Visual recognition and coevolutionary history drive responses of amphibians to an invasive predator

Abstract

During biotic invasions, native prey are abruptly exposed to novel predators and are faced with unprecedented predatory pressures. Under these circumstances, the lack of common evolutionary history may hamper predator recognition by native prey, undermining the expression of effective anti-predatory responses. Nonetheless, mechanisms allowing prey to overcome evolutionary naïveté exist. For instance, in naïve prey history of coevolution with similar native predators or recognition of general traits characterizing predators can favor recognition of stimuli released by invasive predators. However, few studies assessed how these mechanisms shape prey response at the community level. Here, we evaluated behavioral responses in naïve larvae of 13 amphibian species to chemical and visual cues associated with an invasive predator, the American red swamp crayfish (*Procambarus clarkii*). Moreover, we investigated how variation among species responses was related to their coexistence with a similar native crayfish predator. Amphibian larvae altered their behavior in presence of visual stimuli of the alien crayfish, while chemical cues elicited feeble and contrasting behavioral shifts. Activity reduction was the most common and stronger response, whereas in some species we detected more heterogeneous strategies also involving distancing and rapid escape response. Interestingly, species sharing coevolutionary history with the native crayfish were able to finely tune their response to the invasive one, performing bursts to escape. These results suggest native prey can respond to invasive predators through recognition of generic risk cues (e.g., approaching large shapes), still the capability of modulating anti-predator strategies may also depend on their coevolutionary history with similar native predators.

Keywords: amphibian community, anti-predator behavior, history of coexistence, invasive species, predator recognition, prey naïveté

Introduction

Biotic invasions are increasingly shaping ecosystems at the global scale and constitute one of the major drivers of biodiversity loss (Mooney and Cleland 2001, Clavero and Garcia-Berthou 2005, Bellard et al. 2016). Invasive predators have severe impacts on invaded ecosystems, often leading to sharp declines and local extinction of native prey populations worldwide (Rodda et al. 1997, Kats and Ferrer 2003, Salo et al. 2007, Cruz et al. 2008, Doherty et al. 2016), as they expose native species to novel and abrupt predation pressures (Cox and Lima 2006, Sih et al. 2010, Carthey and Banks 2014). Under these circumstances, behavioral responses of native species can be extremely important, as they can constitute a first line of defense for native species towards invasive ones (Holway and Suarez 1999, Weis and Sol 2016). Correct risk assessment is crucial for prey as it is required to foster effective anti-predator responses and finely tune their expression according to the perceived risk (Lima and Dill 1990, Lima and Bednekoff 1999, Ferrari and Chivers 2011). Predator recognition can be mediated by a wide variety of stimuli (Lima and Dill 1990), which depend on the ecological context wherein prey species have evolved, and is favored by the presence of a history of coevolution between predator and prey (Downes and Shine 1998, Sih et al. 2010). Thus, when a non-native predator invades an ecosystem, crucial questions arise on prey capability to withstand the novel threat. How naïve prey respond to the new threat and how responses vary across native prey community? Which mechanisms can favor novel predator recognition?

Native prey can fail to perceive invasive predators as a potential threat or fail to associate cues they release to predation risk, and this generally hampers the expression of adequate anti-predator responses (Salo et al. 2007, Gomez-Mestre and Díaz-Paniagua 2011). Failed predator recognition in native prey is often attributed to the lack of common evolutionary history with the invasive species (Cox and Lima 2006, Sih et al. 2010). This lack of responsiveness due to absence of coevolutionary history is known as evolutionary naïveté (Carthey and Banks 2014, Carthey and Blumstein 2018). However, mechanisms allowing to overcome evolutionary naïveté in prey exist (Cox and Lima 2006, Carthey and Banks 2014) and in some cases native prey can recognize novel predators. On the one hand, when the invasive predator is phylogenetically close or shares similar traits with a native predator, prey can recognize predator archetype and broaden their anti-predator response to the novel species (predator generalization hypothesis) (Griffin et al. 2001, Ferrari et al. 2007, Davis et al. 2012). In other cases, a novel species can be "labelled" as predator by naïve prey when it shares traits that are commonly associated to predator species (e.g., large size; stealthy approaching) (Carthey and Blumstein 2018), inducing a generic anti-predator response in prey (generic response hypothesis) (Mathis and Vincent 2000, Rehage et al. 2009, Wilson et al. 2018). While generic responses are commonly triggered by visual cues (Mathis and Vincent 2000), predator generalization can involve chemical stimuli, as phylogenetically close predators tend to produce similar kairomones (i.e., chemical cues) that can be recognized by native prey (Ferrari et al. 2007, Davis et al. 2012). Finally, it is worth to note that prey response to novel predators may also be modulated by other mechanisms, such as learning through the association of novel stimuli to familiar risk cues (Gonzalo et al. 2007, Nunes et al. 2013) or neophobia (Chivers et al. 2014).

