

1 **Title**

2 How pre- and postcopulatory sexual selection influence male mating decisions in a  
3 promiscuous species

4

5 When females mate multiply, male reproductive success depends on both pre- and  
6 postcopulatory processes, including female choice and sperm competition. However,  
7 these processes can favour different mating tactics in males. Here we use the  
8 Trinidadian guppy (*Poecilia reticulata*) system to understand how this conflict is  
9 resolved. We ask whether knowledge of recent female mating history leads males to  
10 adjust their mating effort in respect of the time devoted to mating activity, and the  
11 frequency and the sequence of mating tactics employed. To do this we quantified male  
12 mating behaviour in three competitive scenarios: 1) Single, when a focal male arrives  
13 near a single female and remains alone with her; 2) First, when a focal male is joined by  
14 a rival male; and 3) Second, when a focal male arrives after a rival male. We  
15 hypothesize that males adjust their behaviour based on arrival order. If female  
16 sequential mate choice is the main process shaping male mating behaviours (favouring  
17 First males in guppies), males should avoid competition and invest most when Single.  
18 Alternatively, if last-male sperm precedence is the major driver of decision making,  
19 males should invest more in mating attempts in the Second scenario. Greatest  
20 investment when First implies an intermediate strategy. We find that order of arrival  
21 influences mating decisions with most mating activity during the First scenario instead  
22 of the Single and Second scenarios. This result suggests that both pre- and  
23 postcopulatory processes influence mating investment, and that individual males make  
24 contingent decisions to maximize both mating and fertilization success.

25

26 **Keywords:**

27 Courtship display; female sequential mate choice; male-male competition; polyandry;

28 sneak; sperm competition; unsolicited attempts.

29

30 When females mate with multiple males within the same breeding season, often referred  
31 as polyandry, males gain more mating opportunities but face, at the same time, the  
32 challenge of cryptic female choice (Eberhard, 1996) and sperm competition (Parker,  
33 1970; 1998). This means that both precopulatory and postcopulatory processes  
34 influence the evolution of male sexual traits.

35         There is considerable interest in the contribution of secondary sexual traits to  
36 male reproductive success both during and after mating, and how they are influenced by  
37 pre- versus postcopulatory processes (reviewed by Evans & Garcia-Gonzalez, 2016).  
38 However, the relative influence of these two selective forces on male mating sexual  
39 traits continues to be debated (Buzatto, Roberts, & Simmons, 2015; Collet, Richardson,  
40 Worley, & Pizzari, 2012; Devigili, Evans, Di Nisio, & Pilastro, 2015; Péliissié, Jarne,  
41 Sarda, & David, 2014; Pischedda & Rice, 2012; Turnell & Shaw, 2015). Recent studies  
42 have focused on physical and sperm traits (e.g. body and sperm length, respectively;  
43 Evans & Garcia-Gonzalez, 2016), but few have considered mating behaviours (Buzatto  
44 et al., 2015; Devigili et al., 2015; Turnell & Shaw, 2015).

45         As with other male sexual traits, mating behaviours are subject to both pre- and  
46 postcopulatory sexual selection pressures (Andersson & Simmons, 2006), and are  
47 correlated with male reproductive success (Buzatto et al., 2015; Devigili et al., 2015;  
48 Fisher, Rodríguez-Muñoz, & Tregenza, 2016; Péliissié et al., 2014; Turnell & Shaw,  
49 2015). Unlike most physical traits however, behaviours can be adjusted in light of the  
50 social context. For instance, mating behaviours can both promote mating and  
51 fertilization success by stimulating or circumventing female mate choice (e.g. courtship  
52 displays and unsolicited mating attempts, respectively; Gross, 1984; Andersson, 1994),  
53 and by avoiding or overcoming mating competition (e.g. mate guarding and sneak,  
54 respectively; Andersson, 1994; Neff & Svensson, 2013).

55           Male order of arrival at or near a female can greatly affect male reproductive  
56 success, both at the pre- and postcopulatory levels (Evans & Garcia-Gonzalez, 2016;  
57 Pélissié et al., 2014; Pischedda & Rice, 2012). For instance, when females choose  
58 sequentially (Jennions & Petrie, 1997; Real, 1990) a male's mating success will vary if  
59 he is the first or last to arrive near a female, depending on whether females are less or  
60 more discriminating (choosy) towards a first than a second male. Similarly, when sperm  
61 competition occurs, male fertilization success can only be maximized if he mates first or  
62 last, depending on whether there is first- or last-male sperm precedence (Birkhead &  
63 Hunter, 1990; Wedell, Gage, & Parker, 2002; Dosen and & Montgomerie 2004; Plath &  
64 Bierbach, 2011). Female mating history can thus play a crucial role in determining  
65 which mating behaviours a male should adopt.

66           In natural conditions males may have little opportunity to evaluate female  
67 mating history (Parker, Ball, Stockley, & Gage, 1997), raising the question of how  
68 males cope with this uncertainty. Considering this, we hypothesize that, if mating order  
69 has no effect on male mating decisions, a male should approach and invest in mating  
70 attempts whenever near a female. In contrast, a male could adjust his behaviour based  
71 on whether he arrives before or after a rival male.

72           Males face a particularly challenging decision when pre- and postcopulatory  
73 processes favour different mating orders. This arises, for example, when females are  
74 less choosy towards the first male they encounter, but where sperm precedence favours  
75 a male that mates afterwards. According to the trade-up hypothesis, females benefit  
76 from being less choosy with a first male, particularly when males are scarce, because  
77 they can ensure the fertilization of all their eggs (Halliday, 1983; Jennions & Petrie,  
78 2000). Females may then become progressively choosier, and mate with any higher-  
79 quality males they subsequently encounter to enhance the genetic quality of their brood.

80 This hypothesis has been supported in species of birds (e.g. Gabor & Halliday, 1997),  
81 mammals (e.g. Klemme, Eccard, & Ylönen, 2006), insects (e.g. Bateman, Gilson, &  
82 Ferguson, 2001), fishes (e.g. guppies; Pitcher, Neff, Rodd, & Rowe, 2003), and reptiles  
83 (e.g. Laloï, Eizaguirre, Fédérici, & Massot, 2011).

84         Here, we ask whether males adjust their mating decisions over a short time scale  
85 based on the order at which they encounter a female. We further examine whether  
86 female mate choice or sperm precedence have the strongest influence on this decision  
87 making. To answer these questions, we studied the mating behaviours of male  
88 Trinidadian guppies (*Poecilia reticulata*) in mixed-sex groups. In these tests we  
89 mimicked the situation in the wild by allowing free interactions between individuals.  
90 First, we determined if a focal male approaches a female before or after another male  
91 (approach decision), and, second, whether his investment in mating behaviours depends  
92 on order of arrival (behavioural adjustment; Figure 1). The Trinidadian guppy is a  
93 freshwater livebearing fish. In this species, the precopulatory process of female mate  
94 choice should favour the first male to approach since females are less discriminatory  
95 towards him than towards subsequent males (Houde, 1997; Liley, 1966; Pitcher et al.,  
96 2003). However, sperm competition (postcopulatory process) favours the last male to  
97 mate since mixed paternity broods (Becher & Magurran, 2004) are predominantly sired  
98 by these males (Evans & Magurran, 2001; Pitcher et al., 2003).

99         Male guppies perform two mating tactics: consensual courtship displays and  
100 unsolicited mating attempts (Magurran, 2005). Courtship displays result in the greatest  
101 paternity success (Evans & Magurran, 2001). Unsolicited mating attempts, on the other  
102 hand, do not require female cooperation (Houde, 1988; Magurran, 2005) and typically  
103 result in the transfer of only modest amounts of sperm (Pilastro & Bisazza, 1999). This

104 mating tactic is more frequent when other males are present (Magellan, Pettersson, &  
105 Magurran, 2005; Magurran, 2005).

106 We hypothesize that, if males are able to evaluate female mating history based  
107 on their own assessment of male-male competition, they will adjust their mating  
108 behaviour based on whether they are with a female alone (Single), approached before  
109 (First) or after (Second) a rival male (Figure 1). In more detail, if female mate choice  
110 gives the most advantage to males, they should avoid competition during mating, and  
111 invest more in following and trying to mate when they are alone with a female (Single),  
112 particularly using the mating tactic that allows them to transfer more sperm (courtship  
113 display). In this case, we expect male guppies to approach females with no rival male  
114 following them, to court more and repeatedly, and to spend more time with a female  
115 when Single. On the other hand, if sperm precedence gives the most advantage to males,  
116 they should invest more when there is competition, particularly in the mating tactic that  
117 transfers fewer amounts of sperm, but may help secure last sperm precedence  
118 (unsolicited attempt). In this case, we predict that male guppies should approach  
119 females with at least one rival male following them, perform more unsolicited mating  
120 attempts, and spend more time with a female when Second. However, if both pre- and  
121 postcopulatory processes are important, males should invest more when First, again due  
122 to potential advantages in terms of female choice, and, simultaneously, to secure sperm  
123 precedence. Figure 1 summarises these scenarios.

124 Alternatively, if information available to males during the current encounter  
125 conveys little fitness benefits to them, then order of arrival should not influence their  
126 behaviour towards the female. Here we expect no difference in mating behaviour if a  
127 male is the only, the first or the second to approach a female (Figure 1).

