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# Invasive predatory crayfish do not trigger inducible defences in tadpoles

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Invasive species cause deep impacts on ecosystems worldwide, contributing to the decline and extinction of indigenous species. Effective defences against native biological threats in indigenous species, whether structural or inducible, often seem inoperative against invasive species. Here, we show that tadpoles of the Iberian green frog detect chemical cues from indigenous predators (dragonfly nymphs) and respond by reducing their activity and developing an efficient defensive morphology against them (increased tail depth and pigmentation). Those defensive responses, however, were not activated against a highly damaging invasive predator (red swamp crayfish). Induced defences increased tadpole survival when faced against either indigenous dragonflies or invasive crayfish, so its inactivation in the presence of the invasive predator seems to be due to failure in cue recognition. Furthermore, we tested for local adaptation to the invasive predator by comparing individuals from ponds either exposed to or free from crayfish. In both cases, tadpoles failed to express inducible defences against crayfish, indicating that *ca* 30 years of contact with the invasive species (roughly 10–15 frog generations) have been insufficient for the evolution of recognition of invasive predator cues.

**Keywords:** biological invasions; phenotypic plasticity; cue recognition; dragonflies; red swamp crayfish; anti-predator defences

## 1. INTRODUCTION

Biological invasions constitute a major threat for biodiversity as they often cause deep impacts on ecosystem function and population declines of indigenous species worldwide [1–4]. Indigenous species possess a repertoire of adaptive defences to local pathogens, parasites, competitors, predators and/or herbivores as a result of ecological interactions over the course of evolution, but depending on the specificity of the defensive trait, such defences may not be useful against newly introduced species.

Phenotypic plasticity may be a key mechanism for an organism to withstand such rapid environmental changes as those imposed by an expanding invasive predator [5,6]. However, because inducible (plastic) defences critically depend upon recognizing predator cues reliably [7], indigenous species may fail to recognize cues from novel invasive species [8–11] and fail to activate inducible defences owing to a lack of joint evolutionary history [12,13]. Alternatively, the physico-chemical properties of the cues may be sufficiently general for organisms to co-opt existing sensory mechanisms adapted to detect indigenous predators into detecting invasive species. Moreover, even if inducible defences are triggered against novel invasive species, they might still be less efficient against the novel threat. The study of plastic traits in the context of biological invasions points towards some key questions of broad interest for species conservation: How often are inducible defences activated against invasive species? If activated, are they equally useful in

increasing fitness against invasive species than against indigenous ones? Can local populations adapt to invasive species quickly enough to avoid extinction?

Many amphibian larvae produce defensive phenotypes in the presence of predators consisting of increased relative tail depth, increased distal tail coloration, reduced overall coloration, reduced activity or various combinations of such responses [14–16]. These inducible defences increase tadpoles' odds of survival against predators either by avoiding being detected or by deflecting predator attacks towards the distal part of the tail and away from the vital organs in the body cavity [17]. Tadpoles from populations exposed to different compositions of predator guilds in nature respond differently to each kind of predator [18], suggesting that local adaptation of predator-specific detection and response evolves locally.

Invasive predators are one of the main factors contributing to amphibian declines worldwide [19,20]. Nonetheless, some amphibian populations display inducible defences against novel predators, based on either reduced activity [8,21,22] or altered morphology [23]. It is in most cases unknown, however, what timeframe is required for the evolution of local adaptation to invasive predators. In order to determine the efficacy of adaptive plastic responses against invasive species, it is important to: (i) assess the extent of phenotypic responses against indigenous and invasive species, (ii) compare the responses of populations or demes with different degrees of historical interaction with the invasive species, and (iii) test the fitness consequences of phenotypic changes (or lack thereof) against each type of predator. This last point is often overlooked, interpreting a lack of phenotypic change as evidence for

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failure in cue detection. It could be, however, that the phenotypic changes induced by indigenous predators were disadvantageous against novel predators, in which case lack of phenotypic response would be favoured despite predator recognition.

