learning phase, guppies made  $11.78 \pm 9.02$  errors before learning to choose  
345 the correct numerosity. In the reversal learning phase, guppies made  $12.39 \pm 4.55$  errors  
346 before learning the new reward contingency. The repeated measures ANOVA did not find a  
347 significant difference between the number of errors made in the two phases ( $F_{1,27} = 0.101$ ,  $P$   
348  $= 0.753$ ).

349 The ANOVA on the number of errors in the initial learning phase found that females made  
350 fewer errors than males (males:  $15.14 \pm 9.36$ ; females:  $8.43 \pm 7.55$ ;  $F_{1,24} = 4.425$ ,  $P = 0.046$ ;  
351 Cohen's  $d = 0.79$ , 95% CI [-0.02, 1.60]; Fig. 3a) with no difference between fish trained to  
352 select the larger numerosity as positive and fish trained to select the smaller numerosity  
353 (respectively,  $9.57 \pm 8.76$  and  $14.00 \pm 9.04$ ;  $F_{1,24} = 1.925$ ,  $P = 0.178$ ). There was not  
354 significant interaction between sex and the numerosity (larger vs. smaller) associated with the  
355 food reward during the training ( $F_{1,24} = 0.451$ ,  $P = 0.508$ ).

356 In the reversal learning phase, the ANOVA on the number of errors did not find any  
357 significant difference, neither between the two sexes (males:  $13.71 \pm 3.60$ ; females:  $11.07 \pm$   
358  $5.12$ ;  $F_{1,24} = 2.330$ ,  $P = 0.140$ ; Cohen's  $d = 0.60$ , 95% CI [-0.20, 1.39]; Fig. 3b) nor between  
359 the numerosity rewarded (larger:  $12.36 \pm 5.60$ ; smaller:  $12.43 \pm 3.41$ ;  $F_{1,24} = 0.002$ ,  $P =$   
360  $0.967$ ). There was not significant interaction between sex and the numerosity (larger vs.  
361 smaller) associated with the food reward during the training ( $F_{1,24} = 0.288$ ,  $P = 0.597$ ). The  
362 Bayesian analysis did not support the absence of sex difference ( $BF = 1.47$ ).

363 In the control test, guppies selected the disc hiding the food reward at chance level ( $0.48 \pm$   
364  $0.13$ ;  $t_{27} = 0.875$ ,  $P = 0.389$ ), showing that they did not use olfactory cues to select the correct  
365 numerosity during the experiment.

366

367 **Meta-analysis of sex differences in reversal learning in guppies**



368           The meta-analysis on all the available data on sex differences in discrimination  
369 reversal learning found evidence of a significant females' greater reversal learning ability ( $P$   
370 = 0.013; Fig. 4a). The bootstrap analysis showed that the mean difference between the  
371 reversal learning performance of males and females observed in the two experiments of this  
372 study could not be obtained by resampling the data of the previous colour reversal learning  
373 experiment (experiment 1:  $P = 0.006$ ; experiment 2:  $P = 0.028$ ). Furthermore, the conditional  
374 probability to obtain two independent experiments giving no sex difference as in this study is  
375 significantly lower than expected based on the colour reversal learning experiment ( $P =$   
376 0.0002; Fig. 4b). This suggests that the difference between the present and the previous study  
377 is not due to subject random sampling.

378

379 **DISCUSSION**

380 A previous study found a large sex difference in guppies' ability to perform a reversal  
381 learning task involving red–yellow colour discrimination, with males making about twice as  
382 many errors as females (Lucon-Xiccato and Bisazza 2014). This could indicate an overall  
383 greater cognitive flexibility of females, as observed in some mammals and birds (Guillamón  
384 et al. 1986; Ha et al. 2011; Roelofs et al. 2017; Rogers 1974). Here, we confronted male and  
385 female guppies in two other reversal learning tasks requiring T-maze and numerical  
386 discrimination, respectively. In both experiments, we found no obvious evidence of sex  
387 difference in discrimination reversal learning. Females' greater performance emerged only  
388 when jointly analysing the present and the previous experiments on guppies' reversal learning  
389 performance.

