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Inducible defences and the underlying sources of information in tadpoles

Indukált védekezés és az alapjául szolgáló információforrások ebihalaknál

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”There is a useless discussion: making a difference between fundamental science and applied science. In my eyes there is good science and bad science. … We have to do good research and innovative research. Whether it is a bit more applied or less, it doesn’t matter. Somewhere down the road something will come out which is usable for whatever: social, commercial or something else.”

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1. General introduction

1.1. Phenotypic plasticity and inducible defences

Phenotypic plasticity is the ability of a given phenotype to produce alternative phenotypes depending on the environment (Bradshaw 1965; Stearns 1989). Some consider phenotypic changes as plasticity only if developmental trajectory shifts are involved, while others accept a much wider definition where plasticity includes also changes in much more labile traits, such as short-term alterations in behaviour or physiology, or even gene regulatory processes which do not necessarily manifest in measurable phenotypic changes (DeWitt and Scheiner 2004). The phenotypic patterns arising from plasticity can be continuous or discontinuous, and the changes can be reversible or irreversible (Piersma and Drent 2003; David et al. 2004). Another important distinction between various forms of plasticity is whether they result from active or passive processes (Smith-Gill 1983; Doughty and Reznick 2004). In the former case, which is usually referred to as adaptive plasticity, individuals are genetically programmed to use (or are able to learn using) environmental cues which trigger alternative responses (e.g., choice of temperature-dependent foraging strategies). In the latter case, phenotypic variation is merely a reflection of environmental variation in the phenotype (e.g., temperature-dependent rate of food-intake). Both types of plasticity have a genetic basis and can therefore evolve as any other trait (Bradshaw 1965; Pigliucci 2001; Sommer 2020).

Adaptive plasticity is a result of selection where the induced phenotype usually delivers a fitness advantage in the presence of the ecological agent inducing the response, while in its absence the costs of expressing the response prevent genetic fixation (Stearns 1989; Harvell 1990). At the same time, the evolution of adaptive plasticity is promoted by high dispersal (Hollander 2008) and by high temporal and spatial variation (Hendry 2016). Once the circumstances are right, and there is sufficient genetic variation for selection to act upon (Via and Lande 1985), another basic prerequisite for the evolution of phenotypic plasticity is that reliable information about the environment has to be accessible and readable by the given individuals (Moran 1992; Getty 1996). However, even if the above criteria are met, costs of producing the induced phenotype, costs of plasticity itself, as well as limits to plasticity can prevent the evolution of plasticity or result in non-adaptive and even maladaptive phenotypic responses (DeWitt et al. 1998; Callahan et al. 2008; Murren et al. 2015).

The importance of phenotypic plasticity lies with its immense effects on ecological and evolutionary processes (Tollrian and Harvell 1999a; Pigliucci 2001; DeWitt and Scheiner 2004). Plastic responses to the environment include changes in physiology, behaviour, morphology, growth and life history. Phenotypic plasticity can manifest during the lifetime of responding individuals (e.g., Van Buskirk and McCollum 2000; Young et al. 2003; Tollrian et al. 2015) or it can become expressed in subsequent generations (Agrawal et al. 1999). Through these multifarious changes, phenotypic plasticity can affect direct and indirect interactions among individuals and their environments and, ultimately, it can influence population dynamics and ecosystem functioning (Lima and Dill 1990; Miner et al. 2005; Fischer et al. 2014; Hendry 2016). By altering ecological interactions and enabling populations to persist under suboptimal or changing conditions, phenotypic plasticity is of fundamental importance for the maintenance of high biodiversity (Schmitz 2003; Kovach-Orr and Fussmann 2013; Hendry 2016) and can provide the basis for adaptive evolution and speciation (West-Eberhard 1989, 2003; Ghalambor et al. 2007; Pfennig et al. 2010). However, it remains debated when phenotypic plasticity hinders evolution by masking genetic variation (‘Bogert-effect’) and when it facilitates adaptive evolution by allowing populations to persist in widely differing conditions, thereby exposing them to different selection regimes (‘Baldwin-effect’), where plastic changes are followed by genetic changes in the same direction (via ‘genetic accommodation’ and ‘genetic assimilation’;

Hendry 2016; Levis and Pfennig 2016; Vinton et al. 2022). The contribution of phenotypic plasticity towards genetic evolution is perhaps most plausible if we consider that the ability of individuals to cope with their environment depends on many traits, some of which are plastic, while others are canalized (Carroll et al. 1997; Parsons and Robinson 2006). When exposed to a drastically altered environment (as in case of invasions), adaptive plasticity in some traits will enhance population persistence and will thereby allow selection to act on other traits that deliver maladaptive responses or are canalized (Reznick and Ghalambor 2001; Ghalambor et al. 2007; Losos et al. 2004). Although relevant evidence is scarce, evolutionary transitions between constitutive and induced defences have been observed in both directions (Thaler and Karban 1997; Heil et al. 2004; Campbell and Kessler 2013).

One type of phenotypic plasticity is when individuals adjust their phenotype to counter threats posed by natural enemies. These phenotypic changes are generally called inducible defences and have evolved to diminish the malign effects of predators, competitors and parasites (Tollrian and Harvell 1999a). Inducible defences have been documented in bacteria (e.g., Rong et al. 2019) and in unicellular eukaryotes (Kuhlmann et al. 1999), and are known to be widespread in multicellular organisms, including plants, fungi, and animals (Doughty and Reznick 2004; Künzler 2018; Wilkinson et al. 2019). The most obvious manifestations of inducible defences are the production of morphological defences, such as spines, thickened shells or altered coloration, the expression of altered behaviour, such as lowered activity or spatial avoidance of threats, or changes in life history, such as shifts in rates of growth or development (Tollrian and Harvell 1999a). Just as in case of phenotypically plastic changes in general, induced defences are expected to be carefully adjusted, so that the benefits are maximized and costs are minimized (Harvell 1990; Houston et al. 1993). The adaptive value of these adjustments critically depends on the availability of reliable and specific cues indicating the type and acute dangerousness of enemies present in the environment (Moran 1992). It is worth noting that the expression of inducible defences can have multifarious consequences, reaching way beyond the interaction between predators and prey, as it can also influence sexual ornamentation and mate choice, and, hence, the process and outcome of sexual selection (Price 2006; Cornwallis and Uller 2010; Ingleby et al. 2010; Frommen et al. 2022), thereby potentially promoting speciation (West-Eberhard 2003).

Larval anuran amphibians and their predators form a relatively well-studied system of enemy recognition and resulting phenotypic adjustments. Tadpoles are known to be able to adjust their defences to the type, density and recent feeding history of predators that are present in their environment (Laurila et al. 1998; Relyea 2001a; Van Buskirk and Arioli 2002; Schoeppner and Relyea 2005). The strength of induced defences has also been related to the dangerousness of predators (e.g., Kusch 1995; Peckarsky 1996; Teplitsky et al. 2004), but this relationship usually remained speculative. The induced defences can deliver survival benefits, irrespective of their generalized (McCollum and Van Buskirk 1996; Laurila et al. 2006) or predator-specific nature (Kishida and Nishimura 2005). At the same time, however, the expression of inducible defences can also incur costs in the form of decreased growth, development rate or fecundity (Van Buskirk 2000; Hoverman et al. 2005; Steiner 2007). Because costs tend to be weak (Steiner 2007) and do not necessarily manifest in all environments, in all (measured) traits, and simultaneously with the induced defence (Van Buskirk and Saxer 2001), they are difficult to detect and often remain elusive (Tollrian and Harvell 1999b). Another factor complicating the study of inducible defences and their consequences is that antipredator-responses of tadpoles can be influenced by several biotic and abiotic environmental factors, including conspecific density, pH or anthropogenic pollution (Bridges 1999; Teplitsky et al. 2007; Van Buskirk et al. 2011; Mikó et al. 2017). Finally, the expression of inducible defences and, thereby, also their detectability, can depend on the

intrinsic state of individuals, such as body size, development state, infection status or hunger level (Lefcort and Eiger 1993; Hoverman et al. 2005; Fraker 2008; Kurali et al. 2018).

1.2. Predator recognition and the underlying sources of information

Responding appropriately to the threat posed by predators is of fundamental importance for individual fitness because failing to do so likely results in death (Sih 1980; Lima and Dill 1990). Knowing what cues prey animals use to detect predators and to adjust the expression of their inducible defences can bring us closer to understanding the underlying mechanisms and the quality, magnitude and, ultimately, the adaptive value of induced responses. For example, in the case of chemical cues the traditional classification lists (1) damage-released cues (cues released passively from injured prey tissue; Chivers and Smith 1998), (2) no-cost disturbance signals (general prey metabolites released at an increased rate in response to predators; Kiesecker et al. 1999), (3) alarm pheromones (special disturbance cues that are costly to produce and are released by prey upon predator attack; Fraker et al. 2009), (4) digestion-released cues (constituents of prey tissue that are released via digestion by the predator; LaFiandra and Babbitt 2004), and (5) kairomones (cues released by the predator unrelated to its recent feeding history; Petranka and Hayes 1998). Kairomones are often referred to as direct cues, whereas cues originating from disturbed, attacked or digested prey are often referred to as indirect cues.

Prey often rely on predator-borne cues to modulate the type of their response, while they take advantage of prey-borne cues to adjust its intensity (Kishida and Nishimura 2005; Teplitsky et al. 2005; Wilson et al. 2005; Schoeppner and Relyea 2008). The use of predator-borne cues when adjusting the type of responses is likely adaptive because predators can vary widely in their activity profile, microhabitat preferences, attack modes or capture mechanisms, consequently, different types of responses may be effective against different predators. Adjusting the intensity of responses to prey-borne cues also makes evolutionary sense because in most cases the concentration / frequency of alarm signals should reliably indicate predation risk. Similarly, digestion-released cues may inform prey about the feeding history of predators, while pre-consumption prey-borne cues could provide information about the current feeding activity of predators. It is important to note that different types of cues in isolation can induce antipredator responses (Petranka and Hayes 1998; Fraker et al. 2009), but usually the simultaneous presence of various cues is necessary to trigger the development of the full suite and magnitude of induced defences (Van Buskirk and Arioli 2002; Schoeppner and Relyea 2005, 2009; Richardson 2006). This is plausible considering the examples that prey individuals focussing only on prey-borne pre-consumption cues (‘alarm pheromones’) would not detect predators that have not fed recently, or, in the other extreme, prey making use of only predator-borne cues may pay unnecessarily high costs of mounting a full-intensity response when the predator feeds on alternative prey. A few studies also established a relationship between the magnitude of phenotypic responses and the dangerousness of predators (e.g., Relyea 2001b; Paper 1), which is most likely detected using a combination of cues of different origins. Finally, the information conveyed by different types of cues can interact in synergistic, complementary or conflicting ways (see Paper 2). For example, a given concentration of damage-released cues may indicate highly differing risks of predation in the presence of predators that chew their prey or swallow it whole. Also, although a high concentration of prey-borne cues normally induces lowered activity, it may pay to increase activity and thereby enhance growth when exposed to gape-limited predators (Urban 2007a,b). However, how important such interactions are between the information delivered by different types of cues is very little known.

Knowing which sensory modalities may be involved in predator recognition can also contribute to our understanding of inducible defences. Relevant cues can be of visual, mechanical, chemical, thermal, electric or acoustic nature, where the relative importance of different sensory modes used is mainly determined by the interplay between the physical characteristics of the environment, the reliability and propagation speed of cues, the ecological traits of the interacting organisms themselves and by the distance between actors (Tollrian and Harvell 1999b; Weissburg et al. 2014). For example, aquatic animals inhabiting turbid ephemeral waters mainly rely on chemical cues to adjust their defences to predators, especially if these adopt sit-and-wait foraging strategies (Kats and Dill 1998; Tollrian and Harvell 1999b; Brönmark and Hansson 2000; see Paper 3). On the other hand, highly mobile prey animals facing fast-moving predators primarily use visual or acoustic cues to detect their enemies, where the primary sensory modality depends on the visual and acoustic transmittance and noise characterizing their environment (Endler 1993; Carr and Lima 2010; Fleishman and Pallus 2010). However, animals relying primarily on chemical cues for predator detection may also heavily rely on visual, acoustic or mechanic cues to avoid actual attacks by predators, and, vice versa, animals that mainly identify predators visually may sense the approach of enemies via olfaction long before the predator becomes visible, if the conditions are right. It also has to be noted that predator recognition may rely on learning (Gonzalo et al. 2007; Fraker 2009; Chivers and Ferrari 2013), but it may also have an innate basis (Petranka and Hayes 1998; Schoeppner and Relyea 2005).

In summary, cues of various origins and modalities may be sensed by prey simultaneously, and the information conveyed by these cues may interactively determine the quality and intensity of plastic antipredator responses. Ambiguities in terminology and differences in its use have hampered advance in this extensively studied field so that more exact and more uniformly used definitions would be needed (see Paper 4). It also has to be recognized that predators and prey are in most cases in a highly dynamic evolutionary arms race (Dawkins and Krebs 1979), where one or the other may temporarily or locally gain the upper hand. For example, prey evolving to become toxic may be safe and do not need to respond to predators, but only until these overcome prey toxicity either via behavioural or physiological adaptations (Holding et al. 2016; Bucciarelli et al. 2022). Similarly, when prey face novel predators, such as in case of biological invasions, prey may not be able to recognize predators or the responses they give may not be effective (Cox and Lima 2006; Banks and Dickman 2007; see Paper 5), which can result in drastic prey vulnerability to predation (Cruz et al. 2006; Arribas et al. 2014). Anyhow, if there is sufficient genetic variation underlying the prey animals' ability to sense the predator, predator recognition will evolve and the advantage of the predator will diminish over time.

Anuran larvae mostly rely on chemical cues for predator detection (e.g., Kiesecker et al. 1996; Laurila 2000; Benard 2006), partly because their habitat is often characterized by turbid water and dense vegetation (Stauffer and Semlitsch 1993; Kiesecker et al. 1996; Jowers et al. 2006; Parris et al. 2006; Saidapur et al. 2009), partly because tadpoles are near-sighted (Hoff et al. 1999). Nonetheless, vision does play a role in intraspecific interactions among tadpoles (Rot-Nikcevic et al. 2005; Gouchie et al. 2008) and there is some evidence that they also use visual cues in predator detection (Jowers et al. 2006; Parris et al., 2006). Anuran larvae respond to direct mechanical stimulation (e.g., Rot-Nikcevic et al. 2005), and a functional lateral line system enables them to sense water movements (Lannoo 1999; Simmons et al. 2004; Schmidt et al. 2011), so that they may use hydraulic cues to detect predators, but relevant studies are scarce (Stauffer and Semlitsch 1993). Tadpoles exhibit a functional inner ear (Lannoo 1999) and some species exhibit intraspecific acoustic communication (Natale et al. 2011; Reeve et al. 2011), but if they use acoustic cues in predator detection is unknown. Anurans lack electroreceptors and are therefore unable to sense electric cues (Lannoo 1999). Despite a large

body of relevant research, it has remained largely unknown to what extent tadpoles use the different sensory modalities, what sources of cues they rely on within sensory modalities, and how they integrate the wealth of acquired information to adjust their induced defences.

From a methodological point of view, there are two conceptually differing experimental approaches to studying predator recognition and the resulting induced defences. Many studies expose prey to cues indicating the presence of predators, while predators are prevented from harming focal prey individuals (i.e., often by constraining predators in some sort of cage). This setup is most suitable for determining whether prey recognize predators, for examining various responses of prey and for assessing associated costs, but does not allow for concluding on survival benefits of induced defences. The other approach is to expose prey to free-ranging predators, where phenotypic changes, survival and costs of expressing defences can be assessed under more natural conditions, but such studies do not allow for discerning between induced responses, thinning and selection by predators (Van Buskirk and Yurewicz 1998; Relyea 2002). To scrutinize the entire series of predator recognition, induced defences, survival benefits and costs, the ideal solution is to combine these two approaches, while measuring several characteristics of larvae and metamorphs to increase the probability of including the most important traits (Relyea 2003).

1.3. Inducibility of chemical defence

Phenotypic plasticity is ubiquitous in living organisms and there is a large body of evidence for its manifestation in various life history traits, including morphology, behaviour and physiology (Tollrian and Harvell 1999a), while phenotypic plasticity in chemical defences has remained severely understudied in many taxa. Plant chemical defences are among the best developed examples. It has long been known that individuals of many species can produce toxic substances in response to herbivores, these chemicals are costly to synthesize, and, therefore, plants only produce them when they are attacked (Cipollini et al. 2003; Heil 2010). Such induced defences are even exploited in agriculture to “immunize” plants against pests (e.g., Karban et al. 1997; Kessler and Baldwin 2004). However, biologists have largely overlooked animals in this respect, even though plenty of species use chemical defences and there is no reason why animals should not be able to produce toxic substances facultatively (see Paper 6).

In animals, toxin production can vary between life stages and among populations, but this has been tentatively attributed to genetically fixed adaptations to predictable temporal and spatial differences in predation pressure rather than to phenotypic plasticity (Kubanek et al. 2002; Fordyce et al. 2006; Hayes et al. 2009). Before we embarked on studying inducibility of chemical defence in animals, plastic responses in toxin production to predators have only been demonstrated in a few taxa of lower animals (for a review see Pohnert 2004), whereas in vertebrates, only two studies had provided suggestive evidence for its presence (Benard and Fordyce 2003; Hagman et al. 2009).

Toxins can not only be produced against predators, but also against competitors. This phenomenon has been intensely studied in algae (Sieg et al. 2011) and in plants (Metlen et al. 2009) and is called allelopathy (Whittaker and Feeny 1971; Rice 1974; Reigosa et al. 2006). Some lower animals and even some vertebrates are also known to contain or release chemicals that can negatively affect growth and survival of competitors (Jackson and Buss 1975; Petranka 1995; Kubanek et al. 2002; Crossland and Shine 2012). However, whether the production of these allelopathic chemicals is plastically adjusted to the abundance of competitors also in animals is little known.

Induced defence against pathogens is perhaps the most intensely studied area within the field of phenotypic plasticity due to its immediate relevance for human medicine. The immune

system responds to pathogens in a plastic and inducible manner (Frost 1999). In some taxa toxins produced by skin glands can contribute to the immune system (Nicolas and Mor 1995; Zasloff 2002; Rinaldi 2002). Nonetheless, few studies have tested whether the production of these toxins are modulated according to the presence, diversity or quantity of pathogens (Miele et al. 1998; Simmaco et al. 1998; Mangoni et al. 2001; Woodhams et al. 2012).

In anuran tadpoles, phenotypic plasticity is a widely studied and well established phenomenon, but despite the presumable importance of poison gland secretions for survival, demography and evolutionary processes, and the general assumption that skin toxins are costly to synthesise (Daly et al. 1997a,b; Wells 2007), there is only very limited information in amphibians on plasticity in this trait. It is known that several peptide, amine and steroid compounds of skin secretions can be toxic and, thus, may constitute effective defences against predators (for a review see Toledo and Jared 1995). In some species already tadpoles produce these toxins (Whittaker and Feeny 1971; Toledo and Jared 1995; Mebs et al. 2007; Hayes et al. 2009; Üveges et al. 2017) and tadpoles of these species are indeed avoided by some predators (Kruse and Stone 1984; Reading 1990; Peterson and Blaustein 1992; Üveges et al. 2019). Nonetheless, before our seminal paper (see Paper 7), there had been only a few published attempts at testing for the existence of adaptive predator-induced changes in the chemical defences of amphibians, and the results these delivered were equivocal (Benard and Fordyce 2003; Hagman et al. 2009; Bucciarelli et al. 2017; Üveges et al. 2017, 2019). Besides predators, inter- and intraspecific competition can also induce a wide range of life-history changes in amphibians (reviewed in Alford 1999), but reliable evidence that chemicals produced by tadpoles play a role in interference competition is scarce (Wells 2007; but see Crossland and Shine 2012), and no study had documented altered toxin production as a response to competition before us (see Papers 8 & 9). Some of the substances produced by poison glands located in the skin of amphibians exhibit activity towards bacteria, fungi, and viruses (Erspamer 1994; Rollins-Smith et al. 2005; Mangoni et al. 2008), and thereby contribute to the defences against pathogens (Nicolas and Mor 1995; Zasloff 2002; Rinaldi 2002). Only four studies (Miele et al. 1998; Simmaco et al. 1998; Mangoni et al. 2001; Woodhams et al. 2012) had investigated induced chemical defences as a response to pathogens in amphibians before us (see Paper 10), and these delivered evidence for an increased synthesis of chemical defences upon exposure to pathogens. However, these studies used adult frogs and nothing was known about similar responses in larvae. All in all, evidence for inducible responses in chemical defences of anuran amphibians was extremely scarce when we embarked on studying this phenomenon.

2. Aims and structure of the thesis

The dissertation contains two large parts, both are based on five papers. The first part concentrates on the sources of information used by anuran larvae to adjust their fine-tuned defensive responses to predators. The second part investigates the inducibility of chemical defences which presumably evolved to attenuate malign effects of environmental threats.

2.1. Predator recognition and the underlying sources of information

Paper 1—We investigated how the dangerousness of predators affected the strength of phenotypic responses and how these translated into benefits and costs of induced defences. We performed an outdoor mesocosm-based study where we raised *R. dalmatina* tadpoles in the presence of free-ranging predators or in the presence of caged predators followed by exposure of predator-naive and predator-experienced tadpoles to free-ranging predators. We used four predators: a leech (*Haemopis sanguisuga*), a water scorpion (*Nepa* sp.), larvae of a dragonfly (*A. cyanea*) and a newt (*L. vulgaris*), assessed their dangerousness and evaluated costs and benefits of responses they induced in *R. dalmatina* tadpoles in terms of survival and several life history traits.

Bibliographic data of the underlying publication:
Hettyey A, Vincze K, Zsarnóczai S, Hoi H, Laurila A (2011): Costs and benefits of defences induced by predators differing in dangerousness. *Journal of Evolutionary Biology*, 24: 1007–1019. (JIF_{2011} = 3.28, D1, N of citations / independent citations: 40 / 28)

Paper 2—We tested how predator species, acute predation risk, the types of chemical cues available as well as their interactions influenced the extent and quality of induced defences. We performed an outdoor experiment where we reared agile frog (*Rana dalmatina*) tadpoles in the presence of caged predators, a newt (*Lissotriton vulgaris*, formerly *Triturus vulgaris*) or larvae of a dragonfly (*Aeshna cyanea*). To manipulate acute predation risk we fed predators one or three tadpoles every other day. To provide different types of prey-borne cues to focal tadpoles, we fed predators outside rearing tanks and placed back predators into the tanks either after washing (to allow only for the presence of digestion-released cues) or along with the water containing remnants of the prey (to allow for the presence of all types of prey-borne cues).

Bibliographic data of the underlying publication:
Hettyey A, Zsarnóczai S, Vincze K, Hoi H, Laurila A (2010): Interactions between the information content of different chemical cues affect induced defences in tadpoles. *Oikos*, 119: 1814–1822. (JIF_{2010} = 3.39, D1, N of citations / independent citations: 34 / 26)

Paper 3—We examined what sources of information anuran larvae use for predator detection besides chemical cues. In a laboratory-based study we assessed behavioural responses of common frog (*Rana temporaria*) tadpoles to chemical, visual, acoustic, and hydraulic cues originating from a dragonfly larva (*A. cyanea*) and a fish (*Gasterosteus aculeatus*). We presented predators to tadpoles in small experimental containers divided by a net (assumed to transmit all cues to focal tadpoles), transparent Plexiglas (assumed to transmit visual cues but blocking chemical, hydraulic, and possibly acoustic cues), or an opaque and thin, freely vibrating polyethylene foil (assumed to transmit acoustic and hydraulic cues, but blocking chemical and visual cues).

Bibliographic data of the underlying publication:
Hettyey A, Rölli F, Thürlimann N, Zürcher A-C, Van Buskirk J (2012) Visual cues contribute to predator detection in anuran larvae. *Biological Journal of the Linnean Society*, 106: 820–827. (JIF_{2012} = 2.41, Q1, N of citations / independent citations: 48 / 45)

Paper 4—We scrutinized how important chemical cues of various origins are for the adjustment of anti-predator defences. In an outdoor mesocosm-based experiment we reared tadpoles of the common frog (*R. temporaria*) in the presence of caged dragonfly larvae (*A. cyanea*). We fed dragonflies outside of tadpole rearing containers with different types and quantities of prey and placed back predators either after washing or along with the water containing remnants of the prey. Predator food contained *Chironomus* midge larvae, *Bufo bufo* tadpoles, *Rana arvalis* tadpoles, or *R. temporaria* tadpoles. We also used starved predators and exposed focal tadpoles to homogenized conspecifics.

Bibliographic data of the underlying publication:
Hettyey A, Tóth Z, Thonhauser KE, Frommen JG, Penn DJ, Van Buskirk J (2015): The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles. *Oecologia*, 179: 699–710. (JIF_{2015} = 2.9, D1, N of citations / independent citations: 80 / 65)

Paper 5—We assessed to what extent the previously investigated mechanisms of predator recognition allow prey to detect invasive alien predators despite the lack of a shared evolutionary history. In a laboratory-based experiment we tested whether predator-naive tadpoles of the agile frog (*R. dalmatina*) displayed antipredator behaviour when exposed to chemical cues produced by native, invasive (established or recent) or allopatric fishes (four predatory perciforms, four predatory siluriforms, and two herbivorous cypriniforms). We further investigated whether the tadpoles' population origin influenced their predator-detection ability by using tadpoles from both fishless hill-ponds and from fish-infested floodplain populations. We also aimed to evaluate to what extent the ability of tadpoles to recognize potential predators depended on the recent feeding history by feeding predators either with bloodworms (larval *Chironomus* sp.) or with *R. dalmatina* tadpoles. We reared focal tadpoles in outdoor mesocosms, exposed them to stimulus water collected from fish tanks in small plastic dishpans individually and compared their behaviour before and after stimulus addition.

Bibliographic data of the underlying publication:
Hettyey A, Thonhauser KE, Bókony V, Penn DJ, Hoi H, Griggio M (2016): Naive tadpoles do not recognize recent invasive predatory fishes as dangerous. *Ecology*, 97: 2975–2985. (JIF_{2016} = 4.81, D1, N of citations / independent citations: 21 / 18)

2.2. Inducibility of chemical defence

Paper 6—We summarized what was documented in the literature about inducible chemical defences in animals. We concentrated on responses to predators, parasites, and competitors, and pointed out large gaps of knowledge in the field.

Bibliographic data of the underlying publication:
Hettyey A, Tóth Z, Van Buskirk J (2014): Inducible chemical defences in animals. *Oikos*, 123: 1025–1028. (JIF_{2014} = 3.44, D1, N of citations / independent citations: 19 / 8)

Paper 7—We tested for inducible changes in the chemical defence of a vertebrate upon exposure to predators. In a laboratory-based experiment we reared larval common toads (*B. bufo*) originating from three permanent and three temporary ponds. We simulated the presence of one of three predators by adding to tadpole rearing containers stimulus water collected from tanks holding dragonfly larvae (*Anax imperator*), newts (*L. vulgaris*) or fish (*Perca fluviatilis*). We fed predators with a mixture of *Tubifex* worms and *R. dalmatina* tadpoles and exposed focal tadpoles also to a homogenate of conspecifics. Controls received clear water. In a previous

experiment we had shown that already young *B. bufo* larvae are capable of producing bufadienolide toxins. We preserved focal tadpoles 20 days after start of the experiment and identified and quantified bufadienolide compounds using high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS).

Bibliographic data of the underlying publication:
Hettyey A, Üveges B, Móricz ÁM, Drahos L, Capon RJ, Van Buskirk J, Tóth Z, Bókony V (2019): Predator-induced changes in the chemical defence of a vertebrate. *Journal of Animal Ecology*, 88: 1925–1935. (JIF_{2019} = 4.55, D1, N of citations / independent citations: 14 / 8)

We published five closely related papers which are not detailed in the dissertation: Kurali et al. 2016 *Biological Journal of the Linnean Society*; Üveges et al. 2017 *BMC Evolutionary Biology*; Tóth et al. 2019 *Journal of Chemical Ecology*; Üveges et al. 2019 *Ecology and Evolution;* Üveges et al. 2023 *Integrative Organismal Biology*.

Paper 8—We examined the possibility that competitors may also induce changes in the chemical defence of vertebrates and that these alterations in toxin production may have negative consequences on competitors via allelopathy. In a previous survey performed on natural populations we found a positive correlation between competitor density and toxin content of *B. bufo* tadpoles. We therefore performed a field based experiment in microcosms where we kept *B. bufo* tadpoles at four different densities with or without admixing various numbers of *R. dalmatina* tadpoles. After three weeks of treatment we preserved *B. bufo* tadpoles for the analysis of bufadienolide content using HPLC-DAD-MS and assessed mortality, growth and development of *R. dalmatina* tadpoles to test for signs of allelopathy.

Bibliographic data of the underlying publication:
Bókony V, Üveges B, Móricz ÁM, Hettyey A (2018): Competition induces increased toxin production in toad larvae without allelopathic effects on heterospecific tadpoles. *Functional Ecology*, 32: 667–675. (JIF_{2018} = 5.04, D1, N of citations / independent citations: 20 / 10)

We published one closely related paper which is not detailed in the dissertation: Bókony et al. 2016 *Journal of Chemical Ecology*.

Paper 9—We investigated how inducible chemical defences are adjusted to the simultaneous presence of predators and high competitor densities. Predator-induced defences are generally predicted to be weaker at high conspecific densities due to risk-dilution and the costs of producing and maintaining defences, but in the special case when chemical defences are also increasingly produced in response to high competitor densities, it was difficult to predict the joint effects of predator presence and varying conspecific densities. We performed an experiment in outdoor microcosms where we raised *B. bufo* tadpoles at three densities in the presence or absence of chemical cues on predation risk. We simulated predation risk by transferring water from tanks holding predatory fish (*P. fluviatilis*) into microcosms holding focal tadpoles and also added a homogenate of conspecifics. Predators were fed with a mixture of *R. dalmatina* and *B. bufo* tadpoles. After two weeks of treatment we preserved focal tadpoles and analysed their bufadienolide content using HPLC-DAD-MS.

Bibliographic data of the underlying publication:
Üveges B, Basson AC, Móricz ÁM, Bókony V, Hettyey A (2021): Chemical defence effective against multiple enemies: Does the response to conspecifics alleviate the response to predators? *Functional Ecology*, 35: 2294–2304. (JIF_{2021} = 5.84, D1, N of citations / independent citations: 5 / 3)

Paper 10—We assessed whether the synthesis of defensive chemicals in the skin is enhanced or suppressed upon exposure to obligate pathogens. In a previous correlative study of natural populations we found a relationship between toxin production in *B. bufo* tadpoles and the

bacterial community structure of their aquatic habitat while in an experimental study we found no effect of antibacterial treatment of the rearing water on toxin production. Here we exposed tadpoles of *B. bufo* and *R. dalmatina* throughout their larval development to an obligate pathogen, the chytrid *Batrachochytrium dendrobatidis*, a fungus causing severe amphibian population declines worldwide. We sampled individuals for their chemical defences in a late larval stage and two weeks after metamorphosis. The bufadienolides synthesized by *B. bufo* tadpoles and the Brevinins produced by *R. dalmatina* tadpoles both have antifungal properties. We measured bufadienolide content using HPLC-DAD-MS, Brevinin-1 DA quantities using nano-UHPLC-MS/MS and infection status and intensity using qPCR.

Bibliographic data of the underlying publication:

Ujszegi J, Ludányi K, Móricz ÁM, Krüzselyi D, Drahos L, Drexler T, Németh MZ, Vörös J, Garner TWJ, Hettyey A (2021): Exposure to *Batrachochytrium dendrobatidis* affects chemical defences in two anuran amphibians, *Rana dalmatina* and *Bufo bufo*. *BMC Ecology and Evolution*, 21: 135. (JIF_{2021} = 3.44, Q1, N of citations / independent citations: 14 / 5)

We published three closely related papers which are not detailed in the dissertation: Ujszegi et al. 2017 *Evolutionary Ecology*; Ujszegi et al. 2020 *Journal of Chemical Ecology*; Kásler et al. 2022 *Journal of Zoology*.

Technical note: The thesis is based on the above ten papers, several of which are supplemented by online appendices. Because these appendices are rather long and do not contain details of central importance regarding the topic of the dissertation, I only included the published main texts here. Supplementary materials are available electronically on the publishers' websites.

3. Papers

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Costs and benefits of defences induced by predators differing in dangerousness

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induced defence;
mortality rate;
phenotypic plasticity;
predation risk;
predator dangerousness;
tadpole.

Abstract

While theoretical studies predict that inducible defences should be fine-tuned according to the qualities of the predator, very few studies have investigated how dangerousness of predators, i.e. the rate at which predators kill prey individuals, affects the strength of phenotypic responses and resulting benefits and costs of induced defences. We performed a comprehensive study on fitness consequences of predator-induced responses by involving four predators (leech, water scorpion, dragonfly larva and newt), evaluating costs and benefits of responses, testing differences in dangerousness between predators and measuring responses in several life history traits of prey. We raised *Rana dalmatina* tadpoles in the presence of free-ranging predators, in the presence of caged predators, and exposed naive and experienced tadpoles to free-ranging predators. Tadpoles adjusted the intensities of their behavioural and morphological defences to predator dangerousness. Survival was lower in the nonlethal presence of the most dangerous predator, while we could not detect costs of induced defences at or after metamorphosis. When exposed to free-ranging predators, small, but not large, tadpoles benefited from exhibiting an induced phenotype in terms of elevated survival when compared to naive tadpoles, but we did not observe higher survival either in tadpoles exhibiting more extreme phenotypes or in tadpoles exposed to the type of predator they were raised with. These results indicate that while predator-induced defences can mirror dangerousness of predators, costs and benefits do not necessarily scale to the magnitude of plastic responses.