Freshwater systems are closely connected habitats that are highly vulnerable to disturbances at network scale, such as damming and fragmentation, and are strongly exposed to invasive species (Leprieur et al. 2008, Strayer 2010). In these habitats, native prey naïveté to introduced predators can thus be particularly frequent (Cox and Lima 2006, Rehage et al. 2009). In aquatic environments, visual stimuli and chemical communication are major cues used by prey for risk assessment (Chivers et al. 2001, Wisenden 2003, Ferrari et al. 2010c, Hettyey et al. 2012). Visual cues primarily allow to locate predators and are involved in rapid predator avoidance (Hettyey et al. 2012), but they can also contribute to refine risk assessment and discriminate between predators actually constituting a threat and non-threating predators (e.g., by assessing predator size) (Chivers et al. 2001). Nonetheless, freshwater environments frequently have poor visibility (e.g., turbid or densely vegetated water), thus visual recognition is often useful only at short distances and cannot prevent predator encounter (Abrahams and Kattenfeld 1997, Ferrari et al. 2010b). Conversely, chemical cues can be perceived before encountering the predator and can elicit anti-predatory responses aimed at preventing exposure to predators (Kats and Dill 1998). Furthermore, chemical stimuli can also provide information on predator diet and density (Schoeppner and Relyea 2005, 2008), allowing to finely tune anti-predator response on the basis of actual predation risk (Benard 2006).

Anti-predator responses against novel predators can have key consequences on the dynamics of invaded communities. Native species recognizing invasive predators as a threat can exhibit more effective anti-predator responses, and this could increase their ability to withstand the impact of invaders. However, understanding inter-specific variation of anti-predator responses can be challenging, because it requires the comparison of a large number of species, potential stimuli and potential responses. As a consequence, very few studies have so far assessed the anti-predator responses to invasive predators at the community level (but see (Rebelo and Cruz 2005, Nunes et al. 2013, Nunes et al. 2014a)).

Here we investigated the capability to recognize a non-native predator and express behavioral responses, across the 13 species composing the amphibian communities of freshwaters in Northern Italy. During behavioral tests, we monitored variation of activity and space use in naïve amphibian larvae exposed to a combination of visual and chemical stimuli from an invasive predator, the red swamp crayfish *Procambarus clarkii* (hereafter American crayfish), which is a major threat to freshwater biodiversity (Nentwig et al. 2018). In so doing, we aimed to assess (i) how the response to the alien predator varies among species; (ii) what is the relative role of predator-released stimuli (i.e., visual and chemical cues) in mediating risk assessment and anti-predator behavior in native amphibian prey; (iii) if interspecific variation in anti-predator responses can be explained by the generalization hypothesis, or by the generic response hypothesis. The generalization hypothesis predicts better anti-predator responses in amphibians that co-evolved with a similar native predator (i.e., the European white-clawed crayfish, *Austropotamobius pallipes*; hereafter European crayfish), while the generic response hypothesis predicts comparable responses across species.

Methods

Study area and collection of individuals

We considered 13 amphibian species, which represent the most common pond-breeding amphibian species in Northern Italy. The study species included five caudates and eight anurans: fire salamander (*Salamandra salamandra*) northern spectacled salamander (*Salamandrina perspicillata*), smooth newt (*Lissotriton vulgaris*), Italian crested newt (*Triturus carnifex*), alpine newt (*Ichthyosaura alpestris*), Italian agile frog (*Rana latastei*), agile frog (*Rana dalmatina*), Italian stream frog (*Rana italica*), European common frog (*Rana temporaria*), green frog (*Pelophylax* kl. *esculentus*), Italian tree frog (*Hyla intermedia*), European common toad (*Bufo bufo*), and European green toad (*Bufo viridis* complex). All the study species were collected in the Po River Valley or in the Northern Apennines (administrative regions: Lombardia, Liguria and Emilia Romagna; see Figure S1). This area hosts a rich hydrographic network where broadleaved forests are intermingled with urban and agricultural areas. In these regions, the native European crayfish, *Austropotamobius pallipes*, which is an amphibian predator generally living in small streams, was historically common (Manenti et al. 2014). Nonetheless, the European crayfish has undergone a rapid decline in the last century, due to habitat modification, fishing and spread of pathogens, and is now extinct in most of its historical range (Holdich et al. 2009, Bonelli et al. 2017, Manenti et al. 2019). To test if the coevolutionary history with the native crayfish allows amphibians to respond towards invasive crayfish, we selected amphibian populations breeding in sites within hydrographic basins that hosted the European crayfish in the past.

Between spring and summer 2018, we collected 12 larvae from two populations for each of the 13 amphibian species (total: 26 populations, 312 individuals). Amphibian larvae were at intermediate developmental stages (for anurans, Gosner's stage 28-33 (Gosner 1960); for caudates, stages 51b-52b according to (Bernabò and Brunelli 2019)) and were all collected from populations where the European crayfish is currently extinct (amphibians sharing a coevolutionary history with the native crayfish) or naturally absent (amphibians without coevolutionary history). All amphibian larvae come from populations uninvaded by the alien crayfish. This allowed to exclude potential effects of individual experience towards any crayfish predator, or the possibility of a recent evolutionary response to the invasive crayfish.

Procambarus clarkii is native of North America but is currently widespread in Northern Italy, even if its distribution is patchy (Lo Parrino et al. 2020). This invasive crayfish has a broad niche and is able to exploit both rivers and lentic environments (Souty-Grosset et al. 2006). The overall morphology and the predatory behavior is similar between the invasive and the European crayfish, even though the invasive one shows a more opportunistic diet and has a better ability to capture prey (Gherardi et al. 2001, Renai and Gherardi 2004). As a consequence, several amphibian populations invaded by the American crayfish underwent recent declines (Cruz et al. 2008, Ficetola et al. 2011), and in some cases the selective pressure posed by this crayfish was strong enough to even trigger rapid adaptation in amphibian populations (Melotto et al. 2020). American crayfish individuals used in this study (n = 40) were collected from a dense population in Lombardy (approx. 45.729°N, 9.237°E).