390 In the first experiment, we tested guppies in a T-maze. Although we did not assess  
391 whether the fish solved the task by learning the correct arm or developing an egocentric  
392 strategy (e.g., turn left), the T-maze performance is usually considered a measure of spatial  
393 discrimination abilities (Elias et al. 1973; Watson and Stanton 2009). Guppies needed  
394 approximately the same number of trials in the learning and reversal phases. We found no sex  
395 difference in the initial learning phase performance, but females outperformed males in one  
396 condition of the reversal learning phase (when the initial reinforced direction was the left  
397 one). Previously, male and female guppies were compared in other spatial tasks (Lucon-  
398 Xiccato and Bisazza 2017a, b). In one experiment, males and females showed similar  
399 learning abilities when tested in a simple spatial task, detouring around a semi-transparent  
400 barrier to reach a social group. In another test, consisting in learning the correct door in two  
401 sequential pairs of binary choices, males outperformed females. An opposite sex difference  
402 was observed in a task consisting of learning a complex maze made of six consecutive T-  
403 junctions. In the initial discrimination of experiment 1, we found reinforced direction had a

404 very strong effect, with both males and females making more errors when the correct choice  
405 was a left turn. The same bias was found in a previous study using the complex maze formed  
406 by six sequential T-junctions (Lucon-Xiccato and Bisazza 2017b). As previously observed in  
407 guppies and other poeciliid fish, the most likely explanation for this turning bias is an effect  
408 of cerebral lateralisation (Bisazza and Brown 2011).

409 The second experiment followed the same procedure as the previous colour discrimination  
410 reversal learning experiment (Lucon-Xiccato and Bisazza 2014), but here, guppies were  
411 trained in a 5 vs. 10 numerical discrimination with stimuli controlled for non-numerical cues  
412 (cumulative surface area, density and convex hull). We found that females were significantly  
413 better than males at learning this discrimination, whereas we did not find significant sex  
414 differences in the reversal phase. This result seems to suggest that females have better  
415 numerical abilities than males. However, this hypothesis must be considered with caution.  
416 Two previous studies did not find sex differences in two diverse numerical tasks, which were  
417 a discrimination of food quantities and a discrimination of the ordinal position (Lucon-  
418 Xiccato et al. 2015; Miletto Petrazzini et al. 2015b). A third study reported females to be  
419 faster than males in discriminating two groups of social companions, but only when the  
420 discrimination was difficult (Lucon-Xiccato et al. 2016). The numerical discrimination used  
421 here (5 vs. 10) is very easy for guppies; therefore, our results could be attributable to abilities  
422 other than a numerical one or to other factors affecting cognitive performance (Griffin et al.  
423 2015; Lucon-Xiccato and Bisazza 2017c).

424 Regarding sex differences in reversal learning, separate analyses of the two experiments  
425 seem to contrast the results of the previous study in which, in spite of a similar ability in  
426 learning colour discrimination, males made twice as many errors as females in the reversal  
427 phase (Lucon-Xiccato and Bisazza 2014). Can we conclude that sex differences in reversal  
428 learning are limited to the specific context of colour discrimination? It is worth noting that in

429 both the experiments reported here, females' average reversal learning performance was  
430 better than that of males, with small to medium effect sizes. Bayesian analysis did not reveal  
431 convincing evidence in favour of the similarity between the two sexes. Furthermore, our  
432 meta-analysis of the three studies on guppies' reversal learning supports an overall  
433 superiority of females. As a consequence, this evidence might suggest that females perform  
434 better than males in reversal learning, but this effect is much smaller in the T-maze and  
435 numerical discrimination reversal learning than in colour discrimination reversal learning.