Introduction

Predator-induced responses are, within the limits of plasticity, predicted to be carefully fine-tuned to the environment and to the intrinsic state of the organism to maximize effectiveness of defences and minimize arising costs (Werner, 1986; DeWitt *et al.*, 1998; Lima & Bednekoff, 1999; Urban, 2007a). Induced defences have been shown to depend on the abundance of predators (Van Buskirk & Arioli, 2002), on the temporal and spatial vicinity of predators (Turner & Montgomery, 2003) on the amount and quality of prey eaten by the predators (Laurila *et al.*, 1998; Schoeppner & Relyea, 2005), or on the size of the predators (Kusch *et al.*, 2004), but also on the size (Fraker, 2008a), energetic state (Hoverman *et al.*, 2005; Fraker, 2008b) or experience (Turner *et al.*, 2006) of prey individuals. The type of predators present in the environment may be among the most important factors influencing defences, as predator species can differ in dangerousness, foraging mode or microhabitat use, and thus, appropriate responses should vary. Empirical studies have indeed delivered many examples of predator-specific responses (e.g. Relyea, 2001a; Bourdeau, 2009; Freeman *et al.*, 2009). The strength of responses has also been related to the dangerousness of predators, that is

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to the rate at which predators kill prey individuals (e.g. Kusch, 1995; Peckarsky, 1996; Teplitsky *et al.*, 2004), but this relationship has often remained speculative, either because dangerousness of predators was not estimated or because only two predators were used. Nonetheless, while some studies have evaluated if benefits are related to the magnitude of phenotypic responses induced by predators differing in dangerousness (e.g. Relyea, 2001b), we know of no similar study testing for a relationship between predator dangerousness and costs of responses.

Predator-induced plastic defences are predicted to evolve when induced defences enhance survival in the presence of enemies and defences are costly to develop or maintain (Harvell, 1990). There is a relatively large number of reports on survival benefits of induced defences (Tollrian & Harvell, 1999). Some investigations have observed generalized responses that lowered mortality in the presence of various predators (McCollum & Van Buskirk, 1996; Laurila *et al.*, 2006; Freeman, 2007), but predator-specific responses enhancing survival probabilities mostly in the presence of the predator that has induced the phenotype have also been documented (Kishida & Nishimura, 2005; Freeman, 2007; Hoverman & Relyea, 2009). Costs, such as a decreased growth, development rate or fecundity (Van Buskirk, 2000; Hoverman *et al.*, 2005; Steiner, 2007), have been proposed to arise from lowered activity (Lima, 1998), from allocation to morphological traits providing protection or enhancing escape ability (Tollrian, 1993; Johnson *et al.*, 2008), or the deleterious effects of the responses to the threat (Slos & Stoks, 2008). However, costs arising from the expression of inducible defences tend to be weak (Steiner, 2007). Also, costs do not necessarily appear in the measured traits, in all environments and simultaneously with the induced defence (Scheiner & Berrigan, 1998; Van Buskirk & Saxer, 2001). Consequently, costs of induced defences have often remained elusive, and detecting them can turn out to be a difficult task (Tollrian & Harvell, 1999). It is, however, important to note that costs may also disappear over evolutionary time, so that not finding a cost does not necessarily mean a contradiction between theory and empirical data (DeWitt *et al.*, 1998).

In this study, our aim was to relate costs and benefits of predator-induced defences to the dangerousness of different types of predators and to the magnitude of the plastic response. Studies using constrained predators are ideal for examining induced phenotypes, but they often do not test for survival benefits of induced defences. On the other hand, studies on the effects of free-ranging predators, where phenotypic changes and survival can be measured under more natural conditions, do not allow discerning between phenotypic effects of induction, thinning and selection by the predator (Van Buskirk & Yurewicz, 1998; Relyea, 2002). To clearly demonstrate survival benefits and costs of induced defences, it is necessary to integrate the two methodological approaches and use a combination of constrained and free-ranging predators. Consequently, we subjected anuran tadpoles, popular models of studies on predator-induced defences (Relyea, 2007), to three experiments: (i) By raising agile frog (*Rana dalmatina*) tadpoles in the presence of free-ranging predators, we estimated the relative dangerousness of the predator species used. (ii) By raising tadpoles in the nonlethal presence of caged predators, we tested the hypotheses that prey respond to different predator species with qualitatively or quantitatively varying induced defences. This experiment also allowed us to test the hypothesis that costs arising from the expression of plastic antipredator responses scale to the magnitude of the responses. By relating tadpole phenotypes developed in the presence of caged predators to relative predator dangerousness, we also tested the hypothesis that the magnitude of phenotypic responses and the costs arising from the expression of the responses scale to predator dangerousness. (iii) By exposing naive tadpoles and tadpoles exhibiting predator-induced phenotypes to free-ranging predators, we tested the hypotheses that the expression of antipredator responses results in benefits in the form of lowered probability of being captured by free-ranging predators and that induced defences are equally effective against all predators as opposed to prey showing the highest survival when facing the predator they were raised with. To provide a full picture on induced defences and, thus, to enhance the probability of observing the most important traits (Relyea, 2003), we examined a relatively large number of tadpole and metamorph characteristics.

Methods

Rana dalmatina breeds in a variety of water bodies, ranging from small ephemeral puddles to large permanent ponds and lakes, varying widely in predator regimes. In early April 2007, we collected 25 freshly laid egg clutches from a breeding site (280 m above sea level, 47°42′ N, 19°02′ E) located in the Pilis-Mountains, 30 km N of Budapest. We further captured at the same locality and at surrounding ponds the following predators: horse leech (*Haemopis sanguisuga*), water scorpion (*Nepa* sp., Hemiptera, Insecta), smooth newt (*Triturus vulgaris*) and dragonfly larva (*Aeshna cyanea*). Leeches feed on anuran eggs and young or injured tadpoles, water scorpions and dragonfly larvae mainly on tadpoles and newts on both eggs and tadpoles (Laurila *et al.*, 2002; Orizaola & Braña, 2003; A. Hettyey, personal observation). These predators are all present in the breeding pond where the eggs were taken from.

We performed two mesocosm experiments. We used 30 small and 30 large rotund tubs placed outdoors in an open field on the outskirts of Budapest belonging to the Plant Protection Institute of the Hungarian Academy of Sciences. Ten days before the start of the experiment,

small and large tubs were filled with tap water (small tubs: diameter = 74 cm, height = 30 cm, 100 L of water; large tubs: diameter = 83 cm, height = 71 cm, 250 L of water). Two days later, we added 7 g rabbit pellets, 150 g dried beech (*Fagus sylvatica*) leaves and 3 L pond water to small tubs and 10 g rabbit pellets, 200 g beech leaves and 3.5 L pond water to large tubs to provide nutrients and cover for tadpoles. We covered experimental units with mosquito nets to prevent colonization of artificial ponds by predators. Large tubs further received four 0.75 L transparent cups with mosquito net bottoms and covers as predator cages and four plastic egg-holding dishes (15 × 15 cm) with mosquito net bottom. Predator cages were hung into tubs. Egg-holding dishes were put afloat with the help of wooden sticks. Both types of subcompartments were partially submerged under water. This design allowed visual and chemical contact between anuran embryos and larvae and the predators. Two days before the start of the experiment, we assigned each tub to one of five treatments in a randomized spatial block design and added ad libitum fed predators to the tubs, whereas in the control treatment we left the predator cages empty. We started experiments on April 4 (day 0) by placing eggs into the tubs. For a timeline of the experiments, see Fig. 1.

Experiment 1 – free-ranging predators

This experiment was designed to estimate natural mortality rates and the relative dangerousness of predators. Small tubs received 20 eggs from each of 15 egg clutches (resulting in 300 eggs per tub) and contained two free-ranging predators of the same species at the start of the experiment. Initial densities were chosen to be high in this experiment to ensure that some tadpoles survive until the end in all treatments. Changing starting conditions (i.e. density) would probably have resulted in slightly different estimates of dangerousness, but we consider the obtained estimates useful for comparing predator dangerousness, especially as densities were within the range of densities readily observable in nature (A. Hettyey, personal observation). Treatments were replicated six times. We monitored survival by counting tadpoles on three intermediate occasions (18, 25 and 33 days after start of the experiment) and at termination (44 days after start), when the first tadpoles were approaching metamorphosis.

Experiment 2 – caged predators

With this experiment, we aimed to determine effects of predators on body size, shape, behaviour and survival of tadpoles, time until and mass at metamorphosis and escape ability of metamorphs, while controlling for density-dependent effects by keeping tadpole numbers constantly low. We placed three eggs from each of ten egg clutches into the four egg-holding dishes of each large tub (resulting in 120 eggs per tub) and one predator into each cage (resulting in four predators of the same species per tub). Treatments were replicated six times. Tubs holding empty cages served as controls. Caged predators were fed two *R. dalmatina* eggs and two tadpoles every other day during the first half of the experiment and two *R. dalmatina* tadpoles during the second half. Seven days after start of the experiment, when more than 90% of hatchlings had left the egg jelly in each tub, we released eleven haphazardly selected healthy tadpoles from each egg-holding dish (resulting in 44 tadpoles per tub) and removed the dishes. Tadpoles could swim around and forage in tubs, and predators could not reach them. Twelve and 27 days after hatching, we removed 11 tadpoles from each tub of each treatment and used them in the predation trials (experiment 3). Surviving tadpoles were not placed back into the large tubs they had been taken from. Thus, the decrease in density occurred simultaneously and at the same extent in all experimental populations of experiment 2 and mirrored decrease in density because of predation or pathogens under natural conditions.

We evaluated behaviour of tadpoles 16, 23, 30 and 37 days after hatching. In each tub, we counted the number of tadpoles swimming in the water column or feeding on the exposed surface of tub walls. Adding these counts together, we obtained the number of tadpoles visible. Thirty-three days after hatching, we caught a random sample of 10 tadpoles per tub, brought them to the laboratory, anaesthetized them with 0.02 g mL^{-1}

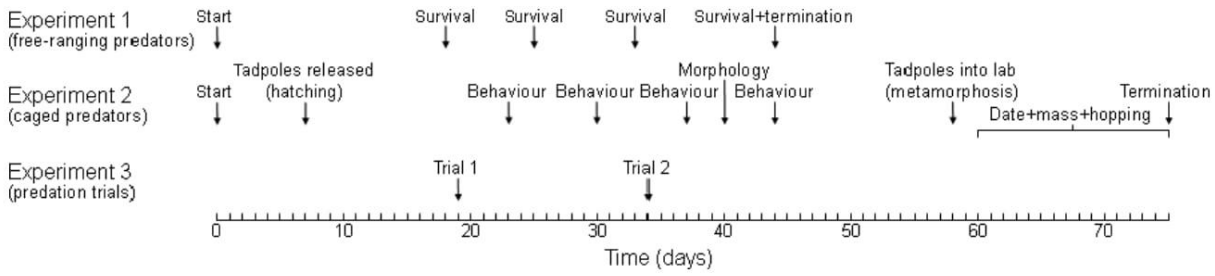

Fig. 1 Timeline of the three experiments.

MS-222 (tricaine, Sigma-Aldrich), photographed them and, after recovery, placed them back into tubs again. Recovery rates were very high (mortality was less than 1%). We later measured body length, tail length, maximum tail muscle depth and maximum tail fin depth using ImageTool 3.0 (UTHSCSA, San Antonio, Texas, USA). These parameters have previously been shown to be phenotypically plastic, and they have been suggested to be of importance for escaping ability (e.g. McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998).

On June 1 (51 days after hatching), when the first tadpoles approached metamorphosis (development stage 41, Gosner, 1960), we emptied outdoor tubs and transported all tadpoles to the laboratory. This was necessary as metamorphosing individuals may have been attacked by less developed tadpoles or may have drowned as they had no possibility for moving out of the water in the rearing tubs, but also because it was logistically not feasible to monitor tubs and transport metamorphosing individuals every day from the outdoor setting to the laboratory. In the laboratory, we maintained tadpoles individually in 0.5-L plastic boxes containing 0.2 L reconstituted soft water and fed them ad libitum with chopped and slightly boiled spinach. Tadpoles were kept at a 10 : 14 dark : light cycle and at a constant 21 °C. We checked metamorphosing individuals every day. As soon as forelimbs of a metamorph emerged (stage 42, Gosner, 1960), we noted the date of metamorphosis and measured mass (to the nearest mg) using an analytical balance (Mettler Toledo PL 303). After measurements, we placed individuals back into their boxes.

When an individual completed tail resorption (stage 45, Gosner, 1960), we measured its hopping ability using the method of Van Buskirk & Saxer (2001) to investigate whether the predator-induced phenotype developing during the tadpole stage affected hopping ability of froglets. Jumping performance was tested in a $50 \times 100 \times 40$ cm arena under laboratory conditions (21 °C). We placed froglets into the middle of the arena and recorded their movements from above using a Sony CyberShot DSC W-50 digital camera. We waited until froglets made at least eight hops. Usually, they hopped spontaneously, but when they did not, we touched them with a brush to induce an escape reaction. We later measured the three longest hops with MB-Ruler 4.0 (Iffezheim, Germany) and used the length of the longest hop in the analyses. When 99% of the tadpoles completed metamorphosis (day 75), we terminated the experiment. Froglets, predators and tadpoles were transported back to the Pilis-Mountains.

In one replicate an escaped dragonfly larva and in two replicates colonizing backswimmers (*Notonecta glauca*) decimated tadpoles. In one further replicate, we observed high tadpole mortality (only five metamorphs) for unknown reasons. These replicates were excluded from analyses that resulted in five replicates for the treatments containing predators and six replicates for the control.

Experiment 3 – predation trials

To assess how the predator-induced phenotypes affect survival, we performed predation trials. Twelve and 27 days after hatching, we caught 11 randomly selected tadpoles from each large tub in Experiment 2. We anaesthetized them with MS-222 and marked them according to the treatment they were taken from with a small incision on the tail fin. This method is used for marking tadpoles and does not largely affect swimming ability (Anholt *et al.*, 1998). To control for potential biases because of the placement of the incision, we varied its location between trials and treatments. Once tadpoles recovered, four marked individuals originating from each of the five treatments were placed into the small tubs used in Experiment 1 (resulting in 20 tadpoles in each tub). Tadpoles reared in the small tubs in Experiment 1 had been moved into 30-L plastic boxes the day before the predation trials and were placed back as soon as these were terminated 1 day later. Free-ranging predators remained in the small tubs and served as predators in the predation trials. On the first occasion (12 days after hatching), we used water scorpions, newts and dragonfly larvae as predators, but not leeches as we assumed that these do not prey on tadpoles (Laurila *et al.*, 2002). In the trials performed 27 days after hatching, we further excluded water scorpions as by that time tadpoles were so large that they could not be caught by these predators. Trials with each predator type were replicated six times. Predation trials were run for 24 h; remaining tadpoles were then removed from small tubs and stored in 10% formalin. We later determined number and origin of survivors using a stereomicroscope.

Statistical analyses

In Experiment 1, data on survival of embryos and tadpoles were not normally distributed and were strongly right-censored. Consequently, we compared survival among treatments using a Cox proportional hazards model (Cox regression) and handled ties using the Breslow method. We entered the four sampling dates as the time variable, event of death for each individual as the status variable, and tub identity and treatment as categorical covariates. We used simple contrasts and entered control treatments as the reference category.

In Experiment 2, where tadpoles were reared in the presence of caged predators, we first investigated if phenotypes systematically varied between treatments. We tested for treatment-dependent differences in behaviour of tadpoles with a multivariate repeated-measures general linear model (GLM), where we entered the ratio of swimming tadpoles and the ratio of tadpoles visible on the four sampling occasions as the dependent variables and treatment as a fixed factor. We calculated the ratios of swimming tadpoles as # swimming/# live tadpoles and ratios of tadpoles visible as # visible/# live tadpoles. We

estimated the number of live tadpoles using linear interpolation between initial and final tadpole numbers for each tub at each occasion. We analysed total tadpole length (body length + tail length) with a linear mixed-effects model (LMM) by entering total tadpole length as the dependent variable, treatment as a fixed factor and tub identity as a random factor. We analysed tadpole shape with a multivariate GLM by entering body length, tail length, maximum tail muscle depth and maximum tail fin depth as dependent variables and treatment as a fixed factor. We used tub averages in this analysis because data based on individuals could not be transformed to yield homogeneous variances. We present results from analyses not correcting for body size as size did not vary significantly between treatments (see Results). Entering body size into the model as a covariate, however, yielded qualitatively similar results. We analysed time until metamorphosis using a GLM with treatment as a fixed factor. We used tub averages in this analysis because data based on individuals could not be transformed to yield normally distributed model residuals. To relate the magnitude of phenotypic responses of tadpoles to predator dangerousness, we entered the average ratio of tadpoles that died in each treatment of Experiment 1 as a covariate and ratio of tadpoles visible, ratio of tadpoles swimming, body length, tail length, tail muscle depth, tail fin depth and time until metamorphosis as dependent variables into a multivariate GLM. This analysis was based on tub averages of measures taken at the morphology sampling (33 days after hatching) and at the third observation on behaviour (30 days after hatching) in Experiment 2.

Second, we searched for potential costs of induced defences in terms of lowered survival, smaller metamorph mass or lowered jumping performance. To investigate whether survival of tadpoles differed among treatments, we used generalized linear modeling (GZLM) procedures with binomial error distribution and logit-link function. Survival (dead or alive) was entered as the dependent variable, treatment as a categorical factor. We also entered tub identity nested within treatment as a categorical variable to control for the nonindependence of data on tadpoles in the same tub. To test for treatment-dependent differences in metamorph mass, we built a LMM with treatment as a fixed factor and tub identity as a random factor. As distances covered by the first three longest hops were strongly correlated within individuals (all pairwise Spearman's $R > 0.77$), we investigated jumping performance of metamorphs by entering the distance covered by the longest hop as the dependent variable, treatment as a fixed factor, tub identity as a random factor and metamorph mass as a covariate into a LMM. To relate dangerousness of predators estimated in Experiment 1 to survival, metamorph mass and jumping performance of metamorphs measured in Experiment 2, we entered survival, metamorph mass and jumping performance as dependent variables and dangerousness of predators as a covariate into a multivariate GLM. This and the following analyses were based on tub averages.

Third, to detect potential effects of phenotypic changes in tadpoles on their survival and on mass and jumping performance of metamorphs, we used a multivariate GLM with survival, metamorph mass and jumping performance as dependent variables, and ratio of tadpoles visible, ratio of tadpoles swimming, tail fin depth and time until metamorphosis as covariates. The covariates entered into the analysis were independent of each other (Pearson's correlations, all $P > 0.22$). Tail fin depth was measured at the morphology sampling (33 days after hatching); behavioural data refer to the third observation period (30 days after hatching).

We analysed the outcome of predation trials (Experiment 3) with GZLM procedures with binomial error distribution and logit-link function. Survival (dead or alive) was entered as the dependent variable; the type of free-ranging predator in the predation trials and the tadpole treatment were entered as categorical factors. We also entered tub identity nested within the type of free-ranging predators as a categorical variable to control for the nonindependence of data on tadpoles used in the same trials.

We included all two-way interactions into initial models and applied a backward stepwise removal procedure to avoid problems because of the inclusion of nonsignificant terms. We re-entered removed variables one by one to the final model to obtain relevant statistics. We fitted linear mixed models using the restricted maximum likelihood approach. All tests were two tailed. Statistics were calculated using SPSS 15.0 for Windows (Somers, New York, USA).

Results

Experiment 1 – free-ranging predators

Survival of embryos and tadpoles was significantly affected by the type of predator present (Cox regression; Wald = 366.3, d.f. = 4, $P < 0.001$; Table 1; Fig. 2). Pairwise comparisons showed that survival was significantly lower in all treatments containing a predator compared to control treatments (all $P < 0.001$) and that newts and

Table 1 Average risk ratios during the four sampling intervals in the treatments containing a predator when compared to the control treatment [RR = p(risk in treatment)/p(risk in control)] in Experiment 1 (free-ranging predators).

	Average risk ratio (relative to control)			
Treatment	Day 0–18	Day 19–25	Day 26–33	Day 34–44
Leech	1.44	1.56	1.30	1.38
Water scorpion	2.05	2.41	0.64	0.64
Newt	5.21	17.74	3.99	1.99
Dragonfly larva	5.64	19.46	5.64	7.55

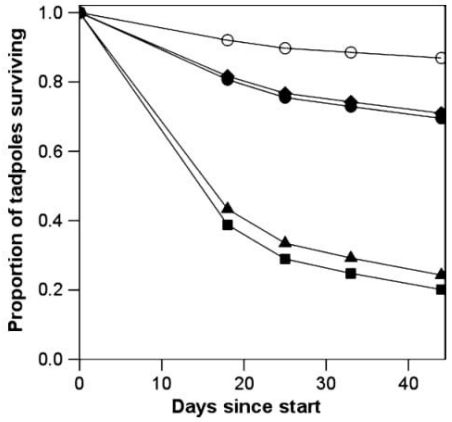

Fig. 2 Changes in the proportion of live tadpoles in Experiment 1 containing free-ranging predators. Treatments are symbolized by ○: control, ●: leech, ◆: water scorpion, ▲: newt, ■: dragonfly larva.

dragonfly larvae were more dangerous predators than leeches and water scorpions (all $P < 0.001$). However, we found no difference in dangerousness between newts and dragonfly larvae and between leeches and water scorpions (both $P > 0.16$). To determine how long the various predators affected tadpole survival, we gradually reduced the time span of the analyses on mortality rates to the last three, two and one monitoring interval. This revealed that in the presence of water scorpions mortality after the first monitoring (18 days after start – d 18) was not different from mortality measured in control treatments ($P = 0.9$). The effect of leeches diminished after the second monitoring (d 25; $P = 0.084$), whereas the effect of newts (Wald = 3.96, d.f. = 1, $P = 0.047$) and dragonfly larvae (Wald = 28.58, d.f. = 1, $P < 0.001$) remained significant even during the last interval (ending on d 44).

Experiment 2 – caged predators

Both the ratio of tadpoles visible and the ratio of tadpoles swimming were significantly affected by treatment and changed over time, but the interaction between treatment and sampling date was also significant (Table 2, Electronic Appendix). Consequent analyses revealed that, except for the ratio of tadpoles swimming on day 30, both measures of tadpole behaviour significantly varied between treatments on all four sampling occasions (Table 2). According to multivariate GLMs performed post hoc on the four sampling dates separately, a higher ratio of tadpoles was visible in the dragonfly than in the control treatment on day 16 ($P = 0.035$), whereas this difference had started to diminish before day 23 ($P = 0.098$) and on day 30 and 37, fewer tadpoles were visible in the dragonfly than in the control treatment (both $P < 0.001$; Fig. 3a). Other treatments did not significantly differ from the control at any sampling occasion (all $P > 0.15$). On day 16, the ratio of tadpoles

Table 2 Impact of predators on the behaviour of tadpoles, as shown by a multivariate repeated-measures general linear model on data from Experiment 2 (caged predators). Behaviour was sampled 16, 23, 30 and 37 days after hatching. The two measures of behaviour were ratio of tadpoles swimming (RTswimming) and ratio of tadpoles visible (RTvisible). We also present results of multivariate general linear models on the effect of treatment at the four sampling dates separately, because the interaction between treatment and sampling date was significant. Bold indicates statistical significance at $P \leq 0.05$.

	Multivariate tests							
Effect	d.f.	Wilk's λ	F	P	Dependent	d.f.	F	P
Repeated-measures GLM						Tests of between-subjects effects		
Treatment	8, 40	0.170	7.114	**< 0.001**	RTvisible	4, 21	15.178	**< 0.001**
					RTswimming	4, 21	3.798	**0.018**
						Tests of within-subjects effects		
Sampling date	6, 16	0.022	116.191	**< 0.001**	RTvisible	2.44	284.517	**< 0.001**
					RTswimming	1.93	70.422	**< 0.001**
Treatment × Sampling date	24, 57.03	0.052	3.162	**< 0.001**	RTvisible	9.78	9.548	**< 0.001**
					RTswimming	7.74	7.319	**< 0.001**
GLM – effect of treatment						Tests of between-subjects effects		
On day 16	8, 40	0.216	5.748	**< 0.001**	RTvisible	4, 21	3.751	**0.019**
					RTswimming	4, 21	16.828	**< 0.001**
On day 23	8, 40	0.241	5.189	**<0.001**	RTvisible	4, 21	6.137	**0.002**
					RTswimming	4, 21	8.848	**< 0.001**
On day 30	8, 40	0.452	2.437	**0.030**	RTvisible	4, 21	4.644	**0.008**
					RTswimming	4, 21	0.876	0.495
On day 37	8, 40	0.248	5.046	**< 0.001**	RTvisible	4, 21	6.733	**0.001**
					RTswimming	4, 21	7.101	**0.001**

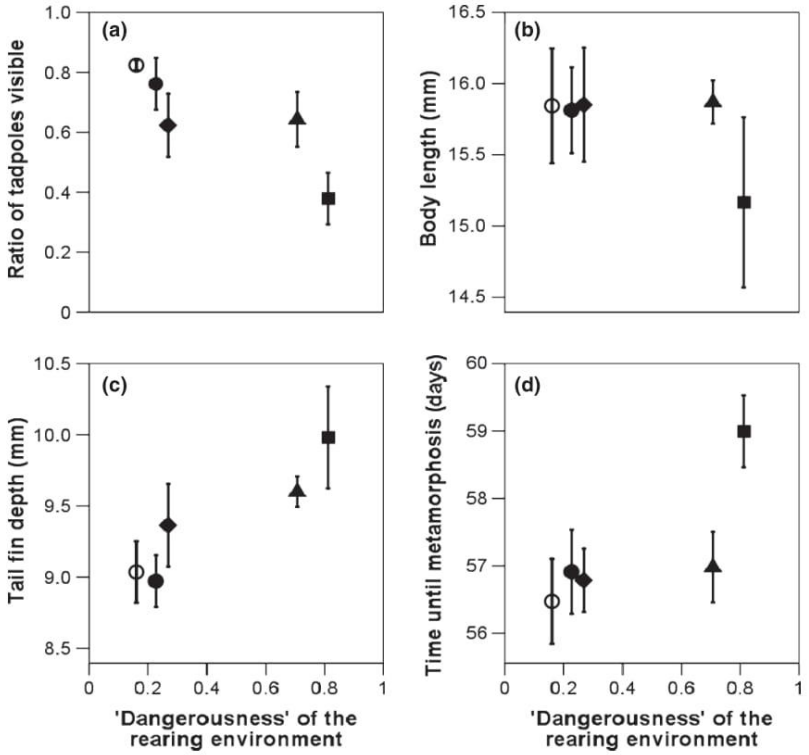

Fig. 3 Relationships between predator dangerousness and the magnitude of responses in behaviour, morphology and development. 'Dangerousness' was estimated by the average ratio of tadpoles that died in the treatments of Experiment 1 (free-ranging predators). Treatments are symbolized by ○: control, ●: leech, ◆: water scorpion, ▲: newt, ■: dragonfly larva. Ratio of tadpoles visible (a) refers to observations made 30 days after hatching. Body length (b) and tail fin depth (c) are measures taken 33 days after hatching in Experiment 2 (caged predators). Time until metamorphosis (d; from start of the experiment to reaching Gosner stage 42) was also estimated in Experiment 2. The figure is based on tub averages; means ± SE are indicated.

swimming was higher in the water scorpion, newt and dragonfly treatment than in the control and leech treatment (all $P < 0.003$). On day 23, only the dragonfly treatment differed from the control and leech treatment (both $P < 0.003$, all other $P > 0.09$), and on day 30, there were no differences among treatments at all (all $P = 1$). On day 37, however, a lower proportion of tadpoles were swimming in the leech, water scorpion and dragonfly than in the control treatment (all $P < 0.01$; newt-control: $P = 0.077$).

Total tadpole length did not differ between treatments (LMM; $F_{4,30.04} = 0.7$, $P = 0.6$). Body shape depended on treatment (GLM; Wilks' $\lambda = 0.11$, $F_{16,55.63} = 3.63$, $P < 0.001$). Tests of between-subjects effects revealed that tail fin depth significantly varied between treatments ($F_{4,21} = 2.89$, $P = 0.048$; Fig. 3c), whereas body length ($F_{4,21} = 0.56$, $P = 0.69$), tail length ($F_{4,21} = 0.27$, $P = 0.89$) and tail muscle depth ($F_{4,21} = 0.38$, $P = 0.82$) did not seem to be largely affected by treatments. Time until metamorphosis differed among treatments (GLM; $F_{4,21} = 3.09$, $P = 0.038$), with tadpoles in the dragonfly treatment taking longer until the start of metamorphosis than those in the control treatment (dragonfly – control: $P = 0.042$) and other treatments being intermediate (all other pairwise $P > 0.13$; Fig. 3d).

Several measures of phenotypic responses in tadpoles were in a close relationship with predator dangerousness (Table 3). Tests of between-subjects effects indicated negative relationships between dangerousness and the ratio of tadpoles visible (Fig. 3a), a positive relationship between dangerousness and tail fin depth (Fig. 3c) and time until metamorphosis (Fig. 3d), whereas there was no significant linear relationship between dangerousness of predators and the ratio of tadpoles swimming, body length, tail length or tail muscle depth (Table 3).

We found among-treatment differences in tadpole survival until metamorphosis (GZLM; Wald $\chi^2 = 12.92$, d.f. = 4, $P = 0.012$; Fig. 4a). Bonferroni-corrected pairwise comparisons of estimated marginal means revealed a significantly lower percentage of tadpoles surviving in the dragonfly treatment (58.18 ± 4.85; mean ± SE) compared to the control (75.76 ± 3.45) and the leech treatments (77.27 ± 5.18; both $P < 0.029$), whereas all other comparisons (water scorpion: 70 ± 7.95; newt: 65.45 ± 9.6) were nonsignificant ($P > 0.34$). Metamorph

Table 3 The relationship between predator dangerousness and the magnitude of phenotypic responses and potential costs of induced defenses and the effect of life history changes in tadpoles on their survival and on metamorph mass and jumping performance. Analyses were performed on tub averages of measures taken at the third observation on behaviour (30 days after hatching) and at the morphology sampling occasion (33 days after hatching) in Experiment 2 (caged predators) using multivariate general linear models. Bold indicates statistical significance at $P \leq 0.05$.

	Multivariate tests				Tests of between-subjects effects					
Effect	d.f.	Wilk's λ	F	P	Dependent variable	d.f.	B	SE	F	P
'Dangerousness'	7, 18	0.254	7.572	**< 0.001**	Ratio of active tadpoles	1, 24	−0.477	0.139	11.718	**0.002**
					Ratio of swimming tadpoles	1, 24	−0.054	0.037	2.159	0.155
					Body length	1, 24	−0.664	0.640	1.075	0.310
					Tail length	1, 24	−0.221	1.272	0.030	0.864
					Tail muscle depth	1, 24	0.094	0.192	0.239	0.629
					Tail fin depth	1, 24	1.292	0.393	10.813	**0.003**
					Time until metamorphosis	1, 24	2.612	0.977	7.147	**0.013**
'Dangerousness'	3, 22	0.459	8.656	**0.001**	Survival until metamorphosis	1, 24	−24.186	10.112	5.721	**0.025**
					Metamorph mass	1, 24	0.125	0.037	11.631	**0.002**
					Jumping performance	1, 24	1.129	12.369	0.008	0.928
Ratio of active tadpoles	3, 20	0.771	1.981	0.149						
Ratio of swimming tadpoles	3, 20	0.874	0.959	0.431						
Tail fin depth	3, 21	0.382	11.321	**< 0.001**	Survival until metamorphosis	1, 23	−5.087	4.443	1.311	0.264
					Metamorph mass	1, 23	0.068	0.013	27.507	**< 0.001**
					Jumping performance	1, 23	12.334	4.644	7.053	**0.014**
Time until metamorphosis	3, 21	0.675	3.367	**0.038**	Survival until metamorphosis	1, 23	−3.963	1.889	4.402	**0.047**
					Metamorph mass	1, 23	0.007	0.006	1.818	0.191
					Jumping performance	1, 23	−2.477	1.974	1.575	0.222
All two-way interactions				> 0.120						

mass also significantly varied among treatments (LMM; $F_{4,20.77} = 3.1$, $P = 0.038$; Fig. 4b), with tadpoles in the dragonfly treatment being larger than those in the control treatment ($P = 0.03$) and no other significant differences (all $P > 0.43$). Metamorphs exhibiting a larger body mass (LMM; $B = 140.63$, SE = 24.08, $F_{1,200.54} = 34.1$, $P < 0.001$) made longer hops; however, neither treatment ($F_{4,19.12} = 0.97$, $P = 0.45$) nor the interaction between treatment and metamorph mass ($F_{4,231.81} = 1.27$, $P = 0.28$) influenced jumping performance.

In the treatments containing more dangerous predators, tadpole survival was lower (Fig. 4a), metamorph mass was larger (Fig. 4b), whereas jumping performance was not affected (Table 3).

