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Anti-predator responses towards an invasive crayfish in amphibian larvae

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CONTENTS

Abstract

Invasive species pose a severe threat to native ecosystems and represent the second cause of biodiversity loss on global scale after habitat destruction.

In particular, invasive predators are major drivers of rapid population declines and local extinctions in native prey. During biotic invasions, native prey become abruptly exposed to novel predators with which they share no history of coevolution. Thus, the lack of common evolutionary history often hampers prey effective response to the novel predation pressures; native prey can both fail to recognise predators as a threat and exhibit anti-predator strategies that are inadequate. Nonetheless, mechanisms such as rapid adaptation and phenotypic plasticity can allow prey to cope with new selective pressures and drive evolutionary changes that can help native species withstanding invasive ones.

Due to their relative ecological isolation, freshwater ecosystems are particularly sensitive to invasive species impacts and likewise are most of the organisms exploiting them like amphibians; aquatic and semi-aquatic amphibians are effective indicators of freshwater habitats conservation with an excellent potential for studying the responses towards invasive species. Indeed, generally, amphibians show high level of developmental and behavioural plasticity and have relatively short life cycles, which could allow the detection of important adaptive patterns even few years after the spreading of invasive organisms.

This thesis investigated how amphibians can respond to a novel predation pressure, by assessing their modulation of anti-predator responses on multiple traits (i.e. behavioural morphological, life-history). In particular, I aimed to shed light on (i) the role of phenotypic plasticity in mediating the expression of anti-predator responses towards invasive predators; (ii) how novel predation pressures can interact with extant selective forces and foster rapid adaptation in native prey; and (iii) which evolutionary mechanisms are involved in allowing predator recognition by naïve prey.

To this extent, I evaluated the expression of anti-predator responses in amphibian larvae towards the American red swamp crayfish (*Procambarus clarkii*). This widespread crayfish is listed among the 100 worst invasive alien species, and is a voracious predator of amphibian eggs and larvae, often associated to strong declines of amphibian populations outside its native range.

In brief, the research consisted in two main experimental studies, the first of which focused on developmental shifts of a single anuran species, while the second one assessed behavioural responses to the invasive crayfish across the amphibian community of Northern Italy.

In the first study, I exposed tadpoles of an endemic frog (*Rana latastei*) from recently invaded (10- 15 years) and uninvaded populations to the non-lethal presence of the invasive crayfish during their ontogenesis. Tadpoles from invaded populations showed rapid adaptation in life-history traits (reduced development time), and this caused the disappearance of pre-existing adaptive divergence between *R. latastei* populations exploiting environments with different climatic regimes. However, even if early metamorphosis in invaded populations probably has great advantages as it reduces exposure to crayfish predation, this shift can produce potential carry-over effects on post-metamorphic traits. Indeed, the observed development acceleration was not without a cost, as faster-developing froglets were smaller and displayed poorer jumping performances.

Besides, experimental exposure to the invasive crayfish revealed tadpoles were also able to modulate both their development time and morphological traits through phenotypic plasticity. An acceleration in development time was observed even in exposed tadpoles. Moreover, tadpoles reared in presence of *P. clarkii* showed shift in body shape together with an increase in tail muscle size, which is a trait associated to faster swim and can increase escape from predators. By contrast, tadpole behaviour was not influenced nor by origin (invaded or not invaded populations) neither by crayfish exposure.

The second study was performed on several species composing Northern Italy amphibian community (five urodele and eight anuran species); for each species I assessed how different stimuli mediated novel predator recognition and elicited the expression of anti-predator responses in naïve species. To this extent, I evaluated multiple larval behavioural traits after brief non-lethal exposure to crayfish-released cues (four treatments: visual cues, chemical cues, contemporary exposure to both cues and control). Moreover, I tested if these responses were influenced by the coevolutionary history some of these species shared with a similar native predator, the European crayfish (*Austropotamobius italicus*). I showed that all species altered their behaviour when exposed to the invasive crayfish while the modality and intensity of response was highly heterogeneous. However, almost all behavioural responses were driven by visual cues, while chemical cues elicited feeble and contrasting outcomes.

Finally, I found no support for a coevolutionary history hypothesis between native amphibians and native crayfish, as responses to the invasive predator were not affected by species coexistence with native crayfish. Instead, behavioural responses observed in naïve species was likely elicited by recognition of general predator traits (e.g. an approaching large shape). The broad implication of my thesis is that amphibian facing invasive predators can both exhibit rapid adaptation to the novel selective pressures and modulate their developmental traits through phenotypic plasticity. Moreover, the expression of these responses is context-dependent and can highly vary in relation to the experienced conditions and across species. For instance, varying typology of risk exposure can produce marked difference in anti-predator response (e.g. activation of short-term behavioural response vs long-term morphological responses). Future studies assessing responses to invasive predators should evaluate multiple traits and carefully consider risk exposure conditions when planning experiments. Finally, the expression of anti-predatory responses of native amphibians, and particularly their effectiveness towards invasive predators, need further extensive investigation, and future conservation plans should take into account both species trends and their responsiveness to global change stressors.

Riassunto

Le specie invasive rappresentano una delle principali minacce per gli ecosistemi e, dopo la distruzione degli habitat, costituiscono la seconda causa di perdita di biodiversità su scala globale.

In particolare, i predatori invasivi sono spesso responsabili di rapidi cali di popolazione e di estinzioni locali delle specie predate negli ecosistemi invasi. Infatti durante le invasioni biotiche le prede native si trovano improvvisamente a dover fronteggiare predatori con i quali non hanno condiviso una storia evolutiva comune. Spesso infatti mancanza di una storia coevolutiva impedisce l'attuazione di risposte efficaci verso nuove pressioni predatorie da parte delle prede che possono non riconoscere il predatore invasivo come una potenziale minaccia oppure attuare strategie anti-predatorie inadeguate. Ciononostante esistono meccanismi evolutivi, come adattamento rapido e plasticità fenotipica, che possono permettere alle prede di opporsi alle nuove pressione selettive e promuovere l'insorgenza cambiamenti evolutivi in grado di favorire la sopravvivenza delle specie native.

A causa del relativo isolamento dal punto di vista ecologico, gli ecosistemi d'acqua dolce sono particolarmente sensibili agli impatti delle specie invasive e ciò vale per la maggior parte degli organismi che li abitano, come gli anfibi. Infatti molti anfibi, sia acquatici che semi-acquatici, possono spesso fungere da indicatori del grado di conservazione degli ambienti d'acqua dolce e costituiscono un ottimo caso di studio per valutare le risposte delle specie native alle specie invasive. Generalmente gli anfibi sono caratterizzati da un rilevante grado di plasticità sia nei tratti comportamentali che in quelli legati allo sviluppo, oltre che da cicli vitali relativamente brevi, caratteri questi che spesso sono in grado di favorire l'insorgenza di rapide risposte adattative anche dopo pochi anni dall'introduzione delle specie invasive.

La presente tesi è volta ad indagare come gli anfibi possano far fronte alle nuove pressioni predatorie imposte dalle specie invasive attraverso la modulazione di diversi tratti implicati nelle risposte anti-predatorie (tratti comportamentali, morfologici e legati allo sviluppo). In particolare si è cercato di far luce su (i) quale sia il ruolo della plasticità fenotipica nel mediare l'espressione di risposte anti-predatorie verso i predatori invasivi; (ii) come le nuove pressioni predatorie possano interagire con le forze selettive esistenti negli ecosistemi invasi e come ciò possa condurre all'insorgenza di rapidi adattamenti nelle specie native; (iii) quali siano i

meccanismi evolutivi coinvolti nel riconoscimento dei predatori invasivi da parte di prede che mancano di una storia coevolutiva con essi (*naif*).

A questo proposito è stata valutata la risposta anti-predatoria nei confronti del gambero della Louisiana (*Procambarus clarkii*) nelle larve di diverse specie di anfibio. Il gambero della Louisiana è largamente diffuso al di fuori del suo areale nativo ed è annoverato tra le cento specie invasive più pericolose per la biodiversità. Si tratta di un vorace predatore sia di uova che di larve di anfibio ed è associato a cali di popolazione ed estinzioni locali delle popolazioni di anfibi in diverse località negli areali invasi.

Il presente lavoro di tesi è basato su due studi sperimentali, il primo dei quali focalizzato su una singola specie che è stata monitorata durante il corso dell'intero sviluppo larvale, mentre il secondo studio è volto a valutare le risposte comportamentali verso il gambero invasivo espresse dalle larve di tredici specie facenti parte della comunità anfibia del nord Italia.

Nel corso del primo studio i girini di una specie endemica (*Rana latastei*) provenienti da popolazioni invase (da 10-15 anni) e da popolazioni non ancora invase dal gambero sono stati esposti alla presenza di questo predatore alloctono per l'intera durata del loro sviluppo.

I girini provenienti da popolazione invase hanno mostrato un rapido adattamento di un tratto legato alla loro sviluppo (ridotta durata dello stadio larvale), che ha causato la scomparsa di preesistenti divergenze adattative nel tempo di sviluppo tra popolazioni di collina e di pianura, registrate in studi precedenti all'invasione del gambero. Sebbene il rapido raggiungimento della metamorfosi possa costituire un rilevante vantaggio adattativo per i girini delle popolazioni invase, consentendo un ridotto tempo di esposizione al gambero predatore, esso può comportare anche potenziali conseguenze negative su diversi tratti post-metamorfici. Infatti negli individui neometamorfosati sono stati registrati costi associati ad una breve durata dello stadio larvale: i girini caratterizzati da un rapido sviluppo presentavano taglia ridotta ed una minore capacità di salto.

Inoltre, l'esposizione sperimentale dei girini alla presenza del gambero durante lo sviluppo, ha rivelato che questi sono in grado di modulare sia la durata dello stadio larvale che diversi tratti morfologici attraverso la plasticità fenotipica. Infatti anche nei girini esposti a *P. clarkii* è stato osservato un aumento della velocità di sviluppo; in aggiunta questi presentavano una variazione nella forma del corpo ed in particolare un aumento relativo del muscolo caudale, un tratto che conferisce ai girini migliori capacità natatorie e maggiori chance di sfuggire ai predatori. Al contrario, per quanto riguarda i tratti comportamentali, non sono state registrate significative variazioni né in relazione alla provenienza da popolazioni invase o non ancora invase, né in relazione all'esposizione al gambero.

Il secondo studio sperimentale è stato condotto su diverse specie che compongono la comunità anfibia del nord Italia (cinque urodeli e otto anuri). Per ogni specie è stato valutato quali stimoli fossero coinvolti nel riconoscimento di un predatore sconosciuto e nell'espressione della risposta anti-predatoria. A questo scopo è stata indagata la variazione di diversi tratti comportamentali in relazione a differenti stimoli rilasciati dal gambero invasivo (quattro trattamenti comprendenti: esposizione a stimoli visivi e olfattivi, soli stimoli visivi, soli stimoli olfattivi, controllo). Inoltre è stata valutata una possibile influenza sulle risposte comportamentali osservate della storia coevolutiva che alcune specie condividono con un predatore nativo simile a *P. clarkii*, come il gambero europeo (*Austropotamobius pallipes*).

I risultati hanno mostrato che tutte le specie testate hanno alterato il proprio comportamento in presenza di *P. clarkii*, anche se con una notevole eterogeneità nella modalità e nell'intensità delle risposte. Una variazione nel comportamento delle larve è stata però registrata quasi unicamente in seguito all'esposizione a stimoli visivi, mentre le risposte agli stimoli olfattivi rilasciati dal gambero sono state scarse e contradditorie. Infine la risposta comportamentale espressa dalle diverse specie non ha risentito della coesistenza di queste con il gambero europeo e quindi non vi è supporto all'ipotesi di una possibile influenza della storia coevolutiva con il predatore nativo. Al contrario le risposte comportamentali riscontrate nelle prede native (e *naif*) sono probabilmente favorite dal riconoscimento di tratti generalmente comuni ai predatori (ad esempio in risposta a grandi figure in movimento o in avvicinamento).

Questo lavoro di tesi evidenzia come gli anfibi possano esibire un elevato potenziale evolutivo ed essere in grado di modulare il proprio sviluppo attraverso la plasticità fenotipica in risposta alle pressioni selettive imposte di predatori invasivi. Inoltre l'espressione di queste risposte è spesso dipendente dal contesto ambientale ed altamente variabile tra le diverse specie. In particolare, diverse tipologie di esposizione a rischio di predazione possono comportare una notevole variazione nella risposta anti-predatoria (ad esempio induzione di risposte comportamentali a breve termine vs risposte morfologiche a lungo termine). Appare perciò evidente l’importanza di includere molteplici tratti legati alla risposta anti-predatoria e valutare attentamente le modalità di esposizione al rischio di predazione negli studi riguardanti le risposte a predatori invasivi. È inoltre importante infine sottolineare la necessità di ulteriori studi che tengano in considerazione l’espressione, ed in particolare verifichino l’efficacia, delle risposte anti-predatorie degli anfibi nativi verso i predatori invasivi, al fine di integrare queste conoscenze alle informazioni riguardanti i trend di popolazione e progettare in tal modo piani di conservazione adeguati.

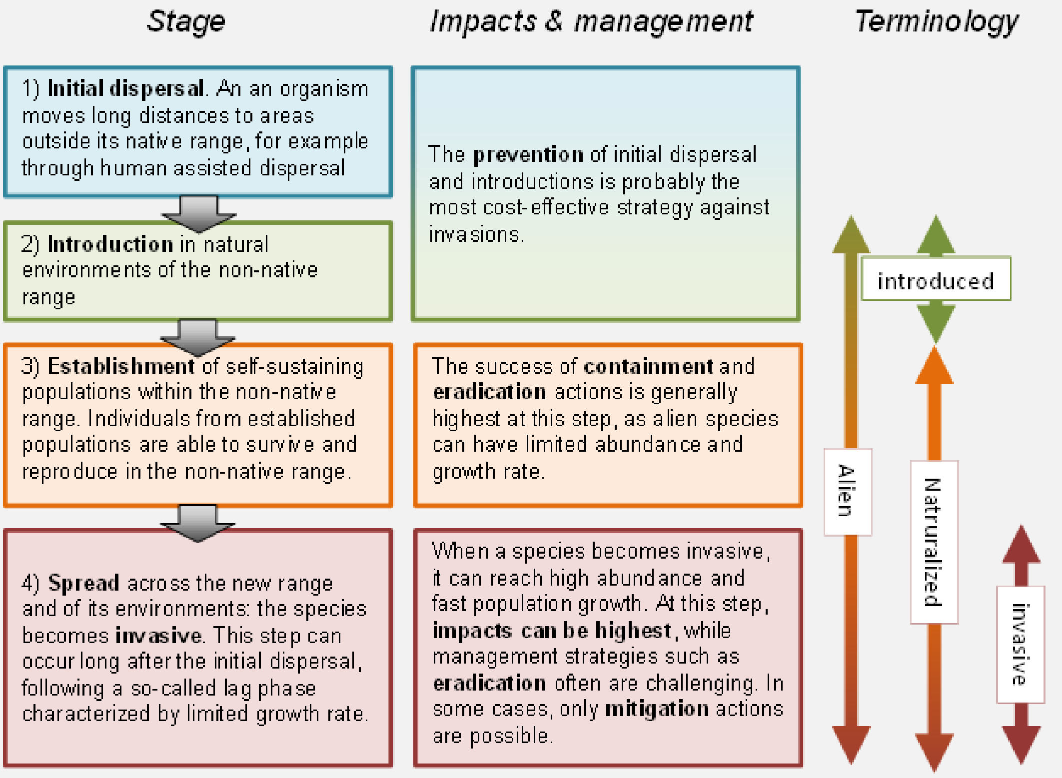
CHAPTER 1

INTRODUCTION

Invasive alien species (IAS) are among the major threats to biodiversity. For instance, more than 800 animal extinctions have been recorded since 1600, and invasive species are implicated in 54% of them (Clavero and Garcia-Berthou 2005). Biological invasions are a complex process (see Box 1 for a conceptual framework), and the impact of IAS on biodiversity is highly heterogeneous both among habitats and geographic areas, with the strongest impacts in islands and in freshwater (Strayer 2010; Spatz et al. 2017). As a consequence, approx. ~16% of amphibian species are threatened by IAS, and species living in some geographic areas (e.g. in the Australasian realm) and with specific life-history strategies (e.g. aquatic larvae) suffer a disproportionate impact by invasive species (Stuart et al. 2008).

Box 1. From introduced to invasive species: how to define a multi-step process

Describing invasions as a multi-step processes, comprising four major phases, allows a better understanding of invasions, provides a unified terminology, and also helps management, given that different approaches can have different efficiency and feasibility across stages (modified after Blackburn et al. 2011). In this review, we generally focus on invasive species, i.e. the category causing the strongest impacts on biodiversity. Nevertheless, when discussing conservation strategies, we also consider the early stages, due to the efficiency of management actions against them (Puth and Post 2005).



Stage	*Impacts & management*
1) **Initial dispersal**. An an organism moves long distances to areas outside its native range, for example through human assisted dispersal	The **prevention** of initial dispersal and introductions is probably the most cost-effective strategy against invasions.
2) **Introduction** in natural environments of the non-native range	
3) **Establishment** of self-sustaining populations within the non-native range. Individuals from established populations are able to survive and reproduce in the non-native range.	The success of **containment** and **eradication** actions is generally highest at this step, as alien species can have limited abundance and growth rate.
4) **Spread** across the new range and of its environments: the species becomes **invasive**. This step can occur long after the initial dispersal, following a so-called lag phase characterized by limited growth rate.	When a species becomes invasive, it can reach high abundance and fast population growth. At this step, **impacts can be highest**, while management strategies such as **eradication** often are challenging. In some cases, only **mitigation** actions are possible.

Despite the impact of IAS on amphibians has long been recognized (see Kats and Ferrer 2003), research on this theme somehow lagged beyond studies focusing on habitat loss, diseases and enigmatic declines. However, in the last decades interest on this topic showed a dramatic growth (Fig. 1). On the one hand, it is increasingly evident that IAS can have a broad range of impacts on amphibians, affecting species and communities through multiple processes. On the other hand, the impact of IAS can be complex and multifaceted, and often interact with other global stressors (e.g. diseases, habitat changes; (Blaustein and Kiesecker 2002; Didham et al. 2007)). The growing awareness of the impacts of IAS also stimulated research on potential conservation strategies, in order to identify management practices that could halt or limit the impact of invasives.

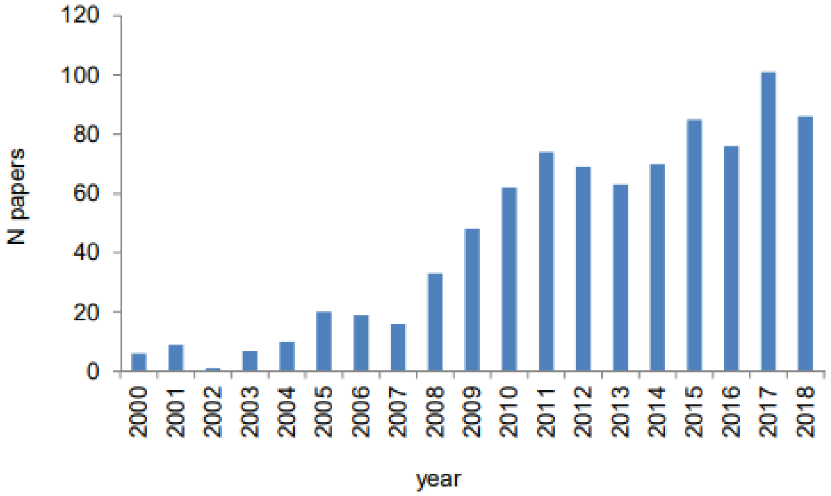


Fig. 1 - Increase of the number of papers on alien species and amphibians, since 2000. The number of papers was obtained from the ISI Web Of Science (June 2019), using the following search terms: TS=*(("invasive species" or "alien species" or "non-native") and (amphibian* or frog or frogs or salamander or salamanders or toad or toads or newt newts or caecilian or caecilians or anura or urodela or caudata or gymnophiona))*, considering the "article" category only.

In this introduction, I reviewed the recent literature to understand the impact of IAS on native amphibians and to evaluate potential mechanisms and strategies that could allow the long-term persistence of native species. First, we describe the multiple pathways through which IAS can influence amphibian populations and communities. Second, we analysed the responses of native amphibians to IAS, to assess how mechanisms such as phenotypic plasticity and rapid evolution could help native species to withstand the impact of IAS and survive even in invaded communities.

IMPACTS OF INVASIVE PREDATORS ON AMPHIBIANS

IAS can affect amphibians through a broad range of pathways including predation, competition, habitat alteration, hybridization, and spread of diseases (Kats and Ferrer 2003; Shin-ichiro et al. 2009; Bucciarelli et al. 2014; Miaud et al. 2016; Nunes et al. 2019). Among them, predation is recognised to be the most severe threat for native amphibians (Nunes et al. 2019), and has been responsible of many cases of population declines and local extinctions in the amphibian communities of invaded regions (Cruz et al. 2008; Ficetola et al. 2011a).

Amphibians with aquatic life-history stages are particularly sensitive to the introduction of predators in the environment. This occurs because many amphibians breed in freshwater ecosystems such as isolated ponds or headwater streams, where large predators are scarce (Cox and Lima 2006). Even though several amphibian species have evolved mechanisms to co-exist with predators such as fish (Van Buskirk 2003), invasive predators can drive local populations to decline or even to extinction, because they directly reduce the abundance of eggs, larvae, or adults (Kats and Ferrer 2003; Bucciarelli et al. 2014; Nunes et al. 2019). Fish are probably the most frequently introduced large predators in freshwaters, and have caused massive loss of amphibian breeding sites in all the continents (e.g. (Knapp and Matthews 2000; Knapp 2005; Tiberti and von Hardenberg 2012)). A remarkable impact is can be exerted not only by large fish, such as trout, as even small-sized fish such as the mosquitofish (*Gambusia holbrooki*) are efficient predators of amphibians (Remon et al. 2016; Miró et al. 2018). Beside fish, many other taxa can exert heavy predation pressure. For instance, carnivorous tadpoles of the Indian bullfrog (*Hoplobatrachus tigerinus*) rapidly preyed on native tadpoles, leading to no survival of larvae of the South-African frogs *Microhyla chakrapanii* and *Kaloula ghoshi* (Mohanty and Measey 2019).

Invasive predators can have impacts over broad (e.g. regional, national) scales. For example, by sampling multiple wetlands in Northern Italy, Ficetola et al. (2011a) found a negative relationship between the invasive red swamp crayfish *Procambarus clarkii* and the abundance of amphibian larvae. Amphibians spending longer periods in aquatic environments (e.g. newts) were completely absent in invaded wetlands, because of the heavy predation. This loss of suitable breeding sites is particularly alarming and caused a rapid decline of newts at the regional scale. Furthermore, other native species such as brown frogs (*Rana latastei* and *R. dalmatina*) continued to breed in invaded wetlands, still the low abundance of their larvae suggest heavy mortality because of predation, with complex effects on meta-population dynamics.

The effects of predators can be complicated by interactions with other factors. Studying the abundance of common frogs (*Rana temporaria*) in Finnish islands, Salo et al. (2010) showed that frog densities where lower in islands invaded by American mink, a highly efficient predator. Additionally, among invaded islands, frog densities where lower in less vegetated islands, indicating that the effect of mink predation was mediated by vegetation cover.

Alien predators do not impact just species occurrence and abundance, as they can also influence intra-specific variation. Paedomorphosis is a remarkable example of intra-specific variation, in which biphasic (metamorphosing) individuals coexist with fully aquatic conspecifics that do not metamorphose (paedomorphs). A long-term field study revealed that fish introductions were the main determinant of extirpation of paedomorphs of two newt species (*Ichthyosaura alpestris* and *Lissotriton graecus*) in Montenegro (Denoël et al. 2019). The decline of paedomorphs was much faster than the one of metamorphic populations, and paedomorphs have lost 99.9% of their area of occupancy in less than 70 years.

Which invasive species impact amphibians?

Negative effects on amphibians have been described for IAS from multiple taxa (Bucciarelli et al. 2014; Nunes et al. 2019). Freshwater fish are perhaps the most frequently described IAS impacting amphibians, because of their dramatic effects on aquatic life stages (Nunes et al. 2019), still IAS from all the classes of vertebrates can exert strong impacts on amphibians, with effects described for several mammals (e.g. American mink; Ahola et al. 2006)), non-native amphibians (Taylor et al. 2017), and reptiles (e.g. viperine water snake, slider turtle; (Moore et al. 2004; Ficetola et al. 2012). Conversely, literature describing the impacts of alien birds on amphibians is rather scarce, despite some invasive birds cause very severe effects on biodiversity (Kumschick and Nentwig 2010). For example, the Canada goose (*Branta canadensis*) is among the IAS with the strongest impact in Europe and can cause the eutrophication of the invaded habitat (Nentwig et al. 2018). Eutrophication can have strong consequences on amphibian populations, such as promoting pathogenic infections and affecting development and survival (Johnson et al. 2007; Peltzer et al. 2008), still evidences of the impact of Canada goose on amphibians remain scanty. Another example is the sacred ibis (*Threskiornis aethiopicus*), a large invasive bird which preys on multiple taxa, including larval and adult amphibians (Yésou and Clergeau 2005); despite this, no study assessed the actual effects of ibis on amphibians. Given impacts of alien birds exert on multiple facets of biodiversity, there is a strong need of studies focusing on the direct and indirect consequences of bird introductions on native amphibians.

Studies assessing the effects of invasive invertebrates on amphibians mostly focused on crayfishes, a group showing particularly strong impacts (Cruz et al. 2006; Gherardi 2006; Ficetola et al. 2011a; Nunes et al. 2019), while less is known about the effects of other invertebrates. For alien plants, described impacts are often indirect, for instance because they can modify habitats or food webs (Watling et al. 2011b; Rogalski and Skelly 2012; Mazerolle et al. 2014).

Finally, it must be remarked that most of these studies focused on aquatic environment (Nunes et al. 2019). However, the majority of amphibians spend their adult lifetime in terrestrial environment, and a many species (especially in tropical areas) are fully terrestrial, without aquatic life-stages (Gomez-Mestre et al. 2012). There is a remarkable lack of information on the impacts of terrestrial IAS (but see (Watling et al. 2011b; Fork et al. 2015; Ransom 2017). Given that survival of adult amphibians can have stronger impact on population dynamics than survival during early life-history stages (e.g. larvae), (Álvarez and Nicieza 2002), there is a need of more studies on the impact of terrestrial IAS (Nunes et al. 2019), with particular focus on the tropical environments where the biodiversity of amphibians is highest.

AMPHIBIAN RESPONSES TO INVASIVE SPECIES

Although it is demonstrated that biotic invasions represent a major driver of global amphibian decline worldwide, ecological responses of amphibians to invasive species and their evolutionary consequences are still relatively understudied and only in the past decades they have become to draw the attention of the scientific community. In any case, assessing capacity of amphibians to withstand invasive species is crucial to implement adequate conservation strategies.

How to recognize an alien predator?

Adaptive traits expressed during interspecific interactions are the result of coevolution between species. However, during biotic invasions, natives face species with which they did not share a coevolutionary history, and this can hinder the expression of effective responses (Cox and Lima 2006; Sih et al. 2010; Carthey and Banks 2014). Predation is probably the major threat posed by IAS to amphibians, and the most studied topic (Fig. 2A). In the context of predatory interactions, failing to recognize a predator can be fatal, still several mechanisms can allow native species identifying IAS as predators and activating adequate responses (Gonzalo et al. 2007; Ferrari et al. 2010a; Davis et al. 2012; Wilson et al. 2018). For example, predator recognition can occur when the IAS shows similar traits or are pylogenetically close to native predators. This pattern was observed for the San Marco salamander (*Eurycea nana*), in which predator-naïve individuals coexisting with native largemouth bass show behavioural response (activity reduction) when exposed to chemical cues of non-native perciform fish (Davis et al. 2012). In freshwater environments, amphibians often detect predators through chemical signals (Ferrari et al. 2010a; Manenti et al. 2016), and a prey might thus identify non-native predators when the kairomones of the invasive species are associated with alarm cues released by preyed conspecifics, or with predator dietary cues (Fig. 2B). Association with alarm cues can be surprisingly effective. For instance, the repeated exposure to a combination of conspecific alarm cues and chemical cues of non-predatory zebrafish elicited in tadpoles of Iberian green frog (*Rana perezi*) a remarkable anti-predator response (activity reduction) to subsequent presentation of zebrafish cues only (Gonzalo et al. 2007). Finally, anti-predator response towards IAS might be mediated by neophobia (Brown et al. 2013) or simply through generic risk cues (e.g. avoidance of large-sized moving shapes) (Wilson et al. 2018).

Some analyses failed to observe any response of amphibians to IAS (Fig. 2). Nonetheless, many experimental studies only tested the effects of one or a few cues, while in nature patterns can be more complex. For instance, the inability to respond to the kairomones of an invasive predator observed in laboratory experiments can be compensated in nature by other chemical signals (e.g. alarm or digestion cues). Moreover, in some habitats, ecological conditions (e.g. composition and variability of predator community) can increase species ability to respond to novelties, such as invasive predators (Ferrari et al.

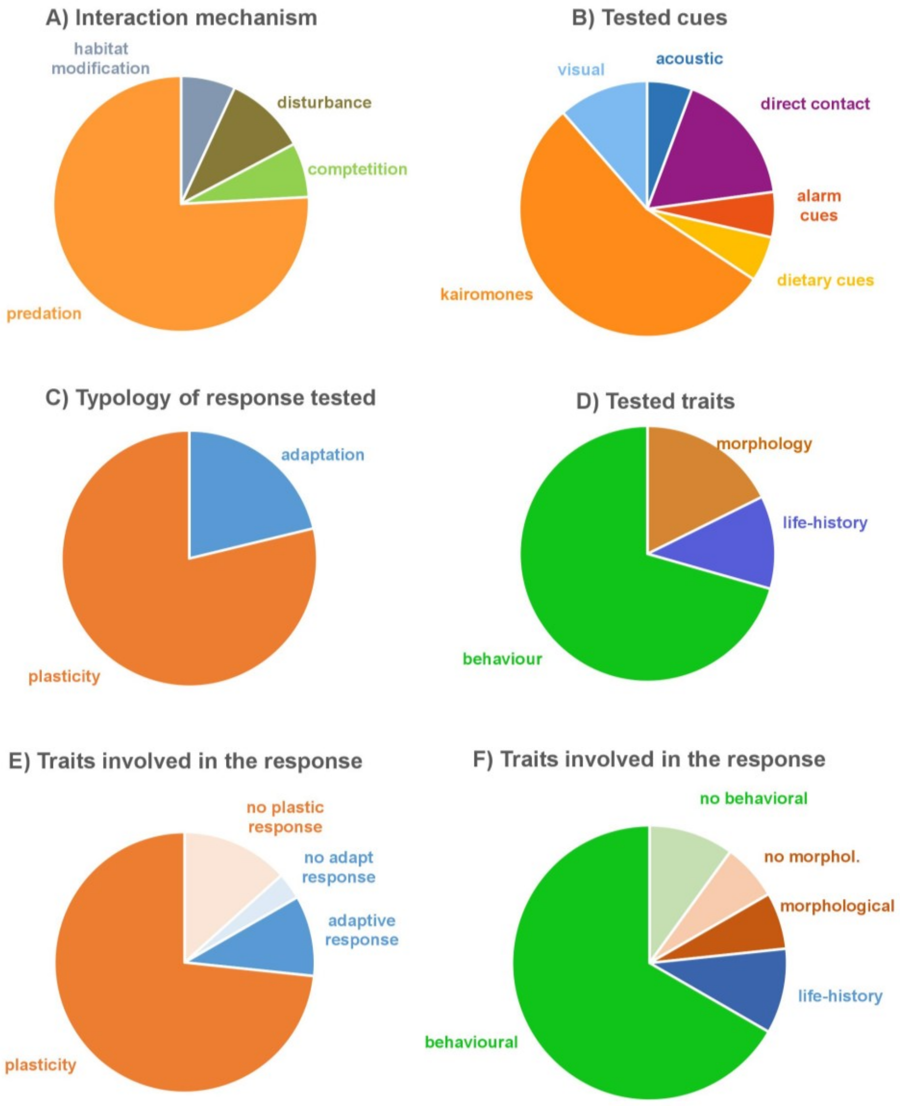


Fig. 2 - Amphibian responses to biological invasions. A) Typology of tested interactions between IAS and native amphibians; B) cues potentially involved in the response. C) frequency of studies assessing phenotypic plasticity and local adaptations in native amphibians, in presence of IAS; D) frequency of studies considering different typologies of traits in native amphibians; E-F) frequency of responses effectively detected in native amphibians. Results are based on the analysis of 99 papers, obtained through the ISI Web of Science (August 2019) using the following search term: TS=*(("invasive species" or "alien species" or "non-native") and (amphibian* or frog or frogs or salamander or salamanders or toad or toads or newt newts or caecilian or caecilians or anura or urodela or caudata or gymnophiona) and ("response" or "defence*") and ("phenotypic plasticity" or behaviour or behavior or "life history" or "rapid evolution" or "contemporary evolution" or "rapid adaptation" or "habitat shift"))*. Results here presented are based on a subset of 26 studies actually testing for amphibian responses.

2015), still knowledge of these mechanisms remains scarce requires investigation in future studies.

The forefront of defence: phenotypic plasticity

Phenotypic plasticity (i.e. the capacity of given genotype to express different phenotypic responses under diverging environmental conditions; Pigliucci 2001) is a key evolutionary mechanism that allows species to persist under unpredictable conditions. For these reason, plasticity can represent a key defence against alien species (Peacor et al. 2006; Berthon 2015). Plasticity in amphibians is well documented, as these organisms are frequently subject heterogeneous and variable ecological pressures (Wells 2007), and IAS often induce plastic responses in native amphibians (Fig. 2C-E). Invasive predators can trigger the activation of multiple inducible defences, including behavioural (e.g. reduced activity, avoidance, microhabitat shift; Gamradt et al. 1997; Nunes et al. 2013), morphological (Nunes et al. 2014b) and life-history shifts (e.g. faster growth or development rate; Nunes et al. 2019; Smith and Harmon 2019) (Fig. 2B). For instance, some populations of *P. perezi* developed deeper tail muscles when reared in presence of invasive crayfish, a trait that can favour faster swim and escape from predators (Dayton et al. 2005; Nunes et al. 2014b), while exposure to an invasive fish (*Gambusia affinis*) elicited faster development in treefrog (*Hyla versicolor*) tadpoles, which metamorphosed and left risky mesocoms sooner compared to control (Smith and Harmon 2019).

Phenotypic plasticity can offer advantages facing both invasive predators and competitors and can help to overcome disturbance on mating or habitat modifications (Watling et al. 2011a; Caut et al. 2013), broadening the range of conditions under which native amphibians can survive (Peacor et al. 2006; Polo-Cavia and Gomez-Mestre 2014; Hossie et al. 2017). Finally, plasticity can offer natural selection a pool of variability and can favour the emergence and the fixation of new adaptive phenotypes through genetic assimilation/canalization (Levis et al. 2018), which could help the long-term persistence of amphibians facing biotic invasions (Peacor et al. 2006; Berthon 2015).

Behavioural responses to invasive species

Behaviour is among the traits showing more rapid variations, and can be both modulated through plasticity according to the environmental conditions experienced. Behavioural responses thus often constitute primary resources when facing fast environmental changes, such as biological invasions (Sih et al. 2010; Sih et al. 2011; Weis and Sol 2016). It is no surprise that a large amount of research has focused on behavioural response of native species, particularly when facing invasive predators (Fig. 2D). In amphibians, common anti-predator behaviours include activity variation or reduction (e.g. to limit predator exposure), space use modification (predator avoidance), shift in micro-habitat (reduction of niche overlap) and aggregation (risk dilution) (Wells 2007). Several studies demonstrates the expression of these behavioural responses in native amphibian larvae exposed to alien predators (Caut et al. 2013; Nunes et al. 2013; Nunes et al. 2014b; Polo-Cavia and Gomez-Mestre 2014), even though the responses to invasive predators can be weaker than the ones to native predators (Nuñez et al. 2019). Nonetheless, in some cases elicited responses proved to be effective: for instance, toad tadpoles that recognize invasive predators reduce activity levels, and such anti-predator behaviour increases their survival (Polo-Cavia and Gomez-Mestre 2014). Fewer studies investigated anti-predator responses in adult amphibians (Winandy and Denoël 2013b; Winandy et al. 2016). Interestingly, Winandy and Denoël (2013b) found that newts exposed to goldfish spend more time in refuges to reduce predation risk, but consequently decreased their courtship activity, with complex impacts on their breeding dynamics. Apart from predation, amphibians can adjust their behaviour also in response of other interactions with IAS, such as disturbance, habitat modification, competition and reproductive interference. For example, males of the marble frog (*Limnodynastes convexiusculus*) adjusted their calls in presence of invasive cane toads, reducing frequency and matching cane toad calling pauses, in order to reduce energetic-expensive overlap with the large invasive toads (Bleach et al. 2015).

What does not kill you make you stronger:

strong selective pressure can foster rapid adaptation

Biotic invasions can be viewed as a global, unintended experiment unravelling mechanisms of natural selection (Strauss et al. 2006; Weis and Sol 2016). Rapid adaptation often occurs when a species is exposed to a strong novel selective force, and has been documented in multiple taxa exposed to alien species. In amphibians, rapid adaptation in response to invasive predators has been described in multiple systems (Kiesecker and Blaustein 1997; Moore et al. 2004; Nunes et al. 2014b; Fig. 2C). For instance, Nunes et al. (2014b) maintained under common conditions green frog tadpoles originating from populations invaded in different time periods by alien crayfish, and uninvaded populations. Long-term invaded populations (~30 years) showed constitutive anti-predator behaviour (constant low activity), and were capable to express morphological defences when exposed to red swamp crayfish, indicating that rapid evolution has occurred in these invaded populations. Nevertheless, reported cases of rapid adaptation to invasive predators in amphibians remain limited, and more studies are needed to ascertain the presence of rapid evolutionary processes and to unravel mechanisms underlying these responses (e.g. identify genetic modifications involved in such adaptations).

Can responses improve amphibian persistence face to invasive species?

Despite many studies show that amphibians can readily respond to biological invasions (Fig. 2), responses to IAS are complex and their outcomes are often context-dependent. In some cases, expressed responses increase amphibian survival and coexistence with invasives (Polo-Cavia and Gomez-Mestre 2014), still most of studies so far assessed whether and how amphibians respond to IAS, while the effectiveness and the costs of responses are rarely tested. In some instances, it is evident that selection has rapidly favoured the fixation of advantageous traits, and this probably helps populations to withstand IAS for long periods (Kiesecker and Blaustein 1997; Nunes et al. 2014b). On the other hand, declines observed in many invaded populations suggest that amphibian responses are often insufficient to face the strong pressures by IAS. Nevertheless, assessing the effectiveness of responses under natural conditions is particularly complex, because multiple abiotic and biotic factors act together in a context-dependent fashion (Blaustein and Kiesecker 2002). Despite these difficulties, understanding whether responses to IAS can help long-term persistence of invaded populations is a key challenge, because it is essential to predict the consequences of invasions across species and habitats, and to identify the conditions under which management actions are the priority, or are most likely to be successful.

AIMS OF THE THESIS

After the seminal review by Kats and Ferrer (2003), we have seen an impressive growth of research on IAS threatening amphibians (Fig. 1; Nunes et al. 2019), and our understanding of their multifaceted impacts is increasingly deep. Nevertheless, multiple open questions remain to be answered. It is evident that native species can show some response to IAS (Fig. 2), but identifying e.g. a behavioral shift in presence of invasive predator does not mean that tadpoles will be able to survive. Even though measuring fitness in nature remains a major challenge of evolutionary biology and ecology, we have now an increasingly complete toolbox (e.g. Dodd 2010) that can allow tackling this challenge.

In this thesis I sought to contribute improving current knowledge about the capability of amphibians to respond to novel and abrupt ecological pressures which they are increasingly exposed as a consequence of biological invasions.

In this regard, I aimed to shed light on the relative role and the interplay between trait adaptability and phenotypic plasticity in shaping anti-predator responses of amphibian larvae facing of invasive predators. Moreover, I examined how novel and extant selective pressures can interact in driving amphibian traits variation in the invaded ecosystems and looked into fitness consequences these shifts can produce in native species. In addition, I devoted special attention in evaluating which are the evolutionary mechanisms that can allow novel predator recognition and which is the variability in the intensity and typology of responses depending on the heterogeneity among species and environmental contexts.

These main issues are addressed through three main study investigating response of amphibian larvae to an invasive and voracious predator, the American red swamp crayfish (*Procambarus clarkii*), which represents a severe predatory pressure for invaded freshwater communities worldwide.

In the first study of this thesis work (chapter 2) I show how predation pressure imposed by an invasive predator on tadpoles of a native frog can foster rapid adaptation in a key ontogenetic trait, both discussing consequences on adaptive divergence among invaded populations and evaluating fitness implications on post-metamorphic stages.