We tested the activation and efficiency of inducible defences in the Iberian green frog *Rana perezi* (= *Pelophylax perezi*) from Doñana National Park (southwest Spain) to either indigenous or invasive predators: dragonfly nymphs (*Anax imperator*) and red swamp crayfish (*Procambarus clarkii*), respectively. The red swamp crayfish is among the most damaging invasive predator in aquatic systems, being a voracious predator of amphibian eggs and tadpoles that has already caused great harm to amphibian assemblages throughout the world [24–26]. It has been argued that the greater the danger a predator poses, the greater should be the prey's ability to generalize predator recognition [27]. This generalization of detection capability to novel predator species, however, gets dampened with increasing phylogenetic distance among predator species [28], and no indigenous crayfish species have been known in the Doñana area. The red swamp crayfish was introduced in the surroundings of Doñana National Park in 1974, and it has since unevenly expanded across marshy areas and into ponds throughout the park [29], although it has not reached the southernmost part of the park. *Rana perezi* is widely extended throughout the park, including a number of isolated ponds to the south that remain free of invasive crayfish. Thus, we compared tadpoles coming from ponds in areas either exposed or naive to invasive crayfish in order to test for local adaptation to the invasive predator. We expected individuals from areas of the park with crayfish and without it to detect and respond to dragonfly nymphs equally because they are indigenous and common, whereas we hypothesized that only individuals from ponds exposed to the invasive crayfish would detect it and respond accordingly. Because tadpoles perceive both predator cues (kairomones) and alarm pheromones emitted by injured prey [28,30], which often act synergistically in triggering tadpoles' induced defences, we exposed *R. perezi* tadpoles to either starved predators or predators fed conspecific tadpoles to distinguish between the tadpole's ability to perceive the predator's presence from the combined effects of predator cues plus injured tadpoles' alarm cues.

## 2. MATERIAL AND METHODS

We conducted two common garden experiments to study the response of *R. perezi* tadpoles to cues from indigenous and from invasive predators. In the first experiment, we analysed predator-induced morphology in tadpoles raised in the presence or absence of caged predators, whereas in the second, we analysed tadpole behavioural responses to water-borne predator cues. In both experiments, we used five treatments: (i) no predator, (ii) unfed dragonfly, (iii) dragonfly fed *R. perezi* tadpoles, (iv) unfed crayfish, and (v) crayfish fed *R. perezi* tadpoles. We applied these treatments to tadpoles from two different areas of Doñana National Park: either exposed to or free from crayfish (ponds in the north or the south of the park, respectively). The northern pond did contain crayfish, whereas both ponds contained aeshnid dragonflies.

### (a) Analysis of morphological inducible defences

We collected four egg clutches from each population and brought them into the laboratory to minimize the risk of exposure to predator cues. We raised tadpoles individually in 4 l plastic buckets with dechlorinated tap water at 20°C and under a 12 L:12 D photoperiod in a climatic walk-in chamber at Estación Biológica de Doñana. Tadpoles were fed 600 mg rabbit chow per week, and water was renewed twice weekly.