Metamorph mass and jumping performance of metamorphs were positively related, and survival was unrelated to tail fin depth (Table 3). Also, survival was negatively related, and metamorph mass and jumping performance were unrelated to time until metamorphosis (Table 3). Neither tadpole survival, metamorph mass nor jumping performance was related to behaviour of tadpoles (Table 3).

Experiment 3 – predation trials

At the first round of predation trials performed 12 days after hatching, when tadpoles taken from Experiment 2 were exposed to free-ranging water scorpions, newts and dragonfly larvae, survival was significantly affected by both the type of free-ranging predators present (GZLM; Wald $\chi^2 = 19.92$, d.f. = 2, $P < 0.001$) and the tadpole treatment (Wald $\chi^2 = 42.02$, d.f. = 4, $P < 0.001$; Fig. 5). The interaction between type of free-ranging predators present and the tadpole treatment had no effect on survival of tadpoles ($P = 0.78$). Bonferroni-corrected pairwise comparisons of estimated marginal means indicated that more tadpoles survived in the presence of free-ranging water scorpions than with the other two predators (both $P < 0.001$), whereas there was no significant difference in survival between treatments containing free-ranging newts or dragonfly larvae ($P = 1$; water scorpions mean ± SE: 87.5 ± 3.55%; newts: 65.83 ± 5.01%; dragonfly larvae: 65.83 ± 5.81%). Also, tadpoles taken from the control treatments had the lowest survival (all pairwise $P < 0.001$), whereas we found no difference between the tadpoles taken from treatments containing predators (all pairwise $P > 0.19$; control: 41.67 ± 6.06%; leech: 76.39 ± 5.14%; water scorpions: 81.94 ± 7.23%; newts: 80.56 ± 5.18%; dragonfly larvae: 84.72 ± 4.11%).

In the second round of predation trials performed 27 days after hatching, tadpoles tended to be more likely to survive trials in the presence of free-ranging newts than in the presence of dragonfly larvae (Wald $\chi^2 = 2.78$, d.f. = 1, $P = 0.095$; newts: 90 ± 2.57%; dragonfly larvae: 82.5 ± 3.42%). We observed no significant differences in survival between tadpole treatments ($P = 0.15$; Fig. 5; control: 75 ± 6.88%; leech: 89.58 ± 3.72%; water

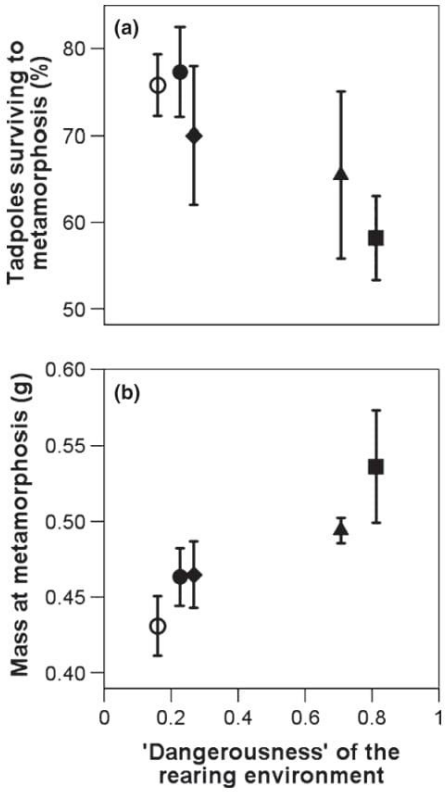

Fig. 4 Relationships between predator dangerousness and the magnitude of potential costs related to induced defences. Percentage of tadpoles surviving until metamorphosis (a) and body mass of individuals at the start of metamorphosis (b) were both measured in Experiment 2 (caged predators). 'Dangerousness' was estimated by the average ratio of tadpoles that died in the treatments of Experiment 1 (free-ranging predators). Treatments are symbolized by ○: control, ●: leech, ◆: water scorpion, ▲: newt, ■: dragonfly larva. The figure is based on tub averages; means ± SE are indicated.

scorpions: 89.58 ± 3.72%; newts: 85.42 ± 4.83%; dragonfly larvae: 91.67 ± 3.55%). As before, the interaction between the type of free-ranging predators present and the tadpole treatment had no effect on tadpole survival ($P = 0.33$).

Discussion

Free-ranging predators

Dangerousness of predators varied both between species and over time. Leeches and water scorpions were less voracious predators at any time than newts and dragonfly larvae, whereas neither leeches and water scorpions nor newts and dragonfly larvae largely differed from each other in dangerousness. Leeches and water scorpions were only dangerous to eggs and/or small tadpoles. Interestingly, leeches did not solely feed on eggs, but were capable of preying upon hatchlings as well. This was also confirmed by observations of predation events. Newts and dragonfly larvae remained effective predators also of large tadpoles and during the last interval, the effect of dragonfly larvae seemed larger than that of newts. Consequently, gape-limited smooth newts may be less effective predators of very large tadpoles than dragonfly larvae. This is also supported by a nonsignificant tendency for large tadpoles being more likely to survive in the presence of newts than in the presence of dragonfly larvae in the second round of our predation trials and has been suggested by previous studies as well (Van Buskirk, 2001; Kishida & Nishimura, 2005).

Caged predators

Behaviour of tadpoles changed over time was strongly affected by treatments and reacted differently to the presence of predators early and late during the larval stage. Patterns in the ratio of tadpoles visible and the ratio of tadpoles swimming were similar. Large tadpoles raised in the presence of predators were less active than tadpoles in the control treatment, which aligns to a generally reported lowered activity as a response to predators. Small tadpoles, however, were more active in the predator treatments compared to controls. This result is surprising, because small tadpoles are generally more vulnerable to predation than large ones (Travis *et al.*, 1985; Semlitsch, 1990; Eklöv & Werner, 2000), and thus, elevated activity is likely to result in higher survival costs in their case (Stoks *et al.*, 2003; Laurila *et al.*, 2006; Takahara *et al.*, 2008). Nonetheless, benefits including elevated growth rates and a resulting early reaching of a size refuge from predation, more resources available for the expression and maintenance of morphological-induced defences, enhanced competitive ability, mating success or fecundity (Urban, 2007a,b; Biro *et al.*, 2005) may result in selection for the maintenance of high levels of activity during early life stages, even if these come at a cost of elevated immediate mortality risk.

Overall, tadpole activity was more strongly affected by the presence of more dangerous predators: smaller tadpoles appeared to increase, whereas large tadpoles to decrease their activity to a larger extent when they were exposed to more dangerous predators. This result also suggests that the benefits of a head start early during ontogeny, achieved by increased food intake resulting from elevated activity (*sensu* Werner & Anholt, 1993), may outweigh the costs. Further, in the presence of caged predators, the observed variation in tadpole activity did not translate into variation in tadpole survival, metamorph mass or jumping performance of froglets. This result supports the notion that changes in activity do not necessarily effect costs through lowered food intake (McPeek, 2004; Relyea & Auld, 2004; Steiner, 2007).

Tadpoles had a deeper tail fin in the presence of more dangerous predators, and body length was shortest in the presence of dragonfly larvae. A short body and high tail

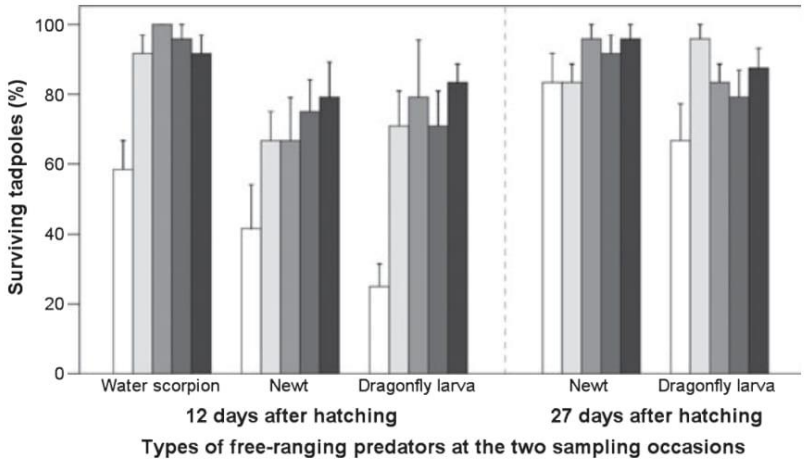

Fig. 5 Percentage of tadpoles surviving (mean ± SE) in the predation trials (Experiment 3), when tadpoles raised in the presence of caged predators (Experiment 2) were exposed to free-ranging water scorpions, newts or dragonfly larvae of Experiment 1. White bars represent tadpoles originating from the control treatment, light grey bars represent tadpoles taken from tubs containing caged leeches, medium grey bars represent tadpoles taken from tubs containing caged water scorpions, dark grey bars represent tadpoles taken from tubs containing caged newts and black bars represent tadpoles taken from tubs containing caged dragonfly larvae.

fin may divert attacks away from the body or enhance swimming performance (Van Buskirk *et al.*, 1997; Doherty *et al.*, 1998; Johnson *et al.*, 2008). We did not find differences in total tadpole length among treatments. This result contradicts the predictions of theoretical models (e.g. Werner, 1986) and is all the more surprising as costs potentially arising from lowered activity and from alterations in morphology should have summed up rather than cancelled each other out. Nonetheless, the lack of an effect of caged predators on tadpole body size accords with previous results (Benard, 2004; Relyea, 2007).

Time until metamorphosis tended to be longer in the presence of predators and was longest in tadpoles raised together with the most dangerous predator. Again, contrary to our results, a theoretical model by Werner (1986) predicted a shortened larval phase in the presence of dangerous aquatic predators, and there is indeed strong selection on the timing of metamorphosis (Richter-Boix *et al.*, 2010). Nonetheless, our results again align to previous experimental studies using caged predators (Benard, 2004; Relyea, 2007). These discrepancies between theoretical predictions and empirical results may be explained by a dependence of responses on the environmental context or constraints imposed by costs that are paid for producing antipredator defences (Relyea, 2007).

We observed significant among-treatment variation in tadpole survival in the experiment containing caged predators, and mortality rates were related to predator dangerousness, mortality being highest in the presence of the most dangerous predator. We cannot be sure what caused elevated mortality rates, but as survival was lowest where morphological responses to predators were strongest and where metamorphosis was delayed, we suggest that elevated tadpole mortality has arisen as a cost of induced defences. Severe costs of the expression of induced defences paid in other life history characters have been documented (Dixon & Agarwala, 1999; Van Buskirk, 2000; Hammill *et al.*, 2008), but organisms should rarely divert so much energy to antipredator defences that this itself puts a risk on survival (Werner & Anholt, 1993; Van Buskirk, 2000). This is one of only a few studies (e.g. McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998) which suggest that expressing antipredator defences may also incur a mortality cost, and further studies are needed to determine the circumstances under which this can arise.

Metamorph mass varied between treatments, and this variation was also related to the dangerousness of predators. However, this relationship is not indicative of a potential cost of antipredator responses, because metamorphs that emerged from tubs containing more dangerous predators were larger, and larger size at metamorphosis is beneficial for fitness (Smith, 1987; Semlitsch *et al.*, 1988; Altwegg & Reyer, 2003).

Even though the jumping performance of froglets depended on metamorph mass, we did not find systematic between-treatment variation in jumping performance. This suggests that escape ability of metamorphosed individuals is not strongly influenced by predators that were present in the aquatic rearing environment. Nonetheless, tadpoles showing strong phenotypic responses in terms of body shape (tail fin depth) developed into large metamorphs that had superior jumping abilities, most probably enjoying enhanced escape ability from predators (for similar results see Van Buskirk & Saxer, 2001). This outcome cannot be interpreted as a

manifestation of costs but rather as a benefit of expressing induced defences.

Predation trials

Small tadpoles exhibiting induced defences had a higher probability of survival in the predation trials than predator-naive tadpoles. This is most probably a result of altered morphology and aligns to results of previous studies (e.g. McCollum & Van Buskirk, 1996; Kishida & Nishimura, 2005; Teplitsky *et al.*, 2005). However, tadpoles taken from tubs that contained more dangerous predators did not exhibit higher survival rates, possibly because positive effects of more pronounced morphological defences were countered by negative effects of elevated activity.

Even though two of the predators involved in the experiment use the sit-and-wait tactic (water scorpion and dragonfly nymph) and the other two (leech and newt) are active foragers, we did not observe qualitatively differing, predator-specific responses, as did Teplitsky *et al.* (2005) in the same tadpole species, probably because our study did not include fish as predators (also see Benard, 2006). The observed antipredator defences rather seem to have been universally effective against the tested predators (McCollum & Van Buskirk, 1996; Van Buskirk, 2001; Laurila *et al.*, 2006), and tadpoles did not have elevated survival when they were exposed to the type of free-ranging predator that they were raised with. Our results, thus, suggest that while the presence of induced defences did provide some protection, variation in the expression level of defences among predator treatments did not have large enough consequences on survival that we could have detected them in our experiment. Large tadpoles in general already had high survival, which was not further elevated measurably by the expression of induced defences. This may have been a result of a size refuge from predation: dragonfly larvae and especially newts may be less effective in capturing large tadpoles (Kishida & Nishimura, 2005; Urban, 2007a,b).

In summary, our results suggest that tadpoles generally reacted more intensely to more dangerous predators both in their behaviour and in their body proportions. We did not observe costs of induced defences manifested at or shortly after metamorphosis in the form of decreased mass at metamorphosis, or lowered jumping performance. Nonetheless, we obtained suggestive evidence for a survival cost suffered during the larval stage, where tadpoles experienced higher mortality in the nonlethal presence of more dangerous predators. When exposed to free-ranging predators, we observed a clear increase in the survival of tadpoles raised in the presence of any predator when compared to naive tadpoles, but did not find benefits of stronger antipredator responses that developed in the presence of more dangerous predators. Our results, thus, suggest that while antipredator responses can be adjusted to the dangerousness of predators, accordingly graded changes in both costs and benefits do not necessarily arise. Consequently, while our experimental set-up did not allow a formal test of this hypothesis, the relationship between costs and benefits appears to be nonlinear. Further studies are needed that simultaneously relate extent, costs and benefits of induced defences to predator dangerousness in other taxa to test the general applicability of our findings. Also, studies directly relating costs and benefits of induced defences to each other will be crucial for testing key assumptions of the theory of predator-induced plastic defences. Finally, it will be interesting to explore the conditions under which varying strengths of induced defences do or do not provide different levels of protection.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Tadpole behaviour (mean ± SE) sampled at four occasions in Experiment 2 (caged predators).

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Interactions between the information content of different chemical cues affect induced defences in tadpoles

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Animals often alter their behaviour, morphology and physiology in the presence of predators. These induced defences can be fine-tuned by a variety of environmental factors such as predator species, acute predation risk or food availability. It has, however, remained unclear what cues influence the extent and quality of induced defences and how the information content of these cues interact to determine the development of antipredator defences. We performed an experiment to study the significance of direct chemical cues, originating from the predators themselves, and indirect cues, released by attacked or consumed prey, for phenotypic responses in *Rana dalmatina* tadpoles. We reared tadpoles in the presence of caged predators (*Triturus vulgaris*, *Aeshna cyanea*) fed either one or three tadpoles every other day outside the tadpole-rearing tanks. Fifteen hours after food provisioning, predators were put back into the tanks containing focal tadpoles either after washing (direct + digestion-released cues) or with the water containing remnants of the prey (direct + all types of indirect cues). Our results suggest that direct cues together with digestion-released cues can be sufficient to induce strong antipredator responses. Induced defences depended on both direct cues, affecting predator-specific responses, and the quantity of indirect cues, resulting in graded responses to differences in predation threat. Moreover, direct and indirect cues interacted in behaviour, resulting in predator-specific graded responses. We also observed a decrease in the extent of predator-induced responses in large tadpoles as compared to small ones. Our results, thus, suggest that prey integrate multiple cues about predators to optimize induced defences and that this process changes during ontogeny.

Life-history theory suggests that predator-induced defences need to be adjusted carefully, since expression of these plastic defences should be costly, and plastic responses that are accurately adjusted should deliver the largest net benefits (Werner 1986, Houston et al. 1993, Werner and Anholt 1993, Lima and Bednekoff 1999, Urban 2007a). Empirical work suggests that fine-tuned antipredator-responses are taxonomically widespread (e.g. protists: Kusch 1995, fishes: Kusch et al. 2004, arthropods: Laforsch et al. 2004, molluscs: Freeman et al. 2009). For example, tadpoles have been shown to adjust defences according to the predator species (Relyea 2001), the type and quantity of prey eaten by predators (Laurila et al. 1998, Schoeppner and Relyea 2005), or to predator density (Van Buskirk and Arioli 2002). Responses to predators may on the other hand be constrained by environmental factors (e.g. presence of competitors: Relyea 2004, pH-level: Teplitsky et al. 2007), and depend on the intrinsic state of the individual (e.g. body size: Fraker 2008).

Despite a growing body of research concerned with predator-induced defences, relatively little is known about the cues that are proximately used by prey species to adjust their phenotypic responses. While tactile and visual cues may sometimes deliver important information (Moore et al. 2004, Rot-Nikcevic et al. 2005), predator-induced defences are usually triggered by chemosensory information in aquatic environments (Tollrian and Harvell 1999). For example, prey may respond to (1) chemical cues passively released from injured prey tissue ('damage-released cues'; Chivers and Smith 1998), (2) general prey metabolites excreted at an increased rate into the environment during stress-response to the predator ('no-cost disturbance signals'; Kiesecker et al. 1999), (3) special disturbance cues that are costly to synthesize and are released actively by prey upon attack by a predator ('alarm pheromones'; Fraker et al. 2009), (4) constituents of prey tissue released through digestion by predators ('digestion-released cues'; LaFiandra and Babbitt 2004), and (5) cues originating directly from the predator unrelated to its recent feeding history ('kairomones'; Petranka and Hayes 1998).

Kairomones deliver information about the predator directly, so these are often referred to as a type of direct cues, whereas cues originating from disturbed, attacked or digested prey indirectly deliver information about the presence of a predator, so these are often referred to as indirect cues. Direct cues may play an important role in the development of fine-tuned responses, as they may allow identifying the predator species, however, the quantity and type of indirect cues may

also deliver important information to prey, especially on the acute predation risk. While direct or indirect cues alone can induce defensive behavioural responses in tadpoles (Petranka and Hayes 1998, Fraker et al. 2009), the presence of all types of chemical cues may be required to develop the full suite and magnitude of morphological defences (Schoeppner and Relyea 2005, 2009). One explanation for this may be that the information content of different types of cues can interact in several ways. Interactions between the information content of different types of cues may be synergistic, complementary or conflicting. For example, kairomones indicating a dangerous predator and the simultaneous presence of large quantities of indirect cues may enhance responses. Also, the same quantity of damage-released cues may indicate very different predation risks in the presence of a chewing or a swallowing predator. Finally, large quantities of indirect cues normally induce lowered activity that can lead to lowered ingestion rates, but in the presence of a gape limited predator, it may pay off to increase activity to enhance growth (Urban 2007a, 2007b). Nonetheless, we currently know very little about the significance of the interactions between the information delivered by the different types of chemical cues.

In the present study, we aimed at disentangling the importance of direct and indirect cues for phenotypic responses in agile frog *Rana dalmatina* tadpoles. More importantly, we studied the effects of interactions between the information content of direct and indirect cues on antipredator responses. We used a gape-limited, actively foraging predator, which swallows its prey whole (the newt *Triturus vulgaris*), and a chewing, sit-and-wait predator (larvae of the dragonfly *Aeshna cyanea*). Due to the differences in feeding mechanism and foraging mode of these predators, we predicted a larger decrease in activity and smaller body sizes in the presence of dragonfly larvae as compared to the presence of newts, arising due to a conflicting interaction between the information delivered by direct and indirect cues. By raising tadpoles in the absence of predators and in the presence of predators receiving low or high food levels, we tested the prediction that the presence of direct cues and increasing quantities of indirect cues induce graded defensive responses, resulting from synergistic interactions between the information content of direct and indirect cues. By including or excluding damage-released cues, no-cost disturbance signals and alarm pheromones, we tested the prediction that direct cues have to be complemented by indirect cues in the presence of the gape-limited predator to induce continually high levels of plastic responses. Finally, by sampling behaviour and morphology of tadpoles repeatedly, we also tested the prediction that, in a stable environment, induced defences remain qualitatively similar during ontogeny.

Methods

Tadpoles of *Rana dalmatina* can be found in most of southern and middle continental Europe (Gasc et al. 1997) in a wide array of temporary and semi-permanent ponds (Nöllert and Nöllert 1992). They co-occur with a variety of invertebrate and vertebrate predators and respond to their presence both morphologically and behaviourally (Lardner 2000, Teplitsky et al. 2004, 2005a, 2005b).

We applied a 2×2×2 factorial randomized block design with predator type (dragonfly larva or newt), predator feeding rate (low or high) and types of cues present (all cues or cue-restricted) as factors. We also had a control treatment receiving no predators. Treatments were replicated once (the control twice) in each of ten spatial blocks with the position of treatments randomized within blocks. We used 100 plastic tanks (42×25×25 cm) covered with mosquito nets as experimental containers, placed out in an open field belonging to the Plant Protection Inst. of the Hungarian Academy of Sciences located on the outskirts of Budapest. Tanks were filled one week before the start of the experiment with 16 litres of aged tap-water and were inoculated with 1 litre of pond water. We also added 2 g of rabbit pellets and 5 g of dried beech (*Fagus sylvatica*) leaves to each tank to enhance algal growth and provide nutrients and cover for tadpoles. Tanks further received a 0.75 litre transparent cup with bottom and cover made of mosquito net as predator cages. Transparency and the net covers allowed visual and chemical contact between tadpoles and predators while cages prevented predators from capturing the focal tadpoles.

In late march 2008 we collected eight freshly laid clutches of *R. dalmatina* from a pond in the Pilis Mountains, Hungary (47°42'N, 19°02'E). This pond is a semi-permanent water body completely desiccating approximately every third year, but usually after *R. dalmatina* metamorphs have left the water. It also supports permanent populations of invertebrate (*Aeshna* sp. larvae, *Notonecta* sp., *Nepa* sp., *Dytiscus* imagos and larvae) and vertebrate (*T. vulgaris*) predators. We brought egg-clutches to the field station of the Plant Protection Inst. and reared embryos in shallow dishes containing 5 litres of aged tap-water until hatching. Families of frog embryos were reared separately at this stage. We also collected 40 *T. vulgaris* males and 40 larvae of *A. cyanea* from nearby water bodies, kept them individually in 0.75 litre plastic cups and fed them two *R. dalmatina* tadpoles every other day until assigning them to experimental containers. *T. vulgaris* males and *A. cyanea* larvae are similarly voracious predators of small *R. dalmatina* tadpoles, whereas for large tadpoles dragonfly larvae appear to be more dangerous (Hettyey et al. unpubl.). Hunting efficiency of newts may decrease with increasing tadpole size due to gape-limitation, as during attempts of swallowing large prey individuals the latter can escape relatively easily, whereas dragonfly larvae are more capable of holding also large prey firmly with their labia while ingesting them piecemeal with their mandibles.

On 25 April, when tadpoles reached a free-swimming state (developmental stage 25–26; Gosner 1960), we started the experiment by randomly distributing predators into the cages hung into the rearing tanks of tadpoles and assigning 16 tadpoles, two per sib-group, to each experimental container. Resulting initial densities (one individual per litre) lie within the range that can be found under natural conditions (Hettyey unpubl.). We fed predators every other day in the following manner: We (1) removed 0.2 l of water from each rearing tank and placed 0.75 l transparent feeding cups filled with 0.2 l of aged tap water on the ground next to the rearing tanks, (2) removed the predators from their cages and placed them into the feeding cups, and (3) added *R. dalmatina* tadpoles to the feeding cups. Next morning (ca 15 h after 1, 2 and 3), we (4) removed tadpoles that were

still alive in the feeding cups and (5) poured predators back into the predator cages either together with the predation water containing remains of food items (all-cues treatment) or put back predators after rinsing and added 0.2 l of aged tap water to rearing tanks (cue-restricted treatment). In the control treatment, we poured 0.2 l of aged tap water into the rearing tanks after each feeding round. Half of the predators received two, the other half six similarly sized tadpoles at a time. After ten days, when tadpoles used as predator food grew bigger, we reduced this amount to one and three tadpoles, respectively.

Ten and 27 days after start of the experiment (5 May and 22 May), we monitored activity of tadpoles three times a day, at 12:00, 14:00 and 16:00 h. For each rearing tank we noted how many tadpoles were swimming in the water column or feeding on the walls of tanks, added these counts together and used the number of tadpoles visible as an estimate of tadpole activity. We calculated the ratio of tadpoles visible by dividing the number of tadpoles visible with the total number of tadpoles in the container. Wherever it was necessary, we corrected for spontaneous mortality in the rearing tanks by means of linear interpolation (mortality averaged 3.5% and was nowhere higher than 19%). The two dates of sampling were selected to obtain measurements for both small and large tadpoles.

On day 11 and 28 (6 May and 23 May), we haphazardly took a sample of ten tadpoles from each tank and anaesthetized them by placing them into 0.02 m/m % MS-222 (tricaine) until they became immobile. We then rinsed tadpoles with aged tap water, photographed them with a digital camera to obtain pictures on their lateral view and, after recovery, put them back into the rearing tanks they were taken from. Mortality during this procedure was very low (< 1%). From the photographs, we later measured four parameters that are known to show plasticity in response to the presence of predators (Laurila et al. 2004, Teplitsky et al. 2004): body length, tail length, maximum tail muscle depth and maximum tail fin depth. Body shape measures were defined following Van Buskirk and McCollum (2000). For digital measurements, we used ImageTool 3.0. After the second sampling occasion, we terminated the experiment, transported tadpoles and predators to the Pilis Mountains and released the animals at their sites of collection.

Statistical analyses

To obtain an overall measure of body size and to be able to control for body size when analyzing body shape, we first performed a principal components analysis (PCA) for each sampling occasion separately. Bivariate correlations between body length, tail length, maximum tail muscle depth and maximum tail fin depth all showed strong positive relationships at both sampling occasions (Pearson correlation; all $r > 0.75$, all $p < 0.001$). The first component explained a large proportion of the variance (first sampling occasion: 84.7%; second sampling occasion: 82.9%) and original variables loaded strongly and positively on PC1 (all $r > 0.88$ at both sampling occasions). We used PC1 scores as measures of body size in subsequent analyses.

When analysing potential effects on body size, body shape and activity, we first performed an analysis involving all treatments and testing for the effect of predator presence/absence. In a second step, we tested for effects of predator type, feeding rate and types of cues present. In these analyses, we had to exclude the control treatment, as feeding rate and types of cues present could not be replicated in the control.

To analyze variation in body size, we used linear mixed effect models (LMM) by entering body size as the dependent variable, rearing tank as a random factor and presence/absence of predators as a fixed factor. We further performed LMM analyses with body size as the dependent variable, predator type, feeding rate and types of cues present as fixed factors and rearing tank as a random factor.

We analyzed variation in body shape of tadpoles using multivariate general linear models (GLM) with body length, tail length, maximum tail muscle depth and maximum tail fin depth entered as dependent variables, predator presence/absence as a fixed factor and body size as a covariate. We further built GLMs with body length, tail length, maximum tail muscle depth and maximum tail fin depth entered as dependent variables, predator type, feeding rate and types of cues present as fixed factors and body size as a covariate. Analyses on body shape were based on measures averaged over individuals within rearing tanks to avoid pseudo-replication.

To investigate tadpole activity, we used a LMM with the arcsine square-root transformed ratio of tadpoles visible entered as the dependent variable, predator presence/absence, and date of sampling occasion as fixed factors and rearing tank as a random factor. Finally, we performed another LMM with activity of tadpoles entered as the dependent variable, predator type, feeding rate, types of cues present and date of sampling occasion as fixed factors and rearing tank as a random factor.

We included all possible interactions into initial models and performed backward removal of terms with $p > 0.1$ to avoid problems potentially arising due to the inclusion of non-significant terms (Engqvist 2005). We re-entered removed variables one by one to the final model to obtain relevant statistics. All tests were two tailed. Statistics were calculated using SPSS 15.0 for Windows.

Results

Size

We did not detect differences in body size between the control treatment and treatments that contained a predator either 11 (LMM; $F_{1,95.5} = 0.02$, $p = 0.89$) or 28 days ($F_{1,97.9} = 0.01$, $p = 0.91$) after start of the experiment (Fig. 1), but observed considerable variation among rearing boxes on both sampling occasions (11 d: Wald $Z = 5.58$, $p < 0.001$; 28 d: Wald $Z = 6.57$, $p < 0.001$).

When analyzing treatments containing a predator, we observed no effect of feeding rate (LMM; 11 d: $F_{1,77} = 0.04$, $p = 0.84$; 28 d: $F_{1,78} = 0.25$, $p = 0.62$) or types of cues present (day 11: $F_{1,77.2} = 0.17$, $p = 0.69$; 28 d: $F_{1,78} = 0.15$, $p = 0.7$). There was a non-significant tendency for predator type to affect tadpole size on day 11 with tadpoles being bigger in the presence of newts than in the presence of dragonfly larvae ($F_{1,77.3} = 3.39$, $p = 0.07$; Fig. 1), this tendency, however, completely diminished by the second sampling occasion

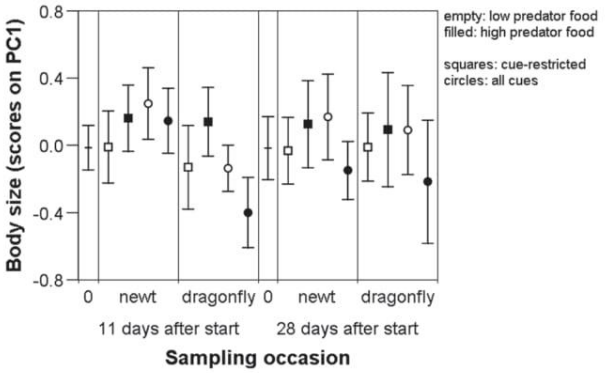

Figure 1. Body size of tadpoles (means ± SE) in the nine treatments at the two sampling occasions. Empty symbols represent treatments where predators received little food, filled symbols represent treatments where predators received much food. Squares represent cue-restricted treatments, whereas circles represent all-cue treatments. The figure is based on rearing tank-averages of factor scores on PC 1 from PCAs on body length, tail length, maximum tail muscle depth and maximum tail fin depth performed for the two sampling occasions separately. Larger values indicate larger body sizes.

($F_{1,78}=0.05$, $p=0.83$; Fig. 1). Effects of all two-way and the three-way interactions were non-significant at both sampling occasions (all $p > 0.17$).

Shape

Relative measures of tadpole shape differed in the presence of predators from that found in the control treatment at both sampling occasions (Table 1, Fig. 2, Appendix 1). Univariate tests revealed that in the presence of predators, the body of tadpoles was relatively shorter and tail fins were deeper on both sampling occasions, whereas tail length was not affected and the tail muscle only tended to be narrower at the first sampling occasion (Table 1, Fig. 2, Appendix 1).

The analysis of treatments containing a predator revealed that predator type affected relative body shape measures on both sampling occasions, whereas feeding rate did not (Table 1, Fig. 2). There was a marginally non-significant tendency for types of cues present influencing shape at the first sampling occasion, this tendency, however, disappeared by the second sampling (Table 1, Fig. 2, Appendix 1). The interaction between predator type and feeding rate tended to affect body shape of tadpoles on day 28, all other two-way interactions were non-significant (Table 1, Appendix 1). Univariate tests suggested that, at both sampling occasions, tadpoles had a shorter tail and a deeper tail fin in the presence of dragonfly larvae than when they were reared together with newts, whereas predator type had no effect on body length or tail muscle depth at either sampling (Table 1, Fig. 2, Appendix 1). Furthermore, at the first sampling occasion, tail fins were deeper and tail muscles tended to be shallower in the cue-restricted treatments as compared to when all cues were present, but types of cues present had no effect on body length or tail length (Table 1, Fig. 2, Appendix 1). At the second sampling occasion, the interaction between predator type and feeding rate had a significant effect on tail fin depth and a marginally non-significant effect on body length with tadpoles in the low food - newt treatments having more control-like phenotypes than in the other predator treatments, but there was no effect on tail length or tail muscle depth (Table 1, Fig. 2, Appendix 1).

Behaviour

Tadpoles were more active in the absence of predators than in their presence and 27 days after the start of the experiment than after 10 days (Table 2, Fig. 3). However, the interaction between predator presence and the date of the sampling occasion was also significant with a smaller decrease in activity during the later sampling occasion (Table 2, Fig. 3).

The analysis of treatments containing a predator indicated that, on average, tadpole activity was lower in the treatments where predators received more food, and repeated a previous result that tadpole activity increased between the first and the second sampling occasion (Table 2, Fig. 3). Tadpoles tended to be more active when all types of cues were present than in the cue-restricted treatments, whereas the main effect of predator type did not have a significant effect on activity (Table 2, Fig. 3). The interactions between predator type and date of sampling occasion and types of cues present and date of sampling occasion were significant (Table 2). During the first sampling occasion, tadpoles were more active in the presence of newts than in the presence of dragonflies, and more active when all types of cues were present than in the cue-restricted treatments, whereas during the second sampling occasion there were no such differences (Table 2, Fig. 3). The interaction between predator type and feeding rate showed a non-significant tendency, whereas all other interactions were non-significant (Table 2).

