

1 **Odour recognition learning of multiple predators by amphibian larvae**

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24 Word count: 6313

25 **Abstract**

26 Many aquatic animals learn to recognize novel predators when they
27 simultaneously perceive the odours of these novel threats paired with alarm cues
28 released by injured conspecifics. Since the odours of several organisms may be present
29 simultaneously in the environment during this process, selection is expected to favour
30 learning mechanisms that allow prey to respond independently to the odour of each
31 species in a mixture of odours. We tested this hypothesis by exposing tadpoles of the
32 edible frog, *Pelophylax esculentus*, to injured conspecific cues paired with either the
33 odour of two fish species (experiment 1) or one fish and one crayfish species
34 (experiment 2). We subsequently tested the ability of tadpoles to respond to each odour
35 separately. We found clear evidence that tadpoles learned to recognize the odour of
36 individual species in the mixture and that the response to each odour of a mixture was
37 equally strong. However, the learned response was weaker overall in tadpoles
38 conditioned with the mixture of fish and crayfish compared to those with the two fish
39 species. Our study reveals that tadpoles can adaptively handle the presence of multiple
40 predator odours in their environment during conditioned learning, but highlights some
41 constraints that may due to the diversity of predators in the mix.

42

43 **Keywords:** Alarm substances; Antipredator behaviour; Cognitive ecology; *Pelophylax*
44 *esculentus*; Predator odour; Predator recognition learning.

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49 **Introduction**

50 It is not uncommon for prey individuals to be vulnerable to a large number of
51 potential predators across their lifespan (Brilot, Bateson, Nettle, Whittingham, & Read,
52 2012; Polis, 1991). Multiple predator species may simultaneously live in the same
53 environment or they may occupy different but adjacent habitats; furthermore, predator
54 abundance often varies among seasons, and some predators feed on specific prey age
55 classes (Ferrari, Sih, & Chivers, 2009b; Hammond, Luttbegg, & Sih, 2007; Schoener,
56 1989; Sih, Englund, & Wooster, 1998). It has been estimated that each prey taxon is
57 exposed, on average, to two to three predator taxa per food web (Schoener, 1989). For
58 individuals of many species, it is therefore paramount to gather information about which
59 species represent a threat and natural selection has equipped these species with
60 sophisticated cognitive mechanisms for predator recognition learning (Brown, 2003;
61 Caro, 2005; Kelly & Magurran, 2003). In aquatic environments, amphibians, fish and
62 invertebrates exploit a learning mechanism based on chemical cues to recognize
63 predators: when an individual perceives a novel odour paired with the chemicals
64 released by an injured conspecific (hereafter ‘alarm cues’), it associates the novel odour
65 with danger and will thereafter respond to that odour by displaying antipredator
66 behaviours (reviewed in Brown, 2003; Ferrari, Wisenden, & Chivers, 2010; Kelly &
67 Magurran, 2003). Indeed, these alarm cues are only released through mechanical
68 damage to the skin, as would occur during a predation event, and hence, represent a
69 reliable indicator of risk for nearby conspecifics (reviewed in Ferrari et al., 2010).

70 Alarm-cue mediated learning is usually studied in controlled settings in which
71 prey are exposed to a single predator cue in clean water, and therefore no (or limited)
72 potential exists for interference from other unknown odours (reviewed in Brown, 2003;

73 Ferrari et al., 2010). Although useful to understand the basic mechanisms of predator
74 recognition learning, the use of such controlled settings might not reflect the complexity
75 of chemical communication in natural environments because several organisms are
76 often present simultaneously in the same microhabitat (Sih et al., 1998). As a
77 consequence, aquatic prey are likely exposed to alarm cues along with a mixture of
78 olfactory cues of different species simultaneously (Darwish, Mirza, Leduc, & Brown,
79 2004), with all or a portion of the odours belonging to the predator species. Selection is
80 expected to favour the evolution of learning mechanisms allowing prey to recognize
81 each odour in the mixture and independently respond with an antipredator behaviour to
82 each odour encountered alone because each of them can belong to the predator. This
83 hypothesis has found support in two tropical fish species. Darwish et al. (2004) exposed
84 glowlight tetras, *Hemigrammus erythrozonus*, to conspecific alarm cues paired with
85 three novel fish odours; in a following testing phase, tetras displayed antipredator
86 responses to each fish odour individually. Similarly, Mitchell, McCormick, Ferrari, &
87 Chivers (2011b) showed that lemon damselfish, *Pomacentrus moluccensis*, can learn to
88 recognize each of four novel predator odours that were simultaneously paired with
89 alarm cues.