All containers had a cage suspended on the side consisting of a 250 ml plastic cup with a mesh bottom. We held eight replicates per treatment combination (two populations of origin  $\times$  five predator treatments, 80 experimental units in total). We allocated two replicates per treatment in each of four shelves in the chamber and considered each shelf a random block. Depending on the treatment, these cups were empty (control) or held either a dragonfly nymph (*A. imperator*) or a red swamp crayfish (*P. clarkii*). Dragonflies were substantially smaller than crayfish, so predator presence was not corrected for predator biomass and the amount of chemical stimulus was probably unbalanced in favour of the invasive predator. Predator cues last approximately 2–4 days [31], so predators in 'fed predator' treatments were fed two *R. perezi* tadpoles from a stock tank every third day. Two sets of predators were cycled in the 'unfed predator' treatments so that any given individual predator starved for a week within the experimental containers while others were being fed in additional containers. Predators were then switched between the housing and experimental containers. Survival was high (91%) and did not vary among rearing treatments ( $\chi^2 = 5.01$ , d.f. = 4,  $p = 0.286$ ). After one month, we photographed each tadpole twice using a Plexiglas tadpole photo booth with a sliding back wall covered with either a 1 mm grid or a white background. We took six standard morphological measurements (total length, body length, body depth, tail length, tail depth and tail muscle depth) from the pictures taken with the scaled background, whereas we used those with a white background to estimate their minimum brightness value as an estimate of tail fin pigmentation. We obtained such values by decomposing each image into an HSB stack (hue, saturation, brightness), delimiting the tail fin area and recording the minimum brightness score. Such scores range from 0 (black) to 256 (white), and were transformed into a percentage (blackness =  $(1 - (\text{brightness}/256)) \times 100$ ) for ease of comparison among treatments. We used general linear models to analyse both morphology (using total length as covariate) and pigmentation. We conducted image analyses in IMAGEJ (National Institutes of Health, USA) and statistical analyses in SAS v. 9.1. (SAS Institute Inc., USA).

### (b) Analysis of tadpole behavioural response to predators

We collected tadpoles in Gosner stages 30–35 [32] from each area of the park (areas with and without crayfish) and maintained them for two weeks at low densities in four housing tanks per area. Tadpoles could thus have had previous encounters with predators. Therefore, if tadpoles exhibited behavioural response against non-indigenous crayfish, we could not distinguish here whether it was a learned behaviour or an innate one, and hence we would be unable to claim adaptive population divergence in predator recognition abilities. Failure to alter their behaviour in the presence of crayfish despite possible previous exposure to crayfish,

