

1 **Presence of invasive crayfish and spawning site selection in brown frogs**

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12

13 **Abstract**

14 Microhabitat selection is a key component of amphibian breeding biology and can be modulated in  
15 response to the features of breeding sites and the presence of predators. Despite invasive alien species  
16 being among the major threats to amphibians, there is limited information on the role of invasive  
17 species in shaping amphibians' breeding microhabitat choice. The invasive red swamp crayfish  
18 *Procambarus clarkii* is a major predator of amphibians' larvae, including those of the brown frogs  
19 *Rana dalmatina* and *Rana latastei*. Although qualitative information about the spawning site  
20 preferences and breeding microhabitat choice of brown frogs is available in the literature, only a few  
21 studies performed quantitative analyses, and the relationship between microhabitat choice and the  
22 presence of alien predators has not been investigated yet. The aims of this study were: 1) to  
23 characterize the microhabitats selected for clutch deposition by *R. dalmatina* and *R. latastei* and 2) to  
24 test if the position and the aggregation of egg clutches differ in sites invaded or not invaded by *P.*  
25 *clarkii*. During spring 2017, we surveyed multiple times 15 breeding sites of both brown frogs in  
26 Northern Italy; in each site we assessed the features of the microhabitat where each egg clutch was  
27 laid, considering its position (distance from the edge, depth of the water column) and the degree of  
28 aggregation of clutches. In each site we also assessed the presence/absence of the invasive crayfish  
29 and the relative abundance in the breeding period. We detected egg clutches in all sites; the crayfish  
30 occurred in eight ponds. Our results showed substantial differences between the spawning  
31 microhabitat features of the two brown frogs: *Rana latastei* clutches showed a higher degree of  
32 aggregation and were associated with deeper areas of the ponds, while *Rana dalmatina* deposited  
33 more spaced out clutches in areas of the ponds that were less deep. For both species, spawning  
34 microhabitat features were not different between sites with and without *P. clarkii*. Although we did  
35 not detect behavioural responses to *P. clarkii* in the choice of spawning microhabitat, additional  
36 studies are required in order to assess whether these frogs modulate other behavioural traits (e.g.  
37 during larval development) in response to the invasive predator.

38

39 **Key words**

40 Alien, amphibians, behaviour, predation, breeding, clutch, egg

41

## 42 **Introduction**

43 Amphibians are among the taxa with the highest ratio of threatened and declining species, and their  
44 global decline has been the focus of many studies with the aim of quantifying and understanding the  
45 causes of this phenomenon (Ficetola 2015; Scheele et al. 2019). The increase of trade and tourism all  
46 over the world, which directly or indirectly facilitates the spread of alien animals and plants, is one  
47 of the strongest threats to native biodiversity (Davis 2003), and amphibians are particularly sensitive  
48 to the impact of alien species (Bellard et al. 2016). Invasive alien species (IAS) can have multiple  
49 impacts on native amphibians. Predatory IAS can feed on both larvae and adults of many amphibian  
50 species, spread diseases, and limit the trophic resources available (Fisher et al. 2009; Hettyey et al.  
51 2016; Kats & Ferrer 2003), potentially leading to detrimental effects on the whole ecosystem of the  
52 invaded sites (Jackson et al. 2016).

53         Adult amphibians are often able to detect the presence of predators and modulate their  
54 breeding activity to limit predation on their offspring. On the one hand, parents may actively select  
55 breeding sites with few predators, and this has been shown to increase offspring fitness (Resetarits  
56 2005; Sadeh et al. 2009). The selection of breeding sites with few predators can also frequent in  
57 amphibians that stay in water for very short periods, and that can detect predators through indirect  
58 cues (e.g. chemical cues) . On the other hand, breeding sites without predators are not always  
59 available, and similarity of habitat preferences between amphibians and their predators may force the  
60 former to breed in sites with predators. However, wetlands can be very heterogeneous environments  
61 with a high number of microhabitats. Within a given breeding wetland the density of predators and  
62 predation risk can vary across microhabitats, thus females can select specific sites to increase the  
63 survival of tadpoles (Ficetola et al. 2006). However, up to now very few studies have investigated  
64 whether amphibians change their patterns of microhabitat selection in response to invasive predators.