90 Larval amphibians show predator recognition learning abilities and mechanisms
91 often similar to those of fish: for example, both groups display generalization of learned
92 predator odours (Chivers, Mitchell, Lucon-Xiccato, Brown, & Ferrari, 2016; Ferrari,
93 Brown, Messier, & Chivers, 2009a), embryonic learning (Atherton & McCormick,
94 2017; Mathis, Ferrari, Windel, Messier, & Chivers, 2008), and latent inhibition (Ferrari
95 & Chivers, 2006; Ferrari & Chivers, 2009). This might be indicative of convergent
96 evolution of alarm-cue mediated predator recognition in aquatic environments. Yet, it is

97 unknown whether larval amphibians conditioned with a mixture of the odour of
98 different species can learn to respond to each individual odour. The main aim of this
99 study was to address this question. To do so, in our two experiments, we conditioned
100 tadpoles of the edible frog, *Pelophylax esculentus*, to alarm cues (or a water control)
101 paired with a mixture of odours from two different species. In experiment 1, we used
102 the odour of two fish species; in experiment 2, with the odour of two species with
103 greater phylogenetic distance, one fish and one crayfish. We then measured the
104 antipredator response of tadpoles when exposed to each predator odour of the mixture
105 individually. If tadpoles can learn multiple predator odours in a mixture, we expected
106 that subjects conditioned with alarm cues would respond to each individual odour more
107 than subjects conditioned with water control. Based on research on odour mixture
108 discrimination in other species (Laska & Hudson, 1993; Livermore & Laing, 1998;
109 Mandairon, Stack, & Linster, 2006; Rabin, 1988), we also expected that tadpoles might
110 learn to recognize the two odours in the same mixture with different accuracy, failing
111 more often to recognize one of the two odours.

112 Lastly, we investigated the effect of the odour mixture on recognition learning
113 by comparing the learned antipredator response of tadpoles from the two experiments.
114 Research on other species has revealed that the type of odours in a mixture have an
115 effect on discrimination performance. For instance, squirrel monkeys, *Saimiri sciureus*,
116 are more efficient in discriminating between odour mixtures in presence of specific
117 components (Laska & Hudson, 1993). Also, in the case of innate reaction to predator
118 odours that do not require previous learning, it has been found that prey respond
119 stronger to the cue of a single predator in a mixture (Eklöv, 2000; Hoverman & Relyea,
120 2007; Smith et al., 2010). Studies on predator recognition learning have suggested that

121 the difference between the odours of two species increases as a function of phylogenetic
122 distance (Ferrari, Gonzalo, Messie, & Chivers, 2007). This bears two different
123 predictions for the results of our experiments. On one hand, if the two odours are highly
124 different, one might expect that they are more distinguishable, and hence result in a
125 better learning of the two cues separately; according to this prediction, we expected
126 greater learned response to the individual odours in experiment 2 compared to
127 experiment 1. On the other hand, if the mix is learned as a unit, a greater divergence
128 between the two cues may lead to a greater mismatch between the conditioning and the
129 testing cue, resulting in a weaker response to each cue separately; this would cause
130 greater learned response to the individual odours in experiment 1 compared to
131 experiment 2.

132

133 **Materials and methods**

134 Animal welfare note

135 Experiments followed institutional guidelines and ethics (D. L. 4 marzo 2014)
136 and were approved by University of Padova ethical committee (protocol n. 51/2016).
137 Care was taken to reduce stress to tadpoles during the experiments. Tadpoles were
138 maintained outdoor in order to expose them to natural conditions. Light:dark regime,
139 temperature, precipitation, and wind were not manipulated. Tadpoles' density matched
140 that observed in their natural environment. Food was constantly presented in the
141 maintenance pails and water was regularly changed. We did not observe signs of
142 distress, diseases or mortality in the tadpoles during captivity. After completion of the
143 experiment, we released all the experimental subjects ($N = 168$) and the tadpoles
144 collected but not used in the experiments of this study (approx. 2000) at their natal

145 environment (i.e., in same the area of the river where eggs were collected). The fish and
146 the crayfish used as odour donors were not subjected to dangerous manipulation. After
147 the completion of the experiments, they were moved back in their maintenance tanks in
148 the laboratory.