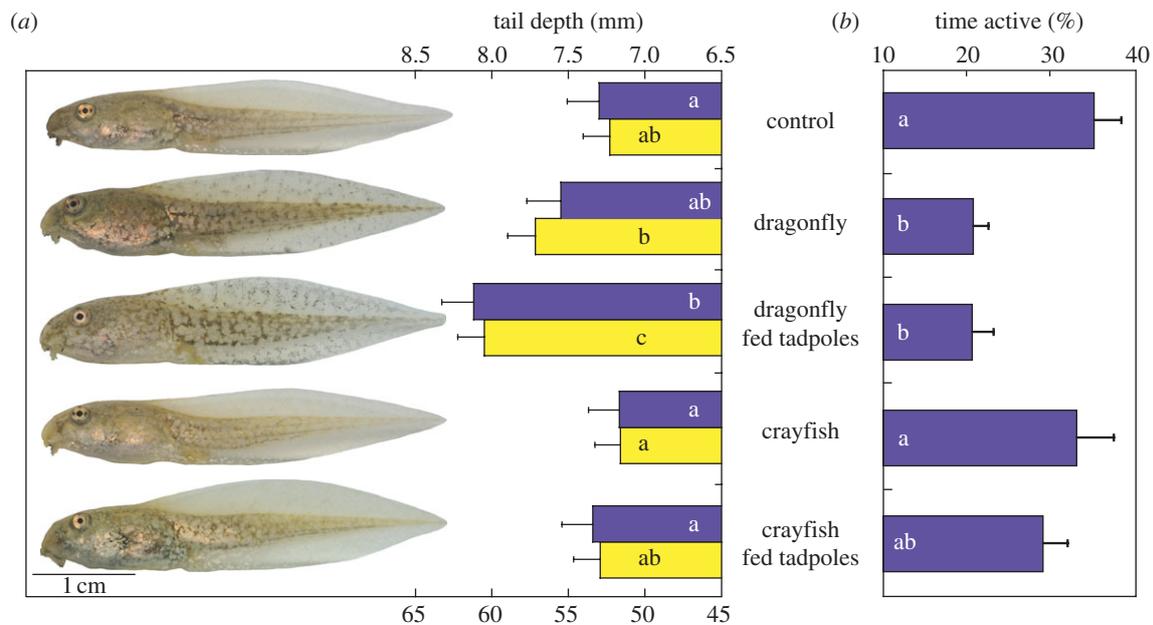


Figure 1. Morphological and behavioural induced responses of *R. perezi* tadpoles to the presence of indigenous or invasive predators (dragonflies or crayfish, respectively) either fed or unfed conspecific tadpoles. (a) Yellow bars show least-squares means for relative tail depth (mm) (correcting for total tadpole length). Purple bars show the amount of pigmentation as the extent of blackness (%). (b) Proportion of time spent active (swimming or foraging) in 20 min focal analyses. Data from both populations studied are here pooled together. In all cases, error bars indicate +s.e. Different letters on the bars within each variable indicate statistical significance of differences of least-squares means (at  $\alpha = 0.05$ ).

however, would strongly suggest that tadpoles do not detect non-indigenous crayfish.

To test for differences in the proportion of time that tadpoles spent active, we used focal sampling of individual tadpoles presented with either clean water or water from containers holding predators: fed or unfed dragonfly larvae or crayfish. To obtain the different chemical stimuli, we filled three buckets of each of five treatments with 21 of clean water and left overnight. Unfed predator tanks held either four dragonflies or one crayfish, whereas in fed predator tanks, we added three *R. perezi* tadpoles. All tadpoles were consumed overnight, presumably adding alarm cues to the predator kairomones present in the water. Water from all three buckets per treatment was mixed and 10 ml aliquots were frozen until use [11]. Tadpoles were fed a combination of rabbit chow and macrophytes from ponds without crayfish. For behavioural trials, tadpoles were placed individually in buckets with 21 of clean water and left acclimating for 10 min. We then randomly added a frozen aliquot from one of the five treatments, waited for it to thaw and recorded for 20 min the time the tadpole spent active (swimming and/or feeding) with JWATCHEX v. 9.0 [33]. We conducted 10 and 9 behavioural assays per treatment for areas of the park with and without crayfish, respectively, for a total of 95 individual tadpoles scored. Observers were unaware of the treatment being given to tadpoles at each trial, and all trials were conducted between 10.00 and 14.00 h.

### (c) Test of efficacy of induced defences

We tested the efficacy of tadpoles' induced responses against indigenous and invasive predators by conducting predation trials in which we presented individual free-ranging dragonflies or crayfish with pairs of tadpoles, one control (raised in the absence of predators) and one raised in the non-lethal presence of a tadpole-fed dragonfly. Trials were hence conducted with pairs of tadpoles showing the largest

possible phenotypic differences (figure 1). We conducted 15 trials per predator species, each conducted in a 4 l bucket and lasting for 24 h, after which we recorded the surviving tadpoles and the treatment to which they belonged. Tadpoles were easily recognized owing to the large phenotypic differences between them.

### (d) Spatial overlap between predators and tadpoles

We tested for geographical association between *R. perezi* and each of the predator species studied using presence/absence data from field surveys. We hypothesized that a negative association between tadpoles and aeshnid dragonflies or crayfish would be indicative of predator avoidance or tadpole exclusion, whereas a positive association would indicate similar habitat preferences. Between 2003 and 2008, 151 ponds were surveyed for *R. perezi*, aeshnid dragonflies and red swamp crayfish. Detectability in temporary ponds varies considerably among species and across years, potentially yielding a high incidence of false absences [34]. Therefore, we used a conservative approach where we only used data from ponds sampled 3 or more consecutive years ( $n = 74$ ), and recorded a species as present if it was found in a pond at anytime during the 3 year span. *Rana perezi* was present in 78.4 per cent of these 74 ponds, whereas indigenous dragonflies and crayfish were found in 35.1 and 51.4 per cent of the ponds, respectively. None of the geographical distributions showed spatial autocorrelation (Moran's  $I = -0.013$ ,  $p = 0.27$ ;  $I = -0.013$ ,  $p = 0.36$ ;  $I = -0.013$ ,  $p = 0.35$ , respectively, for *R. perezi*, crayfish and aeshnid dragonflies).