128

## 129 **Materials and Methods**

### 130 **Experimental setup**

131 We used descendants from wild guppies from the Lower Tacarigua River, in Trinidad.  
132 Following other studies with guppies (e.g. Deacon, Ramnarine, & Magurran, 2011),  
133 observations were carried out in two mesocosm tanks (100 cm x 56 cm x 30 cm).  
134 Behavioural observations in mesocosms have the advantage of allowing individuals to  
135 behave and interact more freely (Devigili et al., 2015). Each mesocosm contained  
136 gravel, an aerating system and two thermostat heaters. The range of temperatures  
137 (24.1°C to 25.7°C) was similar to that found in the wild (Reeve et al., 2014).

138         Inside each mesocosm we placed one mixed sex group of fish: four males and  
139 three females. Wild females are only receptive either as virgins or in few days  
140 immediately following parturition (Liley, 1966; Liley & Wishlow, 1974), thus male  
141 guppies are expected to typically encounter non-receptive females in the wild. This way,  
142 to better simulate natural situations, all females used in our experiments came from a  
143 stock tank, thus likely to be non-virgin and non-receptive.

144         Two groups of three females were haphazardly chosen (standard length  
145 mean  $\pm$  SE: 2.09mm  $\pm$  0.45) from the same stock tank and allocated to each of the two  
146 mesocosms in the afternoon on the day before the observations. This allowed females to  
147 acclimate to the new conditions. Simultaneously, 16 males were transferred from stock  
148 tanks to four maintenance tanks (30 cm x 15 cm x 20 cm). Males were kept in all-male  
149 groups to ensure they were not sperm limited and, therefore, were sexually active during  
150 the observation period. On the day of the observations, males were haphazardly chosen  
151 (standard length mean  $\pm$  SE: 1.48mm  $\pm$  0.15) and assigned to one of the mesocosms 15  
152 minutes before observations started. To avoid familiarity during observations, males  
153 came from different stock and maintenance tanks from that of other males and females.

154 During each observation day, we tested two male groups in each mesocosm with the  
155 same female group, meaning each female group was used twice.

156 All observations were conducted between 9 am and 1 pm. All females were fed  
157 with flake food one hour before the beginning of the observation day, while males were  
158 fed on the previous evening to increase mating behaviour frequency (Sartori &  
159 Ojanguren, n.d.). In total, we tested 152 males and 60 females. At the end of an  
160 observation day, all tested individuals were transferred to a tank identified as observed  
161 group to ensure that males were tested only once, and females were only tested in one  
162 day (with two male groups).

163

#### 164 **Experimental design**

165 The behaviours of the four males in the mesocosm were observed one at a time and  
166 registered using JWatcher v1.0. (Blumstein & Daniel, 2007). To ensure each male was  
167 sampled only once, prior to observation each male was identified based on their unique  
168 colour patterns (Magurran, 2005). Each male behaviour was registered while following  
169 a female, starting with the approach to her and ending when he left that female. As a  
170 result, the duration of each observation was neither controlled by the observer nor had  
171 the same length for each focal male. Instead, the duration of each observation was  
172 determined by the focal male's decision to swim away from the female. Each focal male  
173 experienced one of three possible competitive scenarios: Single, First and Second  
174 (Figure 1). Single was defined as when a male approached a solitary female and  
175 remained as the only male following her the entire time. First was defined as a situation  
176 when the male approached a solitary female, but was subsequently joined by at least one  
177 rival male. Second was defined as a situation where the male approached a female that  
178 was already being followed by at least one rival male. Therefore, we conducted a quasi-



179 experimental design (Cook, 2015) in the sense that males were not allocated to a given  
180 scenario, but scenarios emerged from males (focal and rivals) decisions.

181

## 182 **Behavioural recording**

183 Males made a sequence of mating decisions: (1) whether to approach a female alone or  
184 already being followed by one or more males (approach decision), and (2) whether and  
185 how to adjust their mating behaviour while following the female (behavioural  
186 adjustment) As behavioural adjustment we considered: (a) time spent following the  
187 females, (b) mating tactics performed (courtship displays or unsolicited mating  
188 attempts), and (c) sequence of mating behaviours.

189 We employed two sampling methods: focal and scan sampling. Focal sampling  
190 was used to record male mating decisions and classify the pertaining competitive  
191 scenario. During focal sampling we recorded the total time each focal male followed a  
192 female, the frequency of mating tactics performed (courtship displays or unsolicited  
193 attempts), and the sequence of mating behaviours (from the time the male approached a  
194 female until he swam away from her). Scan sampling was used to calculate the  
195 probability that males had of finding females swimming alone (non-competitive  
196 encounter) or with a rival male (competitive encounter). This method allowed us to have  
197 a proxy of competition. Each focal sampling started when the focal male approached a  
198 female and ended when he left the female. Scan sampling was performed between focal  
199 samplings.

200 We characterized the prevailing competitive scenario during each focal sample  
201 by recording the presence of any other male near the female, as well as the order of  
202 arrival of the focal male. Changes in the competitive scenario during the time a focal  
203 male followed the female were not considered. For example, we considered a scenario

204 as First even if the rival male swam away while the focal male was still following the  
205 female.

206 We started each trial with a scan sample and alternated between scans and focal  
207 observations. Each trial consisted of five scan samples of the females and four focal  
208 observations, one assigned to each male at a time. A total of 184 scans and 152 focal  
209 samples were performed.

210

### 211 **Statistical analyses**

212 All analyses were performed using the software R 3.2.3 (R Core Team, 2015); the  
213 significance alpha was set at  $P = 0.05$ .

### 214 **Approach decision**

215 To evaluate if males preferred non-competitive versus competitive encounters, relative  
216 to a random expectation, we first estimated the frequency of each encounter during the  
217 scan samples. In other words, we estimated the probability of females being alone or  
218 with at least one male following them. These probabilities were calculated by dividing  
219 the frequency of scan samples with females swimming alone, and with females with at  
220 least one male following them, by the total number of scan samples, respectively. A chi-  
221 square goodness-of-fit test was then used to evaluate males' approach decision against  
222 the null expectation.

### 223 **Behavioural adjustment while following a female**

#### 224 **Time following**

225 To test if total time following a female was dependent on the competitive scenarios, we  
226 ran a linear mixed-effects model (LME) from the package nlme (Pinheiro, Bates,  
227 DebRoy, & Sarkar, 2014). The model included time following as the response variable,

228 the competitive scenario (Single, First, or Second) as a fixed term, and the mesocosm  
229 and the female group as random terms, with the female group nested inside the  
230 mesocosm. Diagnostic plots revealed departure in residuals homogeneity in the  
231 response variable. Therefore, time following the female was log-transformed. Models  
232 were compared with a LM model with no random terms using a likelihood ratio test  
233 (LRT).

234         Additionally, we ran an unpaired t-test statistic to compare the time that First  
235 males spent with a female before the approach of another male with the total time  
236 following a female by Single males. This allowed us to infer if time following a female  
237 when in the First scenario was dependent on the male's order of arrival alone, and not  
238 on the time that he previously invested with that female.

239

#### 240 **Mating tactics frequency**

241 We considered the three competitive scenarios to analyse the effect of other males'  
242 presence, and order of arrival, on the focal male's frequency of courtship displays and  
243 unsolicited mating attempts. For the First scenario, only the behaviours performed after  
244 the approach of a rival male were considered. To account for the excess of zeros, the  
245 frequency of mating behaviours was analysed considering separately zeros and non-  
246 zeros using a hurdle model (package pscl; Zuur, Ieno, Walker, Saveliev, & Smith,  
247 2009). This procedure considers a binomial distribution with a logit link function to  
248 model the zeros and a Poisson or a negative binomial distribution to model the non-  
249 zeros. Response variables were the frequency of mating tactics (courtship displays and  
250 unsolicited attempts, analysed separately), while competitive scenario and mesocosm  
251 were added as explanatory variables, both for the count (non-zeros) model and for the  
252 zero model. A likelihood ratio test of nested models with a backward step-wise

253 procedure was used to select the best-fit model (LRTEST from the package lmtest; Zuur  
254 et al., 2009).

### 255 **Mating behaviours sequence**

256 Our aim was to examine if the probability of each behaviour was dependent on the  
257 behaviour that preceded it, and on the competitive scenario the focal male found himself  
258 in. As before, when the focal male was the first to approach a female, we only  
259 considered the behaviours performed after the approach of a rival male. We evaluated  
260 sequences of two behaviours (one followed immediately by another). There were three  
261 possible precedent behaviours: approach, courtship display and unsolicited attempt.  
262 Each of these behaviours was analysed separately. In all cases, a sequence terminated  
263 with either a courtship display, or an unsolicited mating attempt, or a swim away.  
264 Hence, for each analysis three sequence types were considered: precedent behaviour-  
265 courtship display, precedent behaviour-unsolicited attempt, and precedent behaviour-  
266 swim away.

267 Our data do not fulfil the assumptions of Markov Chain Analysis, since the  
268 transition times between behaviours were not homogeneous, and the probability of  
269 behaviours was not uniform (Bakeman & Gottman, 1997). We, therefore, applied  
270 generalized linear mixed-effects models (GLMER from the package lme4; Bates,  
271 Mächler, Bolker, & Walker, 2014), with a logit link function for a binomial distribution  
272 to each precedent behaviour. The response variable was the occurrence/non-occurrence  
273 of each sequence type. This means that in the data set, each focal male was represented  
274 three times, one time for each of the three sequences, using “1” to indicate the  
275 occurrence of one sequence and “0” the non-occurrence. Fixed terms considered were  
276 the competitive scenario and sequence type, as well as their interaction. Random terms  
277 were female group nested in the mesocosm, as well as the repeated observations of each

278 focal male (the pseudo-replicates). Model selection was based on a backward step-wise  
279 procedure with an analysis of variance (anova).