To further dissect the effects of predator type, types of cues present and feeding rate, we performed two more analyses, one for each sampling occasion. At the first sampling occasion, tadpoles were more active in the presence of newts (LMM; $F_{1,76}=4.51$, $p=0.037$), when all types of cues were present ($F_{1,76}=5.4$, $p=0.023$) and when the predators were fed less ($F_{1,76}=5.18$, $p=0.026$, Fig. 3). The interactions were non-significant (all $p > 0.23$). At the second sampling occasion, none of the main effects seemed to influence tadpole activity (all $p>0.17$), but the interaction between predator type and feeding rate was significant ($F_{1,76}=5.39$, $p=0.023$) with tadpoles being more active in the presence of newts than in the presence of dragonfly larvae at low but not at high predator feeding rate (Fig. 3). All other interactions were non-significant (all $p > 0.4$).

Discussion

The observed lack of induced changes in tadpole body size, the present morphological responses and the decrease in activity all agree well with what has previously been observed in other tadpole species (Relyea 2001, Van Buskirk 2002), and in *R. dalmatina* tadpoles specifically (Lardner 2000, Teplitsky et al. 2004, 2005a, 2005b). Apart from delivering further support for these well-documented phenotypic changes, our experimental design also allowed us to draw some conclusions on how interactions between the information content

Table 1. Results of multivariate general linear models on body length (body L), tail length (tail L), tail muscle depth (tail MD) and tail fin depth (tail FD). For the univariate tests, F-values are provided in the cases where the multivariate tests yielded significant results.

	Multivariate tests				Univariate tests				
Effect	DF	Wilk's λ	F	p	DF	body L	tail L	tail MD	tail FD
day 11									
Control incl.									
Body size	4,94	0.00	4.61×10^9	<0.001	1,97	1910.56****	1193.20****	533.51****	1142.99****
P/A of predators	4,94	0.46	27.79	<0.001	1,97	44.63****	0.63	3.19*	76.28****
Control excl.									
Body size	4,73	0.00	347×10^9	<0.001	1,76	1512.47****	990.96****	458.32****	1196.67****
Predator type	4,73	0.74	6.43	<0.001	1,76	0.59	5.72**	1.24	23.13****
Feeding rate	4,72	0.97	0.57	0.687					
Types of cues present	4,73	0.88	2.41	0.057	1,76	0.60	0.33	3.41*	8.22****
Predator type × Feeding rate	4,71	0.98	0.36	0.835					
Predator type × Types of cues present	4,72	0.95	0.88	0.478					
Feeding rate × Types of cues present	4,71	0.94	1.09	0.366					
Predator type × Feeding rate × Types of cues present	4, 68	0.96	0.70	0.593					
day 28									
Control incl.									
Body size	4,94	0.00	1.48×10^10	<0.001	1,97	4287.04****	5827.98****	4020.32****	3309.98****
P/A of predators	4,94	0.84	4.39	0.003	1,97	6.48**	2.25	0.00	13.77****
Control excl.									
Body size	4,72	0.00	1.12×10^10	<0.001	1,75	3604.46****	5255.84****	2844.35****	3053.49****
Predator type	4,72	0.81	4.33	0.003	1,75	2.39	6.60**	0.02	14.37****
Feeding rate	4,72	0.93	1.44	0.230					
Types of cues present	4,71	0.92	1.52	0.206					
Predator type × Feeding rate	4,72	0.89	2.18	0.079	1,75	3.86*	0.00	0.02	4.64**
Predator type × Types of cues present	4,70	0.98	0.44	0.781					
Feeding rate × Types of cues present	4,70	0.93	1.27	0.292					
Predator type × Feeding rate × Types of cues present	4, 68	0.92	1.46	0.225					

*p<0.10, **p<0.05, ***p<0.01, ****p<0.001

of different types of chemical cues may have shaped phenotypic responses.

Young tadpoles were less active and tended to be smaller in the presence of dragonfly larvae than in the presence of newts. This difference may have resulted from a conflicting interaction between the information content of direct and indirect cues in the case of the gape-limited predator, and/or from a synergistic interaction in the case of dragonfly larvae. While the presence of similar amounts of indirect cues should have induced similar responses to both predators, it seems that the direct cues delivering information on the type of predator weakened responses in the case of newts and/or enhanced them in the case of dragonflies. This difference could have evolved because newts are gape-limited and are active foragers, also finding immobile prey. Consequently, it may pay to maintain relatively high activity levels for the returns in growth rate in the presence of newt predators (Kishida and Nishimura 2005, Urban 2007a, 2007b). Larvae of aeshnid dragonflies, however, are not gape-limited and are sit-and-wait predators, so that decreasing activity will lower encounter rates with this predator. Consequently, decreasing foraging activity may be a viable strategy in the presence of dragonfly larvae, even if this comes at a cost of decreased growth (Relyea 2002). An alternative explanation for the weaker responses to the presence of newts compared to that of dragonfly larvae could be that newts pose a weaker threat on tadpoles than dragonflies (Van Buskirk 2001). Our observations, however, suggest that *T. vulgaris* are not less voracious predators of small *R. dalmatina* tadpoles than *A. cyanea* larvae (Hettyey et al. unpubl). As the effect of predator type was not large, further studies are needed to assess the robustness and significance of this result.

A proximate, mechanistic explanation for the observation that dragonfly larvae induce stronger responses than newts may be that as the former chew prey tadpoles and the latter swallow prey without chewing, more alarm substances may be released into the water from tadpoles that are consumed by dragonfly larvae than by newts (sensu Ferrari et al. 2007). Under our experimental conditions, however, this is not a likely explanation for the observed between-predator-treatment differences in the strength of responses, as in that case the interaction between predator type and types of cues present should

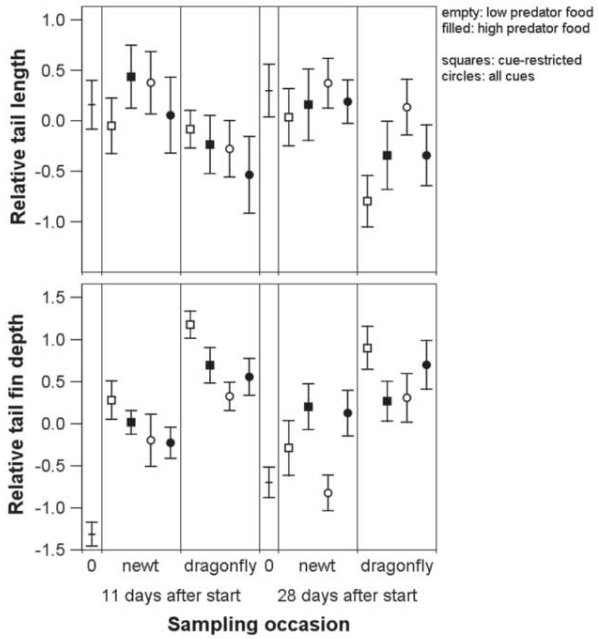

Figure 2. Relative tail length and relative tail fin depth (means ± SE) in the nine treatments at the two sampling occasions. Similar results on relative body length and relative tail muscle depth are presented in an electronic appendix. Empty symbols represent treatments where predators received little food, filled symbols represent treatments where predators received much food. Squares represent cue-restricted treatments, whereas circles represent all-cue treatments. Relative size values are residuals from regressions on body size (the latter calculated as factor scores on PC 1 from PCAs on body length, tail length, maximum tail muscle depth and maximum tail fin depth). The figure is based on rearing tank-averages. Larger values indicate larger sizes relative to overall body size.

Table 2. Results of linear mixed effect models on tadpole activity. Significant effects are highlighted in bold.

Effect	DF	F	p	Wald Z	p
Control incl.					
P/A of predators	1,98	84,14	<**0.001**		
Date	1,498	89,89	<**0.001**		
P/A × date	1,498	88,39	<**0.001**		
Tube identity				4,60	<**0.001**
Control excl.					
Predator type	1,75	2,13	0.148		
Feeding rate	1,75	6,00	0.017		
Types of cues present	1,75	2,97	0.089		
Date	1,397	438.61	<**0.001**		
Predator type × Feeding rate	1,75	3,05	0.085		
Date × Predator type	1,397	7,71	**0.006**		
Date × Type of cues present	1,397	8,01	**0.005**		
All other interactions			>0.1		
Tube identity				3,99	<**0.001**

have been significant: we should have found similar responses to the two predators in the treatments excluding damage-released cues, no-cost disturbance signals and alarm pheromones, and stronger responses to dragonflies than to newts when all types of alarm cues were present. This was, however, not the case for either body size or body shape or behaviour. Consequently, and as food intake of predators was set to an equalized level by controlled feeding, our data indicate that differences in the kairomone-profile, and/or digestion cues provided by the predators may have determined predator-specific responses in tadpoles. Interestingly, kairomones and digestion cues together appear to be sufficient to mount strong inducible defences in behaviour and morphology in *R. dalmatina* (see also LaFiandra and Babbitt 2004, Richardson 2006, Schoeppner and Relyea 2009).

The amount of prey eaten by predators had a clear effect on tadpole activity: when predators were provided with more food, tadpoles generally responded with a larger decrease in activity. Such a graded response to the perceived predation risk has been observed in some studies before in anuran tadpoles (Van Buskirk and Arioli 2002, Teplitsky et al. 2005a, Schoeppner and Relyea 2008) and other taxa (Tollrian 1993, Wiackowski and Staronska 1999, Ferrari et al. 2006). At the second sampling occasion, tadpoles decreased their activity in the presence of little-fed newts less than in the presence of well-fed newts, whereas there was no such difference in the presence of dragonflies. We observed the same pattern in responses in tadpole shape (tail fin depth and body length). Thus, as opposed to our prediction to find synergistic interactions between the information content of direct and indirect cues, these interactions seem to have been complementary: In the presence of newt kairomones, tadpoles fine-tuned their antipredator responses to the concentration of indirect cues informing them about the level of acute predation risk (Kiesecker et al. 2002), whereas such a fine-tuning seemed redundant in the presence of dragonfly kairomones. A possible explanation for this difference is that, apart from infrequent moults, large *Aeshna* larvae are always effective predators of large *R. dalmatina* tadpoles, whereas adult newts are gape-limited and only become dangerous when fully grown, if alternative prey is scarce and breeding activity is low (Griffiths 1985, Kishida and Nishimura 2005, Urban 2007a, 2007b).

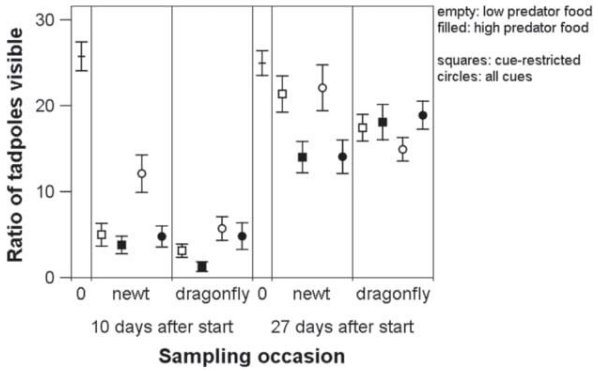

Figure 3. Ratio of tadpoles visible (means ± SE), used as an estimate of tadpole activity, during the first and second sampling occasion. Empty symbols represent treatments where predators received little food, filled symbols represent treatments where predators received much food. Squares represent cue-restricted treatments, whereas circles represent all-cue treatments. For the ease of interpretation, untransformed data are shown.

The presence or absence of alarm cues released during predation-events had relatively weak effects. This result may partly have arisen because some of the tadpole alarm cues have degraded by the time the predation water was poured back together with predators into the tadpole rearing tanks (Turner and Montgomery 2003, Peacor 2006, Ferrari et al. 2008). Nonetheless, young/small tadpoles did respond differentially to the presence or absence of predation-event related indirect cues both morphologically and behaviourally: in their presence, they developed shallower tail fins and deeper tail muscles and lowered activity less than when only digestion-released cues and kairomones were present. These differences in responses disappeared by the second sampling occasion. The diminishing sensitivity of tadpoles to cues on predation threat with increasing size, probably a result of a decrease in tadpole vulnerability to predation (Travis et al. 1985, Semlitsch 1990, Eklöv and Werner 2000), aligns to our other results and to those of previous studies (Van Buskirk 2001, Laurila et al. 2004, Fraker 2008). We expected indirect cues released by the act of predation and ingestion to enhance responses to kairomones and digestion-released cues (also see LaFiandra and Babbitt 2004, Richardson 2006, Schoeppner and Relyea 2009), but observed the opposite. Indeed, our results suggest that the presence of 'old' damage-related and disturbance cues reduce behavioural and morphological defences. However, we can only speculate on the possible reasons for this result and further studies are needed to clarify the causes.

In summary, we observed fine-tuned antipredator responses both in morphology and in behaviour of tadpoles. Induced defences were predator-specific, most likely mediated by kairomones, but a larger amount of indirect cues in the treatments where predators received more food resulted in stronger responses. Our data align to previous studies suggesting that tadpoles use direct cues, and, possibly, also predator-specific digestion-released cues, to adjust the type of responses providing optimal defences against predators using different foraging modes (Kishida and Nishimura 2005, Teplitsky et al. 2005b, Wilson et al. 2005) and indirect cues to adjust the intensity of responses according to the actual predation risk (Van Buskirk and Arioli 2002, Schoeppner and Relyea 2008). Also, large tadpoles reacted only to 'dangerous' newts, probably because these are not always voracious predators of large tadpoles. Information content of direct and indirect cues, thus, seem to have complemented each other. However, small tadpoles did not react to newts as intensively as to dragonfly larvae despite their similar dangerousness, probably because the former is a gape-limited predator. Consequently, the information content of indirect cues indicating the presence of dangerous predators is likely to have interacted antagonistically with the information delivered by kairomones in the case of the newt, and/or synergistically in the case of dragonfly larvae. Finally, we generally observed weaker responses to predators in larger tadpoles, also suggesting that tadpoles optimize their induced defences carefully based on several extrinsic and intrinsic cues, rather than relying on one general cue associated with the act of predation.

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Visual cues contribute to predator detection in anuran larvae

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The ability of prey to detect predators directly affects their probability of survival. Chemical cues are known to be important for predator detection in aquatic environments, but the role of other potential cues is controversial. We tested for changes in behaviour of *Rana temporaria* tadpoles in response to chemical, visual, acoustic, and hydraulic cues originating from dragonfly larvae (*Aeshna cyanea*) and fish (*Gasterosteus aculeatus*). The greatest reduction in tadpole activity occurred when all cues were available, but activity was also significantly reduced by visual cues only. We did not find evidence for tadpoles lowering their activity in response to acoustic and hydraulic cues. There was no spatial avoidance of predators in our small experimental containers. The results show that anuran larvae indeed use vision for predator detection, while acoustic and hydraulic cues may be less important. Future studies of predator-induced responses of tadpoles should not only concentrate on chemical cues but also consider visual stimuli. *Biological Journal of the Linnean Society*, 2012, **106**, 820–827.

ADDITIONAL KEYWORDS: anti-predator behaviour – induced defence – sensory modality – tadpole.

INTRODUCTION

Responding appropriately to predation threat is of fundamental importance for individual fitness (Sih, 1980; Lima & Dill, 1990). The first stage of response involves detecting risk accurately. In aquatic environments, the most important sensory modalities for predator detection are olfaction and vision (Tollrian & Harvell, 1999). Tactile cues, sensed by mechanoreceptors embedded in the skin, can also help detect immediate threats. Further modalities in some taxa include electric and hydraulic cues, sensed by electro- and mechanoreceptors located in the lateral line system. Acoustic cues (also referred to as sonic or auditory cues) may also play a role in predator detection if sound is generated by the predator itself or by prey under attack (e.g. Hoy, 1992; Natale *et al*., 2011; Wilson *et al*., 2011).

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In anuran larvae, chemical cues play a major role in predator detection (e.g. Kiesecker, Chivers & Blaustein, 1996; Laurila, 2000; Benard, 2006), but the importance of other sensory modalities is poorly known and controversial. Tadpoles are near-sighted (Hoff *et al*., 1999) and their habitat often consists of turbid water and dense vegetation; consequently, vision has rarely been studied in the context of predation and is often dismissed as unimportant (Stauffer & Semlitsch, 1993; Kiesecker *et al*., 1996; Jowers *et al*., 2006; Parris, Reese & Storfer, 2006; Saidapur *et al*., 2009). Nonetheless, tadpoles do use vision in other contexts, such as adjusting their swimming movements to those of conspecifics (Wassersug, Lum & Potel, 1981; Rot-Nikcevic, Denver & Wassersug, 2005; Gouchie, Roberts & Wassersug, 2008). Also, anuran larvae respond to tactile stimulation (Rot-Nikcevic *et al*., 2005), and functional mechanoreceptors in the lateral line system allow them to sense water movements (Lannoo, 1999; Simmons, Costa &

Gerstein, 2004; Schmidt, Knowles & Simmons, 2011). One study suggests that hydraulic cues may help tadpoles to detect predators (Stauffer & Semlitsch, 1993). Anurans are unable to sense electric cues because they lack electroreceptors (Lannoo, 1999). Whether tadpoles exploit acoustic cues in predator detection is largely unexplored, although they do have a functional inner ear (Lannoo, 1999) and some species exhibit intraspecific acoustic communication (Natale *et al*., 2011; Reeve *et al*., 2011).

The present study examines whether tadpoles use visual, acoustic, and hydraulic cues for predator detection. Acoustic and hydraulic cues are difficult to separate from each other in practice, so we tested their effects together. We predicted that tadpoles sensing the presence of a predator, regardless of the cue, would decrease activity and move away from the predator (Skelly & Werner, 1990; Stauffer & Semlitsch, 1993; Parris *et al*., 2006). Behavioural responses can vary with the predator species (Van Buskirk, 2001; Teplitsky *et al*., 2005; Hettyey *et al*., 2011), so we included two different predators to increase the likelihood of detecting what we anticipated to be relatively subtle responses to visual, acoustic, and hydraulic cues.

METHODS

Experimental procedures

The prey in our experiment were tadpoles of the European common frog (*Rana temporaria* Linnaeus, 1758), which are known to show strong behavioural responses to many aquatic predators (Laurila, Kujasalo & Ranta, 1997; Van Buskirk, 2001). The predators were larval dragonflies (*Aeshna cyanea* Müller, 1764) and fish (three-spined sticklebacks, *Gasterosteus aculeatus* Linnaeus, 1758), chosen because they are important predators of amphibian larvae, and because they differ in their hunting behaviour: *Aeshna* is a sit-and-wait predator and *Gasterosteus* is an active forager. We collected six freshly laid clutches of *R. temporaria* from a pond in eastern Switzerland (47°02′N, 9°21′E), and held them separately in 10-litre aquaria until hatching. After hatching, we fed tadpoles ad libitum with rabbit chow and changed water every other day. The predators came from ponds near Zurich, Switzerland. We held 45 dragonfly larvae individually in 200-mL plastic cups, and 45 fish in groups of 15 individuals within 80-litre tubs. Predators were fed twice a week with *R. temporaria* tadpoles, but were unfed for 48 h before an experimental trial. We kept all animals in an unheated room with open windows under natural light conditions and water temperatures between 13 and 28 °C.

The experiment had a 3×3 complete factorial design with three combinations of cue crossed with the two species of predator and a predator-free control. Cues were controlled by manipulating a divider that bisected the experimental chambers (polypropylene boxes; 1.0 litre; $20 \times 12 \times 7$ cm) into two parts of equal size (10×12 cm). The divider was either a net with 1.4-mm mesh (assumed to transmit all cues to focal tadpoles), 5-mm-thick transparent Plexiglas (transmitting visual cues but blocking chemical, hydraulic, and possibly acoustic cues), or 0.12-mm opaque and freely vibrating polyethylene foil (assumed to transmit acoustic and hydraulic cues, but blocking chemical and visual cues). Our assumptions about the transmission properties of barriers are untested, but it is reasonable to suppose that a net transmits hydraulic and acoustic cues, Plexiglas blocks both cues, and thin foil transmits these cues to some degree. Experimental chambers were lined with a 0.3-mm polyester filter-paper on the inner surface to minimize sound reflection from the walls or interference from adjacent chambers. Lines drawn on the bottom of each chamber created six equal-sized sectors (1.67 cm wide) at increasing distance from the divider.

Trials were conducted on ten days between 9 and 20 May 2011, 16–27 days after hatching. On each day, we conducted two replicates of the predator-free control for each cue treatment and four replicates of the six combinations of predator species and cue type. These 30 chambers were arranged under two video cameras on a bed of Styrofoam, with treatments assigned at random within each group. The walls of the room were covered with high-frequency-absorbing foam. Animals were acclimatized to the experimental conditions under a dim lamp simulating night for 15 h prior to the trial: tadpoles were in the experimental chambers themselves, and predators were in similar chambers containing a divider of 4-mm opaque Plexiglas. At 09:00 h, we switched on broad-spectrum fluorescent lights, and 5 min later transferred predators to the experimental chambers and turned on the video cameras for 15 min. After each day, we washed experimental and acclimatization chambers and discarded the filter paper. Tadpoles were tested individually and only once, whereas the 45 individual predators of each species were used 2–3 times each, and assigned to treatments haphazardly.

We measured activity and location of both the tadpole and predator at 1-min intervals during the 15 min of trials using the video-recordings. Activity was an appropriate response for this study because much evidence suggests that amphibian larvae react to predation risk by decreasing movement (Lawler, 1989; Skelly, 1994; Van Buskirk & Arioli, 2002). An individual was scored as active if it was visibly feeding or swimming and as inactive if it was motionless. Location was defined as the sector that the animal occupied, with higher values corresponding to increasing dis-

Table 1. The number of trials for each combination of cue treatment and predator treatment available for analyses excluding or including data on predator behaviour

	Predator treatment				
	Without predator behaviour			With predator behaviour	
Cue treatment	Control	Dragonfly	Fish	Dragonfly	Fish
All cues	19	37	34	34	34
Visual cues only	22	39	33	39	32
Acoustic and hydraulic cues	19	27	33	26	33
Total	60	103	100	99	99

The depicted sample sizes represent the number of trials where data on both tadpole activity and tadpole location could be used. Hence, sample sizes in the separate analyses on tadpole activity and on tadpole location were somewhat higher.

tances from the barrier. Several replicates were lost, for various reasons. One chamber with acoustic and hydraulic cues developed a leak and was excluded from all dates. Visual obstructions required us to discard five replicates of tadpole activity, 24 replicates of tadpole location, and five replicates of predator behaviour. In three cases we mistakenly did not add a predator to the chamber, which lowered sample size in the predator treatments and increased the number of trials in the control. In the end, 263 trials were available for analyses of the effects of cue treatment and predator treatment on tadpole behaviour, and 198 trials for analyses of the effects of predator behaviour on tadpoles (details in Table 1).

Statistical analyses

The behaviour of individual tadpoles and predators did not change over the 15-min observation period (all $P \geq 0.29$ in repeated-measures analyses), so analyses were done on averages for each individual. Tadpole activity and location were not highly correlated, and were therefore analysed independently ($r_s = 0.017$, $N = 263$, $P = 0.78$).

First, we investigated whether the two predators differed in their behaviour and whether behaviour depended on cue treatment or date. Predator activity and location were dependent variables in a multivariate linear model with predator species and cue treatment as categorical factors and date as a continuous covariate. Date was included as a covariate to account for potential directional trends in behaviour due to growth and development of tadpoles and predators. Second, we used two separate linear models to test for the effects of predator treatment, cue treatment, and date on tadpole activity and location. Activity and location were the dependent variables, predator treatment and cue treatment were categorical factors, and date was a continuous covariate. Activity was reciprocally transformed to normalize residuals and equalize error variances. To facilitate interpretation, we reversed the sign of the transformed values of tadpole activity in the analyses. Third, we assessed the relationship between predator behaviour and tadpole activity and location in separate analyses in which predator species and cue treatment were factors, and date and residual activity and location of the predator were covariates. The residuals came from a multivariate model with predator species and cue treatment as factors, and date as a covariate. The second analysis did not include covariates representing predator behaviour because the control treatment contained no predator.

We included all two-way interactions into initial models and performed model simplification by applying a backward stepwise removal procedure to avoid problems because of the inclusion of non-significant terms (Engqvist, 2005). Removed variables were re-entered one by one to the final model to obtain relevant statistics. Wherever necessary for the interpretation of results, we performed Bonferroni-corrected pair-wise comparisons. Statistical models were implemented in SPSS 19.0.

RESULTS

Predator behaviour

Predator behaviour depended on predator species, cue treatment, and the interaction between species and cue (multivariate model; species: $F_{2,191} = 594.5$, $P < 0.001$; cue: $F_{4,382} = 9.61$, $P < 0.001$; species × cue: $F_{4,382} = 3.16$, $P < 0.001$). Fish were more active than dragonfly larvae (univariate model; $F_{1,192} = 1004$, $P < 0.001$) and remained further from the divider ($F_{1,192} = 306.7$, $P < 0.001$; Fig. 1). Date and its interactions did not have an effect on predator behaviour (all $P > 0.2$). Subsequent separate analyses for the two predators revealed that the activity of both predators varied among cue treatments (dragonfly larvae: $F_{2,96} = 13.47$, $P < 0.001$; fish: $F_{2,96} = 5.91$, $P = 0.004$):

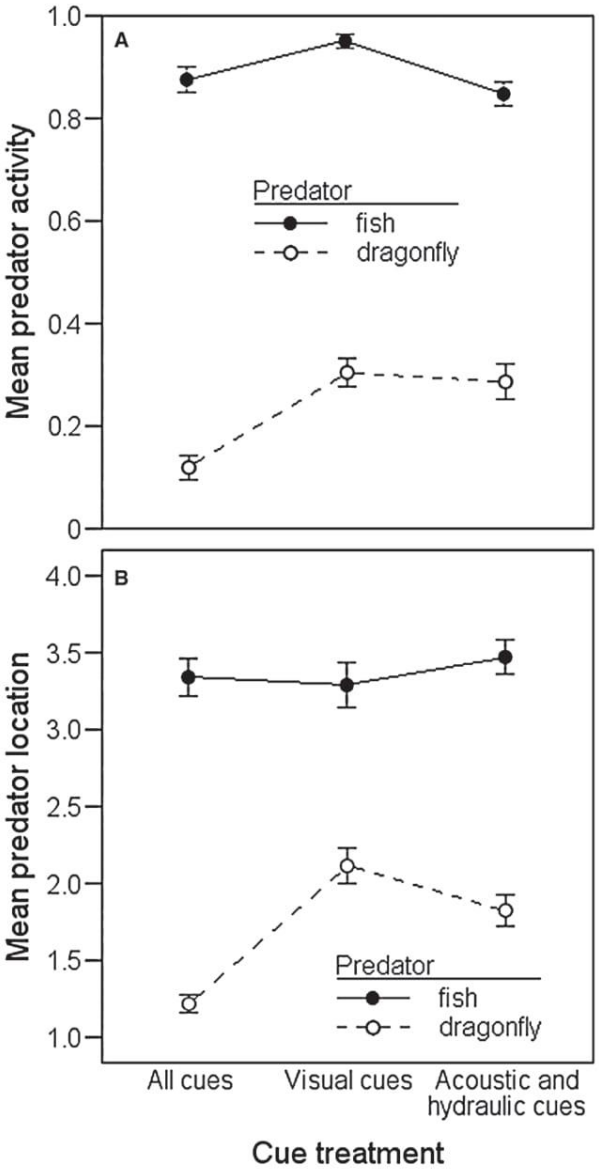

Figure 1. Behaviour of the two predators in the three cue treatments: A, predator activity; B, predator location. Larger values on the *y*-axis indicate higher activity and locations further from the divider. The figure is based on averages calculated for each individual from observations made once every minute over a 15-min period. Means ± SE are indicated.

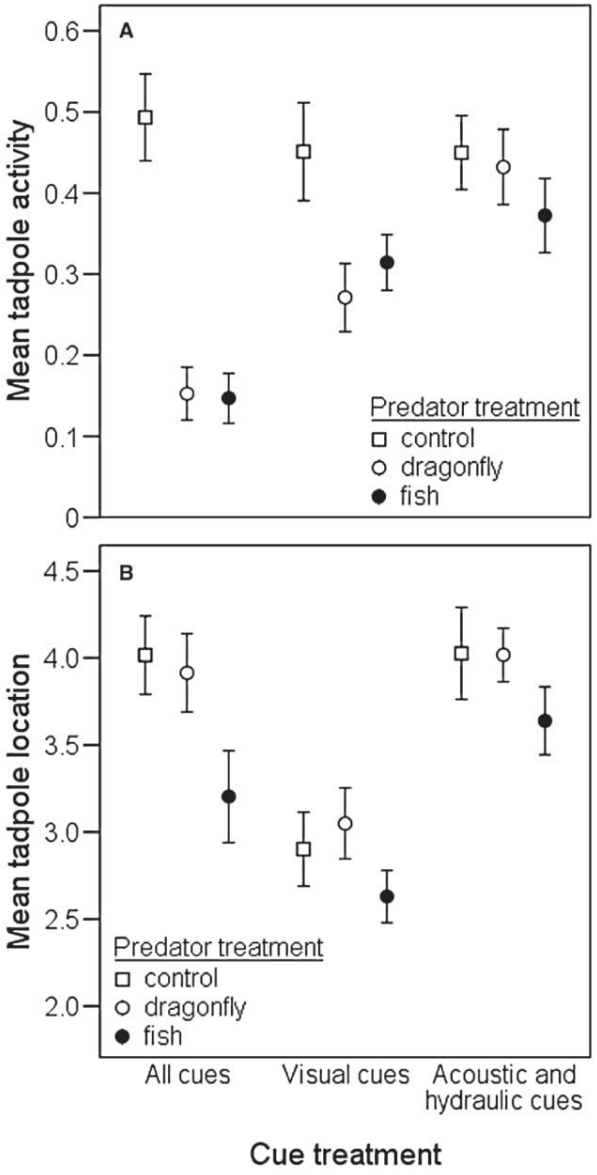

Figure 2. Tadpole behaviour as affected by predator treatment and cue treatment: A, tadpole activity; B, tadpole location. Larger values on the *y*-axis indicate higher activity and locations further from the divider. The figure is based on averages calculated for each individual from observations made once every minute during the 15-min time period of the experiment. For the ease of interpretation, we present untransformed data on tadpole activity. Means ± SE are indicated.

dragonflies were least active when all cues could pass through the divider, and both predator species were most active with only visual cues (Fig. 1A). The location of fish was unrelated to cue treatment ($F_{2,96} = 0.55$, $P = 0.58$), whereas that of dragonfly larvae varied among treatments ($F_{2,96} = 22.58$, $P < 0.001$): the latter were closer to the divider in the all-cues treatment (Fig. 1B).

Tadpole behaviour

Tadpole activity was significantly affected by predator treatment, cue treatment, and their interaction (Table 2; Fig. 2A). When all types of cue were available, activity declined sharply in the presence of either predator (Fig. 2A; control vs. dragonfly larva:

Table 2. Effects of predator treatment, cue treatment, and date on tadpole activity

Effect	d.f.	*B*	SE	*F*	*P*
Overall					
Predator treatment	2, 275	–0.011	0.003	15.436	< **0.001**
Cue treatment	2, 275			10.645	< **0.001**
Date	1, 275			15.027	< **0.001**
Predator treatment × Cue treatment	4, 275			3.871	**0.004**
Predator treatment × Date	2, 273			0.586	0.557
Cue treatment × Date	2, 273			2.448	0.088
All cues					
Predator treatment	2, 93	–0.002	0.005	20.138	< **0.001**
Date	1, 92			0.279	0.599
Visual cues					
Predator treatment	2, 96	–0.017	0.005	3.642	**0.030**
Date	1, 96			12.363	**0.001**
Acoustic and hydraulic cues					
Predator treatment	2, 85	–0.013	0.005	1.299	0.278
Date	1, 87			6.536	**0.012**

Because the interaction between predator treatment and cue treatment was significant, we also present results of three linear models testing the effect of predator treatment and date on tadpole activity in the three cue treatments separately. Significant results are shown in bold type.

$P < 0.001$; control vs. fish: $P < 0.001$; dragonfly larva vs. fish: $P = 1$). When only visual cues were available, activity again declined in the presence of dragonfly larvae compared with the control, whereas tadpole activity was intermediate in the presence of fish (Fig. 2A; control vs. dragonfly larva: $P = 0.025$; control vs. fish: $P = 0.28$; dragonfly larva vs. fish: $P = 0.73$). When only acoustic and hydraulic cues were present, there were no effects of predator treatment on tadpole activity (Table 2; Fig. 2A). The significant effect of date was caused by a general decline in activity over time (Table 2).

Tadpole location was significantly influenced by predator treatment and cue treatment, but not by their interaction (predator: $F_{2,261} = 5.71$, $P = 0.004$; cue: $F_{2,261} = 18.75$, $P < 0.001$; predator × cue: $F_{4,257} = 0.42$, $P = 0.79$; Fig. 2B). Tadpoles moved closer to the divider in the presence of fish (control vs. dragonfly larva: $P = 1$; control vs. fish: $P = 0.035$; dragonfly larva vs. fish: $P = 0.006$; Fig. 2B), and when only visual cues were available (all cues vs. visual cues: $P < 0.001$; all cues vs. acoustic and hydraulic cues: $P = 0.68$; visual cues vs. acoustic and hydraulic cues: $P < 0.001$; Fig. 2B). Tadpoles tended to move closer to the divider during later experiments, although this was not significant ($F_{1,260} = 3.54$, $B = –0.047$, SE = 0.025, $P = 0.061$). All interactions involving date were non-significant ($P > 0.1$).