## 3. RESULTS

### (a) Lack of anti-predator morphology or reduced activity against crayfish

Relative tail depth and tail fin blackness varied significantly across predator presence treatments (figure 1a

Table 1. Results from general linear models on tadpole responses to experimental exposure to predator cues: relative tail height (controlling for total tadpole size), extent of tail pigmentation (% blackness) and proportion of time spent active. Treatments manipulated the presence/absence of two types of predators, indigenous dragonfly larvae or invasive crayfish, each presented either fed or unfed with conspecific tadpoles. Morphological traits were studied using caged predators, whereas activity was studied using water-borne cues. 'Population' refers to a comparison between two areas of Doñana National Park, one infested with crayfish and the other free of it.

source	tail height (mm)				tail blackness (%)				activity (%)			
	d.f.	MS	<i>F</i>	<i>p</i> -value	d.f.	MS	<i>F</i>	<i>p</i> -value	d.f.	MS	<i>F</i>	<i>p</i> -value
treatment	4,66	1.71	5.96	<0.001	4,67	1388.99	3.33	0.015	4,85	0.087	4.80	0.001
population	1,66	1.63	5.69	0.020	1,67	29.26	0.07	0.794	1,85	0.092	5.08	0.027
treat × pop	4,66	0.13	0.46	0.767	4,67	310.36	0.73	0.573	4,85	0.001	0.07	0.990
tadpole length	1,66	87.34	304.23	0.001								

and table 1). Experimental treatments had no effect on tadpole total length ( $F_{4,66} = 0.55$ ,  $p = 0.69$ ), but in the presence of dragonflies, tadpoles averaged an increase in relative tail depth with respect to control tadpoles of 7 and 11 per cent for starved and fed dragonflies, respectively, although only the latter was significant according to differences in least-squares means (starved dragonflies  $p = 0.13$ , statistical power = 43%; fed dragonflies  $p = 0.003$ ). Tadpoles in the presence of starved dragonflies did however have a significantly deeper tail than those in the presence of starved crayfish ( $p = 0.004$ ). Tadpoles also increased their tail fin pigmentation in the presence of fed dragonflies, averaging a 10 per cent increase with respect to the controls in the form of evenly distributed blotches (figure 1a). However, we observed no phenotypic response to the presence of red swamp crayfish, even when these were feeding on *R. perezii* tadpoles. Behavioural responses to the presence of predators were significant and largely concordant with induced morphological changes ( $F_{4,85} = 4.80$ ,  $p = 0.001$ ; figure 1b). Tadpoles significantly reduced the time spent active when exposed to dragonfly cues, whether the dragonflies were fed *R. perezii* tadpoles or not (a 22% reduction relative to the control in both cases), but not when exposed to crayfish cues (figure 1b and table 1). We observed a noticeable reduction in activity in response to tadpole-fed crayfish cues, as this treatment was marginally non-significantly different from the starved and fed dragonfly treatments ( $p = 0.058$  and  $p = 0.056$ , respectively).

Populations differed slightly in average tail height (2.4%) and proportion of time spent active (8%). Nevertheless, both naive and crayfish-exposed populations showed similar responses to indigenous predators and the same lack of response to invasive predators, as indicated by the lack of significance of the 'population × predator treatment' interaction across all traits studied, both morphological and behavioural.

