

## Effects of the Argentine ant venom on terrestrial amphibians

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Keywords:	amphibian decline, chemical weapons, invasive species, impact prioritization, <i>Linepithema humile</i> , predator-prey relationships
Abstract:	<p>Invasive species have major impacts on biodiversity and are one of the major causes of amphibian decline and extinction. Unlike other top ant invaders known to negatively affect larger fauna via chemical defensive compounds, the Argentine ant (<i>Linepithema humile</i>) does not have a functional sting; but nonetheless deploys defensive compounds against competitors and adversaries. We characterised the potential effect of <i>L. humile</i>'s venom on amphibian species, using complementary approaches (field and laboratory) and comparative analyses (<i>L. humile</i> vs. native ant species). We estimated levels of ant aggression directed at three native terrestrial amphibians, the composition and quantities of the toxin used, and the mechanisms of toxicity in juvenile amphibians. To determine the potential scope of the threat faced by amphibians, we employed global databases to estimate the number of terrestrial amphibian species whose ranges overlapped with those of <i>L. humile</i>, looking at the IUCN Red List status of those species. We show how juvenile amphibian, co-occurring spatially and temporally with <i>L. humile</i>, are killed in the ant's trails. Laboratory tests revealed that, upon contact, the invasive ants rapidly spray pygidial-gland venom onto the juveniles' permeable skin. Chemical analyses indicate that iridomyrmecin is the compound responsible: following absorption, it accumulates in brain, kidney, and liver tissue, acting at species-dependent doses. Moreover, we estimated that at least 817 terrestrial amphibian species around the world overlap in range with <i>L. humile</i>, and 6.2% of them are classified as threatened by IUCN. Our findings highlight the high potential of the venom to negatively affect amphibian juveniles and provide a basis for exploring the largely overlooked impacts in this ant in its wide invasive range.</p>

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## 2 **Abstract**

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6 defensive compounds, the Argentine ant (*Linepithema humile*) does not have a  
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25 compound responsible: following absorption, it accumulates

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29 range with *L. humile*, and 6.2% of them are classified as  
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31 potential of the venom to negatively affect amphibian  
32 juveniles and provide a basis for exploring the largely  
33 overlooked impacts in this ant in its wide invasive range.

34

For review only

## 35 INTRODUCTION

36 Amphibians are the most threatened vertebrate taxa worldwide  
37 with 41% of species at risk of extinction  
38 (<https://www.iucnredlist.org/>). Population declines and  
39 species extinctions outpace that of mammals and birds since  
40 the 1980s (Stuart et al. 2004). Habitat alterations and  
41 disease, as well as their synergistic effects with climate  
42 change, are key drivers for extinction, (Kiesecker et al.  
43 2001; Hof et al. 2011). Overwhelmingly, studies suggest that  
44 global amphibian losses are the result of complex  
45 interactions among multiple factors acting together on local  
46 scales, and in a context-dependent manner (Blaustein &  
47 [Kiesecker](#) 2002; Grant et al. 2016). Much of the observed  
48 decline is still attributed to “enigmatic decline” (*sensu*  
49 Stuart et al. 2004) and quantifying lesser known threats to  
50 amphibians is thus important for developing effective  
51 conservation strategies.

52 Invasive species are an additional major causes of  
53 amphibian extinctions, through competition, hybridization,  
54 bearers of diseases and due to predation (reviewed by Kats &  
55 Ferrer 2003). Invasive ants, three species of which are  
56 among the world’s worst invaders, have negative consequences  
57 for local wildlife, including many amphibian species, due to  
58 their opportunistic predation, poisoning, or toxicity  
59 (Holway et al. 2002). For example, the red imported fire

60 ant, *Solenopsis invicta*, is known for its negative effects  
61 on native herpetofauna, birds, and mammals (Allen et al.  
62 2004). Its venom is normally injected by stinging and may  
63 induce anaphylaxis and, at higher doses, paralysis and even  
64 death (Attygalle & Morgan 1984).

65 Chemical defense has evolved in ants and other social  
66 insects to protect their nests. Ants exhibit a plethora of  
67 chemicals with a clear evolutionary pathway from  
68 proteinaceous pain inducer venom to low molecular organic  
69 toxins (Attygalle & Morgan 1984). In addition to their  
70 primary defensive role, they can due to their toxicity also  
71 act in an offensive manner for subduing potential prey. They  
72 also often act, as such or combined with volatile substances  
73 as alarm pheromones, recruiting and eliciting aggression  
74 towards foes (Blum 1996).

75 This is well exemplified in one of the top five ant  
76 invaders, the Argentine ant *L. humile*. Although *L. humile*  
77 lacks visible weapons (e.g., a functional sting or large  
78 mandibles), it produced substances that include volatile  
79 alarm pheromones and defensive allomones (Cavill et al.  
80 1976); and a recent study has established that it deploys  
81 its defensive compounds against native ants (Welzel et al.  
82 2018). Although indirect effects on vertebrates are also  
83 known, such as contributing to the decline of the horned  
84 lizard (Suarez & Case 2002) and the spatial shift in habitat

85 use of amphibians (Alvarez-Blanco et al. 2017), direct  
86 effects, i.e., capacity to subdue vertebrates, which could  
87 explain some of the reported indirect effects, have not been  
88 demonstrated.

89       Here, we reveal that *L. humile*'s use of a toxin can be  
90 lethal to juvenile amphibians. We estimated the levels of  
91 ant aggression directed at different amphibian species in  
92 both the field and the laboratory, and quantified the toxin  
93 used. To determine the potential scope of the threat faced  
94 by amphibians, we employed global databases to estimate the  
95 number of terrestrial amphibian species whose ranges and  
96 habitats overlapped with those of the Argentine ant,  
97 highlighting particularly those species listed as threatened  
98 by the IUCN Red List. Ranges and categories of the Red List  
99 are a global standard for conservation studies, ensuring  
100 consistency across taxa and regions (Betts et al. [in press](#)).  
101 Our results present strong evidence that the venom is  
102 hazardous to the amphibians tested, and due to the ant's  
103 global distribution and extensive overlap with endangered  
104 amphibian species, can have serious implications for  
105 amphibian conservation.

106

## 107 **MATERIALS AND METHODS**

### 108 **Local study site and amphibian species**

109 The Doñana Biological Reserve (RBD, Spain, 36°59.491'N,  
110 6°26.999'W) hosts both terrestrial amphibians and the  
111 invasive *Linepithema humile* (Díaz-Paniagua et al. 2010;  
112 Alvarez-Blanco et al. 2017). We collected individuals of the  
113 three most abundant species: natterjack toads (*Epidalea*  
114 *calamita*), Mediterranean treefrogs (*Hyla meridionalis*), and  
115 western spadefoot toads (*Pelobates cultripes*) (full methods  
116 in Supporting Information). We collected either newly  
117 emerged juvenile amphibians near ponds, or tadpoles (that  
118 were raised to metamorphosis). Juveniles were housed in  
119 groups in terraria, and placed individually in smaller  
120 containers during trials. To compare the effects of *L.*  
121 *humile* on amphibians to those inflicted by other ant  
122 species, we selected two abundant co-inhabiting native ant  
123 species: *Aphaenogaster senilis* (Myrmecinae) and the closely  
124 related *Tapinoma cf. nigerrimum* (Dolichoderinae) (Arnan et  
125 al. 2012). Both ants are generalist feeders including  
126 scavenging on animal and plant remains (Arnan et al. 2012),  
127 similar to the Argentine ant.

128

129 **Spatial and temporal overlap in ant and juvenile amphibian**  
130 **activity at the local scale**

131 During the period when newly metamorphosed *E. calamita*  
132 emerged from ponds, we established two plots separated by  
133 400 m, which encompassed invaded and uninvaded areas

134 surrounding ponds. For two consecutive days, we placed baits  
135 to record the number and species of ants (a 35-m transect  
136 with water-diluted honey and cookie baits placed on 10 pairs  
137 of plastic spoons), and counted the number of toadlets (in  
138 1-m-wide, 50-m-long transect) throughout the day (at 9:00,  
139 12:30, 16:00, 19:30, and 23:00).

140 To demonstrate the direction of the interaction, i.e.,  
141 whether emerging amphibian feed on ants or *L. humile*  
142 predate on them, we inspected, preliminary, relatively permanent  
143 *L. humile* trails near the ponds, revealing dead amphibian in these trails.  
144 Subsequently, for four days per year during three years, we counted the number  
145 of dead juvenile amphibian found along 40 m long and 40 cm  
146 wide trails of *L. humile*.

147

#### 148 **Trail- and foraging-arena exposure experiments**

149 These observations raised two major questions: why juvenile  
150 amphibians did not escape from the trail, and, were native  
151 ants similarly aggressive towards juveniles?

152 We simulate ant-amphibian encounters experimentally in  
153 the field by carefully positioning *P. cultripes* and *H.*  
154 *meridionalis* juveniles 3 cm away from trails of the three  
155 above-mentioned ant species, in perforated cages; either  
156 inverted plastic Petri dish (5.5 cm in diameter, 1.4 cm in height, with holes of 4x4 mm),  
157 or cages (8x8.5x3 cm, with a mesh width of 5x5 mm) for the larger *A. senilis* ant



158 (Supporting Information). Following initial contact with the  
159 ants, the amphibians were kept in place for 2 additional  
160 minutes and then released by carefully removing the cage.  
161 They were then observed for 10 min or until they had moved  
162 at least 1 m away from the trail. During these 10 minutes  
163 individuals could follow normal activity, either escape or  
164 defend themselves from the ants, or get paralyzed; we  
165 observed that paralyzed individuals could either recover or  
166 die. All individuals were subsequently observed in the  
167 laboratory for additional 48 h to monitor the recovery.  
168 Individuals were classified as follows: Individuals that  
169 remained unaffected, those that escaped or were not  
170 paralyzed, were classified as "Alive"; those that recovered  
171 after initial paralysis were classified as "Paralyzed"  
172 whereas those that died within 48h were classified as  
173 "Dead".