The second study (chapter 3) focuses on the role phenotypic plasticity can have in favouring the capability to express responses towards novel selective pressures and how plasticity can act on a broad range of traits differently inducing them depending on the experienced context.

Finally, in the fourth chapter, by means of a multi-species approach, I show how behavioural plasticity allow naïve prey to respond to novel predators through a wide variety of anti-predatory responses, simultaneously shedding light on possible proximate and ultimate causes promoting their expression.

CHAPTER 2

RAPID ADAPTATION TO INVASIVE PREDATORS OVERWHELMS NATURAL GRADIENTS OF INTRASPECIFIC VARIATION

Andrea Melotto[1*], Raoul Manenti[1], Gentile Francesco Ficetola[1,2]

Abstract

Invasive predators exert unprecedented selective pressure over native populations. At the same time, native species are often subjected to natural environmental gradients, which determine intraspecific phenotypic variation. Selective pressures by the invaders are expected to interfere with natural gradients causing rapid adaptive shifts, still understanding such interplay remains challenging. Before and 14 years after the invasion by an alien crayfish, we reared frog tadpoles in common garden conditions to assess how invasive predators interact with extant climatic gradients to determine variation in development rate. Before the invasion, life-history divergence existed among populations exposed to different climatic regimes, with fastest intrinsic development rate in tadpoles from cold areas. Crayfish invasion changed life-history variation in frogs, becoming the dominant driver of intrinsic development rate. Frog populations invaded by the crayfish consistently reduced the time to attain metamorphosis through both constitutive and plastic responses. Ontogenetic acceleration to escape predators was not without a cost, as after metamorphosis fast developing froglets had small size, short hind limbs and poor jumping performance. Selection by invasive species can be stronger than natural environmental gradients, disrupting extant adaptive divergence and causing complex consequences on lifetime fitness.

Keywords: invasive species, amphibian development, inducible defences, rapid evolution, carry-over effects

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Introduction

Invasive alien species (IAS) are a major driver of global biodiversity loss (Mack et al. 2000; Lockwood et al. 2013; Bellard et al. 2016). The decline of native species is often attributed to the lack of common evolutionary history with invasive organisms, which can determine the lack of effective responses (Cox and Lima 2006; Sih et al. 2010). On the other hand, the abrupt selective pressure exerted by invaders can promote the rapid expression of traits improving fitness during interactions with IAS. Such responses include phenotypic plasticity (e.g. developmental, behavioural plasticity), and may lead to prompt adaptations in native species (Skelly and Freidenburg 2000; Phillips and Shine 2006; Nunes et al. 2014b; Cattau et al. 2018).

Nevertheless, native species often inhabit heterogeneous landscapes, and populations exposed to diverging selective pressures can show local adaptations, which allow them to cope with the different environmental challenges and to improve fitness. The new selective forces exerted by IAS are expected to interact with the pressures imposed by the extant environmental context (Leger and Goergen 2017), thus the effectiveness and long-term consequences of evolutionary responses to IAS remain difficult to predict (Mooney and Cleland 2001). To date, research assessing the evolutionary response of native species to IAS rarely take into account the existing environmental heterogeneity and the presence of local adaptations in invaded populations. This is probably related to the complexity of disentangling multiple selective forces. Long-term studies, comparing species responses before and after the introduction of IAS, can provide key insights on how interactions between multiple selective forces can shape evolutionary trajectories.

Development time is a critical life-history trait in ectotherms, and shows both plastic and canalized variation in response to environmental pressures. On the one hand, ectothermic metabolism slows down at cold temperatures, and this is expected to determine slower development. Therefore, populations living in cold environments often evolve faster intrinsic development time, which allows them to partially counteract the dampening effect of low temperature (counter-gradient variation (Berven 1982; Conover et al. 2009). On the other hand, predator presence often selects for a fast development time, which can reduce exposure to predators (Chivers et al. 1999; Kiesecker et al. 2002; Urban et al. 2017). As development time can respond to multiple selective forces with complex patterns, it can be an excellent trait to evaluate the interplay between natural selective gradients, and the pressure by IAS.

Complex life cycles, with larvae strongly different from adults, are pervasive in animals. In these organisms, the analysis of life-history traits is complicated by trade-offs in trait expression between stages, as responses to selective pressures experienced at early stages can cause carry-over effects at later stages (De Block and Stoks 2005). For instance, a fast development in larvae can reduce the time available to harvest trophic resources, thereby limiting investment in morphological structures (e.g. body size, muscles, fat reserves…), with potential fitness consequences after metamorphosis (De Block and Stoks 2005; Relyea 2007).

Here we evaluated whether selective pressure exerted by IAS can produce rapid adaptation in development rate, a trait showing strong variation among populations of native species. To this extent, we assessed how IAS altered the pattern of intra-specific variation in populations of the Italian agile frog, *Rana latastei* exposed to diverging environmental gradients. This frog inhabits both lowland and foothill sites, where tadpoles are exposed to different climatic regimes (cold climate in foothills; Fig. 1B). When reared at the same temperature conditions, individuals showed significant differences in intrinsic developmental time across populations, with tadpoles from cold foothills reaching metamorphosis earlier, as expected under a pattern of counter-gradient variation (Ficetola and De Bernardi 2005). Such adaptive variability was recorded in 2003, immediately before the invasion by the red swamp crayfish (*Procambarus clarkii*). This generalist predator is among the "100 worst" alien species in the world (Nentwig et al. 2018), and determines dramatic predation pressure on aquatic amphibians (Gamradt and Kats 1996; Cruz et al. 2008; Ficetola et al. 2011a). Amphibians can show both plasticity and rapid adaptation in response to recent environmental changes, which can help them to withstand novel challenges (Skelly and Freidenburg 2000; Wells 2007). By repeating common-garden analyses of intrinsic development time before and after IAS invasion (i.e. over 14 years), we evaluated how two distinct selective forces (climatic heterogeneity and invasive predators) interact to determine changes of adaptive variation across space. Given the heavy crayfish predation on frog tadpoles, we predict that selection favours fast development of tadpoles after crayfish invasion. Furthermore, if invasive predators now are a dominant selective force across the landscape, we predict that they would overwhelm the role of climatic gradient in determining among-population variability. Finally, we expect variation in development time induced by the invasive crayfish rate to have costly consequences on post-metamorphic traits and froglet performance.

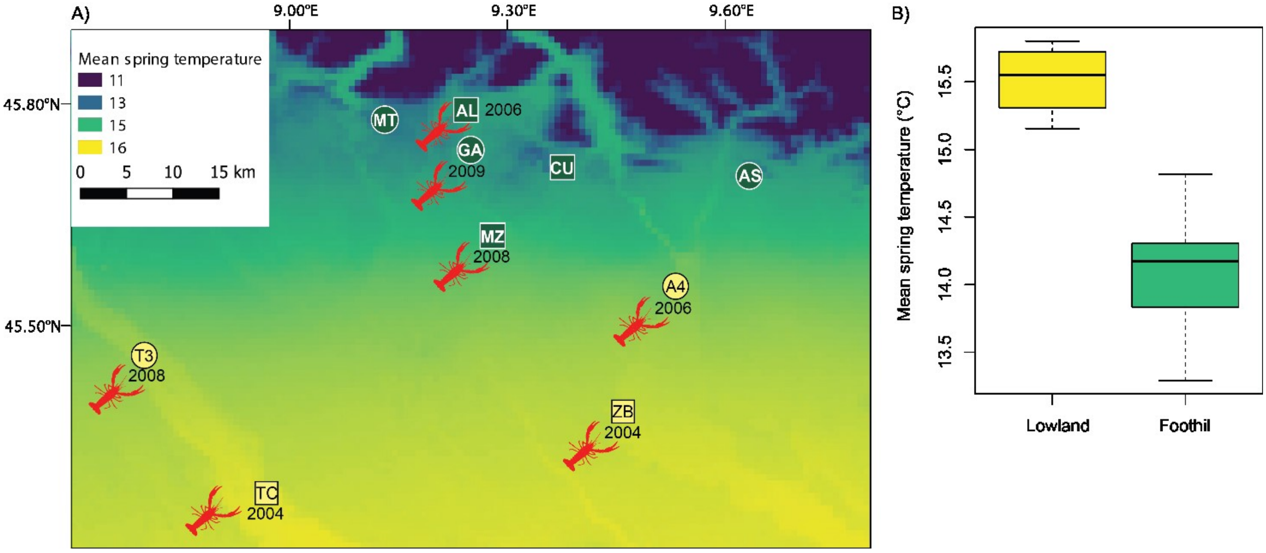

Figure 1 ¬ A) Climatic regime and invasion status in the study area. Squares represent frog populations analysed before crayfish invasion (2003), whereas circles indicates populations added for analyses performed after crayfish invasion (2017). Invaded populations are labelled by a crayfish and for each of them we report the year of first detection of the crayfish. Foothill populations are in dark green; lowlands populations are in pale yellow. The background map also show average spring temperature, from warmer lowlands to colder foothills. B) Boxplot representing differences in spring temperature between in foothill and lowland breeding sites.

Results

Climatic regime determined development differences between populations before crayfish invasion. A common garden experiment was performed before the crayfish invasion in the study area, to measure differences in development time between frogs from diverging climatic regimes. Time to metamorphosis was compared between tadpoles originating from three foothill and two lowland populations (Fig. 1A). Before the invasion, the intrinsic development time of tadpoles from foothill populations was significantly faster than in lowland populations (mixed models, *P* < 0.001; Table 1a); under common environmental conditions foothill tadpoles reached metamorphosis on average 4.1 days earlier (Fig. 2A). This probably occurred because spring temperature was on average 1.4°C colder in foothills, compared to lowland sites ($t_1 = 3.97$; $P = 0.005$; Fig. 1B). The fastest intrinsic development time in foothill populations was therefore interpreted as an evolutionary adaptation to the cold climate (counter-gradient variation) (Ficetola and De Bernardi 2005).

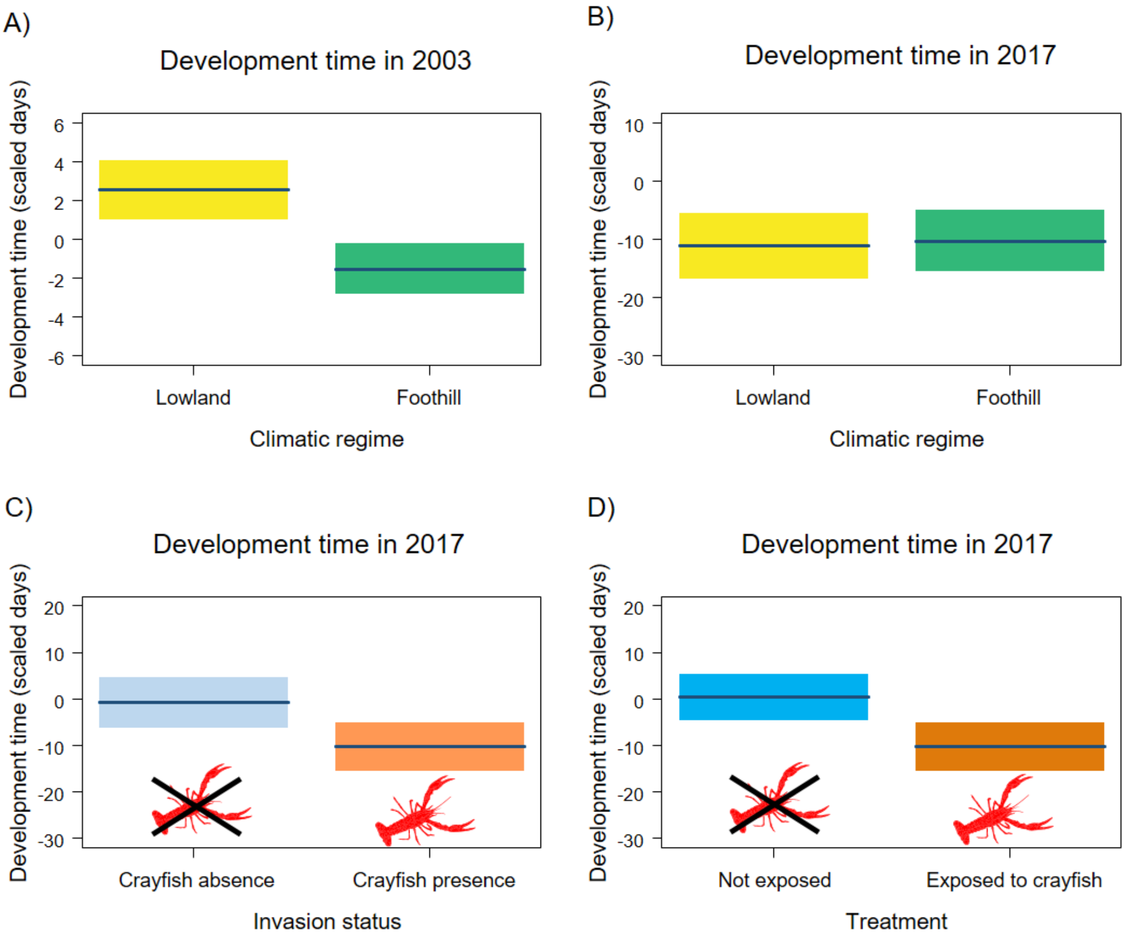

Figure 2 ¬ Factors affecting development time of frog tadpoles. Conditional partial residual plots showing the effect of (A) climatic regime before crayfish invasion; (B) climatic regime after crayfish invasion; (C) invasion status of the population and (D) crayfish exposure during rearing on development time (calculated as the scaled days from Gosner stage 25 to metamorphosis). Shaded areas are 95% confidence bands. In panel A, n = 180 tadpoles; in panels B-D, n = 169 tadpoles.

Crayfish invasion is now a major driver of developmental variation. In the early 2000s, the invasive crayfish was first detected in southern Lombardy (Groppali 2003; Fea et al. 2006), and since then it spread northwards with strong impacts on multiple amphibian species, including agile frogs (Ficetola et al. 2011a). To assess how the new selective pressure posed by the invasive crayfish affected frogs, and how this interacted with the extant selective forces, we repeated the analysis of larval development time 14 years after the onset of the invasion. Among the nine populations analysed in 2017, all the lowland populations and half of the foothill populations were invaded between 2004 and 2009, while the invasive crayfish is still absent in the remaining foothill populations (Fig. 1A). To test the possibility of predator-induced phenotypic plasticity, in this post-invasion experiment, tadpoles were also randomly assigned to two treatments: absence vs. non-lethal presence of the invasive crayfish in the container during rearing.

After crayfish invasion, differences in intrinsic development time between foothill and lowland populations were not significant anymore ($P = 0.786$; Table 1b, Fig. 2B). Conversely, we found significant differences in development time in response to the invasive crayfish. Intrinsic development time was significantly faster in tadpoles from invaded sites ($P = 0.005$; Fig. 2C), suggesting that the novel predation pressure acted as a strong selective force inducing an abrupt shift in the intrinsic development time, becoming a dominant driver of the length of larval stage and overcoming the role of the natural environmental gradient. Furthermore, development time was faster in tadpoles exposed to crayfish ($P < 0.001$; Fig. 2D; no significant interactions with invasion status), suggesting that tadpoles activate an anti-predatory response by modulating their developmental time. Finally, development time was significantly faster in containers where few tadpoles survived until metamorphosis ($P = 0.014$), in agreement with known effects of intraspecific competition (Edge et al. 2016).

Development acceleration induced by invasive predators leads to costly carry-over effects. To assess potential carry-over effects of larval anti-predator strategies, we evaluated whether fast larval development affects the variation of multiple traits after metamorphosis (body length, tibiofibula length and maximum jumping distance). A fast larval development lead to froglets with small body size (*P* < 0.001; Table 2, Fig. 3A) and short hind limbs (*P* < 0.001; Fig. 3B). Froglets with short development time also showed poor locomotory performance, as maximum jumping distance increased in froglets with late metamorphosis (*P* < 0.001; Table 2c).

Fixed effects	F	df	P
a) 2003: before crayfish invasion			
Climatic regime	17.52	1, 16.3	**<0.001**
N of siblings	1.48	1, 28.4	0.234
b) 2017: after crayfish invasion			
Climatic regime	0.07	1, 51.0	0.786
Invasion status	8.52	1, 50.2	**0.005**
Crayfish exposure	15.90	1, 160.6	**< 0.001**
N of siblings	6.13	1, 144.9	**0.014**

Table 1 ¬ Factors determining development time of agile frogs, before (a) and after (b) crayfish invasion: results of generalized linear mixed models. All models included the climatic regime (lowland vs. foothill) as a fixed factor. Experiments performed in 2017 included two additional factors: crayfish invasion in the wetland of origin, and crayfish exposure (presence of crayfish in the container during rearing). All models included the *N* of siblings in the container, to take into account potential effects of tadpole density. Sample size was not identical among treatments, thus degrees of freedom can be not integer. Significant effects are in bold.

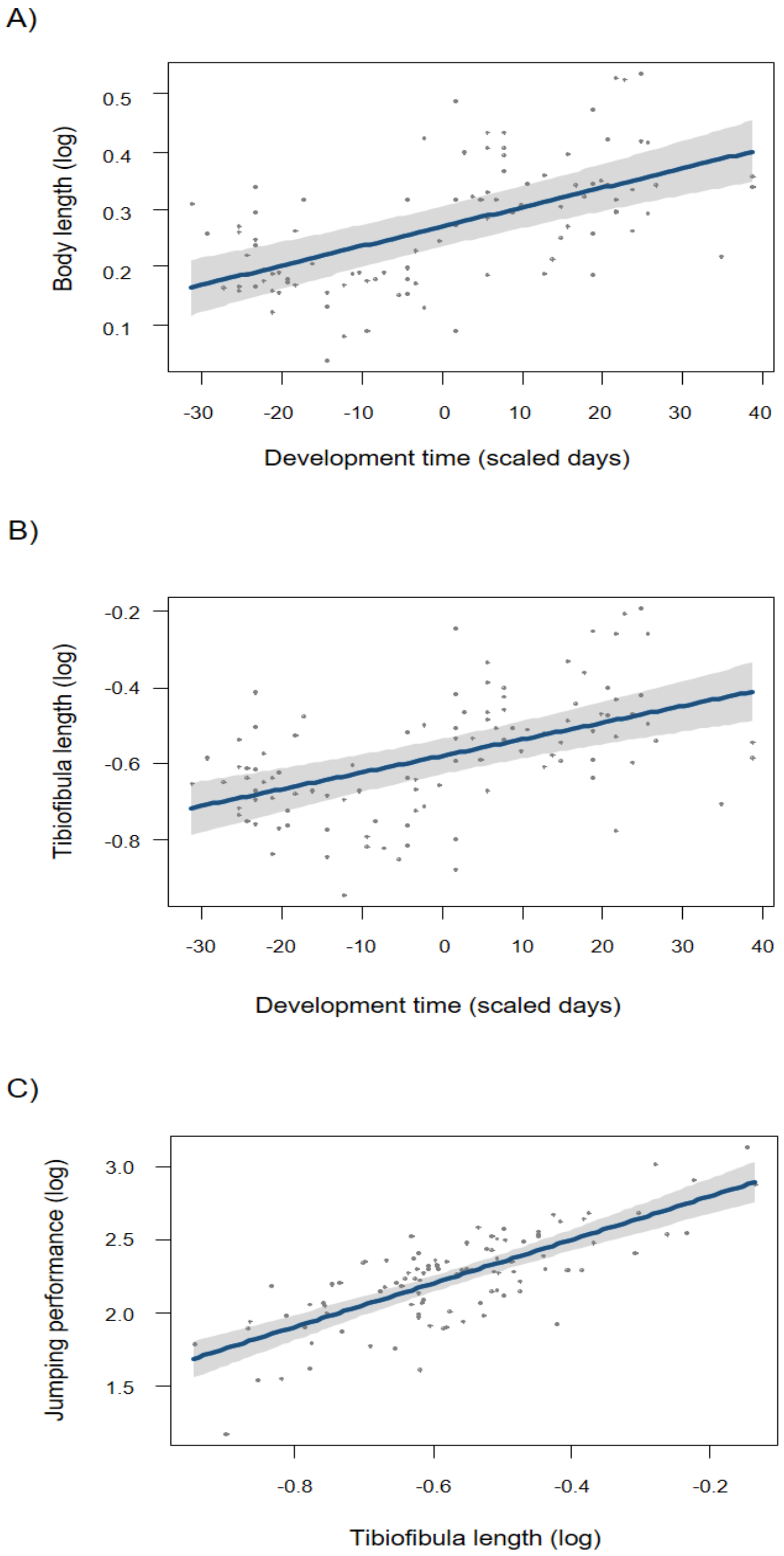

Figure 3 ¬ Factors affecting post-metamorphic traits of froglets. Conditional partial residual plots showing the influence of development time on (A) froglet body length and (B) tibiofibula length, and (C) the effect of tibiofibula length on maximum jumping distance. Shaded areas represent 95% confidence bands. n = 110 froglets.

This occurred because froglets with longer tibiofibula were able to perform longer jumps (*P* < 0.001; Table 2d, Fig. 3C); after taking into account the effect of tibiofibula length, the relationship between development time and jumping performance was not anymore significant (*P* = 0.562).

Finally, we used structural equation models to evaluate the overall consequences of the invasive crayfish (considering both local adaptations and plasticity) on post-metamorphic traits, and how these shifts are mediated by ontogenetic acceleration (Fig. 4). Both crayfish invasion at breeding sites, and exposure to crayfish during development, determined a short larval development time. This, in turn, negatively affected both body and tibiofibula length of froglets which resulted in poor jumping performance of froglets (Fig. 4). Additional complex relationships existed between crayfish exposure, invasion status and post-metamorphic traits, with tadpoles from invaded populations and exposed the crayfish showing slightly larger post-metamorphic traits if the differences in development time were taken into account (Fig. 4; see also Table S1, Supplementary material S1).

Post-metamorphic trait		Fixed effects	F	Df	P	R^2_m	R^2_c
a)	Body length	Development time	29.07	1, 98.2	**<0.001**	0.22	0.31
b)	Tibiofibula length	Development time	39.92	1, 101.3	**<0.001**	0.28	0.40
c)	Max jumping distance	Development time	20.00	1, 105.6	**<0.001**	0.15	0.24
d)	Max jumping distance	Development time	0.34	1, 87.3	0.562	0.55	0.58
		Tibiofibula length	98.34	1, 99.0	**<0.001**		

Table 2 ¬ Effect of development time on post-metamorphic traits of Italian agile frogs: (a) body length; (b) tibiofibula length; (c) maximum jumping distance; (d) combined effect of development time and tibiofibula length on maximum jumping distance. For all tests we report both marginal and conditional determination coefficients (R2m and R2m, respectively (Nakagawa and Schielzeth 2013)). Significant effects are in bold.

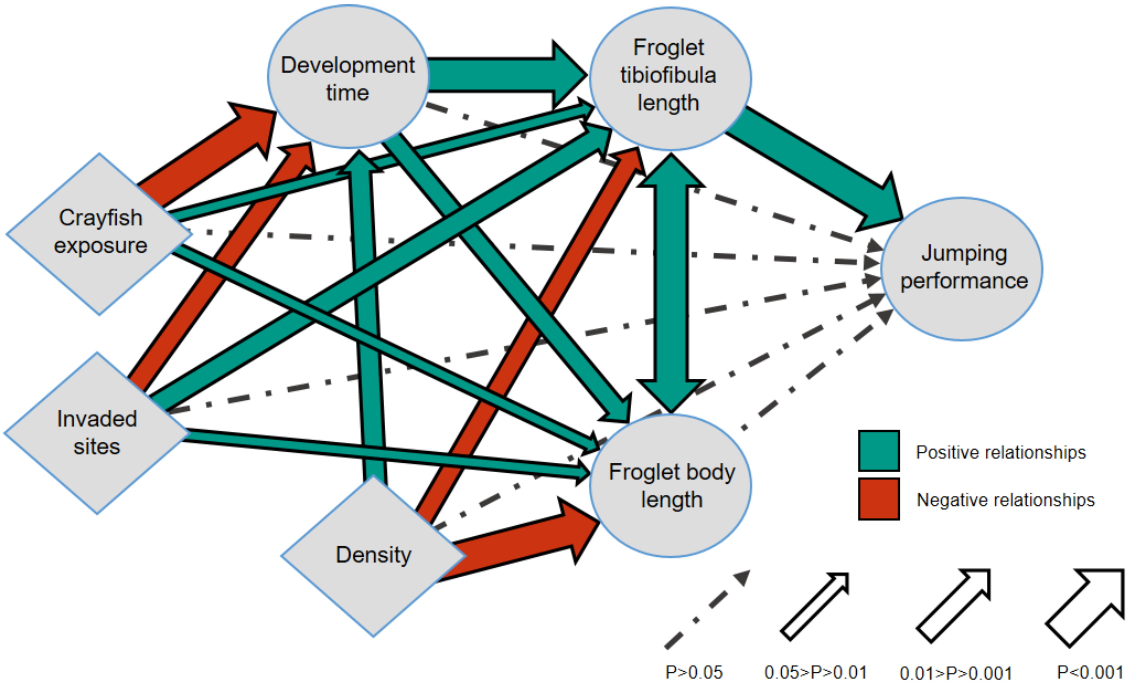

Figure 4 ¬ Structural equation models showing the overall effects of invasive crayfish on tadpole development. Diamonds represents fixed effects, while circles indicate dependent variables. Significance levels of relationships are indicated with arrows. Positive relationships are in green, while negative relationships are in red. The number of sibling per container (Density) was included as a covariate, to take into account the effects of tadpole density. See Supplementary material S2 for coefficients and exact significance values of the paths.

Discussion

Alien species impose severe selective pressures on the invaded ecosystems, interacting with natural selective forces and fostering both plasticity and fast evolution in native species (Mooney and Cleland 2001; Gillis and Walsh 2017). The new predatory pressure posed by the invasive crayfish was strong enough to disrupt in just 8-14 years (3-6 generations) the pre-existing local adaptations of a frog to the regional climatic regime, determining a faster development in tadpoles from invaded sites. However, this development acceleration was not without a cost, as the short larval duration lead to cascading carry-over effects after metamorphosis, such as smaller size and reduced locomotor performance.

Temperature strongly affects amphibian ontogenesis (Wells 2007) and spatial variation of climate often determines adaptive shifts at both species and population level (Berven 1982; Skelly and Freidenburg 2000). Before the crayfish invasion, Italian agile frog populations showed significant divergence for intrinsic development time (Fig. 2A), in response to the existing gradient of temperature (colder climate in foothills; Fig. 1B). Such pattern suggests that climate was a major selective force, and counter-gradient selection caused clear local adaptations, with foothill populations showing a faster development to counteract environmental constraints. However, starting from 2004 the invasive crayfish spread across the study area (Fea et al. 2006), successfully colonizing frog breeding sites and representing an unprecedented selective pressure. Crayfish invasion was particularly intense in lowlands (Manenti et al. 2014) and, after the invasion, differences in development time between foothill and lowland populations were not evident anymore. Instead, we observed a faster development in tadpoles originating from sites invaded by *P. clarkii*, which, on average, metamorphosed ten days before tadpoles from uninvaded sites. Amphibians show an exceptional variety of larval anti-predatory strategies, and their expression can strongly depend on the context they experience (Relyea 2007; Edge et al. 2016; Hossie et al. 2017). A rapid development can allow tadpoles to metamorphose earlier and thus reduces exposure to predators, a strategy particularly relevant where predation pressure is very high (Relyea 2001a; Kiesecker et al. 2002; Urban et al. 2017). The shortening of intrinsic development time suggests that populations underwent quick adaptations in response to the predator pressure imposed by the alien crayfish. This pattern is in agreement with recent evidence of fast evolutionary changes in native species after biotic invasions (Phillips and Shine 2006; Langkilde 2009; Nunes et al. 2014b; Stuart et al. 2014; Thawley and Langkilde 2017), even though our data cannot fully exclude a role for epigenetic or maternal effects (but see also Supplementary material S2).

Beyond the strong differences between invaded and uninvaded populations, development time also shortened when tadpoles were reared in presence of the invasive crayfish, with a consistent pattern across populations. During rearing, exposed tadpoles perceived crayfish presence through both visual and chemical cues, which represent key signals allowing predator detection and modulation of anti-predator responses in aquatic species (Mathis and Vincent 2000; Chivers et al. 2001; Wisenden 2003; Hettyey et al. 2012). The faster development of exposed individuals indicates that tadpoles were able to recognize crayfish cues as a predatory threat, and thus trigger a plastic ontogenetic shift in response to perceived predation risk. Prey are generally able to identify their native predators (Lima and

Dill 1990; Kats and Dill 1998), but the recognition of non-native predators can be more challenging (Gomez-Mestre and Díaz-Paniagua 2011; Hettyey et al. 2016). Responses to invaders can occur if they produce signals (e.g. morphology, chemical cues) shared with similar or related native predators (Ferrari et al. 2007; Epp and Gabor 2008; Davis et al. 2012). For instance, the response of agile frogs to invasive crayfish could arise because *P. clarkii* releases cues similar to the ones of native crayfish species, which in turn have a long coexistence history with frogs and prey upon their larvae (Gherardi et al. 2001).

Acceleration of development can be an effective escape strategy from predators, but life-history theory predicts carry-over effects, with possible trade-offs between the benefits afforded in one trait and the consequences on other traits affecting fitness (Van Buskirk and Relyea 1998; Capellan and Nicieza 2007b; Gervasi and Foufopoulos 2008). In organisms with complex life cycles, the decoupling between different life-history stages is generally incomplete and this can exacerbate the influence of early-development constraints on later stage traits (De Block and Stoks 2005; Ficetola and De Bernardi 2006). Our results show that anti-predatory responses to crayfish produced cascading effects, such as smaller post-metamorphic size and reduced locomotor performance. This can occur because of the reduced time devoted to the acquisition of trophic resources in fast-developing tadpoles (Álvarez and Nicieza 2002). In turn, small size and poor jumping performance can have profound impact on frog fitness, such as reduced survival and limited foraging ability (Altwegg and Reyer 2003; Wells 2007; Tüzün and Stoks 2018). Both the tadpoles exposed to crayfish during ontogenesis, and those originating from invaded sites, experienced a similar acceleration of development and, after metamorphosis, showed a reduced size and jumping performance (Fig. 3, 4). This suggests a consistent cost of development acceleration, independent of the triggering factors. Nevertheless, structural equation models suggested additional, complex relationships between these factors and post-metamorphic traits. When taking into account development time, individuals reared with crayfish or originating from invaded populations, showed slightly larger body size and longer legs at metamorphosis (Fig. 4, SI S2). Amphibian development is influenced by the complex interplay between genetic and environmental drivers, and larvae can show compensatory growth to limit the impact of suboptimal conditions, for instance through differential resource allocation or behavioural plasticity (Capellan and Nicieza 2007a; Relyea 2007). Such partial compensation can explain some apparently contrasting patterns, such as the slightly larger size of froglets reared with the crayfish, when the impact of development time is taken into account (Fig. 4). Overall, predicting the lifetime outcome of carry-over effects is extremely complex. In amphibians, post-metamorphic dynamics often overwhelm the consequences of processes acting on larvae on population dynamics (Vonesh 2005), and additional data are needed to evaluate the overall impact of processes across the different life-history stages.

Invasive predators have been one of the major determinants of extinctions in historical times (Clavero and Garcia-Berthou 2005; Bellard et al. 2016). When native populations persist, the selective force exerted by IAS can swiftly promote the evolution of life-history traits limiting exposure to predation (Langkilde 2009; Nunes et al. 2014b). Our study shows that IAS can pose selective pressures stronger than environmental gradients, driving rapid adaptive shifts in native species and obscuring pre-existing variation among populations exposed to diverging ecological pressures. Nevertheless, such adaptive responses can have major costs at late life-history stages, and could even determine suboptimal phenotypes in some habitats or under specific selective pressures (Thawley and Langkilde 2017). Forecasting the impacts of IAS is notoriously difficult, and is further complicated by the multifaceted adaptive responses of native species. Integrating the complexity of these responses is essential to evaluate how IAS affect population trends, and to assess their longstanding consequences.

Methods

Focal species and study area

The study area is in Lombardy (North-Western Italy, approximately 45.5 N, 9.2 E), and incudes the drainages of the Ticino (West) and Adda (East) rivers (Fig. 1). This region is characterized by a rich hydrographic network and includes both agricultural and urban areas, mainly in the southern lowlands, and hilly relieves scattered with woodlands in the north. We focused on the Italian agile frog, *R. latastei*, which is endemic of lowlands of Northern Italy and adjacent areas and is listed by the IUCN as vulnerable because of habitat loss and fragmentation, together with ongoing population declines caused by multiple factors, including invasive species (Sindaco et al. 2009). *Rana latastei* breeds in ponds and ditches within forest areas, and has a very narrow altitudinal range (from the sea level to approx. 400 m in the foothills of the Alps (Bernini 2004)). Egg-clutches are laid in March, and metamorphosis generally occurs in late spring-early summer (June-July). Previous studies have shown that lowland and foothill populations are exposed to different climatic regimes and show local adaptation to the extant environmental gradients (Ficetola and De Bernardi 2005). In this study, we considered tadpoles originating from multiple foothill and lowland populations, and covering the whole altitudinal range of the species within the study area. To compare climatic regimes between foothill and lowland habitats, we calculated the mean spring temperature (March-June) for all the collection sites from the CHELSA climatic data set at a resolution of 30 arc-seconds (roughly 900 X 650 m within the study area; (Karger et al. 2017).

The alien crayfish, *Procambarus clarkii*, is native of Western North America and was first detected in Lombardy in the early 2000s (Groppali 2003; Fea et al. 2006). Since then the crayfish has successfully colonized a large number of wetlands in the south of the study area. Natural and human-mediated dispersal caused a northward spread of the crayfish, which has colonized many studied breeding sites of *R. latastei* since 2004 (Fig. 1A). This crayfish represents a major threat for the freshwater communities of invaded ecosystems (Gherardi 2006; Cruz et al. 2008), and in the study area is causing the decline of several amphibian species (Ficetola et al. 2011a).

Ethical statement

All the experiments were performed under the authorization of Italian Ministry for Environment (DPN/17391 and Prot. N. 3383/T-A31). After metamorphosis, all the froglets were released in their site of origin. Before releasing froglets, we treated them with Virkon S to clear the eventual occurrence of pathogens and avoid any risk of disease spreading (Jussila et al. 2014; Bosch et al. 2015).

Tadpole rearing before crayfish invasion

In 2003, we reared tadpoles under common conditions to assess differences in development time between lowland and foothill populations. This experiment was performed just before the invasive crayfish colonized the study area. In early March, we collected egg-clutch fragments from five populations (three from foothill: AL CU, MZ; two from lowlands: TC, ZB; Fig. 1A). We sampled five clutches for each population; after hatch, 10 tadpoles from each clutch were randomly selected and placed in containers filled with 1.5 l of aged tap water (total: 250 tadpoles reared). Tadpoles were maintained under common laboratory conditions (12-h light-dark cycles at constant temperature of 20° C) and fed *ad libitum* with lettuce and rabbit pellets. Development time was calculated as the number of days between hatch and metamorphosis (Gosner's stage 45; fully-developed forelimbs and almost complete reabsorption of the tail (Gosner 1960)). Tadpole survival until metamorphosis was similar between lowland and foothill populations ($\chi^2_1 = 0.084$; $P = 0.771$).

Tadpole rearing after crayfish invasion

To assess the response of frogs to the selective pressure posed by the alien crayfish, we repeated the analysis of larval development 14 years after the onset of the invasion. In spring 2017, we collected 54 egg-clutch fragments from nine *R. latastei* populations (4-12 clutches per population). We sampled the same populations analysed in 2003 (except for ZB, where the species suffered local extinction) plus five additional populations (three from foothills: AS, GA, MT; two from lowland: A4, T3; see Figure 1A) in order to increase sample size. Three foothill and all the lowland populations were invaded between 2004 and 2008, while the invasive crayfish was still absent in the remaining foothill populations (Fig. 1A). Clutch fragments were hosted in individual containers at outdoor temperature until hatch.

The 2017 rearing experiment was slightly different from the 2003 one, as it was designed to detect both differences in development time between populations (considering potential effects of both climatic regime and invasion status), and plastic responses to the exposure to the invasive crayfish. At Gosner stage 25, we randomly selected six tadpoles from each clutch and photographed them to measure starting size. Tadpoles of each clutch were divided in two groups of three tadpoles (hereafter triads). Triads were randomly assigned to one of two rearing treatments: absence vs. non-lethal presence of the crayfish. Tadpoles were reared in 0.8 l containers, clustered in six 70 x 48 cm tanks filled with 34 l of aged tap water (hereafter blocks; 18 triads per block). In the crayfish treatment, tadpoles were reared in presence of one adult crayfish, which was separated from the rearing container with a plastic net. Therefore, tadpoles were constantly exposed to non-lethal visual and chemical cues released by the crayfish. Tadpoles in the control treatment were maintained under identical conditions, except for the absence of crayfish. Tadpoles were reared outdoor; tanks were shaded to mimic natural conditions. During rearing, half of the water in the experimental tanks was changed weekly and both tadpoles and crayfish were fed *ad libitum* with rabbit pellets and fish food. When reaching Gosner stage 42 (emergence of the first forelimb), we transferred tadpoles in small individual containers with 5 mm of tap water, and moved them to the laboratory, where they completed metamorphosis. Tadpole mortality was unrelated to climatic regime of origin (generalized linear mixed model: $\chi^2_1 = 1.291$; $P = 0.256$), crayfish presence in the breeding sites ($\chi^2_1 = 0.218$; $P = 0.641$) or rearing conditions ($\chi^2_1 = 0.275$; $P = 0.600$).

Post-metamorphic traits

To assess carry-over effects on post-metamorphic traits, we measured morphology and jumping performance on 110 newly-metamorphosed froglets reared during the 2017 experiment (Gosner stage 45 (Gosner 1960)). We considered two morphological traits that are known to affect survival and locomotory performance of froglets: body length and tibiofibula length (Ficetola and De Bernardi 2006; Cabrera-Guzmán et al. 2013). We photographed froglets on graph paper and measured morphological parameters from photos using ImageJ (Schneider et al. 2012). To assess jumping performance, we placed each froglet on plastic graph paper and stimulated jump by gently pushing its back with a wooden wand. We performed three trials per individual, measuring jumping distance with a ruler. As a measure of jumping performance we considered the maximum distance, since in frogs maximum jumping ability shows high repeatability and is more strongly related to feeding and escape ability than average jumping length (Heinen and Hammond 1997; Semlitsch et al. 1999; Watkins 2001).

Statistical analyses

For both the 2003 and the 2017 experiments, we used linear mixed models to assess the factors affecting development time of tadpoles. For the 2003 data, we used climatic regime (foothill / lowland) as a fixed factor, and both population of origin and clutch identity as random factors. Furthermore, development time is known to be strongly affected by intraspecific competition (Edge et al. 2016), and some tadpoles died during development. Therefore, we also included the number of tadpoles surviving until metamorphosis per container as a covariate.

We used the same models to analyse development time after crayfish invasion (in 2017). Beside climatic regime, these models included two additional factors: invasion status (invaded / not invaded populations), and crayfish exposure during rearing (absence / presence of crayfish); rearing block was an additional random factor. In preliminary tests, we also assessed interactions between invasion at the breeding site and treatment. The interaction was not significant ($F_{1,\,166.2} = 0.996$; $P = 0.320$) and excluded from final analyses.

Tadpole development can be also affected by non-genetic maternal effects; egg provisioning is a major maternal effect in amphibians (Kaplan 1998). To confirm that our results are not biased by differences in egg provisioning, we repeated analyses by including tadpole starting size (a proxy of egg provisioning) as additional covariate into models. Starting size did not show a significant effect on development time neither in 2003 (Ficetola and De Bernardi 2005) neither in 2017 (See supplementary material S2), confirming the robustness of our conclusions.

Mixed models were also used to test the effect of development time on post-metamorphic traits of froglets from the 2017 experiment, and to evaluate relationships between tibiofibula length and jumping performance. In all these models, we considered population of origin, clutch identity and rearing block as random factors.

Finally, we performed a structural equation modelling (SEM) to elucidate the complex relationships between fixed factors, development time, post-metamorphic morphological traits and jumping performance. SEM is a statistical method based on multiple regressions, which allows to test hypotheses regarding multiple causal relationships among predictors, and to estimate their role in explaining the observed variation of the dependent variable (Rosseel 2012). We included as fixed factors the invasion status and crayfish exposure; the number of siblings at Gosner stage 45 was considered as an additional covariate. We did not consider climatic regime of origin, as it did not show any effect on development time (Table 1b). In SEM analyses, just one clustering parameter can be included. We therefore built three separate SEMs using block, population of origin and clutch identity as clustering parameters. In the main results we present the SEM including block as clustering parameter, as this was the analysis showing the most conservative results. Results were highly consistent when using site of origin or clutch identity as clustering parameters (see Table S1 a-c in Supplementary material).