65         The red swamp crayfish *Procambarus clarkii* is among the IAS with the strongest impact both  
66 in Europe and at the global scale (Nentwig et al. 2018). This crayfish is a generalist feeder (Alcorlo  
67 et al. 2004; Whitley & Rabeni 1997), and its global spread affects a growing number of freshwater

68 communities worldwide (Ficetola et al. 2011; Manenti et al. 2019; Ramamonjisoa et al. 2018; Vilà et  
69 al. 2010). *P. clarkii* preys on tadpoles of several amphibian species (Cruz et al. 2008); in northern  
70 Italy crayfish predation is a major cause of local extinctions and tadpole abundances reduction. On  
71 the one hand, the strong predation of *P. clarkii* on frog tadpoles may drive rapid behavioural or  
72 evolutionary responses in invaded communities (Nunes et al. 2014a; Nunes et al. 2014b); on the other  
73 hand, when there is a lack of anti-predatory response to this invasive crayfish, there is a high risk of  
74 extinction of amphibian populations (Nunes et al. 2013). The negative relationship between *P. clarkii*  
75 and tadpole abundance is particularly evident for some brown frog species, such as the agile frog  
76 (*Rana dalmatina*) and the Italian agile frog (*Rana latastei*) (Ficetola et al. 2011). Although these two  
77 species of brown frogs often breed in sites invaded by *P. clarkii*, a study performed a few years after  
78 the invasion showed that very few tadpoles reach metamorphosis in wetlands with high crayfish  
79 density (Ficetola et al. 2011). *Rana dalmatina* and *R. latastei* are species of conservation concern,  
80 and their tadpoles can be the most abundant vertebrates in small wetlands, thus they likely allow a  
81 substantial exchange of biomass from woody patches to the ponds in which they breed and vice-versa  
82 (Barzaghi et al. 2017; Gibbons et al. 2006).

83 In order to assess whether *R. dalmatina* and *R. latastei* are able to modulate microhabitat  
84 selection in the presence of invasive predators, we tested three hypotheses. 1) Shelter hypothesis.  
85 Along the shore, riparian semi-aquatic vegetation, submerged branches and hiding elements are more  
86 abundant, and can offer shelter from aquatic predators (Dodd 2010; Ficetola et al. 2006; Manenti et  
87 al. 2017). Therefore, if the shelter hypothesis is correct, we expect that in invaded ponds, frogs lay  
88 clutches closer to the pond edge.

89 2) Deepness hypothesis. The crayfish is rarely active in the water column, thus laying clutches  
90 in deep water can reduce predation rate on eggs and tadpoles (Cruz & Rebelo 2005). Therefore, this  
91 hypothesis predicts that, in invaded sites, frogs lay clutches in deeper water.

92 3) Schooling hypothesis. When tadpoles hatch, they show high local density during their most  
93 vulnerable stage; thus, by laying clutches nearby other clutches, frogs can create large assemblages

94 of clutches and tadpoles. This grouping strategy can provide advantages under high predation risk  
95 (e.g. confusion, enhanced group vigilance, diluted predation risk (Lima & Dill 1990; Nicieza 1999).  
96 If the schooling hypothesis is right, we expect to find less distance between clutches in invaded  
97 wetlands.

98 To achieve our aims we first characterised the features of the microhabitats selected by the  
99 two frogs; the spawning habits and the microhabitat selection of these two species have been  
100 described in the literature, but quantitative analyses remain limited (Ancona & Capietti 1996; Ficetola  
101 et al. 2006). We then tested if there was significant variation in spawning features between invaded  
102 and non-invaded sites.

103

#### 104 **Materials and Methods**

105 In spring 2017, we surveyed 15 breeding sites in northern Italy for which previous surveys confirmed  
106 the reproduction of at least one brown frog species (*R. latastei* or *R. dalmatina*). All the wetlands  
107 were in the basin of two tributaries of the Po river, i.e. the Lambro and Adda rivers, north of Milan.  
108 Since the early 2000s, wetlands of the Po lowland have been invaded by *P. clarkii* (Fea et al. 2006)  
109 that was first detected in our study area around 2005 (Manenti et al. 2014). Since 2005, *P. clarkii*  
110 spread in the study area colonising approx. 65% of the permanent, large ponds that generally  
111 constitute the breeding sites of these frogs (Manenti et al. 2014). The crayfish impact is heterogeneous  
112 across sub-populations (Siesa et al. 2011). Previous studies have shown *P. clarkii* exerts a heavy  
113 predation pressure on the larvae of *R. latastei* and *R. dalmatina*, strongly reducing their abundance,  
114 and that the impact of crayfish predation is stronger than the effects of native predators such as  
115 dragonflies (Ficetola et al. 2012).