174 In laboratory assays, juveniles of either *P. cultripes*,  
175 or *E. calamita*, or *H. meridionalis* were introduced  
176 individually into the foraging arenas of colonies of each of  
177 the three above-mentioned ant species, for a maximum of 10  
178 min (N = 5 colonies/ant species; see Supporting Information  
179 for colony details). We measured the elapsed time to  
180 juvenile discovery by the ants and the maximum number of  
181 ants found upon it. In cases of apparent harmful effects to  
182 the juveniles (the amphibian remained immobile/paralyzed for

183 one minute or was being dragged off by the ants, risking  
184 serious injury), trials were stopped earlier. After 48 h of  
185 observation, individuals were classified as alive,  
186 paralyzed, or dead, as in the previous experiment.

187

188 **Histological and chemical differences between *L. humile* and**  
189 ***T. cf. nigerrimum***

190 The next step was to determine whether *L. humile* employs a  
191 chemical attack. First, we compared the histology of all  
192 abdominal exocrine glands of *L. humile* and *T. cf.*  
193 *nigerrimum*. Ant gasters were fixed in 2% glutaraldehyde  
194 (buffer: 0.05 M Na-cacodylate and 0.15 M saccharose),  
195 postfixed in 2% osmium tetroxide, and embedded in Araldite.  
196 Semithin sections (thickness of 1  $\mu$ m) were created with a  
197 Leica EM UC6 ultramicrotome and stained with methylene blue  
198 and thionin. These sections were then viewed and  
199 photographed with an Olympus BX-51 microscope. We examined  
200 the sections meticulously in order to identify all known  
201 glands, and look for possible previously undescribed glands.

202       Second, we compared the chemical composition of the  
203 pygidial gland of the two species. We dissected the pygidial  
204 glands of five freeze-killed ants of each species  
205 immediately after death and extracted them in hexane for 24  
206 hrs. Compound identification was achieved via gas

207 chromatography coupled with mass spectrometry (GC-MS), using  
208 an HP-5MS capillary column, temperature programmed from 60°C  
209 (1 min hold) to 320°C at a rate of 10°C.min<sup>-1</sup>. For  
210 iridomyrmecin quantification extracts of **fifty** whole ants  
211 (**10/colony**) were used rather than dissected gland to avoid  
212 possible spillage during dissection. Decyl-alcohol (99%) was  
213 used as the internal standard. Samples were analyzed by gas  
214 chromatography as described above. Calibration curve was  
215 established using synthetic iridomyrmecin (Chauhan & Schmidt  
216 2014; Supporting Information).

217

#### 218 **Iridomyrmecin-exposure experiment**

219 To test iridomyrmecin's toxicity, we applied the synthetic  
220 compound to the backs of *P. cultripes* toadlets (isomers 1  
221 and 2, in a ratio of 1.5:1). We exposed 10 toadlets to each  
222 of three doses of iridomyrmecin dissolved in hexane: 0.1 mg,  
223 1 mg, and 5 mg per toadlet and pure hexane as control. Doses  
224 were calculated from Choe et al. (2012) estimations, to  
225 match naturally occurring concentrations the amphibian would  
226 experience in the field. To avoid skin irritation by the  
227 hexane solvent, the solutions were applied to cavity slides,  
228 allowed the solvent to evaporate, and the slides were rubbed  
229 onto the toadlets' backs. After 48 h of observation,  
230 individuals were classified as alive (not affected),

231 paralyzed (recovered from initial paralysis), or dead, as  
232 described above.

233

#### 234 **Dose-response experiment**

235 To assess the number of ants necessary to elicit an effect,  
236 we constructed dose-response curves for each ant species and  
237 each amphibian species. The number of amphibians was limited  
238 to that necessary to obtain adequate dose-response curves  
239 (Supporting Information).

240 Doses of the toxin were obtained from a given number of either *L. humile* or  
241 *T. cf. nigerrimum* workers that were macerated in a ceramic bowl  
242 using 0.2 ml of dechlorinated water. A single dose of the mash was  
243 immediately applied to the back of an amphibian. After 10  
244 minutes, the individual was gently bathed in dechlorinated water to remove the mash, and  
245 we conducted a clinical evaluation based on the presence (or absence)  
246 of neurological damage: an individual was considered to be  
247 affected by the toxin if an abnormal reaction was displayed  
248 in the motor response, the photopupillary reflex or the  
249 palpebral reflex (Supporting Information)

250

#### 251 **Physiological effects on juvenile amphibians**

252 To elucidate the venom's *modus operandi* and confirm that the  
253 damage was caused by iridomyrmecin, we euthanized the

254 amphibians used in the above experiment after the clinical  
255 evaluation. Half the amphibians were used to quantify  
256 iridomyrmecin levels in tissues: animals were dissected to  
257 remove their brains, livers, and kidneys, which were then  
258 individually extracted in hexane for GC-FID analyses.

259       The other half were used in histological analyses:  
260 individuals were fixed in formalin and dissected to obtain  
261 their livers and kidneys. Tissue samples were embedded in  
262 paraffin wax, sectioned at a thickness of 6  $\mu\text{m}$  using a Leica  
263 RM 2155 microtome, and mounted on glass slides. Sections  
264 were dewaxed through a series of xylene and ethanol washes  
265 (from 100% solution to 100%  $\text{H}_2\text{O}$ ), stained with  
266 hematoxylin/eosin, rehydrated through a series of ethanol  
267 washes (from 70% to 100% solution to 100% xylene), and  
268 mounted with cover slides using DPX. Lesions were evaluated  
269 under the microscope (Axio Imager, A1, Zeiss; objective EC  
270 Plan-NEOFLUAR 20x/0.5,  $\infty$ /0.17), focusing on sensitive areas  
271 such as the periportal spaces in both the liver and the  
272 renal tubules as well as the glomeruli in the kidneys.

273

#### 274 **Potential global effects on amphibians**

275 To quantify the potential spatial overlap of ants and  
276 amphibians at a global scale we obtained a total of 1,407  
277 geographic records on Argentine ant locations from the GBIF

278 (Global Biodiversity Information Facility,  
279 <https://www.gbif.org>), AntWeb (<https://www.antweb.org>,  
280 AntWeb 2018) and GLAD (<http://globalants.org/>) websites.  
281 Amphibian ranges and IUCN status were obtained from the IUCN  
282 Red List (v. 2017-3, [www.iucnredlist.org](http://www.iucnredlist.org)). We used the  
283 function `gContains` in the R package `rgeos` (Bivand & Rundel  
284 2017) to extract amphibian species whose distribution  
285 polygons overlapped with the ranges of any given ant  
286 population. We then filtered this list of species using IUCN  
287 habitat categories to exclude amphibian species that did not  
288 use similar macrohabitats as *L. humile* (Supporting  
289 Information).

290       Ants and amphibians may further be segregated by  
291 differences in microhabitat use. We used the eight  
292 categories of microhabitat, described in Moen & Wiens  
293 (2017), that adults use outside of the breeding period and  
294 included species from our dataset (Supporting Information),  
295 employing habitat descriptions from the IUCN Red List and  
296 the AmphibiaWeb database ([www.amphibiaweb.org](http://www.amphibiaweb.org)). We excluded  
297 any amphibian species that only occur in aquatic, semi-  
298 aquatic, or torrential microhabitats, where *L. humile* is not  
299 likely occur.

300       Juvenile amphibians likely use slightly different  
301 microhabitats from adults (Wells 2010; Duellman & Trueb  
302 1994). We therefore considered the full dataset to be the

303 maximum number of possible amphibian species overlapping  
304 spatially with the ants and the microhabitat-filtered list  
305 to be the minimum. We acknowledge that we may overestimate  
306 risk, which is not solely determined by spatial overlap.  
307 Additionally, the ant's impact will depend on the amphibian  
308 species' biological traits, such as anatomy, behavior, or  
309 physiology.

310 From the full dataset, we determined amphibian species  
311 richness per ant locality. Then, using both the full and  
312 microhabitat-filtered datasets, summarized cumulative  
313 species richness for amphibians co-occurring with ant  
314 populations per continent or section of continent. Finally,  
315 for each of these regions and for both datasets, we assessed  
316 the proportion of amphibian species in the five different  
317 IUCN Red List risk categories.

318

### 319 **Statistical analysis**

320 For analyses, we assumed that paralysis (either in the lab  
321 or the field) is equivalent to death for a juvenile, because  
322 it would ensue if the juvenile remained in the Argentine ant  
323 area. We therefore analyzed the proportion of alive versus  
324 paralyzed+dead individuals using a generalized linear model  
325 employing a binomial distribution and a logit link function  
326 (PROC *Genmod*, SAS 2008); we first tested whether there were

327 differences among amphibian species and among ant species.  
328 We secondly tested the effect of the ant species within each  
329 amphibian species. In this case, we performed planned post-  
330 hoc comparisons (using the *contrast* command in PROC *Genmod*,  
331 SAS 2008), which compared the effect of *L. humile* to each of  
332 the native species.

333         In the foraging-arena exposure experiment, we explored  
334 differences in behavior of *L. humile*, *A. senilis*, and *T. cf.*  
335 *nigerrimum* towards juvenile amphibians. Time to amphibian  
336 discovery and the maximum number of ants found on the  
337 amphibians were analyzed using generalized linear models  
338 employing a gamma distribution and a Poisson distribution,  
339 respectively, and a logit link function (PROC *Genmod*, SAS  
340 2008). Ant species and amphibian species were fixed  
341 independent variables; the number of ants in the foraging  
342 arena at the beginning of the trial and amphibian mass were  
343 covariates (the latter was only used in the model with the  
344 maximum number of ants). When the results were significant,  
345 we performed post-hoc comparisons among ant species, as  
346 explained above.

347         To determine differences in iridomyrmecin quantities we  
348 performed a general linear mixed-effects model (square root  
349 transformed) between *L. humile* and *T. cf. nigerrimum*;  
350 covariance within colonies was included as a random factor.



351 The model was fitted using the *lmer* function in the R  
352 package *lme4* (Bates et al. 2015).

353 The effect of toxic doses on amphibians (affected vs  
354 unaffected) was analyzed using generalized linear models  
355 employing a binomial distribution and a logit link function  
356 (*glm* function in the R package *stats*, R Core Team 2016). Ant  
357 number per gram of amphibian, ant species, and amphibian  
358 species were the independent variables. The toxic dose,  
359 represented by the number of ants per gram of amphibian  
360 expected to elicit a toxic effect for each ant-amphibian  
361 species pair, was calculated using the function *dose.p* in  
362 the R package *MASS* (Venables & Ripley 2002) from the dose-  
363 response curves. Because iridomyrmecin quantities can vary  
364 among sites (Choe et al. 2012) we focused on the ecological  
365 "ant dose", not necessarily on the toxin dose.