<u>Housing and experimental protocol</u>

After collection, larvae were housed in the laboratory in 49 x 35 cm plastic tanks containing 15 L of decanted tap water. Each tank hosted 12 larvae from the same population, which were individually housed in perforated plastic cups (Ø = 8 cm). Larvae were kept under constant oxygenation, and were exposed to room temperature and daily photoperiod. During their housing period, larvae were fed every second day with rabbit pellet (anuran tadpoles), *Chironomus* spp. larvae (*Salamandra* larvae) or *Daphnia* spp. (*Salamandrina* and newt larvae).

After collection, *P. clarkii* individuals (cephalothorax length: mean ± SE = 46.92 ± 0.75 mm) were singularly hosted in plastic tanks (20 x 14 cm, 5 L of decanted tap water), in the same conditions as amphibian larvae and fed with commercial fish food every second day. All larvae were housed in the lab for a minimum of three days before behavioral tests (mean ± SE: 4.7 ± 1 days). After a two-day starvation period, we performed one experimental session for each amphibian population (i.e., 26 experimental sessions). During experimental sessions, each amphibian larva was exposed to the non-lethal presence of the American crayfish with four combinations of cues deriving from the predator (Figure 1): visual and chemical cues (V+C+); visual cues only (V+C-); chemical cues only (V-C+); no risk cues (V-C-). Experiments were conducted in 51 × 18 cm plastic tanks, filled with 8 L of decanted tap water. Experimental tanks were divided in two compartments by a transparent plastic barrier. This barrier was impermeable to water and any unintended chemical cues exchange between the two compartments was prevented. One compartment hosted amphibian larvae (18 x 18 cm, hereafter 'prey compartment'), while the second one hosted the American crayfish (32 × 18 cm, hereafter 'predator compartment'). In all predator compartments, an opaque plastic pot (9 × 9 × 14 cm) was present. Pots hosted the crayfish in treatments without visual cues, while in visual-cue treatments the crayfish was free ranging in its compartment. In treatments with exposure to *P. clarkii* chemical cues, 0.5 cm diameter holes, performed both on the barrier separating larvae from the invasive crayfish (n = 15) and the pot (n = 12 per each of the two lateral sides), allowed chemical cue exchange between compartments. Behavioral tests were conducted between 9 a.m. and 17 p.m.; all individuals from the same population were tested in the same day, while different populations were tested separately. Before experiments started, each larva was inserted in the prey compartment and let acclimatize for three minutes. After acclimatization, we inserted a crayfish in the predator compartment (in the pot for V-C+ and V-C- treatments; out of the pot for V+C+, V+C- treatments). Behavioral tests lasted 7 minutes and larva activity was video-recorded by placing a Nikon d5300 camera (18mm lens) perpendicularly above the prey compartment. For each individual we performed eight behavioral tests (four treatments, each replicated twice). Tests were conducted in a randomized order to minimize the potential bias of exposure sequence (Altmann 1974, Ferrari et al. 2010a, Melotto et al. 2019). During each experimental day, 12 crayfish individuals were randomly selected and assigned to behavioral trials following a randomized protocol, so that each crayfish was used twice for the same condition. We left at least 15-minute recovering time to each animal between consecutive tests. Each tank and pot were assigned to a treatment and then used for that specific treatment only. Tanks and pots were washed multiple times between subsequent trials to minimize traces of cues from preceding tests. In total, we performed 2496 behavioral tests (12 individuals × 26 populations × 4 treatments × 2 replicates). After the conclusion of each behavioral session, all the larvae and lab materials were treated with antifungal disinfectant and all the amphibians were released in their site of origin (see Ethical statement).

Behavioral traits

Behavior and activity of larvae were obtained by extracting individual movements from videos with the video-tracking software *idTracker*. This software allows to track individual identity and position in subsequent frames of a video, by recognizing individual shape basing on its size and chromatic contrast with the background (Pérez-Escudero et al. 2014). We considered three behavioral traits: total distance moved by larvae during the test (hereafter total distance), mean distance from the barrier separating them from the stimulus source (avoidance) and the number of bursts performed by larvae (number of bursts). Two of them, total distance and avoidance, are classical behavioral parameters describing prey activity and space use (Lima and Dill 1990). General decrease of activity and avoidance of risky areas are common anti-predator behavior that are frequently observed in amphibian larvae (Relyea 2001a, Van Buskirk et al. 2012, Winandy and Denoël 2013, Manenti et al. 2016). However, in preliminary observations we noticed that some species show periods of limited movement followed by rapid bursts. These bursts lasted few seconds and allowed larvae to cover large distances, a behavior likely representing an escape attempt (Dayton et al. 2005, Teplitsky et al. 2005). Measuring total movement only could have obscured specific anti-predator strategies, potentially leading to the misinterpretation of behavioral responses. Thus, for all the species we considered the number of bursts performed by larvae during each test as an additional behavioral parameter. For each species, we calculated the mean distance moved during single movements (i.e. continuous movements through time, interspersed with periods of inactivity) and its standard deviation (SD). All the movements exceeding the mean movement + 2SD were defined as bursts. This approach allowed detecting rare movement that considerably differed from the average, while ensuring measure repeatability among species. Correlations among the three behavioral traits analyzed showed that avoidance was weakly correlated to total distance or the number of bursts. Total distance was generally positively related to the number of burst (see Supplementary material, Table S1). This is not surprising as generally larvae covered relatively long distances when performing bursts, thus individuals exhibiting higher bursts frequencies also moved more. However, these behaviors are two distinct aspects of anti-predator responses which can represent different anti-predator strategies (i.e., avoiding predator detection vs actively escaping once detected), and prey responses can be differentially expressed according the perceived risk or show different effectiveness depending on predator hunting strategy (Relyea 2001b, Teplitsky et al. 2005, Rehage et al. 2009, Mogali et al. 2011, Ferrari et al. 2015).