#### (b) Higher survival of predator-induced tadpoles

Survival in real predation trials varied significantly between control tadpoles and dragonfly-induced tadpoles. Deep-and-pigmented tailed tadpoles experienced 80 per cent survival whereas only one tadpole with shallow-and-clear tail survived in trials with dragonflies ( $\chi^2 = 16.43$ ,  $n = 15$ ,  $p < 0.001$ ; figure 2). Likewise, tadpoles with deep-and-pigmented tails experienced 60 per cent survival in predation trials with crayfish, whereas

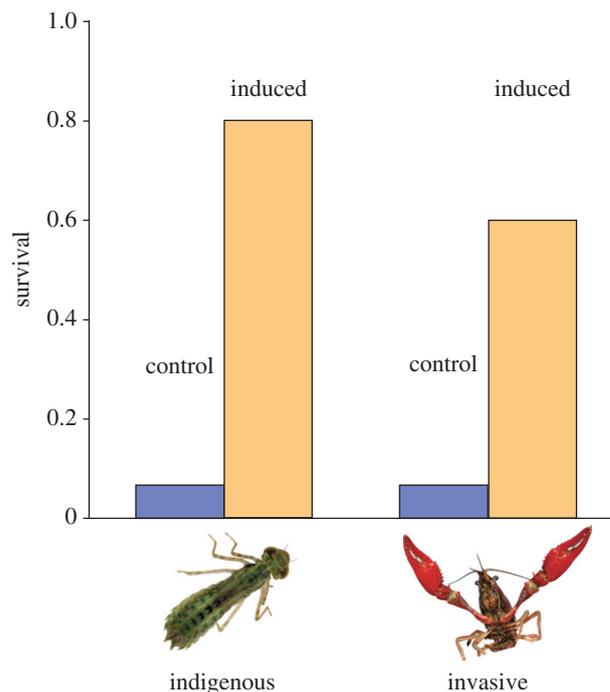


Figure 2. Tadpole survival over 24 h predation trials. Pairs of dragonfly-induced and control tadpoles were exposed to either dragonflies or crayfish ( $n = 15$  trials per predator type). Regardless of the predator, dragonfly-exposed tadpoles (less active, with deeper and more pigmented tails) survived significantly better.

survival was only 7 per cent for shallow-and-clear tailed tadpoles ( $\chi^2 = 9.60$ ,  $n = 15$ ,  $p = 0.002$ ; figure 2).

#### (c) Similar habitat preferences of *R. perezii* and crayfish

Regarding their spatial distribution, we detected a positive and significant geographical association between *R. perezii* and *P. clarkii* ( $n = 74$ ,  $\chi^2 = 14.19$ ,  $p = 0.0002$ ), indicating that both species tend to co-occur. In turn, *R. perezii* showed no significant association with dragonflies ( $n = 74$ ,  $\chi^2 = 0.92$ ,  $p = 0.34$ ).

## 4. DISCUSSION

Tadpoles of the Iberian green frog responded to indigenous dragonflies by developing a deeper, more heavily pigmented tail and reducing their level of activity. However, they failed to develop such anti-predator defences

in the presence of the invasive red swamp crayfish (*P. clarkii*). The red swamp crayfish is a voracious tadpole predator that is causing a deep impact on Iberian amphibian assemblages [35,36]. In Doñana National Park, red swamp crayfish are mainly found in the marsh and neighbouring ponds, mostly long-lasting or permanent, which are also the typical breeding sites of *R. perezi*. Field surveys confirm a high degree of overlap of both species, even if crayfish have not been able to colonize suitable ponds to the south of the park where *R. perezi* breeds. This geographical overlap suggests a high potential impact of the invasive crayfish over *R. perezi*. The association with dragonflies was not significant, so despite their extensive overlap with *R. perezi*, we observed no indication of similar habitat preferences. Thus, pond association between *R. perezi* and the invasive predator is at least as big as with the native predator; and breeding phenology of *R. perezi* is late enough in the season [37] that large crayfish are guaranteed to be present. Hence, we can rule out low encounter probability as a plausible explanation for lack of phenotypic response against crayfish.