280

### 281 **Ethical notes**

282 Before observations all individuals were kept in mixed-sex stock tanks in, with similar  
283 densities. The stock tanks had gravel in the bottom, and an aerating system. The room  
284 was kept at a controlled stable temperature. The period of light was controlled (12:12h  
285 light:dark cycle). Individuals were fed daily. Before the observations, all individuals  
286 were transferred to observation tanks (mesocosms) to acclimate to the new conditions.  
287 Female guppies are often more sensitive to changes than males, because of their  
288 stronger and more stable social bonds (Croft et al., 2006; Griffiths & Magurran, 1998).  
289 Therefore, females were transferred to the mesocosms several hours before the  
290 observations and were kept with other familiar females, while the males were  
291 transferred 15 minutes before observations started. After observations all individuals  
292 were transferred to stock tanks with no fish (labelled as “observed individuals” during  
293 the experiment). All animals were individually and carefully caught and moved between  
294 tanks with a net.

295 All behavioural observations were carried out at the School of Biology at the  
296 University of St Andrews. The premises where the observations were carried out  
297 comply with the UK guidelines for the treatment of animals in behavioural research and  
298 teaching, set by the UK Home Office (PCD 60/2609). All applicable international,  
299 national, and/or institutional guidelines for the care and use of animals were followed.

300

## 301 **Results**

### 302 **Approach decision**

303 In 45.7 % of the scan samples (out of a total of 184) females were not followed by any  
304 male. However, 59.2 % of focal males approached females when they were alone. There  
305 was no significant difference between the frequency with which males chose or avoided  
306 competition relative to the null expectation ( $\chi^2_1 = 1.5$ ,  $N = 152$ ,  $P = 0.220$ ). After  
307 approaching the female, the focal male remained the Single one following the female in  
308 29.6 % of the cases; was the First to approach but other male approached afterwards  
309 29.6 % of the time; and was the Second to approach in 40.8 % of the cases.

310

### 311 **Behavioural adjustment while following a female**

#### 312 **Time following**

313 For the time males spent following females, only the competitive scenario remained in  
314 the best explanatory model ( $F_{2,149} = 16.2$ ,  $N = 152$ ,  $P < 0.001$ ). On average, males spent  
315 25 seconds following a female. No significant difference was found between the time  
316 spent following a female when males were Single versus when they were Second  
317 ( $|t| = 0.6$ ,  $P = 0.568$ ). However, males that First approached a female spent more time  
318 following her compared to males that were Single or the Second to arrive at the female  
319 (Single vs. First:  $|t| = 5.1$ ,  $P < 0.001$ ; First vs. Second:  $|t| = 4.9$ ,  $P < 0.001$ ; Figure 2).  
320 Additionally, the total time males spent following the female when they were First was  
321 independent of the time invested prior to the approach of another male. The time that  
322 First males spent with a female before the approach of another male was not different  
323 from the total time following when males were in the Single scenario (unpaired t-test:  
324  $|t|_{195} = 0.6$ ,  $N_1 = 90$ ,  $N_2 = 107$ ,  $P = 0.555$ ).

325

### 326 **Mating tactics frequency**

327 Most focal males did not perform any courtship display (86.2 %) or unsolicited attempt  
328 (78.9 %) when following females. From those that performed mating attempts, they did  
329 it only once in most of the cases: 81.0 % and 75.0 % of the samples for courtship  
330 displays and unsolicited attempts, respectively. Specifically, for the frequency of  
331 courtship displays, and after model selection, none of the explanatory variables  
332 (competitive scenario and mesocosm) was included in the best-fit model ( $\chi^2_2 = 8.9$ ,  
333  $N = 152$ ,  $P = 0.064$ ; Figure 3a). By contrast, the best-fit model that explained the  
334 frequency of unsolicited attempts included the competitive scenario ( $\chi^2_2 = 8.2$ ,  $N = 152$ ,  
335  $P = 0.017$ ; Figure 3b). Focal males that approached a female First performed more  
336 unsolicited attempts than males that were the Single following the female ( $z = 2.3$ ,  
337  $P = 0.031$ ), or males that were the Second ( $z = 2.3$ ,  $P = 0.031$ ).

338

### 339 **Mating behaviours sequence**

340 For the analyses of the behavioural sequences that started with approach, the best-fit  
341 model ( $\chi^2_4 = 10.2$ ,  $N = 456$ ,  $P = 0.037$ ) excluded the random terms and included the  
342 interaction between sequence type and competitive scenario. Based on the best model,  
343 males had higher probability of swimming away after an approach than of performing  
344 courtship displays or unsolicited attempts across all scenarios (Figure 4a, Table 1).  
345 However, this probability was higher when they were the Second to approach a female  
346 (see in Table 1, for comparison between Second and the other two scenarios).

347 Only 21 focal males performed at least one courtship display, meaning that only  
348 those males were included in the analysis of the behavioural sequences that started with  
349 a courtship display. Males never performed some sequences in some competitive  
350 scenarios (courtship display-courtship display when focal males were Single with the

351 females, and courtship display-unsolicited attempt when males were the First to  
352 approach the females; Figure 4b). Differences between competitive scenarios were not  
353 significant, as the best-fit model included sequence type as the only explanatory  
354 variable ( $\chi^2_2 = 21.9$ ,  $N = 63$ ,  $P < 0.001$ ). Males had significantly higher probability of  
355 swimming away after a courtship display, than of performing a second courtship  
356 ( $z = 3.985$ ,  $P < 0.001$ ) or an unsolicited attempt ( $z = 4.283$ ,  $P < 0.001$ ). But, when males  
357 did not swim away, they were equally likely of performing a courtship and an  
358 unsolicited attempt ( $z = -0.867$ ,  $P = 0.386$ ).

359 For the analyses of the behavioural sequences starting with unsolicited attempts,  
360 only the 32 focal males that performed at least one unsolicited attempt were considered.  
361 The best-fit model excluded the random terms (mesocosm and female group) and  
362 included the interaction between the competitive scenario and the sequence type  
363 ( $\chi^2_4 = 11.0$ ,  $N = 96$ ,  $P = 0.026$ ). The best model revealed that the probability of  
364 swimming away after an unsolicited attempt was higher when males were Single and  
365 Second than when they were First (Figure 4c; Table 1). Additionally, there were no  
366 significant differences between the probabilities of First males swimming away,  
367 performing a courtship display or repeating an unsolicited attempt.

368

## 369 **Discussion**

370 Our findings indicate that males adjust their mating behaviour in response to the order  
371 in which they arrive at a female, investing more on mating behaviours when they  
372 precede a rival. Specifically, males spent more time following females and performed  
373 more and repeated mating attempts when they were the First to arrive at the mating  
374 context, than males that remained Single with the female or arrived Second. The  
375 scenario First was the situation where males' chance of being preferred by females



376 (female sequential mate choice) and of fertilizing more eggs (sperm precedence) could  
377 be compromised by a rival's approach. By investing more in this scenario, males  
378 probably try to reduce such risks. Taken together our results demonstrate that males  
379 make contingent mating decisions depending on the competitive environment.  
380 However, they do this by investing more in unsolicited mating attempts than in  
381 courtship displays.

382

### 383 **Approach decision**

384 Males approached a female regardless of the presence or absence of other males, in line  
385 with the frequency of occurrence of competitive encounters in the population. This  
386 result indicates that order of arrival near a female does not influence a male's decision  
387 to approach a female.

388         Since male guppies express weak social bonds with females and are constantly  
389 moving between groups of females (Croft et al., 2006; Griffiths & Magurran, 1998),  
390 avoiding or seeking encounters with other males may be difficult in the wild. In fact, we  
391 found that, even in cases where a male approached a solitary female (choosing a non-  
392 competitive encounter), he was often joined by a rival male. It is likely that guppies do  
393 not avoid other males, because doing so does not prevent sperm competition, as it has  
394 been shown for the field cricket (*Gryllus campestris*; Fisher et al. 2016). At the same  
395 time, it seems unlikely that male guppies actively seek out females accompanied by  
396 rival males.

397

### 398 **Behavioural adjustment while following a female**

399 We predicted that if a male's order of arrival near a female was important in shaping  
400 male mating behaviour, we would find different patterns of mating behaviour across the

401 three scenarios. More specifically, we expected more investment in mating behaviours  
402 when Single if males were prioritizing female mate choice, or more investment when  
403 Second if males were prioritizing sperm precedence. The First scenario was an  
404 intermediate one, where males would try to secure both the advantage of female choice  
405 and sperm precedence. We found evidence for the First scenario, with First males  
406 investing more in females than Single and Second males. However, they did so through  
407 unsolicited mating attempts instead of courtship display. Additionally, males postponed  
408 the decision to swim away, and invested more on mating attempts (courtship and  
409 unsolicited) after an unsolicited attempt when were First. This indicates that, despite  
410 seemingly being non-selective about their order of arrival, males do not invest equally  
411 in all three competitive scenarios, but invest more in the scenario where neither pre- nor  
412 postcopulatory processes dominate.