149

150 Subjects

151 We collected edible frog eggs from 12 egg masses immediately after spawning
152 in a stream in north-east Italy (45° 32' 30'' N, 11° 53' 40'' E). To prevent any exposure
153 to predators, we raised the eggs and the tadpoles in 20-L pails (50 × 36 cm, water depth
154 12 cm) filled with pond water. Water used in the pails was collected from a nearby
155 artificial pond (6 × 4 m, depth: 60 cm), which was filled 4 weeks prior to the start of the
156 experiments. Plants and algae collected from the sampling site were added to the pond
157 to provide natural cues to the water, while ensuring no predator cues were present. The
158 pond was isolated from any water drainage and free from fish and crayfish. The pails
159 were kept outdoor under natural conditions (light, temperature, precipitation, wind) and
160 underwent a 50% water change every other day. After hatching, tadpoles were fed
161 rabbit pellets (alfalfa) daily to complement the algae present in the pails. We used 168
162 tadpoles randomly selected from the pails for the experiments; these tadpoles were
163 randomly assigned to the two experiments and to the different conditions of each
164 experiment. After completion of the experiment, we released these tadpoles at the
165 sampling site.

166

167 Alarm cues and predator odours preparation

168 We prepared alarm cue following previous studies on amphibian larvae (Ferrari,
169 Vrtělová, Brown, & Chivers, 2012; Lucon-Xiccato, Chivers, Mitchell, & Ferrari 2016).
170 We randomly selected donor tadpoles ($N = 25$) from the pails and we collected them
171 with a small hand net. We sacrificed donor tadpoles with a blow to the head. The use of
172 this standard physical euthanasia (AVMA, 2013) was necessary because chemical
173 methods have been reported to interfere with alarm cue responses (Losey & Hugie,
174 1994). Immediately after euthanasia, the donors were emulsified with a mortar and
175 pestle, and the solution suspended in pond water, to obtain approx. one tadpole per 20
176 mL of water.

177 In experiment 1, we used odours from two fish species from different families,
178 the catfish, *Pangasius hypophthalmus* (family: Pangasiidae), and the common rudd,
179 *Scardinius erythrophthalmus* (family: Cyprinidae). In experiment 2, we used odours
180 from the catfish and the red swamp crayfish, *Procambarus clarkia*. These species were
181 not observed in the sampling site; since tadpoles were maintained in pails filled with
182 pond water with no fish and crayfish, the predators were novel for tadpoles and tadpoles
183 were not exposed to the cues used for conditioning before the experiments. We used 4
184 individuals with the same size (approx. 12 cm) for each species. The fishes were lab-
185 raised and maintained under standard conditions. Their maintenance aquaria (150 L)
186 were provided with gravel bottom, natural plants, water filters, and kept at $26 \pm 1^\circ\text{C}$. Fish
187 were fed three times per day *ab libitum*, alternating commercial fish flakes and *Artemia*
188 *salina* nauplii. Crayfish were collected in a small river one month before the beginning
189 of the experiments, housed individually in 10-L pails (35×24 cm, water depth 12 cm)
190 and fed *ab libitum* with rabbit and shrimp pellets. We prepared predator odours by
191 soaking two individuals of each species in a 10-L tank for 24 h. During these 24 hours,

192 the animals were not fed to avoid confounding effects due to diet cues (Chivers &
193 Mirza, 2001; Mitchell, Ferrari, Lucon-Xiccato, & Chivers, 2016). Water from these
194 tanks was used as odour cues in the experiment.

195

196 Conditioning with odour mixture

197 To study predator recognition learning in tadpoles, we used a well-established
198 tadpole bioassay (Chivers et al., 2016; Ferrari et al., 2009a; Ferrari et al., 2012; Lucon-
199 Xiccato, Chivers, Mitchell, & Ferrari, 2017). Tadpoles were initially conditioned to
200 recognize the predators by exposing them to alarm cues paired with the mixture of
201 predator odours. The tadpoles were then tested for their response to each predator odour
202 alone.