366 Relationships between the concentration of iridomyrmecin  
367 ( $\mu\text{g}\cdot\text{g}^{-1}$  of juvenile) in the brain and the clinical  
368 evaluation (affected vs unaffected) was tested using a  
369 generalized linear model employing a binomial distribution  
370 and a logit function (*glm* function in the R package *stats*, R  
371 Core Team 2016); the model took amphibian species into  
372 account. Then, we examined the relationship (*lm* function in  
373 the R package *stats*) between the concentrations of  
374 iridomyrmecin ( $\mu\text{g}\cdot\text{g}^{-1}$  of juvenile; log transformed) in each  
375 tissue and the quantity of iridomyrmecin ( $\mu\text{g}\cdot\text{g}^{-1}$  of

376 juvenile) applied to each juvenile, which was estimated  
377 based on the species-specific iridomyrmecin contents. We  
378 also tested whether higher doses ( $\mu\text{g}\cdot\text{g}^{-1}$  of juvenile; log  
379 transformed) corresponded to the presence of lesions in  
380 amphibian tissues (liver and kidney); a general linear model  
381 (PROC *genmod*, SAS 2008) was used for each tissue in which  
382 the identity of the amphibian species was taken into  
383 account.

384

## 385 **RESULTS**

### 386 ***Linepithema humile* and juvenile amphibians overlap at the** 387 **local scale**

388 Newly metamorphosed *E. calamita* toadlets emerging from the  
389 temporary ponds in uninvaded areas overlapped with different  
390 species of native ants. Toadlets emerging from invaded ponds  
391 overlapped only with *L. humile*, which was the sole ant  
392 species present. This ant was much more abundant during the  
393 day compared to the abundance of native ants around  
394 uninvaded ponds (Supporting Information).

395

### 396 ***Linepithema humile* preys on and kills juvenile amphibians**

397 During 2013–2018 we performed observation of emerging frogs  
398 from ponds along the surveyed *L. humile* trails. The

399 objectives were to evaluate the direction of the interaction  
400 (not to quantify the impact on amphibian populations). We  
401 observed a total of 46 dead *H. meridionalis* frogs (12 in  
402 2013, 34 in 2014); 6 dead *P. cultripipes* toadlets (3 in 2013,  
403 3 in 2018); 2 dead Iberian painted frogs (*Discoglossus*  
404 *galganoi*; 2018); and 1 dead Iberian parsley frog (*Pelodytes*  
405 *ibericus*; 2018). The ants preyed on the amphibians, which  
406 ranged from being recently dead to being entirely eaten  
407 (skeletons) (Supporting Information).

408

409 ***Linepithema humile* aggressiveness in the ant-trail-exposure**  
410 **experiment**

411 There was a significant detrimental effect of *L. humile* on  
412 juveniles, but neither of *A. senilis* nor *T. nigerrimum* ( $\chi^2 =$   
413 10.10,  $p = 0.006$ ,  $N = 57$ , for differences among ant species,  
414 Fig. 1a). The effects observed -alive versus paralyzed+dead-  
415 also significantly differed among amphibian species ( $\chi^2 =$   
416 6.10,  $p = 0.013$ ,  $N = 57$ ). Specifically, the effects of *L.*  
417 *humile* differed from those of the two native ants in the  
418 case of *P. cultripipes* ( $\chi^2 = 10.10$ ,  $p = 0.006$ ,  $N = 30$ ; planned  
419 comparisons:  $p = 0.010$  in both cases), but not in the case  
420 of *H. meridionalis* ( $\chi^2 = 0.00$ ,  $p = 1.000$ ,  $N = 27$ ), in which  
421 none of the froglets was affected by the ants (they always  
422 escaped). In the *L. humile* trails, 20% of the *P. cultripipes*

423 toadlets died and a further 20% were initially paralyzed but  
424 recovered after approximately 10 min (Fig. 1a).

425

426 ***Linepithema humile* aggressiveness in the foraging-arena-**  
427 **exposure experiment**

428 The native ant *A. senilis* was faster to discover the  
429 amphibians than the invasive ant *L. humile* ( $\chi^2 = 27.0$ ,  $p <$   
430  $0.001$ ,  $N = 290$ ;  $p < 0.001$  for all contrast with *A. senilis*).  
431 Moreover, the amphibians were covered by significantly more  
432 ants of *T. cf. nigerrimum* than of *L. humile* (mean  $\pm$  SE:  
433  $17.9 \pm 1.9$  ants vs  $13.0 \pm 2.0$  ants, respectively;  $\chi^2 =$   
434  $177.22$ ,  $p < 0.001$ ,  $N = 284$ ;  $p < 0.018$  for all contrasts with  
435 *T. cf. nigerrimum*). Notwithstanding, while the attacks by *A.*  
436 *senilis* or *T. cf. nigerrimum* had no obvious effect, those by  
437 *L. humile* ultimately resulted in paralysis and death ( $\chi^2 =$   
438  $88.56$ ,  $p < 0.001$ ,  $N = 294$ , for differences among ant  
439 species, Fig 1b). The effects observed -alive versus  
440 paralyzed+dead- were also significant among amphibian  
441 species ( $\chi^2 = 14.43$ ,  $p < 0.001$ ,  $N = 294$ ). The effects of *L.*  
442 *humile* differed from those of the two native ants on *P.*  
443 *cultripipes* and on *E. calamita* ( $\chi^2 = 44.31$ ,  $p < 0.001$ ,  $N = 94$ ;  
444  $\chi^2 = 39.74$ ,  $p < 0.001$ ,  $N = 125$ , respectively; planned  
445 comparisons:  $p < 0.001$  in all cases), but not on *H.*  
446 *meridionalis* ( $\chi^2 = 4.51$ ,  $p = 0.105$ ,  $N = 75$ ). Exposure to *L.*

447 *humile* caused the strongest effect on *P. cultripes*, with 53%  
448 of juveniles becoming paralyzed, and all but one died within  
449 48 h post-trial (N = 30; Fig. 1b). For *E. calamita*, 38% of  
450 toadlets were paralyzed during exposure but they recovered  
451 ~10 min later and only one died (N = 45; Fig. 1b). Finally,  
452 *H. meridionalis* was the least affected, with only 8% of  
453 froglets paralyzed, all of which recovered within ~10 min (N  
454 = 25).

455

#### 456 **Iridomyrmecin quantities in *Linepithema humile***

457 *Linepithema humile* and *T. cf. nigerrimum* workers have highly  
458 developed pygidial glands (Supporting Information).  
459 Iridomyrmecin (isomer 1) is the main compound found in *L.*  
460 *humile* pygidial glands. In *T. cf. nigerrimum* workers are  
461 isomers of iridodial the main components, accompanied by  
462 smaller amounts of iridomyrmecin (isomers 1 and 2;  
463 Supporting Information). Although *T. cf. nigerrimum* workers  
464 are slightly larger in body size than *L. humile* workers, the  
465 latter contain five times more iridomyrmecin (mean  $\pm$  SE:  
466  $6.416 \pm 0.443 \mu\text{g}$  vs  $1.291 \pm 1.127 \mu\text{g}$ ;  $F = 135.76$ ,  $p < 0.0001$ ,  
467  $N = 100$ ), representing 1.4% of worker fresh body mass in *L.*  
468 *humile* compared to just 0.2% in *T. cf. nigerrimum*.

469

**470 Iridomyrmecin-exposure experiments and toxic doses**

471 According to our quantification and assuming that the ants  
472 eject all their pygidial gland content at once, the three  
473 quantities of iridomyrmecin applied (0.1, 1, and 5 mg) are  
474 equivalent, respectively, to average doses ( $\pm$  SE) ejected by  
475  $8.4 \pm 1.2$ ,  $69.7 \pm 6.4$ , and  $307.5 \pm 30.3$  *L. humile* workers/g  
476 of juvenile. We observed significant differences among  
477 treatments ( $\chi^2 = 25.63$ ,  $p < 0.001$ ,  $N = 42$ , Fig. 1c). The  
478 lower doses were not significantly different from the  
479 control (no treatment,  $p > 0.05$ ), with all individuals alive  
480 at the end of the experiment. However, the highest dose was  
481 different ( $p < 0.001$ ), causing paralysis in 70% of the  
482 juveniles.

483 Amphibians were increasingly affected by greater  
484 numbers of ants in a dose-dependent manner ( $\chi^2 = 26.69$ ,  $p <$   
485  $0.001$ ,  $N = 81$ ). However, the magnitude of the effect  
486 differed, depending on both amphibian species and ant  
487 species ( $\chi^2 = 23.40$ ,  $p < 0.001$ ,  $N = 81$  and  $\chi^2 = 22.92$ ,  $p <$   
488  $0.001$ ,  $N = 81$ , respectively; Fig. 2a). Comparatively,  
489 smaller numbers of *L. humile* caused more dramatic negative  
490 consequences than did larger numbers of *T. cf. nigerrimum*  
491 (Fig. 2b).

492

493 **Iridomyrmecin causes general paralysis and histological**  
494 **lesions**

495 Results of the laboratory evaluations showed that the venom  
496 of the invasive ant *L. humile* had neurological effects,  
497 specifically in the medulla oblongata, pontine nucleus, and  
498 midbrain. The venom caused general paralysis (Fig. 3a),  
499 sometimes accompanied by extraocular paralysis, loss of  
500 photopupillary and palpebral reflexes, and loss of  
501 nociception response. We also observed severe damage to the  
502 skin of juveniles that came in contact with *L. humile* and of  
503 juveniles treated with iridomyrmecin (Fig. 3b).

504 Neurological affected individuals had higher levels of  
505 iridomyrmecin in their brains than did unaffected  
506 individuals ( $\chi^2 = 10.19$ ,  $p = 0.001$ ,  $N = 28$ ). Moreover,  
507 concentrations of iridomyrmecin in brain, liver, and kidney  
508 tissue were significantly correlated with the equivalent  
509 amounts of iridomyrmecin applied (brain:  $F = 17.69$ ,  $p <$   
510  $0.001$ ,  $N = 28$ ; liver:  $F = 14.24$ ,  $p < 0.001$ ,  $N = 27$ ; kidney:  
511  $F = 8.29$ ,  $p = 0.008$ ,  $N = 26$ ; Fig. 3c).