We performed analyses under the R environment (version 3.4.1), using lmerTest, lme4 and MuMIn packages for linear models and lavaan package for SEM analysis (Rosseel 2012). Furthermore, we used visreg package (Breheny and Burchett 2016) to generate conditional regression plots.

Supplementary material

S1 - Model including starting size as an additional covariate

Potential maternal effects on agile frog development time were assessed by including starting size in the preliminary analyses. Starting size was obtained by measuring tadpole total length at Gosner stage 25 (as tadpoles do not feed until stage 25, tadpole length at this stage is strongly related to total egg provisioning (Kaplan 1985). While influence of maternal effects on development time before the crayfish invasion were already excluded (Ficetola and De Bernardi 2005), we checked for the influence of any maternal effects in 2017 experiment by comparing models with or without considering tadpole starting size. We used starting size as a proxy of egg size because in Agile frogs eggs are strictly clumped in clutches and attempts to individually isolate the eggs individually would result in a high risk of damaging embryos (Ficetola and De Bernardi 2009; Ficetola et al. 2011b). Previous studies showed that tadpole starting size provides good information on the impact of maternal effects on tadpole development (Ficetola et al. 2011b). After hatch, tadpoles were kept under the same laboratory conditions (constant 18°C). At Gosner stage 25, tadpoles were photographed to measure starting size. We then repeated analyses using starting size as an additional covariate, to evaluate whether our results are affected by differences in egg-size related maternal effects. When we added starting size as covariate, all results remained unchanged, and we did not detect significant relationships between starting size and development time (Table S1).

Fixed effects	F	Df	P
Climatic regime	0.641	1, 57.5	0.427
Invasion status	6.579	1, 50.4	**0.013**
Crayfish expsoure	13.956	1, 157.2	**< 0.001**
N of siblings	6.953	1, 132.3	**0.009**
Starting size	0.140	1, 59.5	0.700

Table S1 ¬ Factors determining development time of agile frogs in the experiment conducted after crayfish invasion, considering starting size. The model included the climatic regime (lowland vs. foothill), crayfish invasion in the wetland of origin, and crayfish exposure (presence of crayfish in the container during rearing) as fixed factors. Besides, N of siblings in the container and starting size were included in all models, to take into account respectively of potential density and maternal effects on tadpole development time. Significant effects are in bold.

Table S2. Coefficient of structural equation models including block (a), egg-clutch (b) or site (c) as clustering parameters. Coefficient estimates, degrees of freedom, significance values, and determination coefficients are reported for each partial regression. Significant relationships are in bold.

Variances Dependent variable	Fixed effect	estimate	z	P	R^2
Jumping performance	Development time	0.035	0.691	0.49	0.596
	Tibiofibula length	**1.549**	**7.911**	**< 0.001**	
	Froglet length	-0.154	-0.413	0.68	
	Crayfish-invaded site	-0.027	-1.074	0.283	
	Crayfish exposure	0.105	1.92	0.055	
	Number of siblings	-0.054	-1.589	0.112	
Tibiofibula length	**Development time**	**0.114**	**5.674**	**< 0.001**	0.321
	Crayfish-invaded site	**0.09**	**3.745**	**< 0.001**	
	Crayfish exposure	**0.093**	**2.183**	**0.029**	
	Number of siblings	**-0.062**	**-3.096**	**0.002**	
Body length	**Development time**	**0.077**	**5.309**	**< 0.001**	0.304
	Crayfish-invaded site	**0.045**	**2.229**	**0.026**	
	Crayfish exposure	**0.06**	**2.445**	**0.014**	
	Number of siblings	**-0.037**	**-3.713**	**< 0.001**	
Development time	**Crayfish-invaded site**	**-0.55**	**-2.402**	**0.016**	0.216
	Crayfish exposure	**-0.63**	**-8.142**	**< 0.001**	
	Number of siblings	**0.417**	**3.002**	**0.003**	

Covariances var. 1	var. 2	estimate	z	P
Tibiofibula length	**Body length**	**0.01**	**8.146**	**< 0.001**

Table S2a - SEM including block as clustering parameter.

Variances

Dependent variable	Fixed effect	estimate	z	P	R^2
Jumping performance	Development time	0.035	1.02	0.308	0.596
	Tibiofibula length	**1.549**	**5.731**	**0.001**	
	Froglet length	-0.154	-0.357	0.721	
	Crayfish-invaded site	-0.027	-0.595	0.552	
	Crayfish exposure	**0.105**	**2.293**	**0.022**	
	Number of siblings	-0.054	-1.519	0.129	
Tibiofibula length	**Development time**	**0.114**	**6.281**	**0.001**	0.321
	Crayfish-invaded site	**0.09**	**3.024**	**0.002**	
	Crayfish exposure	**0.093**	**2.713**	**0.007**	
	Number of siblings	**-0.062**	**-2.704**	**0.007**	
Body length	**Development time**	**0.077**	**7.047**	**0.001**	0.304
	Crayfish-invaded site	**0.045**	**2.115**	**0.034**	
	Crayfish exposure	**0.06**	**2.777**	**0.005**	
	Number of siblings	**-0.037**	**-2.548**	**0.011**	
Development time	**Crayfish-invaded site**	**-0.55**	**-3.679**	**0.001**	0.216
	Crayfish exposure	**-0.63**	**-3.698**	**0.001**	
	Number of siblings	**0.417**	**3.42**	**0.001**	

Covariances

var. 1	var. 2	estimate	z	P
Tibiofibula length	**Body length**	**0.01**	**6.867**	**< 0.001**

Table S2b ¬ SEM including clutch as clustering parameter.

Variances Dependent variable	Fixed effect	estimate	z	P	R^2
Jumping performance	Development time	0.035	1.399	0.162	0.596
	Tibiofibula length	**1.549**	**8.827**	**< 0.001**	
	Froglet length	-0.154	-0.522	0.602	
	Crayfish-invaded site	-0.027	-0.76	0.447	
	Crayfish exposure	**0.105**	**4.775**	**< 0.001**	
	Number of siblings	-0.054	-1.782	0.075	
Tibiofibula length	**Development time**	**0.114**	**9.337**	**< 0.001**	0.321
	Crayfish-invaded site	**0.09**	**5.106**	**< 0.001**	
	Crayfish exposure	**0.093**	**4.327**	**< 0.001**	
	Number of siblings	**-0.062**	**-3.64**	**< 0.001**	
Body length	**Development time**	**0.077**	**7.842**	**< 0.001**	0.304
	Crayfish-invaded site	**0.045**	**2.059**	**0.039**	
	Crayfish exposure	**0.06**	**4.138**	**< 0.001**	
	Number of siblings	**-0.037**	**-3.496**	**< 0.001**	
Development time	**Crayfish-invaded site**	**-0.55**	**-3.63**	**< 0.001**	0.216
	Crayfish exposure	**-0.63**	**-2.621**	**0.009**	
	Number of siblings	**0.417**	**4.253**	**< 0.001**	

Covariances var. 1	var. 2	estimate	z	P
Tibiofibula length	**Body length**	**0.01**	**7.302**	**< 0.001**

Table S2c ¬ SEM including population as clustering parameter.

CHAPTER 3

PROLONGED EXPOSURE TO AN INVASIVE PREDATOR TRIGGERS MORPHOLOGICAL BUT NOT BEHAVIOURAL PLASTICITY IN TADPOLES OF A THREATENED FROG

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Abstract

During biotic invasions, native communities are abruptly exposed to novel and severe selective pressure, as native species often fail to express effective responses towards invasives and experience rapid population declines. Nonetheless, mechanisms such as rapid adaptation and phenotypic plasticity can allow native species to cope with the new ecological pressures. In particular, phenotypic plasticity often represents a lifeboat mechanism that can provide a first barrier to withstand invasive species. Moreover, plasticity offers natural selection a pool of variability and eventually favour fixation of adaptive phenotypes through canalization. We tested these hypotheses by evaluating behavioural and morphological trait variation in tadpoles of the Italian agile frog (*Rana latastei*) in response to the invasive crayfish predator, *Procambarus clarkii*. To this extent, we collected egg-clutches from recently invaded and uninvaded *R. latastei* populations of Northern Italy. After tadpoles reached Gosner stage 25, we exposed them to two rearing conditions: non-lethal crayfish presence and crayfish absence. After two-month rearing crayfish-exposed tadpoles showed a plastic shift in their body shape and in particular in tail muscle size, a trait that can confer tadpoles higher swimming speed and survival to predators. By contrast, behavioural tests performed after one and two month-rearing showed no effect of crayfish exposure on tadpole behaviour. Furthermore, we did not find significant differences for any behavioural or morphological trait between invaded and uninvaded populations, which suggests that no adaptive fixation of these traits has risen since crayfish arrival (10-15 years). The present study evidences that native prey can respond to new predatory pressures through morphological plasticity, yet the role and adaptive value of these responses remain to be investigated.

Keywords: invasive species, native amphibians, phenotypic plasticity, morphological defences, behavioural defences

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Introduction

Phenotypic plasticity is among the most pervasive and fascinating products of natural selection, and allows a given genotype the possibility to produce different phenotypic outcomes depending on the environmental conditions experienced (Pigliucci 2001). Plasticity is often favoured when the environmental context is less predictable, such as in environments where conditions are heterogeneous highly variable in time and / or space (Pigliucci 2001; Van Buskirk 2017; Reger et al. 2018). Under these circumstances, plasticity can allow species to adjust their responses and match challenges posed by changeable selective pressures. Nevertheless, understanding the role of phenotypic plasticity is generally complex, because plasticity can act on multiple traits (e.g. morphology, physiology and behaviour), can be expressed at different levels in different life-history stages, and often trade-offs exist between modifications of one trait and its effects on other trait regulation (West-Eberhard 2003; Relyea 2007). Furthermore, by interacting with adaptive variation in determining phenotype expression, plasticity broadens the pool of variability on which natural selection acts, and eventually can favour refinement and fixation of traits showing particular adaptive value (Pigliucci et al. 2006; Levis et al. 2018; Levis and Pfennig 2018).

Plasticity can also be important to face the global changes affecting biodiversity. Due to anthropogenic transformation of earth’s biosphere, species are increasingly exposed to new selective forces and unprecedented environmental variability. Introduction of alien species is among the major changes induced by human activities and is a major threat to biodiversity (Clavero and Garcia-Berthou 2005; Bellard et al. 2016). The introduction of non-native species can abruptly impose severe selective pressures for native communities, as the lack of common evolutionary history often hampers the expression of adaptive responses towards the novel invaders (Salo et al. 2007; Sih et al. 2010). Understanding how species cope with new selective pressures experienced during biotic invasions is an urgent challenge for conservation biology, and also represents an unprecedented opportunity to shed light on evolutionary mechanisms of adaptation (Mooney and Cleland 2001).

When a new predator invades a system, multiple outcomes are possible. In some cases, native prey fails to recognise it as a potential threat or adopt ineffective anti-predator strategies, which can result in rapid declines of native populations (Smith et al. 2008; Gomez-

Mestre and Díaz-Paniagua 2011). Second, the prey can respond through phenotypic plasticity (Nunes et al. 2014a; Saura–Mas and Benejam 2019). Plasticity is expected if the invader shares some traits (e.g. trophic niche, hunting strategy) with native predators, or is phylogenetically close to them (Ferrari et al. 2007; Davis et al. 2012). Third, the strong selective pressure can act on existing genotypic variation, favouring rapid adaptations that can allow withstanding the predator (Moore et al. 2004; Langkilde 2009; Bytheway and Banks 2019). The responses of native species are further complicated by potential interplays between plasticity and adaptive variation, which can lead to multiple, and even opposite, outcomes (Levis and Pfennig 2018). On the one hand, natural selection can refine pre-existing phenotypic plasticity, fostering changes in reaction norms of plastic expression of the different phenotypes among environments and fixing them into an adaptive phenotype (canalization hypothesis (Pigliucci et al. 2006; Levis et al. 2018)). Under the canalization hypothesis, we expect lower plasticity in populations subjected to strong predatory pressure by the invader (Levis et al. 2018; Levis and Pfennig 2018). On the other hand, in some circumstances the most plastic individuals can be favoured by novel selective pressures, thus selection can promote the plasticity of populations (increased plasticity hypothesis; (Lande 2009; Chevin and Lande 2010; Manenti et al. 2013). Under the increased plasticity hypothesis, we expect a stronger plastic response in invaded populations, particularly when environmental heterogeneity is strong (e.g. only part of the population is invaded), and / or when invasive predators are not the dominant selective force (Crispo 2008; Crispo and Chapman 2010).

The response of native species to invasives can be essential to their long-term survival in a changing world. However, predicting the actual impact of invasives on natives is challenging. This requires assessing both plastic and adaptive responses, but only a limited number of studies are designed to achieve this task.Even though there is growing evidence that exposure to strong selective forces during invasions can determine rapid adaptive shifts (Phillips and Shine 2006; Langkilde 2009; Nunes et al. 2014b), knowledge of the mechanisms and dynamics leading these processes is far from exhaustive.

Among vertebrates, amphibians are highly sensitive to invasive predators, which often represent a primary cause of their decline (Kats and Ferrer 2003; Cruz et al. 2008; Ficetola et al. 2011a). Nonetheless, amphibians can also show an impressive capacity to tune their responses to environmental variation through phenotypic plasticity (Relyea 2001b; Warkentin 2011; Van Buskirk 2017; Levis and Pfennig 2018), which makes them an excellent model to study responses towards invasive species. During their larval stage, amphibians are generally subjected to heavy predation pressure (Wells 2007), and predation risk can trigger the expression of a wide variety of anti-predator strategies in amphibian larvae, which show a striking capability to modulate them through plasticity (Relyea 2007; Kishida et al. 2009; Hossie et al. 2017). Furthermore, larval amphibians can show rapid adaptations to novel predators (Kiesecker and Blaustein 1997; Moore et al. 2004; Nunes et al. 2014b), and this sometimes helps species coexistence (Moore et al. 2004). For instance, tadpoles of the frog *Rana aurora* evolved the ability to recognize invasive predators (bullfrogs) in less than 60 years, increasing their refuge use in presence of bullfrog chemical cues (Kiesecker and Blaustein 1997), and adaptations can be even faster when predation pressures are particularly heavy (Nunes et al. 2014b).

In the present study, we assessed anti-predatory responses in tadpoles of the Italian agile frog (*Rana latastei*) towards a recently introduced non-native predator, the invasive crayfish *Procambarus clarkii*. This crayfish is present in the study area since the early 2000s (Groppali 2003; Fea et al. 2006), and currently shows a fragmented distribution, probably because of barriers and of complex dispersal mechanisms (Gherardi et al. 2000; Manenti et al. 2014). This study system includes both frog populations invaded by the crayfish since 10-15 years, and uninvaded populations (Ficetola et al. 2011a). We evaluated tadpole response to the invasive crayfish by assessing an extensive panel of both behavioural and morphological features, as different traits can be expressed differently through the ontogeny and can be activated by distinct stimuli and conditions (Van Buskirk and Arioli 2002; Relyea 2003; Kishida et al. 2010). This also allowed us to shed light on the relative role different ontogenetic traits can play in the expression of responses towards an invasive predator. In particular, we evaluated (i) if non-lethal continuous exposure to the invasive predator during larval development triggers plastic variation in morphological or behavioural traits and (ii) if fixed divergence in tadpole traits exists between invaded and non-invaded populations. Furthermore, we evaluated the possibility of interplay between plasticity and rapid adaptation. Specifically, iii) if invasive predators have canalized phenotypic variation (canalization hypothesis), we expect lower plasticity in invaded populations, while iv) if invasive predators have favoured plasticity (increased plasticity hypothesis), we expect a higher plasticity in invaded populations. Finally, we evaluated (v) which is the relative role of plastic larval traits involved in the expression of responses towards an invasive predator during the ontogenesis of a native frog.

Methods

Study area and target species

The study area is located in Lombardy, NW Italy, between the Ticino and the Adda rivers (approx. 45.5 N, 9.2 E). This region is characterized by a dense hydrographic network, and hosts several populations of the Italian agile frog. This species is endemic of lowlands of Northern Italy and West Slovenia (Lanza et al. 2009; Sillero et al. 2014), and is classified as vulnerable by the IUCN, with populations declining because of habitat loss, fragmentation, and invasive species (Sindaco et al. 2009). The American red swamp crayfish, *Procambarus clarkii* is among the most threatening invasive alien predators at the global scale (Gherardi 2006; Nentwig et al. 2018). This crayfish preys upon both amphibian eggs and larvae and has been associated to several cases of amphibian declines (Rodríguez et al. 2005; Cruz et al. 2008; Ficetola et al. 2011a). The crayfish was introduced in the study in the early 2000s (Groppali 2003; Fea et al. 2006) and is now widespread, causing decrease of abundance in several amphibians, including the Italian agile frog (Ficetola et al. 2011a; Manenti et al. 2020)

Collection and rearing condition

We collected egg-clutches of the Italian agile frog in early spring (March) 2017, immediately after laying. We selected six *R. latastei* populations breeding in sites invaded by the *P. clarkii* (since 10-15 years), and three populations breeding in sites where the crayfish is absent. In total, we collected 36 clutches from invaded populations and 18 from uninvaded populations (3-11 clutches per population). Animal sampling was performed under the authorization of Italian Ministry for Environment (DPN/17391 and Prot. N. 3383/T-A31). During sampling, we carefully removed a small portion from each clutch (less than 30%, 30-250 eggs). After collection, each sample was individually placed in tanks filled with decanted tap water and maintained at outdoor conditions. At hatch, tadpoles were maintained under the same conditions until reaching Gosner's stage 25 (Gosner 1960). At this stage, we randomly selected six tadpoles from each clutch and divided them in two groups of three individuals (hereafter called triads). Selected individuals (N = 324) were dorsally photographed on graph paper to measure total length (from the tip of the snout to the tail tip) using the software *ImageJ* (Schneider et al. 2012). After measurement, the two triads from each egg clutch were assigned to a different rearing treatment: non-lethal exposure to *P. clarkii*, and absence of the invasive crayfish during ontogenesis (Figure 1A). During rearing, tadpoles were hosted in six 70 x 48 cm plastic tanks (three for each rearing condition; hereafter called blocks) filled with 34 l of aged tap water. Each block contained 18 triads, which were separately housed in 0.8 l containers. In the crayfish-exposure blocks an adult *P. clarkii* was enclosed in the central section of the tank (24 x 28 cm), which was surrounded by tadpole containers; a thin plastic net (Ø = 0.2 cm) separated triads from the container with the crayfish so that tadpoles were exposed to both visual and chemical cues of the crayfish, but any predation attempt was prevented. In blocks where tadpoles were not exposed to crayfish, conditions were identical except for the absence of the crayfish. The experimental blocks were kept outdoor and placed under tree shade, in order to mimic conditions at natural breeding sites. During rearing, water was changed weekly and both tadpoles and crayfishes were fed with fish food and rabbit pellets. Tadpoles were reared until metamorphosis and then released in their respective sites of origin.

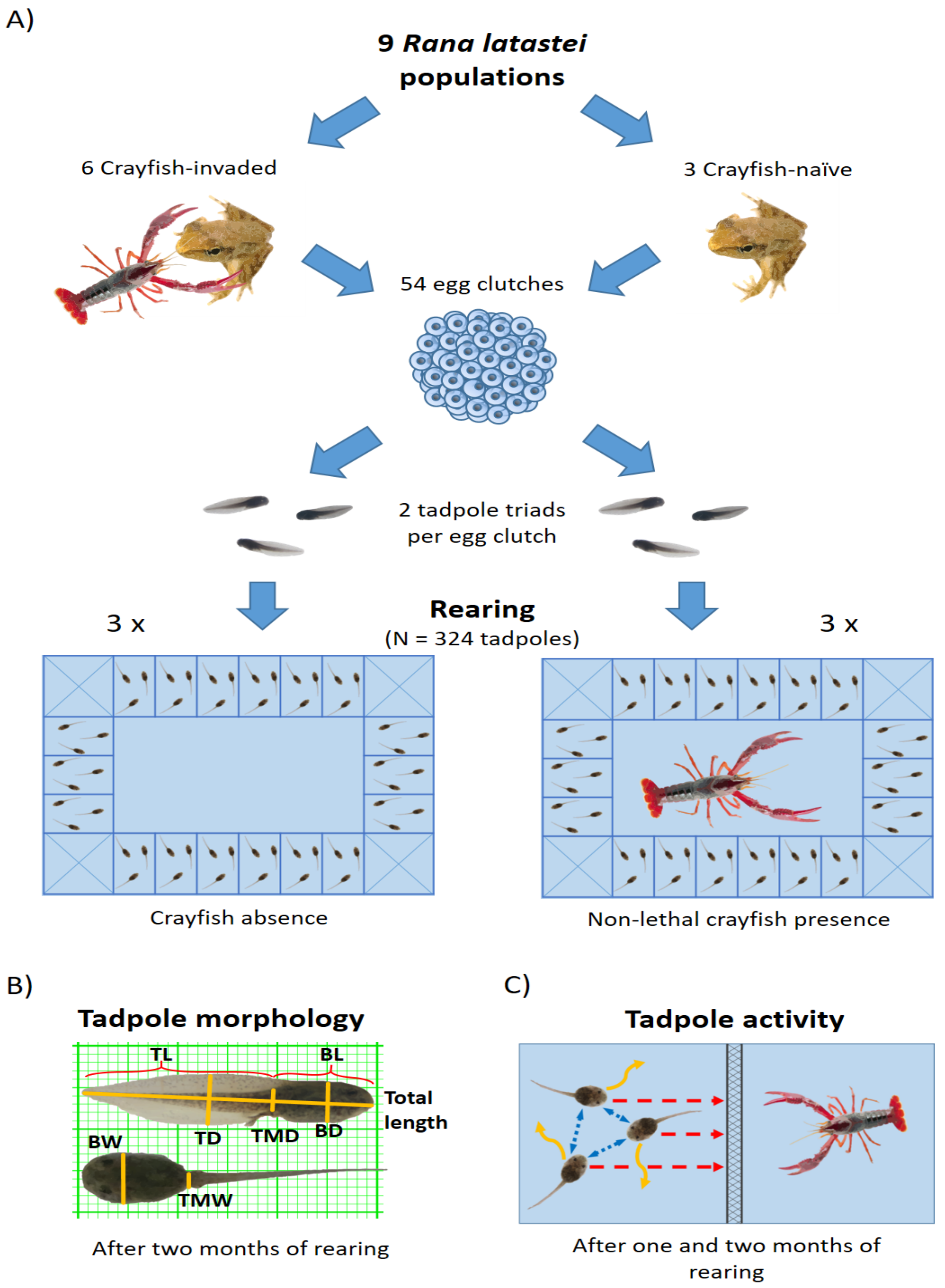

Figure 1 – Experimental plan. Sample collection and rearing conditions (A). We collected 54 egg clutches from nine *Rana latastei* populations (six invaded by the American red swamp crayfish and three uninvaded). At Gosner's stage 25, six tadpoles from each egg clutch (N = 324) were split into two triads and assigned at two rearing treatments: non-lethal exposure to the crayfish vs crayfish absence. Tadpole morphological traits (B) were measured at the end of the second behavioural session: total length, body depth (BD), body width (BW), body length (BL), tail depth (TD), tail length (TL), tail muscle depth (TMD) and tail muscle width (TMW). Tadpole activity (C) was assessed during two behavioural sessions after one and two months of rearing by registering distance moved (solid arrows in yellow), crayfish avoidance (dashed arrows in red) and sibling aggregation (two-headed dashed arrows in blue). Behavioural traits were calculated as the mean score of tadpoles in each triad.

Morphological traits

After two months of rearing (67.5 ± 0.1 days), we measured morphological features of surviving tadpoles (Figure 1B). Tadpoles were briefly transferred in small transparent boxes and photographed with plastic graph paper. Each tadpole was photographed both dorsally and laterally. Eight morphological traits were measured with *ImageJ* (Schneider et al. 2012): total length (from snout tip to tail tip), body depth, body length, body width, tail depth, tail length, tail muscle depth and tail muscle width (Relyea 2001b). Body width, body depth and tail depth were measured at their maximum width and depth, while muscle width and muscle depth were measured at the basis of the tail.

Tadpole activity

During rearing, we performed two test sessions to record tadpole activity and assess the occurrence of anti-predator behavioural response. The first session took place after approx. four weeks of rearing (28.9 ± 0.2 days from stage 25), while the second one was performed approx. two months from stage 25 (67.5 ± 0.1 days; immediately before morphological measurements). In each session, we placed a Canon SX40 HS camera above the container of each triad and recorded their activity in a five-minute trial. All videos were recorded on the same day between 9 a.m. and 5 p.m., with two trials per triad (one in the morning and one in the afternoon) during each session. Videos were subsequently analysed with the video-tracking software *idTracker*. This software can distinguish the shape of the focal animals on the basis of both their size and their contrast with the background, recording their displacements in the experimental arena (in our settings, the 7 x 11 cm triad container) (Pérez-Escudero et al. 2014). *idTracker* returns a set of the coordinates corresponding to the position of each focal animal in the arena during each frame of the video. Basing on this set of coordinates, we reconstructed the trajectories of individuals in the R environment (R 2019) and recorded three behavioural traits (Figure 1C) that are commonly involved in the anti-predatory response: total distance covered by tadpoles (*Distance moved*), the mean distance from the net separating tadpoles from the crayfish (*Avoidance*) and the mean distance among tadpoles (*Aggregation*). All behavioural traits were calculated as the mean score of tadpoles in each triad. Tadpole survival after one month of rearing was 0.73, with no significant difference between treatments ($\chi^2_1 = 0$; $P = 0.946$) or origin (invaded vs. non-invaded populations: $\chi^2_1 = 0.13$; P = 0.723). At the second behavioural session, tadpole survival was 0.57, with no significant differences between treatments ($\chi^2_1 = 0.55$; P = 0.457) or origin ($\chi^2_1 = 0.11$; P = 0.735). As a consequence, the first behavioural session was performed on 235 tadpoles from 90 triads (N = 172 tests), while the second session was performed on 186 individuals from 82 triads (N =154 tests).

Statistical analysis

Both behavioural and morphological traits were analysed using Linear Mixed Models (LMMs). For each behavioural trait, we included two fixed factors as independent variables: treatment (crayfish presence vs crayfish absence during rearing) and origin (crayfish-invaded populations vs uninvaded populations). Population of origin, egg-clutch identity and block as random factors. Moreover, we included average total length of tadpoles and number of surviving siblings in the triad (one to three) as covariates, since size and presence of conspecifics can affect tadpole activity (Wells 2007). Behavioural traits analysed in the model were square-root transformed to improve normality. We built separate models for the two sessions of behavioural tests.

Similarly, we used LMM to analyse variation at the morphological traits (total length, body depth, body width, body length, tail depth, tail length, tail muscle depth, tail muscle width). All traits were log transformed prior to perform analyses. We included the same fixed and random factors considered for behavioural traits; as covariates we included number of siblings and tadpole starting size and total length. Tadpole starting size was intended to take into account potential non-genetic maternal effects, since starting size is mostly determined by the amount of yolk provided by the mother to the eggs (Kaplan 1998). For all traits, except for total length, we included tadpole total length as a further covariate, to assess morphological differences after taking into account the variation of body size.

In all models, we tested two-way interactions between origin and treatment to assess the potential joint effects; no significant interactions were excluded from the final models. All analyses were performed in R environment (R version 3.6.0), using packages *lmerTest*, *lme4*, and *MuMIn*; partial regression plots were built using the *visreg* package.

Results

Morphological traits of tadpoles

We assessed variation at eight morphological traits in 186 tadpoles reared under regimes of exposure vs absence of the invasive crayfish. 65% of tadpoles originated from populations invaded by the crayfish, while 35% originated from uninvaded populations. Seven tadpoles were excluded from the analyses because of insufficient quality of pictures. Total length of tadpoles was not significantly affected by exposure to the crayfish or origin (Table 1, Figure 2A), while it was positively related to starting size, and decreased significantly in triads with more surviving siblings (Table 1).

Treatment had a significant effect on multiple morphological traits (Table 1); the impact of crayfish exposure was particularly evident for traits describing tail muscle. Specifically, tadpoles exposed to crayfish showed an increase in relative body depth (Figure 2B), body width (Figure 2C), tail muscle depth (Fig. 2G) and tail muscle width (Fig. 2H). We did not find any significant difference between invaded and uninvaded populations (all $P > 0.3$; Table 1, Fig. 2A-G). Nonetheless, we found a significant interaction between treatment and origin for body depth (Table 1, Fig. 2B; $P = 0.028$). When exposed to the crayfish, tadpoles from invaded populations increased body depth more, compared to tadpoles from non-invaded populations. No further significant interactions between treatment and origin were observed for any of the other morphological traits. After taking into account the effect of body size, starting size was not related to any morphological trait (all $P > 0.4$). The number of surviving siblings in the triad negatively affected tadpole body width (Table 1), but not the other morphological traits (all $P > 0.05$).

Trait	Fixed effect	df	F	P
Total length	Origin	1, 3.5	2.99	0.169
	Treatment	1, 3.4	0	0.956
	Starting size	**1, 46.7**	**6.49**	**0.014**
	N siblings	**1, 119.8**	**15.07**	**< 0.001**
Body depth	Origin	1, 2.1	1.10	0.402
	Treatment	**1, 3.9**	**8.53**	**0.045**
	Treatment*origin	**1, 159.4**	**4.92**	**0.028**
	Total length	**1, 168.4**	**201.84**	**< 0.001**
	Starting size	1, 22	0.78	0.387
	N siblings	1, 126.3	0.01	0.938
Body width	Origin	1, 5.5	1.23	0.314
	Treatment	**1, 169.1**	**8.56**	**0.004**
	Total length	**1, 164.4**	**192.85**	**< 0.001**
	Starting size	1, 43.1	0.09	0.771
	N siblings	**1, 119.7**	**5.29**	**0.023**
Body length	Origin	1, 171.8	0.14	0.705
	Treatment	1, 4.0	1.83	0.248
	Total length	**1, 169.5**	**351.39**	**< 0.001**
	Starting size	1, 170.4	0.08	0.783
	N siblings	1, 172	0.56	0.454
	Origin	1, 2.5	1.66	0.302
	Treatment	1, 61.1	2.58	0.114

Tail depth	Total length	**1, 171.9**	**322.79**	**< 0.001**
	Starting size	1, 26.4	0.28	0.601
	N siblings	1, 149.7	0.10	0.754
	Origin	1, 172.2	0.15	0.697
	Treatment	1, 4.0	1.73	0.258
Tail length	Total length	**1, 166.2**	**2436.51**	**< 0.001**
	Starting size	1, 170.7	0.20	0.654
	N siblings	1, 172.4	0.78	0.378
	Origin	1, 4.5	0.05	0.828
	Treatment	**1, 165.8**	**20.90**	**< 0.001**
Tail muscle depth	Total length	**1, 164.2**	**266.57**	**< 0.001**
	Starting size	1, 58.3	0.59	0.444
	N siblings	1, 113.9	3.19	0.077
	Origin	1, 4.7	0.29	0.617
	Treatment	**1. 166.8**	**30.35**	**< 0.001**

Table 1 – Influence of the independent variables on tadpole morphological traits. Results of LMMs showing the effect of origin (crayfish invaded vs crayfish uninvaded populations) and treatment (crayfish exposure vs crayfish absence) on tadpole morphological traits: total length, body depth, body width, body length, tail depth, tail length, tail muscle depth and tail muscle width. In all models, total length at stage 25 (starting size), the number of surviving siblings in the triad and tadpole total length at the test (except for the latter in the model testing total length) were included as covariates. Significant results are reported in bold.

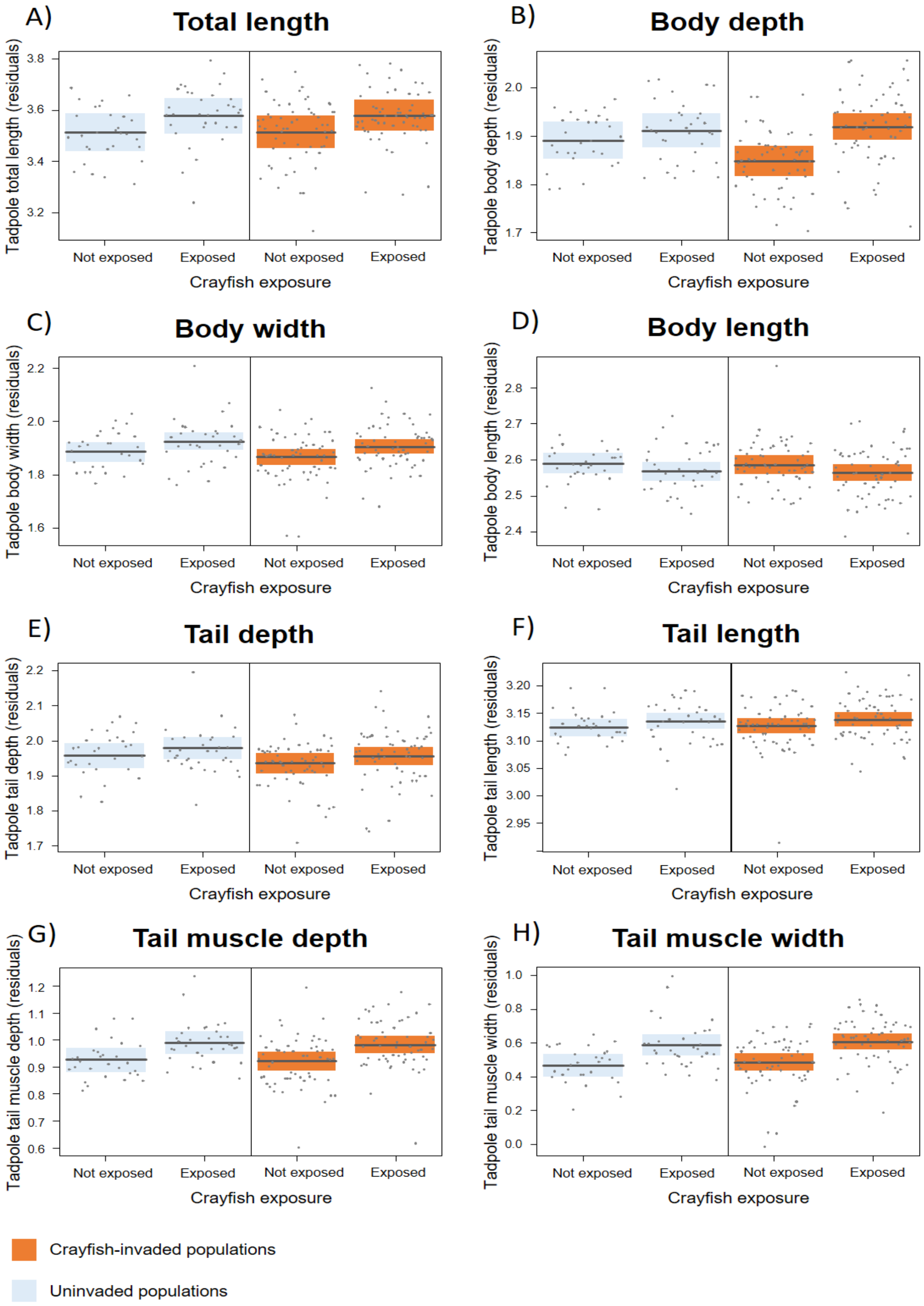

Figure 2 – Effect of origin and treatment on tadpole morphological traits. Partial residual regression plots showing the relative influence of origin (crayfish invaded vs crayfish uninvaded populations) and treatment (crayfish exposure vs absence) on tadpole morphological traits (from A to H): total length, body depth, body width, body length, tail depth, tail length, tail muscle depth and tail muscle width. Boxes in orange represents invaded populations, while light blue boxes stands for uninvaded populations. Shaded areas represents 95% confidence bands.

Tadpole activity

We measured tadpole activity in two behavioural sessions, after one and after two months of rearing. During the first behavioural session we performed 172 tests on 235 tadpoles (N = 90 triads). The average distance moved by tadpoles during trials was 14.4 ± 1.2 cm (range: 0-92.3 cm). We detected no significant effect of origin or treatment on the total movement (Table 2; Figure 3A), neither of any of the covariates (Table 2). The average distance from tadpoles from the crayfish sector (avoidance) was 5.3 ± 0.2 cm (range: 0.1 to 11.2 cm), and was not significantly affected by origin, treatment or any of the covariates (Table 2; Figure 3B). Aggregation was only calculated on triads with more than one surviving individual (N = 86 triads; 164 tests). The mean distance between siblings was 5.9 ± 0.2 cm (range: 1-11.2 cm), without any effect of origin, treatment or any of the covariates included in the model (Table 2; Figure 3C). The interaction between treatment and origin was never significant for any of the observed behavioural traits (Table 2).

In the second behavioural session, we performed 154 tests on 186 individuals (N = 82 triads) that yielded nearly identical results, with no effect of either treatment or origin on any of the traits measured (Table 2; Figure 3D-3F). Aggregation was calculated for 72 triads with more than one surviving tadpole. Also during the second behavioural session, we did not detect significant interactions between treatment and origin for any behavioural traits (Table 2).

		One month			Two months		
Trait	Fixed effect	df	F	P	df	F	P
Distance moved	Origin	1, 33.6	0.74	0.397	1, 43.6	0.15	0.699
	Treatment	1, 8.0	0.02	0.885	1, 7.7	0.80	0.399
	Total length	1, 63.6	0.26	0.612	1, 107.5	0.11	0.737
	N siblings	1, 73.3	0.37	0.544	1, 115.4	3.10	0.081
Avoidance	Origin	1, 3.3	0.24	0.656	1, 5.5	0.28	0.615
	Treatment	1, 3.6	0.60	0.487	1, 142.7	1.52	0.220
	Total length	1, 93.5	0.46	0.499	1, 72.4	0.01	0.934
	N siblings	1, 79.5	1.81	0.182	1, 87.3	2.41	0.124
Aggregation	Origin	1, 4.9	0.11	0.752	1, 3.9	0.01	0.916
	Treatment	1, 139.4	0.36	0.551	1, 128.8	< 0.01	0.994
	Total length	1, 118.4	0.03	0.869	1, 92.6	< 0.01	0.965
	N siblings	1, 123.6	0.60	0.441	1, 60.8	0.04	0.838

Table 2 – Influence of the independent variables on tadpole activity. For each behavioural session (after one month or two months of rearing), we report results of LMMs showing the effect of origin (crayfish invaded vs crayfish uninvaded populations) and treatment (crayfish exposure vs crayfish absence) on tadpole behavioural traits: distance moved, distance from the crayfish (avoidance) and distance among siblings (aggregation). All values of behavioural traits are the mean score of tadpoles composing the triad. In all models, the number of surviving siblings in the triad and mean tadpole total length at the test were included as covariates. Significant results are reported in bold.