116 In both frog species, the deposition period begins in early spring and each female lays only  
117 one clutch. Detectability of clutches is high and the two species can be identified on the basis of their  
118 morphological characteristics (Ambrogio & Mezzadri 2018). For each site we performed two surveys  
119 at the peak of frog breeding activity (March), one during daytime and one during night. During

120 daytime surveys, we assessed the features of the microhabitat of deposition of each egg clutch of *R.*  
121 *latastei* and *R. dalmatina*. For each clutch we measured three variables describing the position and  
122 the degree of aggregation of clutches: distance from the edge of the pond, distance from the closest  
123 conspecific clutch, and depth of the water column. We measured distance from the pond shore as the  
124 minimum distance between the clutch and the closest edge of the breeding site. In the study ponds,  
125 aquatic vegetation was nearly absent, while semi-aquatic vegetation and submerged branches were  
126 most abundant near the shoreline, thus distance from the pond shore is a good proxy of the availability  
127 of shelters for tadpoles. In order to assess the distance from the closest conspecific clutch, for each  
128 clutch we identified the closest conspecific clutch and measured the distance between them. When  
129 two clutches were in contact, the distance was recorded as zero. We measured depth of the water  
130 column as the total depth of the water column at the spawning point, also if the clutch was underwater  
131 (as often occurs for *R. latastei*). Moreover, we measured the maximum depth and the surface of each  
132 wetland in order to compare pond features among invaded and not invaded sites. The total number of  
133 clutches per site and surface were then used to calculate clutch density at each breeding site. A few  
134 clutches were clearly laid several days before sampling, and, especially for the agile frog, drift could  
135 have modified their position; for this reason, they were not considered for microhabitat  
136 measurements.

137 To verify the occurrence of the red swamp crayfish and to measure the relative abundance of  
138 crayfishes in ponds active during breeding period, we performed one visual encounter survey during  
139 night-time to maximize the detection probability of the crayfish. Recent studies showed that during  
140 nocturnal surveys the per-visit detection probability is very high (>95%; (Manenti et al. 2019)).  
141 Surveys were performed using night lamps along the whole perimeter of the ponds and lightening the  
142 inner sectors as much as possible.

143 Invasive species often have strongest impacts in sites where they attain the highest abundance. In this  
144 case, just measuring the presence/absence of invasives can obscure patterns caused by variation of  
145 abundance. Therefore we estimated relative abundance of crayfishes across sites using CPUE (Catch

146 Per Unit Effort) index applying the following formula:

$$147 \quad CPUE = \frac{N_{crayfish}}{m \times t \times N_{obs}}$$

148 where N crayfish is the number of individuals observed, m is the distance travelled, t the time spent  
149 in the survey and Nobs the number of observers participating in the survey.

150

151 Each survey lasted at least 20 minutes, and total survey time was proportional to the surface of ponds;  
152 particular attention was paid to the occurrence of small young individuals. Additional surveys were  
153 performed in the same sites during late spring – middle summer of 2017 and of 2019 and confirmed  
154 the absence of alien crayfish detection in the sites considered as no-invaded during this study  
155 (Manenti et al. 2019).

156

### 157 *Statistical Analyses*

158 Before running analyses, distance from the closest clutch, clutch density and distance from the edge  
159 were log-transformed, while water depth was square-root transformed to improve normality.

160 First, we used t-tests, assuming heterogeneous variance, to assess whether pond features (pond  
161 surface, maximum depth and clutch density) were significantly different between invaded and non-  
162 invaded ponds. Then, we used linear mixed models (LMMs) to assess whether the study species select  
163 different microhabitat features. We ran three separate LMMs with the different dependent variables  
164 (distance from pond edge, distance from the closest conspecific clutch and water depth) and with  
165 species identity as the independent variable; site was included as a random factor to take into account  
166 the non-independence of clutches within the same site.