512 The histological samples revealed liver and kidney  
513 damage, indicating the toxin's acute effects on these  
514 tissues. In the liver, we found inflammatory cell  
515 infiltrates (heterophils) around the hepatic artery (Fig.  
516 3d, e). These lesions were observed in 16 cases ( $N = 33$ , all  
517 species combined). There was no significant relationship

518 between the quantity of iridomyrmecin per gram of amphibian  
519 and the presence of lesions ( $\chi^2 = 0.12$ ,  $p = 0.727$ ,  $N = 33$ ),  
520 which could be due to the individuals' short exposure to the  
521 toxin (only 10 min; see Methods). In the kidney, we found  
522 inflammatory cell infiltrates (lympho-plasmocitary cells) in  
523 the renal tubules, which indicated tubulo-interstitial  
524 nephritis (Fig. 3f, g). There were lesions in just five  
525 cases ( $N = 32$ , all species combined); these were found in  
526 individuals who had received mean doses of 0.674, 0.665, and  
527 1.167 mg of iridomyrmecin per gram of amphibian for *E.*  
528 *calamita*, *P. cultripes*, and *H. meridionalis*, respectively.

529

### 530 **Potential global impacts on amphibians**

531 We examined the overlap across the globe between the  
532 distribution ranges of amphibian species and the locations  
533 of 1,407 *L. humile* populations, 61 of which were native,  
534 while the rest were invasive. There were only 51 *L. humile*  
535 populations (all invasive) that were not associated with any  
536 amphibians.

537 Using the full dataset, we determined that, worldwide,  
538 *L. humile* populations co-occurred with a total of 813  
539 amphibian species (based on the 6,513 terrestrial amphibian  
540 species with spatial data in the IUCN Red List database),  
541 and only 9 of these amphibians exclusively co-occurred with



542 native *L. humile* populations. Outside of its native range,  
543 *L. humile* potentially co-occurs with a mean ( $\pm$ SE) of 11.06  
544 ( $\pm$  0.23) amphibian species per locality (range: 1-86, N =  
545 1295; Fig. 4). When filtering the amphibian species by  
546 microhabitat, *L. humile* populations co-occurred with a total  
547 of 693 amphibian species, and, with a mean ( $\pm$ SE) of 7.22 ( $\pm$   
548 0.20) amphibian species per locality (range: 1-78, N = 1287)  
549 outside its native range.

550

## 551 **DISCUSSION**

552 Here, we provide empirical evidence demonstrating the  
553 detrimental effect of *L. humile* ants, through their  
554 iridomyrmecin toxin, in killing juvenile terrestrial  
555 amphibians. The effect is dose- and species-dependent, and  
556 specific to *L. humile*. Although the three tested amphibian  
557 species are listed as of least concern (*H. meridionalis* and  
558 *E. calamita*) and near-threatened (*P. cultripes*;  
559 <https://www.iucnredlist.org>), they represent a broad  
560 phylogenetic spectrum and some of the most geographically  
561 widespread families. Worldwide, a total of 813 amphibian  
562 species overlap in range and macrohabitat with the Argentine  
563 ant and could therefore be affected by the species' chemical  
564 weaponry. Of these species, 6.27% are classified as  
565 threatened by IUCN. At the regional level, this percentage  
566 is as high as 16.39% (in Australia).

567           Although the most tolerant *H. meridionalis* was able to  
568 escape from the ant trails in the field soon after contact,  
569 more subtle effects were observed when confined with the  
570 ants for longer periods. These findings suggest that, unlike  
571 the two other amphibian species, the jumping behavior of  
572 this frog could enable its quicker escape. Similar escape  
573 behavioral strategies have been described for juvenile  
574 *Sceloporus undulatus* lizards when encountering the red  
575 imported fire ant *S. invicta* (Langkilde et al. 2009).  
576 Moreover, juveniles of several *Hyla* species were observed  
577 feeding on Argentine ants without any apparent negative  
578 effects (although the researchers did not look for them; Ito  
579 et al. 2009), hinting at further tolerance.

580           The dose-response experiments confirmed the high  
581 susceptibility of *E. calamita* and *P. cultripes* toadlets to  
582 *L. humile* attack. For example, *E. calamita*, exhibiting a  
583 mean mass ( $\pm$  SE) of  $0.45 \pm 0.05$  g after metamorphosis,  
584 required only 20 attacking *L. humile* to result in a  
585 detrimental effect. In contrast, more than 150 workers of  
586 the native ant *T. cf. nigerrimum* would have been required to  
587 achieve such an effect. We attribute this difference to the larger quantities of  
588 iridomyrmecin in *L. humile* compared to in *T. cf. nigerrimum*.  
589 Besides its greater toxicity, the augmented threat from *L. humile* arise  
590 from its high abundance and monopolization of invaded areas, e.g., around ponds  
591 (Angulo et al. 2011; Alvarez-Blanco et al. 2017). Consequently, emerging *E. calamita*

592 have little chances of surviving in ant invaded areas. Moreover, this species is also  
593 especially sensitive to other drivers of global change, such as climate warming (Bosch et  
594 al. 2018).

595 The role of *L. humile* as a predator is not apparent and  
596 ill studied. It is mostly considered as scavenger (Angulo et al.  
597 2011), and reports on its predation habits are scanty (Suarez et al. 2005). This is  
598 probably due to the lack of a functional sting and the  
599 ineffectiveness of its venom to humans or mammals (Pavan & Ronchetti 1955).  
600 Moreover, it may have a delayed detrimental effect on amphibians, thus there is no  
601 obvious association between their death and the ants.

602 The iridomyrmecin-exposure experiment revealed its high  
603 toxicity to amphibians, indicating that *L. humile* can cause amphibian  
604 mortality, and describes the proximate mechanisms involved  
605 (behavioral and chemical). Understanding the mechanisms that  
606 underlie the impacts of invasive species helps scientists to  
607 assess their potential magnitude, which is essential when  
608 prioritizing and managing invasions, as made clear in the  
609 Aichi targets of the Convention of Biological Diversity  
610 (Strategic Plan 2020,  
611 [www.cbd.int/sp/targets/rationale/target-9](http://www.cbd.int/sp/targets/rationale/target-9)). Here we reveal  
612 the potential magnitude of this impact, based on the global  
613 spread of the Argentine ant (Bertelsmeier et al. 2018), and  
614 when acting together with other drivers of amphibian decline  
615 (Grant et al. 2016). We highlight the need for new research along two broad  
616 lines: determining the factors underlying venom toxicity to other amphibians (e.g., skin

617 permeability or life-history traits, such as developmental type or breeding strategy); and  
618 examining whether the venom effect could scale to demographic effects (because  
619 population persistence is highly sensitive to the survival of juveniles in pond-breeding  
620 amphibians, Pittman et al. 2014), in order to accurately understand and contend with the  
621 worldwide impact of this invasive ant on amphibians.

622

### 623 **Supporting Information**

624 Extended information on methods (Appendix S1), the  
625 functional ecology of iridomyrmecin (Appendix S2), the  
626 temporal and spatial overlap of *L. humile* ants with  
627 amphibians (Appendix S3), the identification of *L. humile*  
628 venom (Appendix S4), [and the list of amphibian species](#)  
629 [across the globe overlapping with \*L. humile\* populations](#)  
630 [\(Appendix S5\)](#) are available online. The authors are solely  
631 responsible for the content and functionality of these  
632 materials. Queries (other than absence of the material)  
633 should be directed to the corresponding author.

634

### 635 **Literature Cited**

636 Allen CR, Epperson DM, Garmestani AS. 2004. Red imported  
637 fire ant impacts on wildlife: a decade of research. The  
638 American Midland Naturalist **152**:88-103.

- 639 Alvarez-Blanco P, Caut S, Cerdá X, Angulo E. 2017. Native  
640 predators living in invaded areas: responses of  
641 terrestrial amphibian species to an Argentine ant  
642 invasion. *Oecologia* **185**:95-106.
- 643 Arnan X, Cerdá X, Retana J. 2012. Distinctive life traits  
644 and distribution along environmental gradients of  
645 dominant and subordinate Mediterranean ant species.  
646 *Oecologia* **170**:489-500.
- 647 Attygalle AB, & Morgan ED. 1984. Chemicals from the glands  
648 of ants. *Chemical Society Reviews* **13**:245-278.
- 649 Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting  
650 linear mixed-effects models using lme4. *Journal of*  
651 *Statistical Software* **67**:1-48.
- 652 Bertelsmeier C, Ollier S, Liebhold AM, Brockerhoff EG, Ward  
653 D, Keller L. 2018. Recurrent bridgehead effects  
654 accelerate global alien ant spread. *Proceedings of the*  
655 *National Academy of Sciences* **115**: 5486-5491.
- 656 Betts, J., Young, R. P., Hilton-Taylor, C., Hoffmann, M.,  
657 Rodríguez, J. P., Stuart, S. N., & Milner-Gulland, E. J.  
658 In press. A framework for evaluating the impact of the  
659 IUCN Red List of threatened species. *Conservation*  
660 *Biology*.

- 661 Bivand R, Rundel C. 2017. rgeos: Interface to Geometry  
662 Engine - Open Source ('GEOS'). R package version 0.3-26.  
663 <https://CRAN.R-project.org/package=rgeos>
- 664 Blaustein AR, Kiesecker JM. 2002. Complexity in  
665 conservation: lessons from the global decline of  
666 amphibian populations. *Ecology Letters* **5**:597-608.
- 667 Blum MS. 1996. Semiochemical parsimony in the Arthropoda.  
668 *Annual Review of Entomology* **41**:353-374.
- 669 Bosch J, Fernández-Beaskoetxea S, Garner TW, Carrascal LM.  
670 2018. Long term monitoring of an amphibian community after  
671 a climate change and infectious disease driven species  
672 extirpation. *Global Change Biology* **24**:2622-2632.
- 673 Cavill GWK, Houghton E, McDonald FJ, Williams PJ. 1976.  
674 Isolation and characterization of dolichodial and related  
675 compounds from the Argentine ant, *Iridomyrmex humilis*.  
676 *Insect Biochemistry* **6**:483-490.
- 677 Chauhan KR, Schmidt W. 2014. Biorational synthesis of  
678 iridomyrmecin diastereomers from catnip oil. *Tetrahedron*  
679 *Letters* **55**:2534-2536.
- 680 Choe DH, Villafuerte DB, Tsutsui ND. 2012. Trail pheromone  
681 of the argentine ant, *Linepithema humile*  
682 (Mayr) (Hymenoptera: Formicidae). *PLoS One* **7**:e45016.