413         There is evidence that female guppies choose higher displaying males  
414 (Magurran, 2005) and that male reproductive success is linked to the frequency of  
415 courtship displays (Evans & Magurran, 2001). Since we found that focal males did not  
416 change the frequency of courtship displays between competitive scenarios, this could  
417 indicate that investment on courtship displays is more dependent on female traits, like  
418 receptivity (Farr, 1980; Guevara-Fiore, Stapley, & Watt, 2010), than on the male's order  
419 of arrival. Males typically achieve mating after courtship display only if females are  
420 receptive (Magurran, 2005). Given that receptive females are more responsive to males  
421 that approach them first (Pitcher et al., 2003) and males are able to distinguish among  
422 receptive and non-receptive females (Guevara-Fiore, Stapley, Krause, Ramnarine, &  
423 Watt, 2010), a higher investment in courtship displays could still be expected with  
424 receptive females in scenarios where males were alone with the females or the first to  
425 approach. Although we did not control for female receptivity, our experimental

426 approach mimics closely female-male dynamics in nature, where receptive females are  
427 rare (Liley, 1966; Liley & Wishlow, 1974). In such natural conditions, our results  
428 indicate that courtship displays are not used differently across the three competitive  
429 scenarios, suggesting that reproductive advantages are obtained from such displays  
430 irrespective of whether males are Single, First or Second with a female. Future studies  
431 could examine the relevance of female receptivity in male mating behaviours relative to  
432 order of arrival.

433         In contrast to courtship displays, both the frequency of unsolicited mating  
434 attempts and the sequence of behaviours performed after these attempts were dependent  
435 on the order of arrival. Males that were the First to approach the female performed more  
436 unsolicited attempts, and were more likely to repeat that behaviour or to court than to  
437 swim away than Single and Second males. It has been previously shown that male  
438 guppies increase unsolicited mating attempts in response to increased mate competition  
439 (Magellan et al., 2005; Magurran, 2005). Indeed, males can mate more rapidly and  
440 repeatedly without female cooperation by performing unsolicited attempts (Houde,  
441 1988; Magurran, 2005). Furthermore, by re-mating, males ensure a higher chance of  
442 being the last one to mate or of transferring more sperm. The greater proportion of  
443 unsolicited mating attempts found in our study when males were the First to approach  
444 suggests that males are less willing to abandon the female in that scenario. This could  
445 represent a strategy either to transfer more sperm (correlated with unsolicited attempts  
446 frequency; Matthews, Evans, & Magurran, 1997), to be the last to transfer sperm and  
447 ensure last sperm precedence, or to inform rival males that the female has mated.  
448 Supporting this last hypothesis, a theoretical study suggests that males should only  
449 transfer such information to rival males when they are in disadvantage on sperm  
450 competition (Engqvist & Taborsky, 2017). In fact, the First scenario is the one where

451 the risks are higher, as males have more to lose than those who approached Second or  
452 those that were alone with a female the entire time.

453 To assess if First males tried to be the last to mate, we performed additional  
454 statistical analyses on the order by which males left the females and found that males  
455 left the females later than rivals more frequently when they were the First to arrive than  
456 when they were Second (see Appendix Table A1). We also found that males invest  
457 more time and make more mating attempts when they left the female after the rival male  
458 in both First and Second scenarios (see Appendix Table A1, Figures A1 and A2),  
459 suggesting that First males tried to secure sperm precedence. These findings are  
460 compatible with higher investment in mating attempts (more sperm transference) in  
461 high sperm competition scenarios predicted by theoretical models (Parker et al., 1997),  
462 and supported by empirical studies in several other species (e.g. dominant versus  
463 sneaker males, Collet et al. 2012; and absence versus presence of competitor males,  
464 Kelly & Jennions 2011). Sperm competition is potentially high in guppies, as females  
465 can store sperm of multiple males for months (Herdman, Kelly, & Godin, 2004;  
466 Magurran, 2005) and a single brood is generally fathered by two or more males (Becher  
467 & Magurran, 2004; Devigili et al., 2015; Elgee, Ramnarine, & Pitcher, 2012). Males'  
468 investment in time and energy with a female, when they are the First to approach the  
469 female, could give them a significant fitness advantage relative to sperm competition. In  
470 fact, previous studies found that male guppies invest less in mating behaviours when  
471 they lose the opportunity of mating first with a female (Dosen & Montgomerie, 2004;  
472 Jeswiet, Lee-Jenkins, Ramnarine, & Godin, 2011).

473 Our study provides additional support for the finding by Magris and colleagues  
474 that, when female cryptic choice is controlled (by artificial insemination), there is first-  
475 instead of last-male sperm precedence (Magris, Cardozo, Santi, Devigili, & Pilastro,

476 2017). The greater mating investment by First males reported in our study may increase  
477 a male's chances during cryptic female choice. Moreover, sperm competition could  
478 explain why First males performed more unsolicited attempts and repeated more mating  
479 attempts after this tactic. This was why sperm precedence was reversed in Magris et al.  
480 (2017) study where males' equal ejaculate size competed for fertilization.

481 For female sequential mate choice, we predicted higher investment when males  
482 were Single, than when they were First and Second. We failed to detect an increase time  
483 following and in the frequency of courtship displays when males were Single (as  
484 discussed above). What we did find was that males spent more time with females when  
485 they were First and invested more in mating attempts (both unsolicited and courtship)  
486 after an unsolicited attempt than in the other scenarios. This suggests that female  
487 sequential mate choice is not the main mechanism shaping male mating behaviours, but  
488 that in concert with sperm precedence it selects for male mating tactics. We cannot  
489 exclude the possibility that males also improved their chances of being preferred by the  
490 females when arriving First. The fact that males invested in courtship displays after an  
491 unsolicited mating attempt supports this possibility, as do studies showing that female  
492 guppies cryptic choice favours the sperm of preferred males (Gasparini & Pilastro,  
493 2011; Pilastro, Simonato, Bisazza, & Evans, 2004).

494

## 495 **Conclusions**

496 Our work advances understanding of how pre- and postcopulatory sexual processes  
497 shape male mating behaviours, when the order in which males approach a female  
498 (before or after a rival male) provides contrasting competition advantages. We provide,  
499 for the first time, empirical evidence about the interplay between male uncertainty about  
500 long-term female mating history, short term male behavioural flexibility, and

501 antagonistic pre- and postcopulatory processes. We show that male guppies use  
502 information based on the order in which they approach a female and invest more in  
503 mating attempts when the risk of losing both female preference and sperm precedence is  
504 higher. In light of the growing evidence of female multiple mating across taxa (Arnqvist  
505 & Nilsson, 2000; Barbosa & Magurran, 2006; Parker & Birkhead, 2013; Taylor, Price,  
506 & Wedell, 2014), we suggest that future studies (including comparative approaches) ask  
507 how males (and females; Shuster, Briggs, & Dennis, 2013) shape their mating decisions  
508 based on their knowledge of their mates' mating history. Such new studies should  
509 consider the degree of polyandry in a population (based on the number of females that  
510 mate multiply, as well as the maximum average number of matings per female; Taylor  
511 et al. 2014); the degree of uncertainty about female mating history; whether sequential  
512 mate choice is in place or not; and the degree of sperm precedence (if partial or total;  
513 see Turnell & Shaw, 2015). For example, in species like guppies that have high  
514 frequency of female multiple mating, but unlike guppies have total sperm precedence,  
515 postcopulatory pressures may have a higher contribution to the evolution of male  
516 mating behaviours. In such cases, uncertainty about female mating history and order of  
517 arrival should be irrelevant, if males assume that they are always the first or the last to  
518 mate, and have strategies to control sperm competition (e.g. mate guarding; Ridley,  
519 1980, 1989), or to avoid sperm competition (e.g. mating plugs; Dougherty, Simmons, &  
520 Shuker, 2016). On the other hand, precopulatory selection may act more strongly on  
521 male mating behaviours in species with partial sperm precedence and less polyandry  
522 than in guppies (Turnell & Shaw, 2015). Indeed, as we have shown here, the outcome of  
523 selection on male behaviour is subject to subtle drivers that vary in intriguing and  
524 complex ways amongst species.

525

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538

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- 754

755 **Tables**

756

757 **Table 1.** Summary table of the GLMER models for the behavioural sequences on

758 different competitive scenarios.