167 Finally, we tested whether spawning site selection is different between invaded and non-  
168 invaded sites. Given that we generally found differences in microhabitat selection between the two  
169 frog species (see results), we analysed them separately. For each species, we used LMMs to assess  
170 whether distance from the shore, distance from the closest conspecific clutch and water depth are  
171 significantly different between invaded and non-invaded sites. For the analysis of water depth, we

172 included the maximum water depth in each pond as a covariate; for the analysis concerning distance  
173 from the shore and distance from conspecific clutches we included clutch density as a covariate. In  
174 some cases, the variance of dependent variables showed heteroscedasticity between groups (*R.*  
175 *dalmatina* vs. *R. latastei* clutches; invaded vs uninvaded sites; see supplementary materials for  
176 details). Therefore, we compared LMMs assuming homogeneous variance with models assuming  
177 heterogeneous variance between invaded and uninvaded sites. Models were fit using the VarIdent  
178 argument of the lmer function in R (Pinheiro & Bates 2000). We used a likelihood ratio test to assess  
179 if the model assuming heterogeneous variance performed significantly better than the one with  
180 homogeneous variance. The model with heterogeneous variance was then used since it provided a  
181 significantly better fit. We used a likelihood ratio test to evaluate whether the model with  
182 heterogeneous variance better fit the data. This analysis was also repeated for each frog species using  
183 crayfish relative abundance (CPUE) instead of occurrence/absence as independent variable. This  
184 allowed us to test the robustness of our conclusion to variation of crayfish abundance across ponds.  
185 Analyses were performed in environment R using the packages “lme4” (Bates et al. 2015) and “nlme”  
186 (Pinheiro et al. 2016; RDevelopment 2010).

187

## 188 **Results**

189 Overall, we measured spawning site features for 498 clutches of the two frog species (332 *R.*  
190 *dalmatina* and 166 *R. latastei* clutches) in the 15 surveyed sites. Ponds showed an average ( $\pm$  SD)  
191 surface of  $94.6 \pm 87.1$  m<sup>2</sup>, a maximum depth in average ( $\pm$  SD) of  $48.9 \pm 24.7$  m. We detected the red  
192 swamp crayfish in eight sites. Regarding *Rana dalmatina*, we detected clutches in 13 sites, 6  
193 uninvaded and 7 invaded by crayfish, with a mean ( $\pm$  SD) number of clutches per site of  $26 \pm 7$ . For  
194 *Rana latastei* we detected clutches in 8 waterbodies, 6 uninvaded and 2 invaded (average:  $21 \pm 8$   
195 clutches per site). Ponds invaded and not invaded by *P. clarkii* showed similar environmental  
196 variables. There were no significant differences for pond surface ( $t_{9,2} = 2.05$ ,  $P = 0.07$ ), max depth ( $t_{11} =$   
197  $-1.46$ ,  $P = 0.17$ ), or for clutch density of the two frog species (*R. latastei*:  $t_{6,20} = -0.75$ ,  $P = 0.48$ , *R.*

198 *dalmatina*:  $t_{12,4} = -1.05$ ,  $P = 0.31$ ). The CPUE index (abundance) of *P. clarkii* was generally low with  
199 a mean of  $0.02 \pm 0.03$  for *R. dalmatina* breeding sites and  $0.008 \pm 0.02$  for *R. latastei* breeding sites.

200  
201 *Spawning microhabitat differences between R. dalmatina and R. latastei*

202 *Rana latastei* clutches were more grouped than the *R. dalmatina* ones. The average distance ( $\pm$  SD)  
203 from the closest clutch was  $24.7 \pm 34.1$  cm for *R. latastei*, and  $98.3 \pm 122.3$  cm for *R. dalmatina* ( $F_{1,482}$   
204  $= 29.1$ ;  $P < 0.001$ ; Fig. 1a). Furthermore, *R. latastei* clutches were laid in deeper sectors of ponds than  
205 *R. dalmatina* ( $F_{1,482} = 5.33$ ;  $P = 0.02$ ; Fig. 1b); the mean depth ( $\pm$  SD) of water column was  $22.8 \pm$   
206  $7.8$  cm for *R. latastei* clutches while it was  $18.5 \pm 6.8$  cm for *R. dalmatina* clutches.