- 683 Díaz-Paniagua C, et al. 2010. Temporary ponds from Doñana  
684 National Park: a system of natural habitats for the  
685 preservation of aquatic flora and fauna. *Limnetica* **29**:41-  
686 58.
- 687 Duellman WE, Trueb L. 1994. *Biology of amphibians*. JHU  
688 press.
- 689 Grant EHC, et al. 2016. Quantitative evidence for the  
690 effects of multiple drivers on continental-scale  
691 amphibian declines. *Scientific Reports* **6**:25625.
- 692 Hof C, Araújo MB, Jetz W, Rahbek C. 2011. Additive threats  
693 from pathogens, climate and land-use change for global  
694 amphibian diversity. *Nature* **480**:516-519.
- 695 Holway DA, Lach L, Suarez AV, Tsutsui ND, Case TJ. 2002. The  
696 causes and consequences of ant invasions. *Annual Review*  
697 *of Ecology and Systematics* **33**:181-233.
- 698 Ito F, Okaue M, Ichikawa T. 2009. A note on prey composition  
699 of the Japanese treefrog, *Hyla japonica*, in an area  
700 invaded by Argentine ants, *Linepithema humile*, in  
701 Hiroshima prefecture, western Japan (Hymenoptera:  
702 Formicidae). *Myrmecological News* **12**:35-39.
- 703 Kats LB, Ferrer RP. 2003. Alien predators and amphibian  
704 declines: review of two decades of science and the  
705 transition to conservation. *Diversity and Distributions*,  
706 **9**:99-110.

- 707 Kiesecker JM, Blaustein AR, Belden LK. 2001. Complex causes  
708 of amphibian population declines. *Nature Methods* **410**:681-  
709 684.
- 710 Langkilde T. 2009. Invasive fire ants alter behavior and  
711 morphology of native lizards. *Ecology* **90**:208-217.
- 712 Moen DS, Wiens JJ. 2017 Microhabitat and climatic niche  
713 change explain patterns of diversification among frog  
714 families. *The American Naturalist* **190**:29-44.
- 715 Pavan M, Ronchetti G. 1955. Studi sulla morfologia esterna e  
716 anatomia interna dell'operaia di *Iridomyrmex humilis* Mayr  
717 e ricerche chimiche e biologiche sulla iridomirmecina.  
718 *Atti Soc It Sc Nat* **94**:379-477.
- 719 Pittman S, Osbourn M, Semlitsch R. 2014. Movement ecology of  
720 amphibians: a missing component for understanding  
721 population declines. *Biological Conservation* **169**:44-53.
- 722 R Core Team. 2016. R: A language and environment for  
723 statistical computing. R Foundation for Statistical  
724 Computing, Vienna, Austria.
- 725 SAS/STAT® 2008. 9.2 User's Guide SAS Inst, Cary, NC.
- 726 Strategic Plan 2020, target 9.  
727 <https://www.cbd.int/sp/targets>. (Accessed May 2020).
- 728 Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL,  
729 Fischman DL, Waller RW. 2004. Status and trends of



730 amphibian declines and extinctions worldwide. *Science*,  
731 **306**:1783–1786.

732 Suarez AV, Case TJ. 2002. Bottom-up effects on persistence  
733 of a specialist predator: Ant invasions and horned  
734 lizards. *Ecological Applications* **12**:291–298.

735 Suarez A, Yeh P, Case TJ. 2005 Impacts of Argentine ants on  
736 avian nesting success. *Insectes Sociaux* **52**:378–382.

737 Venables WN, Ripley BD. 2002. *Modern Applied Statistics with*  
738 *S*, Fourth Edition ed. Springer, New York.

739 Wells KD. 2010. *The ecology and behavior of amphibians*.  
740 University of Chicago Press.

741 Welzel KF, Lee SH, Dossey AT, Chauhan KR, Choe D-H. 2018.  
742 Verification of Argentine ant defensive compounds and  
743 their behavioral effects on heterospecific competitors  
744 and conspecific nestmates. *Scientific Reports* **8**:1477.

745

#### 746 **Data accessibility**

747 Data will be deposited in the institutional repository.

748

749 **Figure legends (in color for online version)**

750 **FIGURE 1.** Effects of ant contact and iridomyrmecin on  
751 juvenile amphibians. (a) Effects on juveniles of two  
752 amphibian species that spent 2-10 min in contact with ants  
753 on their trails in the field. (b) Effects on juveniles of  
754 three amphibian species that spent up to 10 min in contact  
755 with ants in the foraging arenas of laboratory nests. (c)  
756 Effects on *Pelobates cultripes* toadlets whose backs were  
757 rubbed with three different concentrations of iridomyrmecin  
758 (0.1, 1, or 5 mg/toadlet, equivalent to  $8.15 \pm 1.13$ ,  $67.86 \pm$   
759  $6.78$ , or  $307.62 \pm 30.30$  *Linepithema humile* workers/g of  
760 toadlet; mean  $\pm$  SE). Sample size is indicated in the center  
761 of each pie chart.

762

763 **FIGURE 2.** Dose-response curves for three amphibian species  
764 (ant numbers per g of juvenile amphibian): *Epidalea calamita*  
765 (red), *Pelobates cultripes* (blue), and *Hyla meridionalis*  
766 (green). (a) Numbers of amphibians that were affected (1) or  
767 unaffected (0) (normal or abnormal reactions observed during  
768 clinical evaluation, see Methods) 10 min after application  
769 of mashes of different numbers of ants of the invasive *L.*  
770 *humile* (straight lines/circles) or the native *Tapinoma* cf.  
771 *nigerrimum* (dashed lines/triangles). (b) Toxic dose,  
772 comprising the mean number  $\pm$  SE of ants (and the equivalent

773 amount in mg  $\pm$  SE of iridomyrmecin) per g of juvenile  
774 amphibian that elicited an effect. SE are only shown when  
775 meaningful. Equivalent amounts of iridomyrmecin were  
776 calculated using the species-specific contents:  $6.416 \pm$   
777  $0.443 \mu\text{g}$  for *L. humile* and  $1.291 \pm 1.127 \mu\text{g}$  for *T. cf.*  
778 *nigerrimum*.

779

780

781

782 **Figure 3. Effects of iridomyrmecin on toadlets and toadlet**

783 **tissues.** *Pelobates cultripes* toadlet with **(a)** an abnormal  
784 motor response (ataxia, paresis, and flaccidity) **and (b)**  
785 with skin ulcers (marked with **an** arrow). **(c)** Relationship  
786 between the dose applied (equivalent amounts of  
787 iridomyrmecin estimated from the number of *Linepithema*  
788 *humile* [in red] or *Tapinoma cf. nigerrimum* [in blue] applied  
789 to toadlets) and the concentration of iridomyrmecin measured  
790 in toadlet tissues after treatment. Model fit was determined  
791 using the combined data for all the amphibian species and  
792 ant species. **(d)** Unaffected liver. **(e)** Damaged liver with  
793 lymphoplasmocytic inflammatory infiltrates in the periportal  
794 space (circled). **(f)** Unaffected kidney. **(g)** Damaged kidney  
795 with acute tubulo-interstitial nephritis (marked with  
796 arrows).

797

798 **Figure 4. World map showing records of native and invasive**  
799 ***L. humile* populations** (native range in light blue). Point  
800 color reflects the number of co-occurring amphibian species  
801 (1:green to 86:red; 0: gray) based on spatial and  
802 macrohabitat overlap, and the pie charts show regional  
803 species richness (range of cumulative number of species for  
804 the full dataset [top] and for the microhabitat-filtered  
805 dataset [bottom]) and the proportion of species in non-  
806 threatened (black) and threatened (gray) IUCN Red List  
807 categories (for full dataset). The following regions were  
808 defined: the native range of *L. humile*, the rest of South  
809 America, North America (plus Japan), Europe (including  
810 European island colonies in North Africa), Sub-Saharan  
811 Africa, and Oceania. The bar charts for each region show the  
812 number of species that are vulnerable (VU), endangered (EN),  
813 and critically endangered (CR) for both the full (hashed)  
814 and microhabitat-filtered (filled) datasets.

815

816 **Figure legends (black and white for printed**  
817 **version)**

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837 10 min after application of mashes of different numbers of  
838 ants of the invasive *L. humile* (straight lines/circles) or  
839 the native *Tapinoma cf. nigerrimum* (dashed lines/triangles).

840 (b) Toxic dose, comprising the mean number  $\pm$  SE of ants (and  
841 the equivalent amount in mg  $\pm$  SE of iridomyrmecin) per g of  
842 juvenile amphibian that elicited an effect. SE are only  
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844 were calculated using the species-specific contents:  $6.416 \pm$   
845  $0.443 \mu\text{g}$  for *L. humile* and  $1.291 \pm 1.127 \mu\text{g}$  for *T. cf.*  
846 *nigerrimum*.