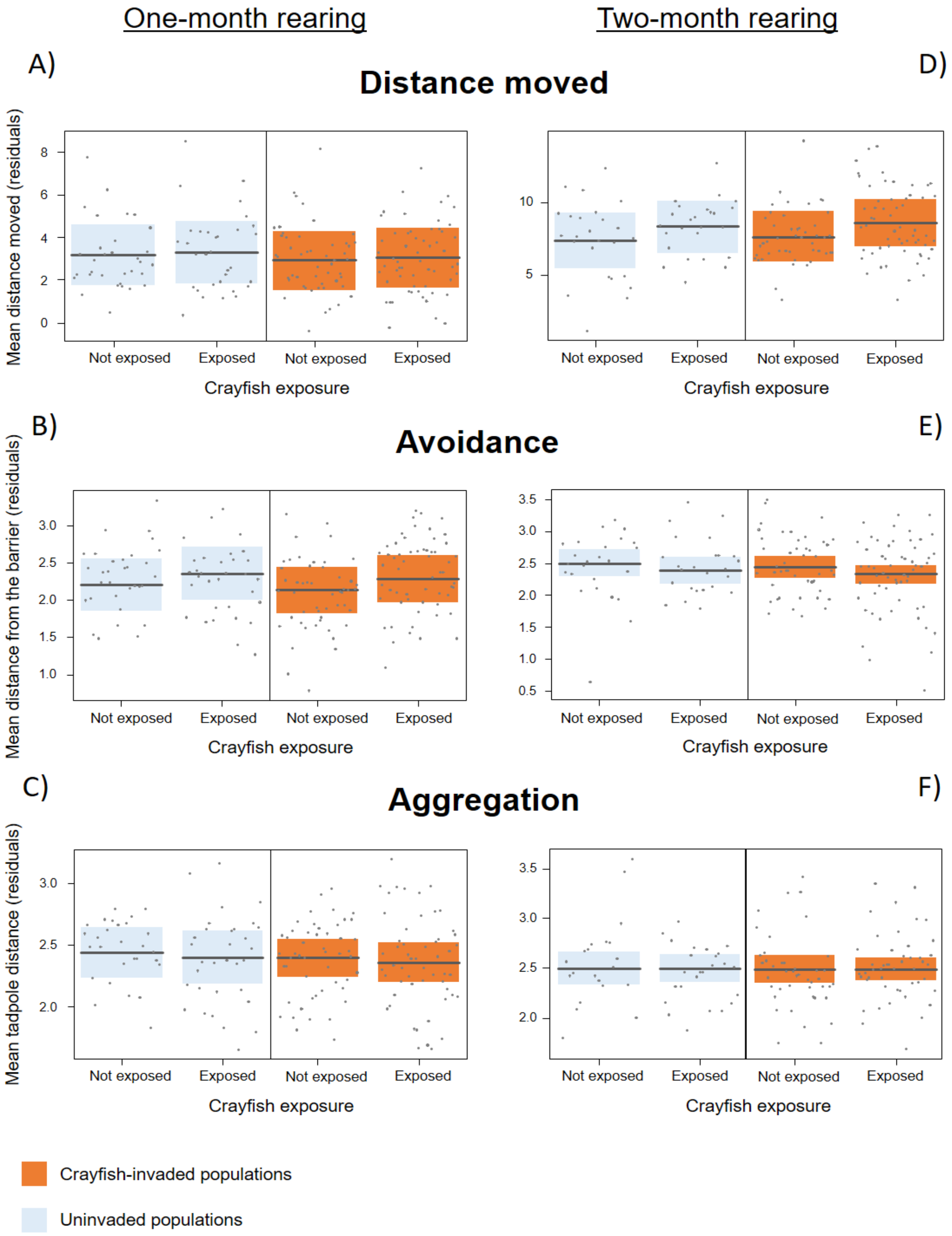

Figure 3 – Effect of origin and treatment on tadpole behavioural traits. Partial residual regression plots showing the relative influence of origin (crayfish invaded vs crayfish uninvaded populations) and treatment (crayfish exposure vs absence) on distance moved (A, D), avoidance (B, E) and aggregation (C, F). Results for each behavioural session are reported: after one month of rearing (left plots) and after two months of rearing (right plots). Boxes in orange represents invaded populations, while light blue boxes stands for uninvaded populations. Shaded areas represents 95% confidence bands.

Discussion

Amphibian larvae can respond to alien predators by activating a suite of trait modifications through phenotypic plasticity. We observed a complex phenotypic response in *Rana latastei* tadpoles, as the invasive crayfish fostered the plastic expression of morphological traits, while we did not detect any behavioural response. Overall, plastic responses to the crayfish occurrence were much stronger than differences between invaded and uninvaded populations, suggesting that plasticity currently is the strongest response to the invasive crayfish for these traits.

Tadpoles reared with the alien crayfish showed clear morphological plasticity at multiple body features, such as increased muscle depth and width of tail muscle (Table 1). The plastic response was evident at four out of eight morphological traits (Table 1; Figure 2B-C, G-H), suggesting that tadpoles are able to express complex and generalized modifications when invasive predators are present. Increased tail muscle is a typical plastic response observed when tadpoles are exposed to native predators (Relyea 2001b; Van Buskirk 2009), which is associated to faster swimming performance, and can improve the ability to escape predators (Dayton et al. 2005; Pease and Wayne 2014). Thus, such a modification in tail shape can also improve the survival in wetlands invaded by the American crayfish, potentially improving the resistance to this invasive predator. Furthermore, in crayfish exposed tadpoles we also observed a slight increase in relative body dimension (relative body depth and width). These plastic modifications have been reported by some studies assessing responses to native and invasive predators, still are not commonly observed across species, suggesting that they can provide a complex or context-dependent advantages (Van Buskirk 2009; Nunes et al. 2014a). Conversely, morphological differences between invaded and not-invaded populations were weak. Tadpoles can have a remarkable ability to adapt under strong selective pressure by invasive species and other global change stressors (Kiesecker and Blaustein 1997; Moore et al. 2004; Nunes et al. 2019), and some studies have shown the rapid evolution of morphological modifications in response to invasive predators (e.g. (Moore et al. 2004; Nunes et al. 2014b)). However, in our study case tadpoles from invaded populations did not show significant differences for any of the recorded morphological traits, thus providing no support to the local adaptation or canalization hypotheses, at least for the measured traits. The lack of fixed differences among populations could occur because of multiple reasons. First, the study populations have only been invaded a few generations ago (approx. 3-5). Even though rapid evolution has been observed multiple times in larval amphibians, it is possible that more time is needed before significant differences among populations can arise. Second, the Italian agile frog is a threatened species with high inbreeding and low genetic diversity (Garner et al. 2004; Ficetola et al. 2007), and this can limit the adaptive potential of populations (Frankham et al. 2004). Finally, morphology constitutes just a small part of the multiple traits of tadpoles, and tadpole survival and fitness can be caused by the complex interplay between morphology, environmental variation, and other traits. This makes it very difficult the prediction of which traits are most likely to evolve in response to invasive species, and in which direction. Interactions between crayfish exposure and origin were generally weak. We only observed a significant interaction between treatment and origin for body depth, as tadpoles from invaded populations increased their body depth more when exposed to the crayfish, compared to tadpoles from non-invaded populations. In principle, this observation is in agreement with the hypothesis that, in heterogeneous landscapes, invaded populations may evolve a higher plasticity (Nunes et al. 2014a). Nevertheless, the effect size of this interaction was rather weak (Table 1), and we did not detect interactions for any of the other morphological traits. Overall, morphological analyses are in agreement with the idea that, shortly after invasions, species responses to the new selective pressures mostly occur through plasticity (Sih et al. 2011), while local adaptation and canalization could have a higher importance in the long-term (Kiesecker and Blaustein 1997; Moore et al. 2004). Our study system can be excellent to test this hypothesis, as the same experiment can be repeated multiple times in the future, evaluating how each population evolves through time in response to invasive species.

Though the plastic morphological response observed in *R. latastei* tadpoles suggests that the invasive crayfish is perceived as a threat, the mechanisms through which predator recognition occurred are less clear. During rearing, tadpoles were continuously exposed to both chemical and visual cues released by the non-native predator. On the one hand, the capacity to exhibit anti-predatory responses towards to these cues can be favoured when prey share a common evolutionary history with native predators which presents a certain degree of similarity or phylogenetic proximity with the invasive one (Ferrari et al. 2007; Davis et al. 2012). For instance, in our study area, *R. latastei* historically coexisted with the native Italian freshwater crayfish (*Austropotamobius italicus*), which shares similar trophic niche and predatory attitudes with *P. clarkii*. On the other hand, observed plastic variation in *R. latastei* tadpoles can be the result of a general anti-predatory response to large approaching or moving shapes (Mathis and Vincent 2000; Wilson et al. 2018). Overall, even if plastic shifts in tadpole morphology after crayfish exposure were evident, mechanisms allowing crayfish recognition need further investigation.

Behavioural traits are commonly considered highly plastic, as their modulation is generally faster, more reversible and less energetically demanding than morphological trait shifts (Schoeppner and Relyea 2005; Weis and Sol 2016). However, anti-predator behaviour often involves balancing trade-offs between escaping predation risk and devote time to meet other fitness-related demands (e.g. foraging and mating) (Lima and Dill 1990; Ferrari et al. 2009; Winandy and Denoël 2013b). For instance, predator avoidance and activity reduction are common anti-predator behaviours, but they are often associated to decreased foraging or exploitation of suboptimal trophic niches (Lima and Dill 1990). However, prey has to feed to grow and develop, thus activity reduction can be not sustainable over long-term. In fact, when predation risk is prolonged or constantly high, anti-predator responses can become weaker and can even be suppressed to permit foraging and favour rapid growth (Turner 1997; Lima and Bednekoff 1999; Ferrari et al. 2009). In these cases, other anti-predatory strategies, such as morphological and life-history trait modifications (e.g. defensive structures and faster development) can be favoured. In our study, the absence of behavioural responses, together with the contemporary expression of morphological traits facilitating escape, matches this prediction. Thus, constant exposure to predation risk can have rapidly reversed benefits deriving from behavioural avoidance of predator (e.g. hiding or decrease activity), while may have favoured differential resource allocation promoting traits enhancing survival in case of predator encounter, such as an increase in tail muscle dimension.

Besides, the absence of behavioural shift may also have occurred because tadpoles were not exposed to all the cues associated to predation risk. Aquatic prey rely on different suites of predation-related cues which can play a key role in favouring recognition and fostering the expression of anti-predator responses, including alarm cues released by stressed or wounded conspecifics and foraging cues related to predator diet (Kats and Dill 1998;

Schoeppner and Relyea 2005; Ferrari et al. 2010c). These cues can facilitate prey risk assessment through learning, by mediating the association of predator kairomones perception with a potential threat, both with native and non-native predators (Marquis et al. 2004; Mandrillon and Saglio 2005; Gonzalo et al. 2007; Polo-Cavia and Gomez-Mestre 2014). Moreover, predation cues (i.e. foraging and alarm cues) can be perceived by prey as signals of a transient peak in predation risk and thus are often required to trigger short-term and reversible responses, such as behaviour (Van Buskirk and Arioli 2002; Schoeppner and Relyea 2005). In our study, predation cues were absent and this may further explain the observed lack of behavioural response. Still, under natural conditions the crayfish successfully preys on tadpoles and predation cues may cause short-term behavioural anti-predatory responses that were unrecorded in our study.

Even though we observed significant plastic responses of tadpoles to the crayfish, it is unclear whether such responses actually improve tadpole fitness and enhance their possibility to withstand biological invasions. Few studies have assessed the effectiveness of the crayfish-induced variation in native preys, and only rarely observed positive effects of plastic response on tadpole survival (Polo-Cavia and Gomez-Mestre 2014), while the decline of amphibian communities induced by *P. clarkii* is well documented (even in plastic-responsive species, see for example (Cruz et al. 2008; Ficetola et al. 2011a; Nunes et al. 2013). Even if invaded frog populations are not necessarily declining, the crayfish has been shown to reduce recruitment with complex effects on metapopulation dynamics, and such reduced recruitment could cause the broad-scale collapse of populations (Manenti et al. 2020). Assessing if plastic responses really increase fitness under natural conditions is particularly complex, because multiple abiotic and biotic factors act together in a context-dependent fashion (Blaustein and Kiesecker 2002; Kiesecker et al. 2002). Still, understanding which responses can help long-term persistence of invaded populations is essential to predict the consequences of invasions across species and habitats, and to identify the conditions under which management actions are the priority, or are most likely to be successful.

The results of our study shed new light on the key role played by phenotypic plasticity in allowing quick responses of native species facing the spreading of new predators. Phenotypic plasticity can be one of the major drivers of evolutionary change, but is also a lifeboat mechanism allowing species persistence in a global scenario of rapid ever-changing selective pressures. Even if understanding the complex morphological and behavioural responses induced by predation risk can be extremely challenging, considering multiple traits is essential to assess predator-prey interactions between native and non-native species. As the response can be detectable only at one or few traits, focusing on one feature only can lead to underestimate the prey response to invasive predators. Integrated analyses, combining morphological and behavioural observations prove to be necessary to unravel the complexity of responses to major global change stressors.

Ethical statement

The present study was performed under the authorization of Italian Ministry for Environment (DPN/17391 and Prot. N. 3383/T-A31). After the end of the experiments, all the individuals were released in their site of origin. Before releasing, all individuals were treated with Virkon S to prevent any risk of disease spreading (Johnson et al. 2003; Jussila et al. 2014; Bosch et al. 2015).

CHAPTER 4

FEARFUL AT FIRST SIGHT: VISUAL STIMULI FROM AN INVASIVE PREDATOR ELICIT BEHAVIOURAL RESPONSES IN LARVAE OF NATIVE AMPHIBIANS

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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 in native prey, undermining the expression of effective anti-predatory responses. However, mechanisms allowing prey to overcome evolutionary naïvete exist. For instance, history of coevolution with similar native predators or recognition of general traits characterizing predators can favour recognition of stimuli released by invasive predators in naïve prey. In the present study, we exposed naïve larvae of 13 amphibian species to chemical and visual cues released by an invasive predator, the American red swamp crayfish (*Procambarus clarkii*), and evaluated behavioural responses to different stimuli. Moreover, we compared occurrence and intensity of response among species with their coexistence with a similar predator, the native European crayfish. Larvae of all the amphibians altered their behaviour, especially reducing activity, in presence of visual stimuli of the alien crayfish, even if intensity and modality of response was highly heterogeneous among species. Conversely, few feeble and contrasting behavioural shifts were elicited by the exposure to crayfish chemical cues. We found no correlation between species coevolutionary history with the native crayfish and their response to the invasive crayfish. These results suggest that the plastic behavioural response observed in naïve amphibian larvae exposed to the invasive crayfish are mainly driven by recognition of general stimuli associated to predators (e.g. approaching large shapes), rather depending on their coevolutionary history with another crayfish predator.

Keywords: invasive species, anti-predator behaviour, amphibian community, predator recognition, history of coexistence

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Introduction

Biotic invasions are increasingly shaping ecosystems on 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 and have led 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). Behavioural responses of native species to invasive predators can be extremely important, as they can constitute a first line of defence for native species towards invasives (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 (e.g. intensity and modality), while missing predator recognition or incorrect evaluation of risky situations can often prove fatal (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. However, predator recognition generally requires a history of coevolution between predator and prey (Downes and Shine 1998; Sih et al. 2010). Therefore, when a non-native predator invades an ecosystem, crucial questions arise on prey capability to withstand the novel threat. How naïve prey responds to the new threats and how responses vary across native prey community? Which mechanisms can favour novel predator recognition?

Native prey often 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). The 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 recognise novel predators. On the one hand, when the invasive predator is phylogenetically close or shares similar traits with a native predator, prey can recognise 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 (Carthey and Blumstein 2018), when it shares traits that are commonly associated to predator species (e.g. shape, stealthy approaching, large size), inducing a generic anti-predator response in prey (generic response hypothesis) (Mathis and Vincent 2000; Rehage et al. 2009; Wilson et al. 2018). Finally, novel predator recognition can be favoured when prey experience the unfamiliar stimuli paired to predation-related cues (e.g. an unknown odour and alarm cues released by conspecifics), which in turn can favour the association of the novel stimuli to predation context (association hypothesis) (Gonzalo et al. 2007; Nunes et al. 2014a; Bytheway and Banks 2019).

Freshwater systems are particularly threatened by invasive species (Leprieur et al. 2008; Strayer 2010) and, due to their isolation, prey naïveté to introduced predators can be 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, visual stimuli often cannot prevent predator encounter and have limited reliability in environments with scarce visibility (e.g. turbid or highly vegetated water) (Abrahams and Kattenfeld 1997; Ferrari et al. 2010b). By contrast, chemical cues are generally perceived before encountering the predator and can elicit anti-predatory responses aimed at preventing exposure to predators (Kats and Dill 1998). On the basis of chemical stimuli, prey can also gather information on predator diet and density (Schoeppner and Relyea 2005; Schoeppner and Relyea 2008), and finely tune anti-predator response to specific predators (Benard 2006).

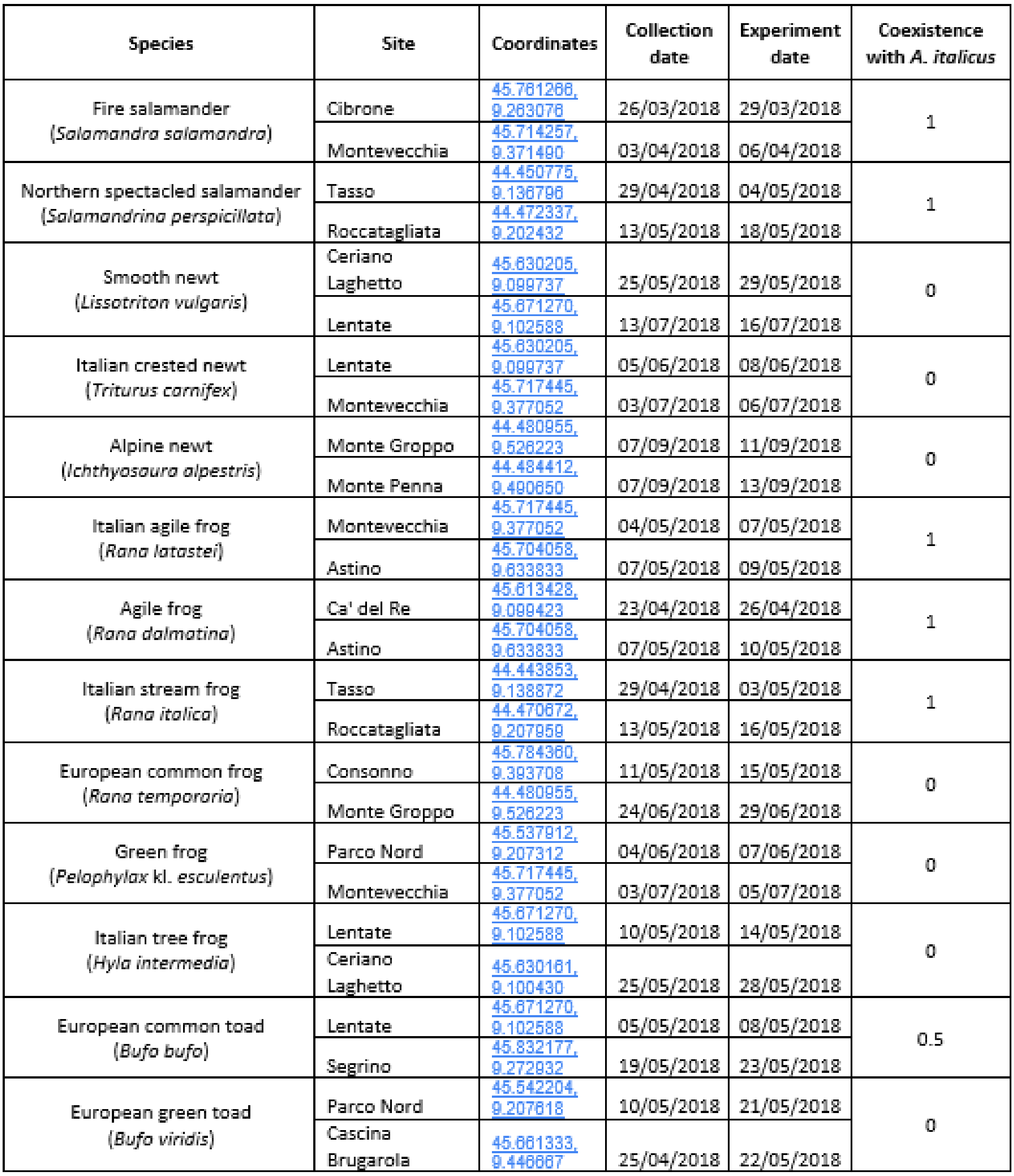
Anti-predator response against novel predators can have key consequences on the dynamics of invaded communities. Native species recognizing invasive predators can have more effective anti-predator responses, and this could increase their ability to withstand the impact of invaders. However, understanding 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)).

The present study investigates the capability to recognise a non-native predator, such as the invasive American red swamp crayfish (*Procambarus clarkii*) and express behavioural responses, in larvae of 13 species (five caudates and eight anurans) composing the amphibian community living in freshwaters of Northern Italy. Selected species were 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*). During behavioural tests, we exposed amphibian larvae from naïve populations (i.e. not invaded by *P. clarkii*) to a combination of visual and chemical stimuli from the invasive crayfish and monitored variation in activity and space use. In so doing, we aimed to assess (i) how the occurrence and modality of responses 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 behaviour in native amphibian prey; (iii) if the coevolutionary history of some species with a similar native predator (the European white-clawed crayfish, *Austropotamobius italicus*) can influence the capability to express anti-predator behaviours towards the invasive crayfish.

Methods

Study area and collection of individuals

Amphibian larvae were collected in two main areas located in Northern Italy. Three species (*Rana italica*, *Ichthyosaura alpestris* and *Salamandrina perspicillata*) were collected in low-mountainous forested sites of the Northern Apennines (located in Liguria and Emilia Romagna regions). Conversely, all other species were collected in Lombardy, in the area of the Po Valley ranging from the basins of Adda river on the East and Ticino river on the West (Table 1). This region hosts a rich hydrographic network where woodland cover is highly intermingled with densely populated urban or agricultural areas. In these regions, the native white-clawed crayfish, *Austropotamobius italicus*, was common in the past. This crayfish is an amphibian



Species	Site	Coordinates	Collection date	Experiment date	Coexistence with *A. italicus*
Fire salamander (*Salamandra salamandra*)	Cibrone	45.761266, 9.263076	26/03/2018	29/03/2018	1
	Montevecchia	45.714257, 9.371490	03/04/2018	06/04/2018	
Northern spectacled salamander (*Salamandrina perspicillata*)	Tasso	44.450775, 9.136796	29/04/2018	04/05/2018	1
	Roccatagliata	44.472337, 9.202432	13/05/2018	18/05/2018	
Smooth newt (*Lissotriton vulgaris*)	Ceriano Laghetto	45.630205, 9.099737	25/05/2018	29/05/2018	0
	Lentate	45.671270, 9.102588	13/07/2018	16/07/2018	
Italian crested newt (*Triturus carnifex*)	Lentate	45.630205, 9.099737	05/06/2018	08/06/2018	0
	Montevecchia	45.717445, 9.377052	03/07/2018	06/07/2018	
Alpine newt (*Ichthyosaura alpestris*)	Monte Groppo	44.480955, 9.526223	07/09/2018	11/09/2018	0
	Monte Penna	44.484412, 9.490650	07/09/2018	13/09/2018	
Italian agile frog (*Rana latastei*)	Montevecchia	45.717445, 9.377052	04/05/2018	07/05/2018	1
	Astino	45.704058, 9.633833	07/05/2018	09/05/2018	
Agile frog (*Rana dalmatina*)	Ca' del Re	45.613428, 9.099423	23/04/2018	26/04/2018	1
	Astino	45.704058, 9.633833	07/05/2018	10/05/2018	
Italian stream frog (*Rana italica*)	Tasso	44.443853, 9.138872	29/04/2018	03/05/2018	1
	Roccatagliata	44.470672, 9.207959	13/05/2018	16/05/2018	
European common frog (*Rana temporaria*)	Consonno	45.784360, 9.393708	11/05/2018	15/05/2018	0
	Monte Groppo	44.480955, 9.526223	24/06/2018	29/06/2018	
Green frog (*Pelophylax* kl. *esculentus*)	Parco Nord	45.537912, 9.207312	04/06/2018	07/06/2018	0
	Montevecchia	45.717445, 9.377052	03/07/2018	05/07/2018	
Italian tree frog (*Hyla intermedia*)	Lentate	45.671270, 9.102588	10/05/2018	14/05/2018	0
	Ceriano Laghetto	45.630161, 9.100430	25/05/2018	28/05/2018	
European common toad (*Bufo bufo*)	Lentate	45.671270, 9.102588	05/05/2018	08/05/2018	0.5
	Segrino	45.832177, 9.272932	19/05/2018	23/05/2018	
European green toad (*Bufo viridis*)	Parco Nord	45.542204, 9.207618	10/05/2018	21/05/2018	0
	Cascina Brugarola	45.661333, 9.446667	25/04/2018	22/05/2018	

Table 1 – Studied species, sampling site and date, test date and coexistence with the European crayfish. Date and site of collection are reported for each populations of the studied species, together with the date of experimental test. Studied populations were selected among ones occurring in the hydrographic network that the past hosted the European crayfish *Austropotamobius italicus* in, and that are still uninvaded by the invasive crayfish at present. Coexistence degree with native crayfish predator is reported for all species (1 = coexisting, 0.5 = partially coexisting, 0 = non-coexisting).

predator primarily associated to fast-flowing lotic habitats. Nonetheless, this native crayfish has undergone a rapid decline in the last century, due to habitat modification, direct fishingand 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 for potential influence of coevolutionary history with the native crayfish on the response towards *P. clarkii*, we selected amphibian populations located in hydrographic basins that in the past hosted *Austropotamobius italicus*. Between spring and summer 2018, we collected 12 individuals from two populations for each of the thirteen amphibian species. Amphibian larvae were at intermediate developmental stages (approx. 1.5 months after hatch) and were all collected from populations originating from sites where the native crayfish is now extinct and where the invasive one is not present. On the one hand, this allowed to exclude effects of individual experience towards the two predators on larva behavioural responses. On the other hand, by selecting uninvaded populations, we avoided potential influence of recent coevolutionary history with the invasive crayfish.

Procambarus clarkii is native of North America but is currently widespread in Northern Italy, even if its distribution is patchy (Barbaresi and Gherardi 2000; Groppali 2003; Fea et al. 2006; Manenti et al. 2014). The invasive crayfish has a wide niche, and is able to exploit both rivers and lentic environments. The overall morphology and the predatory behaviour is similar between the invasive and the European crayfish, even though the American crayfish 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. 2011a). *P. clarkii* used in this study (N = 40 adult individuals) were collected from a dense population steadily inhabiting flooded areas adjacent to Lambro river (Lombardy, approx. 45.729, 9.237).

Housing and experimental protocol

After collection, larvae were housed in laboratory in 49 x 35 x 16 cm plastic tanks containing 15 l of decanted tap water. Each tanks hosted 12 larvae from the same population, which were singularly 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 (salamander larvae) or *Daphnia* spp. (*S. perspicillata* and newt larvae).

After collection, *P. clarkii* individuals (mean cephalothorax length = 46.92 ± 0.75 mm) were singularly hosted in plastic tanks (20 x 14 cm), 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 behavioural tests (mean: 4.7 ± 1 days). After two-day starvation period, we performed one experimental session for each amphibian population. During experimental sessions, each amphibian larva was exposed to four combinations of predation-risk related to the non-lethal presence of *P. clarkii* (Fig. 1): visual and chemical cues (V+C+); visual cues only (V+C-); chemical cues only (V-C+); no risk cues (V-C-).

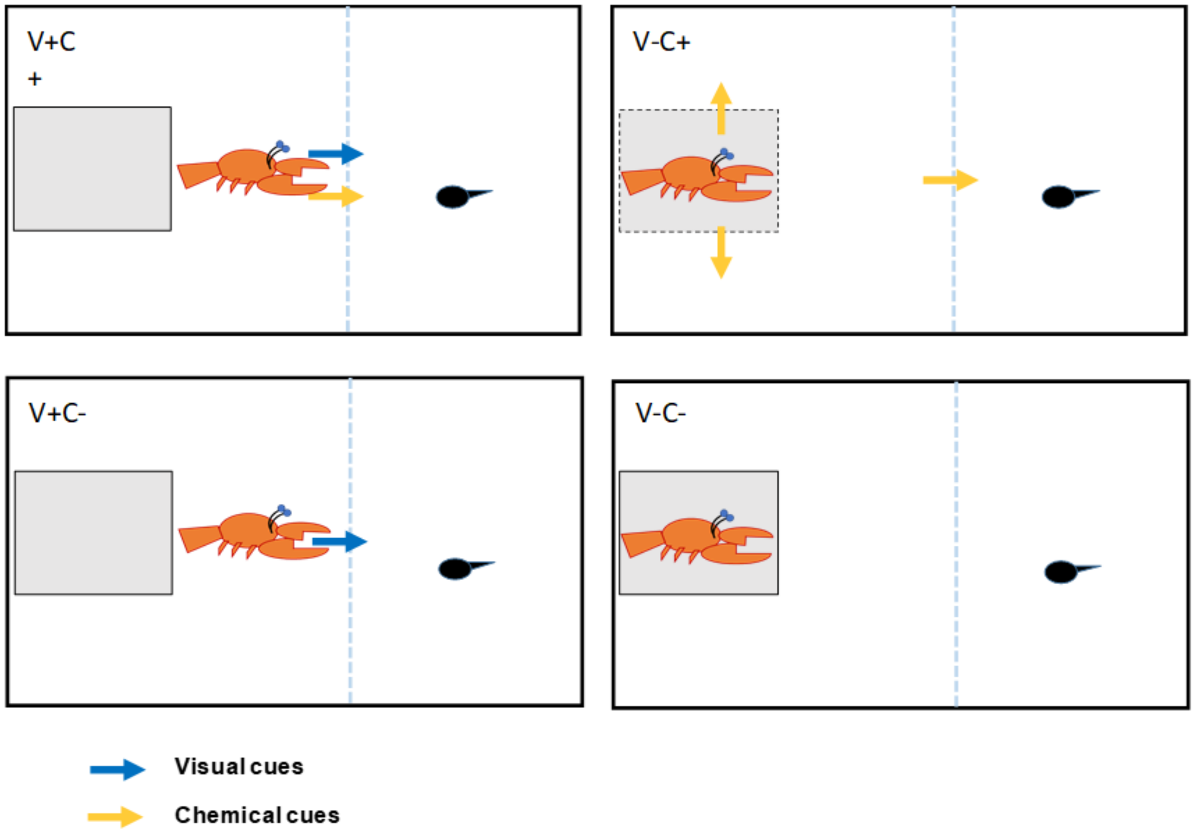


Figure 1 – Experimental scheme Activity of amphibian larvae was tested in behavioural trials with the exposure to four treatments: contemporary presence of visual and chemical stimuli of *P. clarkii* (V+C+); presence of chemical cues only (V-C+); presence of visual cues only (V+C-); absence of crayfish cues (V-C-). During the tests, tadpoles were housed in one side of a two-sided experimental tanks (prey compartment). The other side (predator compartment) was separated by a transparent plastic barrier hosted an adult red swamp crayfish individual. The crayfish was placed in an opaque pot in treatments excluding visual stimuli (V-C+ and V-C-), while was free ranging in treatments with exposure to visual stimuli (V+C+ and V+C-). Chemical cue exposure was allowed (treatments V+C and V-C+) by means of small holes in the barrier and in the pot hosting the crayfish, whereas holes were absent in treatments excluding exposure to crayfish cues (V+C- and V-C-). Behavioural tests lasted seven minutes and each larvae (N = 24 individuals per species) was exposed to each treatment in two replicates.

Experiments were conducted using 49 x 16 x 16 cm plastic tanks, filled with 8 L of decanted tap water. Experimental tanks were divided in two compartments by a transparent plastic barrier. One compartment was used to host amphibian larvae (16 x 16 cm, hereafter 'prey compartment'), while the second hosted one American crayfish (32 x 16 cm, hereafter 'predator compartment'). In all predator compartments, an opaque plastic pot (9 x 9 x 14 cm) was present. These pots hosted the crayfish in treatments excluding visual cue exposure, while in visual-cue treatments the non-native predator was free ranging in its compartments. In treatments with exposure to *P. clarkii* chemical cues, 0.5 cm diameter holes, present 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,. Behavioural tests were conducted between 9 a.m. and 17 p.m.; all individuals from the same population were tested in the same day. Before experiment started, each larva was inserted in the prey compartment and let acclimatize for three minutes. After acclimatization, we inserted an invasive crayfish in the predator compartment (in the crayfish pot for V-C+ and V-C- treatments, while out of the pot for V+C+, V+C- treatments). Behavioural test lasted 7 minutes and larvae activity was video-recorded by placing a Nikon d5300 camera (18mm lens) perpendicularly above the prey compartment. For each individual we performed eight behavioural tests (two replicates for each treatment). Tests were conducted in a randomized order so as minimizing the potential bias of exposure sequence (Altmann 1974; Ferrari et al. 2010a; Melotto et al. 2019). Similarly, crayfishes used during each test were randomly selected. We left at least 15-minute recovering time to each individuals between consecutive tests. Tanks and pots assigned to a particular treatment were not used for any other. Furthermore, tanks and pots were washed multiple times between subsequent trials to minimize traces of cues from preceding treatments. In total, we performed 2496 behavioural tests (12 individuals X 26 populations X 4 treatments X 2 replicates). After the conclusion of each behavioural 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).

Behavioural display

Larvae behaviour and activity 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 five behavioural displays. 1) total distance moved by larvae during the test (hereafter total distance); 2) mean distance from the barrier separating them from the stimulus source (avoidance). General decreased of activity and avoidance risky areas are common anti-predator behaviour that are frequently observed in amphibian larvae (Relyea 2001b; Van Buskirk et al. 2012; Winandy and Denoël 2013a; Manenti et al. 2016). However, after observing larvae behaviour, we noticed that some species have periods of limited movement followed by rapid bursts. These bursts lasted few seconds and allowed larvae to cover large distances, a behaviour which appear to be an escape attempt (Dayton et al. 2005; Teplitsky et al. 2005). Consequently, measuring total movement only could have obscured specific anti-predator strategies, leading to underestimation or misinterpretation of behavioural response in some species. Thus, we considered as additional behavioural parameters: 3) the total number of bursts (number of bursts), 4) the total distance travelled while performing bursts (burst distance) and 5) the distance moved without considering bursts (no-burst distance).

To identify bursts, we plotted the frequency distribution of distance moved in single movements (i.e. continuous movements through time). We observed a slightly bi-modal distribution of frequencies that was consistent through species, with most of movements ≤ 1 cm per second, and a second peak of movements > 4 cm per second. Therefore, bursts were defined as all the movements faster than 4 cm/s. Burst distance was obtained by summing distance covered in movements identified as bursts, while no-burst distance was calculated as the difference between total distance and burst distance. Correlation among the five behavioural display analysed for each species are reported in Supplementary material S3.

Statistical analysis

The effect of treatments on amphibian behaviour were analysed through Linear Mixed Models (LMMs) and Generalised Linear Mixed Models (GLMMs). These models allow to consider the influence of fixed effects on the dependent variable while taking into account non-independence of observations (e.g. repeated observations on the same individual or on the same population (Pinheiro and Bates 2000)). In all models, we included treatment (chemical or visual cue exposure) as fixed factor. Potential non-additive effects between chemical and visual cues were assessed by considering statistical interactions among these treatments; non-significant interactions were excluded from the final models. Together with treatment, we included in all models air temperature (°C) and day time (minutes from midnight) as covariates, as they are known to affect amphibian activity (Wells 2007). All continuous variables were scaled (mean = 0, SD = 1) before analyses. Moreover, a few videos were slightly shorter, thus we also included video duration as an additional covariate in all models. Video duration was not considered for the fire salamander, as for this species all the videos lasted 7-minutes. All models also included individual identity, population of origin and test replicate (first or second exposure to a single cue) as random factors.

For the number of bursts, which was not normally distributed and showed overdispersion, thus we used GLMMs with negative binomial distributions. All other behavioural displays were log-transformed and analysed with LMMs (Gaussian error).

To test for the hypothesis that coevolution with the native crayfish could increase the ability to detect the invasive crayfish, we assigned each species to different categories on the basis of their habitat overlap with the native crayfish (coexistence degree): complete overlap, partial overlap, virtually no overlap (Table 1). To compare the effect size of the response to the American crayfish across species, we converted the test statistics (Fisher's F or χ^2 values) to Fisher's z values. Fisher's z is a measure of effect size that allows comparisons among statistical tests (Field et al. 2012). For number of bursts models failed to converge in one species (*Hyla intermedia*), thus we excluded this display from the analysis. Overall, for each species we considered eight effect sizes (4 behavioural displays X 2 fixed factors, visual and chemical stimuli). We used redundancy analysis to test whether species with high niche overlap with the native crayfish have a stronger response to the invasive crayfish. (Legendre et al. 2011). Significance of redundancy analysis was calculated through permutation test (10000 iterations).

All statistical analyses were performed using R (version 3.6.0). For mixed models, we used packages "nlme", "lmerTest", "MuMIn" and "glmmTMB", whereas effect sizes were obtained through "compute.es" package. Finally, we used "vegan" package to perform to redundancy analyses.

Results

Behavioural displays by larvae were highly heterogeneous among species (Fig. 2). A comprehensive table showing complete results of mixed models is reported in supplementary material S1, while and significant effects of treatments for each behavioural display in each species are shown in Fig. 3.

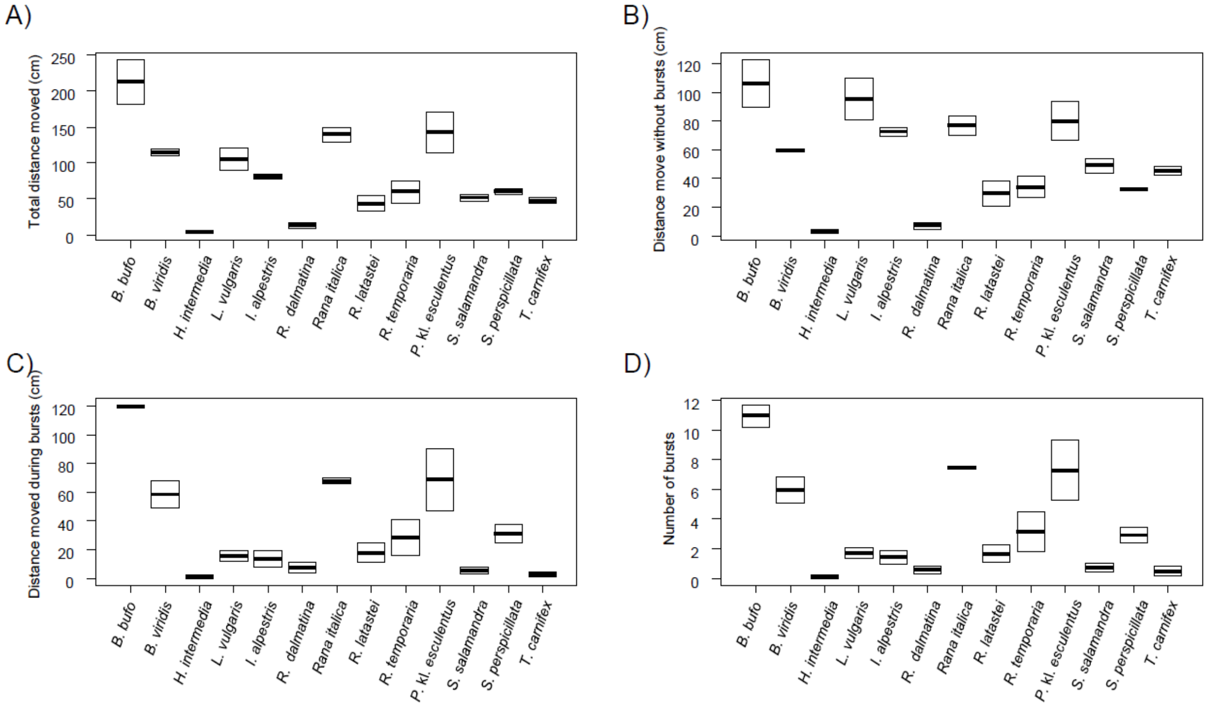
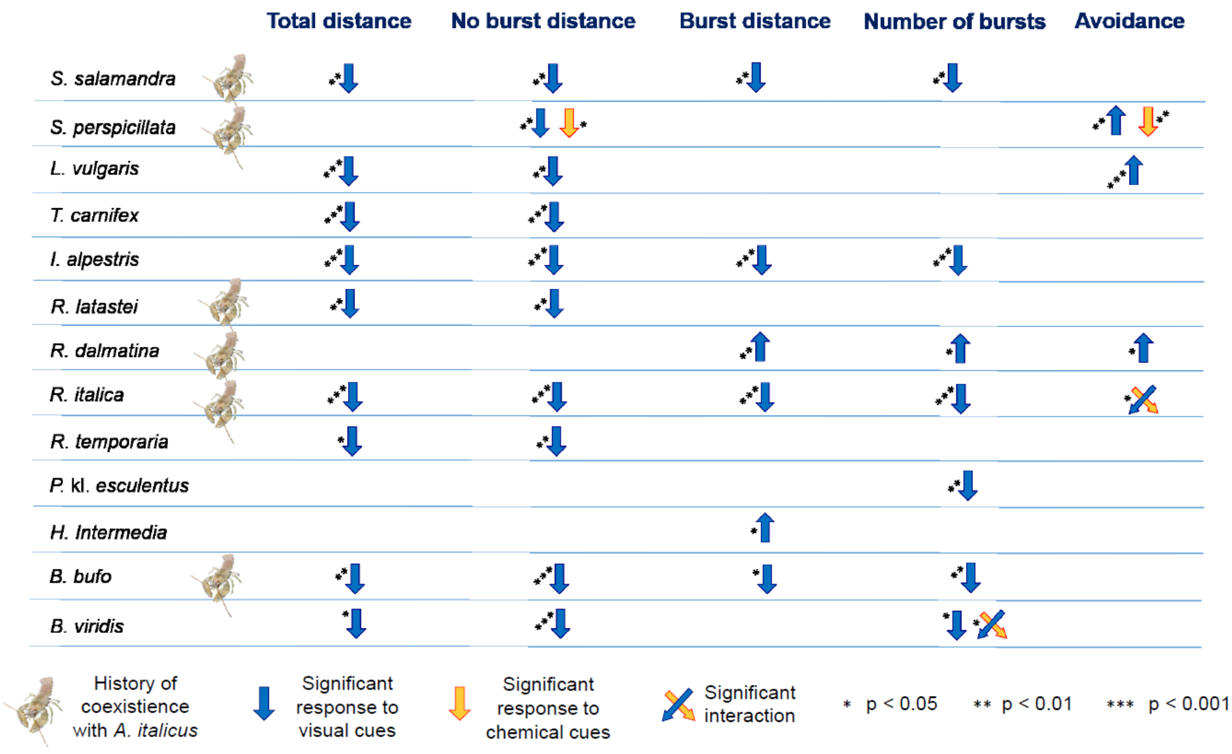


Figure 2 – Variability of activity among studied species. Activity during behavioural tests is reported for larvae of all studied species. Boxplots show the general median and confidence interval (95%) of behavioural displays describing larvae movement and activity: total distance moved (A); no-burst distance (distance covered excluding rapid bursts) (B); burst distance (distance covered during bursts) (C); number of bursts performed by larvae (D).