Precedent Behaviour	Sequence type		Competitive scenario	Z-value	P-value
Approach					
	Approach- Swim away	vs	Approach- Courtship display	Single First Second Single vs First First vs Second Single vs Second	-5.013 < <b>0.001</b> -4.795 < <b>0.001</b> -6.232 < <b>0.001</b> -0.057 0.955 -2.015 <b>0.044</b> -2.108 <b>0.035</b>
			Approach- Unsolicited attempt	Single First Second Single vs First First vs Second Single vs Second	-4.429 < <b>0.001</b> -3.500 < <b>0.001</b> -6.743 < <b>0.001</b> 0.854 0.393 -2.532 <b>0.011</b> -1.620 0.105
Unsolicited attempt					
	Unsolicited attempt- Swim away	vs	Unsolicited attempt- Courtship display	Single First Second Single vs First First vs Second Single vs Second	-2.948 <b>0.003</b> 0.000 1.000 -1.736 0.083 2.586 <b>0.010</b> -1.332 0.183 1.515 0.130
		vs	Unsolicited attempt- Unsolicited attempt	Single First Second Single vs First First vs Second Single vs Second	-2.948 <b>0.003</b> -0.409 0.682 -2.128 <b>0.033</b> 2.384 <b>0.017</b> -1.423 0.155 1.185 0.236
	Unsolicited attempt- Unsolicited attempt	vs	Unsolicited attempt- Courtship display	Single First Second Single vs First First vs Second Single vs Second	0.000 1.000 0.409 0.682 0.514 0.608 0.198 0.843 0.152 0.879 0.296 0.768

759 Analyses were conducted separately based on the behaviour that initiated the sequence  
 760 type (precedent behaviour): approach to a female ( $N = 152$ ; all males approached a  
 761 female) or unsolicited attempt ( $N = 32$ ; number of focal males that performed the  
 762 unsolicited tactic). Each sequence type includes the precedent behaviour and the  
 763 following behaviour (courtship display, unsolicited attempt, or swim away). Sequences  
 764 that started with courtship display are not shown because the best-fit model did not  
 765 include the interaction between sequence type and competitive scenario. P-values in  
 766 bold represent significant differences.

767

768 **Table A1.** Summary table of the best models encountered to explain male mating  
 769 investment relative to their chance of winning last sperm precedence.

Response variable	Model type		Distribution	Explanatory variables	Contrasts	Z-value/ T-value	P-value
Leaving order	GLMER		Binomial	Competitive scenario	First vs Second	-2.2	0.025
Time following	LMER		Normal (log-transformed)	Competitive scenario	First vs Second	-3.8	<0.001
				Leaving order	Earlier vs Later	4.8	<0.001
Courtship display	Hurdle	Zero model	Poisson	Leaving order	Earlier vs Later	5.0	<0.001
Unsolicited attempt	Hurdle	Count model	Negative Binomial	Competitive scenario	First vs Second	-2.2	0.0310
		Zero model		Leaving order	Earlier vs Later	5.0	<0.001

770

771 Two competitive scenarios during which focal males faced competition were considered  
 772 (First and Second). Response variables were: leaving order (i.e., leave the female  
 773 earlier/later than rival male), frequency of courtship displays, frequency of unsolicited  
 774 attempts, and time following females. Explanatory variables considered for all the  
 775 models were: competitive scenario (First and Second), mesocosm, female group (nested

776 inside the mesocosm) and leaving order (except when it was used as the response  
777 variable). For leaving order (as the response variable) the best-fit model included the  
778 competitive scenario ( $\chi^2_1 = 5.0$ ,  $N = 107$ ,  $P = 0.025$ ). Both leaving order and the  
779 competitive scenario were included in the best-fit model for time following  
780 ( $F_{104,149} = 23.5$ ,  $N = 107$ ,  $P < 0.0001$ ; Figure A1). For frequency of courtship displays,  
781 only leaving order (as explanatory variable) influenced this behaviour (included in the  
782 zero model;  $\chi^2_1 = 30.0$ ,  $N = 107$ ,  $P < 0.001$ ; Figure A2a). In relation to frequency of  
783 unsolicited attempts, the competitive scenario was included in the count model and  
784 leaving order was included in the zero model ( $\chi^2_1 = 29.2$ ,  $N = 107$ ,  $P < 0.001$ ; Figure  
785 A2b). None of the best models included the interaction between leaving order and  
786 competitive scenario.

787

788 **Figure legends**

789

790 **Figure 1.** Questions, hypotheses and predictions on male mating decisions. Observed  
791 competitive scenarios: Single ( $N = 45$ ), the focal male (black) was the single male  
792 following a female (grey) for the entire time; First ( $N = 45$ ), the focal male was the first  
793 to approach the female and a rival male (white) arrived later; Second ( $N = 62$ ), the focal  
794 male approached the female after the rival male. Males make mating decisions at  
795 different points: (1) whether to approach a female or not (approach decision), (2) after  
796 approaching, they decide how and how much to invest (time spent following, mating  
797 tactics and mating behaviours sequence). The scenario below each of the hypothesis  
798 represents the predicted outcome. In addition, the framework identifies the mating tactic  
799 we expect to dominate under each scenario. Drawings are adapted from Liley (1966).

800

801 **Figure 2.** Time spent by focal males following a female under the three competitive  
802 scenarios: when the focal male was the single male following a female the entire time  
803 (Single,  $N = 45$ ); when he was the first to approach a female (First,  $N = 45$ ); and when  
804 he was the second (Second,  $N = 62$ ). Pairwise comparisons of time spent following  
805 between competitive scenarios were obtained from the best-fit LME model. Asterisks  
806 and associated lines above the plots show which sets of competitive scenarios differed  
807 significantly from one another ( $*P < 0.05$ ). In each boxplot the internal line represents  
808 the median. Lower and upper edges represent the 25% and the 75% quantile,  
809 respectively. Whiskers below and above the box edges represent, respectively, the  
810 minimum and the maximum points within the 1.5 interquartile range. Circles represent  
811 outliers.

812

813 **Figure 3.** Frequency of mating tactics in relation to competitive scenario. Mating tactics  
814 were either (a) courtship displays (on the left side of the figure) or (b) unsolicited  
815 mating attempts (on the right side). There were three scenarios: when the focal male  
816 was the single male following a female the entire time (Single,  $N = 45$ ); when he was  
817 the first to approach a female (First,  $N = 45$ ); and when he was the second (Second,  
818  $N = 62$ ). The diameter of the circles is proportional to the sample size. Pairwise  
819 comparisons between competitive scenarios of the frequency of each mating tactic were  
820 obtained from the best-fit hurdle models. Asterisks and associated lines above the plots  
821 show which sets of competitive scenarios differed significantly from one another  
822 ( $*P < 0.05$ ).

823

824 **Figure 4.** Mating behaviours sequences in three different competitive scenarios. The  
825 three scenarios were (from left to right; with focal male represented in grey): when the  
826 focal male was the single male following a female the entire time (Single,  $N = 45$ );  
827 when he was the first to approach a female (First,  $N = 45$ ); and when he was the second  
828 (Second,  $N = 62$ ). Each arrow represents the sequence of two behaviours. Each  
829 sequence could start with (a) approach to a female, (b) courtship display, or (c)  
830 unsolicited attempt; and end with (b) courtship display, (c) unsolicited attempt, or (d)  
831 swim away. Arrow width represents the estimated probability of that sequence.  
832 Sequences that were not observed or were rare (with an estimated probability lower than  
833 0.1) were not included. For each scenario, continuous arrows represent behavioural  
834 sequences that were significantly more likely to occur than sequences represented by  
835 dashed arrows. We obtained the probability estimates of each behaviour sequence from  
836 the best-fit statistical GLMER models. The diameter of the circles is proportional to the  
837 frequency of each behaviour for each scenario.

838

839 **Figure A1.** Time spent by focal males following a female between scenarios with  
840 different advantage in relation to sperm precedence. Two competitive scenarios related  
841 with order of arrival near a female were considered: when the focal was the first to  
842 approach a female (First); and when he was the second (Second). And, within these  
843 competitive scenarios, males were divided in two additional scenarios depending on the  
844 order they left the female (i.e. probability of mating at last): when the focal male leave  
845 the female earlier than the rival (lower probability of mating after the rival - Earlier);  
846 and when the focal leave the female later than the rival (higher probability of mating  
847 after the rival - Later). Pairwise comparisons of time spent following between scenarios  
848 were obtained from the best-fit LME model. Asterisks and associated lines above the  
849 plots show which sets of competitive scenarios differed significantly from one another  
850 ( $*P < 0.05$ ). In each boxplot the internal line represents the median. Lower and upper  
851 edges represent the 25% and the 75% quantile, respectively. Whiskers below and above  
852 the box edges represent, respectively, the minimum and the maximum points within the  
853 1.5 interquartile range. Circles represent outliers.

854

855 **Figure A2.** Frequency of mating tactics between scenarios with different advantage in  
856 relation to sperm precedence. Mating tactics were either (a) courtship displays (on the  
857 left side of the figure) or (b) unsolicited attempts (on the right side). Two competitive  
858 scenarios related with order of arrival near a female were considered: when the focal  
859 was the first to approach a female (First); and when he was the second (Second). And,  
860 within these competitive scenarios, males were divided depending on the order they left  
861 the female (i.e. probability of mating at last): when the focal male leave the female  
862 earlier than the rival (lower probability of mating after the rival - Earlier); and when the

863 focal leave the female later than the rival (higher probability of mating after the rival -  
864 Later). The diameter of the circles is proportional to the sample sizes. Pairwise  
865 comparisons of the frequency of each mating tactic between scenarios were obtained  
866 from the best-fit hurdle models. Asterisks and associated lines above the plots show  
867 which sets of competitive scenarios differed significantly from one another ( $*P < 0.05$ ).