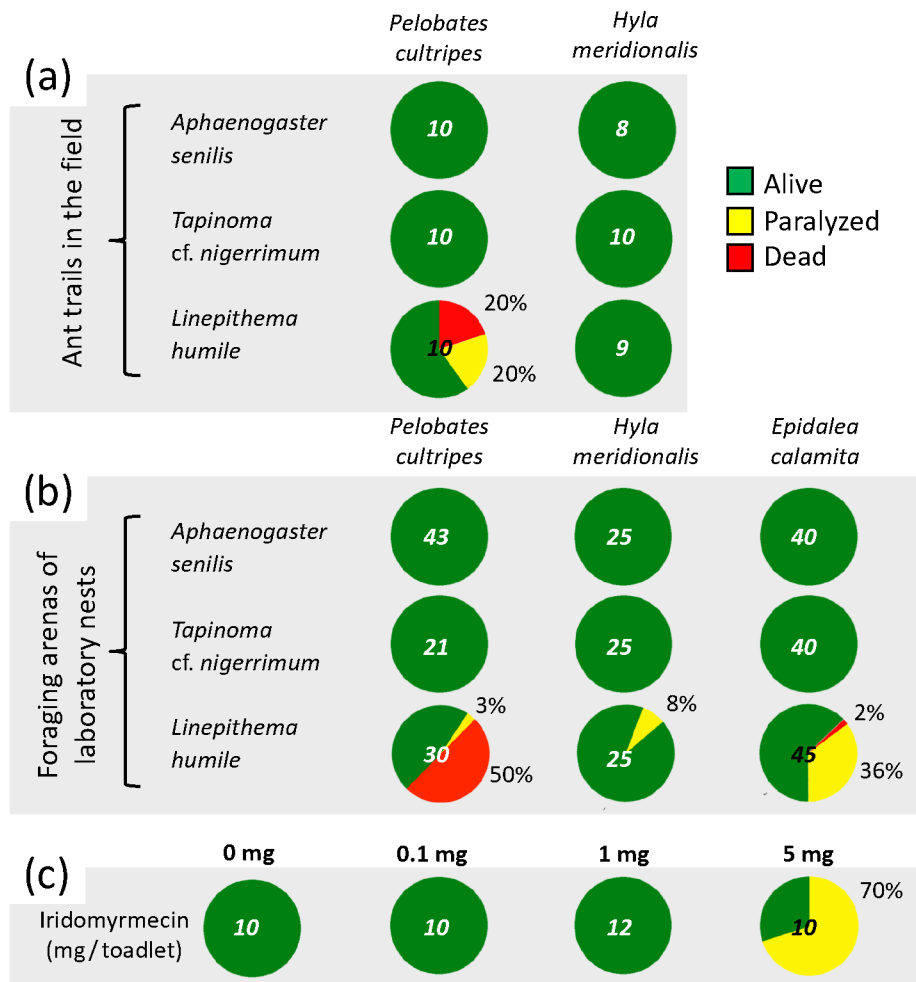
847

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861 Damaged kidney with acute tubulo-interstitial nephritis  
862 (marked with arrows).

863

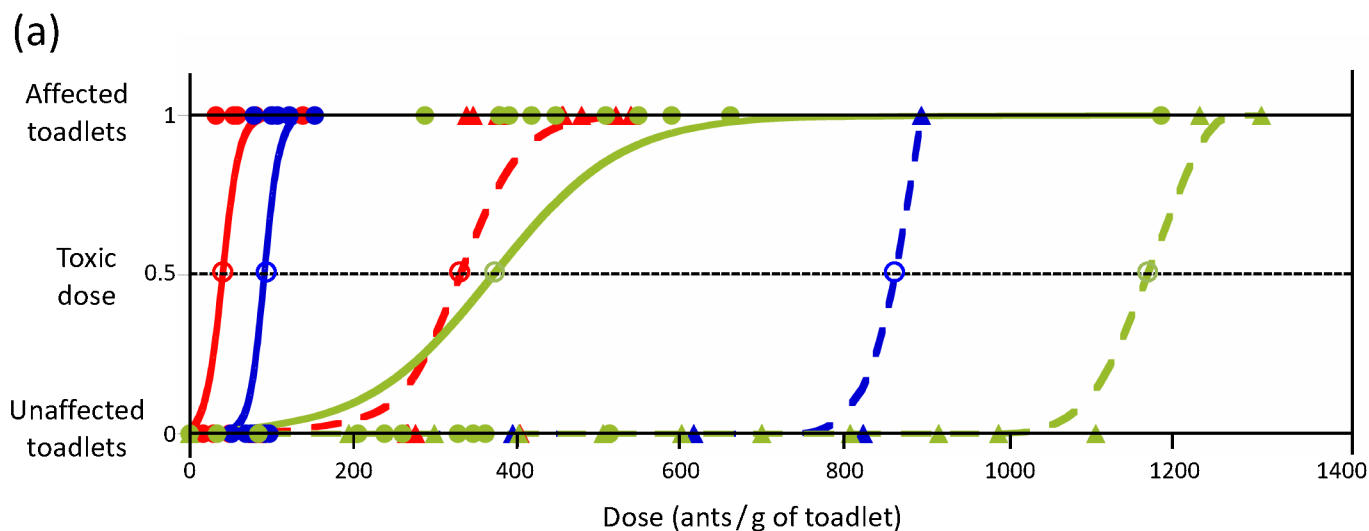
864 **Figure 4. World map showing records of native and invasive**  
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867 0:hollow, gray outline only) based on spatial and  
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870 the full dataset [top] and for the microhabitat-filtered  
871 dataset [bottom]) and the proportion of species in non-  
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879 and critically endangered (CR) for both the full (hashed)  
880 and microhabitat-filtered (filled) datasets.




881



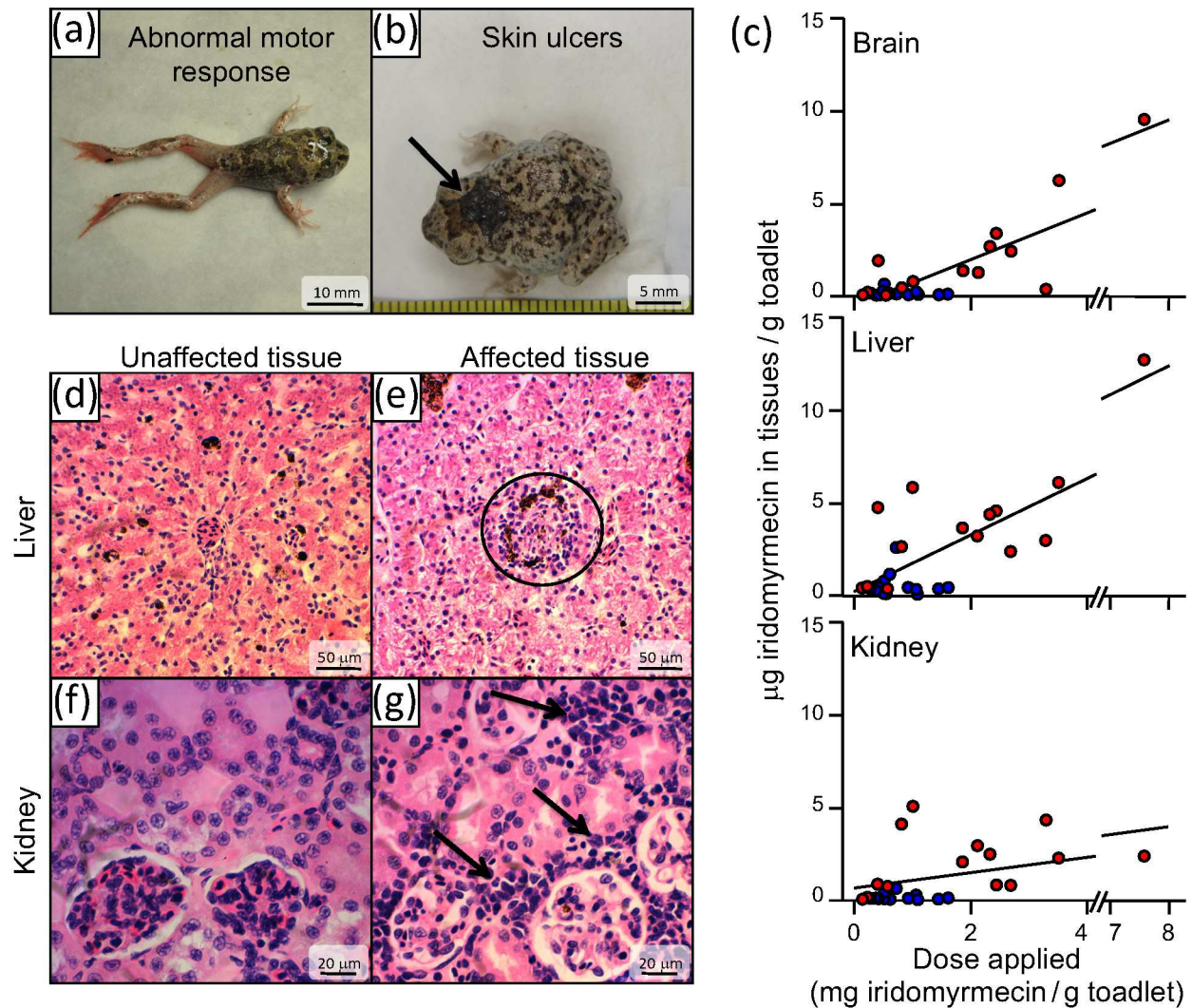
**Figure 1. Effects of ant contact and ant chemicals on juvenile amphibians.** (a) Effects on juveniles of two amphibian species that spent 2–10 min in contact with ants on ant trails in the field; (b) Effects on juveniles of three amphibian species that spent up to 10 min in contact with ants in the foraging arenas of laboratory nests; (c) Effects on *Pelobates cultripes* toadlets whose backs were rubbed with three different concentrations of iridomyrmecin (0.1, 1, or 5 mg/toadlet, which was equivalent to  $8.15 \pm 1.13$ ,  $67.86 \pm 6.78$ , or  $307.62 \pm 30.30$  *Linepithema humile* workers/g of toadlet; mean  $\pm$  SE). Sample size is indicated in the center of each pie chart.