	Total distance	No burst distance	Burst distance	Number of bursts	Avoidance
S. salamandra 🦞	**↓	**↓	**↓	**↓	
S. perspicillata 🦞		**↓ ⇩*			**↑ ⇩**
L. vulgaris	***↓	**↓			***↑
T. carnifex	***↓	***↓			
I. alpestris	***↓	***↓	***↓	***↓	
R. latastei 🦞	**↓	**↓			
R. dalmatina 🦞			**↑	*↑	*↑
R. italica 🦞	***↓	***↓	***↓	***↓	*⤨
R. temporaria 🦞	*↓	**↓			
P. kl. *esculentus*				**↓	
H. Intermedia			*↑		
B. bufo 🦞	**↓	***↓	*↓	**↓	
B. viridis	*↓	***↓		*↓ *⤨	

🦞 History of coexistence with *A. italicus*
↓ Significant response to visual cues
⇩ Significant response to chemical cues
⤨ Significant interaction
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Figure 3 – Effect of exposure to visual and chemical cues released by *P. clarkii* on amphibian larva behaviour
Results of LMMs and GLMMs on behavioural displays of amphibian larvae exposed to visual and chemical stimuli released by the American crayfish. Only significant results are showed; asterisks indicate the significance levels. Blue arrows indicate significant response to visual cues; yellow arrows indicate significant response to chemical cues. Up arrows represent positive effects, while down arrows negative ones. Crossed arrows indicates significant interactions between chemical and visual cues. Amphibian species coexistence with a native predatory crayfish (*Austropotamobius italicus*) is indicated by the crayfish symbol beside species name.

Total distance

Among anurans, total travelled distance was largest for the common toad (average: 244.1 ± 13.4 cm) and the Italian stream frog (149.7 ± 9.4 cm). Among caudates, the longest distances were covered by smooth newt (121.5 ± 6.9 cm) (Fig.2A; Supplementary S2). Conversely, in some species total distance moved was limited and particularly in the Italian tree frog (2.5 ± 0.4 cm) and the agile frog (8.7 ± 1.3 cm). Nevertheless, in these species the total travelled distance was highly variable among individuals (range: 0 – 23.7 cm and 0 – 113.3 cm, respectively). In most of the species (except the spectacled salamander, the Italian agile frog, the green frog and the Italian tree frog), total distance was negatively influenced by exposure to crayfish visual cues (see Fig. 3 and Supplementary S1). Conversely, no significant effect of chemical cue treatment was observed for this display for any species (all $p > 0.1$).

No-burst distance

Distance moved without bursts showed a pattern nearly equivalent to total of distance in most of caudates, except for the spectacled salamander (Fig. 2B; Supplementary S2). Among anurans, no-burst distance was very similar to total distance in the Italian tree frog and in the Italian agile frog for which constituted more than two third of total distance moved. No-burst distance was negatively influenced by visual cue treatment in all species except for the Italian agile frog, the green frog and Italian tree frog (Supplementary S1; Fig. 3). In spectacled salamanders, exposure to crayfish chemical cues caused a significant reduction in no-burst distance ($F_{1, 159.2} = 4.86$; $p = 0.029$, Table S1). By contrast, no significant effect of chemical cues on no-burst distance was detected for the other species (all $p > 0.05$).

Burst distance

Common toad, green toad and spectacled salamander covered the largest burst distances (Fig.2C; Supplementary S2), which constituted nearly half of their total distance moved during tests, while burst distance was lower in other species. Among anurans, burst distance was particularly low in the Italian tree frog (average: 0.4 ± 0.1 cm), while among caudates it was least in the Italian crested newt (ranging from 0 to 45.7 cm, but being on average 1.2 ± 0.4 cm). Burst distance was significantly affected by exposure to crayfish visual cues in six species (Fig. 3; Supplementary S1). In particular, exposure to *P. clarkii* visual cues significantly increased the burst distance in the agile frog and in the Italian tree frog ($F_{1, 163.2} = 8.10$; $p = 0.005$ and $F_{1,159.5} = 6.76$; $p = 0.010$, respectively), while caused a decrease in burst distance in fire salamander ($F_{1, 161.3} = 7.01$; $p = 0.009$), alpine newt ($F_{1, 161.2} = 19.66$; $p < 0.001$), Italian stream frog ($F_{1, 159.0} = 20.63$; $p < 0.001$) and in the common toad ($F_{1,154.8} = 4.10$; $p = 0.045$). We did not detect any significant effect of chemical cues on the burst distance performed by any of the studied species (Fig. 3; Supplementary S1).

Number of bursts

Among anurans, Italian stream frog, green frog and both toad species performed on average more than five burst per test (Fig. 2D; Supplementary S2), while in caudates the highest number of bursts was observed in spectacled salamander, which performed on average 2.4 ± 0.3 per test. The number of burst was significantly affected by the visual cue treatment in multiple species (Fig. 3; Supplementary S1). In the agile frog, the number of bursts significantly increased in the visual cue treatment ($F_1 = 6.2$; $p = 0.013$). Conversely, in six species (fire salamander, alpine newt, Italian stream frog, green frog, common toad and green toad) visual exposure to the invasive crayfish caused a general reduction in the number of burst performed by larvae (all $p < 0.02$). Chemical cue treatment produced no significant effect on number of burst in any species, even though for this display we detected a significant interaction between visual and chemical cues in green toad tadpoles. Specifically, the simultaneous exposure to both visual and chemical cues caused significant reduction in the number of bursts ($F_1 = 4.27$; $p = 0.039$). For the remaining species we found no significant interactions.

Avoidance

Visual cue treatments caused a significant increase in avoidance in spectacled salamander ($F_{1, 164.4} = 10.35$; $p = 0.02$), smooth newt ($F_{1, 144.3} = 44.47$; $p < 0.001$) and agile frog ($F_{1, 163.2} = 4.40$; $p = 0.37$). In these species, the mean distance between the larva and the barrier increased when the invasive crayfish was visible (Fig. 3). No significant effects of visual cue treatment on avoidance was observed for any other studied species (Fig. 3; Supplementary S1). Exposure to chemical cues significantly reduced the distance from the barrier in spectacled salamander larvae ($F_{1, 162.8} = 8.74$; $p = 0.004$). Finally, we detected a significant interaction between chemical and visual cues on agile stream frog, with tadpoles of this species reducing their mean distance from the barrier when both cues were simultaneously present ($F_{1, 161.9} = 5.68$; $p = 0.018$).

Is behavioural response higher in species coexisting with the native crayfish?

Redundancy analysis performed on overall larvae behavioural responses did not show any difference between species coexisting and not coexisting with the native crayfish (permutation test: $F_4 = 0.48$; $p = 0.985$). All effect size coefficients of larva behavioural responses are reported in supplementary material S4.

Discussion

Naïve larvae of amphibian species were generally able to modulate their behaviour in presence of an invasive predator, even though the modality and intensity of response were highly heterogeneous among species. Behavioural response was mainly triggered by the visual exposure to the invasive crayfish, whereas exposure to its chemical cues only caused feeble and contrasting effects on larvae behaviour. Finally, we found no correlation between the capacity of species to alter their behaviour towards the invasive predator and their potential coevolutionary history with a similar crayfish predator, such as *Austropotamobius italicus*.

Crayfish presence seems to be generally recognised as risky, as most of species altered their behaviour expressing classical anti-predator responses (e.g. reduced activity, predator avoidance, escaping behaviour) that can favour prey survival (Skelly 1994; Relyea 2001c; Teplitsky et al. 2005). Indeed, when exposed to *P. clarkii*, ten out thirteen species reduced distance moved (i.e. total distance and no-burst distance), while other species increased their escape behaviour (number of burst and burst distance covered during tests) and some of them avoided areas in proximity of the predator. These responses were mostly expressed when native prey could perceive visual cues of the invasive crayfish. Conversely, exposure to its chemical cues elicited scarce behavioural shifts, some of which was inconsistent with expected anti-predator responses. For example, when exposed to the olfactory cues released by the crayfish, spectacled salamander larvae reduced their activity (no-burst distance), a frequent behavioural response to the predators. However, crayfish chemical cues also caused spectacled salamanders to approach the crayfish sector (Fig. 3), and this contrasting behaviour hardly appears as an anti-predator response to the invasive crayfish kairomones. Rather, as the crayfish was fed fish food, it is possible that foraging cues it released resulted attractive for a predatory species, such as *S. salamandrina*.

Moreover, we found no significant correlation between capability to express behavioural response towards *P. clarkii* and species co-occurrence with the native crayfish, and this result does not support the hypothesis of coevolutionary history influence. On the one hand, it is unknown to what extent amphibians differ in their ability to respond to the cues of the native crayfish. On the other hand, even though the two crayfish share similar morphology and trophic niche, and both prey upon amphibian larvae (Renai and Gherardi 2004), these species belong to different families (*P. clarkii*: Cambaridae; *A. italicus*: Astacidae), and the large phylogenetic distance between the two crayfish species could limit the likelihood of a predator generalization by native prey.

Generalization of predator recognition is highly variable among species and closely depends on their evolutionary history (Carthey and Blumstein 2018), however the results of the present study suggest no generalization towards the red swamp crayfish in native amphibians. Rather, behavioural responses elicited by visual cues of the crayfish resemble a non-specific anti-predator behaviours towards generic risk cues (Mathis and Vincent 2000; Rehage et al. 2009; Carthey and Blumstein 2018), supporting the predictions of the generic response hypothesis. In fact, large approaching figures have been observed to produce similar responses in amphibian larvae (Wilson et al. 2018).

The incapability of larvae to recognise chemical stimuli released by the invasive crayfish may weaken the effectiveness of their responses, as risk assessment is based on incomplete information. Indeed, under some circumstances visual stimuli can allow risk assessment only when the predator is in close proximity, thus hampering predator avoidance and undermining a prompt anti-predatory response. For instance, predator recognition based on visual cues can be particularly ineffective in environmental contexts where the visibility is scarce, such as in turbid or highly-vegetated habitats, where the quality of visual information received by the prey dramatically decreases (Abrahams and Kattenfeld 1997; Ferrari et al. 2010b). Conversely, in these environments chemical cues may allow a better forewarning of predator presence (Chivers and Smith 1998; Chivers et al. 2001) and prey can strongly rely on these stimuli for risk assessment. However, it is also worth noting that native prey can learn to recognise predator kairomones and refine their anti-predator response through experience. Indeed, in nature, naïve prey become often exposed 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; Polo-Cavia and Gomez-Mestre 2014). However, ethical issues hampered to set up experiments involving the direct predation of amphibian larvae by the crayfish.

Even though most of species responded to the visual stimuli of the crayfish, response modality was highly heterogeneous among species (Fig. 3). The most frequent behavioural shift we observed was reduction in activity, while predator avoidance was observed in fewer cases (smooth newt, spectacled salamander, and agile frog). Total distance and no-burst distance were generally highly correlated and significantly decreased with the exposure to crayfish. Instead, shifts in the number of bursts and distance covered during bursts were highly variable across species. The tendency to perform rapid movements decreased together with general decrease of the activity in some cases, suggesting shift in these displays were consistent or part of a general activity reduction (i.e. in fire salamander, alpine newt, green frog and toads). Conversely, in other species (i.e. agile frog and Italian tree frog) bursts frequency and burst distance were increased by crayfish presence. These species showed generally low activity levels compared to others, but showed no further reduction of distance moved when exposed to crayfish. In these species, rapid bursts may constitute escape responses, which, for instance, could have been activated when the crayfish got closer. For spectacled salamanders, behavioural response to the predator was only visible when we separately considered distance covered without bursts. However, it is worth noting that the activity of spectacled salamanders is characterized by high burst frequency. This could have inflated total distance covered and thus possibly prevented observation of any activity reduction when considering total distance moved only. Such a variation among strategies exhibited by different species underlines the importance of finely evaluating species behavioural displays when considering their anti-predator responses.

Behavioural responses to predation risk are typically short-term reversible strategies (Turner 1997; Relyea 2001b), and often constitute the first line of defence native species can rely on when facing invasive predators (Holway and Suarez 1999; Sih et al. 2010; Weis and Sol 2016). However, the actual effectiveness of the observed responses towards the invasive crayfish remains to be tested. Few studies has investigated the effectiveness of behavioural responses of amphibian larvae to this crayfish, and to our knowledge only a study has found positive effects on larvae survival in one species (Polo-Cavia and Gomez-Mestre 2014), while in the majority of cases there was no significant correlation between behavioural responses and survival (Rebelo and Cruz 2005). In fact, the severe impact of *P. clarkii* on multiple native amphibians has led to rapid declines and local extinctions in invaded populations worldwide (Gamradt and Kats 1996; Cruz et al. 2008; Ficetola et al. 2011a; Liu et al. 2018), suggesting that the responses of native species can be often insufficient to withstand the predatory pressure posed by this voracious predator.

The present study demonstrated that naïve amphibian larvae have a striking capability to alter their behaviour in presence of novel predators and shed light on ecological mechanisms allowing non-native predator recognition. Moreover, by unravelling a relevant heterogeneity of behavioural responses across species, with the presence of even contrasting strategies, we highlight the importance of considering multiple traits when investigating predator-prey interactions between native and non-native species. Even though non-native predators triggered the expression of a relevant behavioural plasticity in native species, still the potential of these responses in promoting their persistence and coexistence with invasive species has to be ascertained. Further research should thus investigate the effectiveness of behavioural responses of native prey in withstanding invasive predators and compare capability to express anti-predator behaviour and species population trends.

Ethical statement

Larva collection, treatments and behavioural tests were authorized by Italian Ministry for Environment (DPR 357/97 and Prot. N. 12969/T-A31). To prevent potential pathogen exchange between amphibian populations or conveyed by the invasive crayfish, after each behavioural sessions and before individual releasing, all experimental and housing material was carefully washed with 1% Virkon S solution (Bosch et al. 2015), while amphibian larvae were treated with 0.5 mg/L for five minutes. This antifungal is a powerful disinfectant that 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 has no significant effect on individual survival and growth and can be used to treat amphibian larvae (von Rütte et al. 2009; Hangartner and Laurila 2012). However, after treatment with Virkon S, larvae were monitored for one day before releasing, and we observed no mortality or visible change in their behaviour.

Supplementary material

Table S1 – Results of mixed models for larva behavioural responses in each species. Influence of the independent variables on each behavioural display in each species. Total distance, no-burst distance, burst distance and avoidance were Gaussian-distributed and LMMs were used for the analysis of these displays (test statistic: F). Number of bursts presented a negative binomial distribution and were analysed with GLMMs (test statistic: χ^2). Test duration was not included for *Salamandra salamandra*, as we had no shorter trials for this species. Significant effects are reported in bold.

Species	Display	Independent variable	df	test statistic	p value	effect
Salamandra salamandra	Total distance	chemical cues	1, 161.2	0.02	0.885	-
		visual cues	**1, 161.2**	**9.00**	**0.003**	**-**
		temperature	**1, 174**	**18.3**	**< 0.001**	**+**
		day time	**1, 54.8**	**4.53**	**0.038**	**-**
	No-burst distance	chemical cues	1, 161.2	0.12	0.725	-
		visual cues	**1, 161.2**	**7.45**	**0.007**	**-**
		temperature	**1, 174.1**	**20.05**	**< 0.001**	**+**
		day time	**1, 55.0**	**5.78**	**0.02**	**-**
	Burst distance	chemical cues	1, 161.3	0.66	0.418	+
		visual cues	**1, 161.3**	**7.01**	**0.009**	**-**
		temperature	1, 19.4	0.12	0.734	+
		day time	1, 37.8	0.31	0.582	+
	Number of bursts	chemical cues	1	0.28	0.597	+
		visual cues	**1**	**7.43**	**0.006**	**-**
		temperature	1	0.04	0.841	+
		day time	1	0.69	0.406	+
	Avoidance	chemical cues	1, 160.1	0.02	0.894	-
		visual cues	1, 160.1	0.01	0.905	-
		temperature	1, 14.1	4.19	0.060	-
		day time	1, 28.1	1.87	0.182	+
Salamandrina perspicillata	Total distance	chemical cues	1, 159.4	2.25	0.136	-
		visual cues	1, 160.3	1.51	0.220	-
		temperature	1, 23.7	0.14	0.716	-
		day time	1, 25.7	0.17	0.685	-
		test duration	1, 177.6	0.97	0.325	-
	No-burst distance	**chemical cues**	**1, 159.2**	**4.86**	**0.029**	**-**
		visual cues	**1, 160.1**	**7.62**	**0.006**	**-**
		temperature	1, 44.0	0.44	0.508	-
		day time	1, 41.9	0	0.946	-
		test duration	1, 177.4	0.05	0.822	-
	Burst distance	chemical cues	1, 161.5	0.70	0.405	-
		visual cues	1, 162.6	1.38	0.242	+
		temperature	1, 26.9	0	0.997	-
		day time	1, 27.4	0.63	0.434	-
		test duration	1, 180.9	3.15	0.078	-
		chemical cues	1	0.74	0.390	-

	Number of bursts	visual cues	1	0.34	0.562	+
		temperature	1	0.06	0.803	+
		day time	1	1.20	0.272	-
		test duration	1	2.58	0.108	-
	Avoidance	**chemical cues**	**1, 162.8**	**8.74**	**0.004**	**-**
		visual cues	**1, 164.4**	**10.35**	**0.002**	**+**
		temperature	1, 25.3	1.59	0.219	-
		day time	1, 25.5	1.43	0.243	+
		test duration	1, 182.2	1.73	0.190	+
Lissotriton vulgaris	Total distance	chemical cues	1, 141.8	1.96	0.164	-
		visual cues	**1, 142.6**	**10.42**	**0.002**	**-**
		temperature	1, 24.1	0.28	0.599	-
		day time	1, 24.0	0.31	0.581	+
		test duration	**1, 150**	**9.67**	**0.002**	**+**
	No-burst distance	chemical cues	1, 141.7	2.87	0.093	-
		visual cues	**1, 142.5**	**14.76**	**< 0.001**	**-**
		temperature	1, 24.4	0.35	0.559	-
		day time	1, 23.9	0.36	0.553	+
		test duration	**1, 149.8**	**6.95**	**0.009**	**+**
	Burst distance	chemical cues	1, 141.6	1.34	0.248	+
		visual cues	1, 142.7	2.11	0.149	+
		temperature	1, 20.4	0.22	0.644	-
		day time	1, 20.3	0.57	0.459	+
		test duration	**1, 155.2**	**6.53**	**0.012**	**+**
	Number of bursts	chemical cues	1	0.34	0.561	+
		visual cues	1	3.36	0.067	+
		temperature	1	0.16	0.686	-
		day time	1	0.22	0.639	+
		test duration	**1**	**7.58**	**0.006**	**+**
	Avoidance	chemical cues	1, 143.2	0.01	0.915	+
		visual cues	**1, 144.3**	**47.44**	**< 0.001**	**+**
		temperature	1, 21.4	0.59	0.450	-
		day time	1, 28.2	0.19	0.668	-
		test duration	1, 156.6	0.02	0.899	+
Triturus carnifex	Total distance	chemical cues	1, 152.2	0.28	0.598	+
		visual cues	**1, 152.4**	**20.90**	**< 0.001**	**-**
		temperature	**1, 17.4**	**25.71**	**< 0.001**	**+**
		day time	**1, 21.2**	**24.26**	**< 0.001**	**-**
		test duration	1, 159.4	1.94	0.165	-
	No-burst distance	chemical cues	1, 152.2	0.17	0.682	+
		visual cues	**1, 152.5**	**21.31**	**< 0.001**	**-**
		temperature	**1, 17.4**	**26.37**	**< 0.001**	**+**
		day time	**1, 21.2**	**24.38**	**< 0.001**	**-**
		test duration	1, 159.4	1.72	0.191	-
	Burst distance	chemical cues	1, 161.7	1.23	0.269	+
		visual cues	1, 162.5	0.03	0.873	-
		temperature	1, 2.2	0.20	0.693	-
		day time	1, 4.5	0.42	0.549	-
		test duration	1, 177.6	0.39	0.535	+
		chemical cues	1	0.62	0.430	0

	Number of bursts	visual cues	1	0.55	0.457	-
		temperature	1	3.50	0.061	+
		day time	**1**	**5.68**	**0.017**	**-**
		test duration	1	0.37	0.542	+
	Avoidance	chemical cues	1, 163.5	0.32	0.572	-
		visual cues	1, 164.7	1.56	0.214	+
		temperature	1, 18.1	2.50	0.131	+
		day time	1, 28.3	3.54	0.070	-
		test duration	1, 183	1.62	0.205	-
Ichthyosaura alpestris	Total distance	chemical cues	1, 160.4	0.40	0.530	-
		visual cues	**1, 160.8**	**20.15**	**< 0.001**	**-**
		temperature	1, 156.8	0.12	0.726	-
		day time	1, 99.1	1.28	0.261	+
		test duration	**1, 169.3**	**8.85**	**0.003**	**+**
	No-burst distance	chemical cues	1, 160.2	0.58	0.446	-
		visual cues	**1, 160.6**	**20.03**	**< 0.001**	**-**
		temperature	1, 162.8	0.17	0.685	-
		day time	1, 102.3	1.87	0.175	+
		test duration	**1, 168.4**	**8.31**	**0.004**	**+**
	Burst distance	chemical cues	1, 160.3	0.87	0.352	-
		visual cues	**1, 161.2**	**19.66**	**< 0.001**	**-**
		temperature	1, 89.7	0.19	0.664	+
		day time	1, 59.2	2.49	0.120	-
		test duration	1, 177.5	4.01	0.047	+
	Number of bursts	chemical cues	1	1.88	0.170	-
		visual cues	**1**	**19.54**	**< 0.001**	**-**
		temperature	1	0.52	0.471	+
		day time	1	3.44	0.064	-
		test duration	**1**	**7.63**	**0.006**	**+**
	Avoidance	chemical cues	1, 162	0.35	0.557	-
		visual cues	1, 163.2	2.93	0.089	+
		temperature	1, 68.8	0.37	0.544	+
		day time	1, 56.2	1.27	0.265	-
		test duration	**1, 181.3**	**7.83**	**0.006**	**-**
Rana latastei	Total distance	chemical cues	1, 138.4	0	0.949	-
		visual cues	**1, 141.2**	**7.57**	**0.007**	**-**
		temperature	**1, 23.0**	**8.23**	**0.009**	**-**
		day time	1, 19.1	0	0.975	-
		test duration	1, 162	1.28	0.259	-
	No-burst distance	chemical cues	1, 138.8	0.07	0.794	-
		visual cues	**1, 141.3**	**17.66**	**< 0.001**	**-**
		temperature	**1, 7.7**	**7.97**	**0.023**	**-**
		day time	1, 16.3	0.10	0.762	-
		test duration	1, 160.2	1.97	0.163	-
	Burst distance	chemical cues	1, 142.2	0.01	0.927	+
		visual cues	1, 145.4	2.41	0.123	+
		temperature	**1, 46.5**	**6.09**	**0.017**	**-**
		day time	1, 22.2	0.08	0.786	+
		test duration	1, 166.9	0.61	0.436	-
		chemical cues	1	0.02	0.888	+

	Number of bursts	visual cues	1	0.86	0.353	+
		temperature	**1**	**4.11**	**0.043**	**-**
		day time	1	0	0.953	+
		test duration	1	1.12	0.289	-
	Avoidance	chemical cues	1, 172.9	1.84	0.177	-
		visual cues	1, 172.9	0.09	0.759	-
		temperature	1, 172.9	0.74	0.390	+
		day time	1, 172.9	3.10	0.080	-
		test duration	1, 172.9	0.48	0.487	+
Rana dalmatina	Total distance	chemical cues	1, 161.8	1.13	0.290	+
		visual cues	1, 162.5	0.28	0.597	+
		temperature	**1, 23.8**	**13.4**	**0.001**	**-**
		day time	1, 23.5	3.87	0.061	+
		test duration	1, 182.1	1.50	0.223	-
	No-burst distance	chemical cues	1, 161.4	0.58	0.448	+
		visual cues	1, 162	0.52	0.472	-
		temperature	**1, 15.7**	**11.48**	**0.004**	**-**
		day time	1, 23.8	1.71	0.204	+
		test duration	1, 182	1.34	0.248	-
	Burst distance	chemical cues	1, 162.5	0.55	0.458	+
		visual cues	**1, 163.2**	**8.10**	**0.005**	**+**
		temperature	1, 24.6	6.54	0.017	-
		day time	1, 24.6	3.72	0.065	+
		test duration	1, 182.7	0.53	0.466	-
	Number of bursts	chemical cues	1	0.31	0.580	+
		visual cues	**1**	**6.2**	**0.013**	**+**
		temperature	1	3.85	0.050	-
		day time	1	2.85	0.091	+
		test duration	1	0.25	0.620	-
	Avoidance	chemical cues	1, 162.5	2.14	0.145	+
		visual cues	**1, 163.2**	**4.40**	**0.037**	**+**
		temperature	1, 24.1	0.16	0.693	+
		day time	1, 23.8	0.10	0.755	+
		test duration	1, 181.8	0.11	0.744	-
Rana italica	Total distance	chemical cues	1, 158.9	2.28	0.133	-
		visual cues	**1, 158.9**	**25.91**	**< 0.001**	**-**
		temperature	**1, 45.2**	**4.87**	**0.032**	**+**
		day time	1, 36.1	0.70	0.409	-
		test duration	1, 167.9	0.82	0.368	+
	No-burst distance	chemical cues	1, 158.8	2.60	0.109	-
		visual cues	**1, 158.8**	**30.93**	**< 0.001**	**-**
		temperature	**1, 51.7**	**5.81**	**0.020**	**+**
		day time	1, 37.2	1.33	0.256	-
		test duration	1, 167.8	1.10	0.295	+
	Burst distance	chemical cues	1, 159	1.09	0.298	-
		visual cues	**1, 159**	**20.63**	**< 0.001**	**-**
		temperature	**1, 38.4**	**5.29**	**0.027**	**+**
		day time	1, 34.9	0.06	0.807	-
		test duration	1, 167.4	0.04	0.839	-
		chemical cues	1	0.48	0.489	-

	Number of bursts	**visual cues**	**1**	**20.29**	**< 0.001**	**-**
		temperature	1	1.46	0.227	+
		day time	1	0.02	0.901	+
		test duration	1	0.13	0.716	-
	Avoidance	chemical cues	1, 162.2	0.14	0.706	-
		visual cues	1, 162.3	0.31	0.576	-
		temperature	1, 25.5	0.71	0.407	-
		day time	1, 24.7	1.32	0.261	+
		test duration	1, 180.5	0	0.962	-
		chemical * visual cues	**1, 161.9**	**5.68**	**0.018**	**+**
Rana temporaria	Total distance	chemical cues	1, 149.4	0.04	0.842	+
		visual cues	**1, 149.5**	**6.14**	**0.014**	**-**
		temperature	**1, 18.5**	**13.66**	**0.002**	**-**
		day time	1, 23.7	0.15	0.698	+
		test duration	1, 154.5	0.25	0.619	-
	No-burst distance	chemical cues	1, 148.9	0.19	0.660	-
		visual cues	**1, 149.1**	**7.73**	**0.006**	**-**
		temperature	1, 0	12.73	0.957	-
		day time	1, 20.3	0.09	0.766	+
		test duration	1, 153.9	0.03	0.861	-
	Burst distance	chemical cues	1, 152.2	3.53	0.062	+
		visual cues	1, 152.3	0.67	0.413	-
		temperature	**1, 20.1**	**11.69**	**0.003**	**-**
		day time	1, 23.7	0.80	0.379	+
		test duration	1, 159.9	0.25	0.619	-
	Number of bursts	chemical cues	1	2.40	0.122	+
		visual cues	1	1.18	0.277	-
		temperature	**1**	**5.62**	**0.018**	**-**
		day time	1	0.48	0.488	+
		test duration	1	0.11	0.736	-
	Avoidance	chemical cues	1, 155.3	3.08	0.081	-
		visual cues	1, 155.7	2.22	0.138	+
		temperature	1, 0.5	0	0.973	+
		day time	1, 23.3	1.79	0.194	+
		test duration	1, 171.2	1.52	0.220	+
Pelophylax kl. *esculentus*	Total distance	chemical cues	1, 151.9	0.32	0.574	+
		visual cues	1, 151.6	0.8	0.371	-
		temperature	1, 167.3	0.08	0.775	-
		day time	1, 24.2	0.03	0.873	-
		test duration	**1, 168.1**	**5.32**	**0.022**	**+**
	No-burst distance	chemical cues	1, 152.4	0.48	0.489	+
		visual cues	1, 152	0.95	0.332	-
		temperature	1, 168	0.22	0.641	-
		day time	1, 24.8	0.12	0.727	-
		test duration	**1, 168.7**	**5.66**	**0.019**	**+**
	Burst distance	chemical cues	1, 151	0.05	0.821	+
		visual cues	1, 150.9	2.07	0.153	-
		temperature	1, 162.3	0.44	0.508	+
		day time	1, 26.2	0.02	0.877	-

		test duration	1, 163.2	3.25	0.073	+
	Number of bursts	chemical cues	1	0.29	0.592	-
		visual cues	**1**	**8.31**	**0.004**	**-**
		temperature	1	0.07	0.797	+
		day time	1	0.60	0.438	-
		test duration	1	2.98	0.084	+
	Avoidance	chemical cues	1, 173.1	2.43	0.121	-
		visual cues	1, 173	0.05	0.815	+
		temperature	1, 170.3	0.27	0.606	+
		day time	1, 156.4	2.86	0.093	-
		test duration	1, 173.6	0.25	0.619	-
Hyla intermedia	Total distance	chemical cues	1, 153	0.81	0.370	+
		visual cues	1, 153.9	0.62	0.434	+
		temperature	**1, 44.2**	**10.59**	**0.002**	**-**
		day time	1, 49.8	2.68	0.108	+
		test duration	1, 168.6	0.85	0.358	-
	No-burst distance	chemical cues	1, 152.6	0.38	0.538	+
		visual cues	1, 153.4	0.04	0.838	+
		temperature	**1, 42.8**	**10.41**	**0.002**	**-**
		day time	1, 48.8	2.16	0.148	+
		test duration	1, 167.2	0	0.998	-
	Burst distance	chemical cues	1, 158.2	2.28	0.133	+
		visual cues	**1, 159.5**	**6.76**	**0.010**	**+**
		temperature	1, 1.4	1.57	0.371	-
		day time	1, 3.9	1.46	0.293	+
		test duration	**1, 176**	**4.09**	**0.045**	**-**
	Number of bursts	chemical cues	NA	NA	NA	NA
		visual cues	NA	NA	NA	NA
		temperature	NA	NA	NA	NA
		day time	NA	NA	NA	NA
		test duration	NA	NA	NA	NA
	Avoidance	chemical cues	1, 156.4	0.61	0.435	-
		visual cues	1, 157.9	1.44	0.232	+
		temperature	1, 0.6	0.31	0.708	-
		day time	1, 23.3	0.06	0.814	-
		test duration	1, 176.7	0.06	0.813	-
Bufo bufo	Total distance	chemical cues	1, 154.2	0	0.998	+
		visual cues	**1, 154.5**	**8.25**	**0.005**	**-**
		temperature	1, 40.6	0.26	0.615	-
		day time	1, 40.3	1.74	0.194	+
		test duration	1, 161.7	1.53	0.217	+
	No-burst distance	chemical cues	1, 154.4	0.23	0.629	-
		visual cues	**1, 154.8**	**14.61**	**< 0.001**	**-**
		temperature	1, 39.8	1.63	0.209	-
		day time	1, 38.6	4.07	0.051	+
		test duration	1, 162.6	0.75	0.387	+
	Burst distance	chemical cues	1, 154.5	1.37	0.244	+
		visual cues	**1, 154.8**	**4.10**	**0.045**	**-**
		temperature	1, 15.0	0.67	0.425	+
		day time	1, 23.3	0	0.955	+

		test duration	1, 161.8	0.42	0.518	+
	Number of bursts	chemical cues	1	2.83	0.092	+
		visual cues	**1**	**8.02**	**0.005**	**-**
		temperature	1	0.53	0.468	+
		day time	1	0.02	0.882	-
		test duration	1	2.77	0.096	0
	Avoidance	chemical cues	1, 156.7	0.02	0.890	+
		visual cues	1, 157.6	0.55	0.461	+
		temperature	**1, 24.3**	**6.99**	**0.014**	**-**
		day time	1, 23.9	2.32	0.141	+
		test duration	1, 172.5	0.01	0.943	+
Bufo viridis	Total distance	chemical cues	1, 156	0.07	0.792	+
		visual cues	**1, 156.2**	**6.06**	**0.015**	**-**
		temperature	**1, 92.4**	**7.73**	**0.007**	**+**
		day time	**1, 49.4**	**9.31**	**0.004**	**-**
		test duration	1, 158.9	1.37	0.244	+
	No-burst distance	chemical cues	1, 156.5	0	0.965	-
		visual cues	**1, 156.7**	**12.88**	**< 0.001**	**-**
		temperature	**1, 77.0**	**7.02**	**0.010**	**+**
		day time	**1, 44.8**	**9.60**	**0.003**	**-**
		test duration	1, 159.8	1.24	0.267	+
	Burst distance	chemical cues	1, 154.8	0.85	0.357	+
		visual cues	1, 155	0.02	0.888	-
		temperature	1, 6.3	2.27	0.180	+
		day time	1, 30.7	3.60	0.067	-
		test duration	1, 158.1	1.05	0.308	+
	Number of bursts	chemical cues	1	0.64	0.425	+
		visual cues	**1**	**6.44**	**0.011**	**-**
		temperature	1	1.03	0.310	+
		day time	1	2.93	0.087	0
		test duration	1	0.47	0.494	+
		chemical * visual cues	**1**	**4.27**	**0.039**	**-**
	Avoidance	chemical cues	1, 156.9	0.24	0.625	+
		visual cues	1, 157.8	0.32	0.573	+
		temperature	1, 25.2	4.12	0.053	+
		day time	1, 26.4	0.62	0.437	-
		test duration	1, 173.3	0.01	0.912	-

Table S2 – Comparison of behavioural display value among species. Table S2 shows mean values, standard error and range (minimum – maximum value) for each behavioural display in each species.

Species	Display	mean	standard error	range
Salamandra salamandra	Total distance	56.8	3.4	0.3 - 204.3
	No-burst distance	53.6	3.2	0.3 - 187.7
	Burst distance	3.2	0.6	0 - 53.9
	Number of bursts	0.5	0.1	0 - 6
	Avoidance	6.3	0.3	1.3 - 16.6
Salamandrina perspicillata	Total distance	56.7	4.6	0 - 308.1
	No-burst distance	32.0	2.4	0 - 148.4
	Burst distance	24.7	2.7	0 - 212.4
	Number of bursts	2.4	0.3	0 - 15
	Avoidance	8.5	0.3	0.8 - 16.1
Lissotriton vulgaris	Total distance	121.5	6.9	0 - 365.5
	No-burst distance	109.6	6.3	0 - 356.6
	Burst distance	11.8	1.5	0 - 119.3
	Number of bursts	1.4	0.2	0 - 11
	Avoidance	8.9	0.3	0.9 - 16.1
Triturus carnifex	Total distance	43.3	3.7	0 - 307.1
	No-burst distance	42.1	3.5	0 - 261.5
	Burst distance	1.2	0.4	0 - 45.7
	Number of bursts	0.2	0.1	0 - 8
	Avoidance	7.6	0.3	1.4 - 16.2
Ichthyosaura alpestris	Total distance	83.3	5.6	0.1 - 408.1
	No-burst distance	75.7	5.0	0.1 - 348.5
	Burst distance	7.6	1.4	0 - 206.4
	Number of bursts	1.0	0.1	0 - 13
	Avoidance	8.1	0.2	1.4 - 15.9
Rana latastei	Total distance	32.3	4.0	0 - 264.8
	No-burst distance	21	2.9	0 - 222.6
	Burst distance	11.4	1.9	0 - 130.2
	Number of bursts	1.1	0.2	0 - 11
	Avoidance	11.4	0.4	2.7 - 19.3
Rana dalmatina	Total distance	8.7	1.3	0 - 113.3
	No-burst distance	5.1	0.7	0 - 56.9
	Burst distance	3.6	0.8	0 - 88.7
	Number of bursts	0.3	0.1	0 - 6
	Avoidance	10.6	0.4	3.4 - 19
Rana italica	Total distance	149.7	9.4	0 - 507.2
	No-burst distance	83.6	5.1	0 - 247.4
	Burst distance	66.1	5.1	0 - 310
	Number of bursts	7.5	0.5	0 - 29
	Avoidance	12.9	0.3	3.3 - 19.7
Rana temporaria	Total distance	43.1	5.6	0 - 517.1
	No-burst distance	26.9	3.1	0 - 209.4
	Burst distance	16.2	3.0	0 - 307.7
	Number of bursts	1.8	0.3	0 - 34
	Avoidance	10.5	0.4	2.1 - 18.9

Pelophylax kl. *esculentus*	Total distance	114	12.7	0 - 736.7
	No-burst distance	66.7	6.9	0 - 434.1
	Burst distance	47.4	6.7	0 - 447.8
	Number of bursts	5.3	0.7	0 - 45
	Avoidance	12.0	0.4	3.4 - 18.9
Hyla intermedia	Total distance	2.5	0.4	0 - 23.7
	No-burst distance	2.1	0.3	0 - 23.7
	Burst distance	0.4	0.1	0 - 14.4
	Number of bursts	0	0	0 - 1
	Avoidance	10.9	0.4	2.8 - 19.7
Bufo bufo	Total distance	244.1	13.4	0 - 750.2
	No-burst distance	123.0	6.6	0 - 360.3
	Burst distance	121.1	8.8	0 - 538.4
	Number of bursts	11.8	0.8	0 - 42
	Avoidance	11.7	0.3	3.2 - 18.7
Bufo viridis	Total distance	108.8	8.7	0 - 529.7
	No-burst distance	59.9	4.3	0 - 251.5
	Burst distance	48.9	5.0	0 - 323.2
	Number of bursts	5.1	0.5	0 - 31
	Avoidance	10.9	0.3	2.4 - 18.3

Table S3 – Correlation among behavioural displays in each species. Correlation matrixes show relations among behavioural displays in each species. Correlation value (r = correlation coefficient) and significance value of the correlation (p) are reported.