Statistical analysis

The effects of crayfish exposure on amphibian behavior were analyzed through Bayesian multivariate Generalized Linear Mixed Models (GLMMs). These models allow to consider the influence of fixed effects on the dependent variable while taking into account the covariation between multiple dependent variables and the non-independence of observations (e.g., repeated observations on the same individual or on the same population (Pinheiro and Bates 2000, Bürkner 2018). In this global model, we considered the three behavioural traits (total distance, number of bursts and avoidance) as dependent variables. We included treatment (chemical or visual cue exposure) as fixed factors to assess the effect of crayfish exposure on larva behavior. Moreover, to test the hypothesis that coevolution with the native crayfish could increase the ability to detect the invasive crayfish, amphibians were classified according to their history of co-existence with the native crayfish (species living in habitats once hosting *A. pallipes* vs species exploiting different habitats; see Figure 3 and Supplementary Figure S1). History of coexistence with the European crayfish was included in the model as an additional fixed factor, while we used 2-way interactions between coexistence, chemical and visual cues to assess if responsiveness to a particular stimulus from the invasive crayfish was affected by the coevolutionary history with the native crayfish. Air temperature (°C) and day time (minutes from midnight) as covariates, as they can affect amphibian activity (Wells 2007). All continuous independent variables were standardized before analyses. Moreover, a few videos were slightly shorter, thus we also included video duration as an additional covariate in all models. As random factors we included species identity, population of origin, individual identity and test replicate (first or second exposure to a single cue) as random factors. In mixed models, we took into account the nested structure of random factors (individual, population and species identity) (Zuur et al. 2009). The multivariate GLMM was run with three MCMC chains using 2,000 iterations and a burn-in of 1,000 in the brms package in R (Bürkner 2018). 48. For all variables, c-hat was <1.01, indicating convergence. The number of bursts showed strong overdispersion, therefore we used a negative binomial distributions (Bolker et al. 2012, Brooks et al. 2017). All other behavioral traits were transformed using log(x+0.01) and analyzed with Gaussian error.

In a second step, to finely investigate specific responses expressed by species, we built standard univariate mixed models assessing the behavioral traits of each species (hereafter: single-species models). These models were intended as post-hoc analyses of the main models, therefore interactions that were non-significant in the main model were excluded (i.e., chemical x visual cues). Video duration was not considered in fire salamander models, as for this species all the videos lasted 7 min. All results describing covariate effects on larva behavior are reported in the Supplementary material (Table S2). In single-species models a large number of statistical tests were performed ($n = 39$; 3 traits $\times$ 13 species), and this can inflate the rate of type I errors, thus we used the false discovery rate (fdr) method to recalculate significance values for each trait. fdr recalculates the significance values of related parameters on the basis of the distribution of null hypothesis rejections among them, and enables limiting false discoveries (type I errors), while minimizing type II errors compared to other classical methods (Strimmer 2008). In the Supplementary material we report both the uncorrected significance values, and those recalculated using false discovery rate (Table S2).

We used Pagel's lambda to confirm that our results are not biased by phylogenetic relationships between species. To compare the response to the American crayfish across species, we converted the effect sizes of species responses to the crayfish at single species level (F or χ^2 values) to Fisher's z (see Supplementary material: Table S4). Fisher's z is a measure of effect size that allows comparisons among statistical tests (Field et al. 2012); information on phylogenetic relationships between species was obtained from the tree of (Pyron and Wiens 2011) (see Figure S2 in Supplementary material). Overall, we obtained six sets of effect sizes (one per each crayfish cue effect on each behavioral trait) for the 13 species. The model for number of bursts failed to converge in one species (*Hyla intermedia*), thus we excluded tree frogs for this display. We measured the phylogenetic signal of each set of effect sizes using Pagel's lambda (Orme et al. 2012). For each set of effect sizes, phylogenetic signal was extremely low, and confidence intervals always included zero (all lambdas $\leq$ 0.13; see Supplementary material S5), suggesting the effect of phylogenetic signal on larva responses was negligible. Basing on these results we decided not included phylogenetic relatedness in our mixed models.

All statistical analyses were performed using R (version 3.6.0). Bayesian multivariate GLMMs we run in STAN using the *brms* package (Bürkner 2017), while for mixed models we used packages nlme, lmerTest, MuMIn and glmmTMB (Bates et al. 2012, Barton and Barton 2015, Kuznetsova et al. 2017, Magnusson et al. 2017). The effect sizes were obtained through the compute.es package (Del Re 2013). We assessed phylogenetic signal with the *caper* package (Pyron and Wiens 2011, Orme et al. 2012, Oksanen et al. 2013). Finally, we used fdrtool package to perform to false discovery rate analyses (Klaus et al. 2015).