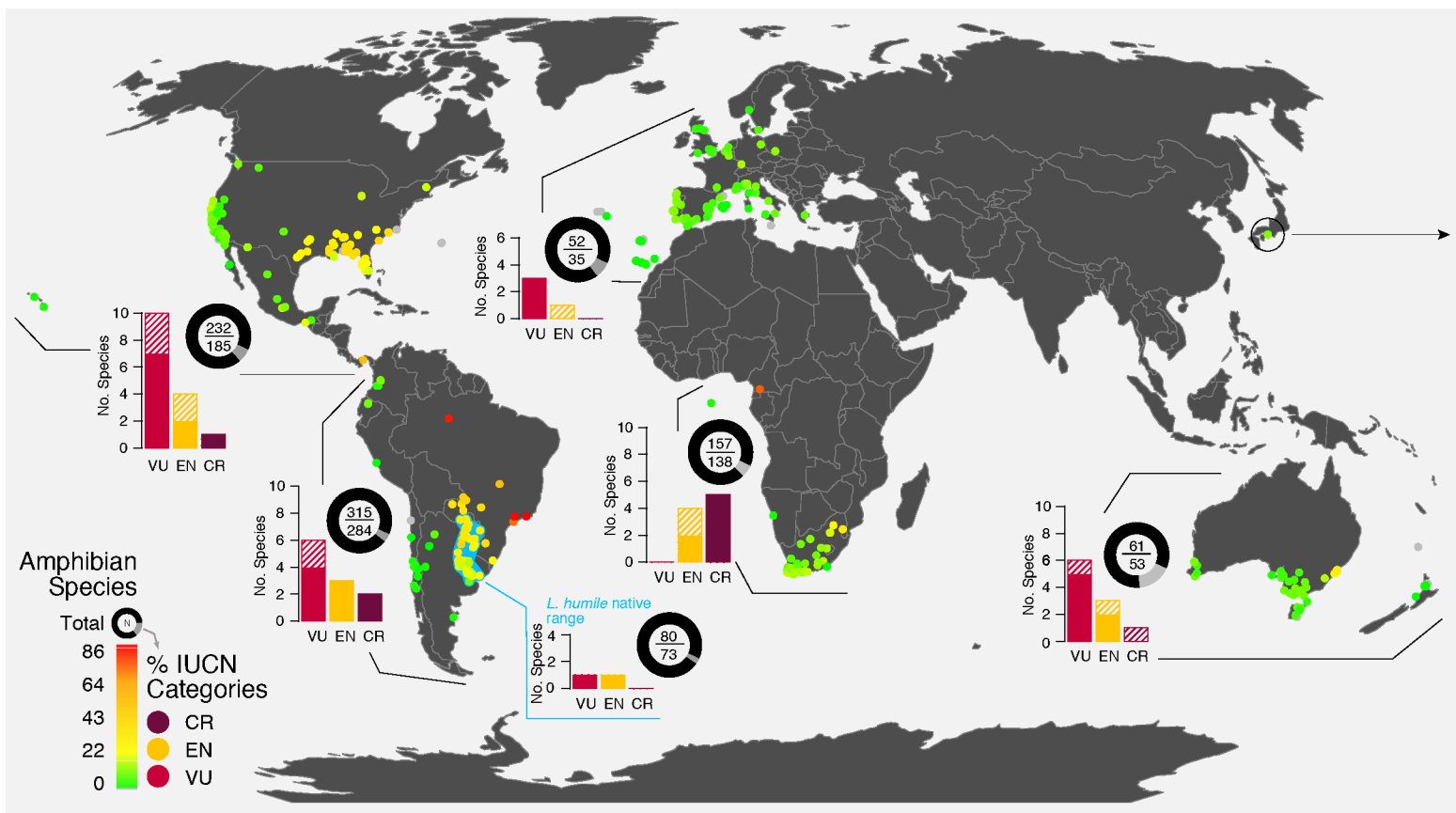


	TOXIC DOSE	Number of ants / g toadlet		mg iridomyrmecin / g toadlet	
		<i>L. humile</i>	<i>T. cf. nigerrimum</i>	<i>L. humile</i>	<i>T. cf. nigerrimum</i>
 <i>Epidalea calamita</i>		40.1 ± 9.5	328.0 ± 29.9	0.257 ± 0.061	0.423 ± 0.039
 <i>Pelobates cultripes</i>		90.6 ± 6.5	849.7	0.582 ± 0.042	1.097
 <i>Hyla meridionalis</i>		367.8 ± 44.5	1158.5	2.360 ± 0.285	1.496

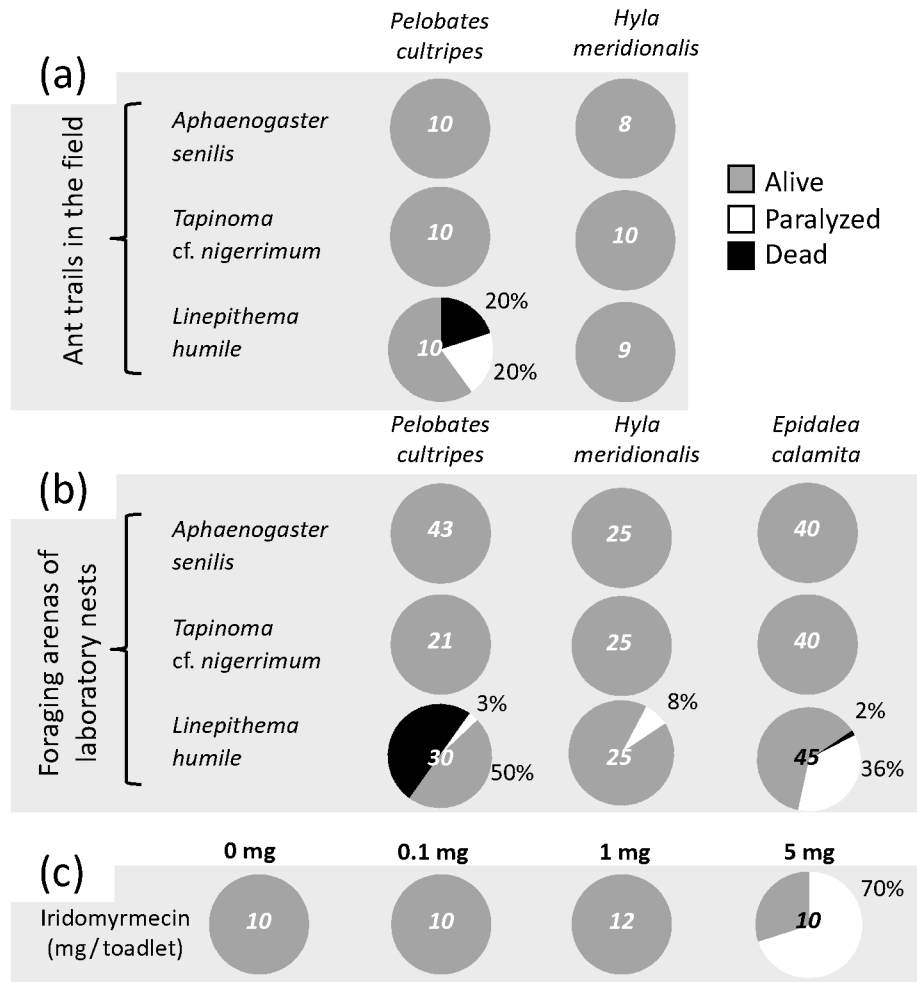
**Figure 2. Dose-response curves for three amphibian species (ant numbers per g of juvenile amphibian):** *Epidalea calamita* (red), *Pelobates cultripes* (blue), and *Hyla meridionalis* (green). **(a)**, Numbers of amphibians who were affected (1) or unaffected (0) (normal or abnormal reaction observed during clinical evaluation, see Methods) after spending 10 min in contact with a mash of different numbers of ants of the invasive *Linepithema humile* (straight lines/circles) or the native *Tapinoma cf. nigerrimum* (dashed lines/triangles). **(b)** Toxic dose, which was the mean number ± SE of ants (and the equivalent amount in mg ± SE of iridomyrmecin) per g of juvenile amphibian that elicited an effect. SE are only shown when meaningful. Equivalent amounts of iridomyrmecin were calculated using the species-specific contents: 6.416 ± 0.443 mg for *L. humile* and 1.291 ± 1.127 mg for *T. cf. nigerrimum*.



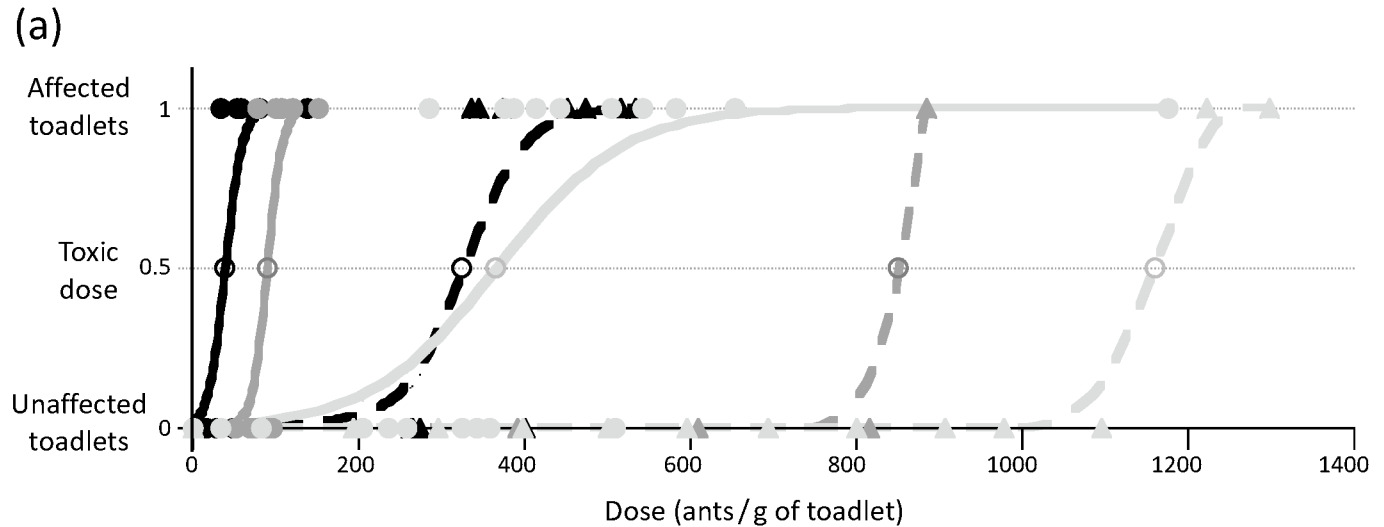
**Figure 3. Effects of iridomyrmecin on toadlets and toadlet tissues.** *Pelobates cultripes* toadlet with (a) an abnormal motor response (ataxia, paresis, and flaccidity) and (b) with skin ulcers (marked with an arrow). (c) Relationship between the dose applied (equivalent amounts of iridomyrmecin estimated from the number of *Linepithema humile* [in red] or *Tapinoma cf. nigerrimum* [in blue] applied to toadlets) and the concentration of iridomyrmecin measured in toadlet tissues after treatment. Model fit was determined using the combined data for all the amphibian species and ant species. (d) Unaffected liver. (e) Damaged liver with lymphoplasmocytic inflammatory infiltrates in the periportal space (circled). (f) Unaffected kidney. (g) Damaged kidney with acute tubulo-interstitial nephritis (marked with arrows).