203 For the conditioning, we moved each individual tadpole into a 0.5-L cup filled
204 with pond water. After a 1-h acclimation, we injected 5 mL of predator odour mixture
205 paired with either 5mL of alarm cues or 5 mL of water as a control. The predator odour
206 mixture was prepared by mixing 2.5 mL of each of the two odours. Sample sizes of
207 experiment 1 were as follow: alarm cues + fish mixture: 52; control water + fish
208 mixture: 32. Sample sizes of experiment 2 were as follow: alarm cues + fish/crayfish
209 mixture: 48; control water + fish/crayfish mixture: 36. Several learning studies with this
210 protocol showed not learned response in the control group (Chivers & Ferrari, 2013;
211 Chivers et al., 2016; Ferrari et al., 2009a; Lucon-Xiccato et al., 2016; Lucon-Xiccato et
212 al., 2017); thus, we used reduced control groups to minimise the number of wild
213 animals necessary for the study (Mitchell et al., 2016). The tadpoles were exposed to the
214 mixture for 1 h; then, tadpoles were moved into 16 holding pails (approx. 10 individuals
215 per pail), fed and left undisturbed.

216

217 Predator recognition test

218 Predator recognition was assessed the day after conditioning. We moved each
219 individual tadpole to a 0.5-L cup and left them to acclimate for 30 min. The bioassay
220 used to measure antipredator responses is identical to that used in previously published
221 studies on tadpole antipredator responses (Lucon-Xiccato et al., 2016; Lucon-Xiccato et
222 al., 2017; Mitchell et al., 2016). In the predator recognition test, each subject was
223 exposed to a single predator cue. In experiment 1, the experimental cue administered to
224 each subject was either catfish (alarm-cue treatment tadpoles $N = 26$; water-control
225 tadpoles $N = 16$) or rudd cue (alarm-cue treatment tadpoles $N = 26$; water-control
226 tadpoles $N = 16$); in experiment 2 the experimental cue administered to each subject
227 was either catfish (alarm-cue treatment tadpoles $N = 24$; water-control tadpoles $N = 18$)
228 or crayfish (alarm-cue treatment tadpoles $N = 24$; water-control tadpoles $N = 18$). We
229 measured the activity of each tadpole for 4 min prior to (baseline) and 4 min after the
230 injection of the experimental cue. Activity was assessed by counting the number of
231 times the tadpoles crossed a line that bisected the bottom of the cup in half. We
232 considered the line was crossed when the entire body of the tadpole crossed the line.
233 The baseline observation period and the post-injection observation period were
234 separated by a 30-sec injection period, where we slowly injected 5 mL of the cue (fish
235 or crayfish odours) on the side of the cup, to minimize disturbance. The experimenter
236 was blind regarding the treatment of the subject because each subject was coded with a
237 number before behavioural observation. If tadpoles conditioned with alarm cues learned
238 to recognize the individual odours in the conditioning mixture, they were expected to
239 show a marked reduction in activity between the initial baseline and the post-injection

240 period. Indeed, a decrease in activity is a common antipredator response in larval
241 amphibians after conditioning with both fish cues (Chivers et al., 2016; Ferrari, Crane,
242 & Chivers, 2016) and crayfish cues (Lucon-Xiccato et al., in preparation; Mitchell et al.,
243 2016). Conversely, tadpoles exposed to water instead of alarm cues in the conditioning
244 phase were expected to not show such activity reduction.

245

246 Statistical analysis

247 We performed statistical analysis in R version 3.4.0 (The R Foundation for
248 Statistical Computing, Vienna, Austria, <http://www.r-project.org>). Data were checked
249 for normality before the analysis using Kolmogorov-Smirnov test. For both
250 experiments, we initially tested whether the response to the individual odours in the
251 predator recognition test was different between tadpoles conditioned with alarm cues
252 and those exposed to water control in the conditioning phase. To do this, we used
253 ANOVAs fitted with the percentage decrease of activity between baseline and post-
254 injection measurement (calculated as: $[(\text{post-injection number of median line crossing} -$
255 $\text{baseline number of median line crossing}) / \text{baseline number of median line crossing}] \times$
256 100) as dependent variable, and conditioning cue (alarm cues versus water control) and
257 testing cues (each of the two species used in the conditioning odour mixture) as factors.
258 We expected a significant effect of conditioning cue if tadpoles learned to respond to
259 the predator odours. A significant interaction between conditioning cue and testing cue
260 would indicate that the identity of the cue donor affects the response pattern of the
261 tadpoles. Because this last prediction (the different recognition performance between
262 the two odours in the mixture) did not find statistical support (i.e., significant
263 conditioning cue \times testing cue interaction), we tried to remove the possibility of