207 *R. latastei* also laid clutches more distant from the edge of the pond than *R. dalmatina* ( $F_{1,482}$   
208  $= 4.16$ ;  $P = 0.04$ ; Fig. 1c); the mean ( $\pm$  SD) distance between the shore and *R. latastei* egg-clutches  
209 was  $164.6 \pm 201.6$  cm, while it was  $131.4 \pm 101.8$  cm for *R. dalmatina* clutches.

210  
211 *Clutch microhabitat features in invaded and not invaded sites*

212 For both frog species, the spawning microhabitat features were similar between invaded and non-  
213 invaded ponds (Table 1). We did not detect any significant relationship between crayfish occurrence  
214 and microhabitat neither regarding the distance between the clutches, nor the depth of the water  
215 column, nor the distance from pond edge. Results were identical when we consider variation of  
216 crayfish abundance, as we did not detect any significant relationship between crayfish CPUE and  
217 spawning microhabitat features (Table 2).

218  
219 **Discussion**

220 The differences observed between *Rana latastei* and *R. dalmatina* highlight the importance of  
221 microhabitat selection for these frog species that adopt different strategies and select different  
222 spawning sites. Nevertheless, we did not detect any relationship between the spawning microhabitat  
223 choice and the occurrence of the alien predator *Procambarus clarkii*. Our results did not conform

224 with the predictions of either the shelter, the deepness or the schooling hypothesis, suggesting that  
225 the choice of the spawning position by adults is not modulated in order to minimize predation risk of  
226 eggs and tadpoles by the alien crayfish.

227         There are many reported cases of dramatic effects of alien species introduction on native  
228 species, which range from behavioural shifts of native species (Tiberti & von Hardenberg 2012;  
229 Winandy & Denoël 2013) to cascading effects on entire ecosystems, and can lead to the extirpation  
230 of entire species or communities (Arribas et al. 2014; Bonelli et al. 2017). Semi-aquatic organisms  
231 like amphibians can play important roles for nutrient exchanges between aquatic and terrestrial  
232 habitats (Barzaghi et al. 2017; Gibbons et al. 2006), thus invasive crayfish are expected to deeply  
233 affect the biotic community of both lentic and lotic environments (Ficetola et al. 2012; Ficetola et al.  
234 2011; Gherardi & Acquistapace 2007; Manenti et al. 2019; Shin-ichiro et al. 2009). If the breeding  
235 microhabitat selected by adult amphibians is important for their breeding success, we expect a  
236 modulation of habitat choice in response to the presence of IAS. Evolutionary and plastic changes in  
237 invaded communities can arise at different levels and might be difficult to detect (Nunes et al. 2014a).  
238 In our study area previous research detected brown frogs breeding in sites with red swamp crayfish  
239 (Ficetola et al. 2011). Given the high tadpole mortality in invaded wetlands, a plastic selection of  
240 breeding habitats and microhabitats could limit predation on eggs or tadpoles. However, adult brown  
241 frogs have continued to breed for several years in these ponds, selecting the same microhabitat  
242 features as in non-invaded waterbodies. This suggests that, contrary to what happens in urodeles  
243 (Cabrera-Guzman et al. 2019; Winandy et al. 2017), predation pressure of the alien crayfish does not  
244 lead to rapid shifts or responses in adult brown frogs spawning behaviour.

245         The ability of native prey to assess risk and adopt appropriate behavioural responses depends  
246 on different factors such as the experience accumulated during lifespan, their learning ability and also  
247 their evolutionary history and ecology (Kovacs et al. 2012). Several factors can explain the lack of  
248 microhabitat shifts in brown frogs. First, contrary to urodeles that invest considerable time in  
249 courtship and eggs laying, brown frogs invest relatively little time in egg laying, and frogs remain in