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Figure

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Questions	Hypotheses			
	Yes			No
Males adjust to short-term order of arrival?				No
Which selective process has higher contribution to male reproductive success?	Female mate choice (precopulatory)	Sperm precedence (postcopulatory)	Both	-
	Predictions			
<b>Male mating decisions</b>				
(1) Approach decision (at this point only scenarios Single and Second occur)	Single	Second	Single	Single, Second
(2) Behavioural adjustment			First	Single, First, Second
(a) Time spent following				
(b) Mating tactics	Courtship display	Unsolicited attempt		
(c) Mating behaviours sequence	(transfer more sperm)	(transfer sperm in last)		

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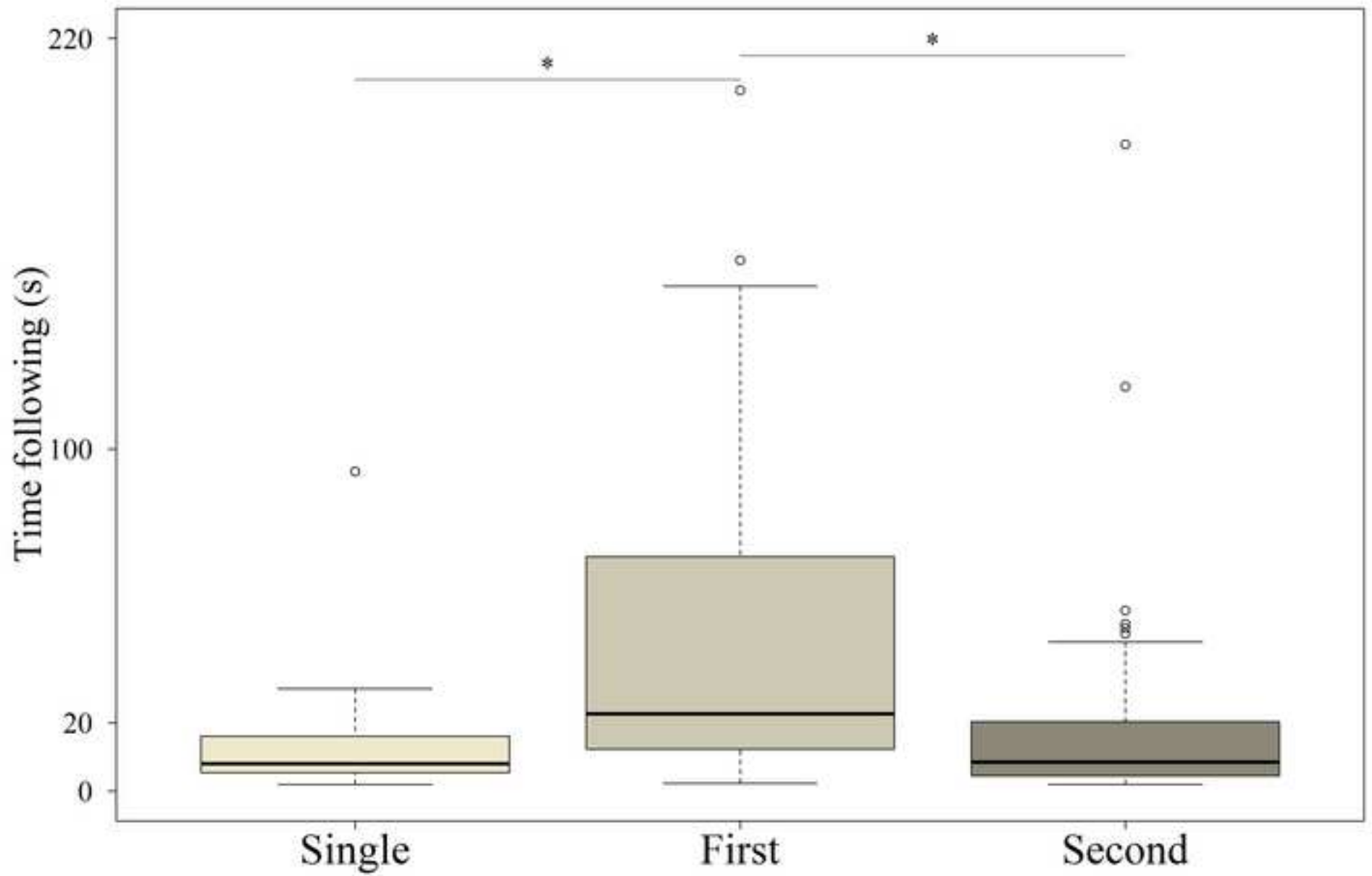


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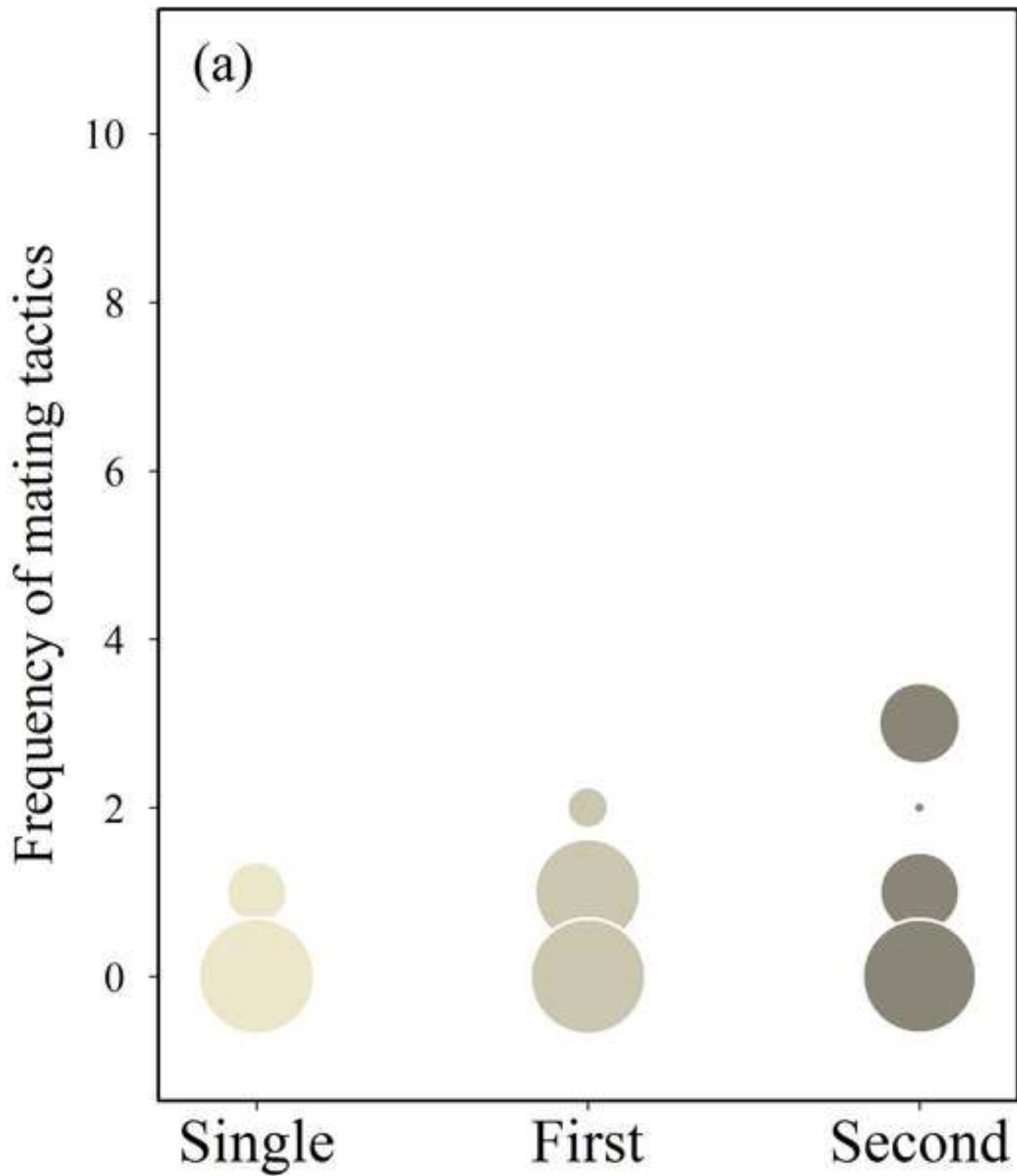