Residual predator activity was positively related to tadpole activity ($F_{1,188} = 5.4$, $B = 0.153$, SE = 0.066, $P = 0.021$). The main effect of residual predator location ($F_{1,188} = 0.5$, $P = 0.48$) and the interaction terms were non-significant (all $P > 0.09$), except for the interaction between predator type and residual predator location ($F_{1,188} = 4.95$, $P = 0.027$). In the presence of dragonfly larvae, tadpole activity was positively related to residual predator activity ($F_{1,91} = 7.58$, $B = 0.217$, SE = 0.079, $P = 0.007$; Fig. 3) and negatively to residual predator location ($F_{1,91} = 4.29$, $B = –0.047$, SE = 0.023, $P = 0.041$). In the presence of fish, tadpole activity was not related to residual predator activity or location (both $P > 0.24$). Tadpole location was unrelated to residual predator activity or location ($P > 0.15$).

DISCUSSION

Tadpoles of *R. temporaria* reduced activity when they detected predators, as reported in many other anuran species (Lawler, 1989; Van Buskirk, 2002; Laurila, Pakkasmaa & Merilä, 2006). The strongest anti-predator responses have been found when chemical cues are available to tadpoles (Stauffer & Semlitsch, 1993; Kiesecker *et al.*, 1996; Parris *et al.*, 2006). Our results agree with this, because the greatest decline in activity occurred in the treatment with chemical cues, in addition to visual, acoustic, and hydraulic cues. The strength of the response to fish and dragonfly larvae was similar, perhaps because both are important predators of *R. temporaria* (Relyea,

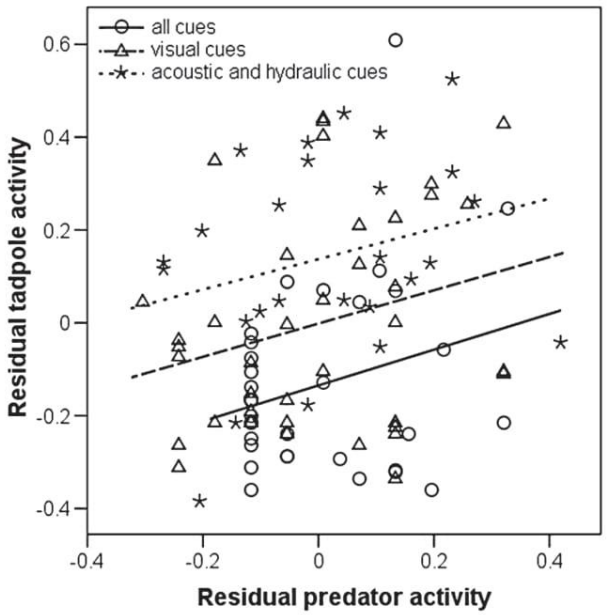

Figure 3. Relationships between tadpole activity and residual predator activity in the presence of dragonfly larvae. Residual values of predator activity originate from the analysis of predator behaviour, whereas residual values of tadpole activity originate from a regression of tadpole activity on date.

2001a; Teplitsky, Plénet & Joly, 2004; Hettyey *et al*., 2011).

Our most noteworthy result was that when only visual cues were available, tadpole activity was lower in the presence of both predators than in the control (Fig. 2A). Previous studies have found either weak evidence for use of visual cues in anuran predator detection (Stauffer & Semlitsch, 1993; Kiesecker *et al*., 1996; Jowers *et al*., 2006; Parris *et al*., 2006) or no support at all (e.g. Saidapur *et al*., 2009). Improvements in study design may account for our results. For example, we used smaller experimental chambers to accommodate the near-sightedness of tadpoles (McDiarmid & Altig, 1999), and we used somewhat older animals than had been used previously because tadpole vision improves throughout the larval stage (Lannoo, 1999). Young tadpoles may not use visual cues, but our results suggest that as they become larger they can recognize predators visually.

When only acoustic and hydraulic cues were available, activity was no different from that observed in the control treatment. This agrees with the single previous study that has tested for the use of acoustic and hydraulic cues (Stauffer & Semlitsch, 1993). Stauffer & Semlitsch (1993) argued that water movements may provide information on predator location that augments chemical information on predator presence. In our study, as well, we cannot exclude the possibility that these cues function in combination with other types of cues during predator recognition and localization. It is also possible that the foil divider in our study weakened or otherwise altered acoustic or hydraulic cues such that they could not be recognized by tadpoles. Thus, further experiments may be necessary to validate our conclusion that acoustic and hydraulic cues are not important.

The relationship between activity of dragonfly larvae and tadpole activity did not differ between cue treatments, as indicated by a lack of a significant interaction between residual predator activity and cue treatment (Fig. 3). A possible interpretation of this result is that tadpoles are able to sense the movements of predators when only visual and when only acoustic and hydraulic cues are available to them, but they recognize predators only using chemical and visual cues but not from acoustic or hydraulic cues. Alternative interpretations are that predators adjust their behaviour to that of tadpoles or that both predators and prey react to other unknown variables. Direct manipulation of predator activity would be required to verify a causal relationship.

We observed no spatial predator avoidance, and in fact tadpoles moved closer to the divider in the presence of fish. Previous studies, conducted in larger arenas, almost always report spatial avoidance of predators (Skelly & Werner, 1990; Relyea, 2001b; Parris *et al*., 2006). We can only speculate that the small size of the experimental chambers used in the present experiment did not leave much space for tadpoles to express spatial avoidance (also see Parris *et al*., 2006). The observation that tadpoles were significantly closer to the divider when only visual cues were available might indicate that they misjudged the size of the container in the presence of the completely transparent Plexiglas divider, and attempted to explore and use all of the apparently available space.

Vision may be important for tadpoles living in oligotrophic habitats with little vegetation and clear water, or at very close range in meso- and eutrophic habitats with dense vegetation and murky water. On a longer timescale, chemical cues may provide information on the types of predators present, and on their abundance and dangerousness. Prey clearly adjust their phenotypic responses according to chemical signals from different densities and species of predator (e.g. Van Buskirk & Arioli, 2002; Teplitsky *et al*., 2005; Hettyey *et al*., 2011). However, when a predator approaches and an immediate threat develops, the concentration of chemical cues in the water is less relevant than the visual cues immediately available to the prey. Our results suggest that vision does indeed play an important role in eliciting anti-predator behaviour in anuran larvae, at least when

the predator is at close range. Thus, future studies on predator-induced defences of tadpoles should not concentrate solely on chemical stimuli, but also take visual cues into consideration. After all, while chemical cues alone can induce defensive responses in tadpoles (Petranka & Hayes, 1998; Schoeppner & Relyea, 2005, 2009; Fraker *et al*., 2009), both chemical and visual cues may be required to develop the full suite and magnitude of defences.

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BEHAVIORAL ECOLOGY - ORIGINAL RESEARCH

The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles

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Abstract Chemical cues that evoke anti-predator developmental changes have received considerable attention, but it is not known to what extent prey use information from the smell of predators and from cues released through digestion. We conducted an experiment to determine the importance of various types of cues for the adjustment of anti-predator defences. We exposed tadpoles (common frog, *Rana temporaria*) to water originating from predators (caged dragonfly larvae, *Aeshna cyanea*) that were fed different types and quantities of prey outside of tadpole-rearing containers. Variation among treatments in the magnitude of morphological and behavioural responses was highly consistent. Our results demonstrate that tadpoles can assess the threat posed by predators through digestion-released, prey-borne cues and continually released predator-borne cues. These cues may play an important role in the fine-tuning of anti-predator responses and significantly affect the outcome of interactions between predators and prey in aquatic ecosystems. There has been much confusion regards terminology used in the literature, and therefore we also propose a more precise and consistent binomial nomenclature based on the timing of chemical cue release (stress-, attack-, capture-, digestion- or continually released cues) and the origin of cues (prey-borne or predator-borne cues). We hope that this new nomenclature will improve comparisons among studies on this topic.

Keywords Alarm signal · Inducible defence · Kairomone · Phenotypic plasticity · Predator labelling

Introduction

Prey have evolved a variety of mechanisms that lessen the threat of predation, including behavioural, physiological and morphological responses. These responses are not necessarily present at all times, but can be induced by signals indicating predation risk. The expression of inducible defences is expected to be optimally adjusted, within the limits of plasticity, such that protection is maximized and costs are minimized (Harvell 1990; DeWitt et al. 1998; Tollrian and Harvell 1999). Adaptively adjusting inducible defences requires that prey are able to detect reliable cues regarding the type, abundance and dangerousness of predators present in the environment (Moran 1992). Different kinds of cues may be favoured in different ecological contexts, but in aquatic environments—and especially in turbid waters—chemical cues are considered the most important sensory modality for detecting predators (Kats and Dill 1998; Tollrian and Harvell 1999; Brönmark and Hansson 2000).

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Table 1 A tabulated summary of the suggested terminology and classification of chemical cues of predation threat

Timing of release	Popular term	Suggested term	Constituents
	Indirect cues	Prey-borne cues	
Pre-consumption	No-cost disturbance signals	Stress-released cues	General prey metabolites
	Alarm pheromones	Attack-released cues	Alarm pheromones
	Damage-released cues	Capture-released cues	Alarm pheromones, tissue fragments
Post-consumption	Digestion-released cues	Digestion-released cues	Constituents of digested prey
	Direct cues	Predator-borne cues	
Pre-consumption	–	Capture-released cues	Saliva
Post-consumption	Kairomones/digestion-released cues	Digestion-released cues	Digestive fluids, digestive tract tissue, gut flora
Continuously	Kairomones	Continually released cues	Chemicals and tissue fragments from integument

Many studies have demonstrated the induction of antipredator defences mediated through chemical cues, but drawing general conclusions about the underlying mechanisms has been hampered by ambiguities and differences in terminology and definitions (see Appendix, Box 1). Therefore, we suggest a new terminology and a classification of terms regarding chemosensory-mediated predator detection (Table 1), which we hope will help clarify our study and future studies as well. Henceforth, we use this new terminology. We collectively refer to stress-, attack- and capture-released prey-borne cues as pre-consumption prey-borne cues throughout the text because the experimental design does not allow us to differentiate among their effects.

Numerous studies demonstrate the role of pre-consumption prey-borne cues in the induction of antipredator responses (for a review see Chivers and Smith 1998), but similarly comprehensive and convincing studies of continually released predator-borne cues and digestion-released prey- or predator-borne cues are scarce. One recurring problem is the uncertainty about whether prey-borne cues are present. Studies designed to investigate effects of predator-borne cues often do not report how long predators were deprived of food before exposing them to focal prey. Even if the duration of food restriction is known, it is not always clear that prey-borne cues are completely absent. The rate of degradation of pre-consumption prey-borne cues has been measured (Peacor 2006; Ferrari et al. 2008; Van Buskirk et al. 2014), but predators may defecate long after they consumed prey and digestion-released prey-borne cues may therefore persist. Observed prey responses may therefore not be attributed solely to continually released predator-borne cues (but see Petranka and Hayes 1998; Schoeppner and Relyea 2009). Also, the results of studies that investigate whether prey exploit information contained in digestion-released cues are inconclusive (e.g. Laurila et al. 1997, 1998; Schoeppner and Relyea 2005, 2009; Richardson 2006; Ferrari et al. 2007; Ferland-Raymond et al. 2010). Differences among treatments cannot unambiguously be assigned to effects of digestion-released cues, because pre-consumption prey-borne cues or continually released predator-borne cues are not always eliminated, or a synergistic effect between these two cannot be excluded.

Clarifying the origin of chemical cues is critical for understanding the proximate mechanisms through which aquatic prey detect predators and express antipredator defences. It has been argued that prey use predator-borne cues to adjust the type of response, and prey-borne cues to adjust the intensity of response (Kishida and Nishimura 2005; Teplitsky et al. 2005; Wilson et al. 2005; Schoeppner and Relyea 2008; Hettyey et al. 2010). Also, while predator-borne and prey-borne cues can induce behavioural responses in isolation in some species (Petranka and Hayes 1998; Fraker et al. 2009), both types of chemical cues may be necessary for developing the full suite and magnitude of induced defences (Van Buskirk and Arioli 2002; Schoeppner and Relyea 2005, 2009; Richardson 2006; Hettyey et al. 2010).

In theory, predator-borne cues and digestion-released cues could provide prey with very specific information on the abundance, location and recent feeding habits of the predators in their environment, while pre-consumption prey-borne cues could provide more general information about the whereabouts and overall feeding activity of predators. Also, prey that rely solely on pre-consumption cues would not detect predators that have not fed recently. Finally, we expect predator-borne and digestion-released cues to be used by prey when adjusting their phenotypic responses to predation threat because different responses vary in their effectiveness against different types of predators, and predators may differ in their activity profile and in their food and microhabitat preferences.

This study was designed to disentangle effects of continually released predator-borne cues and digestion-released prey- or predator-borne cues on the antipredator responses of prey. We used combinations of different cue types, which allowed us to evaluate the relative importance of the cues and estimate the suite of cues necessary for the induction of the full intensity of inducible defences. We used tadpoles of

the common frog (*Rana temporaria*), which are known to adjust their behaviour and morphology in response to predators (e.g. Laurila et al. 1997; Van Buskirk 2001; Teplitsky and Laurila 2007). By using predator-naive tadpoles, we excluded any confounding effects of learning (e.g. Gonzalo et al. 2007; Fraker 2009; Chivers and Ferrari 2013). We addressed the following main hypotheses:

1. Continually released predator-borne cues and digestion-released cues interact with pre-consumption cues and with each other in eliciting a response.
2. Effects of digestion-released cues are graded according to the phylogenetic distance between focal tadpoles and prey.
3. Varying quantities of digestion-released cues result in graded responses in tadpoles.

Materials and methods

Experimental design

We performed an outdoor mesocosm experiment in which *R. temporaria* tadpoles were exposed to ten treatments, each replicated ten times in a randomized spatial block design. A high level of replication was necessary to deliver the power to evaluate hypotheses where previous studies failed to provide decisive answers [e.g. effect of continually released predator-borne cues in isolation (Schoeppner and Relyea 2009)]. The ten treatments exposed focal tadpoles to chemical cues of different sources and kinds (Table 2):

- A no-predator control provided baseline data for the description of the predator-naive tadpole phenotype (T1).
- Predators fed with live conspecific prey provided all types of chemical cues (T2).
- Homogenized tadpoles in the absence of predators exposed focal tadpoles to pre-consumption prey-borne cues (T3).
- A starved predator allowed only continually released predator-borne cues (T4).
- Homogenized tadpoles together with a starved predator provided a combination of pre-consumption prey-borne cues and continually released predator-borne cues while excluding digestion-released cues (T5).
- Predators fed with *Chironomus* midge larvae, *Bufo bufo* tadpoles, *Rana arvalis* tadpoles, or *Rana temporaria* tadpoles, respectively, and subsequently washed to remove pre-consumption prey-borne cues, so that a combination of continually released predator-borne cues and digestion-released cues was present; the digestion-released cues originated from four prey taxa that differed in their phylogenetic relatedness to the focal tadpoles (T6-T9).
- Predators fed twice as much conspecific prey and subsequently washed, to provide elevated levels of digestion-released cues (T10).

Table 2 summarizes which kinds of cues were present in each treatment.

The experimental design allowed us to make three kinds of comparisons. (a) We tested whether *cue type* affected antipredator responses by comparing T1-T5 and T9. These

Table 2 A list of procedures and the types of cues present in the ten treatments [predator (*P*), only handling of an empty cage (*no P*), *Rana temporaria* tadpoles (*Rt*), homogenized *Rana temporaria* tadpoles (*Rt mix*), chironomid larvae (*Ch*), *Bufo bufo* tadpoles (*Bb*), *Rana arvalis* tadpoles (*Ra*), double amount (*2×*), predator washed three times after feeding (*wash*)]

Treatment code	Procedure	Prey-borne cues		Predator-borne cues	
		Pre-consumption	Digestion-released	Digestion-released	Continually released
T1	No P	−	−	−	−
T2	P fed Rt	+	+	+	+
T3	Rt mix	+	−	−	−
T4	Starved P	−	−	−	+
T5	Starved P + Rt mix	+	−	−	+
T6	P fed Ch + wash	−	+	+	+
T7	P fed Bb + wash	−	+	+	+
T8	P fed Ra + wash	−	+	+	+
T9	P fed Rt + wash	−	+	+	+
T10	P fed 2 × Rt + wash	−	+	+	+

This design does not distinguish between various types of pre-consumption prey-borne cues, but rather focuses on digestion-released cues of both origins and on continually released predator-borne cues. We do not list pre-consumption predator-borne cues (present only in T2) because we know very little about them and the design does not support conclusions regarding their importance

analyses investigated whether prey-borne and predator-borne cues interacted with each other and with pre-consumption cues in determining the strength of responses. These comparisons were also suitable for assessing if all types of cues were necessary to mount the full intensity of inducible defences. (b) We assessed the hypothesis that *prey type* matters for inducible defences by comparing T4 and T6-T9. These analyses tested whether effects of digestion-released cues are graded according to the phylogenetic distance between focal species and the prey consumed by the predator, as had been observed for pre-consumption cues (Laurila et al. 1997, 1998; Schoeppner and Relyea 2005; Fraker 2009). Finally, (c) we investigated the importance of *prey quantity* based on T4, T9, and T10. These comparisons tested whether varying quantities of digestion-released cues resulted in graded responses in focal tadpoles, as they do when all cues are available (Van Buskirk and Arioli 2002; Ferrari et al. 2005; Fraker 2008; McCoy et al. 2012). A positive result would provide another line of evidence for sensitivity to digestion-released cues by tadpoles.

Experimental procedures

The experiment was conducted in rectangular plastic mesocosms (29 L, 0.18 m^2), covered with mosquito netting and placed outdoors at the Konrad Lorenz Institute of Ethology, Vienna. We used relatively small rearing containers to be able to obtain adequate sample sizes and statistical power (see above). Mesocosms were filled with tap water 2 weeks before the start of the experiment. Two days later we stocked mesocosms with 15 g of dried leaves (*Fagus sylvatica*) to provide shelter and nutrients for tadpoles, and added to each mesocosm 1 L of water containing phyto- and zooplankton from a nearby pond to enhance algal growth and maintain water quality. Each mesocosm was fitted with a predator cage made of opaque plastic tube; a double net bottom allowed free exchange of chemical cues while preventing predators from injuring focal tadpoles. Visual and tactile cues may also play a role in predator detection (Stauffer and Semlitsch 1993; Hettyey et al. 2012), but chemical cues seem to be the most important for tadpoles, and strong antipredator responses have been reported when only chemical cues were available to focal individuals (Stauffer and Semlitsch 1993; Ferland-Raymond et al. 2010; Winkler and Van Buskirk 2012).

The experimental animals were hatched from eggs deposited in captivity by ten pairs of adult *R. temporaria* collected at a pond near Vienna (48°13′N, 16°17′E). Clutches were reared separately in containers placed outdoors until the experiment began, and tadpoles were completely naive to predators. Predators in this study were larvae of the dragonfly *Aeshna cyanea*, because these are abundant and important predators of anuran tadpoles in central European wetlands (e.g. Van Buskirk 2009; Hettyey et al. 2011). The *A. cyanea* dragonfly larvae (instars F-1 and F-2) came from a pond in Hungary (47°44′N, 19°01′E) and food for the dragonflies came from ponds in Styria (46°46′N, 15°39′E; *R. arvalis*) and Vienna (48°12′N, 16°15′E; *B. bufo*), or from a local pet shop (live chironomids). The predators were kept individually in 0.3-l cups and fed on chironomid larvae and *R. temporaria* tadpoles until 3 days before the start of the experiment. In a preliminary study, we confirmed that *A. cyanea* larvae do not defecate after being deprived of food for 3 days. It has been claimed that not only defecation may generate digestion-released prey-borne cues (Brown et al. 1995), but cues that label the predators as dangerous are known to degrade within 48 h or less (Peacor 2006; Ferrari et al. 2008; Van Buskirk et al. 2014).

Predators were fed every day, except when we performed behavioural observations (see below). At feeding events, we brought predators to the laboratory and fed them in 100-ml cups with 25 mg of the appropriate prey. The two ground-tadpole treatments were prepared 1 h after the predators were fed, by placing 600 mg of *R. temporaria* tadpoles into 120 ml of aged tap water and grinding them with a mixer. Tadpoles were dead within seconds of turning on the mixer, but we intentionally did not anaesthetize them to ensure that anaesthetics were not present and that tadpoles were not unconscious preceding death. Homogenized tadpoles have been used in similar studies as a source of pre-consumption prey-borne cues and are well known to induce clear responses in prey (e.g. Petranka and Hayes 1998; Schoeppner and Relyea 2005; Ferrari et al. 2008; but see Fraker et al. 2009). Feeding cups in T2 and T4 each received 5 ml of this tadpole mixture. Two hours after the start of feeding, we removed any uneaten prey from the cups and washed predators in T6-T10 by pouring out and refilling feeding cups three times. In previous studies, pre-consumption cues were reduced below detectability by washing predators once (LaFiandra and Babbitt 2004) or twice (Richardson 2006) or by changing the predator water after feeding and waiting for 24 h (Ferland-Raymond et al. 2010). Consequently, it seems likely that washing predators three times effectively excluded pre-consumption cues of predation. The contents of all feeding cups were poured into the respective predator cages 4 h after the start of feeding. To equalize disturbance caused by feeding, we handled cages at the beginning of feeding in T1 and T2 and added 100 ml of tap water at the end. Once a week we rotated predators within treatments to minimize variation arising from individual predators. Starved predators in T3 and T4 were exchanged after 11 days with dragonflies that had not been fed for 3 days.

We started the experiment when *R. temporaria* tadpoles were free-swimming [stages 25, 26 (Gosner 1960)].

We first fed the *Aeshna* and added one to each cage, where appropriate. Then we added ten tadpoles to each mesocosm, one from each of the ten *R. temporaria* sibships. The resulting density of 57 tadpoles/m^2 lies well within the range found under natural conditions (Van Buskirk 2009).

We observed tadpole behaviour on days 9, 18 and 27 after starting the experiment, by visiting each mesocosm four times between 1100 and 1500 hours. On each visit, we noted the number of tadpoles that were located in the third of the mesocosm closest to the predator cage, the number of active tadpoles and the number of tadpoles visible. A tadpole was scored as being active if it was swimming or moving its tail while feeding (for similar methods, see Laurila et al. 2006; Schoeppner and Relyea 2005, 2009; Winkler and Van Buskirk 2012).

We made morphological measurements at the end of the experiment [days 28 and 29, when tadpoles were at about stage 32 (Gosner 1960)]. All tadpoles were removed, anaesthetized lightly with 0.02 m/m % tricaine, weighed to the nearest milligram, and photographed in lateral and ventral view with a digital camera. From the photographs we later measured head length, head depth, head width, tail length, tail fin depth and tail muscle depth (using UTHSCSA ImageTool version 3.0). These morphological measures together define the general head and tail shape of a tadpole and are sensitive to the presence of predators (Laurila et al. 2004; Teplitsky et al. 2004). The six size measurements were defined following Van Buskirk and McCollum (2000) except that tail muscle depth was measured at the location of maximum tail fin depth. We did behavioural observations and morphological measurements blindly with respect to treatment.

Statistical analyses

We tested for treatment effects on the survival, body mass, behaviour and body shape of tadpoles. Survival was the arcsine-square-root transformed proportion alive at days 28, 29. In six mesocosms there were 11 survivors, suggesting that we added more than ten tadpoles to some mesocosms when setting up the experiment. In these six cases we set survival to 1. The error was random with respect to treatments, but our survival results must nevertheless be interpreted with some caution. Body mass was log-transformed mass on days 28, 29, after excluding seven extremely small tadpoles with mass <300 mg (compared with an average of 746 mg ± 131 SD for the remaining 953 survivors). The proportions of live tadpoles close to the predator cage, active, and visible above the leaf litter were calculated assuming a linear mortality curve. The behavioural data were arcsine-square-root transformed, averaged for each date, and subjected to principal components (PC) analysis (PCA) to produce a single component that explained 83.2 % of the variance. The original measures of behaviour loaded strongly and positively on the first component (PC1; near predator cage, 0.87; activity, 0.92, visibility, 0.95). Low values corresponded to mesocosms in which tadpoles were far from the predator cage, moved little, and hid frequently under the leaf litter. This combination of behaviours is characteristic of tadpoles that are threatened by predators (Kats and Dill 1998), and is associated with elevated survival under predation threat (McCollum and Van Buskirk 1996; Laurila et al. 2006; Takahara et al. 2008).

We derived a single biologically relevant index of body shape from the six measures of head and tail. The measures were regressed against the square-root of mass, and the mesocosm-means of residuals were subjected to PCA. The first component (PC1) explained 62.5 % of the variance and all original shape measures loaded strongly on it (head length, −0.86; head depth, 0.81, head width, 0.80; tail length, −0.84; tail fin depth, 0.85; tail muscle depth, 0.53). A large value of PC1 corresponded to a short tadpole with a wide and high head and deep tail fin and muscle. This combination of traits is typical of tadpoles exposed to odonate predators (Van Buskirk 2002; Relyea 2003; Laurila et al. 2004) and confers enhanced survival under predation (McCollum and Van Buskirk 1996; Teplitsky et al. 2005; Hettyey et al. 2011). PC2, mainly representing variation in tail muscle depth, responded to treatments qualitatively the same as PC1; for the sake of simplicity, we present only the results of the first component.

All responses were analysed using general linear models (GLM) with treatment and spatial block as fixed factors. The analysis of mass included the number of tadpoles as a covariate to control for variation in resource availability. The analysis of behaviour observed over three dates was a repeated-measures GLM, and when the time-by-treatment interaction was significant we fitted separate models for each date. We designed three sets of planned contrasts to address the hypotheses outlined above. A contrast among treatments T1–T5 and T9 tested the effects of cue type; that among treatments T4 and T6–T9 tested the effects of prey type; that among treatments T4, T9, and T10 tested the effect of prey quantity. Within the planned contrasts, we used Tukey's honest significant difference (HSD) tests for pairwise comparisons among treatments and for delineating homogeneous subsets of treatments. Appendix A gives the full list of pairwise comparisons. Multivariate analyses on the three original measures of tadpole behaviour and on the six original tadpole-shape measures yielded qualitatively very similar results (Appendices B, C). Statistical models were implemented in IBM SPSS Statistics 20.

Results

Survival and body mass

Survival averaged 0.955 and varied significantly among blocks (GLM; $F_{9,81} = 9.85$; $P < 0.001$) and treatments ($F_{9,81} = 2.55$; $P = 0.012$): it was lowest in mesocosms containing a starved dragonfly larva (mean ± SE 0.89 ± 0.03), and was similar in all other treatments (range 0.94–1.0). Average tadpole mass also varied among blocks (GLM; $F_{9,80} = 8.8$; $P < 0.001$) and treatments (GLM; $F_{9,80} = 5.44$; $P < 0.001$). Mass was, on average, around 770 mg in all treatments (range 752–783 mg), except for the treatments receiving ground *R. temporaria* tadpoles (683 ± 16 mg) and those containing a dragonfly larva fed with *R. temporaria* tadpoles not subjected to washing after feeding (665 ± 25 mg). The number of tadpoles in the mesocosm at termination had a significant negative effect on body mass ($F_{1,80} = 5.74$; $B = -0.062$; SE 0.026; $P = 0.019$).

Behaviour

Analysis of cue type (T1–T5 and T9) revealed that tadpoles were least active when exposed to predators fed on *R. temporaria* tadpoles (Fig. 1a–c). Repeated-measures analysis indicated that behaviour changed over time and varied significantly among treatments, and that the interaction between time and treatment was significant (time, $F_{2,90} = 35.32$; $P < 0.001$; treatment, $F_{5,45} = 64.2$; $P < 0.001$; time × treatment, $F_{10,90} = 3.46$; $P = 0.001$). Effects of block and its interaction with time were non-significant (block, $F_{9,45} = 1.9$; $P = 0.077$; time × block, $F_{18,90} = 1.54$; $P = 0.094$). The overall pattern of behaviour was similar on the three sampling dates. Tadpoles reacted most strongly to predators fed conspecific tadpoles (T2). The response to starved predators and homogenized conspecifics (T5) was intermediate between the control and T2, and differed from both (T5 vs. T1 and T2; all $P < 0.002$). Also, tadpoles exposed to homogenized tadpoles or to starved predators tended to show induced behaviour as compared to the control on all sampling dates (T3 and T4 vs. T1; 9 days after start, both $P < 0.085$; 18 days after start, both $P < 0.004$; 27 days after start, T3 vs. T1, $P < 0.001$, T4 vs. T1, $P = 1$). However, the responses to homogenized tadpoles or starved predators were at times weaker, and at times similar to those of tadpoles exposed to both homogenized tadpoles and starved predators (T3 and T4 vs. T5; 9 days after start, both $P < 0.02$; 18 days after start, both $P > 0.39$; 27 days after start, T3 vs. 5: $P = 1$, T4 vs. 5, $P < 0.001$). Washing the predator had no effect on the behavioural response on the first two sampling dates (T2 vs. T9; both $P > 0.7$), whereas it weakened the response on the third date ($P = 0.003$).

The analysis on the behavioural effects of prey type (T4 and T6–9) revealed that tadpoles reacted more strongly to predators feeding on conspecific or phylogenetically closely related prey than to starved predators or to predators feeding on phylogenetically distantly related prey (Fig. 1d–f). Time-dependent changes in behaviour and among-treatment differences were again significant, while the interaction of time and treatment was significant as well (repeated-measures GLM; time, $F_{2,72} = 55.61$; $P < 0.001$; treatment, $F_{4,36} = 33.08$; $P < 0.001$; time × treatment, $F_{8,72} = 2.9$; $P = 0.007$). Block and its interaction with time were again non-significant (block, $F_{9,36} = 1.78$; $P = 0.11$; time × block, $F_{18,72} = 1.41$; $P = 0.16$). Behaviour of tadpoles exposed to predators fed with phylogenetically unrelated or distantly related prey (*Chironomus* or *B. bufo* larvae) and subsequently washed did not differ significantly at any sampling occasion from that of tadpoles exposed to starved predators (T6 and 7 vs. T4; all $P > 0.16$), while tadpoles exposed to predators fed with conspecific prey and subsequently washed showed stronger induced changes (T4, 6 and 7 vs. T9; all $P < 0.004$). The behaviour of tadpoles exposed to predators fed with conspecific or phylogenetically closely related prey did not differ significantly at any sampling occasion (T8 vs. 9; 9 days after start, $P = 0.99$; 18 days after start, $P = 0.89$; 27 days after start, $P = 0.063$).

In the analysis of the effects of prey quantity, there was a strong reaction to predators fed conspecific tadpoles but no effect of the quantity of food consumed by the predators (Fig. 1g). Repeated-measures analysis revealed significant effects of treatment and time, but no interaction between them (treatment, $F_{2,18} = 48.44$; $P < 0.001$; time, $F_{2,36} = 34.06$; $P < 0.001$; treatment × time, $F_{4,36} = 0.23$; $P = 0.92$). Tadpoles exposed to predators fed conspecifics showed stronger responses than those exposed to starved predators (T4 vs. T9; $P < 0.001$). Doubling the amount of prey did not further elevate behavioural responses (T9 vs. T10; $P = 0.54$). The effect of block and its interaction with time were both non-significant (block, $F_{9,18} = 2$; $P = 0.1$; block × time, $F_{18,36} = 0.8$; $P = 0.69$).

Body shape

Planned contrasts testing effects of cue type (T1–T5 and T9) revealed that homogenised tadpoles induced no change in the shape of the head and tail, and feeding predators induced a stronger change than starved predators (Fig. 2a). There was significant overall variation among treatments (GLM; $F_{5,45} = 89.38$; $P < 0.001$) and blocks ($F_{9,45} = 6.08$; $P < 0.001$). Tukey's HSD post hoc tests indicated that control tadpoles did not differ significantly from tadpoles exposed solely to homogenized conspecifics ($P = 0.89$), whereas all remaining pairwise comparisons among treatments were significant (all $P < 0.007$).