Results

Bayesian multivariate models showed that the three analyzed behavioral traits were affect different by the crayfish stimuli used (Table 1).

Total distance

Total distance travelled strongly varied among species (Figure S3A in Supplementary material). Among anurans, total distance was largest for the common toad (244.1 ± 13.4 cm per trial; mean ± SE, here and afterwards) and the stream frog (149.7 ± 9.4 cm). Among caudates, the longest distances were covered by the smooth newt (121.5 ± 6.9 cm) (see Supplementary material: Table S3 and Figure S3A). Conversely, some species moved for very limited distances, particularly the tree frog (2.5 ± 0.4 cm) and the agile frog (8.7 ± 1.3 cm). In these species the total travelled distance was highly variable among individuals (range: 0 – 23.7 cm and 0 – 113.3 cm, respectively).

Total distance was strongly affected by visual stimuli from the invasive predator, with amphibian larvae showing a general decrease in their activity when exposed to crayfish (B = -0.20, 95% CI = -0.255, -0.154; see Table 1 and Figure 2A). By contrast, no significant effect of chemical stimuli (β = -0.02 [-0.065, 0.033]), coexistence (β = 0.27 [-0.197, 0.535]) or any covariate was detected (95% confidence intervals always overlapped zero). The global model did not detect any interactive effect between visual and chemical cues, or between cues and coexistence (Table 1).

Post-hoc single-species models indicated that the exposure to the visual cues of the invasive crayfish clearly reduced the distance covered in nine species: fire salamander, smooth newt, crested newt, alpine newt, Italian agile frog, common frog, common toad and green toad (see Figure 3 and Supplementary material Table S2).

Number of bursts

Similar to total distance, the number of bursts performed by larvae was highly variable (see Figure S3B in Supplementary material). Among anurans, the stream frog, green frog, and common toad performed on average more than two bursts per test (Supplementary material: Table S2 and Figure S3B), while in caudates the highest number of bursts per test was observed in the smooth newt, which performed on average 3.7 ± 0.4 bursts per test. The number of bursts performed by amphibian larvae was not affected by the exposure to cues (0.04 [-0.032, 0.110]) of the invasive predator (see Table 1, Figure 2B). Visual cues tended to reduce the number of bursts (-0.06 [-0.137, 0.013]), but 95% Cis slightly overlapped zero. However, the model revealed a positive interaction between coexistence and visual cues (0.09 [0.018, 0.168]), showing that larvae with a history of coexistence with the native crayfish increased the number of bursts in presence of visual stimuli of the invasive one (Table 1, Figure 2B). All other fixed effects showed 95% CI overlapping zero (Table 1; Figure 2B).

Post-hoc single-species models revealed the number of bursts was affected by the visual cue of the American crayfish in multiple species, with contrasting responses across species (Figure 3; Supplementary material Table S2). In both the agile frog and the Italian agile frog, the visual cues treatment increased the number of bursts performed (see Figure 3 and Table S2), while in five species (fire salamander, alpine newt, crested newt, stream frog, green frog) visual exposure to the invasive crayfish caused a reduction in the number of bursts (Figure 3 and Table S2). The chemical cue treatment produced no significant effect on number of bursts in any species (Table S2). In one case (tree frog), the univariate mixed model failed to converge, probably because three frogs performed very few bursts (average number of bursts per test = 0.04).

<u>Avoidance</u>

Both crayfish chemical and visual stimuli affected larvae tendency to avoid the invasive predator (Table 1). The two stimuli produced opposite effect, as distance from the predator increased if larvae were able to see the crayfish (β = 0.05 [0.027, 0.065]) but slightly decreased with chemical cues (β = -0.02 [-0.039, -0.0004]; Figure 2C). Conversely, we detected no influence of coexistence and other fixed effect or interactions (all confidence intervals overlapping zero; Table 1; Figure 2C).

Single-species models suggested that exposure to the visual cues of the American crayfish affected avoidance particularly in spectacled salamanders and smooth newts (Table S2). In these species, the mean distance between the larva and the barrier increased when the invasive crayfish was visible (Figure 3). In single-species models, chemical cues significantly reduced the distance from the barrier in spectacled salamander larvae only, while no clear effects of chemical cues on avoidance was detected for the other species (Figure 3; Table S2).

Discussion

Naïve larvae of amphibian species were generally able to modulate their behavior in presence of an invasive predator. Behavioral responses were mostly triggered by the visual exposure to the invasive crayfish, while its chemical cues only caused a feeble and unclear effect on avoidance. Overall, our results suggest that responses to a novel predator across the whole community are dominated by the response to generic risk cues associated to predator presence. Still the modality and intensity of responses were heterogeneous among species, and the capacity of larvae to alter their behavior towards the invasive predator was to some extent affected by species coevolutionary history with a similar, native crayfish predator.