		Display									
Species	**Display**	Total distance		No-burst distance		Burst distance		Number of bursts		Avoidance	
		r	p	r	p	r	P	r	p	r	p
Salamandra salamandra	Total distance	1	< 0.001	0.99	< 0.001	0.44	< 0.001	0.49	< 0.001	-0.18	0.048
	No-burst distance	0.99	< 0.001	1	< 0.001	0.29	< 0.001	0.34	< 0.001	-0.19	0.04
	Burst distance	0.44	< 0.001	0.29	< 0.001	1	< 0.001	0.97	< 0.001	0	1
	Number of bursts	0.49	< 0.001	0.34	< 0.001	0.97	< 0.001	1	< 0.001	0	1
	Avoidance	-0.18	0.016	-0.19	0.01	0	0.956	0	0.968	1	< 0.001

Salamandrina perspicillata	Total distance	1	< 0.001	0.87	< 0.001	0.9	< 0.001	0.93	< 0.001	-0.06	1
	No-burst distance	0.87	< 0.001	1	< 0.001	0.57	< 0.001	0.7	< 0.001	-0.11	0.467
	Burst distance	0.9	< 0.001	0.57	< 0.001	1	< 0.001	0.94	< 0.001	0	1
	Number of bursts	0.93	< 0.001	0.7	< 0.001	0.94	< 0.001	1	< 0.001	-0.06	1
	Avoidance	-0.06	0.416	-0.11	0.117	0	0.976	-0.06	0.421	1	< 0.001
Lissotriton vulgaris	Total distance	1	< 0.001	0.98	< 0.001	0.49	< 0.001	0.53	< 0.001	-0.48	< 0.001
	No-burst distance	0.98	< 0.001	1	< 0.001	0.3	< 0.001	0.36	< 0.001	-0.52	< 0.001
	Burst distance	0.49	< 0.001	0.3	< 0.001	1	< 0.001	0.93	< 0.001	-0.04	0.652
	Number of bursts	0.53	< 0.001	0.36	< 0.001	0.93	< 0.001	1	< 0.001	-0.08	0.652
	Avoidance	-0.48	< 0.001	-0.52	< 0.001	-0.04	0.569	-0.08	0.326	1	< 0.001
Triturus carnifex	Total distance	1	< 0.001	1	< 0.001	0.58	< 0.001	0.62	< 0.001	-0.12	0.402
	No-burst distance	1	< 0.001	1	< 0.001	0.5	< 0.001	0.56	< 0.001	-0.12	0.402
	Burst distance	0.58	< 0.001	0.5	< 0.001	1	< 0.001	0.95	< 0.001	-0.05	0.76
	Number of bursts	0.62	< 0.001	0.56	< 0.001	0.95	< 0.001	1	< 0.001	-0.06	0.76
	Avoidance	-0.12	0.106	-0.12	0.1	-0.05	0.536	-0.06	0.38	1	< 0.001
Ichthyosaura alpestris	Total distance	1	< 0.001	0.97	< 0.001	0.54	< 0.001	0.68	< 0.001	-0.2	0.015
	No-burst distance	0.97	< 0.001	1	< 0.001	0.32	< 0.001	0.55	< 0.001	-0.21	0.013
	Burst distance	0.54	< 0.001	0.32	< 0.001	1	< 0.001	0.76	< 0.001	-0.05	0.927
	Number of bursts	0.68	< 0.001	0.55	< 0.001	0.76	< 0.001	1	< 0.001	-0.05	0.927
	Avoidance	-0.2	0.005	-0.21	0.003	-0.05	0.475	-0.05	0.463	1	< 0.001
Rana latastei	Total distance	1	< 0.001	0.91	< 0.001	0.78	< 0.001	0.88	< 0.001	0.05	1
	No-burst distance	0.91	< 0.001	1	< 0.001	0.45	< 0.001	0.65	< 0.001	0.03	1
	Burst distance	0.78	< 0.001	0.45	< 0.001	1	< 0.001	0.91	< 0.001	0.06	1
	Number of bursts	0.88	< 0.001	0.65	< 0.001	0.91	< 0.001	1	< 0.001	0.05	1
	Avoidance	0.05	0.529	0.03	0.72	0.06	0.412	0.05	0.518	1	< 0.001
Rana dalmatina	Total distance	1	< 0.001	0.78	< 0.001	0.86	< 0.001	0.9	< 0.001	0.05	1
	No-burst distance	0.78	< 0.001	1	< 0.001	0.35	< 0.001	0.51	< 0.001	0.02	1
	Burst distance	0.86	< 0.001	0.35	< 0.001	1	< 0.001	0.93	< 0.001	0.05	1

	Number of bursts	0.9	< 0.001	0.51	< 0.001	0.93	< 0.001	1	< 0.001	0.05	1
	Avoidance	0.05	0.535	0.02	0.748	0.05	0.506	0.05	0.462	1	< 0.001
Rana italica	Total distance	1	< 0.001	0.92	< 0.001	0.92	< 0.001	0.95	< 0.001	0.04	1
	No-burst distance	0.92	< 0.001	1	< 0.001	0.7	< 0.001	0.81	< 0.001	0.06	1
	Burst distance	0.92	< 0.001	0.7	< 0.001	1	< 0.001	0.94	< 0.001	0.02	1
	Number of bursts	0.95	< 0.001	0.81	< 0.001	0.94	< 0.001	1	< 0.001	0.03	1
	Avoidance	0.04	0.546	0.06	0.385	0.02	0.807	0.03	0.672	1	< 0.001
Rana temporaria	Total distance	1	< 0.001	0.92	< 0.001	0.92	< 0.001	0.94	< 0.001	-0.01	1
	No-burst distance	0.92	< 0.001	1	< 0.001	0.7	< 0.001	0.75	< 0.001	-0.02	1
	Burst distance	0.92	< 0.001	0.7	< 0.001	1	< 0.001	0.98	< 0.001	-0.01	1
	Number of bursts	0.94	< 0.001	0.75	< 0.001	0.98	< 0.001	1	< 0.001	-0.01	1
	Avoidance	-0.01	0.877	-0.02	0.837	-0.01	0.938	-0.01	0.862	1	< 0.001
Pelophylax kl. *esculentus*	Total distance	1	< 0.001	0.93	< 0.001	0.93	< 0.001	0.95	< 0.001	-0.03	1
	No-burst distance	0.93	< 0.001	1	< 0.001	0.72	< 0.001	0.8	< 0.001	-0.01	1
	Burst distance	0.93	< 0.001	0.72	< 0.001	1	< 0.001	0.98	< 0.001	-0.05	1
	Number of bursts	0.95	< 0.001	0.8	< 0.001	0.98	< 0.001	1	< 0.001	-0.05	1
	Avoidance	-0.03	0.664	-0.01	0.91	-0.05	0.482	-0.05	0.503	1	< 0.001
Hyla intermedia	Total distance	1	< 0.001	0.93	< 0.001	0.52	< 0.001	0.48	< 0.001	0.05	1
	No-burst distance	0.93	< 0.001	1	< 0.001	0.16	0.189	0.14	0.325	0.03	1
	Burst distance	0.52	< 0.001	0.16	0.031	1	< 0.001	0.95	< 0.001	0.05	1
	Number of bursts	0.48	< 0.001	0.14	0.065	0.95	< 0.001	1	< 0.001	0.04	1
	Avoidance	0.05	0.537	0.03	0.668	0.05	0.52	0.04	0.598	1	< 0.001
Bufo bufo	Total distance	1	< 0.001	0.82	< 0.001	0.9	< 0.001	0.94	< 0.001	-0.18	0.038
	No-burst distance	0.82	< 0.001	1	< 0.001	0.5	< 0.001	0.68	< 0.001	-0.11	0.13
	Burst distance	0.9	< 0.001	0.5	< 0.001	1	< 0.001	0.92	< 0.001	-0.18	0.038
	Number of bursts	0.94	< 0.001	0.68	< 0.001	0.92	< 0.001	1	< 0.001	-0.19	0.036
	Avoidance	-0.18	0.017	-0.11	0.13	-0.18	0.013	-0.19	0.009	1	< 0.001
Bufo viridis	Total distance	1	< 0.001	0.92	< 0.001	0.94	< 0.001	0.96	< 0.001	0.01	1

	No-burst distance	0.92	< 0.001	1	< 0.001	0.74	< 0.001	0.83	< 0.001	0.06	1
	Burst distance	0.94	< 0.001	0.74	< 0.001	1	< 0.001	0.96	< 0.001	-0.03	1
	Number of bursts	0.96	< 0.001	0.83	< 0.001	0.96	< 0.001	1	< 0.001	-0.02	1
	Avoidance	0.01	0.856	0.06	0.445	-0.03	0.728	-0.02	0.81	1	< 0.001

Table S4 – Treatment effect size on behavioural displays. Effect size values (z values) of treatment effect on tested behavioural displays were calculated from the corresponding mixed model F values. Effect sizes of responses to both stimuli are reported: chemical cues (A) and visual cues (B).

A)		**Responses to chemical cues**				
Species	**Coexistence with *A. italicus***	**Effect size (z)**				
		Total distance	**No-burst distance**	**Burst distance**	**Number of bursts**	**Avoidance**
Salamandra salamandra	1	0.01	0.03	0.06	0.04	0.01
Salamandrina perspicillata	1	0.12	0.17	0.07	0.06	0.23
Lissotriton vulgaris	0	0.12	0.14	0.10	0.05	0.01
Triturus carnifex	0	0.04	0.03	0.09	0.06	0.04
Ichthyosaura alpestris	0	0.05	0.06	0.07	0.1	0.05
Rana latastei	1	0.01	0.02	0.01	0.01	0.10
Rana dalmatina	1	0.08	0.06	0.06	0.04	0.11
Rana italica	1	0.12	0.13	0.08	0.05	0.03
Rana temporaria	0	0.02	0.04	0.15	0.12	0.14
Pelophylax kl. *esculentus*	0	0.05	0.06	0.02	0.04	0.12
Hyla intermedia	0	0.07	0.05	0.12	NA	0.06
Bufo bufo	0.5	0	0.04	0.09	0.13	0.01
Bufo viridis	0	0.02	0	0.07	0.06	0.04
B)		**Responses to visual cues**				
Species	**Coexistence with *A. italicus***	**Effect size (z)**				
		Total distance	**No-burst distance**	**Burst distance**	**Number of bursts**	**Avoidance**
Salamandra salamandra	1	0.23	0.21	0.21	0.2	0.01
Salamandrina perspicillata	1	0.10	0.22	0.09	0.04	0.25
Lissotriton vulgaris	0	0.27	0.32	0.12	0.14	0.54
Triturus carnifex	0	0.36	0.36	0.01	0.05	0.10
Ichthyosaura alpestris	0	0.35	0.35	0.34	0.33	0.13
Rana latastei	1	0.23	0.35	0.13	0.07	0.02
Rana dalmatina	1	0.04	0.06	0.22	0.18	0.16

Rana italica	1	0.39	0.43	0.35	0.34	0.04
Rana temporaria	0	0.20	0.23	0.07	0.08	0.12
Pelophylax kl. *esculentus*	0	0.07	0.08	0.12	0.22	0.02
Hyla intermedia	0	0.06	0.02	0.20	NA	0.09
Bufo bufo	0.5	0.23	0.30	0.16	0.21	0.06
Bufo viridis	0	0.20	0.28	0.01	0.19	0.04

CHAPTER 5

DISCUSSION AND CONCLUSIONS

In an era in which biotic invasions are ever more dominant in shaping global biodiversity, the scientific community is struggling to answer questions about how species may adapt to novel conditions, such as: how will the performance of individuals and populations react to environmental changes? What is the respective role of plasticity and evolution in allowing populations survival and adaptation? On the one hand, there is a pressing urge to increase our knowledge about interactions between native and invasive species and the potential outcomes of these dynamics in an evolutionary perspective, as this can provide crucial information for biodiversity conservation. On the other hand, biotic invasions also constitute an enormous unintended experiment shedding light on mechanisms of species evolution and their adaptation to novel and changeling selective pressures (Mooney and Cleland 2001).

Amphibians represent a taxon particularly sensitive to invasive predators and cases of rapid declines following their introduction are well documented (Kats and Ferrer 2003; Nunes et al. 2019). However, amphibians possess a striking capability to respond to novel or variable selective pressures, as have been observed in all the three study cases presented in this thesis work. For instance, when the populations maintain sufficient genetic variability and the novel selective pressure is particularly strong (e.g. invasive crayfish predation), traits that confer adaptive advantages withstanding the new selective force can become positively selected and undergo rapid fixation. For instance, as I showed in chapter 2, this was the case of *Rana latastei* populations invaded by the non-native crayfish, in which few generations were sufficient to foster the fixation of fast larval development. Indeed, rapidly attaining of metamorphosis can reduce time of exposure to this aquatic predator and thus considerably favour survival to predation at larval stage.

However, amphibians have complex life cycles and have to face multiple environmental pressure. Thus, a shift in response to a novel selective force may traduce in suboptimal response to extant environmental pressure or produce negative consequences on other traits related to fitness (e.g. carry-over effects that may affect later life stages (Capellan and Nicieza 2007a). This may ultimately result in poorer persistence also in species that show an adaptive response to novel ecological pressure posed by invasive predators. Thus, evaluating trade-offs among prey capability to match different selective pressures and indirect effects of invasive predators on native prey fitness, must be considered when assessing predator-prey interactions during biotic invasions.

Furthermore, as I showed in the three studies here presented, amphibians have a great capability to modulate their response to novel selective pressures through phenotypic plasticity. Plasticity is favoured in these organisms, as having a complex life, cycle they are often bounded to specific and different niches across life stages and are thus frequently subjected to heterogeneous and variable ecological pressures (Wilbur 1980; Benard 2004). Plastic responses elicited by invasive predators may represent a life-boat mechanism for native species. Nonetheless, this strictly depend on the effectiveness of trait plasticity induced. For instance, adaptiveness of anti-predatory strategies may vary according to predators typology (Kishida and Nishimura 2005) and responses of native prey which are effective in contrasting native predators may fail towards invasive species (e.g. differing in predatory strategy (Ferrari et al. 2015). Morphological plasticity triggered by crayfish exposure in *R. latastei* tadpoles, (i.e. increased tail muscle size) is a common inducible defence that can grant prey increased swimming performances and can enhance chances to escape predators (Teplitsky et al. 2005). Even though, the effectiveness of this trait variation in escaping crayfish predation still has to be investigated. However, we did not observe differences in tail muscle size or plasticity among invaded and uninvaded populations, which may suggest a lower adaptive advantage in escaping predation from the invasive crayfish when compared to decreased development time, which, by contrast, rapidly became positively selected in the invaded populations. Even though, this may also depend on other constraints unrelated directly to predation pressure (e.g. limited time or genetic variation, trait differential evolvability).

Furthermore, plasticity can act on multiple traits and its expression and modulation can be complex. Behavioural traits are commonly thought to benefit from a high degree of plasticity and thus represent a major defence for native species during biotic invasions (Holway and Suarez 1999; Weis and Sol 2016). Nonetheless, in the context of predation, several studies demonstrated also morphological plasticity can be readily inducible and highly reversible (see (Relyea 2003; Kishida and Nishimura 2004; Kishida et al. 2010) and their importance in the context of responses to invasive predators can be underestimated. Moreover, as prey live in complex habitats and must match diverse ecological pressures, often they rely on multiple sets of traits that can be finely tuned depending on the experienced conditions. For instance, varying risk intensity or risk duration can produce different outcomes in prey responses (Lima and Bednekoff 1999; Ferrari et al. 2007). In this regard, if we compare responses expressed by native prey in all the three studies summarized in this thesis, we can observe that different typology of risk elicited diverging anti-predatory strategies and different traits. Prolonged exposure to risk prompted in *R. latastei* (chapters 2 and 3) elicited the expression of long-term morphological and life-history responses, but did not involve a reduction in tadpole activity. Conversely, brief exposure to crayfish activated short-term behavioural responses in larvae of all species, and even in *R. latastei*. Indeed, behavioural responses, such as reduced activity, are often not sustainable over the long term since prey has to forage and develop (Turner 1997). The observation that *R. latastei* originating from uninvaded populations (chapters 3 and 4) show differential response accordingly to perceived risk suggests that even naïve prey may finely modulate their anti-predator strategies when facing novel predators.

Besides, as the invasive crayfish induced behavioural responses in larvae of tested native species (see chapter 4), some potential shifts are not excluded to act in shaping other traits (e.g. morphological or life-history traits), which might be concomitantly triggered by the prolonged exposure to this invasive predator (i.e. in invaded breeding sites, see for instance (Nunes et al. 2014b; Saura–Mas and Benejam 2019).

However, when considering larvae responses to *P. clarkii*, it is noteworthy to remember they were essentially triggered by visual recognition of the predator (chapter 4). Recognition of predators through chemical stimuli is a key factor in aquatic environments (Kats and Dill 1998; Ferrari et al. 2010c). The incapability to recognise relevant stimuli associated to predation risk, such as novel predator kairomones, can lead to delayed and poorly effective responses. For instance, visual predator recognition generally implies closeness to predator and can be ineffective in certain environmental contexts (Abrahams and Kattenfeld 1997). Thus, naïve amphibian prey seems to rely on an incomplete risk assessment, and this can potentially produce lethal consequences in natural environments (Feminella and Hawkins 1994). Indeed, even if in nature individuals can learn to associate novel predator cues to risk through experience (e.g. when paired to other predation cues (Gonzalo et al. 2007), innate predator recognition can be extremely advantageous, especially for species subjected to heavy predation risk during early stages of their life-history, as amphibian larvae.

This thesis work highlights that responses of amphibian larvae to invasive predators can be complex and highly heterogeneous among species and may strongly depend on the experienced conditions. For instance, tadpoles of *H. intermedia* and *R. dalmatina* showed peculiar escape response rather than activity reduction when exposed to crayfish, while *R. latastei* tadpoles demonstrated a great capability to finely-tune their behaviour accordingly to the duration of risk exposure. Thus, limiting analyses to behavioural traits only in the study on *R. latastei* (chapters 2-3) or to common anti-predator behaviours in the study on the amphibian community (chapter 4) would have underestimated species capability to recognise and express responses towards the invasive crayfish. Future studies should thus embrace a broad perspective and consider variability across multiple traits, multiple species and typology of risk exposure when investigating anti-predator response of amphibian larvae towards invasive predators.

Finally, the present studies showed native amphibians exhibit a great capability to respond to novel predators and stress the necessity of increasing knowledge about the adaptiveness of native species responses, as a general understanding of how plastic responses may promote evolution of native populations facing invasive species is lacking. Further investigations should address assessing the actual effectiveness of anti-predator strategies exhibited by amphibian larvae in withstanding invasive predators. Moreover, information on implications the shifts induced by non-native predators can have on species life-history is needed. Studying amphibian interactions with invasive predators may contribute to reveal causes of species declines, explain observed population trends and provide insights on long-term evolutionary consequences, and ultimately can furnish key information for their conservation, favouring identification of more threatened species and fostering proper management plans.

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Collateral publications

Invasive species and amphibian conservation

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Abstract

The recognition that alien invasive species (IAS) are among the greatest threats to biodiversity has stimulated growing interest on the impacts of IAS on amphibians. Here we reviewed the recent literature to describe the multifaceted consequences of biological invasions on native amphibians, and to identify potential mechanisms and strategies that could allow the long term persistence of populations and species. First, we describe the multiple pathways through which IAS can influence amphibian fitness, populations and communities. IAS can have major direct impacts through predation, competition and hybridization. Furthermore, the consequences of indirect impacts such as habitat alteration, spread of diseases and context-dependent effects can be particularly severe in natural populations. Second, we analysed the responses of native amphibians to IAS, and show that amphibians strongly modulate behaviour, morphology and life history in presence of IAS. Nevertheless, it is still unclear to what extent phenotypic plasticity and rapid evolution actually help native species to withstand the impacts of IAS and survive in invaded communities. Finally, we provide indication on practical management strategies. Prevention, monitoring and early control are the most effective approaches approach and should be prioritized in pro-active conservation plans, still eradications and mitigation approaches are feasible and can greatly improve the status of populations. Our study highlights complex impacts of IAS on amphibians, and identifies multiple open questions for both research and practical conservation.

Key words: amphibian decline, biological invasions, contemporary evolution, fitness, horizon scanning; impacts, invasive fish, management strategies phenotypic plasticity.

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ORIGINAL ARTICLE

Safe as a cave? Intraspecific aggressiveness rises in predator-devoid and resource-depleted environments

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Abstract

Intraspecific aggressiveness can be affected by multiple environmental pressures. In several cases, aggressiveness can grade into full-scale cannibalism, particularly when resources are scarce. However, limited information exists on how intraspecific aggressiveness varies among populations experiencing different environmental pressures, and on the role intraspecific predation plays for the exploitation of harsh habitats. The fire salamander, *Salamandra salamandra*, is an excellent model species to study factors affecting intraspecific aggressiveness, because of its ability to breed in habitats with contrasting food resources and predation pressure. Here, we evaluated the influence of predation risk and habitat of origin on aggressive interactions. To this extent, we reared larvae from cave (scarce resources; nearly-absent predators) and surface (abundant resources and predators) populations under different risk conditions and measured aggressive behavior towards conspecifics. During behavioral trials, larvae were exposed to different combinations of predator and wounded conspecific chemical cues. Intraspecific aggressiveness increased in large and late-development larvae. Larvae from all the populations significantly reduced aggressiveness under both typologies of risk experienced during rearing (constant presence of predator; pulses of high predation risk), and also when stimulated by predator cues. However, larvae from cave populations exhibited a more pronounced aggressiveness, especially when exposed to wounded conspecific cues. Intraspecific aggressiveness can be modulated by the complex interaction between multiple variables, and both behavioral plasticity and local adaptations can determine its variation across populations. Our findings reveal that aggressive interactions are favored in cave environment, suggesting intraspecific predation can play a key role in the exploitation of resource-depleted habitats.

Significance statement

In this study, we investigated how intraspecific aggressiveness of salamander larvae is shaped under predation risk in populations originating from contrasting environments, such as cave and surface habitats. Larvae experiencing predator presence during their development or exposed to predator cues significantly reduced their aggressive interactions, both in cave and surface populations. Interestingly, cave-originating individuals reacted to wounded conspecific cues by increasing the frequency of their aggressive displays, suggesting cannibalistic behavior is locally enhanced in populations from resource-depleted habitats. The present study offers new insights on the importance of intraspecific aggressiveness for the adaptation to harsh environments.

Keywords Intraspecific aggressiveness · Cannibalism · Chemical signaling · Cave environment · Predation risk · *Salamandra*

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Introduction

Intraspecific aggressiveness is a plastic behavioral trait that can deeply modulate interactions with conspecifics. Intraspecific aggressiveness can have profound impact on populations, by modulating competition and determining access to resources, and often has cascading effects on fitness and population dynamics (Reques and Tejedo 1996; Whitehouse 1997; Arnott and Elwood 2008). This is particularly relevant in contexts where conspecifics become a potential trophic resource, and aggressive interactions often result into full-scale cannibalism (Wise 2006; Manenti et al. 2015). Cannibalistic behavior can, at the same time, provide additional foraging resources to the cannibals and release them from intraspecific competition pressure, with potential benefits such as a faster development rate and better survival (Polis 1981; Crump 1983). These benefits are particularly relevant in environmental contexts where resources are limited, conditions are harsh, or risk is elevated (Fox 1975; Crump 1983). Therefore, aggressive interactions can be more frequent in ephemeral habitats, when food availability is scarce or conspecific densities are elevated (Reques and Tejedo 1996; Wildy et al. 2001; Amat et al. 2008; Cooper et al. 2015; Manenti et al. 2015). Moreover, intraspecific aggressiveness can be higher in age- or size-structured populations (e.g., where cohorts from different breeding seasons coexist) and, in these conditions, predation on conspecifics by large, late-stage individuals is facilitated by size asymmetry (Ziemba and Collins 1999; Eitam et al. 2005; Wissinger et al. 2010).

The benefits of intraspecific aggressiveness are often context-dependent. Aggressiveness is frequently a plastic or conditional strategy which is subjected to multiple ecological trade-offs (Fox 1975; Polis 1981; Pizzatto and Shine 2008). The occurrence of predators is often a major determinant of intraspecific aggressiveness, nonetheless its effects on cannibalism occurrence may be complex and difficult to predict. First, predators directly affect the availability of conspecifics through consumption, thereby reducing intraspecific encounter rate (Polis 1981). Second, predation pressure often determines non-consumptive effects in prey populations, such as behavioral responses that allow minimizing predation risk (Peckarsky et al. 2008; Davenport and Chalcraft 2013). Non-consumptive effects can affect the incidence of aggressiveness in diverse ways. On the one hand, predation risk is known to determine the decrease of activity level across multiple taxa (Lima and Dill 1990; Anholt et al. 2000; Barbosa and Castellanos 2005), which in many cases can limit or even suppress the occurrence of cannibalistic behavior (Wissinger et al. 2010; Kishida et al. 2011). For instance, dragonfly larvae are predators that can cause a dramatic reduction of activity and aggressive interactions in the Ezo salamander (*Hynobius retardatus*) larvae, thus inhibiting the occurrence of cannibalistic individuals (Kishida et al. 2011).

On the other hand, when the risk of being predated is constantly elevated, a prolonged reduction of activity can be too costly, as it would hamper foraging (Lima and Bednekoff 1999; Ferrari et al. 2009). Under persistent risky conditions, an increase in foraging may even result more advantageous, favoring faster growth and rapid development, which can limit exposure to predators of most vulnerable stages or size-classes (Ferrari et al. 2009; Kishida et al. 2015; Manenti et al. 2016). An increase in cannibalistic behavior under heavy predation risk was observed in spadefoot toads, which showed a higher frequency of cannibalistic tadpoles in sites where their main predators (salamanders) were present (Ghioca-Robrecht et al. 2009).

Non-consumptive effects and the degree of anti-predatory responses strongly depend on the ability of prey to perceive predator presence and to assess predation risk (Lima and Dill 1990; Palmer et al. 2017). In aquatic environments, risk is typically perceived through chemical cues released by the predator (kairomones) (Chivers and Smith 1998; Kats and Dill 1998). Chemical signals can be also released by conspecifics, for instance when they are stressed, wounded, or eaten by a predator (stress, alarm, and diet cues) (Mirza and Chivers 2001; Wisenden 2003; Ferrari et al. 2010b). The simultaneous perception of predator kairomones and conspecific cues often produces synergic effects on prey behavior and can induce or enhance antipredator responses (Bryer et al. 2001; Keppel and Scrosati 2004; Dalesman et al. 2007). Moreover, anti-predator response can be modulated on the basis of experienced conditions (Wisenden and Millard 2001; Gonzalo et al. 2007; Epp and Gabor 2008) thus previous encounters with predators or risk cues can determine refined responses (McCollum and VanBuskirk 1996; Martin and Lopez 2003; Ferrari et al. 2007; Ferrari et al. 2008). However, when exposure to predators is continuous, predator-associated stimuli can lose their effectiveness and the intensity of anti-predator behavior can decrease (Turner 1997; Ferrari and Chivers 2011; Gonzalo et al. 2013). Overall, mechanisms regulating cannibalism under predation risk are difficult to disentangle, and complex trade-offs likely determine its occurrence and intensity (Nilsson et al. 2011; Kishida et al. 2015). Despite environmental conditions being expected to affect the benefits of cannibalism, only a few studies have analyzed the variation of aggressive interactions between populations subjected to considerably diverging predatory and environmental pressures (Griffiths 1994; Nilsson et al. 2011).

In our study, we evaluated how predation risk affects intraspecific aggressiveness of salamander larvae in populations originating from extremely diverging habitats, such as surface and cave environments. The fire salamander (*Salamandra salamandra*) typically breeds in surface streams, where predators are abundant (Lanza et al. 2009; Manenti et al. 2009b, 2016). Nevertheless, several populations breed in underground streams or pools (Manenti et al. 2009a, 2011). These environments are virtually devoid of interspecific predators, are characterized by limited trophic resources, and can host high salamander densities (Manenti et al. 2015). Theory predicts that cannibalistic behavior is favored under these conditions (Polis 1981; Crump 1983).

Cannibalism is frequent in fire salamander larvae (Joly 1968) and is known to occur both in surface and cave populations (Manenti et al. 2015). In this species, the frequency of aggressive interactions is associated with cannibalism intensity, thus intraspecific aggressiveness represents a good proxy for the occurrence of this behavior (Markman et al. 2009; Limongi et al. 2015; Manenti et al. 2015). To evaluate the complex interplay among factors determining cannibalism, we investigated the plasticity and variability of aggressive displays linked to cannibalistic behavior in salamander larvae from cave and surface populations, after the exposure to risk conditions and risk-associated cues. We predict that (i) long-term exposure to predators during development decreases the occurrence of aggressive interactions among larvae; (ii) the degree of anti-predator behavior is affected by the temporal pattern of experienced conditions (e.g., less pronounced response under constant risk regime compared to periodical exposure); moreover, (iii) acute exposure to chemical cues associated to predation risk should affect the perceived level of risk, decreasing intraspecific aggressiveness; finally, (iv) we expect higher aggressiveness in larvae facing environmental conditions that favor intraspecific predation (e.g., cave populations). Understanding mechanisms regulating cannibalism occurrence and intensity in natural environments can offer important insights on the relative role played by cannibalism in favoring ecological plasticity and the exploitation of harsh, resource-deprived habitats.

Methods

Collection of larvae

The study individuals were collected in the districts of Como and Lecco (Lombardy, NW Italy. Approximately 45.8° N, 9.2° E). This area is characterized by hilly and mountainous reliefs with scattered woodlands, predominantly composed of deciduous trees and a dense hydrographic network. In this region, karstic areas are frequent, with numerous cavities constituting a suitable habitat for multiple terrestrial and aquatic invertebrates. In this area, the fire salamander is common and gives birth to fully-aquatic larvae both in surface sites (e.g., pools and small streams (Manenti et al. 2009b)) and in underground springs (Manenti et al. 2009a). In underground sites, larvae prey upon cave-dwelling invertebrates, but food availability is scarce (Romero 2009). Therefore, development rate is generally slower than in surface sites (Manenti et al. 2011; Limongi et al. 2015), still salamanders are able to successfully complete metamorphosis.

In spring 2014, 142 newborn fire salamander larvae (developmental stage 1, Zakrzewski 1987) were collected from six underground pools ($N = 56$ individuals, average 9.3 individuals per site; range 5–12) and from eight surface sites ($N = 86$, average 10.7 individuals per site; range 7–12; Fig. 1), located between 250 and 970 m a.s.l. The distance between sites ranged between 350 m and 29 km. Nearby sites were separated by natural barriers like deep valleys with steep slopes, thus larvae from different sites belong to separate populations. All underground sites were emitting springs inside caves; therefore, no accidental drift of larvae from outdoor sites was possible (Manenti et al. 2009a; Manenti and Ficetola 2013). For the surface sites, only habitats with permanent water were selected, because temporary wetlands have very different selective pressures (Reinhardt et al. 2013). Fire salamanders are ovoviviparous; thus, larvae could only be collected after deposition. To limit age differences and minimize the effect of possible prior experiences, we daily monitored breeding sites and collected newborn larvae immediately after deposition.

Rearing conditions

Immediately after collection, each larva was photographed to allow individual identification through the unique pattern of their tail (Eitam and Blaustein 2002). Larvae were then individually hosted in transparent plastic containers (10 cm diameter, 15 cm height), and arranged in large plastic tanks (40 × 50 cm), filled with 5 cm of aged tap water (blocks). All the individual containers were perforated (2 mm perforations); therefore, water freely flowed between the tank and the individual containers. Water temperature (15 °C) and oxygenation were kept constant and larvae were exposed to natural photoperiod. Five days after collection, cave and surface larvae were equally divided into three rearing conditions differing in risk exposure: constant predator exposure with no direct predation risk (No-contact), short periodical predator encounters (Contact), and a control condition with no exposure to predators (Fig. 1). The predator used for the experiment was a large (35 mm) dragonfly (*Cordulegaster bidentata*) larva. Dragonfly larvae were collected from surface sites, where they represent a common predator of salamander larvae. Dragonfly larvae can also occur in cave environments, but their frequency is extremely low (Manenti et al. 2013b). In the No-contact conditions ($N = 43$ larvae, subdivided in two blocks), two dragonfly larvae were added to the tanks hosting the containers with salamanders and allowed to freely move inside the tank for the whole rearing period. In this condition, predation was impossible but larvae were constantly exposed both to predator's visual and chemical cues. In the Contact conditions ($N = 49$ larvae, two blocks), individuals were subjected to brief pulses of risk with an abrupt exposure to predator presence (with the simultaneous perception of visual, chemical, and tactile stimuli),

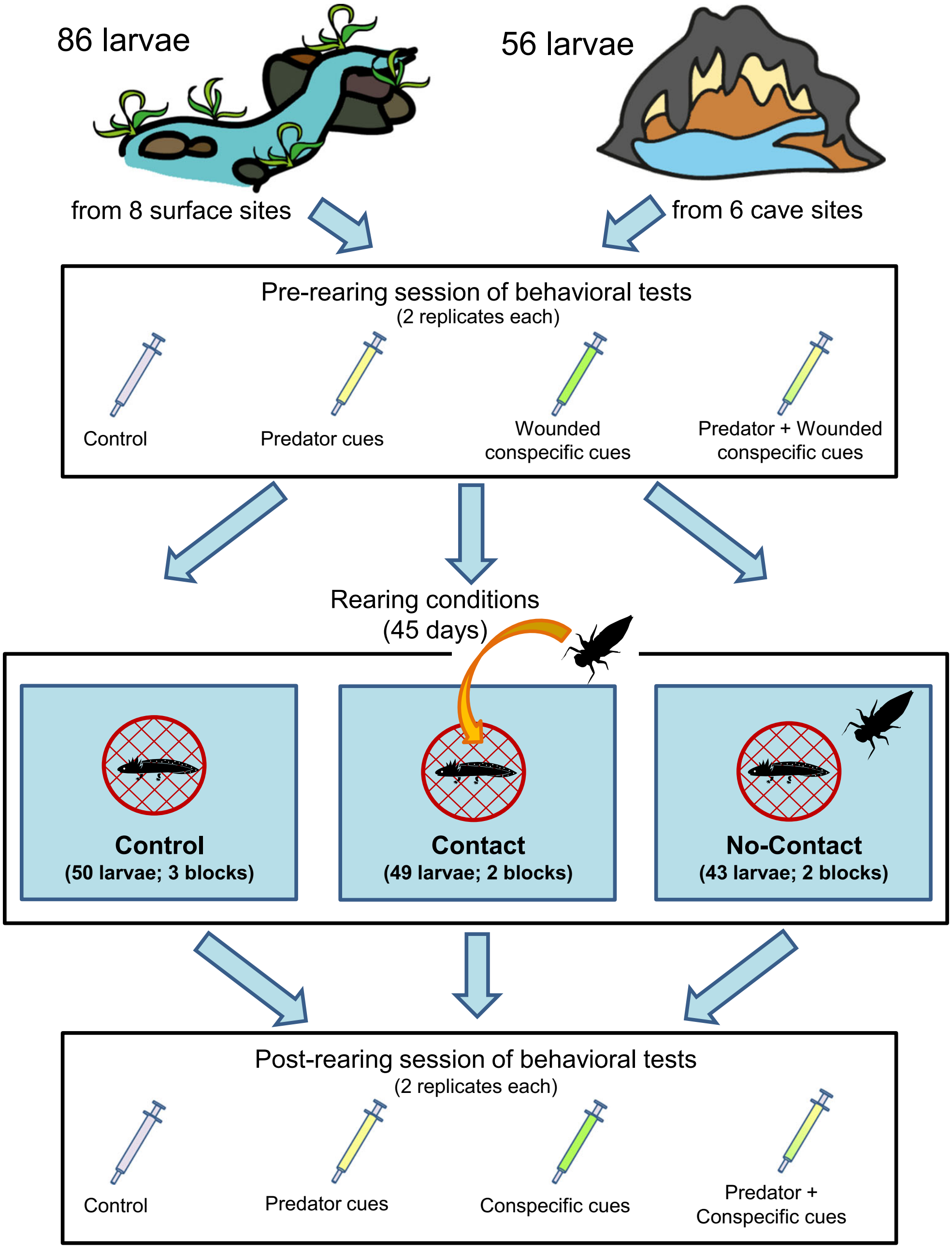

Fig. 1 Experimental design and sample sizes. We collected 142 fire salamander larvae (*Salamandra salamandra*) from six caves ($N = 56$) and eight surface ($N = 86$) populations. Larvae were exposed for 45 days to three rearing conditions: Control, absence of predator ($N =$ 18 cave larvae + 32 surface larvae); Contact, periodical exposure to 30-s encounter with predator ($N = 20$ cave larvae + 31 surface larvae); No-contact, constant to non-lethal exposure to predator ($N = 18$ cave larvae + 23 surface larvae). We conducted two sessions of behavioral tests: before rearing period and after rearing period. During each behavioral test, the focal larva was exposed to each chemical treatment in two replicates ($N = 8$ tests per larva per session)

larvae were exposed every 4 days to direct encounters with predators, by inserting one dragonfly larva in the plastic container for 30 s (total: 11 encounters throughout the rearing period). This brief exposure prevented predation attempts but was sufficient to be perceived as a threat by salamander larvae, which showed startled escape responses when the dragonfly larva was inserted into their container. The escape response was observed even at the last exposure, suggesting no habituation to this treatment. Predator exposure in Contact and No-contact conditions differed both in time and modality: while Contact-reared animals experienced risk during limited but acute stress events, individuals reared in No-contact conditions were exposed to persistent and but less pronounced risk conditions. Contact and No-contact conditions aimed at comparing differential risky conditions, which could differentially affect behavior (Turner 1997; Lima and Bednekoff 1999; Sih and McCarthy 2002). Finally, larvae under Control conditions never experienced predator presence ($N = 50$ larvae, three blocks). Rearing lasted 45 days, and during this period, both fire salamander and dragonfly larvae were fed ad libitum every second day with fresh *Chironomus* spp. larvae.

Chemical cues

To test the influence of predation-related cue exposure, two chemical treatments were prepared: predator cues and wounded conspecific cues. Cue extraction was performed before rearing and behavioral tests as in Manenti et al. (2016). Previous studies have demonstrated that salamander larvae perceive dragonfly larvae as a threat, as they heavily reduce activity (Manenti et al. 2016). Therefore, predator chemical cues were obtained leaving 6 *C. bidentata* specimens in 1.5 l of decanted tap water for 24 h. Conversely, in order to obtain cues from a wounded conspecific, the tail tip (< 30%) of a fire salamander larva was cut off with a sterilized scalpel, and the individual was left in 1.5 l of decanted tap water for 24 h. Tail loss is very frequent in natural populations (up to 40% of salamander larvae in populations with abundant predators) (Manenti et al. 2013b). Moreover, salamander larvae are able to quickly regenerate tail, and this operation does not impact larval survival or subsequent performance (Segev et al. 2015). The wounded individual was separately reared; its conditions were monitored for 40 days, and it was released in its site of origin. The two cue solutions were collected, divided into 10 ml aliquots, and stored at − 20 °C until the behavioral tests. Tap water was collected using the same procedure and used as control treatment. Salmander and dragonfly larvae used during this procedure were not used for any rearing treatment or behavioral trial, and were maintained separated from the individuals used for the experiment.

Behavioral tests

The experimental design consisted of two behavioral sessions: before and after the 45-day rearing period. During these sessions, the aggressive behavior of individuals was measured by different cue exposures. At each behavioral session, each individual was tested in two replicates for all the combinations of cue exposures (predator; wounded conspecific; predator + wounded; control; see Fig. 1). In total, individuals were subjected to 16 behavioral trials ($N = 8$ trials per individual per session); in each session, the order of cues was randomized. This procedure made it possible to perform all the test combinations in a reasonable and comparable time; the randomization of test order allows the minimization of potential biases related to the sequence of cue exposure (Altmann 1974; Ferrari et al. 2010a). Aggressiveness trials were performed after a 3-day starvation period to promote the occurrence of aggressive interactions and standardize individual motivation to attack. Before each experimental session, all larvae were photographed on graph paper to measure total length. Behavioral tests were conducted under daylight conditions in a 13.5 × 18.3-cm plastic arena filled with 250 ml of decanted tap water. To test for intraspecific aggressiveness, the focal individual was exposed to a so-called “prey larva” in the experimental arena. The prey larva was a newborn fire salamander larva originating from a different site of collection, and not subjected to any rearing treatment. Both the focal and the prey larvae were gently introduced at the opposite side of the same arena and let acclimatize for a 5-min period. During acclimation, larvae were kept separated by means of a removable plastic barrier, which divided the arena into two equal-size sectors. After the acclimation phase, 1 ml of the selected cue treatment was injected by the same experimenter in the prey larva sector, then the plastic barrier separating the two larvae was cautiously removed and the test started.

Aggressiveness tests lasted 7 min. During tests, three behavioral displays were recorded for focal larvae: total number of bites or biting attempts towards the prey larva (Attack), the latency of the first movement towards the prey (Latency), and the time spent by the focal larvae approaching the prey larva (Following). An Attack was any effective bite, a snap trying to catch the conspecific, or a sudden rush towards the prey larva. Latency was the time occurring until the first approach, such as when the larva performed an attack or at least two consecutive movements towards the prey larva. Following was the total duration of approaching events towards the prey larva (approaching events were three or more consecutive movements towards the prey larva). Data were not blindly recorded to avoid the risk of mismatching errors.

Between trials, both focal and prey larvae were placed in their plastic boxes for a 15-min recovery time, while arenas were carefully washed twice to remove cues; previous studies showed that this procedure is sufficient to detect the effect of exposure to different cues (Manenti et al. 2016). In total, we used 47 prey larvae and randomly assigned them to the focal larva during each trial to minimize repeated encounters. For each trial, we also calculated size difference between larvae (i.e., the total length difference between focal and prey larva). During the trials, two clear cannibalism attempts occurred. In both cases, the consumption was promptly interrupted, and the trial stopped. The prey larvae were replaced, separately allowed to recover and never used in any subsequent trial.