250 breeding wetlands from few hours to at maximum one day (Ambrogio & Mezzadri 2018). Such a  
251 period can be too short to allow them to acquire enough experience on the risk determined by the red  
252 swamp crayfish occurrence. Nevertheless, it is important to remark that multiple studies evidenced  
253 that amphibians are able to detect predator chemical cues in water, and can modulate the breeding  
254 habitat selection even when they use breeding sites for few hours (Resetarits 2005; Sadeh et al. 2009).  
255 Second, brown frogs breed at the end of winter, when water temperatures are low. The activity of the  
256 red swamp crayfish is generally high during warm periods (Holdich et al. 2009); thus, the individuals  
257 may not be particularly active when adult frogs are in water, with limited consumptive effects. The  
258 worst effect of the crayfish probably happens only later in the season when temperatures rise up, and  
259 the crayfish mostly impacts the tadpoles. Third, it is possible that the time since the arrival of the red  
260 swamp crayfish was not enough for frog populations to develop appropriate antipredator adaptations.  
261 Recent studies show that even if the ability to recognise invasive predators may evolve quickly, agile  
262 frog populations can be vulnerable to alien fish due to their inability to recognize them as a threat  
263 (Hettyey et al. 2016).

264         The lack of response in our target species can thus be explained by naïveté towards a novel  
265 predator (Sih et al. 2010). The first detection of the red swamp crayfish in the study area dates back  
266 to 2005 (Manenti et al. 2014), indicating that frogs coexisted with the crayfish for approx. 3-4  
267 generations (Guarino et al. 2003; Racca 2003; Weddelling et al. 2005). Rapid local adaptations of  
268 brown frogs when selective pressure is strong are known (Ficetola et al. 2011; Skelly & Freidenburg  
269 2000), still amphibians responses to invasive species are mostly known to occur at the tadpole stage  
270 (Hettyey et al. 2016; Nunes et al. 2014b; Nunes et al. 2013). Future studies are required to investigate  
271 if behavioural responses may occur in the larval stages of the study species. We should also underline  
272 that, especially for *R. latastei*, the number of uninvaded ponds was low, because the crayfish has  
273 already invaded the majority of breeding sites of this frog. To confirm the generality of our results it  
274 would be thus interesting to increase the number of uninvaded breeding sites, for instance in regions  
275 where *P. clarkii* is less widespread.

276

277 **Conclusion**

278 Our study characterized the spawning microhabitat of the agile frog and the Italian agile frog,  
279 considering also sites in which they are syntopic and quantifying differences in spawning  
280 microhabitat between these two species. Italian agile frog females lay eggs in significantly deeper  
281 areas of the ponds, and with a strongly aggregated pattern. The clustering of Italian agile frog clutches  
282 has been repeatedly described in the herpetological literature, and it is known that dozens of females  
283 can attach their egg-clutches to the same submerged woods (Ambrogio & Mezzadri 2018; Pozzi  
284 1980). However, there are few data on the causes of microhabitat selection. First, differences in  
285 microhabitat could reduce the frequency of interactions with heterospecific males, which in turn can  
286 reduce the fertility of clutches (Ficetola & Bernardi 2005; Hettyey & Pearman 2003; Hettyey et al.  
287 2014). Second, tadpoles could exploit different microhabitats within the wetlands, for instance  
288 because they have different thermal optima (Balogová & Gvoždík 2015). Nevertheless, very limited  
289 information exists so far on differences in microhabitat use between tadpoles, and future studies are  
290 required to understand the factors allowing the syntopy between these frog species, and the relative  
291 effect of micro and macro-ecological determinants. A better knowledge of behaviour of these species  
292 is an important starting point for a better understanding of the strategies of these animals in response  
293 to biological invasions.

294 The invasive crayfish is widespread in the study area (Manenti et al. 2019) and is likely to  
295 attain high density, making eradication programs almost impossible. Under these circumstances, it is  
296 essential to identify the processes that can allow long-term persistence of native species, such as  
297 behavioural changes or the selection of specific microhabitats. However, such processes can be  
298 complex and can occur at multiple levels, and this can make their identification challenging. On the  
299 one hand, it will be important to integrate analysis performed on the microhabitat-scale with research  
300 performed on the landscape level, with long term analysis including the metapopulation-scale. On the  
301 other hand, additional studies are required to assess whether native frogs can modulate other

302 behavioural traits when interactions with the crayfish are more frequent, such as during larval  
303 development.

304

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