The presence of the invasive crayfish was generally recognized as risky by native amphibians, as exposed larvae altered their behavior expressing classical anti-predator responses (e.g., activity reduction, predator avoidance) that can favor prey survival (Skelly 1994, Relyea 2001b, Teplitsky et al. 2005). However, these responses were only expressed when native prey could perceive visual cues of the invasive crayfish. Conversely, exposure to chemical cues of this predator elicited some feeble and contrasting behavioral shift in avoidance, causing larvae to decrease their distance from the crayfish, which was inconsistent with the expected anti-predator response (Figure 3). Such a behavioral reaction to crayfish odor might represent a maladaptive response to unknown cues or, alternatively, a response towards a potential trophic source. Indeed, prior to experiments the crayfish have been fed with classical fish food (composed by insects, crustaceans and other animal proteins); it is thus possible that foraging cues they released was attractive for some predatory amphibian. This effect was particularly evident in the spectacled salamander (see results and Table S2 in the Supplementary material), which is a mesopredator. Overall, the general absence of response to chemical stimuli by *P. clarkii* suggests that naïve amphibian larvae are incapable of recognizing kairomones of this invasive crayfish as a signal of predation risk. This lack of responsiveness was found also in amphibians that coexisted with native predator, suggesting that the incapability to perceive chemical cues of the invasive crayfish as a threat was unrelated to the coevolutionary history of the species with the European crayfish. Even though the two crayfishes share similar morphology and trophic niche, and both use a similar strategy (i.e., active search at bottom of waterbodies alternated to ambush predation) when preying upon amphibian larvae (Gherardi et al. 2001, Renai and Gherardi 2004, Rebelo and Cruz 2005, Gonçalves et al. 2011, Manenti et al. 2019), the phylogenetic distance between them is large, as these species belong to different families. This may have hampered the recognition of kairomones of the American crayfish even in amphibians sharing a coevolutionary history with the native one. Indeed, even though generalization of predator recognition is highly variable among prey species (Carthey and Blumstein 2018), close proximity between novel and native predators is generally required for their chemical recognition (Ferrari et al. 2007). It is also worth noting that the extent amphibians differ in their ability to respond to the cues of this predator currently is unknown, still predation on amphibian larvae by the native crayfish is well documented (Gherardi et al. 2001, Renai and Gherardi 2004), thus a complete incapability of larvae to respond to their native predator is unlikely. In our study, we were not able to the responses of amphibian larvae to the European crayfish due to its endangered status and the risk of pathogen spread, as our tests involved the American crayfish, which quickly spreads crayfish plague to the native ones (Manenti et al. 2014). Future studies assessing the capability of amphibian larvae to respond to cues released by to the European crayfish may further contribute to shed light on the role history of coexistence can play in determining amphibian anti-predator to these predators.

In contrast to chemical stimuli, exposure to visual cues of American crayfish elicited pronounced shifts in the behavior of nearly all the species. Anti-predator responses included a general decrease in activity (particularly a reduction of the distance moved) together with an overall avoidance of the predator, which represent typical anti-predator strategies to avoid detections from predators (Relyea 2001a, Teplitsky et al. 2005). The general behavioral responsiveness elicited by visual cues of the American crayfish can be interpreted as a non-specific anti-predator behavior towards generic risk cues (Mathis and Vincent 2000, Rehage et al. 2009, Carthey and Blumstein 2018), supporting the predictions of the generic response hypothesis. Indeed, large approaching figures have been already observed to trigger similar responses in amphibian larvae (Mathis and Vincent 2000), and can drive prey behavioral shifts even in presence of unknown predators (Rehage et al. 2009, Wilson et al. 2018).

Moreover, amphibians sharing a history of coexistence with the European crayfish tended to increase the number of rapid bursts they performed when exposed to visual cues more compared to the other species, which generally reduced activity (Fig. 2). This suggests that species that faced predation from the native crayfish during their evolutionary history were able to show a distinct anti-predator behavior when experiencing a similar invasive predator. The capability to combine classic activity reduction and rapid bursts in larvae from these species suggests that complex behavioral tuning, involving apparently contrasting patterns, might be advantageous to withstand crayfish predators. For instance, traits promoting rapid escape ability are positively selected in amphibian larvae facing active-search or pursuing predators, as this strategy can improve their survival (Teplitsky et al. 2005). Our findings support the idea that coevolutionary history can play a role in shaping behaviors of species facing novel predation pressures, in agreement with the generalization of predator hypothesis. Future studies should investigate the adaptive value of such responses and assess potential divergence in resilience to the American crayfish of species that have or have not experienced similar predators during their evolutionary history.

Finally, we emphasize that anti-predator strategies were highly heterogeneous among species, with different combinations of behavioral responses to the visual stimuli of the American crayfish (Figure 3). Most amphibians responded by reducing their activity (i.e., distance moved and number of bursts), while predator avoidance was observed in fewer species. The response was particularly heterogeneous for the number of bursts, with some species privileging escape responses, and others reducing rapid movements consistently with the general decrease of their activity, supporting a role of evolutionary coexistence of species with the native predator (Table 1). The Italian agile frog is a striking example of the ability of fine-tuning anti-predator strategies, as it accompanied a marked reduction of overall activity to rapid escape responses (Figure 3). The strong variation among strategies exhibited by different species is often forgotten and underlines the importance of finely evaluating prey behavioral traits, for instance through multiple behavioral parameters, when assessing anti-predator responses. Species capability to express responses towards novel selective pressures may intimately depend on their evolutionary history. Investigating the remote causes and mechanisms underlying the rise of these differences, and unravelling the adaptive value they entail, constitutes an intriguing new area of study, and can provide key insights on species capability to face new threats in a rapidly changing world.