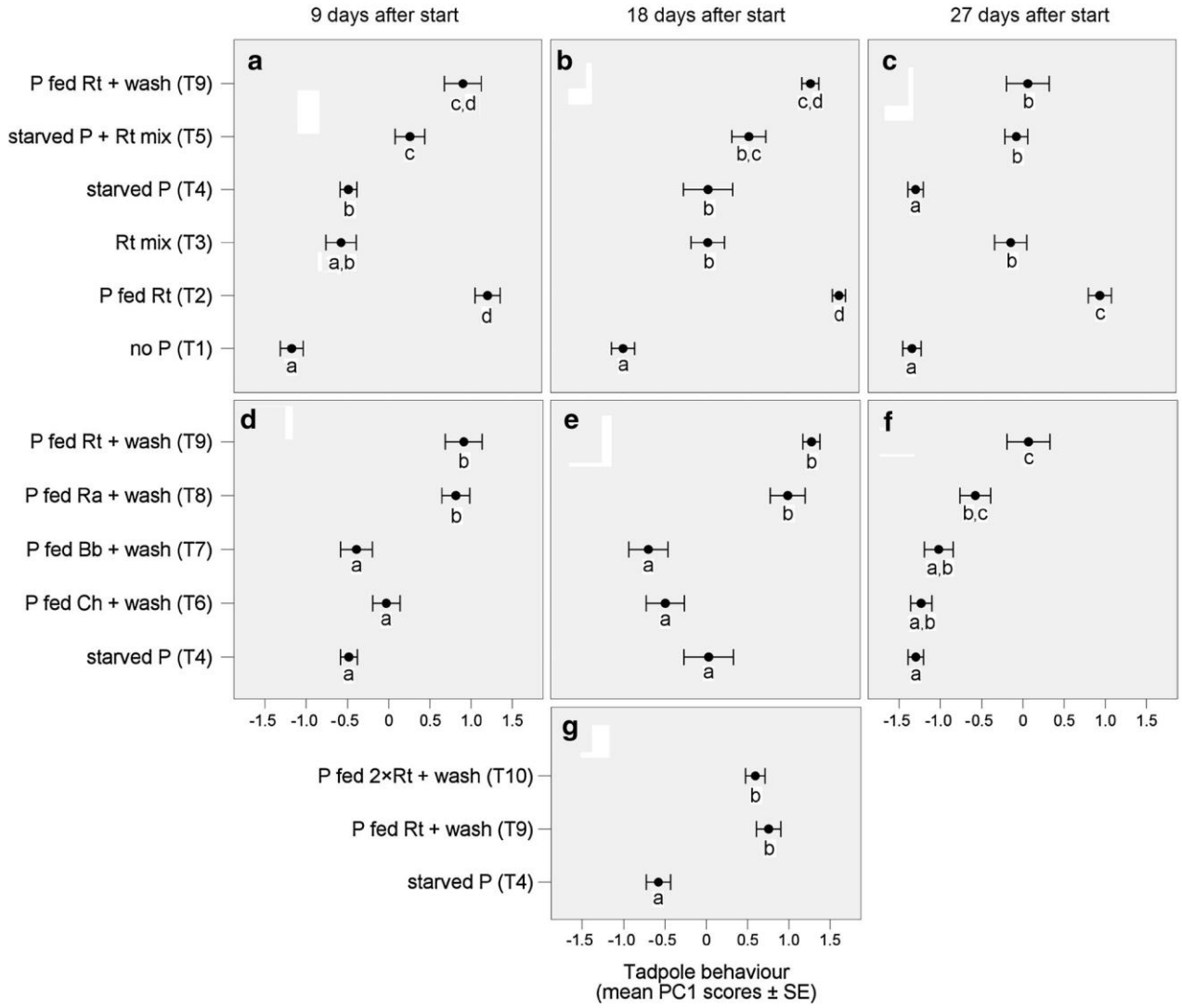

Fig. 1 Behaviour of tadpoles observed 9, 18 and 27 days after the start of the experiment ($n = 10$ in all treatments). Behaviour was calculated as principal component (PC)1 scores of a PC analysis (PCA) on percent active, percent close to the predator cage, and percent visible. To facilitate comparison between treatment effects on behaviour and morphology, we depict component scores after multiplication by −1, so that high values on the behaviour axis correspond to low activity, few tadpoles close to the predator cage, and few tadpoles visible. Symbols are mean ± SE. *Letters* depict homogeneous subsets calculated using Tukey's honest signficant difference (HSD) tests in planned comparisons on the effects of **a–c** cue type, **d–f** prey type and **g** prey quantity. The effect of prey quantity is depicted only on *one panel* because it was similar on all sampling occasions. *P* Predator, *no P* only handling the empty cage, *Rt Rana temporaria* tadpoles, *Rt mix* homogenized *Rana temporaria* tadpoles, *Ch* chironomid larvae, *Bb Bufo bufo* tadpoles, *Ra Rana arvalis* tadpoles, *2×* double amount, *wash* predator washed three times after feeding

Analysis on the effects of prey type (T4 and T6-T9) indicated that tadpoles had deeper tails and shorter heads when exposed to predators fed with *R. arvalis* or *R. temporaria* prey (Fig. 2b). Body shape varied among treatments and blocks (treatment, $F_{4,36} = 27.73$; $P < 0.001$; block, $F_{9,36} = 5.3$; $P < 0.001$). Post hoc tests showed that shape was similar in treatments where tadpoles were exposed to the smell of starved predators or predators fed with chironomid or *B. bufo* prey (Tukey's HSD pairwise comparisons among T4, T6 and T7, all $P > 0.18$) and when predators fed on *R. arvalis* or *R. temporaria* (T8 and T9, $P = 0.83$). All pairwise comparisons between these two sets of treatments were significant (all $P < 0.001$).

Doubling the quantity of prey consumed had no further impact on tadpole morphology (Fig. 2c). Comparison of T4, T9 and T10 revealed significant variation in body shape among treatments ($F_{2,18} = 39.3$; $P < 0.001$) and no block effect ($F_{9,18} = 1.59$; $P = 0.19$). Post hoc tests showed that

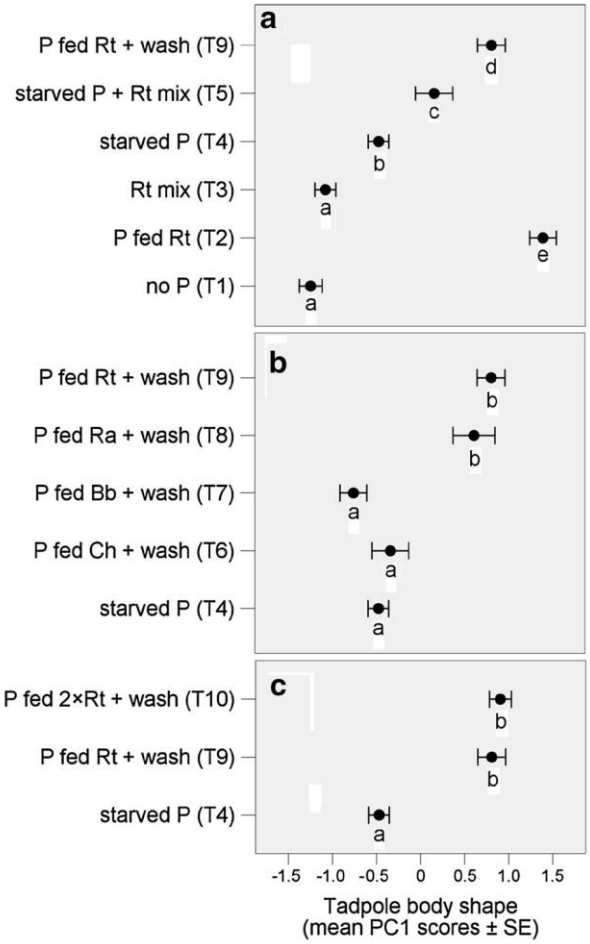

Fig. 2 Mean head and tail shape (±SE) of tadpoles sampled 28 days after the start of the experiment. The figure is based on mesocosm means, consequently, sample sizes equalled ten in all treatments. Shape is a score on the first axis of a PCA on six measures of the head and tail after correcting for mass. Higher PC1 scores represent a relatively short total length with a wide and high head and deep tail fin and tail muscle, which corresponds to a tadpole reacting to the presence of an odonate predator. *Letters* depict homogeneous subsets calculated using Tukey's HSD tests in planned comparisons on the effects of **a** cue type, **b** prey type and **c** prey quantity. For abbreviations, see Fig. 1

tadpoles exposed to the smell of predators fed with conspecific tadpoles had higher values of PC1 than those exposed to starved predators (T4 vs. T9 or T10, both $P < 0.001$), while the amount of prey did not make a significant difference (T9 vs. T10, $P = 0.84$).

Discussion

Our study demonstrates that anuran larvae use the information encoded in both continually released predator-borne cues and digestion-released prey-borne cues to adjust antipredator responses. Both types of chemical cue proved to be necessary for the induction of the full intensity of inducible defences in *R. temporaria* tadpoles. Results for behaviour and morphology were consistent. Treatment effects on body mass were relatively weak and inconclusive, which agrees with previous reports (e.g. Relyea and Werner 2000; Van Buskirk 2001; Schoeppner and Relyea 2009). While the main results affirm those of previous studies (LaFiandra and Babbitt 2004; Schoeppner and Relyea 2005, 2009; Richardson 2006; Hettyey et al. 2010), our study supports a range of further conclusions regarding the relative importance of different types of cues, of prey type and of prey quantity.

Analysis of the types of cues that were available to focal tadpoles revealed that pre-consumption prey-borne cues in isolation induced behavioural defences but no change in body shape (T1 vs. T3). This is somewhat surprising because some predators have evolved behavioural or physiological adaptations impeding the use of predator-borne cues and digestion-released cues (Brown et al. 1995; Chivers and Smith 1998), so that pre-consumption prey-borne cues may be the only cues available for prey to adjust their defences. However, prey may reserve development of morphological changes—which take time to be expressed and can be costly—for situations in which reliable information about the predator species is available. Also, in comparison with behavioural responses to predation, morphological responses may be effective only when they are specific to the type of predator. Consequently, if only pre-consumption prey-borne cues are present and there is no information available about the predator, morphological changes may not be induced. Accordingly, studies of several other taxa agree that pre-consumption prey-borne cues in isolation elicit weak responses, frequently affecting only prey behaviour [cladocerans (Pijanowska 1997); bryozoans (Harvell 1986); snails (Turner 1996); tadpoles (Petranka and Hayes 1998); Schoeppner and Relyea (2005, 2009)].

Continually released predator-borne cues in isolation elicited both morphological and behavioural responses (T1 vs. T4). This suggests that *A. cyanea* larvae, and probably many other predators as well, release olfactory cues more or less constantly and not only when they chew or digest prey, and these can indeed be used by prey to detect predators and adjust their level of response. Evidence from previous studies is inconclusive on this point: some studies report no detectable response to predator-borne cues alone (e.g. McCollum and Leimberger 1997; Schoeppner and Relyea 2009), while others observed changes in both behaviour and morphology (e.g. Pettersson et al. 2000; Petranka and Hayes 1998; Schoeppner and Relyea 2005). This discrepancy may partly be due to differences in the traits measured, because behavioural responses may be induced by pre-consumption cues, whereas less plastic

changes in morphology may only develop in the presence of predator-borne cues (Van Buskirk and Arioli 2002; for further references see above). Also, predator recognition may involve learning in some prey species or in the presence of some predator species [damselflies (Wisenden et al. 1997); fishes (Brown 2003); tadpoles (Gonzalo et al. 2007; Fraker 2009; Chivers and Ferrari 2013)], whereas it must be at least partly innate in many other prey or in relation to other types of predators [snails (Turner 1996); fishes (Vilhunen and Hirvonen 2003); tadpoles (Petranka and Hayes 1998; Schoeppner and Relyea 2005; Hettyey et al. 2012; this study)].

Stronger responses to continually released predator-borne cues than to pre-consumption cues may have partly resulted from our experimental methodology: predators emitting continually released cues were present almost all the time, whereas pre-consumption cues were added only once a day. However, phenotypic responses to these two types of cues in isolation tended to be weaker than when they were both available to focal tadpoles (T3, T4 vs. T5). The only comparable study found that the combined presence of pre-consumption and continually released predator-borne cues did not elicit stronger responses in tadpoles than when these cues were available in isolation (Schoeppner and Relyea 2009). This discrepancy may be attributed to methodological differences between the studies. For example, we added pre-consumption cues more frequently (seven vs. three times a week), thereby potentially causing more pronounced responses. In any case, the data indicate that detectable quantities of intact pre-consumption prey-borne cues were transferred into the mesocosms in T2, T3 and T5, because responses to a combination of pre-consumption prey-borne cues and continually released predator-borne cues were stronger than to continually released predator-borne cues alone (T4 vs. T5).

Our results deliver several lines of evidence for the importance of digestion-released cues. First, effects of digestion-released cues added to the effects of pre-consumption cues and continually released predator-borne cues (T2 vs. T5) (for similar results see Jacobsen and Stabell 2004; Schoeppner and Relyea 2009). This also supports the hypothesis that all types of chemical cues are necessary to induce the full suite and magnitude of inducible responses in anuran larvae (Van Buskirk and Arioli 2002; LaFiandra and Babbitt 2004; Richardson 2006; Schoeppner and Relyea 2005, 2009). Second, morphological responses were stronger when continually released predator-borne cues were combined with digestion-released cues than when combined with pre-consumption cues (T5 vs. T9). There was a similar tendency in behavioural responses (also see Ferrari et al. 2007). However, the process of homogenization in a blender may not allow tadpoles to produce or release large quantities of pre-consumption cues before death (Fraker et al. 2009). Hence, the difference between T2 and T5 may be attributed to lower concentration of pre-consumption cues in T5. Nonetheless, while the temporal pattern of attack-released cue synthesis is largely unknown, we are inclined to dismiss this possibility because previous studies using similar homogenization methods have induced strong behavioural responses in focal animals (Hews 1988; Schoeppner and Relyea 2005; Ferrari et al. 2008). Another explanation may be that continually released predator-borne cues are released in lower quantities by starved predators than by well-fed individuals. According to both explanations, the discrepancy between T5 and T2/T9 may simply result from differences in the concentration of predator-borne cues. Consequently, these lines of evidence for the importance of digestion-released cues require further investigation.

We found that prey type affected tadpole phenotype via digestion-released cues. The response of tadpoles exposed to cues released from phylogenetically distantly related prey via digestion by the predator was weaker than the response to digestion-released cues after consumption of phylogenetically closely related prey. Indeed, the response to predators fed with phylogenetically distant prey did not differ significantly from that of tadpoles exposed to starved predators releasing no digestion-released cues. That is, tadpoles showed no detectable difference in response to unfed and fed predators if pre-consumption cues were excluded and the predators had been fed with phylogenetically distant prey. In previous experiments, *R. temporaria* tadpoles responded similarly to predators that had been fed either conspecifics or *B. bufo* tadpoles (Laurila et al. 1997, 1998). However, Laurila et al. (1997, 1998) did not exclude pre-consumption cues, and our data show that in the absence of these cues the response to predators consuming *B. bufo* was weaker than to predators fed conspecifics and similar to the response to starved predators. Therefore, *R. temporaria* tadpoles respond to attack-released cues emitted by *B. bufo*, but not to digestion-released cues from predators that had fed on toad larvae. This suggests that at least some types of pre-consumption cues emitted by attacked prey are conservative and universal and thus less dependent on the phylogenetic relationship between the sender and receiver (see Kiesecker et al. 1999), while the cues released through digestion contain more taxon-specific information (Ferland-Raymond et al. 2010), allowing for a relaxation of responses to predators feeding on alternative prey.

Other studies also agree that tadpoles perceive predators feeding on their conspecifics or close relatives as more dangerous than predators that have not fed recently on these types of prey (Laurila et al. 1997, 1998; Schoeppner and Relyea 2005; Richardson 2006; Fraker 2009). The usual interpretation is that by not responding to predators that are feeding on other species, prey can spare the cost of induced

defences without experiencing elevated predation risk (Persons et al. 2001). We also find that tadpoles may use digestion-released cues to detect predators that had fed on conspecifics or close relatives but recently have not attacked further prey [also see Richardson 2006; Ferland-Raymond et al. 2010; for similar results in damselflies and fish see Mathis and Smith (1993); Chivers et al. (1996); Ferrari et al. (2007)]. Our results demonstrate especially clearly that tadpoles adjust their responses to digestion-released prey-borne cues originating from different types of prey because we held constant the quantity of digestion-released predator-borne cues [digestive fluids, gut tissue fragments, or the predator's gut microflora (Pettersson et al. 2000; Ferrari et al. 2007; Schoeppner and Relyea 2009; Ferland-Raymond et al. 2010)].

Antipredator phenotypic responses were stronger to predators fed conspecifics than to starved predators, but a larger quantity of prey consumed by predators did not further enhance induced changes. That is, elevated amounts of digestion-released cues did not lead to increased responses. The absence of a graded dosage response may reflect an all-or-nothing reaction to digestion-released cues originating from conspecifics, or may indicate that our lowest treatment level was already too high to detect the graded phase. Comparison with earlier work suggests that the latter was probably not the case. McCoy et al. (2012) observed that the dose–response curve had levelled off already in response to 0.2 mg tadpole tissue L^{-1} day^{-1} fed to the predators, which is lower than the concentrations applied in our experiment (1.7–3.3 mg L^{-1} day^{-1}). Nonetheless, Van Buskirk and Arioli (2002) and Hettyey et al. (2010) noted graded responses to cue concentrations as high as 5.6 and 8.8 mg L^{-1} day^{-1}. The latter study, like the present one, also excluded pre-consumption cues. While many studies have documented that prey animals use the concentration of pre-consumption prey-borne cues and continually released predator-borne cues to adjust their antipredator-responses in other taxa as well [insects (Kesavaraju et al. 2007); fishes (Ferrari et al. 2005)], further research will be necessary to uncover the importance of the quantity of digestion-released cues.

In summary, our results support conclusions about the relative importance of several types of chemical cues of predation threat. Most importantly, however, we have clearly demonstrated that continually released predator-borne cues and digestion-released cues are used by tadpoles for the adjustment of antipredator defences. Using continually released predator-borne cues and digestion-released cues may enhance survival probabilities of prey by providing specific information on the type, location, abundance and recent feeding history of predators. This information could be only partially derived from pre-consumption prey-borne cues. Also, continually released predator-borne cues allow prey to detect recently relocated or unfed predators, and to recognize them as dangerous even when pre-consumption prey-borne cues are absent. While these are long-standing and widely recognized theoretical considerations (e.g. Laurila et al. 1998), our study provides the most compelling and detailed empirical evidence available that continually released predator-borne cues and digestion-released cues are used by larvae of anuran amphibians.

Author contribution statement A. H. and J. V. B. conceived and designed the study. A. H., Z. T., K. E. T. and J. G. F. collected data. A. H. and J. V. B. performed statistical analyses. A. H. and J. V. B. wrote the first draft and Z. T., K. E. T., J. G. F., and D. J. P. substantially improved the manuscript.

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Box 1: Clarifying terminology and classifying mechanisms for chemosensory-mediated predator detection

Many studies report the use of chemical cues to detect predators, but they employ widely different definitions and classifications of types of cues. The same terms are used sometimes as synonyms, at other times they refer to different phenomena, and definitions are often missing. For example, 'diet-released cues' can refer to those that originate from digested prey (e.g., Ferrari et al. 2007; Ferland-Raymond et al. 2010), but sometimes they also include cues that are released by prey upon attack (e.g., Laurila et al. 1997; El-Balaa and Blouin-Demers 2013). Many authors use the term *kairomone* in reference to cues from a predator that are independent of its recent feeding history (e.g., Brönmark and Hansson 2000; Hettyey et al. 2010), others state that kairomones include digestion-released cues (e.g., Kats and Dill 1998; Schoeppner and Relyea 2005, 2009), and still others use the term kairomone whenever the receiver is a heterospecific (e.g., Chivers and Smith 1998).

A second example of inconsistent terminology is the classification of cues as indirect or direct. Indirect cues originate from prey and have evolved to alert other prey to predation threat. They include several kinds of chemicals: general prey metabolites that are excreted actively upon stress ('no-cost disturbance signals'; Wisenden et al. 1995; Kiesecker et al. 1999), special disturbance cues that are costly to produce and are released by prey actively upon attack ('alarm pheromones'; Fraker et al. 2009), cues that are passively released from injured prey tissue ('damage-released cues'; Chivers and Smith 1998), and cues that are released from prey by digestion ('digestion-released cues', also referred to as 'predator-labelling'; Mathis and Smith 1993; Chivers and Smith 1998; Ferrari et al. 2007). Direct cues, on the other hand, originate directly from the predator and represent the smell of the predator itself that is independent from its recent feeding history. These cues are released 'unintentionally', alerting potential prey to predation threat and lowering the predator's chance of successful attack. Direct cues include chemicals and tissue fragments that are released more or less continually from the integument of the predator ('kairomones'; Petranka and Hayes 1998; Brönmark and Hansson 2000), saliva released during capture and consumption of prey (we know of no study demonstrating this), and digestive body fluids of the predator, tissue fragments of the predators' digestive tract and samples of the predators' gut flora released during excretion ('digestion-released cues'; Mathis and Smith 1993; Ferrari et al. 2007). As can be seen from the above list, excrements of predators may contain both indirect and direct cues. Furthermore, kairomones may be released not only continually from the integument of predators, but also during defecation (fractions of 'digestion-released cues'). This further confuses functional and physiological/mechanistic classification. Finally, some of the current nomenclature is based on functionality and some on the timing of release, while cue origin is only implicitly understood.

To improve clarity, help avoid misunderstandings and facilitate comparability of results, we propose a new terminology for the cues involved in chemosensory-mediated predator detection. We suggest using a binomial nomenclature and classification based on the timing of cue release (stress-, attack-, capture-, digestion- or continually-released cues) in combination with cue origin (prey-borne versus predator-borne cues) (Table 1).

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Naive tadpoles do not recognize recent invasive predatory fishes as dangerous

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Abstract. Invasive alien predators (IAP) are spreading on a global scale—often with devastating ecological effects. One reason for their success may be that prey species fail to recognize them due to a lack of co-evolutionary history. We performed a comprehensive test of this "prey naiveté" hypothesis using a novel approach: we tested whether predator-naive tadpoles of the agile frog (*Rana dalmatina*) display antipredator behavior upon encountering chemical cues produced by native, invasive (established or recent) or allopatric fishes (four perciforms, four siluriforms, and two cypriniforms). We studied the influence of population origin on predator-detection ability by presenting chemical cues to predator-naive tadpoles that originated from fishless hill-ponds or fish-infested floodplain populations. Before trials, we fed fishes with tadpoles or an alternative food to test whether direct chemical cues from the predator's diet influences the tadpoles' recognition of potential predators. Tadpoles reduced their activity upon exposure to cues from native and long-established invasive perciforms, but not in response to recent invaders, allopatric predators, or to any siluriforms. Also, predators that were previously fed with tadpoles did not universally induce behavioral defensedefenses upon first encounter. Finally, tadpoles originating from isolated hill-ponds exhibited higher baseline activity and responded in weaker fashion than their conspecifics from floodplain populations, which co-exist with predatory fishes. Our results indicate that tadpoles may be vulnerable to invading predatory fishes due to their inability to recognize them as dangerous, though their ability to recognize invasive IAP may evolve rapidly, in fewer than 30 generations.

Key words: antipredator behavior; history of coexistence; inducible defense; invasive species; predator recognition.

Introduction

One would expect invasive species to be poorly adapted to novel environments upon their arrival, and yet an increasing number of invasive alien species (IAS) are spreading on a global scale. In fact, IAS are one of the leading causes of global ecological problems, impacting native species and communities through predation, vectoring diseases, genetic introgression, reproductive interference, habitat modification, and competition (Clavero and García-Berthou 2005, Davis 2009, McGeoch et al. 2010). Negative impacts can sometimes be mitigated (Davis 2009, McGeoch et al. 2010), but invasions are notoriously difficult to counteract (Davis 2009, Blackburn et al. 2010, Tabak et al. 2015).

The spread of IAS is a "natural experiment" that provides an opportunity to determine why some but not other invading species successfully become established. IAS are typically ecological generalists with short generation times, high rates of growth and reproduction, and high dispersal and competitive abilities (Whitney and Gabler 2008, van Kleunen et al. 2010). Successful IAS may also exhibit high levels of phenotypic plasticity and evolvability, allowing them to rapidly adapt to new conditions (Daehler 2003, Whitney and Gabler 2008). The lack of a shared evolutionary history with native organisms may also affect the success of IAS. This "enemy release hypothesis" suggests that after invading a new environment, IAS escape many of their parasites, predators, and competitors (Keane and Crawley 2002). The "prey naiveté hypothesis" suggests that invasive alien predators (IAP) may not be recognized as enemies by native prey, or that effective antipredator responses may be absent (Cox and Lima 2006, Banks and Dickman 2007), resulting in greater hunting efficiency of predators (Kiesecker and Blaustein 1997, Gomez-Mestre and Díaz-Paniagua 2011), and potentially devastating effects for prey populations (Cruz et al. 2006, Arribas et al. 2014). It is often assumed that prey naiveté largely explains the success of IAP and their harmful ecological impacts, but this idea has only recently become the focus of empirical research (Cox and Lima 2006, Paolucci et al. 2013).

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Previous attempts to test the prey naiveté hypothesis have provided contradictory results (for a review see: Sih et al. 2010), but there are several potential problems that need to be considered. First, previous studies generally relied on testing native prey species' response to only one IAP species compared to specimens of only one native predator species. This one-to-one comparison is problematic because prey can respond differently even to various native predators (e.g., Relyea 2001*a*, Freeman et al. 2009). Second, many studies compared responses of prey to native and invasive predators that were phylogenetically unrelated and ecologically divergent, even though the strength and type of optimal responses may vary depending on the predators' ecological characteristics, such as their foraging mode, prey-capture mechanism, dangerousness, or microhabitat preferences (sensu Hettyey et al. 2011, Miehls et al. 2014). Third, only a few studies considered the time since the invasion occurred, even though effects of IAP are expected to vary with time since invasion due to phenotypic plasticity and genetic adaptations both in the IAP and the native prey species (Strauss et al. 2006, Hawkes 2007, Mitchell et al. 2010). Finally, many previous studies measured antipredator responses to IAP in only one population, although these responses may vary spatially due to their dependence on the local intensity of selection and on the genetic variation available for selection to act on (Strauss et al. 2006). For these reasons, more comprehensive tests of the prey naiveté hypothesis are needed to allow for better forecasting effects of biological invasions.

To test the prey naiveté hypothesis, we conducted a common garden experiment using an aquatic prey species—larvae of the agile frog (*Rana dalmatina*)—collected as eggs from several different populations, and we compared their antipredator responses to several native and invasive predatory fish species. In the aquatic environment, chemical cues are considered the most important sensory modality of predator recognition (Kats and Dill 1998), and therefore, we examined the behavioral responses of tadpoles presented with chemical cues from different potential predatory fishes. We assessed the importance of the history of coexistence between fish predators and their anuran prey, while controlling for ecological similarities and phylogenetic relationships among predators. We did this by exposing tadpoles to the smell of fishes that have been present in the region for varying time periods and by studying behavioral responses of tadpoles originating from fish-exposed and fish-free habitats. Because the recent feeding history of predators may strongly influence their conspicuousness towards prey (Laurila et al. 1997, Schoeppner and Relyea 2005, Hettyey et al. 2015), we also tested whether the diet of fishes affected the ability of tadpole prey to recognize native, as well as invasive fishes as predators.

We tested the prediction that the tadpoles' antipredatory response should vary along two gradients of naiveté: (1) the longer the fish species has been present in the amphibians' habitat, the stronger response its cues should elicit (from allotopic through recent invasive, then established invasive to native), and (2) tadpoles from fish-exposed (floodplain) habitats should respond more strongly than tadpoles from fish-free (hill) habitats. We also predicted that behavioral responses of tadpoles to chemical cues on predators should vary along the gradient of invasiveness similarly in different clades of predatory fishes. Further, if the fright response to fishes is not specific to predators but generalized, we expected native herbivorous fishes to also elicit stronger responses than invasive herbivorous fishes. Finally, we predicted that the ability to recognize IAP as dangerous and respond behaviorally should be facilitated if the predator recently consumed conspecific tadpoles.

Methods

Experimental design

To assess whether tadpoles of *Rana dalmatina* recognize invasive predators upon first encounter, we reared tadpoles in the absence of predators and exposed them to chemical cues originating from various types of fish predators. We used predator-naive tadpoles to exclude potentially confounding effects of learning (sensu Chivers and Smith 1998). We used members of two orders of predatory fishes, Perciformes and Siluriformes. From both orders, we used specimens of a native, an established invasive, a recent invasive, and an allopatric species from a Central European point of view (for details, see Table 1). We used the native species to confirm that tadpoles respond to this predator (positive controls), and we predicted that responses to predators would diminish through the established and recent invasive to the allopatric ones. Additionally, we tested whether there are generalized responses to fishes independently of their dangerousness by also exposing tadpoles to chemical cues originating from a native and an invasive species of herbivorous Cypriniformes.

To examine the importance of the history of coexistence more closely, we collected tadpoles from two types of habitats. We sampled egg-clutches laid in three semi-permanent water bodies on the floodplain of the Danube river and in three temporary ponds in the hills of the Vienna Woods, Austria (Fig. 1; Appendix S1). Larval habitats on the floodplain were located in the alluvial forest alongside the river, less than 1.5 km away from the main river arm and known to come into contact a few times every year during times of high water. The recent evolutionary history of these amphibian populations is thus shared with fishes, including invasive species soon after their arrival. Hill habitats were located at higher elevation and at least 1.5 km away from larger streams or permanent water bodies that could connect the local frog populations to others exposed to fish. Hence, amphibians in these localities live isolated from fishes. Due to the differences in the presence of fishes in their original habitat, we expected to observe weaker responses to fishes in

TABLE 1. A list of the fish species used in the experiment.

Order	Species	Type	Origin	*N*	Mass	Treatment
–	–	Aged tapwater control	–	6	–	1
Cypriniformes	*Scardinius erythrophthalmus*	Native	Commerce	3	26.7 ± 14.1	2
Cypriniformes	*Ctenopharyngodon idella*	Invasive (~45 years*)	Fishery in A	3	34.0 ± 8.5	3
Perciformes	*Perca fluviatilis*	Native	Commerce	6	42.5 ± 11.7	4, 12
Perciformes	*Lepomis gibbosus*	Invasive (~120 years†)	Commerce	6	31.2 ± 17.7	5, 13
Perciformes	*Neogobius melanostomus*	Invasive (~10 years‡)	Danube in A	6	12.5 ± 6.6	6, 14
Perciformes	*Lepidiolamprologus elongatus*	Allopatric	KLIVV	6	18.0 ± 8.6	7, 15
Siluriformes	*Silurus glanis*	Native	Commerce	6	27.5 ± 4.4	8, 16
Siluriformes	*Ameiurus nebulosus*	Invasive (~120 years§)	Commerce	4	41.8 ± 6.0	9, 17
Siluriformes	*Ameiurus melas*	Invasive (~15 years¶)	Fishery in H	6	18.2 ± 2.5	10, 18
Siluriformes	*Clarias batrachus*	Allopatric	Commerce	5	6.6 ± 3.4	11, 19

Notes: Cypriniform fishes were used as herbivorous controls, perciform and siluriform fishes as predators. We chose fishes to represent native, established invasive, recent invasive or allopatric species and obtained them either commercially from aquarist shops, or caught them from the Danube in Lower Austria or from fisheries in Hungary (H) or Austria (A). Differences in the number of fish per species are due to mortality before the start of experimental trials and, in the case of the herbivorous cyprinids, to only one type of food provided. Treatment number in the case of the predatory fishes depended on the food (bloodworms or tadpoles). Mean mass (g) ± SD are presented.

* Hauer (2007).
† Muus and Dahlström (1981).
‡ Wiesner et al. (2000).
§ Arnold (1990).
¶ Schmutz et al. (1995).

general and especially to invasive fish species in tadpoles originating from hill populations than in tadpoles from the floodplain populations (for analogous results on responses by prey that are syntopic or allotopic with predators see Kiesecker and Blaustein 1997, Hartman and Lawler 2014, Nunes et al. 2014*a*).

To further investigate the factors that facilitate predator recognition, we manipulated the recent feeding history of predators by feeding half of the predatory fish with bloodworms (larval *Chironomus* sp.), and the other half with *R. dalmatina* tadpoles. Tadpoles are known to respond behaviorally to chemical cues originating from injured conspecifics, but their responses are generally weak to similar cues released by phylogenetically unrelated prey (Laurila et al. 1997, Schoeppner and Relyea 2005, Hettyey et al. 2015). We predicted that tadpoles would respond to chemical cues originating from all predators fed with conspecifics (for a review, see Chivers and Smith 1998). In the case of bloodworm-fed predators, we expected tadpoles to respond to native predators if they were able to perceive continually released predator-borne cues (Schoeppner and Relyea 2005, Hettyey et al. 2015), and perhaps to established invasive predators, but not to recent invasive or allopatric predators (Marquis et al. 2004, Nunes et al. 2013).

We used tadpoles originating from six populations and presented them with 19 types of chemical stimuli, comprising the 19 treatments: aged tap water (control, treatment 1), stimulus water from two types of spinach-fed herbivores (treatments 2–3), from eight types of bloodworm-fed predators (treatments 4–11), and from eight types of tadpole-fed predators (treatments 12–19; Table 1). We tested for a decrease in tadpole locomotor activity, which usually enhances prey survival by lowering prey detectability and predator encounter rates, and is a frequently observed behavioral response to predators in prey in general and in *R. dalmatina* tadpoles specifically (Lima and Dill 1990, Teplitsky et al. 2003, Hettyey et al. 2011).

Collection and maintenance of animals

We collected 50 eggs from each of ten freshly laid egg-clutches of *R. dalmatina* from each of three floodplain and three hill populations, all located in Lower Austria (Fig. 1; Appendix S1). Populations were relatively large (>80 egg clutches laid in the same water body) and were separated from each other by >10 km. Eggs were laid a few days later in the hill populations than in the floodplain populations, but this did not translate into systematically earlier developmental stages at the time when we performed trials (in both groups, developmental stage: range = 28–30, median = 29; Mann–Whitney $U = 215.5$, $N = 42$, $P = 0.94$). We transported eggs from the field to the Konrad Lorenz Institute of Ethology (KLIVV) in Vienna.