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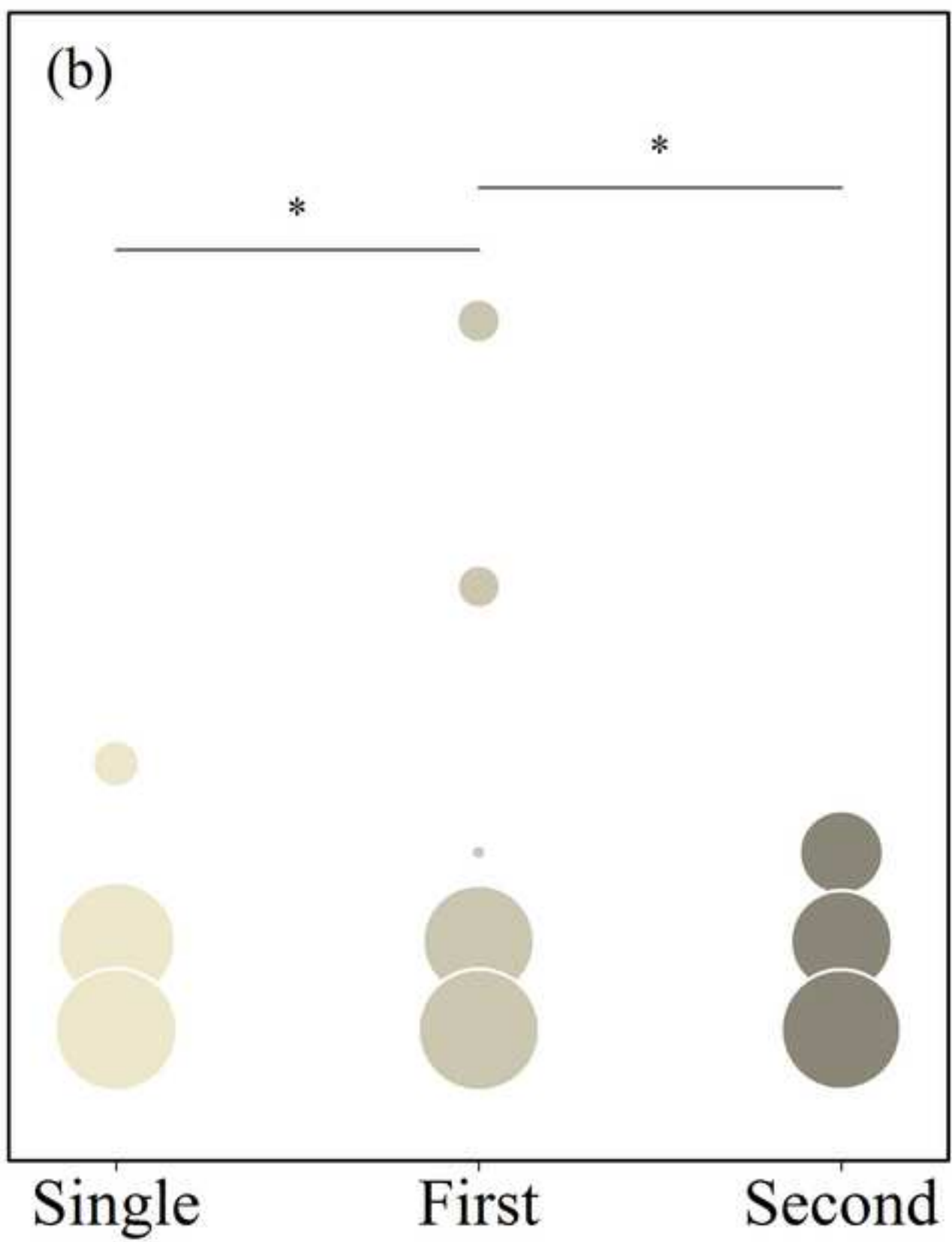