264 negative results based on a lack of statistical power by performing a Bayesian analysis
265 directly testing for the absence of effects (Barchard, 2015; Dienes, 2014). We computed
266 an approximated Bayes factor (BF) in favour of the absence of the interaction (Schwarz,
267 1978; Wagenmakers, 2007) using the Bayesian information criterion (BIC) of the model
268 with (BIC_1) and without (BIC_0) the interaction term as: $BF = \exp((BIC_1 - BIC_0) / 2)$. For
269 example, if $BF = 10$, our data are ten times more likely to fit the model without the
270 interaction term. Lastly, we compared the intensity of the learned antipredator response
271 between the two odour mixture conditions (experiment 1 and experiment 2). We pooled
272 the data of the tadpoles conditioned with alarm cues from both experiments and we
273 compared their activity decrease using two-samples t test.

274

275 **Results**

276 Experiment 1

277 In the ANOVA on the activity decrease of tadpoles conditioned with the mixture
278 of two fish species, we found a significant effect of conditioning cue (alarm cues versus
279 water control: $F_{1,80} = 41.461$, $P < 0.0001$). This indicates that tadpoles conditioned with
280 alarm cues responded to the injection of individual predator odours more than control
281 tadpoles exposed to water during condition phase (Fig. 1a). We found no significant
282 effect of testing cue (catfish versus rudd: $F_{1,80} = 0.758$, $P = 0.387$; Fig. 1a).

283 Furthermore, we found no significant interaction between conditioning cue and testing
284 cue ($F_{1,80} = 0.025$, $P = 0.875$; $BF = 9.05$; Fig. 1a), indicating that tadpoles' response to
285 the odour of the two individual fish species was similar.

286

287 Experiment 2

288 We found similar results in the ANOVA on the activity decrease of tadpoles
289 conditioned with the mixture of a fish and a crayfish species. The effect of conditioning
290 cue (alarm cues versus water) was significant ($F_{1,80} = 21.405, P < 0.0001$), indicating
291 that tadpoles conditioned with alarm cues responded to the injection of individual
292 predator odours more than control tadpoles exposed to water during condition phase
293 (Fig. 1b). We also found no significant effect of testing cue (catfish versus crayfish:
294 $F_{1,80} = 3.088, P = 0.083$; Fig. 1b); this might indicate a differential response that is
295 difficult to interpret because it is based on the cumulative the score of the learned
296 response and that of control tadpoles conditioned with water. The interaction between
297 conditioning cue and testing cue was not significant ($F_{1,80} = 0.918, P = 0.341$; $BF =$
298 5.68 ; Fig. 1b), indicating that tadpoles' response to the odour of the two individual
299 species was equal.

300

301 Comparison between experiment 1 and experiment 2

302 When we compared the learned response to the individual odours between the
303 two experiments, we found that tadpoles responded more to the individual odours when
304 conditioned with the two fish species than when conditioned with fish and crayfish (t_{98}
305 $= 3.551, P = 0.0006$; Fig. 2).

306

307 **Discussion**

308 Aquatic species are often exposed to odour cues from many different species,
309 including their predators. Our study demonstrated that amphibian larvae can handle this
310 complexity and learn to recognize the odour of multiple predator species via association
311 with conspecific alarm cues. Edible frog tadpoles exposed to a mixture of odours

312 belonging to two novel species paired with alarm cues subsequently responded to each
313 individual odour with a similar antipredator response; conversely, control tadpoles
314 exposed to the same odour mixture paired with water instead of alarm cues showed no
315 or little response to the individual odours of the two ‘predator’ species. Furthermore,
316 tadpoles responded stronger to the individual odours when the two odours in the
317 conditioning mixture were derived from two fish species (experiment 1) than when the
318 two odours in the mixture were derived from a fish and a crayfish (experiment 2).