436 The different effect size observed in the three reversal learning experiments can have  
437 different explanations. First, despite our sample size was the same as that used in the previous  
438 study on guppies' reversal learning and larger than that of most studies on cognitive sex  
439 differences in other non-human animals (e.g., Gaulin and FitzGerald 1986; Liu et al. 2017), it  
440 might not be sufficient to ensure effect size stability (Lakens and Evers 2014). However, our  
441 simulation based on bootstrap seems to exclude this possibility: the results observed in this  
442 study could not be obtained by randomly resampling the scores of the guppies tested in the  
443 reversal learning experiment by Lucon-Xiccato and Bisazza (2014).

444 A second possibility is that methodological factors have reduced or masked sex  
445 differences in the two experiments of this study. We have followed, as far as possible, the  
446 same procedure used in the previous study on colour discrimination (Lucon-Xiccato and  
447 Bisazza 2014). However, one difference between the experiments is clear: the colour  
448 discrimination was learned with a few errors by guppies and thus appears much easier than  
449 the two discriminations studied here, which were acquired with more than ten errors. So, the  
450 degree of training received by subjects may differ between the experiments included in this  
451 study and the previous one. Some studies reported that overtraining facilitates reversal  
452 learning (rats: Mackintosh 1965), and other studies reported the contrary effect (chicks:  
453 Mackintosh 1965; paradise fish: Warren 1960; goldfish: Mackintosh et al. 1966), although it

454 is not clear to which extent this inconsistency of results was due to species rather than  
455 procedural differences (Mackintosh et al. 1966). There is no available data on overtraining  
456 for guppies or for our specific settings. However, the longer training performed by subjects in  
457 the present experiments may have prevented the appreciation of sex differences in reversal  
458 learning. Another methodological issue to consider concerns the strong effect of the rewarded  
459 arm's direction in experiment 1. Sex differences in lateralisation are commonly found in  
460 vertebrates (Bisazza et al. 1998; Reddon and Hurd 2009; Rilea et al. 2004; Tommasi and  
461 Vallortigara 2004). If the turning bias in the T-maze was due to cerebral lateralisation, this  
462 effect might differ between male and female guppies and might increase performance  
463 variance reducing power to detect learning sex differences.

464 The third, and perhaps more interesting, explanation for the different effect sizes of the sex  
465 difference observed in the present and the previous study regards the presence of sex-specific  
466 selective pressures. More flexible responses in colour discrimination learning might have  
467 been selected in females, which use colour discrimination for mate choice. Females show an  
468 exceptional ability to rapidly estimate and memorize the colour features of the males they  
469 encounter, and they use these cues to comparatively evaluate their prospective mates (Houde  
470 1997). They are also extremely flexible in their mate preferences and, for example, prefer  
471 males which differ in colour compared to males they were previously exposed to (Eakley and  
472 Houde 2004; Hughes et al. 1999). Furthermore, female guppies revert their mate preference  
473 after seeing less preferred males chosen by another female (Dugatkin and Godin 1992; Godin  
474 et al. 2005) and modify their colour preferences under perceived predation hazard (Gong and  
475 Gibson 1996). Even males have been shown to compare the colouration of potential rivals  
476 (Gasparini et al. 2013), but there is no evidence that this ability is highly flexible in this sex.  
477 Flexibility in colour discrimination could also undergo sex-specific selective pressures during  
478 foraging. Guppies of both sexes have a strong tendency to search for small carotenoid-rich