**Figure 4. World map showing records of native and invasive *L. humile* populations** (native range in light blue). Point color reflects the number of co-occurring amphibian species (1:green to 86:red; 0: grey) based on spatial and macrohabitat overlap, and the pie charts show regional species richness (range of cumulative number of species for the full dataset [top] and for the microhabitat-filtered dataset [bottom]) and the proportion of species in non-threatened (black) and threatened (gray) IUCN Red List categories (for full dataset). The following regions were defined: the native range of *L. humile*, the rest of South America, North America (plus Japan), Europe (including European island colonies in North Africa), Sub-Saharan Africa, and Oceania. The bar charts for each region show the number of species that are vulnerable (VU), endangered (EN), and critically endangered (CR) for both the full (hashed) and microhabitat-filtered (filled) datasets.



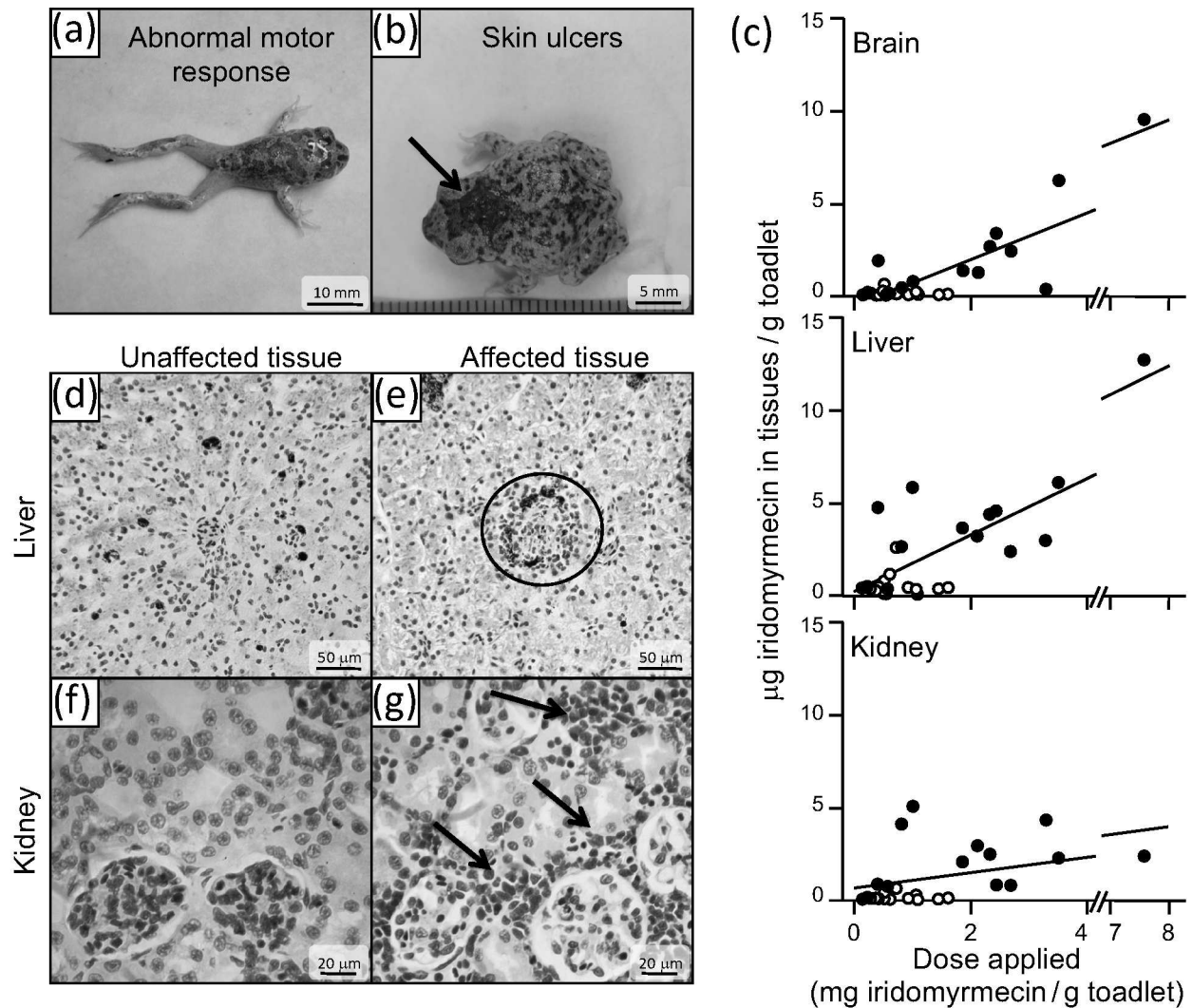
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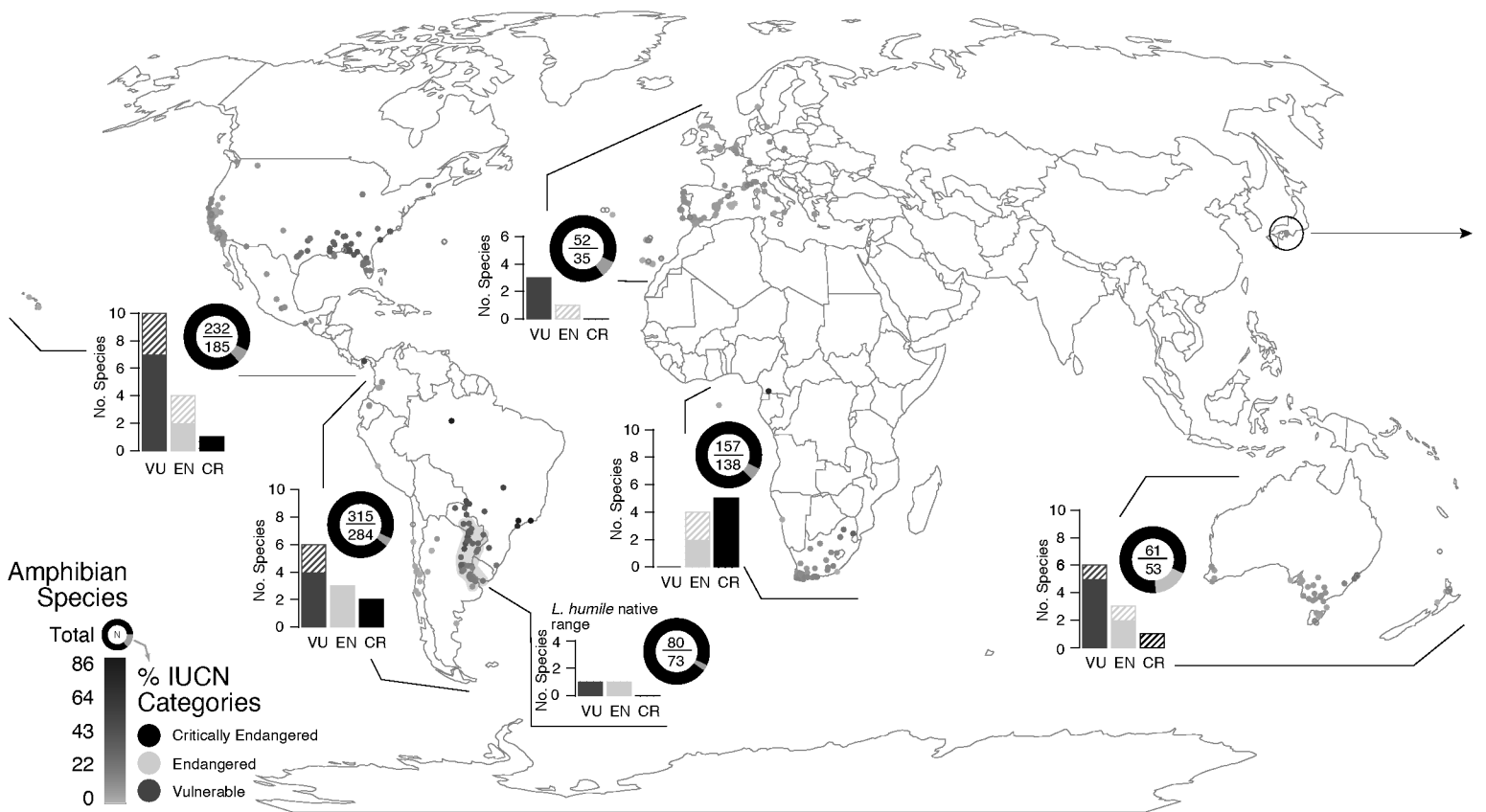
(b)

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**Figure 2. Dose-response curves for three amphibian species (ant numbers per g of juvenile amphibian):** *Epidalea calamita* (black), *Pelobates cultripes* (dark grey), and *Hyla meridionalis* (light grey). (a) Numbers of amphibians who were affected (1) or unaffected (0) (normal or abnormal reaction observed during clinical evaluation, see Methods) after spending 10 min in contact with a mash of different numbers of ants of the invasive *Linepithema humile* (straight lines/circles) or the native *Tapinoma cf. nigerrimum* (dashed lines/triangles). (b) Toxic dose, which was the mean number ± SE of ants (and the equivalent amount in mg ± SE of iridomyrmecin) per g of juvenile amphibian that elicited an effect. SE are only shown when meaningful. Equivalent amounts of iridomyrmecin were calculated using the species-specific contents: 6.416 ± 0.443 mg for *L. humile* and 1.291 ± 1.127 mg for *T. cf. nigerrimum*.



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**Figure 4. World map showing records of native and invasive *L. humile* populations.** Point color reflects the number of co-occurring amphibian species (1:white to 86:black; 0:hollow, gray outline only) based on spatial and macrohabitat overlap, and the pie charts show regional species richness (range of cumulative number of species for the full dataset [top] and for the microhabitat-filtered dataset [bottom]) and the proportion of species in non-threatened (black) and threatened (gray) IUCN Red List categories (for full dataset). The following regions were defined: the native range of *L. humile*, the rest of South America, North America (plus Japan), Europe (including European island colonies in North Africa), Sub-Saharan Africa, and Oceania. The bar charts for each region show the number of species that are vulnerable (VU), endangered (EN), and critically endangered (CR) for both the full (hashed) and microhabitat-filtered (filled) datasets.