319 Learning of multiple predator odours has been previously reported in two fish
320 species (Darwish et al., 2005; Mitchell et al., 2011b). The finding that tadpoles can also
321 learn multiple predator odours aligns with other studies in suggesting that convergent
322 selective pressures might have shaped the cognitive processes involved in novel
323 predator recognition of different aquatic vertebrates (Atherton & McCormick, 2017;
324 Chivers et al., 2016; Ferrari & Chivers, 2006). It is worth noting that the
325 aforementioned fish species are tropical and found in highly biodiverse environments,
326 where the high number of sympatric species would favour the evolution of such
327 learning abilities in small-bodied, highly vulnerable prey. Our results in tadpoles
328 suggest that odour learning of multiple predators also exists in temperate species that
329 are typically exposed to a lower density of novel predators at once. It remains unknown
330 whether tropical species of amphibians possess the ability to handle a larger number of
331 predator odours simultaneously, given the higher biodiversity of tropical environments.
332 Also, it is possible that amphibians learn multiple odours only at the larval stage, when
333 the number of potential predators is higher; ontogenetic changes in the response to
334 predators have already been reported in other taxa (Deecke, Slater, & Ford, 2002).

335 The ability to handle multiple odours of potential predators is likely adaptive: if
336 one odour or few odours in the mixture belong to a predator, this ability allows
337 individuals to recognize each potential predator species when they perceive its odour
338 alone or in combination with the odour of other species; if all the odours in the
339 conditioning mixture belong to predators, this ability allows recognition of multiple
340 threats in a single conditioning event, rather than identifying them individually. In
341 addition, individuals learning multiple threats are at a survival advantage compared to
342 their unconditioned counterpart (Darwish et al., 2005), hereby providing a selective
343 mechanism for this ability.

344 One could argue that prey learning to respond to all the odours in a mixture will
345 likely overestimate the number of predator species in their environment because some
346 odours might belong to non-predator species. This might bear fitness costs because the
347 prey will express antipredator responses, such as cover seeking or reduced movements,
348 in presence of non-predator species and the expression of unnecessary antipredator
349 behaviours constrains other fitness related activities such as foraging (Sih, 1990).
350 However, given the unforgiving nature of predation, overestimating risk, as in a “play it
351 safe” strategy, may be less risky than underestimating the risk (Bouskila, Blumstein, &
352 Mangel, 1995). In addition, other learning processes are in place to avoid such mis-
353 association. Latent inhibition is a learning process whereby the repeated exposure to a
354 stimulus in the absence of negative reinforcement results in the individual recognizing it
355 as non-threatening. This means that a subsequent attempt to learn this ‘safe’ stimulus as
356 risky, via pairing with alarm cues, will be unsuccessful (Ferrari & Chivers, 2009;
357 Hazlett, 2003; Mitchell, McCormick, Ferrari, Chivers, 2011a), thereby keeping the
358 occurrence of fortuitous pairing to a minimum.

359 In the recognition learning test, tadpoles responded equally to the two odours in
360 each mixture, as evinced by the non-significant interaction between conditioning cue
361 and testing cue that we observed in both experiment 1 and 2. This contrasts with studies
362 on innate responses to predator cues: naïve tadpoles and snails exposed to cues of two
363 predators respond to one of the cues only, typically the one of the more ‘dangerous’
364 predator (Eklöv, 2000; Hoverman & Relyea, 2007; Smith et al., 2010). Also research on
365 odour learning in other contexts, suggests increased response to one or few cues in a
366 mixture. For example, honeybees, *Apis mellifera*, trained to recognize a mixture of 14
367 floral odorants showed a strong learned response to few key odorants and little response
368 to the remaining compounds in the mixture (Reinhard, Sinclair, Srinivasan, &
369 Claudianos, 2010). This effect on learning might be due to the fact that the different
370 odours in a mixture have different salience because of their perceptual characteristics
371 (e.g., one of them being more intense than the others, because of previous experience of
372 the individual, or because of innate predisposition to pay more attention to or be more
373 sensitive to particular cues (Laska & Hudson, 1993; Livermore & Laing, 1998;
374 Mandairon et al., 2006; Rabin, 1988). As a consequence, the more salient stimulus
375 should evoke a greater learning response, thereby overshadowing learning of the less
376 salient stimulus (Rescorla, 1988). Alternatively, general recognition learning might
377 have been selected to be ‘fast and frugal’ (Todd, 2001). That is, even if the different
378 compounds in the mixture are equally salient, animals might learn only one or few key
379 odours in the mixture to minimize the information, and thus the neuronal resources,
380 required for mixture recognition. We cannot completely exclude that the lack of
381 differences between the responses of tadpoles to the two odours in the mixture was due
382 to lack of power, though Bayesian analysis did not support this hypothesis and