Despite their striking capacity to respond to the novel threat, predator recognition in naïve amphibian larvae was mostly mediated by visual stimuli by the alien crayfish, while its kairomones were not perceived as risky. Risk assessment based on incomplete information may result in weakened effectiveness of prey responses. For instance, in many freshwater environments, visual stimuli only allow the detection of nearby predators, thus hampering predator avoidance and limiting an effective anti-predatory response. Predator recognition based on visual cues can be particularly ineffective in turbid or highly-vegetated wetlands, where the quality of visual information received by the prey dramatically decreases (Abrahams and Kattenfeld 1997, Ferrari et al. 2010b). In these environments, chemical cues may allow a better detection of predator presence (Chivers and Smith 1998, Chivers et al. 2001) and prey can strongly rely on these stimuli for risk assessment. It is also worth noting that native prey can learn to recognize predator kairomones and refine their anti-predator response through experience (Gonzalo et al. 2007). In nature, naïve prey are often exposed to predation cues (e.g., conspecific alarm cues or predator foraging cues) and thus they can learn to associate unfamiliar cues of non-native predators to dangerous situations, potentially refining their anti-predator response through experience (Gonzalo et al. 2007, Chivers et al. 2014, Polo‐Cavia and Gomez‐Mestre 2014, Falaschi et al. 2020). In our experimental design larvae came from crayfish-deprived populations, still potential experience of other predators acquired during early life stages could have been possible, and, in principle, we cannot exclude this might affect their capability to respond to a new predator. Nevertheless, innate predator recognition is a major component of behavior in amphibian larvae and responses to novel predators with no prior exposure are often evident (Epp and Gabor 2008, Wilson et al. 2018). In particular, laboratory-reared larvae (i.e., naïve to predators or any predation cues) of multiple amphibian species have been observed to show anti-predator responses when exposed to the American crayfish (Nunes et al. 2014b, Nunes et al. 2014a). Thus, while prior experience might have refined larva risk assessment, it is unlikely that the responses to the crayfish we observed were mostly caused by previous exposure to other predators. Furthermore, for each species we considered two distinct populations, inhabiting different habitats and thus probably with different predator communities. Further research is needed to assess the role of experience and learning in mediating the responses of amphibian larvae to cues associated to invasive predators, and if these mechanisms may favor the survival of native species.

Behavioral responses to predation risk are typically short-term reversible strategies (Turner 1997, Relyea 2001a, Westrick et al. 2019), and often constitute the first line of defense native species can rely on when facing invasive predators (Holway and Suarez 1999, Sih et al. 2010, Weis and Sol 2016, Falaschi et al. 2020). However, the actual effectiveness of the observed responses remains to be tested. Few studies have investigated behavioral responses of amphibian larvae to this invasive crayfish, and to our knowledge only one of them showed that behavioral shifts can improve the survival of larvae (Polo-Cavia and Gomez-Mestre 2014), while others suggested that reduction of activity and/or altered microhabitat use do not necessarily reduce vulnerability (Rebelo and Cruz 2005). In fact, multiple native amphibians underwent rapid declines and local extinctions after the invasion of the American crayfish (Gamradt and Kats 1996, Cruz et al. 2008, Ficetola et al. 2011, Liu et al. 2018, Falaschi et al. 2021), suggesting that the responses of native amphibians can be insufficient to withstand the predatory pressure posed by this voracious crayfish, at least for some species.

This study demonstrated that naïve amphibian larvae have a striking capability to alter their behavior in presence of novel predators and shed light on the mechanisms allowing the recognition of alien predators. The heterogeneity of behavioral responses across species and the presence of diverse and even contrasting anti-predator strategies highlight the importance of considering multiple traits when investigating predator-prey interactions. This heterogeneity of anti-predator strategies responses was partially related to amphibian coexistence with a similar predator, suggesting species history may influence their responsiveness towards novel selective pressures. However, even though non-native predators can trigger the expression of a striking behavioral plasticity in native species, the potential of these responses in promoting species persistence during biological invasions remains to be ascertained. The linkage between behavioral responses measured in the lab, and the dynamics of wild populations, remain a major question if we want to predict the long-term impact of invasive species (Falaschi et al. 2020). Further research should investigate the effectiveness of behavioral responses of native prey in withstanding invasive predators and test whether population trends are related to species capability to express anti-predator behavior.

Ethical statement

Collection of larvae, treatments and behavioral tests were authorized by Italian Ministry for Environment (DPR 357/97 and Prot. N. 12969/T-A31). To prevent potential pathogen exchange and spread, after each behavioral sessions and before individual releasing all experimental and housing material was carefully washed with 1% Virkon S solution (Bosch et al. 2015), while larvae were treated with 0.5 mg/L for five minutes at the end of experiments. This antifungal is highly recommended in studies involving collection or translocation of individuals (Johnson et al. 2003; Bosch et al. 2015), and moderate concentrations of Virkon S have no significant effects on individual survival and growth and can be used to treat amphibian larvae (von Rütte et al. 2009; Hangartner and Laurila 2012). After treatment with Virkon S, larvae were monitored for one day before releasing, and we observed no mortality or visible change in their behavior.

Data availability statement

The datasets generated during andr analyzed during the current study will be available in the fisgshare repository (https://figshare.com/).

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Conflict of interest

The authors declare no conflicts of interest.