To provide semi-natural conditions during embryonic and larval development, we constructed outdoor mesocosms 2 weeks before egg collection. We filled 60 rectangular 45-L boxes placed outdoors at the KLIVV with 25 L of tap water and covered them with mosquito netting. Two days later, we added 10 g of dried beech leaves (*Fagus sylvatica*) to each mesocosm to enhance spatial heterogeneity and provide nutrients for tadpoles,

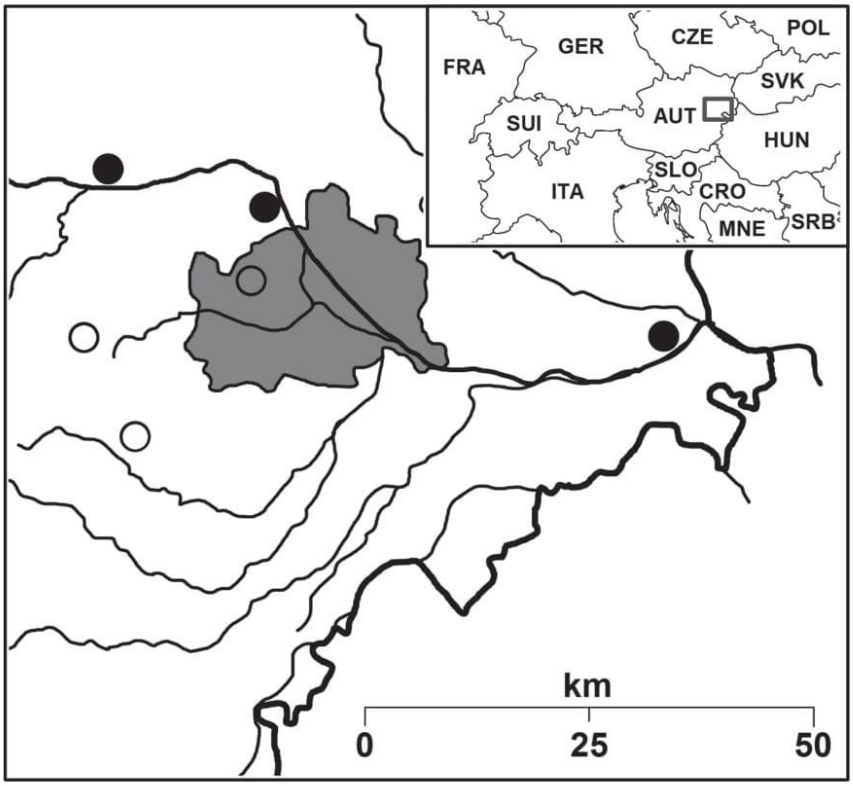

FIG. 1. Geographic location of the sampled populations around Vienna (shaded area), Austria. Floodplain populations are represented by filled circles, hill populations by empty circles.

and 1 L of pond water containing phyto- and zooplankton to enhance algal growth and maintain high water quality. One day before egg collection, we hung an egg-holding dish (16 × 13 × 13 cm) made of a plastic frame and mosquito-net walls into each mesocosm. After collecting eggs, we placed partial clutches into egg-holding dishes and reared embryos until hatching. Each box received eggs of only one family, and boxes were arranged into 10 spatial blocks, where each block received eggs from one family per sampled population. Three days after hatching, when tadpoles reached the free-swimming state (developmental stage 25; Gosner 1960), we released 30 haphazardly chosen healthy-looking tadpoles from egg-holding dishes into each mesocosm. We removed egg-holding dishes and transferred surplus tadpoles into large plastic containers, where we maintained them until using them as predator food.

We obtained fish from various sources (see Table 1) and maintained them in 10 aerated 100-L tanks, each tank holding six individuals of one species. By ordering specimens of similar sizes and later selecting individuals from the stock populations that were as similar in size across species as possible, we aimed to minimize among-species differences in body size. However, some variation was unavoidable due to logistical constraints (see Table 1). Nonetheless, predatory fish were large enough to pose an immense threat for tadpoles, as they consumed several tadpoles within a few minutes during feeding immediately before commencement or after termination of trials. We fed predatory fish with bloodworms and cypriniforms with spinach daily ad libitum and changed water every other day. Two days before the start of experimental trials, we separated fish and placed them individually into aquaria of 4, 12, 20, or 45 L, depending on the size of the fish. We filled aquaria with ~0.5 L aged tap water/g fish body weight and fed fish with ~13 mg food/g fish body weight (2–14 tadpoles or 7–47 bloodworms or 108–756 mg spinach). We adjusted aquarium size, water volume, and the quantity of food to the size of predators to obtain roughly similar cue concentrations in all treatments. We used the fish holding water in the aquaria as stimulus water in experimental trials.

Experimental procedures

We started the experimental trials 4 weeks after hatching, when the tadpoles were between developmental stages 28–30 (Gosner 1960). On the day preceding trials, we fed fish at 17:00 as described previosuly. On the day of trials, we removed leftover food from fish tanks between 8:30 and 9:00. Subsequent procedures were very similar to those employed successfully in previous studies (e.g., Ferrari et al. 2008, Mathis et al. 2008). We set up 19 dishpans (16 × 12 × 7.5 cm), corresponding to the 19

treatments, under a USB-camera and filled them with 0.3-L aged tap water. We captured 19 haphazardly selected tadpoles from one mesocosm at a time and entered them individually into the dishpans. Tadpoles were haphazardly allocated to treatments. Each population was represented by 10 tadpoles originating from 10 different clutches, resulting in 10 replicate tadpoles per population in each treatment. We let tadpoles acclimate for 45 min. Ten minutes before the start of behavioral recordings, we took 3 mL of stimulus water using 10 mL syringes, each one assigned to one treatment. Resulting concentrations of chemical cues in the experimental dishpans corresponded to 0.26 mg/L consumed tadpole tissue and 20 mg/L fish, exceeding concentrations that have previously been shown to elicit antipredator responses (e.g., Mathis et al. 2008, McCoy et al. 2012). We took stimulus water from one randomly selected fish-holding tank within each treatment, and did this immediately before addition to focal tadpoles' dishpans to prevent cue degradation (Van Buskirk et al. 2014). Once the 45-min acclimation period was over, we video-recorded the movements of tadpoles for 5 min, thereafter adding the stimulus water and recording movements for another 5 min. We recorded tadpoles' behavior by photographing them every 2 s, and from these images, we counted the number of positional changes between frames to estimate tadpole activity. The assistant scoring tadpole movement was blind with respect to treatments. After video-recording them, we over-anesthetized tadpoles and fixed three haphazardly chosen individuals from each group of 19 in 30% ethanol for later determination of developmental stage using a binocular microscope. Tadpoles were very similar in their developmental stage within families, because tadpoles were only exposed to different treatments during the 10 min of video-recording and before that they were raised together under identical conditions at relatively low density. We tested tadpoles collected from two randomly selected mesocosms simultaneously in two parallel experimental setups (two sets of 19 dishpans arranged on two separate tables overseen by two webcams) to increase throughput and thereby avoid large differences in the developmental state of tadpoles. We performed this procedure with tadpoles taken from all rearing mesocosms over the course of three consecutive days. After termination, we euthanized fish and remaining tadpoles with MS-222.

Statistical analyzes

We tested 1,083 tadpoles from 57 families, which was fewer than planned, as embryos failed to develop in one clutch from each hill population. For the stimulus water, we aimed to use three individual fish in each feeding × species combination, but three individuals died before the start of trials (two *A. nebulosus* and one *C. batrachus*), leaving 51 individual fish (see Table 1). By chance, two more fish (one *P. fluviatilis*, one *A. melas*) were not selected by the randomization on any day. Four fish did not eat tadpoles (two *L. elongatus*, one *S. glanis*, one *P. fluviatilis*) and one did not eat bloodworms (*L. elongatus*) at the feeding preceding trials. Consequently, we excluded experimental trials from the analyzes in which stimulus water originated from these five individual fish, thus we analyzed the data of 988 tadpoles.

From the recordings, we excluded the first minute after start, and 1 min before and 1 min after the addition of stimulus water to avoid the inclusion of tadpole movements potentially due to disturbance, i.e., while observers were present in the experimental room. Consequently, we counted movements over 3 min prior and 4 min after the addition of chemical cues. We used the mean activity over the 3-min prestimulus period ("prestimulus activity") as a measure of baseline activity, and the mean activity over the 4-min poststimulus period ("poststimulus activity") as a measure of response to the stimulus. To validate that tadpoles responded to the stimuli in general by decreasing their activity, we compared pre- and poststimulus activity by a linear mixed-effects (LME) model with the nested random-effects structure of tadpole ID in family in population. To test whether baseline activity was similar in all treatment groups, we analyzed prestimulus activity as a dependent variable in a LME model which included treatment type as a fixed factor, and tadpole family nested in population of origin as random factors. We used a similar LME model to compare the prestimulus activity of hill and floodplain tadpoles.

To answer our main research questions, we analyzed poststimulus activity as a dependent variable using LME models, in which we included tadpole family nested in population of origin as random factors. The initial model contained treatment type (treatments 1–19) and tadpole habitat (floodplain or hill) as fixed factors, prestimulus activity as a covariate, and all two-way and three-way interactions of these three predictors. Additionally, the model contained the following fixed effects as potentially confounding variables: date as a fixed factor, and time of day and fish mass as covariates. We reduced the initial model stepwise by excluding the term with the largest *P*-value in each step until only significant ($P < 0.05$) variables and interactions remained in the final model. We checked our models by diagnostic plots and found no outliers or deviation from normality and homoscedasticity of the residuals.

For the treatments that elicited an antipredatory response according to the LME results, we calculated linear contrasts to compare the treatment effects separately in floodplain and hill tadpoles. Specifically, we estimated the slopes of relationship between pre- and poststimulus activity for each habitat × treatment group combination from a LME model that included the three-way interaction of treatment type, habitat type, and prestimulus activity, and also contained the confounding effects that were significant in our final model. From these slopes, we estimated the control-treatment difference for each treatment group, separately in floodplain and hill tadpoles. All analyses were run in R 3.1.0

(R Core Team 2014), using the package 'nlme'; for linear contrasts we used the 'lsmeans' package. *F*-tests were calculated with 'anova' (type-III sum of squares); qualitatively identical results were obtained from *F*-tests based on Kenward-Roger approximation, using the 'pbkrtest' package (Appendix S2).

Results

Tadpoles' mean activity decreased from 11.37 ± 0.62 movements per minute prestimulus to 7.02 ± 0.62 movements per minute poststimulus (mean ± SE; LME: $t_{987} = 23.9$, $P < 0.001$), and there was no significant difference in prestimulus activity among treatment groups (LME, $F_{18,913} = 1.23$, $P = 0.228$). Although hill tadpoles tended to show higher prestimulus activity (12.73 ± 0.33) than floodplain tadpoles (10.16 ± 0.31), this difference was nonsignificant (LME, $F_{4,931} = 4.73$, $P = 0.095$).

Variation in poststimulus activity was explained by date, time of day, and interactions of prestimulus activity with treatment type and tadpole habitat type (Table 2; Appendix S3). The tadpoles responded to the stimulus by reducing their activity, i.e., the slopes of poststimulus activity with prestimulus activity were <1 in all but one of the treatment groups (Fig. 2; Appendix S4 and Appendix S5). For tadpoles with zero prestimulus activity, poststimulus activity was also low and did not differ significantly between the control group and the other treatment groups, as shown by the nonsignificant differences in the intercept values (lines 7–24 in Appendix S3; Fig. 2). However, as the prestimulus activity increased, the difference between certain treatment groups increased, which is shown by the differences of the slopes (given by the interaction parameters in lines 26–43 of Appendix S3; Fig. 2). For most treatment groups, neither the intercept nor the slope differed from the control, meaning that poststimulus activity did not differ from control at any value of prestimulus activity. However, two treatment groups had significantly different slopes compared to the control group (lines 36–37 in Appendix S3), i.e., as prestimulus activity increased, the difference in poststimulus activity increased, as shown by the increasingly divergent regression lines in Fig. 2 (comparing panel "a" to panels "d1–d2") and Fig. 3. Specifically, tadpoles that received cues from the tadpole-fed native perciform fish had significantly reduced poststimulus activity compared to the control group when they made more than ~8 prestimulus movements per min (Fig. 3), while tadpoles exposed to cues from the tadpole-fed established-invasive perciform fish had significantly reduced poststimulus activity compared to the control group when they made more than ~15 prestimulus movements per min (Fig. 3). Thus, ~65% and 40% of tadpoles clearly responded to these respective two treatments; the remaining tadpoles were either unresponsive or their response was not detectable due to their low prestimulus activity. Other treatments (i.e., cues from herbivorous Cypriniformes, predators that had been fed with bloodworms, all Siluriformes, and recent invasive and allopatric Perciformes that had been fed with tadpoles) did not elicit significantly stronger responses than the addition of tap water (Fig. 2; Appendix S3 and Appendix S4).

Table 2. Analysis-of-variance table calculated from Wald-tests for the final LME model (in bold) of poststimulus activity, including tadpole family ($n = 57$) nested in tadpole population ($n = 6$) as random factors ($n = 988$ tadpoles). Statistics for nonsignificant terms (in italics) were calculated by re-including them into the final model.

	F	df	*P*
Date	**5.15**	**2, 48**	**0.009**
Time of day	**5.62**	**1, 48**	**0.022**
Prestimulus activity	**35.09**	**1, 893**	**<0.001**
Treatment	**0.34**	**18, 893**	**0.996**
Habitat	**3.03**	**1, 4**	**0.157**
Prestimulus activity × Treatment	**2.37**	**18, 893**	**0.001**
Prestimulus activity × Habitat	**8.18**	**1, 893**	**0.004**
Treatment × Habitat	*0.95*	*18, 875*	*0.511*
Prestimulus activity × Treatment × Habitat	*1.45*	*18, 857*	*0.099*
Fish mass	*0.30*	*1, 892*	*0.583*

Hill and floodplain tadpoles did not differ significantly in poststimulus activity if their prestimulus activity was zero (line 6 in Appendix S3), but the slopes with prestimulus activity were significantly less steep for floodplain tadpoles than for hill tadpoles (line 25 in Appendix S3; Fig. 2), meaning that poststimulus activity became increasingly higher in hill tadpoles than in floodplain tadpoles as prestimulus activity increased. This habitat difference was relatively consistent across treatments, i.e., the three-way interaction between treatment, habitat, and prestimulus activity was nonsignificant (Table 2). In the two treatments that elicited an antipredatory response, linear contrasts showed that floodplain tadpoles responded significantly stronger to the predator cues than to tap water in the treatments with tadpole-fed native Perciformes (difference between slopes: 0.36 ± 0.18, $P = 0.048$) and established invasive Perciformes (0.34 ± 0.17, $P = 0.045$), whereas the response of tadpoles from hill populations to the same two treatments was marginally nonsignificant (0.32 ± 0.18, $P = 0.076$) and nonsignificant (0.16 ± 0.16, $P = 0.305$), respectively. Poststimulus activity increased over the day but decreased with date (Appendix S3), whereas it was unrelated to fish mass (Table 2).

Discussion

Predator-naive larvae of *Rana dalmatina* responded to chemical cues of native or established invasive perciform fish predators, though only if the fish had recently been fed conspecific larvae, whereas the tadpoles ignored chemical stimuli from recent invasive and allopatric perciforms, even when cues of injured tadpoles were present

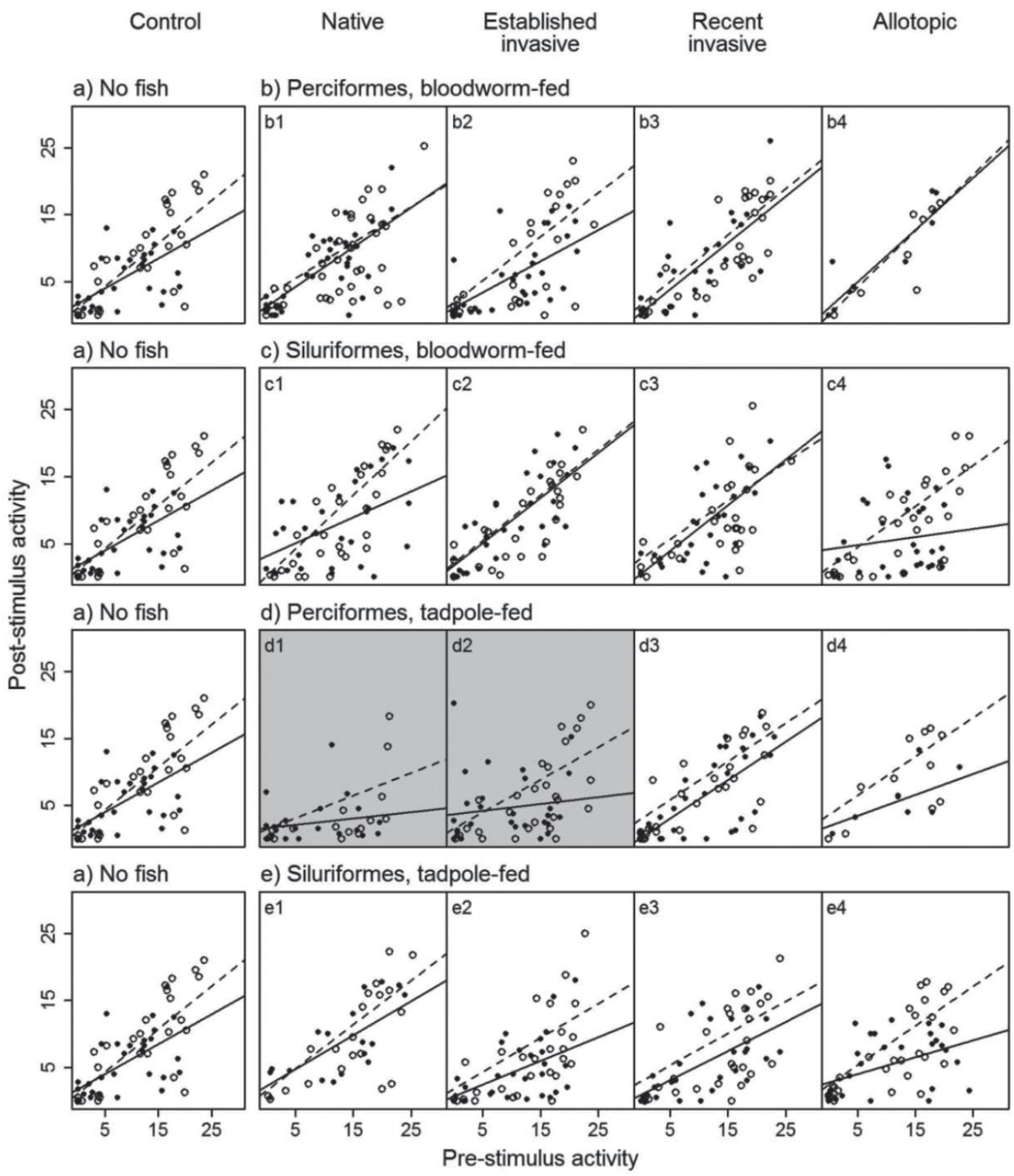

FIG. 2. Poststimulus activity in relation to prestimulus activity (number of movements per min) of tadpoles originating from floodplain (filled symbols, solid lines) and hill (empty symbols, dashed lines) populations in the control and the 16 treatments including exposure to chemical cues from predatory fishes (for nonsignificant responses to chemical cues from herbivorous fishes see Appendix S4). The control group ("no fish") is repeated in each row to facilitate control–treatment comparisons. Slopes are fitted from an LME model allowing for a three-way interaction between prestimulus activity, treatment type, and habitat type. Grey background indicates treatments in which the slope of the relationship differed significantly between the treatment group and the control group.

(see Fig. 2, panels d1–d4). Surprisingly, tadpoles did not respond to any of the siluriform predators (Fig. 2, panels e1–e4). Further, tadpoles did not decrease activity in response to cues from predators feeding on bloodworms or to herbivorous fishes fed with spinach (Fig. 2, panels b1–c4; Appendix S4). Finally, tadpoles originating from hill populations showed slightly higher prestimulus activity and significantly weaker responses to all stimuli

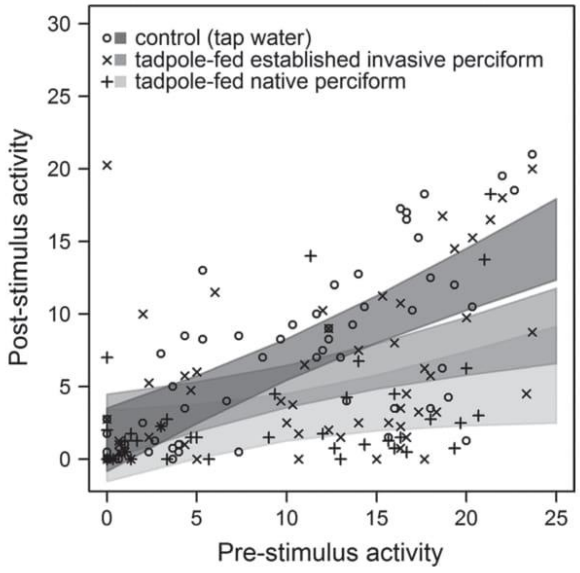

FIG. 3. Poststimulus activity in relation to prestimulus activity (number of movements per min) of tadpoles exposed to tap water (control) or the olfactory cues of native or established invasive Perciformes that had been fed with tadpoles. The shaded polygons show the 95% confidence band of the regression line in each treatment group, predicted from the final model in Appendix S3 (hill and floodplain tadpoles were combined). Where two bands do not overlap, the average response of tadpoles differs significantly between the respective two groups.

than floodplain tadpoles. Thus, our findings on perciform predators support the prey naiveté hypothesis, and show that antipredator responses of tadpoles were not elicited upon a first encounter with IAPs, not even in the presence of direct prey-borne chemical cues, unless the prey species and the IAPs share a sufficiently long co-evolutionary history.

The question arises, why didn't tadpoles respond to all predators fed with conspecifics, even though prey-borne chemical cues were clearly present. Relying on general cues of predation risk may have costs, such as reacting unnecessarily, and a response that is effective against one predator may enhance susceptibility to another (Soluk and Collins 1988, Sih et al. 1998). Consequently, there may be selection for using predator-specific cues (Sih et al. 2010). Indeed, tadpoles show weak responses to general preconsumption prey-borne cues alone (e.g., Petranka and Hayes 1998, Schoeppner and Relyea 2005, Hettyey et al. 2015), and several species seem to require a combination of general prey-borne cues and specific predator-borne cues to respond strongly to the threat of predation (Schoeppner and Relyea 2005, Hettyey et al. 2015). Thus, upon a first encounter, without the benefit of experience, tadpoles may not respond behaviorally to alien predators that are not recognized innately, not even if prey-borne cues are present. Additionally, while recognition and responses to some IAP may be effective if phylogenetically related native taxa are present in the environment (sensu Ricciardi and Atkinson 2004, Cox and Lima 2006, Sih et al. 2010), phylogenetically related native and invasive predators are not necessarily similar in terms of chemical cues or foraging mode (Chalcraft and Resetarits 2003). Taken together, these factors may explain the observed responses to tadpole-fed native and established invasive perciform predators, and the ignorance of cues from recent invasive and allopatric perciforms.

To our surprise, tadpoles did not respond to any of the siluriform predators, not even native or established invasive fishes. We do not know how to explain this unexpected finding, though we suggest it may be due to differences in the feeding behavior of the predators. There is a striking difference between the oral apparatus and the food intake mechanism of perciform and siluriform fishes in general. Both groups use suction for capturing prey, but whereas siluriform fishes engulf their prey whole, the more gape-limited perciforms often bite their prey before eventually engulfing them. Consequently, concentrations of capture-released, prey-borne cues may be higher in the presence of feeding perciform predators than in that of siluriform fishes, hence the stronger response to the former (sensu Ferrari et al. 2007). Also, siluriform fishes may have evolved stealth adaptations for inactivating prey-borne cues during digestion (Feminella and Hawkins 1994, Chivers and Smith 1998, Miller et al. 2016), hence the inability of tadpoles to sense that conspecifics had been consumed and digested by siluriform predators. These are merely speculations and the lack of behavioral responses to siluriform predators requires further investigation.

Tadpoles did not reduce their activity in response to chemical cues from the herbivorous fishes or to any of the bloodworm-fed predators (controls). These findings further support the conclusion that *R. dalmatina* tadpoles lack a generalized fright response to fishes, unlike some other prey taxa (e.g., Langerhans and DeWitt 2002, Gherardi et al. 2011). When predators search for and feed on alternative prey, unresponsive prey may spare costs arising from lowered activity without elevating the risk of being preyed upon (Lima and Dill 1990, Wilson and Lefcort 1993, Persons et al. 2001). Indeed, several studies on tadpoles have found weak or no responses to predators that had been feeding on phylogenetically distantly related prey (Laurila et al. 1997, Schoeppner and Relyea 2005, Hettyey et al. 2015). Hence, a response to chemical cues from recent invasive predators may only be elicited in the simultaneous presence of attack-, capture-, or digestion-released prey-borne cues originating from conspecific or closely related prey (Marquis et al. 2004, Nunes et al. 2013).

The lower activity and stronger inducible responses of floodplain tadpoles may be explained by local adaptation, as anurans on the floodplain are exposed to fishes, whereas hill populations do not coexist with fishes. The low baseline activity and strong inducible responses to fishes may be selectively maintained in floodplain populations as an adaptation to lower encounter rates with

fish predators, which are the most voracious aquatic predators of anuran larvae (Semlitsch 1993, Relyea 2001*b*). At the same time, the lack of predatory fishes in hill populations may lead to an evolutionary loss of fearfulness (for similar results see Magurran 1990, Kiesecker and Blaustein 1997). It is unlikely that these differences among tadpole populations were due to adaptations to microclimatic differences arising from varying altitudes, because hill ponds were located only ~250 m higher than floodplain populations on average. Variation in tadpole age could not explain our findings either, as there were no systematic differences in the developmental state of tadpoles originating from lowland and from hill populations at the time when we performed trials.

The spread of IAP may be viewed as natural experiments, which can be used to assess the pace of contemporary microevolution (sensu Hendry and Kinnison 1999). For example, recognition of IAP and adaptive inducible responses have been reported to appear after only 15 yr in a native mussel and invasive crab system (Freeman and Byers 2006). Nunes et al. (2013, 2014*a*, *b*) documented constitutive and inducible changes in behavior, morphology and life history in the larvae of several frog species in response to an invasive crayfish within 30 years. These and other reports (for reviews see Hendry and Kinnison 1999, Strauss et al. 2006) suggest that IAP impose evolutionary changes in their prey within a few generations. Given that the generation time of *R. dalmatina* is ~4 yr (Riis 1997, Sarasola-Puente et al. 2011), their antipredatory response to the established invasive perciform predator (present for ~120 years) has apparently evolved within 30 generations. Taking advantage of the recent linear spread of invasive fish predators along water drainage systems would allow for more precisely estimating the speed of predator recognition evolution.

In summary, our study shows that the innate ability of tadpole prey to detect and respond to IAP depends upon how long the predator and prey have been in contact with each other. Tadpoles did not respond to chemical cues of any recent invasive or allopatric predators, whereas they lowered their activity when exposed to native and established invasive perciforms, but not to any siluriforms. This result indicates that time since arrival of the IAP to the geographic region may be an important factor; significant evolutionary changes in the predator-recognition ability of prey may evolve in fewer than 30 generations but appear to take more than 3–4 generations. Also, tadpoles originating from hill ponds devoid of fish predators exhibited weaker responses towards all fishes than tadpoles originating from fish-exposed floodplain populations. This result supports the hypothesis that the presence of predators that are phylogenetically and ecologically similar to the IAP may precondition prey, lowering their mortality upon arrival of the IAP. Further, the observation that tadpoles did not respond to any of the siluriform predators while they did lower their activity in response to some perciforms emphasizes the need for a careful selection of multiple study species in similar studies. Finally, since tadpoles ignored all predators that had not fed on conspecifics, but responded to some of those that had consumed conspecifics, it appears that the diet of predators is also crucial for predator-recognition and triggering off a response. Our results have important implications for the interpretation of previous studies and for the design of future investigations. More importantly, however, even though learning may enhance the ability of prey to recognize predators (Chivers and Smith 1998), our results are consistent with suggestions that prey naiveté contribute to the success of IAP, facilitating their spread into new environments. Nonetheless, if prey populations avoid extinction shortly after the arrival of IAP, recognition of predators and effective antipredator defensedefenses may evolve within a few generations and contribute to the co-existence of once invasive predators and their native prey.

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Forum

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Inducible chemical defences in animals

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Phenotypic plasticity is extremely widespread in the behaviour, morphology and life-history of animals. However, inducible changes in the production of defensive chemicals are described mostly in plants and surprisingly little is known about similar plasticity in chemical defences of animals. Inducible chemical defences may be common in animals because many are known to produce toxins, the synthesis of toxins is likely to be costly, and there are a few known cases of animals adjusting their toxin production to changes in environmental conditions. We outline what is known about the occurrence of inducible chemical defences in animals and argue that there is immense potential for progress in this field. Possible directions include surveying diverse taxa to explore how general its occurrence may be and testing for selection acting on inducible chemical defences. Data on inducible chemical defences would provide insight into life-history tradeoffs by enabling novel tests of how time-costs and resource-costs affect life-history. If the synthesis of toxic compounds by animals proves accessible to manipulation, as it is in plants and fungi, this will open the way to refined estimates of the fitness costs of defence, ultimately providing a clearer picture of how plasticity evolves and is maintained in nature.

Synthesis

Inducible changes in the behaviour, morphology, and life-history of animals are extremely widespread, but surprisingly little is known about similar changes in the production of defensive chemicals. We outline what is known about the occurrence of inducible chemical defences in animals and argue that there is immense potential for progress in this field. Possible directions include surveying diverse taxa to explore how general its occurrence may be and testing for selection acting on inducible chemical defences. Data on inducible chemical defences would provide insight into life-history tradeoffs by enabling novel tests of how time-costs and resource-costs affect life-history. If the synthesis of toxic compounds by animals proves accessible to manipulation, we will be able to estimate the fitness costs of defence more precisely, and ultimately provide a clearer picture of how plasticity evolves and is maintained in nature.

Individual organisms often adjust their phenotype in response to environmental stimuli, a process known as phenotypic plasticity. Natural selection favours the evolution of plasticity if the induced phenotype is costly to produce but enhances fitness in some (but not all) environments (Via and Lande 1985, Van Tienderen 1991, Scheiner 1993, DeWitt et al. 1998, Sultan and Spencer 2002, Urban 2007). Plasticity has been a focus of research for decades due to its obvious contribution to morphological and behavioural diversity. Plasticity is also important for the development and maintenance of ecological patterns and processes (Miner et al. 2005), and it may contribute to speciation (West-Eberhard 1989, 2003, Pfennig et al. 2010).

Plasticity induced by natural enemies – such as parasites, predators, or competitors – can help defend individuals and increase survival. This form of plasticity is termed an inducible defence (Harvell 1990). Research on animals has identified inducible defences principally in behaviour, morphology, and life-history (Harvell 1990, Tollrian and Harvell 1999), but adaptive plasticity could also occur in chemical defences, a type of inducible physiological response to enemies. One reason to expect the presence of inducible chemical defences in animals is that constitutive chemical defences are so widespread. Many animals are known to synthesize and store toxic secondary metabolites that defend effectively against predators and parasites (Toledo and Jared 1995, Schmid-Hempel 2005, Kicklighter 2012). These chemicals are termed constitutive in the sense that they are (supposedly) always produced, regardless of the presence or proximity of their target. Many animals therefore possess the genetic and physiological capability to produce defensive toxins. Moreover, the synthesis of toxins requires the maintenance and operation of specialized biochemical machinery and is presumably costly. Consequently, important conditions for the evolution of plasticity, including fitness benefits of chemical defence in the presence of

enemies and costs in the absence of enemies, may often be met (Harvell 1990, Tollrian and Harvell 1999). According to this hypothesis, induced chemical defences evolved by increasing the environmental sensitivity of ancestral constitutive defences. This idea is implicit in models of the evolution of phenotypic plasticity (Karban and Baldwin 1997, p. 221). Available evidence is limited, but macro-evolutionary transitions in both directions between constitutive and induced defences have been observed (Thaler and Karban 1997, Heil et al. 2004, Campbell and Kessler 2013). In sum, it seems likely that inducible changes in the production of chemical defences are widespread in animals and may be just as important as other types of plasticity.

Inducible chemical defences may have been overlooked in animals because behavioral and morphological responses to enemies are so important and conspicuous that they have, in effect, distracted us from noticing more subtle changes in physiology or chemical composition occurring at the same time. Methodological difficulties associated with the analysis of minute samples of unknown chemicals represent an additional hurdle, but this has been alleviated by the availability of HPLC-DAD-ESI-MS (Hayes et al. 2009, Hagman et al. 2009). Recent technological advances enabling fast and efficient separation (e.g. ultra-HPLC, monolythic or core-shell technology column) and allowing for accurate and sensitive mass-selective detection (e.g. high resolution MS, quadrupole time-of-flight tandem MS) further facilitate the identification of new components in small tissue samples.