383 suggested substantial evidence of similarity between the response to the two odours.
384 Also previous studies on predator recognition learning did not find such differences
385 even with mixture of 4 odours (Mitchell et al., 2011b). Thus, it appears plausible that in
386 the case of predator recognition, learning processes have been shaped to give equal
387 weight to all the odour compounds in the mixture. This can be adaptive given the cost of
388 failing to recognize a predator.

389 Our comparison of the two experiments indicates that the species making the
390 mixture affect the learning of the mixture, a phenomenon that, to the best of our
391 knowledge, has never been studied. One could argue that the overall reduced learning of
392 the mixture of experiment 2 might only be due to one of the two odours (e.g., crayfish).
393 This explanation appears unlikely because in experiment 2 the response to crayfish
394 odour was similar to the response to catfish odour. A more likely explanation for our
395 result is that large phylogenetical difference between the species in the mixture used
396 during conditioning reduces the recognition performance of tadpoles. Support for this
397 hypothesis comes from both behavioural and chemical studies. In behavioural studies
398 on generalization of predator odours (Ferrari et al., 2007; Ferrari et al., 2009; Mitchell,
399 McCormick, Ferrari, Chivers, 2013), prey are conditioned to recognize a specific
400 species as a predator. The prey is then tested for their response to the learned predator
401 odour, but also the odour of species more or less related to the known predator. Results
402 across taxa concur that the response to the different predator species decreases as the
403 phylogenetic distance from the initial predator species increases (Ferrari et al., 2007).
404 This result has been interpreted as evidence that the odours of the different species
405 diverge with increasing phylogenetic distance. Regarding chemical studies, there is
406 limited information about tadpoles' reaction to specific odorants. Studies on rodents,

407 however, have suggested that the odour of a predator is composed by several individual
408 odorants activating specific receptors in prey olfactory epithelium (Kobayakawa et al.,
409 2007). Some of these individual odorants that cause the response in prey have been
410 found in many species of the same predator order (i.e., carnivora) but were absent in
411 species of different orders (Ferrero et al., 2011). Chemical data align with behavioural
412 data in suggesting that odour differences between predator species increases as a
413 function of phylogenetic distance. This provides an interpretation for the results of our
414 experiment: when the predators in the mixture are largely different, the degree of
415 matching between the conditioning mixture and the test cue (one species only) is low,
416 leading tadpoles to display weaker responses. Further work should investigate the effect
417 of species composition on learning of multiple predator cocktails using different species
418 and testing a larger number of species combinations or using individual chemicals to
419 simplify the study system.

420 In conclusion, our study reveals the presence of a cognitive mechanism for
421 learning to recognize multiple predator odours simultaneously in tadpoles. Since
422 tadpoles have been shown to rely on predator recognition learning for survival (Ferrari
423 et al., 2010), and since they are likely exposed to multiple olfactory cues during the
424 learning process, our study raises several questions regarding the extent and limits of
425 recognition learning of multiple predators. Future studies should try to address whether
426 tadpoles' learning of more than two cues is reduced because of receptors replenishment
427 dynamics or attentional limits of the cognitive system, and whether greater relative
428 concentration and greater intensity of one odour improves its learning at the costs of the
429 other odours in the mixture.

430

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611 **Figure captions**

612 Fig. 1

613 Mean \pm SE percentage change in activity between baseline and post-injection period

614 during the predator recognition test for tadpoles exposed to the mixture between odour

615 of (a) two fish species (experiment 1) and (b) one fish and one crayfish (experiment 2)

616 during the conditioning. Dark grey bars represent tadpoles exposed to alarm cues and

617 odour mixture during the conditioning phase; light grey bars represent tadpoles exposed

618 to water control and odour mixture during the conditioning phase.