Statistical analysis

Data were analyzed using linear mixed effects models (LMMs) and generalized linear mixed effects models (GLMMs), which take into account random factors determining non-independence of observations (Pinheiro and Bates 2000). Gaussian LMMs were used to analyze the factors determining Following and Latency displays, while Poisson GLMMs were used for the number of attacks. As fixed effects, we considered size difference between focal and prey larva, period (before or after rearing), origin (cave or surface population), rearing condition (No-contact, Contact, Control), and chemical treatment. The two sessions of behavioral trials (before and after rearing) were analyzed simultaneously; we

Table 1 Influence of independent variables on Attack, Latency, and Following displays performed by fire salamander larvae. For Attack, we used Poisson GLMMs (test statistics: χ^2); for Latency and Following, we used Gaussian LMMs (test statistics: F value). Significant effects are in italic

Display	Fixed effects	df	Test statistic	*P*
Attack	Size difference	1	4.160	*0.041*
	Period	1	4.212	*0.040*
	Origin	1	3.099	0.078
	Rearing	2	14.962	*< 0.001*
	Predator cues	1	0.003	0.960
	Conspecific cues	1	2.016	0.156
	Period × Predator cues	1	4.351	*0.037*
	Rearing × Origin	2	16.372	*< 0.001*
	Rearing × Predator cues	2	8.735	*0.013*
	Rearing × Conspecific cues	2	8.875	*0.012*
Latency	Size difference	1, 126.9	5.68	*0.019*
	Period	1, 91.64	1.33	0.253
	Origin	1, 81.6	0.53	0.467
	Rearing	2, 100.4	1.64	0.199
	Predator cues	1, 168.0	0.001	0.972
	Conspecific cues	1, 176.3	0.02	0.893
	Origin × Conspecific cues	1, 172.1	7.32	*0.008*
Following	Size difference	1, 73.8	6.47	*0.013*
	Period	1, 48.8	3.52	0.067
	Origin	1, 151.99	5.43	*0.021*
	Rearing	2, 120.31	8.79	*< 0.001*
	Predator cues	1, 148	4.81	*0.029*
	Conspecific cues	1, 151.99	0.19	0.666
	Origin × Conspecific cues	1, 150.7	4.93	*0.028*

included period in the mixed models to take into account differences occurring through time. We also included length difference between larvae as a covariate. Random factors were larva identity, rearing block, and population of origin. Preliminary analyses including the identity of prey larva as a further random factor showed higher AIC values and were qualitatively identical.

We used orthogonal contrasts (or “planned comparisons”) to perform the comparison between rearing conditions. Orthogonal contrasts allow pairwise comparisons without increasing type I and type II errors, as it would occur when using post-hoc tests (Field et al. 2012). We first used contrasts to compare predator-rearing conditions (Contact- and No-contact) against Control-larvae. Subsequently, we tested the significance of differences between Contact and No-contact conditions. For each behavioral display, two-way interactions between period, origin, rearing condition, and chemical treatment were tested; non-significant interactions were not included in the final models. Testing statistical interactions between chemical stimuli (predator and wounded conspecific cues) was used to assess the possibility of joint effects between them. The analysis of latency and following behaviors was performed on the subset of trials in which larvae approached or followed conspecifics (i.e., 196 and 160 trials, respectively; see Appendix S1).To visualize the effects of GLMMs, we built conditional partial regression plots using visreg 2.4 (Breheny and Burchett 2017). All analyses were performed under the R 3.4.1 environment using the packages lmerTest and lme4 (Pinheiro and Bates 2000), while the visreg package was used to generate plots presented hereafter.

The datasets generated during and/or analyzed during the current study available from the corresponding author on reasonable request.

Results

Overall, we performed 1520 behavioral trials. During trials, differences between focal larva and prey larvae for the total length ranged between −5 and 21 mm (mean = 3.44, SD = 5.37 mm). None of the correlations between behavioral displays was strong. We detected a significant correlation between the duration of Following and the number of Attacks and ($r = 0.472$, $P < 0.001$), while correlations between Attacks and Latency ($r = 0.002$, $P = 0.979$) and between Following and Latency ($r = -0.111$, $P = 0.120$) were weak and non-significant. Survival rate after rearing was 70.4%. The mortality of salamander larvae in nature is often > 90% even in

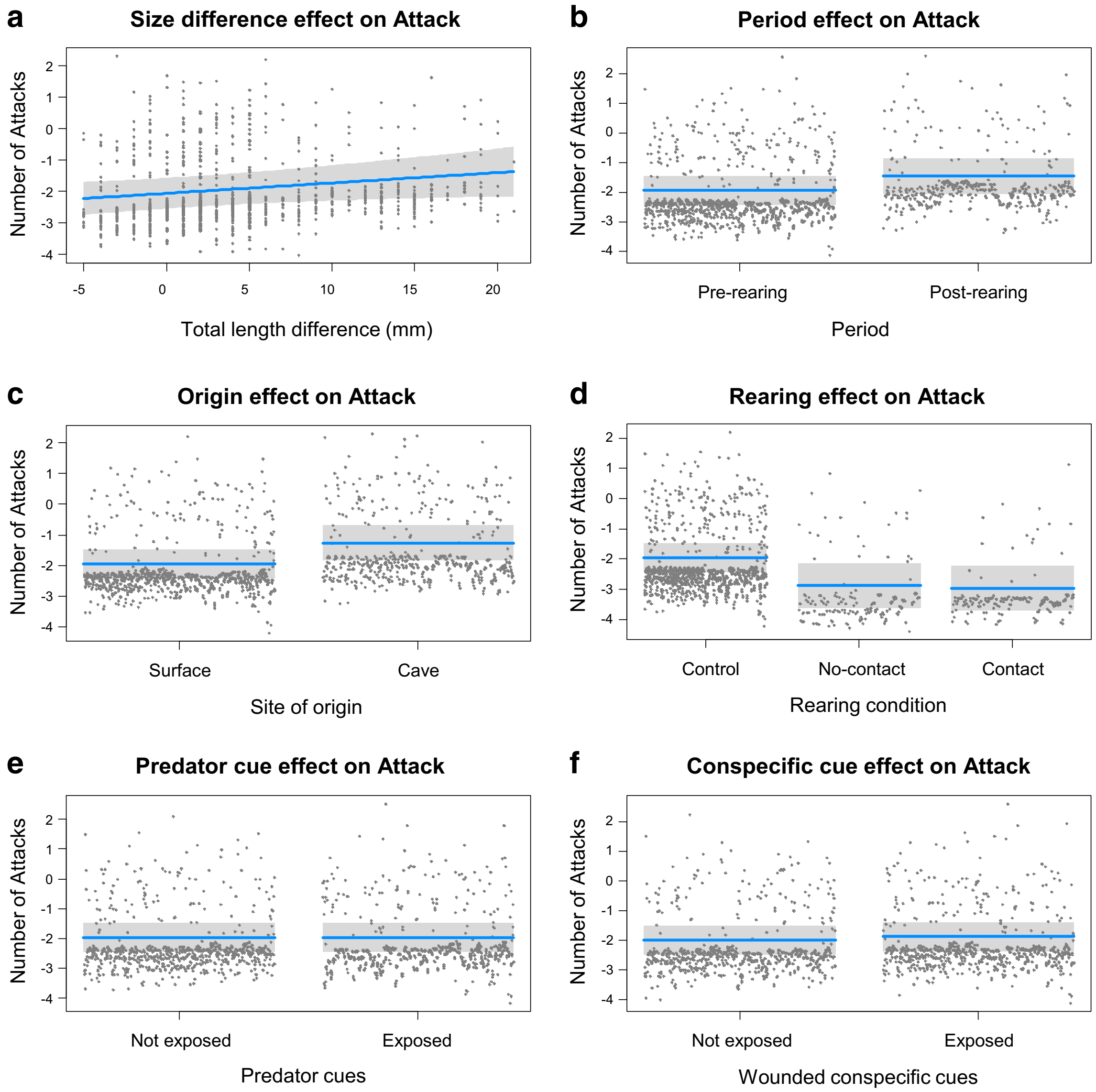

Fig. 2 Conditional partial residual plots, showing the relative influence of size difference (**a**), period (**b**), origin (**c**), rearing (**d**), predator cues (**e**) and conspecific cues (**f**) on fire salamander Attack display. Shaded areas are 95% confidence bands

environments with limited predators (Limongi et al. 2015); therefore, the observed values are not unexpected. Mortality was not significantly different between rearing conditions or origin (binomial generalized linear mixed model; all $P > 0.05$).

Attack

During behavioral tests, mean (± SD) Attack rate was 0.34 ± 0.88, and the total number of attacks ranged from 0 to 6 per trial. The number of attacks significantly increased when size differences among larvae were largest ($P = 0.041$), and after 45 days ($P = 0.040$; Table 1; Fig. 2a, b). Furthermore, rearing under risky conditions significantly decreased the Attack rate ($P < 0.001$; Fig. 2d). Orthogonal contrasts showed that both rearing conditions significantly reduced Attack rate compared to Controls ($\chi^2_1 = 11.37$; $P < 0.001$), while we did not detect differences between the Contact and the No-contact conditions ($\chi^2_1 = 0.08$; $P = 0.781$). We found no significant effect of origin, predator cues, or wounded conspecific cues on this display (all $P > 0.05$; Table 1, Fig. 2c–f).

We also detected multiple significant interactions between independent factors (Fig. 3, Table 1). First, larvae reduced their Attack rate in the presence of predator cues, but only after 45 days of rearing ($P = 0.037$; Fig. 3a). Second, cave larvae reared under No-contact conditions reduced Attack rate more than surface larvae ($P < 0.001$; Fig. 3b). Finally, larvae reared under Contact conditions reduced their aggressiveness less when exposed to predator ($P = 0.013$; Fig. 3c) and to conspecific cues ($P = 0.012$; Fig. 3d), compared to the other

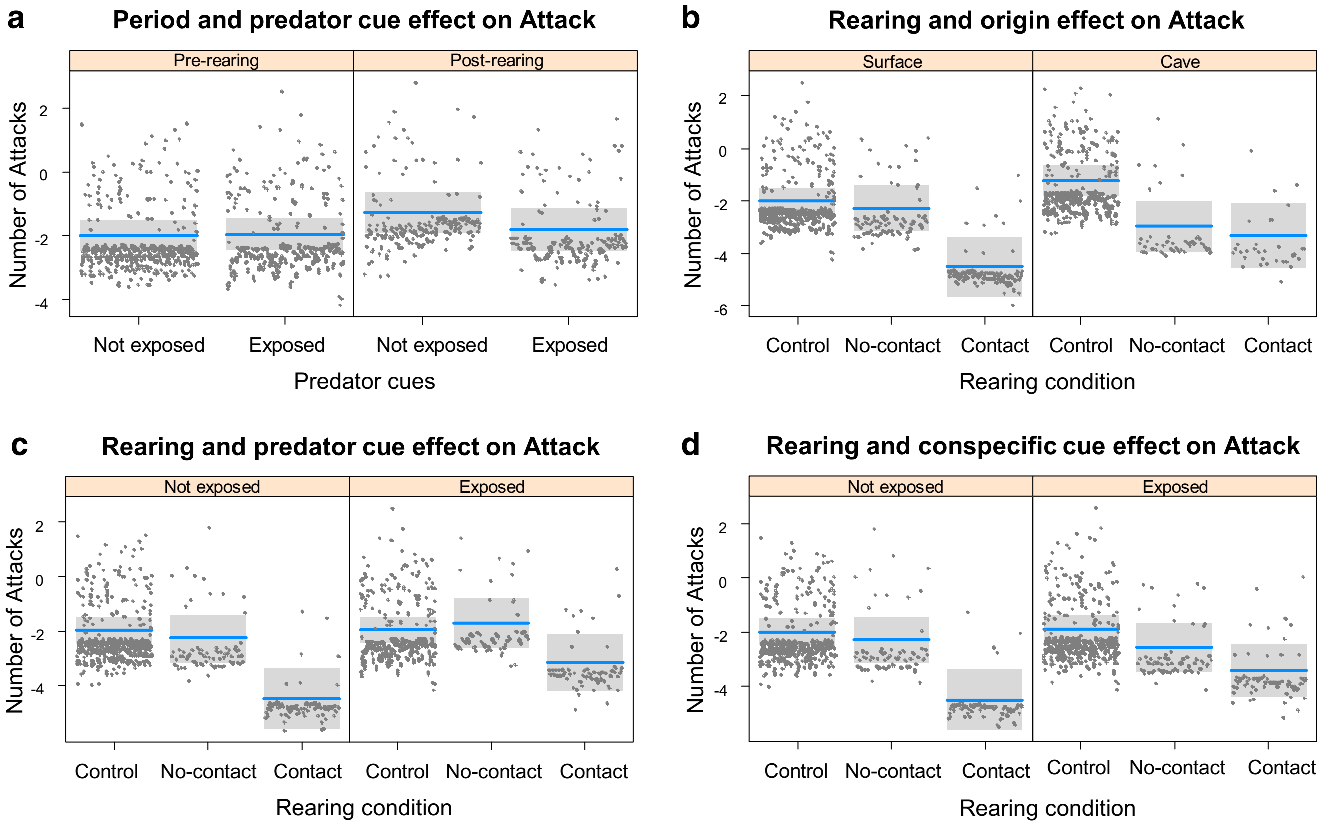

Fig. 3 Conditional partial residual plots, showing the significant interaction effects on fire salamander Attack display: period and predator cues exposure (**a**), rearing conditions and origin (**b**), rearing conditions and predator cues exposure (**c**), rearing conditions, and conspecific cues exposure (**d**). Shaded areas are 95% confidence bands

conditions. We found no significant interaction of the paired exposure to the two chemical cues nor between any other fixed factor.

Latency

Approaches towards prey larvae were detected in 12.9% of trials, with Latency time ranging between 0 and 417 s (mean = 189.22 ± 127.85 s). The time before approaching prey was significantly shorter when size differences between the two larvae were largest ($P = 0.019$; Table 1, Fig. 4a). By contrast, we did not detect a significant effect of period, origin, rearing, predator cues, or conspecific cues (all $P > 0.05$; Table 1, Fig. 4b–f). Besides, when exposed to wounded conspecific cues, cave larvae showed a shorter time to first approach the prey larvae, compared to surface larvae ($P = 0.008$; Fig. 5a). No other interactions between factors showed significant effects on latency.

Following

The Following behavior occurred in 10.5% of trials. The total time pursuing prey larvae ranged from 10 to 267 s (mean = 43.53 ± 46.48 s). The Following time was significantly longer when length differences between larvae were largest ($P = 0.013$; Table 1; Fig. 6a). Furthermore, less prolonged Following was observed in larvae from surface populations ($P = 0.021$; Fig. 6c), in the presence of predator cues ($P = 0.029$; Fig. 6e), and in larvae reared with predators ($P < 0.001$; Fig. 6c). Larvae reared under both risk conditions showed significantly shorter Following behavior, compared to Controls (orthogonal contrasts: $F_{1,\ 118.4} = 13.9$; $P < 0.001$; Fig. 6d), while differences between the Contact and No-contact conditions were not significant ($F_{1,\ 150.7} = 0.74$; $P = 0.390$). We did not detect significant effects of period or wounded conspecific cues on Following (all $P > 0.05$; Fig. 6b, f). Finally, a significant interaction between origin and wounded cues indicated that only cave larvae increased their Following activity when exposed to wounded cues ($P = 0.028$; Fig. 5b). No other interaction between fixed factors significantly affected Following duration.

Discussion

Environmental pressure can determine strong variation of aggressive behavior, and we observed increased agonistic

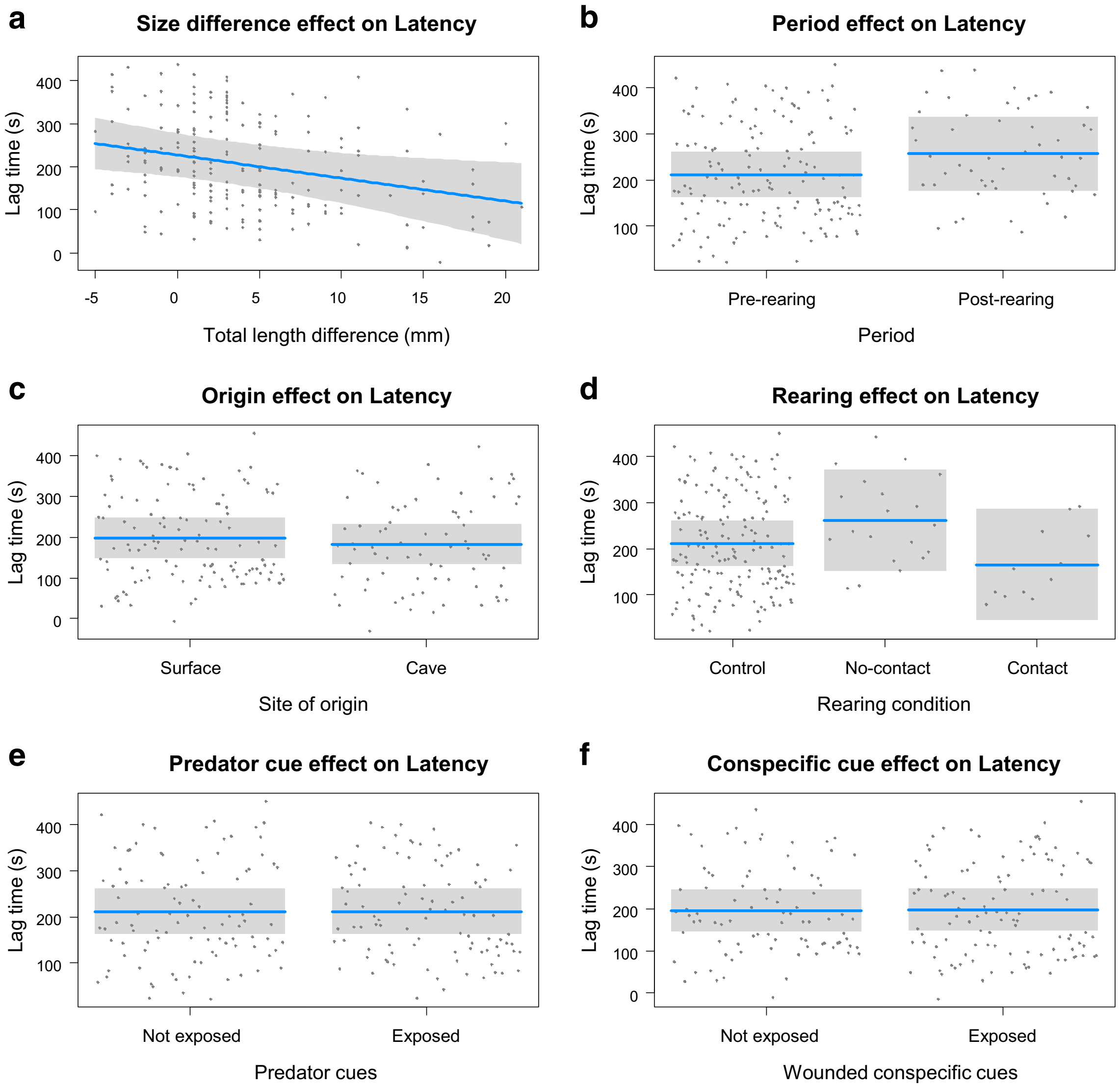

Fig. 4 Conditional partial residual plots, showing the relative influence of size difference (**a**), period (**b**), origin (**c**), rearing (**d**), predator cues (**e**), and conspecific cues (**f**) on fire salamander Latency display. Shaded areas are 95% confidence bands

interactions in larvae from cave populations, where the fitness advantages of aggressive individuals can be particularly effective. Our study confirms that the expression of aggressive behavior can be affected by habitat pressures, and that populations can show differences in their responses to environmental conditions. Different typologies of risk exposure (constant presence of predator; pulses of high predation risk; presence of chemical cues) yielded a consistent reduction of aggressiveness through all the populations, still larvae from cave populations exhibited a more pronounced aggressiveness, especially when exposed to wounded conspecific cues. Individuals from cave populations also tended to follow more actively the perspective prey larvae, suggesting a stronger propensity to start agonistic interactions. This supports the prediction that aggressive interactions are favored in cave environment and suggests a key role of intraspecific predation on the exploitation of resource-depleted habitats.

The higher aggressiveness of cave larvae is probably related to their peculiar environment. Theory predicts that food scarcity, high conspecific density, and absence of predators shall enhance cannibalistic tendency (Fox 1975; Polis 1981), and these conditions are generally experienced by salamander larvae from cave populations (Limongi et al. 2015; Manenti et al. 2015). Moreover, when exposed to wounded conspecific cues, cave larvae considerably decreased their Latency to approach prey larvae, and increased their Following duration. Examples of conspecific cues eliciting an active foraging strategy are described for some cannibal species (Harvey and Brown 2004; Tran 2014; Carlson et al. 2015), and resource-poor environments, such as caves, may further favor this response. Cannibalism is often favored in size- or age-structured populations (Fox 1975; Reques and Tejedo 1996). As expected, size differences promoted aggressive displays by increasing the number of attacks and the following intensity, and by

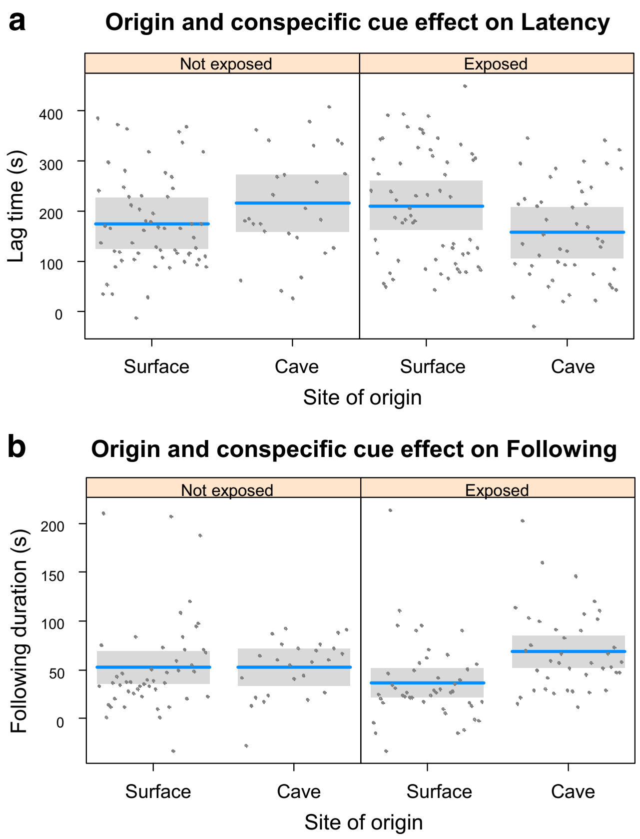

Fig. 5 Conditional partial residual plots, showing the significant interaction effects of origin and conspecific cues exposure on fire salamander Latency (**a**) and Following (**b**) displays. Shaded areas are 95% confidence bands

decreasing the latency of aggressive behaviors. Moreover, an increased aggressiveness was detected after 45 days of rearing, in agreement with studies suggesting that aggressive behaviors and cannibalism are more frequent in older larvae (Sadeh et al. 2009; Wissinger et al. 2010). In cave environments, prey are scarce and new-born conspecifics likely constitute an important resource for older, larger, and more aggressive larvae to survive and successfully achieve metamorphosis. Our results suggest that larvae from cave populations can be particularly prone to adopt risky behaviors and actively search for prey (Blecha et al. 2018), and perceive the cues of wounded conspecifics as a signal of trophic opportunity rather than an alarm signal.

The different behavioral strategies between cave and surface larvae (Figs. 5, and 6c) support the hypothesis of local adaptation in populations facing different environmental pressures (Baur 1994; Caspers et al. 2015). High foraging plasticity and cannibalism are traits that can facilitate the exploitation of resource-depleted environments (Polis 1981) and favor the colonization of novel habitats such as caves (Romero 2009; Manenti et al. 2013a). Moreover, in these environments, strategies offering access to alternative trophic resources, such as intraspecific predation and active search for the prey, should be positively selected and can become established as local adaptations (Hüppop 1987; Manenti and Ficetola 2013). Cave and surface populations often live nearby; therefore, some gene flow between populations is possible. Nevertheless, local adaptations might be maintained by multiple processes, such as the strong difference in selective pressure between the two environments and assortative mating (Caspers et al. 2009).

Predation pressure can determine complex anti-predatory responses. A reduction of activity and higher refuge exploitation are frequent non-consumptive effects allowing to limit encounter rate with predators (Lima and Dill 1990; Davenport and Chalcraft 2013; Manenti et al. 2016); thus, we expected a reduction of intra-specific aggression in salamander larvae exposed to predators. Our findings were in agreement with this prediction, as a strong decrease of aggressiveness was evident in salamanders from both cave and surface populations experiencing predator presence during rearing (i.e., Contact and No-Contact conditions), or exposed to predator cues during behavioral tests.

Attack rate and Following duration were significantly reduced in larvae reared under both Contact and No-contact conditions, confirming the hypothesis that risk exposure can lead to important non-consumptive effects in fire salamander (Manenti et al. 2016). On the one hand, the strong risk pulses of Contact were expected to determine a pronounced increase of anti-predator responses and thus limit the occurrence of aggressive interactions. On the other hand, a continuous exposure to predator, without direct experience of negative consequences as in the No-contact condition, was supposed to cause a lower anti-predator response (Turner 1997; Lima and Bednekoff 1999; Sih and McCarthy 2002). Nevertheless, we did not detect significant differences between the Contact and No-contact conditions, as both caused a similar reduction in aggressiveness. While such lack of difference might also be favored by the good nutritional regime of larvae, which can limit the need for an active search of resources, the reduction of aggressiveness in No-contact larvae strongly suggests that any occurrence of habituation to predator presence was unlikely. In contrast, these results support the idea that perceived predation risk, even if extremely differing both in time and modality, can elicit a strong anti-predator response causing deep consequences on behavioral interactions between conspecifics. Moreover, we detected complex interactions between chemical cues and rearing conditions, as larvae reared under Contact condition showed a lower reduction in their aggressiveness when exposed to cues of predators and wounded conspecifics (Fig. 3). During the Contact condition, animals experienced pulses of risk with the direct presence of the predator (with co-occurring visual, tactile, and chemical stimuli). In contrast, during behavioral trials, animals were exposed to just the chemical cues released by predators. The lower response to predator cues in Contact-reared animals seems to suggest that animals experiencing the contemporary exposure to multiple stimuli

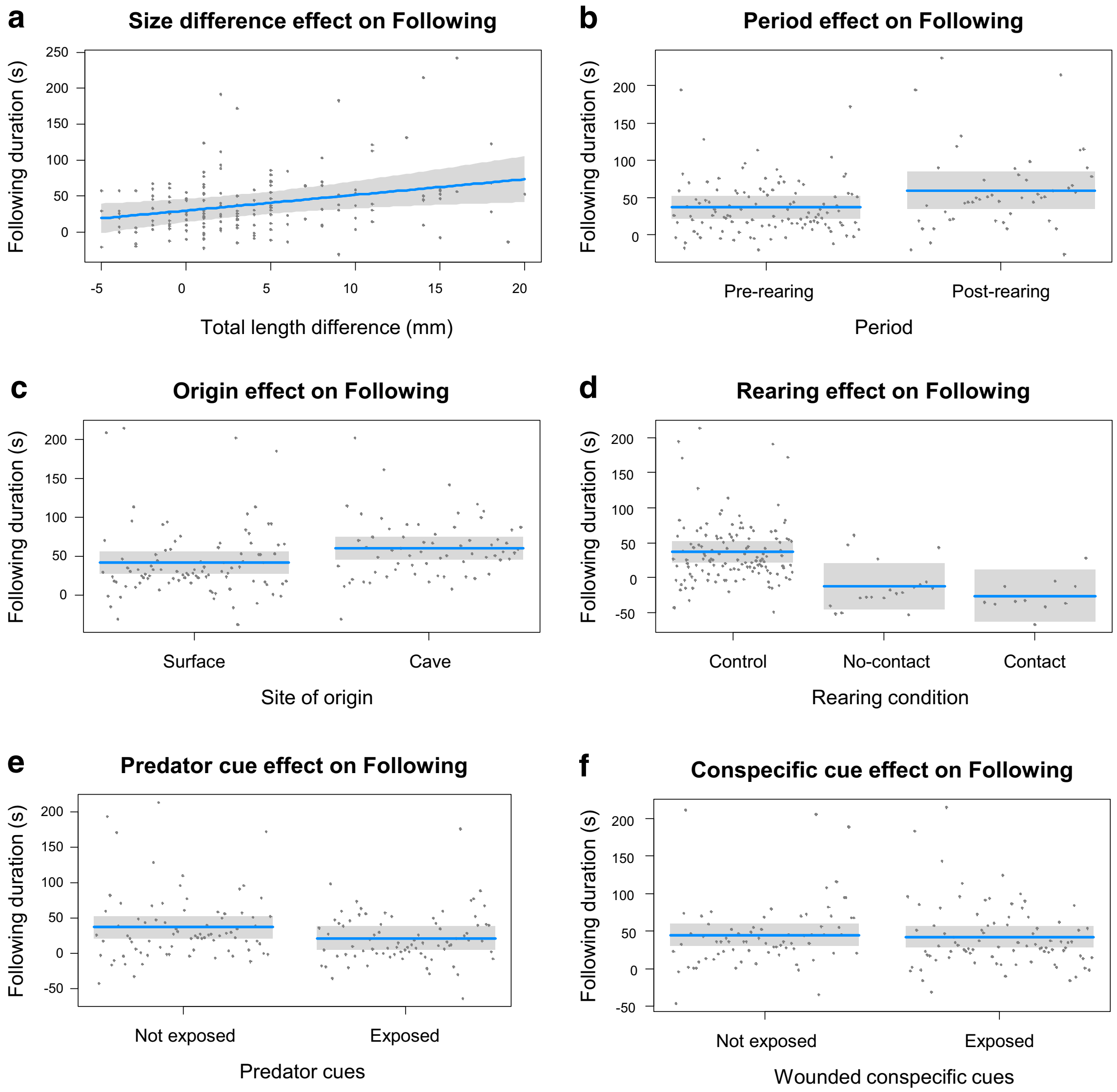

Fig. 6 Conditional partial residual plots, showing the relative influence of size difference (**a**), period (**b**), origin (**c**), rearing (**d**), predator cues (**e**), and conspecific cues (**f**) on fire salamander Following display. Shaded areas are 95% confidence bands

could have improved their capability to discriminate between stimuli associated to predators, compared to individuals that never experienced direct contact with predators. However, the assessment of this hypothesis would require focused tests.

Chemical signals are assumed to be the most common cues used for risk perception by amphibian larvae (Chivers and Smith 1998). Our study showed that chemical signals are important also for fire salamanders, as a small amount of cues from predators and/or conspecifics was enough to entail significant behavioral responses. While predator cue exposure determined a general anti-predator response, with a significant reduction of some aggressive displays, cues of wounded conspecifics did not determine anti-predator behaviors, suggesting that in fire salamander, they are not perceived as alarm cues potentially inhibiting intraspecific aggressiveness. Several studies showed that other amphibian larvae reduce activity rate when exposed to the cues of wounded conspecifics (Gonzalo et al. 2007; Ferrari et al. 2010b). However, most of these studies have been performed on anuran tadpoles, which rarely are predators (Wells 2007), and more studies are necessary to unravel the complex effects of wounded conspecifics in aquatic predators.

Both exposure to predators during rearing and the presence of predator cues during behavioral trials led to pronounced decreases of intraspecific aggressive interactions. Aggressiveness decreased in both cave and stream larvae, and in both newborn and older larvae. Such a reduction of aggressiveness led by predator occurrence may result in a waning or even a complete suppression of cannibalism in risky habitats (Kishida et al. 2011; Líznarová et al. 2018). As cannibalism plays a key role in many aquatic ecosystems (Rudolf 2008; Wissinger et al. 2010; Takatsu and Kishida 2015; Takatsu et al. 2017), its suppression may impact the survival and life-history of individuals (Kishida et al. 2011), with potential broad consequences on population dynamics (Claessen et al. 2004; Rudolf 2008).

Behavioral decisions under predation risk are driven by a complex interplay between multiple conditions (e.g., background level of risk, experience, prey energetic state). Their expression can be fine-tuned by the multifaceted nature of the environmental pressures experienced, but is also affected by adaptations to local conditions. Integrated analyses, considering multiple behavioral displays, are essential to better understand how prey responses are modulated under the complex environmental variation that can occur in nature.

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Compliance with ethical standards

The study design was approved by the ethical committee of the Lombardy Region Authority and was authorized as complying with the regional law 10/2008, p.n.: F1.2013.0002091. Larvae were subjected to the same rearing conditions as in Manenti et al. (2016). The aim of the present study was to evaluate the non-consumptive effects of dragonfly larvae on salamander larvae aggressive behavior. Consequently, care was taken to plan the experiment accordingly and, thus, to avoid any larvae being exposed to any actual predation. To this end, during rearing, direct contact with predator was restricted to 30-s encounters, which were short enough to prevent predation events. Whatever the case, we were always ready with tweezers to block snaps or stop them from extending their mouthparts. Similarly, full cannibalistic interactions were not allowed. During behavioral trials, when an aggressive interaction lasted more than a single bite event, conspecifics were promptly separated, thus, preventing injuries and intraspecific predation events. It should be also noted that in natural conditions, fire salamander larvae show very often aggressive interactions (Joly 1968), facing much more severe outcomes (e.g., being cannibalized) than those experienced in the study design that we adopted during behavioral trials and that was approved by the ethical committee. Conspecific cues were extracted from a single larva by removing the tail tip (far less than 30% of the tail) with sterilized scissors, a procedure that has been assessed to have no impact on individual survival or subsequent performance (Segev et al. 2015). After tail removal, the conditions of this individual were monitored and it perfectly recovered during a 40-day rearing period before releasing it in the site of origin. Overall, the survival rate of larvae was 70.4%, being much higher than that observed in natural populations (Limongi et al. 2015). All individuals were checked daily and fed every 2 days (see also Winandy and Denoel 2013, 2015). All the larvae were released at their site of origin at the end of the study, following the recommendations of the permit.

Conflict of interest The authors declare that they have no conflict of interest.

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Even worms matter: cave habitat restoration for a planarian species increased environmental suitability but not abundance

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Abstract Invertebrates living in underground environments often have unusual and sometimes unique adaptations and occupy narrow ranges, but there is a lack of knowledge about most micro-endemic cave-dwelling invertebrate species. An illustrative case is that of the flatworm *Dendrocoelum italicum*, the first survey of which was performed 79 years after its description. The survey revealed that the underground stream supplying water to the pool from which the species was first described had been diverted into a pipe for human use, thus severely reducing the available habitat for the species. Here we describe the results of what we believe is the first habitat restoration action performed in a cave habitat for the conservation of a flatworm. The water-diverting structure was removed, with the involvement of local protected area administrators, citizens and volunteers from local organizations. The intervention resulted in the restoration of a large, stable pool inside the cave, thus creating an optimal habitat for this threatened planarian, with increased availability of prey and a stable population. This report of habitat restoration for a neglected invertebrate offers insights for the protection of other micro-endemic species.

Keywords Cave, *Dendrocoelum italicum*, flatworm, freshwater, hypogean, Italy, planarian, restoration, subterranean

Introduction

Subterranean environments such as caves, shallow subterranean habitats and underground aquifers are among the most vulnerable habitats, often requiring conservation attention (Culver & Pipan, 2009; Pipan et al., 2010). Increasingly, studies are emphasizing the importance of conservation approaches based on habitat restoration (Perring et al., 2015; Chazdon et al., 2017; Canedoli et al., 2018). Ecologically important areas such as ecotones, ponds and wetlands have been restored successfully (Bergmeier et al., 2010; Romano et al., 2010; Lü et al., 2012; Merenlender & Matella, 2013) but there are not, to our knowledge, any documented cases of freshwater restoration involving cave habitats.

Subterranean environments generally exhibit environmental stability and are heterotrophic systems of key importance for the surrounding surface habitats (Culver & Pipan, 2009; Barzaghi et al., 2017): they work as recharge sites for surface waters, control water flux and exchanges with the surface, and provide shelter for key organisms such as bats, which sustain a variety of ecosystem services, including pollination, seed dispersal and pest control (Souza Silva et al., 2015). Cave environments are sensitive to threats such as climate change and groundwater pollution, but are also affected by other local or regional factors such as quarrying (Di Maggio et al., 2012), irresponsible exploitation for tourism (Ivanova, 2017), and water harnessing and diversion (Achurra & Rodriguez, 2008). All of these threats are likely to affect cave and subterranean ecosystems significantly, but they have been poorly studied.

Underground habitats are of special importance for biodiversity conservation because they often host a highly specialized fauna (Culver & Pipan, 2009; Romero, 2009). Moreover, most organisms that live in caves are highly restricted geographically and low in abundance (Zagmajster et al., 2008; Delić et al., 2017). Predators, sunlight and environmental variations occurring on the surface may be important environmental conditions for a number of cave-adapted organisms (Culver & Pipan, 2009). Micro-endemic invertebrate species, both stygobionts (specialized aquatic cave-dwelling organisms) and troglobionts (specialized terrestrial cave-dwelling organisms), are in some cases known only from one or a few caves (Culver & Pipan, 2014; Mammola & Isaia, 2016; Wei et al., 2017).

However, endemic invertebrate species are rarely considered in conservation programmes (Cardoso et al., 2011; Noreika et al., 2015) despite their ecological importance. Cave-dwelling endemic invertebrates can play an important functional role in karst ecosystems, facilitating nutrient recycling and biomass exchange (Schneider et al., 2011). Among cave invertebrates, planarians are free-living flatworms generally characterized by zoophagous feeding behaviour, preying mainly on small living invertebrates and grazing on decaying organisms (Reynoldson & Young,

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2000). Most planarian species are particularly sensitive to organic matter pollution and water quality (Horvat et al., 2005; Alonso & Camargo, 2006; Manenti, 2010). Nearly 200 species, including marine and freshwater species, have been recorded in cave habitats (Romero, 2009). Most of the species described are blind and depigmented, thus showing a strong adaptation to cave life (Stocchino et al., 2013). Cave-dwelling planarians occur in subterranean habitats worldwide (Gourbault, 1972; Carpenter, 1982; Sluys et al., 2009), but most of the described species have extremely narrow ranges, in many cases being confined to a single cave (De Beauchamp, 1932). For most known cave-dwelling planarians no further study has been conducted beyond the description of the species. Generally, no additional information has been reported after collection of the specimens used for description, and even the information reported with the description is often secondhand. In many cases descriptions were performed by anatomists specialized in Platyhelminthes, who never entered the caves but received specimens from speleologists. Moreover, cave-dwelling planarians are adapted to interstices in which they shelter, and even when cave pools or streams host abundant populations, planarians are not easily detected (Gourbault, 1972) and, consequently, it is difficult to assess their conservation status.

This situation is exemplified by the flatworm *Dendrocoelum italicum* (Benazzi, 1982), which was described in 1937 in the Bus del Budrio cave in the Italian Prealps (Vialli, 1937). The description of the species includes information about the habitat where it was found: a large subterranean pool below a small waterfall. In 2016 we discovered that the species was threatened by a structure that was diverting water from the waterfall into a pipe. Water was no longer reaching the pool, which had completely dried up, and *D. italicum* was confined to a short rivulet and a couple of tiny secondary pools.

We therefore began what we believe is the first habitat restoration action performed in a cave habitat (Plate 1) for the conservation of a flatworm species, aiming to investigate whether the restoration technique applied was appropriate. In particular, we predicted that (1) the abundance of this cave-dwelling planarian would increase shortly after the restoration, and (2) habitat restoration would increase the occurrence of the invertebrate prey of *D. italicum*.

Study site

The Bus del Budrio cave is in the Italian Prealps (Fig. 1) in the regional protected area of Cariadeghe, in northern Italy. The cave consists of an elongated chamber (43 m long, 6.5 m high), which opens at the cave entrance and is connected to an elevated narrow and sinuous corridor located on the north-western side. A small stream runs along the corridor and after 20 m forms a waterfall. In 1937, when *D. italicum* was described, a small waterfall filled a large pool, with a surface area of at least 5 × 5 m (Fig. 1). During the 1980s the water was diverted to a farm close to the cave (GT, pers. obs.). This left only a small trickle flowing down to form a bipartite rivulet 6 m long and 50 cm wide (Fig. 2a). As a result of this environmental alteration, the pool dried up permanently.

Plate 1 The restored pool in the Bus del Budrio cave (Fig. 1) in February 2018. Photograph by Luana Aimar.

Methods

Habitat restoration

In April 2016 we observed that the planarian *D. italicum* still occurred in the small rivulet. The rivulet appeared to be suboptimal, having a consistent flow and apparently poor prey occurrence, whereas the typical requirements of *Dendrocoelum* planarians are for standing waters (Gourbault, 1972) with relatively abundant invertebrate prey (Reynoldson & Young, 2000).