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		Effects	*B*	CI 95%	R^2
Total distance	**Fixed**	Chemical cues	-0.02	-0.065 - 0.033	0.64
		Visual cues	**-0.20**	**-0.255 - -0.154**	
		Temperature	-0.04	-0.298 - 0.226	
		Daytime	-0.05	-0.160 - 0.063	
		Test duration	0.04	-0.015 - 0.087	
		Coexistence	-0.27	-1.097 - 0.535	
		Chemical cues x Visual cues	-0.03	-0.077 - 0.018	
		Chemical cues x Coexistence	-0.02	-0.072 - 0.028	
		Visual cues x Coexistence	0.00	-0.047 - 0.051	
	Random	Individual identity	0.77	0.69 - 0.87	
		Population	0.96	0.62 - 1.53	
		Species	1.16	0.29 - 2.13	
		Trial	1.36	0.3 - 4.08	
N bursts	**Fixed**	Chemical cues	0.04	-0.032 - 0.110	0.48
		Visual cues	-0.06	-0.137 - 0.013	
		Temperature	-0.23	-0.629 - 0.176	
		Daytime	0.03	-0.150 - 0.196	
		Test duration	0.03	-0.045 - 0.112	
		Coexistence	-0.39	-1.164 - 0.423	
		Chemical cues x Visual cues	0.01	-0.065 - 0.074	
		Chemical cues x Coexistence	0.02	-0.060 - 0.091	
		Visual cues x Coexistence	**0.09**	**0.018 - 0.168**	
	Random	Individual identity	0.89	0.76 - 1.03	
		Population	1.15	0.71 - 1.76	
		Species	0.98	0.09 - 1.97	
		Trial	1.55	0.3 - 4.89	
Avoidance	**Fixed**	**Chemical cues**	**-0.02**	**-0.039 - -0.0004**	0.19
		Visual cues	**0.05**	**0.027 - 0.065**	
		Temperature	0.00	-0.048 - 0.041	
		Daytime	-0.01	-0.032 - 0.016	
		Test duration	-0.01	-0.027 - 0.013	
		Coexistence	-0.03	-0.173 - 0.103	
		Chemical cues x Visual cues	0.00	-0.013 - 0.022	
		Chemical cues x Coexistence	0.00	-0.024 - 0.015	
		Visual cues x Coexistence	-0.01	-0.031 - 0.006	
	Random	Individual identity	0.08	0.03 - 0.12	
		Population	0.04	0 - 0.1	
		Species	0.24	0.15 - 0.4	
		Trial	0.33	0 - 1.75	

TABLE 1 – Results of Bayesian multivariate GLMMs showing the effect of exposure to the alien crayfish and the history of coexistence with a native crayfish on behavioral traits of amphibian larvae. In these models, responses from all the 13 species to invasive crayfish cues were analyzed altogether, while as dependent variable we included three behavioral traits: Total distance (distance moved by larvae during tests), Number of bursts (rapid movements performed by larvae), and Avoidance (mean distance from the invasive crayfish during tests). Model estimates (*B*), and the 95% credible intervals for both fixed and random effects are reported. The Bayesian R^2 for regression models is also reported. Effects with 95% CI not overlapping zero are in bold.

Figure legends

FIGURE 1 – Experimental scheme Activity of amphibian larvae was tested in behavioral trials with the exposure to four treatments: contemporary presence of visual and chemical stimuli of the American crayfish (V+C+); presence of chemical cues only (V-C+); presence of visual cues only (V+C-); absence of crayfish cues (V-C-). During the tests, tadpoles were housed in one compartment of two-sided experimental tanks (prey compartment). The predator compartment was separated by a transparent plastic barrier and hosted an adult American crayfish. The crayfish was placed in an opaque pot in treatments excluding visual stimuli (V-C+ and V-C-), while it was free ranging in treatments with exposure to visual stimuli (V+C+ and V+C-). Exposure to chemical cues (treatments V+C and V-C+) was allowed by means of small holes in the barrier and in the pot hosting the crayfish, whereas holes were absent in treatments excluding exposure to chemical cues (V+C- and V-C-). Behavioral tests lasted seven minutes and each larva (n = 24 individuals per species) was exposed to each treatment in two replicates.

FIGURE 2 – Forest plots showing the global influence of fixed effects on amphibian larva behavior. Larva behavioral responses to the invasive crayfish exposure were assessed through Bayesian multivariate GLMMs, analyzing all the 13 species together and using three behavioral traits as dependent: Total distance (A); Number of bursts (B); Avoidance (C). For each fixed effect, horizontal lines are 95% credible intervals, while dots represent model estimates (*B*).

FIGURE 3 – Effect of exposure to visual and chemical cues by the alien crayfish on larva behavior of each amphibian species. Results of single-species models relating behavioral traits to the exposure to visual and chemical stimuli by the American crayfish *(Procambarus clarkii*). In this analysis, a separate model was built for each species. Blue arrows indicate significant responses to visual cues; yellow arrows indicate significant responses to chemical cues. The direction of arrows represents positive (up) vs. negative (down) effects. Amphibian species historically coexisting with the native European crayfish (*Austropotamobius pallipes*) are indicated by the crayfish symbol nearby species names. Asterisks represent of significance after fdr analysis (* = 0.01 ≤ p < 0.05; ** = 0.001 ≤ p 0.01); *** = p < 0.001).