619

620 Fig. 2

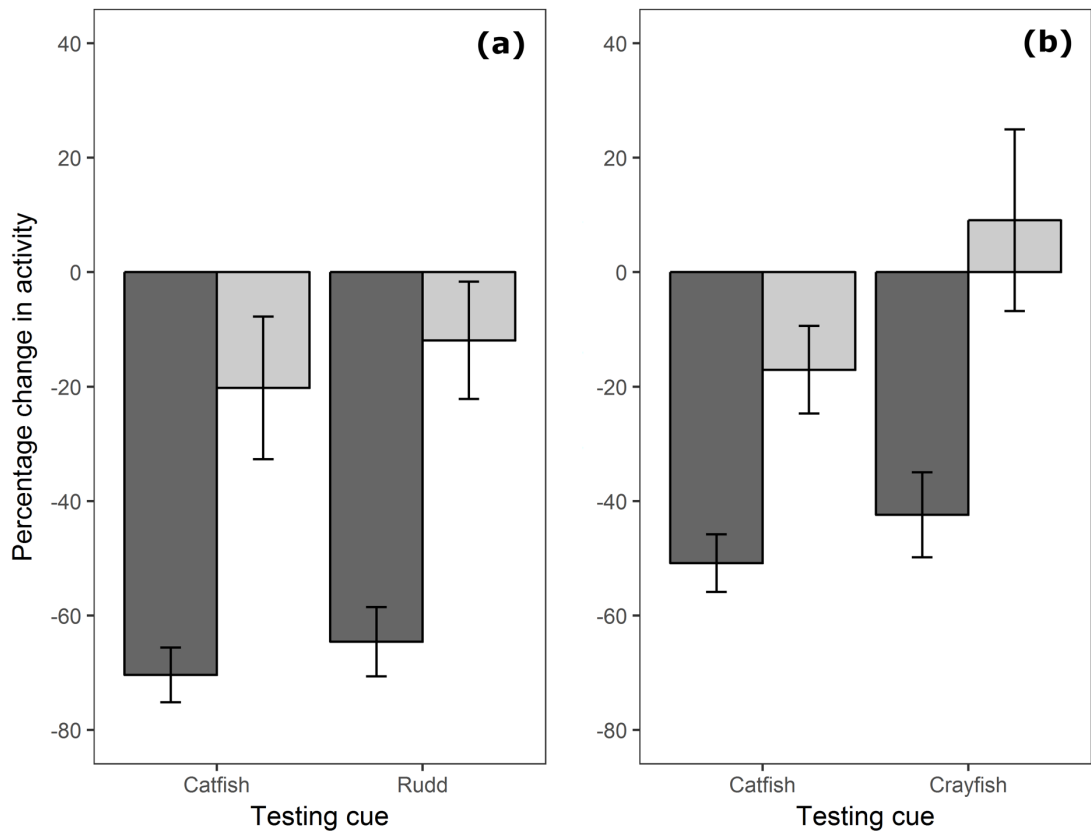
621 Mean \pm SE percentage change in activity between baseline and post-injection period for

622 tadpoles exposed to the odour of two species paired with conspecifics alarm cues

623 (pooled data from the two experiments).

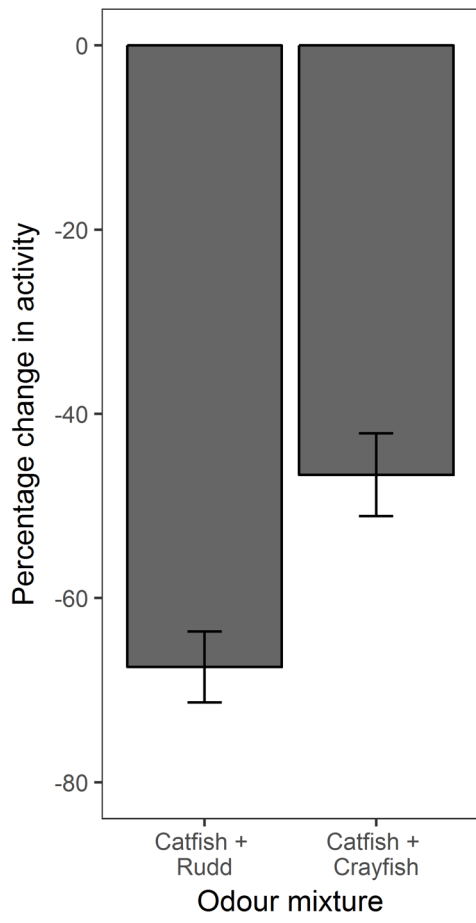
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