479 fruits that drop from the forest canopy into the rivers of Trinidad (Rodd et al. 2002).  
480 However, for females, fruits enrich the diet with micronutrients that affect physiological  
481 functioning, whereas males gain an additional selective advantage because accumulating  
482 carotenoids increases their chances of being chosen by females and thus increases male  
483 reproductive success (Kodric-Brown 1989). As a consequence, natural and sexual selection  
484 could have shaped different foraging strategies and underlying learning mechanisms between  
485 the two sexes. For example, it might be that once a source of carotenoids is discovered, it  
486 pays for a male to look for similar objects, while ignoring other stimuli. Food imprinting, i.e.  
487 foraging bias following previous experience with a specific food type, has been reported in a  
488 large number of species (Persons and Rypstra 2000; Burghardt and Hess 1966; Rabinowitch  
489 1969), and there is evidence that the “search image” (Tinbergen 1960) can be based on colour  
490 (Croze 1970). Conversely, for females, who are called on to maximize embryo production, it  
491 might be more important to exploit caloric intake, opportunistically utilizing every source of  
492 food, and this could have promoted the switch from one type of food to another (Laland and  
493 Reader 1999). More research is needed to disentangle the possible explanations for task-  
494 specific sex differences in reversal learning.

495 In conclusion, despite the absence of clear sex differences in the two reversal learning  
496 experiments presented here, this study does not overtly exclude the hypothesis that female  
497 guppies are generally more flexible than males in reversal learning as observed in other  
498 vertebrates. Notably, our study suggests that guppies’ sex differences in reversal learning  
499 might vary across cognitive tasks. This points out the importance of investigating sex  
500 differences in fish cognitive flexibility with different reversal learning tasks controlling for  
501 methodological factors that can affect flexibility; also, it would be important to use other  
502 types of tests to measure cognitive flexibility.

503

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508

509 **COMPLIANCE WITH ETHICAL STANDARDS**

510 **CONFLICT OF INTEREST**

511 The authors declare that they have no conflict of interest.

512

513 **ETHICAL STANDARD**

514 Experiments were conducted in agreement with the law of our country (Italy, D.L. 4  
515 Marzo 2014, n. 26). The Ethical Committee of Università di Padova reviewed and approved  
516 the experimental procedures (protocol n. 33/2015 and n. 22/2016). None of the subjects  
517 showed sign of distress. At the end of the experiments, all subjects were released into the  
518 maintenance tanks.

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710

711 **FIGURE CAPTIONS**

712

713 **Fig. 1** Experimental apparatuses. (a) Experiment 1: The experimental apparatus was a T-  
714 maze inserted in a tank filled with water. The maze consisted of a starting chamber, a central  
715 arm and two identical lateral arms connected to the external tank. The terminal part of the  
716 arms was S shaped to prevent the subjects from seeing the exit from inside the maze. (b)  
717 Experiment 2: The apparatus was divided into the back compartment and the front  
718 experimental compartment. The start box was inserted between the compartments. Stimuli  
719 consisted of arrays of black dots on a white background placed orthogonally to the green  
720 panel in the experimental compartment. The holes in front of each stimulus were completely  
721 covered with a pair of yellow discs

722

723 **Fig. 2** Results of Experiment 1. (a) Number of errors of females (dark column) and males  
724 (light column) in the discrimination learning phase. (b) Number of errors in the reversal  
725 learning phase as a function of sex and the side of the arm reinforced. Bars represent the  
726 standard error of the means

727

728 **Fig. 3** Results of Experiment 2. (a) Number of errors of females (dark column) and males  
729 (light column) in the discrimination learning phase. (b) Number of errors of females and  
730 males in the reversal learning phase. Bars represent the standard error of the means

731

732 **Fig. 4** Results of the comparison between experiments on guppies' sex differences in reversal  
733 learning. (a) Mean differences (and 95% CI; logarithmic transformation) between the number  
734 of errors made by males and females in the colour discrimination reversal learning (Lucon-  
735 Xiccato and Bisazza 2014), the spatial (T-maze) and numerical discrimination reversal



736 learning (present study) and estimated from the meta-analysis. (b) Histogram of the mean  
737 difference between the number of errors (logarithmic transformation) made by males and  
738 females obtained with bootstrap from the data of the colour discrimination reversal learning  
739 (Lucon-Xiccato and Bisazza 2014); black, grey and dotted grey line are observed difference  
740 between males and females in the colour, spatial and numerical discrimination reversal  
741 learning, respectively.

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