Different predators, however, can select for different optimal defensive phenotypes. Some amphibian species or populations alter their phenotype to different extents and even in opposite directions depending on the predator guilds they are naturally exposed to [18,38–40]. In particular, hour-glass frog tadpoles produce a shallow, clear-finned phenotype in the presence of fish but a deep tail with a heavily pigmented tip in the presence of dragonfly nymphs [40]. Dragonfly nymphs are ambush predators, whereas crayfish actively seek and chase *R. perezi* tadpoles much as some fishes would do, so inactive tadpoles could be equally or at greater risk than active ones. Nonetheless, our predation trials confirmed that less active tadpoles with deeper and more pigmented tails as expressed in the presence of indigenous dragonflies also survive better in the presence of crayfish. This result discards the possibility that a shallow and clear-finned phenotype could be advantageous against crayfish and supports that *R. perezi* tadpoles simply do not recognize the cues from the invasive crayfish.

Moreover, we observed no differences between areas of the park exposed to and free from invasive crayfish in their phenotypic response against either type of predator. It then follows that no local adaptation in the cue recognition system has yet evolved to allow detection of the crayfish. This may be unsurprising because the time since invasive crayfish irrupted in the park is still short, just about 30 years. Nevertheless, although this time translates into only 10–15 *R. perezi* generations, it seems as if such adaptation would only require a seemingly simple co-option of an already existing inducible defence mechanism, highly efficient against indigenous predators. Hence, there is likely to be a complete new set of cues associated with red swamp crayfish to which tadpoles need to adapt de novo. Few other systems have been able to test for plastic responses against introduced predators in the context of a relatively well-dated introduction event, but we are now beginning to have an idea about the timeframe required for adaptive recognition of novel predator cues in amphibians. Thus, *Rana aurora* populations that have been syntopic with the introduced predatory tadpoles of *Rana catesbeiana*

for about 60 years reduce their activity in the presence of the latter, whereas allotopic populations do not [8]. Also, *R. catesbeiana* tadpoles introduced to the Pacific northwest ca 110 years ago already respond to reddsideshiners (*Richardsonius balteatus*) and northern pikeminnows (*Ptychocheilus oregonensis*), local fish predators and hence novel to *R. catesbeiana*. Furthermore, Mallorcan midwife toads reduce activity in response to snakes (*Natrix maura*) introduced to the island ca 2000 years ago, but not to conspecific snakes from populations in continental Spain [41]. Midwife toad tadpoles also alter tail depth and body length in response to the snakes [23]. In view of these and our own results, it is tempting to speculate whether amphibian behavioural responses to novel predators could evolve faster (on the range of decades) than morphological responses (which could take hundreds or a few thousand years). We need, however, a better functional understanding of the cue-recognition system and the developmental response, as in many cases both behavioural and morphological responses could be orchestrated by the same regulatory pathways.

Invasive species are a global threat to biodiversity. Here we show that their disruptive potential may be in part due to indigenous species' inability to detect invasive species cues and their concomitant failure to express inducible defences that would otherwise contribute to reduce mortality. This effect is due to a lack of joint evolutionary history with the invasive species, and it could be attenuated through local adaptation if the indigenous species harboured enough genetic variation, held large-enough populations and the generation time of indigenous species could keep up with that of the invasive species, allowing for adaptive recognition of non-indigenous cues to evolve [12,42,43]. Moreover, there could be two other factors contributing to compensate the lack of recognition of a novel predator. First, native predators would be inducing behavioural and morphological responses in tadpoles, especially if conspecific tadpoles are being consumed, that seem to have the potential to also improve survivorship against the novel predators, as in our system. Hence, induction by native predators could have some degree of trait-mediated indirect effect on survivorship against the novel, unrecognized predator [44], especially given that dragonflies and crayfish co-occur in 16.6 per cent of *R. perezi* breeding ponds in Doñana (C.D.-P., 2011, unpublished data). Second, *R. perezi* tadpoles have been shown to learn to recognize new predators from their association with attacked conspecifics' alarm cues, behaviourally responding to the new threat and remembering such association for several days [45,46]. Whether these mechanisms can buy time for adaptive recognition of the novel predator to evolve or not may largely determine the likelihood of invasive predators driving local populations to extinction. Our results highlight the importance of considering the role of phenotypic plasticity in the study of biological invasions, as it can provide functional explanations for the outcome of interactions between invasive and indigenous species.

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