Firstly, we assessed the status and distribution of the species at the site (see below). Secondly, we informed the administrators of the protected area about the critical condition of the species and the need for immediate conservation action. Thirdly, we helped the protected area administrators to raise awareness about the species amongst citizens that use the reservoir for recreational activities and to obtain the agreement of the farmer responsible for diverting the water. Fourthly, we involved a local caving group, who volunteered to remove the structure diverting the water flow. On 3 December 2016 we collected all the planarians occurring in the rivulet, stored them in plastic tanks inside the cave, and removed both the duct feeding the small rivulet and the barrier that conveyed the water into it above the waterfall. Removal required 3 hours of work by five people. All artificial material was removed from the cave. When the water started to flow along its natural course we used shovels to compact the substrate under the waterfall, to

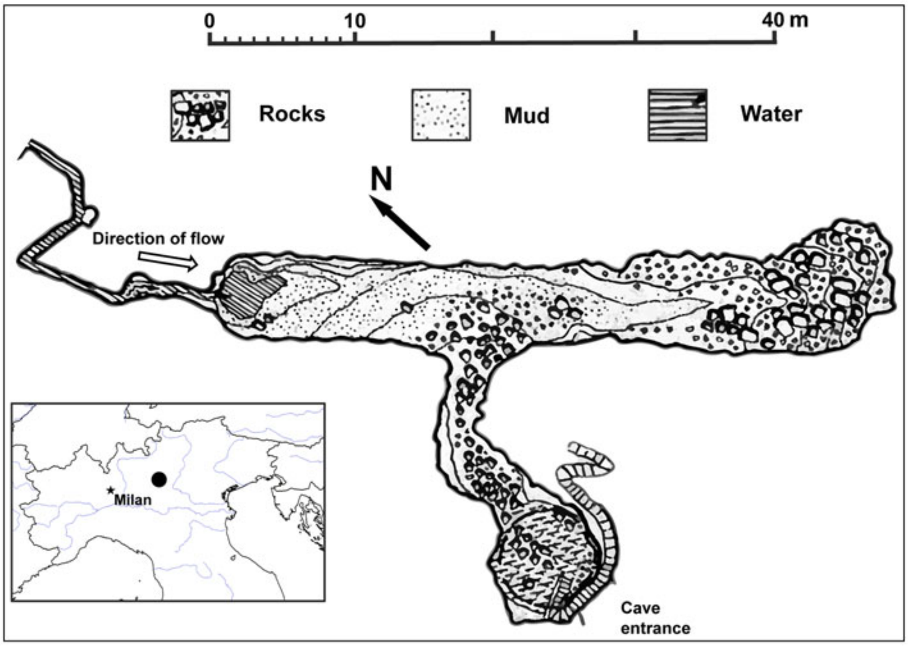

FIG. 1 Plan of the Bus del Budrio cave redrawn from the original survey performed by Allegretti and Trevisani of the Gruppo Grotte Brescia (19 September 1926) and subsequently published by Vailati (1979). The black circle on the inset indicates the location of the cave in northern Italy.

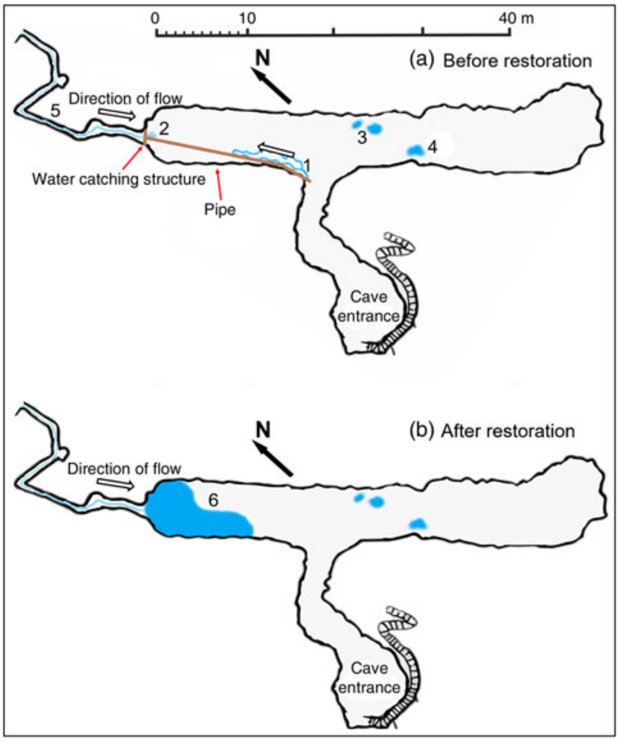

FIG. 2 The extent of the freshwater habitats of the Bus del Budrio cave (Fig. 1), (a) before and (b) after habitat restoration. 1, a small rivulet formed by an artificial water-catching structure installed in the 1980s; 2–4, pools resulting from drips; 5, small subterranean stream; 6, a pool fed by the subterranean stream after removal of the water–catching structure.

avoid water infiltration and to facilitate the filling of the former pool (Fig. 2b). In the following months of December and January the water column was highly turbid because of sediment suspension.

Dendrocoelum italicum assessment and relocation

We performed 18 surveys during February 2016–January 2018 to assess the status of *D. italicum* (Table 1): six before the habitat restoration, three during the restoration activities, and nine after restoration. At each survey the same (one or two) observers used the 30-minute visual encounter survey technique to count the number of planarians in all the freshwater habitats of the cave (Gourbault, 1972). After the intervention, the restored large pool replaced the small rivulet and a pool fed by drips. Surveys covered exactly the same area before and after the restoration. When the original pool started to fill from the fully restored waterfall, on 4 December 2016, we released all the collected planarians into it. In February 2016 and February 2018 we assessed the density of the potential invertebrate prey by performing 10 consecutive samplings with a thin-mesh dip net. The invertebrate surveys were repeated at three locations, chosen randomly.

Data analysis

We used random-effect Poisson generalized linear mixed models to assess the relationships between the relative abundance of *D. italicum* and habitat restoration activities (Barker et al., 2018). Poisson generalized linear mixed models produce reliable estimates of the relationships between the relative abundance of species and habitat variation. Simulations performed by Barker et al. (2018) showed that the estimates obtained through Poisson generalized linear mixed models are consistent with those obtained using alternative approaches (N-mixture models, double Poisson models), and are particularly appropriate for species for

TABLE 1 Details of surveys of the planarian *Dendrocoelum italicum* in Bus del Budrio cave in the regional protected Aarea of Cariadeghe, northern Italy (Fig. 1), before the intervention to remove a structure diverting the water flow, during the stabilization period and after the intervention.

Date	Time of survey	No. of *D. italicum* observed	Air temperature (°C)	No. of observers
Before the intervention				
4 Feb. 2016	Day	49		1
22 Apr. 2016	Day	69		2
27 July 2016	Night	109	10.7	1
15 Nov. 2016	Day	41	10.6	2
18 Nov. 2016	Day	8		2
27 Nov. 2016	Day	43		2
Stabilization period (turbid water)				
3 Dec. 2016	(planarians removed)	73	5.2	1
4 Dec. 2016	Day[1]	6	5.6	2
25 Jan. 2017	Day	7	−3.8	2
After the intervention				
19 Mar. 2017	Day	52	9.4	2
22 Mar. 2017	Night	109		1
30 Mar. 2017	Day	26	6.1	2
30 Mar. 2017	Night	58	6.2	2
31 Mar. 2017	Day	43	6.9	2
31 Mar. 2017	Night	91	6.2	2
1 Apr. 2017	Day	31	6.6	2
24 Jan. 2018	Day	65	0.5	2
24 Jan. 2018	Night	81	0.5	2

[1] After all collected planarians had been returned to the water.

TABLE 2 Density of invertebrate prey of *D. italicum* in Bus del Budrio cave (Fig. 1) before (February 2016) and after (February 2018) habitat restoration.

	Density ± SE (individuals/m^2)	
Taxon	Before restoration	After restoration
Pisidium personatum (Mollusca)	32.7 ± 6.48	88.6 ± 0.88
Haplotaxidae	0	11.25 ± 0.74
Niphargus sp. (Crustacea)	0.7 ± 0.35	0.82 ± 0.47

which detection probability of individuals is low. The dependent variable was the number of planarians observed in the whole cave in each survey. We included the time of observation (day/night) as a covariate. The period of observation (before, during stabilization or after the intervention) was included as a fixed factor, and the date of the survey was included as a random factor, as some surveys were conducted at different times on the same date. We distinguished between the after intervention and stabilization periods because after the remediation action the water was highly turbid, which probably affected planarian detectability. We assessed the significance of variables using a likelihood ratio test. The model did not exhibit overdispersion (residual deviance = 9.8; residual degrees of freedom = 11). A Tukey post-hoc test was used to determine the significance of differences in abundance in the intervention periods (before, during stabilization, and after intervention). A t test was used to assess differences between the density of potential invertebrate prey before and after the intervention. Analyses were performed in *R v.3.3.2* (R Development Core Team, 2016) using the package *lme4*.

Results

The restoration was successful in achieving stable hydrological conditions in the pool inside the cave (21 m^2 surface area, 15 cm mean depth). At the time of restoration, 73 planarians (58 adults, 15 juveniles) were removed from the rivulet and released the next day in the restored pool, when its conditions became stable. The number of planarians detected per survey was highly variable among surveys: 8–109 individuals before the restoration, and 31–109 individuals after the restoration (Table 1). Comparing winter surveys, the density and variability of available prey was significantly higher after the restoration (Table 2). In particular, after the restoration we detected significantly more bivalves *Pisidium personatum* ($t = 8.55$, $P = 0.01$), and annelids of the family Haplotaxidae that were not present before ($t = 15$, $P < 0.01$). A small, non-significant increase (from 0.7 to 0.8 individuals/m^2) was detected in the number of crustaceans of the genus *Niphargus*.

The number of planarians was higher during night surveys (Poisson generalized linear mixed model:

$\chi^2 = 28.17$, df = 1, $P < 0.0001$). During the period after the intervention we did not detect significant differences in abundance (Tukey's post-hoc test: P = 0.85).

Discussion

As far as we are aware, this is the first case of restoration of a cave habitat hosting a planarian species. The intervention helped to restore the original habitat conditions in the type locality of the endemic planarian *D. italicum*. As we detected no significant difference in the number of planarians before and after the restoration, the population at least remained stable. However, environmental conditions in the cave changed considerably: a large shallow lentic habitat oxygenated by the waterfall became available and the density and diversity of suitable invertebrate prey increased.

The few available studies suggest that cave-dwelling planarians prefer shallow standing pools (Ginet & Puglisi, 1964; Gourbault, 1972). The epigean species of the genus *Dendrocoelum* are also normally reported from standing waters (Reynoldson & Young, 2000), and therefore the restored conditions probably provide more suitable environmental conditions for *D. italicum* than those prevailing before the restoration. Restoration of the water flow rapidly recreated a large pool in the western part of the cave. The restored environmental features are also suitable for species that need oxygenated waters, such as *Niphargus* sp., and for species of standing water, such as the molluscs and annelids observed. The increased availability of potential prey following habitat restoration has augmented the amount and quality of the trophic resources available to the planarians.

The number of planarians was significantly lower in the 2 months immediately after the restoration. In this period water turbidity was high and probably limited detection. The similarity in planarian density before and after restoration suggests that more time is needed to assess the effects of habitat restoration on population dynamics. Breeding cycles are generally long in *Dendrocoelum* species, which are considered to be semelparous (Young & Reynoldson, 1965), and cycles may be even more prolonged in cave-dwelling species of the genus (Gourbault, 1972).

Planarians were more active at night. Although most freshwater planarians are known to be photonegative (De Beauchamp, 1932; Reynoldson & Young, 2000), their diel rhythms have rarely been quantified. Two non-exclusive hypotheses may explain the differential activity of *D. italicum*. Firstly, although they do not have eyes, *D. italicum* individuals may be able to perceive some light from the cave entrance. Secondly, the species may have retained circadian rhythms from a surface ancestor.

To our knowledge, the only other case of a worm that involved zoological studies, citizens' awareness and conservation actions is that of *Macrocotyla glandulosa* (Romero, 2009). This species is also the only cave-dwelling planarian that is monitored regularly for conservation purposes (Wicks et al., 2010). Our study demonstrates that it is possible to establish a successful conservation programme for a neglected invertebrate species. Generally, little attention is given to worms and other invertebrate species that have limited appeal to the public or protected area administrators (Cardoso et al., 2011). In our project, however, the involvement of administrators of the regional protected area of Cariadeghe and local citizens was central to its success. The citizens demonstrated an interest in the uniqueness of this planarian species and, together with the farmer responsible for previously diverting the water, approved the decision of the protected area administrators to restore the cave habitat. Most cave invertebrates have narrow ranges, and although they attract the interest of taxonomists, they are otherwise little studied (Culver & Pipan, 2014). This situation often results in large gaps in knowledge following species' description, especially for species described long ago (Leroux & Schmiegelow, 2007). It is likely that numerous micro-endemic invertebrate species are in a similar situation to *D. italicum*, and our results provide insights for the conservation of micro-endemic invertebrate species and cave-dwelling flatworms.

In particular, our study provides two messages: the feasibility of including neglected invertebrate taxa in restoration programmes that lead to general habitat improvement, and the role that endemism can play in fostering people's empathy towards invertebrates. Conservation management of cave invertebrate species may be encouraged by drawing attention to the unique role of such species.

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Author contributions Concept, data collection and writing: RM; data collection and habitat restoration: BB; provision of logistics, materials and site access, and organization of habitat restoration: GT; data analysis and writing: GFF; data collection, analysis and writing: AM.

Conflicts of interest None.

Ethical standards The research and intervention were approved by the Monumento Naturale Regionale Altopiano di Cariadeghe.

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SCIENTIFIC REPORTS

N-mixture models reliably estimate the abundance of small vertebrates

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Accurate measures of species abundance are essential to identify conservation strategies. *N*-mixture models are increasingly used to estimate abundance on the basis of species counts. In this study we tested whether abundance estimates obtained using *N*-mixture models provide consistent results with more traditional approaches requiring capture (capture-mark recapture and removal sampling). We focused on endemic, threatened species of amphibians and reptiles in Italy, for which accurate abundance data are needed for conservation assessments: the Lanza's Alpine salamander *Salamandra lanzai*, the Ambrosi's cave salamander *Hydromantes ambrosii* and the Aeolian wall lizard *Podarcis raffonei*. In visual counts, detection probability was variable among species, ranging between 0.14 (Alpine salamanders) and 0.60 (cave salamanders). For all the species, abundance estimates obtained using *N*-mixture models showed limited differences with the ones obtained through capture-mark-recapture or removal sampling. The match was particularly accurate for cave salamanders in sites with limited abundance and for lizards, nevertheless non-incorporating heterogeneity of detection probability increased bias. *N*-mixture models provide reliable abundance estimates that are comparable with the ones of more traditional approaches, and offer additional advantages such as a smaller sampling effort and no need of manipulating individuals, which in turn reduces the risk of harming animals and spreading diseases.

Estimating species abundance is a pivotal task of species monitoring. Unfortunately, in most of cases detecting individuals of the target species can be challenging. Very often we are not able to detect all individuals present in a given place and time, and this may happen for several reasons, such as their elusive behaviour, cryptic habits or simply because of the limited ability of surveyors[1]. Therefore, the number of observed individuals rarely represents a reliable estimation of the number of individuals occurring in a given area.

Multiple approaches have been developed to estimate the true number of present individuals. Among them, approaches requiring multiple sessions of capture have a considerable success. For instance, in capture-mark-recapture (CMR) approaches animals of a population are captured, individually marked or photographed to allow identification, and released at the capture site. The frequency of marked individuals observed in subsequent capture sessions is then used to estimate abundance[1,2]. Removal sampling (sometimes named catch-effort model) is an alternative approach, which requires the systematic capture and removal of individuals. Population size is then estimated on the basis of the decline in catch size during sequential capture sessions[2–4]. These approaches

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have been particularly useful to obtain reliable estimates of population size, and have revealed long-term temporal trends, allowing to identify the factors determining the survival of individuals and the decline of populations[5–9].

However, approaches requiring the capture and identification are generally labour intensive, as many individuals need to be captured and identified to obtain reliable population estimates. Therefore, the broad scale monitoring of the abundance of wildlife has been a challenge for decades[10]. In the last years formal approaches have been proposed to estimate animal abundance from repeated counts at fixed sites, without marking individuals to identify them[11,12]. The number of individuals detected in a given site is counted using standard monitoring techniques (e.g., point counts, observation in small plots, fixed area transects), and each site is generally surveyed in multiple occasions. The repeated counts in a given site are then used to jointly estimate the detectability of individuals and population size based on *N*-mixture models[11–14]. As they do not require capture or manipulation of individuals, such models might allow collecting abundance information over larger areas compared to traditional approaches, can be appropriate for protected species, and have been proposed for broad-scale assessment of populations[13,15,16]. The usefulness of *N*-mixture models to estimate abundance of amphibians and reptiles is advocated since several years[1,13,17] and, given their high cost-effectiveness, these approaches have also been suggested to obtain broad scale estimates of the population trends of amphibians and reptiles[16]. For instance, repeated counts analysed with *N*-mixture models have been proposed for the periodic monitoring of several species of amphibians and reptiles listed in the EU Habitat Directive at the national scale[18].

Nevertheless, approaches based on *N*-mixture models are not yet widely used to estimate population abundance, perhaps because practitioners remain unsure about their efficiency compared to more traditional techniques requiring capture. Recent analyses casted doubts about the usefulness of *N*-mixture models, because the loss of information resulting from not marking animals can make problematic the joint estimation of abundance and detection probability[19]. Moreover, these models are sensitive to violations of their assumptions, and unmodeled heterogeneity in abundance or detection probability can cause substantial biases[20,21]. Thus, real-world studies are required to verify the estimates from *N*-mixture models under a range of conditions. Recently, Kéry[22] found excellent agreement between estimates under *N*-mixture and those from different approaches (i.e., multinomial *N*-mixture models), which are a generalization of CMR that do not suffer from borderline estimability of parameters[19]. Furthermore, some studies comparing the performance of mixture models with more traditional approaches (e.g., CMR) found similar abundance and density estimates [e.g.[23,24]] but, until now, such comparative analyses have focused on a limited range of species.

In this study, we compared population estimates obtained using *N*-mixture models with estimates obtained applying more traditional approaches, i.e., removal sampling and capture-mark-recapture. We focused on three threatened species of amphibians and reptiles endemic of Italy and adjacent areas: the Lanza's Alpine salamander *Salamandra lanzai*, the Ambrosi's cave salamander *Hydromantes ambrosii* and the Aeolian wall lizard *Podarcis raffonei*. All these species are threatened, and both salamanders are fully terrestrial and do not require water for reproduction[25], thus other traditional approaches to estimate the abundance of amphibians (e.g., egg counts, monitoring of breeding sites) cannot be used. Therefore, the reliability of monitoring approaches based on the observation of unmarked active individuals is a key aspect to provide effective information for management plans.

Results

Lanza's Alpine salamander. During 63 repeated surveys, we obtained 63 salamander detections (range: 0–9 individuals per plot in each survey). In *N*-mixture models, we used a zero-inflated Poisson model as it showed AIC values lower than the Poisson model (model without covariates: AIC: 180.9 vs. 207.2). *N*-mixture models estimated an average detection probability of 0.14 (95% CI: 0.02–0.62). The model including hour of survey as covariate showed a higher AIC value than the model without hour (AIC = 182.5), and hour did not have a significant effect on salamander detection ($z = -0.20$, $P = 0.608$), therefore we kept the model without covariates. The estimated number of individuals ranged between 0.4 and 14.7 individuals per plot (Fig. 1).

Studies using capture-mark-recapture[26,27] estimated a density of ~300 individuals/ha. If we only consider the six plots within the CMR study area, the average density of salamanders estimated using *N*-mixture models was 141 individuals/ha (95% CI: 38–450 individuals/ha). However, if we also include the plots nearby the CMR study area, the average density of salamanders was higher, and closer to the estimates obtained using CMR. For instance, if we also consider plots within 250 m, the average density was 254.3 individuals/ha (95% CI: 130–544).

Ambrosi's cave salamander. During 20 repeated counts, we obtained 599 salamander detections (range: 0–123 detections per cave in each survey). In *N*-mixture models, we used a Poisson error distribution as it showed AIC values lower than zero-inflated Poisson models (AIC: 510.3 vs. 512.4). *N*-mixture models estimated an average detection probability of 0.62 (95% CI: 0.59–0.76); empirical Bayes estimates indicated population abundances between 13 and 135 individuals/site (Table 1).

During the removal experiment ($N = 29$ capture sessions), we captured 507 individuals (range: 0–99 individuals per cave in each session). In removal models, estimates of population size ranged between 13 and 244 individuals per cave (Table 1). The depletion method was unable to estimate population size for the two cavities with less captured individuals (False Snake and Ambrosi's sinkhole), in which zero individuals were captured during the second and third capture sessions.

Overall, *N*-mixture models and removal sampling provided highly correlated and consistent estimates of population densities (Fig. 1), with similar values in most of populations (Table 1, Fig. 2). In the populations with more individuals, *N*-mixture models tended to estimate smaller population sizes (Table 1). Nevertheless, a linear regression model, relating log-transformed abundances estimated with the two approaches revealed a strongly significant relationship ($R^2 = 0.91$; $F_{1,6} = 57.2$, $P < 0.001$), with a slope not significantly different from one ($B = 1.32$, 95% CI = 0.89/1.74) and an intercept not significantly different from zero ($k = -1.00$, 95% CI = −2.63/0.61).

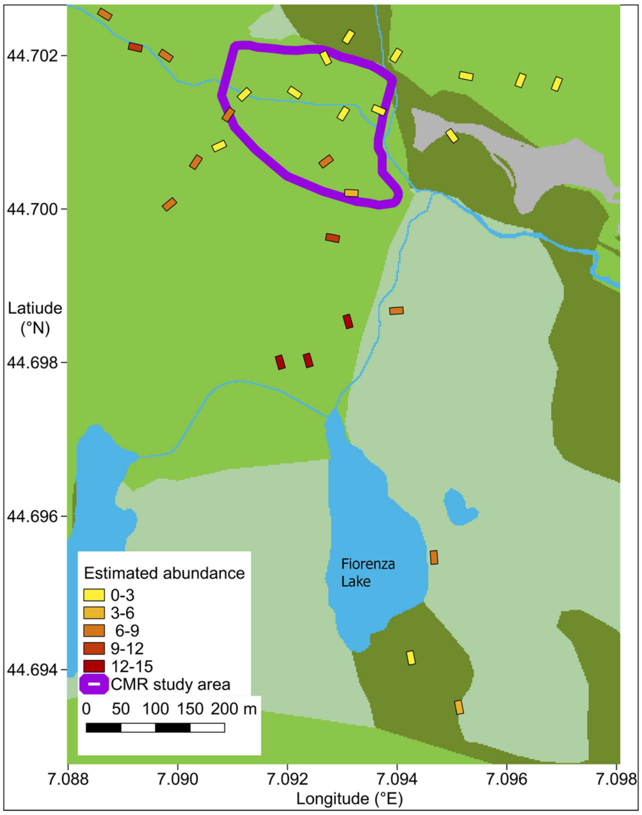

Figure 1. Plots used to assess the abundance of *Salamandra lanzai*, and spatial variation of abundance estimates. The violet line is the approximate limit of the area sampled with capture-mark-recapture[42]. The map was generated by GFF using the open-source software QGis 2.18 (QGIS Development Team, 2016. QGIS Geographic Information System. Open Source Geospatial Foundation Project. www.qgis.org); background colors represent land use (grey: built-up; green: pasture; pale green: sparse vegetation; dark green: high-altitude pasture and moorland; blue: water).

	N max	*N*-mixture models		Removal sampling		
Cave		**Abundance**	**95% CI**	**Capture rate**	**Abundance**	**95% CI**
Pignone left entrance	27	33.8	29/39	0.5	24	*
Pignone right entrance	38	50.1	45/56	0.702	53	51/63
Pignone main cave	38	53.3	48/59	0.636	59	57/70
Pignone – False snake's hole	5	11.0	7/16	†	†	
Pignone – Ambrosi's sinkhole	3	8.7	5/13	†	†	
Fornace	30	43.3	38/49	0.38	76	*
Fornace left entrance	15	23.8	19/29	0.6	20	19/33
Pignone abandoned mine	52	57.8	54/63	0.386	114	92/240
Spelerpes	6	13.1	9/18	0.426	13	*
Alta di Castè	123	144.5	138/152	0.382	244	219/300

Table 1. Abundance estimates in ten populations of *Hydromantes ambrosii*, obtained with different approaches. *N* max: max number of individuals detected in one single survey session. [†]The method was unable to estimate population size. [*]Estimation of 95% CI was not available.

Aeolian lizard. During 11 repeated counts, we obtained 85 detections of adult lizards in four replicated transects. On average, we observed 11.8 lizards/man-hour of survey. In *N*-mixture models, we selected the model with Poisson error, as it showed AIC values lower than the respective ZIP model (69.4 vs. 71.4). The model including hour of survey as covariate showed the lowest AIC value, and suggested that detection probability was highest in early morning (Table 2). At 9.30 a.m., the average detection probability was 0.18 (SE = 0.78). *N*-mixture models yielded a population size estimate of 274 individuals within the transects (95% CI: 221–334), indicating an average lizard density of 0.35 individuals/m^2 (95% CI: 0.28–0.43). Assuming homogeneous density in the survey area, this yielded a total estimate of 1050 individuals (95% CI: 847–1280).

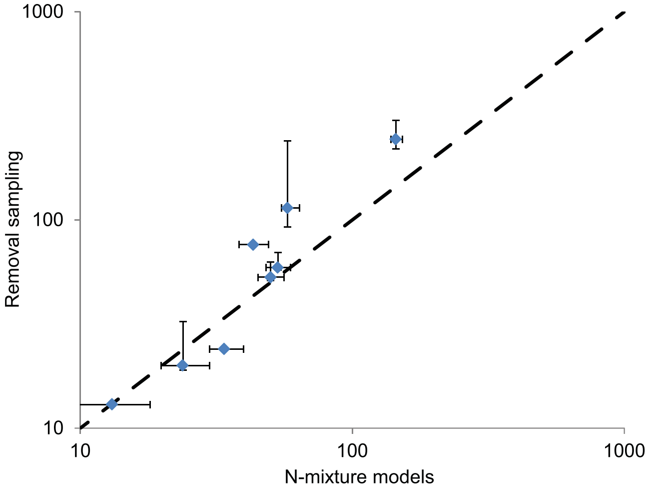

Figure 2. Abundance of *H. ambrosii*: comparison between removal sampling and *N*-mixture models. Error bars are 95% confidence intervals of each estimate, the black dashed line has intercept = zero and slope = 1.

Variables in the model	K	AIC
hour of survey (−)	3.00	53.5
hour of survey (−); sampling effort (+)	4.00	55.1
None	2.00	69.4
sampling effort (+)	3.00	71.1

Table 2. Candidate *N*-mixture models on factors determining the detection probability of Aeolian lizards. Signs after variable names indicate the sign of regression coefficients. Models are ranked on the basis of their AIC values. K: number of parameters in the model.

During the removal sessions ($N = 5$ capture sessions), we captured 131 adult lizards (63 males and 68 females; range: 7–18 males per capture session). Average capture rate was 2.9 individuals/man-hour. Males were temporarily maintained in terraria for the removal experiment, while most of females were immediately released in nature. The removal method estimated a total population size of 538 males (lambda: 0.003). If we assume a 1:1 sex ratio, the overall abundance of *P. raffonei* estimated using removal sampling was ~1080 individuals. The number of captured males per hour only slightly decreased from the first to the last capture session, therefore confidence intervals for this estimate were not available. Genetic analyses are currently ongoing to ascertain whether these lizards are pure *P. raffonei*, or are hybridized with non-native species.

Discussion

Accurate estimates of population size provide baseline data for many studies on population ecology, are essential to assess the conservation status of populations, and allow to identify management priorities. For instance, in the European Union the Habitat Directive protects several hundreds of animal species, and requires regular reports on the conservation status of all species protected by the directive. Such reports should include measures of population size and trends for all these species across the continent. Obtaining quantitative measures of population size of hundreds species over broad areas requires considerable monitoring efforts, and volunteers are a key resource for such a broad scale monitoring[13,15,28–31]. Approaches based on the capture of individuals such as capture-mark-recapture or removal sampling can provide reliable estimates of population size, but also have drawbacks. First, the capture of many individuals often requires more time than just observing their presence, therefore it can be less effective if we need monitoring many populations. For instance, for lizards the detection rate was four times higher than the capture rate. Similarly, for cave salamanders the removal sampling required approx. 80 man-days of work, while only 28 man-days were required for the visual surveys of *N*-mixture models. Second, some techniques used to mark amphibians and reptiles are expensive, or can harm individuals and pose ethical issues (e.g., toe clipping)[32]. Finally, the manipulation of specimens can pose threats to the study populations, such as the risk of transmission of infectious diseases[33]. Actually, European salamanders currently face the risk of infection by the chytrid fungus, *Batrachochytrium salamandrivorans*, which is lethal to most salamanders and is causing dramatic declines in several populations[34–36]. Under these circumstances, protocols requiring the capture of individuals by a large number of volunteers cannot be advocated. *N*-mixture models just require the observation of individuals, and thus are a promising alternative that does not require the manipulation of individuals. Our study shows that this approach can provide reliable estimates of population size, which are highly comparable with the ones obtained by more traditional approaches.

Simulations suggest that population sizes estimated through *N*-mixture models generally have a limited bias if model assumptions are met[16,20], but the accuracy of these analyses still requires assessment. If detection probability is ~0.15 (as we recorded for the Lanza's salamander) and sites are surveyed only a few times, simulations suggested that the relative bias of mixture models may be 45–50%[16], i.e., they can over- or underestimate population size by approx. 50%. The accuracy of mixture models increases in easily detectable species and, if detection probability is 0.5, the expected bias is ~12–20%, and the correlation between true and estimated population size is expected to be ~0.9 [Fig. S2 in[16]]. Testing the validity of these predictions is difficult, as in real populations we hardly know the true population size. Nevertheless, if we compare mixture models with CMR and removal sampling, we obtain measures of bias that are in agreement with these predictions. For Lanza's Alpine salamanders, the differences between CMR and mixture models was 17–52% (depending if we consider all transects nearby the CMR study area, or only transects within the CMR study area; see below). For cave salamanders, the average relative bias was 26%, and the correlation between the two population size estimates was 0.95. The match between empirical data and simulations confirms the robustness of conclusions obtained through the virtual ecologist approach[37], and supports mixture models as a reliable tool for the analysis of monitoring data.

Nevertheless, the reliability of *N*-mixture models heavily depends on meeting model assumptions, and recent simulations suggested that emigration, double counts or variation of detection probability among sampling occasions can produce biased results, yielding substantial overestimates of species abundances[20,21]. In our study, detection probability of lizards was not constant, and declined in surveys performed during late morning, as commonly observed for reptiles[38]; actually, the hour of survey was included in the best-AIC model (Table 2). It should be remarked that abundance estimates would be heavily biased, if such variation of detection probability was not incorporated into models. In fact, a model without detection covariates (e.g., model 3 in Table 2) would provide density estimates of 1.3 individuals/m^2, which are three-times larger than the ones obtained through the removal experiment. The correct incorporation of heterogeneity of detection probability is thus essential to obtain useful estimates[20], and appropriate knowledge of species biology can be extremely important to identify and accurately measure the variables that can allow describing this variability.

In the Lanza's Alpine salamander example, the two population estimates (*N*-mixture vs. CMR) were not performed in the same year. It is well known that salamander populations can undergo strong temporal variation, for instance in response to habitat modifications, climatic variation and variation of biotic factors (e.g.[7,39,40]), and population fluctuations can occur even in absence of evident habitat changes[41]. Despite we do not have quantitative data on population dynamics, the available information suggests that the study populations did not undergo strong variations of abundance through time. For instance, for Alpine salamanders, CMR estimates of abundance obtained in 1992 and in 2003 were very similar[27,42]. Furthermore, the study area is a protected site, for which no major habitat modifications occurred in the last 20 years. Differences between CMR and *N*-mixture models were stronger if we only consider the eight plots falling within the CMR area. However, only eight plots were inside the target area, and sampled just 1,600 m^2, which represent 3.9% of the surface sampled by CMR. Therefore, the imperfect match between the estimates possibly occurred because the sampling effort inside the target area was too low. Conversely, if all the plots nearby the Andreone *et al.*[25,27] study area are considered, *N*-mixture models sampled a much larger surface (5,400 m^2). Salamander distribution is not homogeneously distributed across the landscape (Fig. 1), and the more intense effort probably allows a better representation of the overall landscape. The quality of population estimates generally increases at high sampling efforts[43] which, in this case, is related to both the number of surveys per plot, and the total area covered by plots. When planning surveys, both these parameters must be defined a priori. Increasing the surface of each plot, and increasing the number of plots, are alternative approaches to increase sampling efforts. Deciding the best strategy (a few large or several small plots) strongly depends on parameters such as population density, detection probability, spatial heterogeneity and logistic constraints, and should be decided a priori, on the basis of study aims and resources availability. For instance, the number of individuals that are detected at each survey is generally higher in larger plots. Therefore, large plots and/or a large number of surveys per plot are a more effective strategy for species with limited detectability, while surveying several small transects can be preferable if populations have high detection probability[16,44–46].

With cave salamanders, detection probability estimates were very high ($\geq$0.4) using both approaches. High detection probability has already been demonstrated in other species of cave salamanders, particularly during their underground activity phase[5,47–49], and this favors studies on the ecology and dynamics of cave salamander populations (e.g.[39,48,49]). The match between mixture models and removal sampling was excellent for the caves with fewer salamanders. Mixture models tended to underestimate population size in the two caves where removal estimated more individuals (Table 1, Fig. 2). It should be remarked that capture rate, estimated by removal sampling, is unrelated to both cave depth and salamander abundance ($|r| \leq 0.4$ and $P > 0.25$ for both correlation), suggesting that this does not occur because sampling quality was weaker in larger caves and/or in caves with more salamanders. Overall, the slope of the regression between population sizes estimated with the two approaches was not significantly different from one, and in the majority of cases abundance estimates were extremely similar confirming that, in most of sites, *N*-mixture models are an excellent approach to estimate the abundance of these animals. Nevertheless, additional analyses are needed to understand the performance of *N*-mixture models when variation of abundance among sites is strong.

We showed that *N*-mixture models can provide effective measures of the abundance of populations for small vertebrates with very different habits and living in a wide range of habitats, from nocturnal salamanders living in alpine meadows, to lizards living in Mediterranean islands. However, just measuring abundance provides limited information for conservation. An additional advantage of *N*-mixture models is the possibility of including covariates as potential predictors of species abundance also at very fine spatial scale[17]. Assessing the factors that can determine differences in abundances among sites, or differences in abundance in a site surveyed during different years would provide more complete information and, for instance, can allow the identification of threatening factors that should be targeted by conservation strategies[50,51]. During surveys, experienced observers can also record parameters representing habitat quality or threats[51] that can be successfully integrated within *N*-mixture models to provide quantitative management indications (e.g.[17]). The elaboration of comprehensive monitoring schemes, that allow the integration of species abundance data with information on habitat features and on drivers of abundance is not easy[52], but will provide essential information for more effective management.

Methods

Species, study areas and sampling.

Lanza's Alpine Salamander Salamandra lanzai. *Salamandra lanzai* is endemic of a small area of the Alps, between NW Italy and E France, and lives at altitudes of 1200–2650 m. This salamander is viviparous, and individuals are active at the surface from late spring until early autumn, mostly at night and during humid periods[26]. The study was performed at the Pian del Re area (approx. 44.70°N, 7.10°E; altitude: 2000–2150 m; Fig. 1), which is an alpine meadow with multiple small streams, and is the type locality of *S. lanzai*.

Capture-mark-recapture data were obtained from previously published studies performed in 1992–2003, which sampled a surface of approx. 41,000 m^2 (Fig. 1 [27,42]). *Repeated counts*. We defined 28 rectangular (20 × 10 m) plots, across the whole Pian del Re. Each plot was surveyed by a 6–8 people team, actively searching and counting all the salamanders at the surface for 10–15 min. Plots were surveyed 2–3 times (average: 2.3 surveys per plot) in the period 16–19 August 2015 after dusk, between 9.00 pm and 1.00 am. We positioned the 28 plots performed in 2015 as follows: eight were inside the study area where Andreone *et al.* (refs[27,42]) performed their CMR study, 17 were nearby the Andreone *et al.* (refs[27,42]) study area (<250 m from the area), and three were 500–750 apart (Fig. 1).

Cave salamander Hydromantes ambrosii. The Ambrosi's cave salamander *H. ambrosii* (see[53] for discussion on nomenclature) is endemic of a small area of peninsular Italy. This terrestrial salamander does not live exclusively in caves, as from autumn to spring it is often active at the surface. However, during the dry and hot Mediterranean summer it often moves to underground refugia, where its detectability is highest[48,54]. In June 2017, we monitored ten cavities (caves and abandoned mines) in Central Italy using both repeated counts and removal sampling. We considered the Spelerpes cave (44.13°N, 9.78°E), six cavities within the Pignone karst Area (44.18°N, 9.72°E) and the Alta di Castè cave (44.12°N, 9.77°E). Explored depth of caves ranged between 9 and 48 m. For repeated counts, each cave was monitored by 6–7 observers during daytime in sunny, dry days, by actively searching and counting all the salamanders visible on both cave walls and floor, as described by Lunghi, *et al.*[48]. Each cave was surveyed two times within 3–10 days, to ensure meeting assumption of population closure. Survey effort was approx. 0.5 man/hour for each m of explored cave. Subsequently, we performed a three-sample removal experiment[4]. Immediately after the end of the last count survey, 6–7 people collected and stored salamanders in specific fauna boxes (40 × 25 × 15 cm) which were left inside caves. Removal session ended after 10 minutes without captures. At the end of the third session of capture, animals were released in the same cave areas in which they were found. Individuals were manipulated with disposable nitrile gloves, and all the equipment was disinfected following guidelines for preventing the spread of infectious diseases[55].

Aeolian wall lizard Podarcis raffonei. The Aeolian wall lizard *Podarcis raffonei* is endemic of the Aeolian Archipelago (Southern Italy). The species is critically endangered and is undergoing a quick decline; the most likely factors determining lizard decline are the competition/hybridisation with non-native lizards, and habitat modifications. Currently, only four populations of this species are known to persist[56,57]. During spring 2017 (end of April-beginning of May) we carried out field surveys in the Capo Grosso Peninsula (approx. 38°25′N, 14°56′E; surface area available for surveys: 2990 m^2) in order to estimate the size and the status of the last population of *P. raffonei* on the Vulcano Island using repeated counts (visual strip transects) and removal sampling. First, we established four linear transects (length: 60–75 m; width: 1.5 m) covering the whole peninsula, and used visual encounter surveys to count the number of active lizards. Transects were >5 m apart, to avoid double counts of the same individual, and were performed between 9.00 and 12.00 a.m. by one-two observers. Only adult lizards were considered; each transect was repeated 2–3 times (average: 2.75). Second, individuals were noose-captured through the whole peninsula. Females were mostly released immediately after capture, while all the captured males were temporarily transported in terraria. Overall, we performed five capture sessions; the sampling efforts of capture sessions ranged between six and 12.5 man/hours.

Data analysis.

For all the species, repeated counts were analysed using *N*-mixture models for closed populations[11]. We used Akaike's Information Criterion[58] to select the most appropriate error distribution (Poisson or zero-inflated Poisson); we did not consider negative binomial errors as can produce infinite abundance estimates, particularly when detection probability is limited[59]. In models, we used 100+ the maximum observed species abundance as upper bound to approximate an infinite summation of the likelihood, since preliminary analyses suggested that this value provides robust estimates[16]. For Lanza's Alpine salamanders, activity is often higher early after dusk[42], thus we considered hour of survey as a covariate potentially affecting detection probability; all surveys were conducted within four days, with constant meteorological conditions (similar temperature; no rain). For Aeolian lizards, all surveys were performed within one week, during sunny days without wind. However, activity of lizards is generally higher in early morning, and survey effort was variable (range: 0.33–0.66 man/hours per transect), therefore we tested models including hour of survey and survey effort as detection covariates. For all the species we then used empirical Bayes methods to estimate the posterior distribution of the abundance (mean and 95% Bayesian credible intervals)[60]. *N*-mixture models were run using the unmarked package in R 3.3.3[61].

To estimate population size from removal sampling of *H. ambrosii* and *P. raffonei*, we used the sampling coverage estimator for heterogeneous models of Chao and Chang[4], which is able to estimate population size with low bias, assuming that capture rate can be different among individuals. For *P. raffonei*, the length of sampling session (men-hours) was included as a measure of sampling effort. In preliminary analyses, we also tried using methods assuming homogeneous detection probabilities[4], and obtained very similar estimates.

Availability of data and materials. The dataset supporting the conclusions of this article is included within the additional files.

Ethics statement. All research involving animals was performed in accordance with the national regulations, and was conducted under the authorization of National Authorities (Ministero dell'Ambiente della Natura e del Mare; *H. ambrosii*: 9384/PNM/2015, 20624/PNM/2016; *S. lanzai*: 14382/PNM/2015, 12273/PNM/2015; *P. raffonei*: 4602/PNM/2017).

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Author Contributions

G.F.F., E.L., F.A. and R.M. conceived the study; G.F.F., B.B., A.M., M.M., E.L., C.C., E.L.P., V.N., I.S.R., A.U., M.A.C., D.S., S.S., G.S., R.P., F.A. and R.M. performed the research; G.F.F. prepared the images and wrote the first draft of the manuscript, with subsequent contribution by all the co-authors.

Additional Information

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