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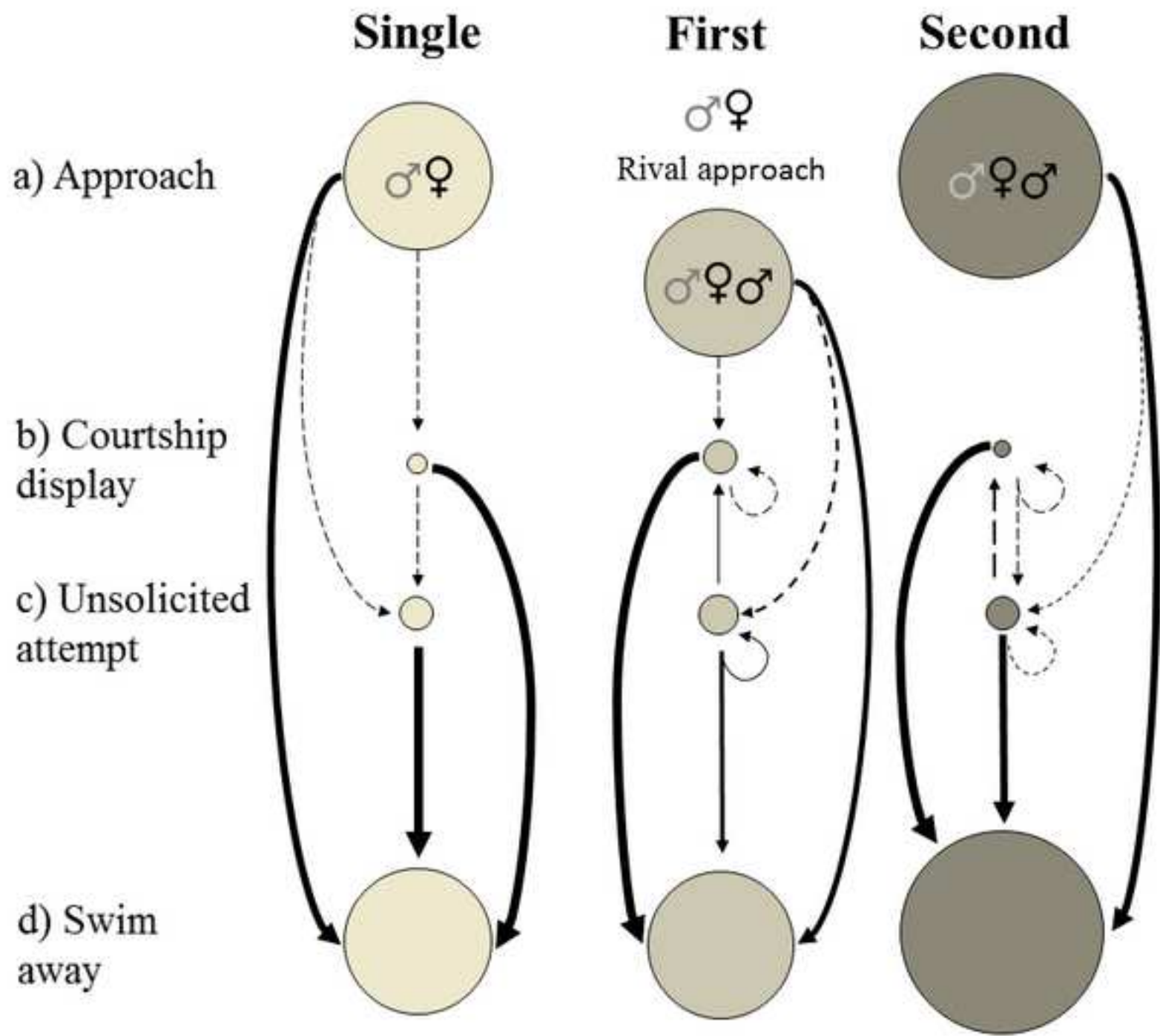
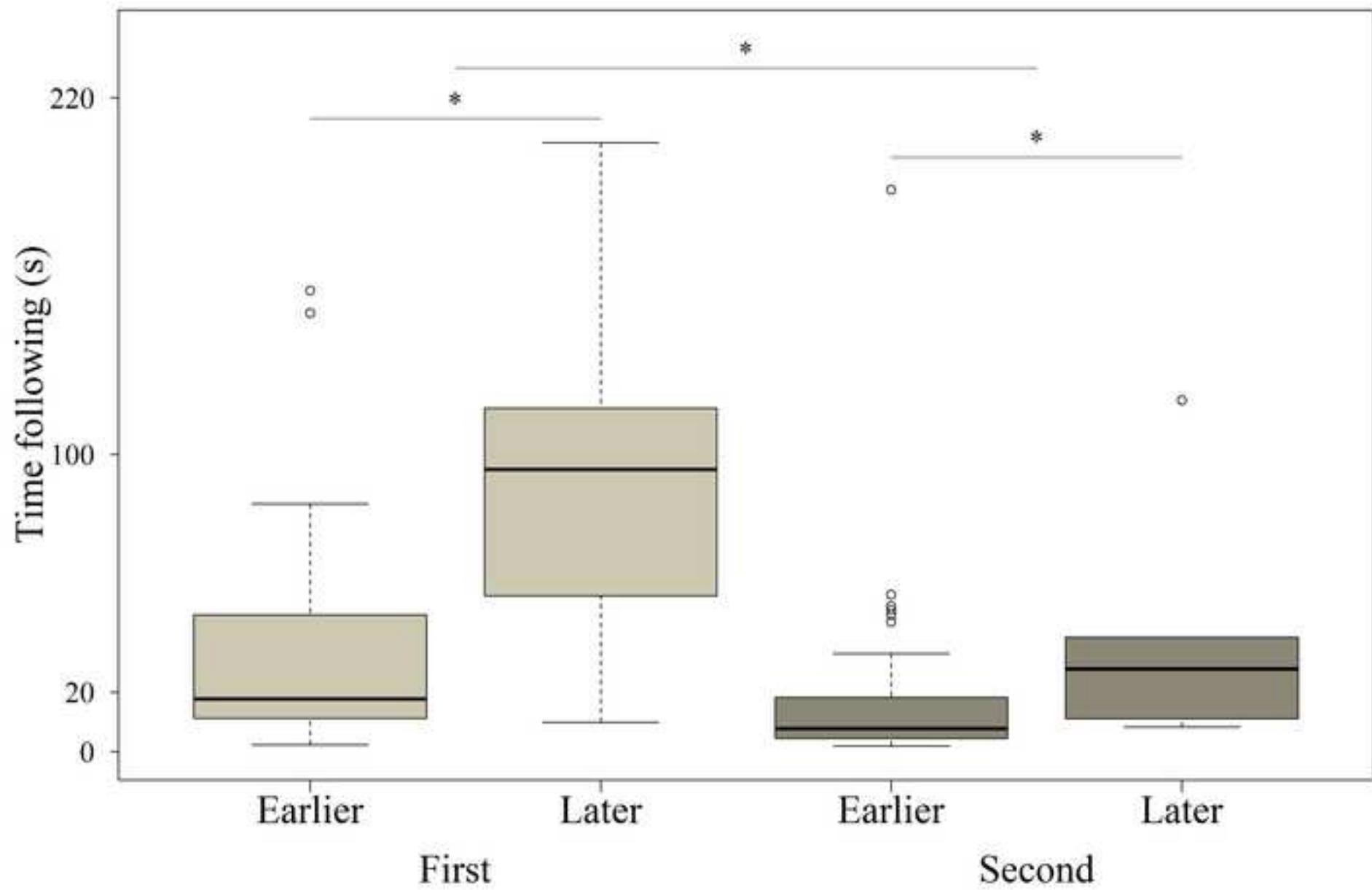
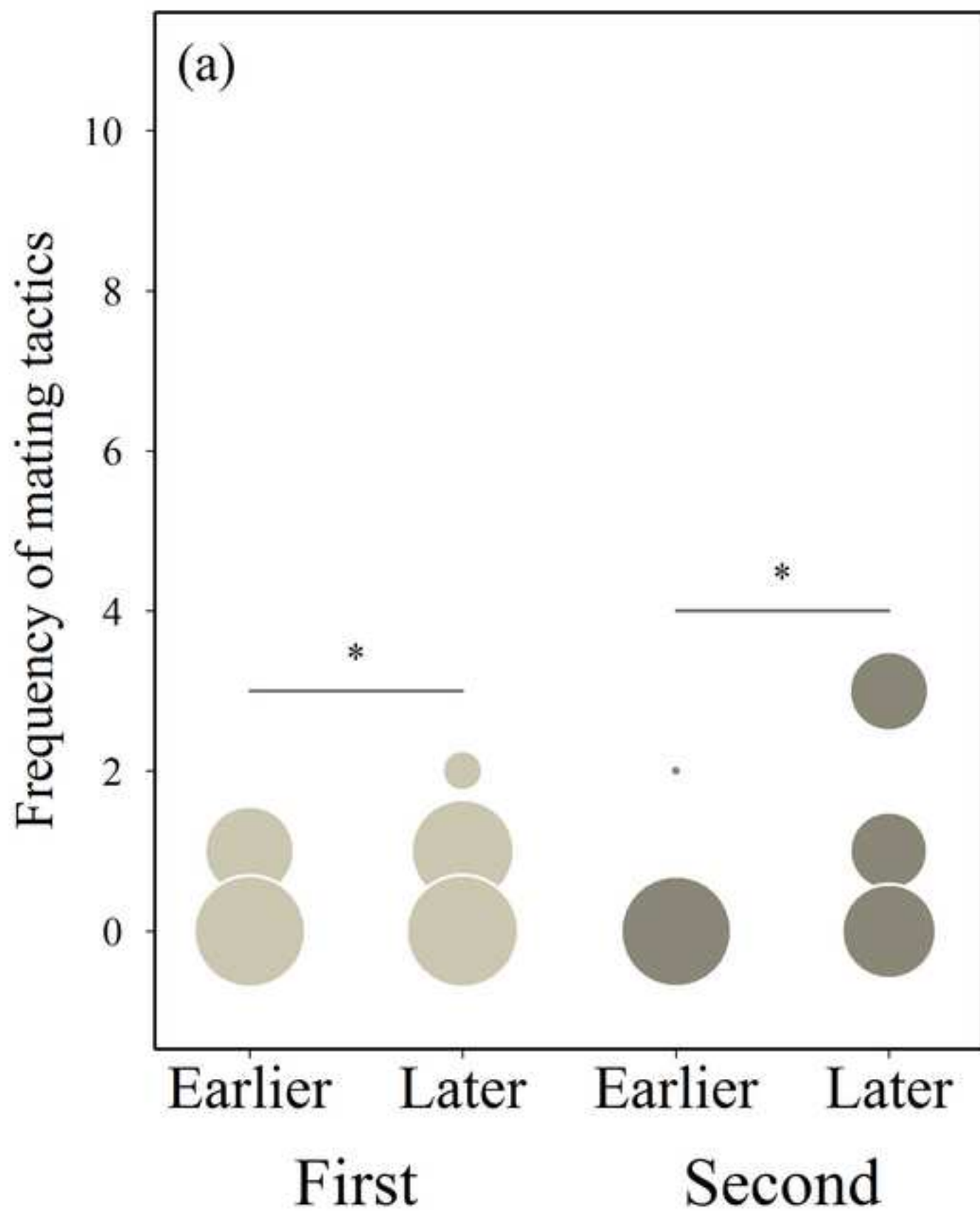
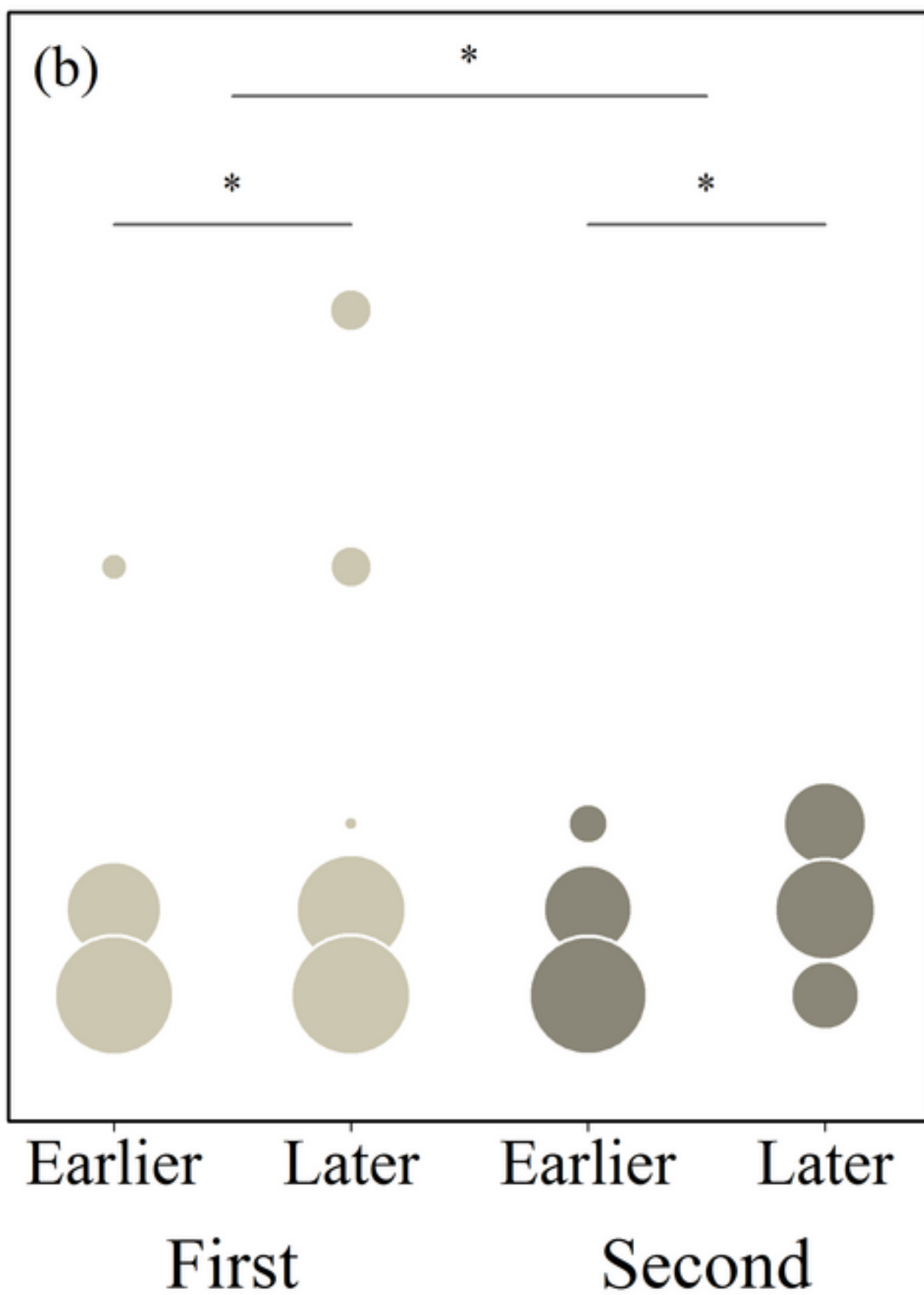


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## **Ethical notes**

Before observations all individuals were kept in mixed-sex stock tanks in, with similar densities. The stock tanks had gravel in the bottom, and an aerating system. The room was kept at a controlled stable temperature. The period of light was controlled (12:12h light:dark cycle). Individuals were fed daily. Before the observations, all individuals were transferred to observation tanks (mesocosms) to acclimate to the new conditions. Female guppies are often more sensitive to changes than males, because of their stronger and more stable social bonds (Croft et al., 2006; Griffiths & Magurran, 1998). Therefore, females were transferred to the mesocosms several hours before the observations and were kept with other familiar females, while the males were transferred 15 minutes before observations started. After observations all individuals were transferred to stock tanks with no fish (labelled as “observed individuals” during the experiment). All animals were individually and carefully caught and moved between tanks with a net.

All behavioural observations were carried out at the School of Biology at the University of St Andrews. The premises where the observations were carried out comply with the UK guidelines for the treatment of animals in behavioural research and teaching, set by the UK Home Office (PCD 60/2609). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.