This essay highlights recent discoveries of inducible chemical defences in three general contexts in which they could be important in the animal kingdom and describes the opportunities and benefits of future work on chemical response to predators.

Chemical defences induced by parasites and pathogens

Induced defence against parasites and pathogens is perhaps the most intensely studied area within the field of phenotypic plasticity due to its immediate relevance for human (and non-human) medicine. In vertebrates, the adaptive immune system responds to pathogens in a plastic and inducible manner, enabling hosts to recognize and quickly counteract diseases and parasite infections (Frost 1999). Outside the adaptive immune system, though, little is known about induced chemical defence against parasites. Such mechanisms are usually mentioned as part of the innate immune system in previous studies (Rollins-Smith 2001, Zasloff 2002). Many animal species, including vertebrates, employ non-specific chemical defences that can act as broad-spectrum antibiotics. These can exhibit activity towards bacteria, fungi, viruses, and are effective even against multidrug-resistant strains of pathogens (Nicolas and Mor 1995, Rinaldi 2002, Zasloff 2002, Rollins-Smith et al. 2005, Schmid-Hempel 2005, Mydlarz and Harvell 2007, Mangoni et al. 2008). We know of only two studies that have tested for plasticity in such non-specific chemical defences as a response to pathogens in vertebrates. Miele et al. (1998) discovered that adult *Bombina orientalis* toads increase the production of skin peptides after experimental exposure to the bacterium *Aeromonas hydrophila*. Conversely, Mangoni et al. (2001) observed a sharp decrease in peptide synthesis in *Rana esculenta* frogs kept in sterile water as compared to control animals in naturally bacterium-rich water. With so few examples in vertebrates, it is far from clear how widespread pathogen-inducible chemical defences will prove to be.

Chemical defences induced by predators

Predators represent the second context in which inducible chemical defences may be important. Here again, there is abundant evidence of constitutive expression of toxic or unpalatable chemicals in a variety of vertebrates and invertebrates, and these very often serve as effective deterrents of predation (Toledo and Jared 1995, Kicklighter 2012). Furthermore, toxin levels can vary between life stages and among populations, and this has been interpreted as an adaptation to predictable temporal and spatial differences in predation risk and to the presumably high costs of toxin production (Kubanek et al. 2002, Fordyce et al. 2006, Hayes et al. 2009). Predator-induced changes in toxin production are well-known in planktonic taxa and a few benthic invertebrates (Pohnert 2004). For example, Slattery et al. (2001) observed changes in the production of defensive metabolites in soft corals (*Sinularia* sp.) after transplantation among sites exhibiting different levels of predation. Thornton and Kerr (2002) demonstrated that a cnidarian (*Pseudopterogorgia elisabethae*) produced more pseudopterosins, which may act as predator-deterrents, when attacked by a mollusc predator. Curiously, pseudopterosin production remained unchanged when animals were wounded artificially or preyed upon by a fish (Thornton and Kerr 2002). We know of only two reports of induced toxin production in vertebrates in response to predators. Toad metamorphs (*Anaxyrus boreas* and *Rhinella marina*) that had been raised with predators during the larval stage produced more toxins than their predator-naive conspecifics (Benard and Fordyce 2003, Hagman et al. 2009), illustrating that synthesis or storage of defensive chemicals can be environmentally-induced in vertebrates. However, these studies leave open the question of adaptive significance of induced antipredator responses in chemical defences because the risks of predation in the aquatic and terrestrial stages are not necessarily related.

Chemical defences induced by competitors

One mechanism of indirect interference competition involves chemicals produced and released by individuals that suppress growth or survival of competitors. This phenomenon – called allelopathy (Rice 1974, Reigosa et al. 2006) – has been demonstrated to play an important role in interactions among sponges, cnidarians, bryozoans and ascidians (Jackson and Buss 1975, Thacker et al. 1998, Engel and Pawlik 2000, Kubanek et al. 2002, Pawlik et al. 2007, Chaves-Fonnegra et al. 2008). In these organisms, at least some of the compounds responsible for the allelopathic activity may be produced by surface-associated microbes (Lam 2006). Allelopathy is also described in freshwater zooplankton (Folt and Goldman 1981, Burns 2000). Interference competition among larvae of anuran amphibians has long been termed 'chemical interference' (Petranka 1989), although it is a unicellular alga (*Prototheca*) that suppresses the growth

of competitors: the proliferation of *Prototheca* in the intestines of tadpoles increases dramatically with the density of competing individuals and the scarcity of food resources, so the effect is one of competition-induced growth suppression (Griffiths et al. 1993). However, a recent study reported that the exposure to large toad tadpoles (*Rhinella marina*) during early, non-feeding developmental stages lowered survival and body mass of younger conspecifics (Crossland and Shine 2012), suggesting that allelochemicals may indeed play a role in interference competition among anuran larvae. Nonetheless, it remains unknown whether production of allelochemicals is generally induced by the appearance of competitors.

Outlook

A huge diversity of invertebrates, but also many fishes and amphibians that contain defensive toxins, could serve as model organisms for studies of inducible chemical defences. For example, anuran amphibians are known for depositing their eggs in a wide variety of water bodies, exposing larvae to unpredictably varying abundances of predators, competitors, and pathogens. This then creates conditions ideal for the evolution of phenotypic plasticity (West-Eberhard 1989, Harvell 1990). Species that show relatively weak inducible responses in behaviour or morphology, such as bufonid toads (Laurila et al. 1998, Lardner 2000, Van Buskirk 2002), may instead be relying on defensive skin secretions when facing parasites, predators or competitors (Toledo and Jared 1995, Wells 2007) and may be especially promising model organisms in studies of inducible chemical defences.

Studies on inducible chemical defences will expand our understanding of how animals respond to their environment. It is important to involve more animal taxa in studies testing for inducible chemical defence to determine how general its occurrence may be. To verify the presence of adaptive inducible chemical defences, we need experiments testing whether individuals facultatively adjust their toxin production to their environment, and whether induced changes enhance the fitness of individuals despite costs related to production or storage of toxins.

Data on inducible chemical defences will refine our understanding of life-history tradeoffs and the evolution of plasticity. Theory suggests that behavioural and morphological modes of response to enemies can have distinct fitness consequences, or may interact synergistically in their effects on fitness (Steiner and Pfeiffer 2007, Cressler et al. 2010, Higginson and Ruxton 2010). The two modes are involved in different kinds of tradeoffs, involving constraints in either time or resource acquisition, and probably function at different stages of the predation sequence. Chemical defence may prove to be similar to morphology, because it is costly to express and is therefore involved in a resource acquisition tradeoff. If so, inducible chemical defences will provide opportunities for testing predictions about how time-costs and resource-costs affect life-history transitions and overall investment in defence (Steiner and Pfeiffer 2007, Higginson and Ruxton 2010).

A sharper understanding of inducible chemical defences could also help establish a platform for performing more mechanistic experiments on the evolution of plasticity. In common with all types of inducible defence, the response begins with perception of environmental cues reflecting the risk of encountering enemies (Harvell 1990). Subsequent steps, involving synthesis of the toxic compounds, may prove accessible to biochemical analysis and manipulation. In plants and fungi, the enzymes in question and their production pathways are currently the objects of intense study (Tag et al. 2001, Mao et al. 2011, Ahuja et al. 2012). Knowledge of expression pathways has enabled direct experimental manipulation of chemical production, yielding refined estimates of the fitness costs of defence (Meldau et al. 2012, Yang et al. 2012). Studying the underlying mechanism of toxin production and its genetic basis in animal model systems has similar potential to reveal how genetic variation for plastic responses arises, to test competing hypotheses about the adaptive basis of plasticity and, thus, should lead to a clearer picture of how plasticity evolves and is maintained in nature (Windig et al. 2004). In addition, because studies of plasticity in chemical defences target biogenic and bioactive chemicals, results may provide new insights in medicine, pharmacology, physiology, and agriculture (Daly et al. 1999, Proft 2009, Ujváry 2010). Thus, research on inducible chemical defences could spark both basic and applied research.

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RESEARCH ARTICLE

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Predator-induced changes in the chemical defence of a vertebrate

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Abstract

1. Inducible defences are ubiquitous in the animal kingdom, but little is known about facultative changes in chemical defences in response to predators, especially so in vertebrates.
2. We tested for predator-induced changes in toxin production of larval common toads (*Bufo bufo*), which are known to synthesize bufadienolide compounds.
3. The experiment included larvae originating from three permanent and three temporary ponds reared in the presence or absence of chemical cues of three predators: dragonfly larvae, newts or fish.
4. Tadpoles raised with chemical cues of predation risk produced higher numbers of bufadienolide compounds and larger total bufadienolide quantities than predator-naive conspecifics. Further, the increase in intensity of chemical defence was greatest in response to fish, weakest to newts and intermediate to dragonfly larvae. Tadpoles originating from temporary and permanent ponds did not differ in their baseline toxin content or in the magnitude of their induced chemical responses.
5. These results provide the first compelling evidence for predator-induced changes in chemical defence of a vertebrate that may have evolved to enhance survival under predation risk.

KEYWORDS
among-population variation, antipredator defence, anuran amphibian, local adaptation, phenotypic plasticity, poison

1 | INTRODUCTION

Inducible responses to predators are ubiquitous in the animal kingdom (Tollrian & Harvell, 1999). They evolve because they confer a survival advantage against predators that exceeds whatever fitness costs they may carry. Inducible responses shape ecological patterns and processes and thereby contribute to the diversity, stability and persistence of communities, populations and species (Miner, Sultan,

Morgan, Padilla, & Relyea, 2005), and may pave the way for speciation (Pfennig et al., 2010; West-Eberhard, 1989, 2003). Inducible defences can manifest in many forms, including altered behaviour, morphology and life history (Tollrian & Harvell, 1999). However, whether animals are capable of plastically adjusting their chemical defences to the risk of predation, as many plants are (Karban & Baldwin, 1997), is poorly known (Hettyey, Tóth, & Buskirk, 2014).

Chemical defences are found in many animal taxa, and toxins can be effective in deterring predators (Kicklighter, 2012; Toledo & Jared, 1995). Toxicity is known to vary among populations and life stages within species (Bókony et al., 2016; Fordyce, Nice, & Shapiro, 2006; Hayes, Crossland, Hagman, Capon, & Shine, 2009; Kubanek et al., 2002; Ujszegi, Móricz, Krüzselyi, & Hettyey, 2017; Üveges et al., 2017), which may indicate that the physiological machinery of toxin synthesis is flexible. Also, a handful of studies suggest that plastic responses in animal chemical defences may be induced by the appearance of pathogens (Mangoni, Miele, Renda, Barra, & Simmaco, 2001; Miele, Ponti, Boman, Barra, & Simmaco, 1998), competitors (Bókony et al., 2016; Bókony, Üveges, Móricz, & Hettyey, 2018) and even by anthropogenic pollutants (Bókony, Üveges, Verebélyi, Ujhegyi, & Móricz, 2019; Bókony, Zs, Móricz, Krüzselyi, & Hettyey, 2017). Although changes in toxin levels in response to predators are known to exist in some lower invertebrates (e.g. a sponge: Ebel, Brenzinger, Kunze, Gross, & Proksch, 1997; cnidarians: Slattery, Starmer, & Paul, 2001; Thornton & Kerr, 2002), evidence in vertebrates is scarce and controversial.

Benard and Fordyce (2003) and Hagman, Hayes, Capon, and Shine (2009) showed that juvenile toads of two species altered their toxin synthesis after having been raised in the presence of chemical cues indicating predation risk during the larval stage. However, the adaptive significance of these delayed environment-induced changes in toxin production is unclear because predation risk in the terrestrial habitat of juveniles is unlikely to be correlated with that experienced during the aquatic larval stage. Further, Benard and Fordyce (2003) and Üveges et al. (2017, Üveges, Szederkényi,et al.2019) found no effect of predation risk on toxin synthesis in toad larvae. Bucciarelli, Shaffer, Green, and Kats (2017) reported an increase in the quantity of tetrodotoxin in *Taricha torosa* newts resulting from repeated invasive skin sampling. Although they claimed that these changes represented predator-induced responses in chemical defence, this interpretation is uncertain because no natural predators were used in the experiment, and environmental stressors unrelated to predation can also stimulate the production of chemical defences (Bókony et al., 2018, 2017; Mangoni et al., 2001). It is also unclear whether newts, or indeed metazoans in general, are capable of synthesizing tetrodotoxin (Bane, Lehane, Dikshit, O'Riordan, & Furey, 2014; Chau, Kalaitzis, & Neilan, 2011; Magarlamov, Melnikova, & Chernyshev, 2017).

Predation risk can vary among habitats, so that local adaptation can lead to considerable among-population variation in the expression of defences and in the magnitude of its inducible component (Hettyey et al., 2016; Kishida, Trussell, & Nishimura, 2007; Van Buskirk, 2014). The few studies testing the effects of predators on amphibian chemical defences (Benard & Fordyce, 2003; Hagman et al., 2009; Üveges et al., 2017, Üveges, Szederkényi, et al.,2019) used individuals originating from only one or two populations, and may not have been able to detect plastic responses in chemical defences due to accidental choice of populations with low levels of inducibility. This hypothesis is supported by the observation of Bucciarelli et al. (2017) that the changes in the toxin content of repeatedly injured *T. torosa* newts differed between members of the two studied populations.

To perform a comprehensive test of predator-induced changes in the chemical defences of a vertebrate, we conducted an experiment with an anuran amphibian, the common toad (*Bufo bufo* Linnaeus, 1758), which produces bufadienolide toxins (cardiotoxic steroids) starting early in the larval stage (Üveges et al., 2017). We collected freshly laid eggs from three permanent and three temporary ponds, reared the hatched larvae in either the absence or presence of cues of predation risk and assessed their bufadienolide toxin content after 20 days. We simulated predation risk by exposing developing tadpoles to chemical cues originating from injured conspecifics combined with the chemical cues of either dragonfly larvae, newts or fish. Dragonfly larvae and newts are typical top predators of smaller, temporary water bodies, while fishes dominate the predator fauna of permanent ponds and lakes.

We expected to observe elevated bufadienolide content in tadpoles reared in the presence of predator cues. We also predicted that variation in the magnitude of induced changes in toxin production would depend on the danger represented by the predator species and whether it is sensitive to bufadienolides. Of the three predators used in this experiment, fishes are considered the most dangerous to anuran larvae in general, followed by aeshnid dragonfly larvae and newts (Relyea, 2001; Semlitsch, 1993). However, chemical defences of common toad tadpoles appear to be most effective against fish and newts and less effective against invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019). These relationships led us to predict a strong induced chemical defence against fish (dangerous and sensitive), a weaker response to dragonfly larvae (fairly dangerous but not very sensitive) and the weakest response to newts (sensitive but not very dangerous). Further, we expected to find signs of local adaptation to differences in predation risk (Kawecki & Ebert, 2004) in the form of variation among populations in baseline toxin content (i.e. the number and amount of bufadienolides produced when developing in a predator-free environment) and in the intensity of antipredator responses in toxin synthesis. One reason to expect among-population differences is that continuously high predation risk imposed by fishes in permanent ponds may select for higher baseline toxin production and/or more intense plastic responses than weaker and more variable predation risk in temporary water bodies. Analogous findings have been reported for behavioural and morphological defences (Åbjörnsson, Hansson, & Brönmark, 2004; Herczeg, Turtiainen, & Merilä, 2010; Hettyey et al., 2016; Kishida et al., 2007; Magurran, 1990). However, constantly high predation risk may also purge plasticity in toxin production by selecting for constantly high levels of chemical defences (Crispo, 2007; Pfennig et al., 2010; West-Eberhard, 2003). Also, high

baseline levels of toxin production may hinder a further increase in bufadienolide synthesis because of physiological constraints. Therefore, we predicted that compared to tadpoles from temporary ponds, tadpoles from permanent ponds would exhibit higher baseline toxin levels, and perhaps (but not necessarily) also more intense antipredator responses in toxin production.

2 | MATERIALS AND METHODS

2.1 | Experimental procedures

In early spring 2016, we collected 50 eggs from each of ten *B. bufo* egg strings (families) from each of six water bodies located in the Pilis–Visegrádi Mountains, Hungary. Three of these water bodies are permanent ponds inhabited by fish: Apátkúti-tó (P1; 47°46'1.55"N, 18°58'53.11"E), Garancsi-tó (P2; 47°37'25.38"N, 18°48'26.18"E) and Határréti-tó (P3; 47°38'46.90"N, 18°54'31.82"E), while the other three are temporary ponds lacking fish: Jávor-tó (T1; 47°42'50.32"N, 19°1'10.74"E), Békás-tó (T2; 47°34'34.72"N, 18°52'8.06"E) and Szárazfarkas-belső (T3; 47°44'4.12"N, 18°49'7.04"E). We transferred eggs to the experimental station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences) in Budapest, where we kept them in the laboratory until hatching. Each family was kept in 0.5 L reconstituted soft water (RSW; 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \times 2\ H_2O$, 61 mg/L $MgSO_4 \times 7H_2O$ and 2 mg/L KCl dissolved in reverse-osmosis filtered tap water and treated with UV). We set room temperature to 20°C during daylight hours and 17°C at night. Lighting was set to a 13.5:10.5-hr light:dark cycle in the beginning; day length was increased by half an hour each week to simulate natural changes in the photoperiod.

The experiment had a 6 × 4 complete factorial design with 10 replicates. Tadpoles from the six source ponds were exposed to four predator treatments (described below), with one tadpole in each predator treatment taken from each of ten replicate families per pond. Two days after the tadpoles hatched, we haphazardly selected four from each family, placed them individually into 2-L rearing containers filled with 0.7 L RSW and assigned them randomly to treatments. Containers were arranged in ten spatial blocks in the laboratory; the 24 containers within each block were assigned positions at random. We changed water twice a week and fed tadpoles on these occasions with a 1:100 mixture of finely ground Spirulina alga powder and slightly boiled, chopped spinach ad libitum.

The four treatments were a predator-free control, chemical cues of adult male smooth newts (*Lissotriton vulgaris*), late-instar emperor dragonfly larvae (*Anax imperator*) and adult European perch (*Perca fluviatilis*). Apparent predation risk was manipulated in the experiment by adding stimulus water to the rearing containers of toad tadpoles twice a week. Stimulus water contained chemical cues originating from the respective predators, their prey (see below) and injured conspecific *B. bufo* tadpoles.

To ensure similar concentrations of chemical cues in the three predator treatments, we adjusted the quantity of water and food provided to predators as follows. We maintained six newts together in a 40-L container (57 × 39 × 28 cm, length × width × height) filled with 8 L RSW. Six late-instar (F-1) dragonfly larvae were kept individually in 2-L containers filled with 1 L RSW and equipped with a plastic perching stick. Six fish were housed together in a 140-L tub (82 × 58 × 30 cm) filled with 105 L aerated RSW (which was later lowered to 95 L; see below). These procedures ensured a constant ratio of predator mass to water volume across all predator species, averaging 1.344 g body mass/L ± 0.021 *SD* at the beginning of the experiment. A few predators were replaced during the experiment because they transformed to the terrestrial form (newts), refused to eat (dragonfly larvae) or spawned (fish). We took care to use similar-sized individuals and adjusted water levels if necessary to ensure a constant concentration of cues. Five times a week we fed predators with one agile frog (*Rana dalmatina*) tadpole (a preferred prey of all three predators) and ca. five *Tubifex* worms for every 2 L of RSW. Thus, the group of six fish received a total of 52 tadpoles on each feeding occasion (47 after readjustment of the water volume), the six newts received four tadpoles, and each dragonfly larva received one tadpole on every other feeding occasion. We did not weigh the tadpoles used as food, but chose similar-sized individuals at each feeding. *Rana dalmatina* tadpoles were used to guarantee that predators consumed prey and generated equal amounts of prey-borne cues in all treatments. This would not have been feasible if we had fed predators with toad tadpoles, because smooth newts are reluctant to feed on *Bufo* tadpoles and perch consume *Bufo* but avoid them if possible, while *Anax* larvae readily feed solely on *Bufo* (Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019).

Toad tadpoles in the predator treatments also received chemical cues originating from injured and killed conspecifics. We homogenized 138.5 ± 2.4 mg (mean ± *SD*) common toad tadpoles using a blender in 150 ml water and added the homogenate to 2 L water taken from the housing container(s) of each predator species. Five times a week we pipetted 20 ml freshly prepared stimulus water into the rearing containers of *Bufo* tadpoles assigned to the respective predator treatments. Simultaneously, we added 20 ml RSW into rearing containers of tadpoles in the control treatment. Tadpoles homogenized using a blender perish almost instantly, and therefore may not produce or release all types of chemical cues in the same quantity as during a natural predation event (Fraker et al., 2009), but similar methods have been used before and result in strong induced responses in tadpoles (Hagman et al., 2009; Hettyey et al., 2015; Schoeppner & Relyea, 2005). The procedure described above resulted in 8.3 mg conspecific tadpole tissue per L per week in the rearing containers of focal tadpoles. Similar and also lower concentrations of chemical cues of predation risk have been shown to induce plastic responses in amphibian larvae (Hettyey et al., 2015; McCoy, Touchon, Landberg, Warkentin, & Vonesh, 2012; Van Buskirk & Arioli, 2002). After preparation of stimulus water, we filled the containers of predators with RSW to the original level.

To be able to assess treatment effects on toxin content of toad tadpoles, we preserved all 240 individuals in HPLC-grade absolute

methanol 20 days after the start of the experiment, when tadpoles were at developmental stage 35 (Gosner, 1960). We chose this age to give tadpoles enough time to respond to treatments, to grow large enough to enable the quantification of toxin content, and because other work suggests that bufadienolide content of *Bufo* tadpoles is highest when they are about three weeks old (Ujszegi et al., 2017; Üveges et al., 2017). All tadpoles survived to the end of the experiment.

2.2 | Analysis of toxin content

We used high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS, Model LC-MS-2020; Shimadzu) to identify and quantify bufadienolide compounds. Tadpoles were homogenized with an IKA S12N-7S dispersing tool attached to a VWR VDI 12 homogenizer. We then dried samples *in vacuo* at 45°C using a Büchi Rotavapor R-134 rotary evaporator and measured dry mass to the nearest 0.1 mg using an analytical balance. Samples were re-dissolved in 1 ml HPLC-grade absolute methanol, facilitated by brief exposure to ultrasound in a Tesla UC005AJ1 bath sonicator. We filtered samples using FilterBio nylon syringe filters (pore size: 0.22 μm). We identified compounds as bufadienolides by inspecting the UV (Benard & Fordyce, 2003; Bókony et al., 2016; Hagman et al., 2009) and HRMS/MS spectra of peaks using a QTOF Premier mass spectrometer (Waters Corporation, Manchester, UK) in positive electrospray mode, and by comparing them to the following commercially acquired bufadienolides as standards: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals) and digitoxigenin (Santa Cruz Biotechnology). Identification of compounds present in low quantities as bufadienolides was further aided by the chemical analysis of a pooled sample obtained from 49 juvenile *Bufo* by massaging their parotoid glands.

The HPLC-MS system (Model LC-MS-2020; Shimadzu) was equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector and a single-quadrupole mass analyser with electrospray ionization (ESI/MS). We injected 10 μl of each sample at 35°C on a Kinetex C18 2.6-μm column (100 mm × 3 mm i.d.; Phenomenex) protected by a C18 guard column (4 mm × 3 mm i.d.; Phenomenex). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid, and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.6 ml/min, and the gradient was as follows: 0–2 min: 10%–20% B; 2–15 min: 20%–32% B; 15–21 min: 32%–60% B; 21–21.5 min: 60%–100% B; 21.5–26 min: 100% B; and 26–30 min: 10% B. We set ESI conditions as follows: interface temperature: 350°C; desolvation line (DL) temperature: 250°C; heat block temperature: 400°C; drying N_2 gas flow: 15 L/min; nebulizer N_2 gas flow: 1.5 L/min; and positive ionization mode. We recorded full-scan spectra in the range of 350–800 m/z and also performed selected-ion monitoring (SIM) acquisition detecting the base peak of bufadienolides we previously found in common toads (Bókony et al., 2016; Üveges et al., 2017). We acquired and processed data using the software LabSolutions 5.42v (Shimadzu Corp.).

2.3 | Statistical analysis

One sample was lost during preparation for HPLC-DAD-MS analysis, resulting in a sample size of 239 tadpoles. We determined the number of bufadienolide compounds (NBC) for each tadpole by assuming that a compound was present if the signal-to-noise ratio (S/N) of its peak, calculated from appropriate SIM chromatogram by the LabSolutions software, was at least three. We estimated the quantity of each bufadienolide compound from the area of its chromatogram peak using the calibration curve of the bufotalin standard, and we obtained estimates of total bufadienolide quantity (TBQ) for each tadpole by summing up these values. This approach yields only a rough estimate of TBQ, but due to the unavailability of standards for the majority of bufadienolides, there is currently no better alternative for toxin quantification. This method has been successfully applied in similar studies (Benard & Fordyce, 2003; Bókony et al., 2016, 2018, 2019, 2017; Hagman et al., 2009; Tóth, Kurali, Móricz, & Hettyey, 2019; Üveges et al., 2017). We calculated mass-corrected total bufadienolide quantities (mcTBQ) by dividing TBQ values by tadpole dry mass. We calculated mcTBQ to estimate individual investment (i.e. proportion of resources allocated to toxin synthesis), while TBQ is more likely to be relevant for the actual outcome of predatory interactions.

We investigated if predator treatments affected growth and development rates by analysing variation in tadpole body mass (dry mass at toxin sampling) using a linear mixed-effects model, entering predator treatment as a fixed factor and family nested within population crossed with block as random factors. Because developmental stage (Gosner, 1960) in our sample had only a few discrete values (79% of individuals were in stage 35 or 36), we used Mann–Whitney U tests to compare developmental stage of tadpoles in the control treatment to those in each predator treatment. We corrected *p*-values for the number of comparisons by applying the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). There was a reduced sample size for developmental stage because we assessed this trait in only a subset of individuals (N = 48, i.e. two replicates for each combination of predator treatment by population). Tadpoles showed little variation in stage (range: 33–37), and in a previous experiment where we used tadpoles in very similar developmental stages (range: 32–35), we found no correlation between developmental stage and toxin content (Bókony et al., 2017).

We ran three linear mixed-effects models, one for NBC, one for TBQ and one for mcTBQ, entering predator treatment and population and their interaction as fixed factors, and block crossed with family as random factors. From each model, we calculated the following pre-planned comparisons (linear contrasts; for R scripts, see supplementary material). First, we assessed among-population differences in baseline toxin production, that is in the absence of predator cues, by comparing the control group's estimated confidence intervals between the six ponds. We also compared baseline toxin levels between permanent and temporary ponds by estimating the difference between the mean of the three permanent ponds and the mean of the three temporary ponds in the absence of predator cues.

Second, to test for predator-induced responses in toxin production, we first estimated among-treatment differences irrespective

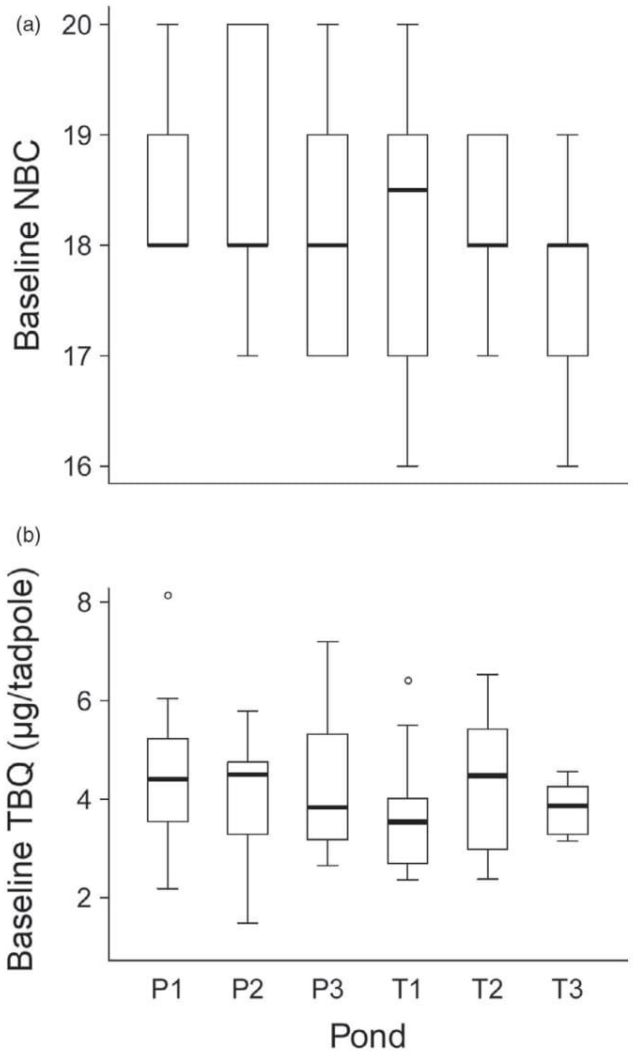

FIGURE 1 The number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in control tadpoles reared in the absence of cues of predation risk separated by their population of origin. Thick horizontal lines represent medians, boxes represent the interquartile ranges, and whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points. Numbering of permanent (P) and temporary (T) ponds of origin corresponds with that in the 'Methods' section

of among-population differences, calculating the overall difference between the control group (mean of six populations) and each predator treatment (mean of six populations). Next, to assess among-population differences in antipredator responses, we calculated differences in toxin production between the control and each predator treatment within each population. Finally, we calculated the difference between permanent and temporary ponds in the response to each predator (i.e. difference between the control and the respective predator treatment), as a linear contrast of the within-population contrasts (i.e. comparing the average response of the three permanent-pond populations to the average response of the three temporary-pond populations). In each step, we corrected *p*-values for the number of comparisons by the FDR method.

Throughout the statistical analyses on toxin content of tadpoles, we used the approach of planned comparisons (Ruxton & Beauchamp, 2008), in which we first estimated the mean of each population (i.e. mean baseline toxin content, or mean response in toxin content in response to each predator) in a linear model and then estimated the effect of pond permanence as the difference between the mean of the three permanent ponds and the mean of the three temporary ponds. This approach has two main advantages over using pond permanence as fixed effect and pond as random effect. First, because ponds are nested within the two permanence categories, and there were only three ponds of each category, a mixed model with pond as random effect would have low power for testing the fixed effect of pond permanence. Second, estimating the variance component due to a random effect is reliable only when the number of levels is large (Bolker et al., 2009), so we could not evaluate variance among populations if pond were included as a random effect. Therefore, pond was a fixed effect as detailed below.

We confirmed that our data fit the assumptions of analyses by inspecting residual plots. Mixed-effects models were fitted using the 'lmer' function of the 'lme4' package (Bates, Mächler, Bolker, & Walker, 2015) in R v. 3.4.0 (R Core Team, 2017). Satterthwaite approximation was used to calculate degrees of freedom. For calculating linear contrasts, we used the 'lsmeans' package (Lenth, 2016). We report least-squares means with standard errors (*SE*s) and with 84% confidence intervals (CIs) to facilitate comparisons between populations, because the lack of overlap between two 84% CIs indicates a significant difference, that is is equivalent to a 95% CI around the difference not including zero (Julious, 2004).

3 | RESULTS

3.1 | Treatment effects on body mass and development

At the termination of the experiment, body mass of tadpoles raised in the presence of chemical cues from dragonfly larvae and fish was significantly lower than in predator-naïve tadpoles, while the mass of tadpoles exposed to cues from newts did not differ from that of controls (Table S1, Figure S1). Tadpoles exposed to cues from newts tended to be more developed than control tadpoles (Mann–Whitney *U* test; $W = 40.5$, $p = .07$; Figure S1), whereas those raised in the presence of cues from fish were slightly less developed than controls ($W = 104$, $p = .07$; Figure S1), and tadpoles exposed to dragonfly cues did not differ in developmental stage from controls ($W = 82.5$, $p = .52$; Figure S1).

3.2 | Baseline toxin content

The analysis on control tadpoles reared in the absence of cues of predation risk revealed no significant variation among populations either in NBC or in TBQ (Table S2, Figure 1). Linear contrasts indicated that baseline NBC and TBQ did not differ between tadpoles originating from permanent and temporary ponds (difference in

TABLE 1 Estimates of linear contrasts and their *p*-values corrected for false discovery rate, comparing the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) between treatments. Significant differences are highlighted in bold

Trait	Contrasts	Estimate ± *SE*	*df*	84% CI	*t*	*p*
NBC	Control versus newt	**0.889 ± 0.139**	**156.60**	0.693, 1.086	**6.39**	**<.001**
	Control versus dragonfly	**1.100 ± 0.138**	**156.01**	0.904, 1.296	**7.94**	**<.001**
	Control versus fish	**1.367 ± 0.138**	**156.01**	1.171, 1.562	**9.87**	**<.001**
	Newt versus dragonfly	0.211 ± 0.139	156.60	0.014, 0.407	1.51	.132
	Newt versus fish	**0.477 ± 0.139**	**156.60**	0.281, 0.674	**3.43**	**.001**
	Dragonfly versus fish	0.267 ± 0.138	156.01	0.071, 0.462	1.93	.067
TBQ	Control versus newt	**494.13 ± 191.93**	**123.56**	222.81, 765.44	**2.57**	**.014**
	Control versus dragonfly	**541.89 ± 190.81**	**123.29**	272.16, 811.61	**2.84**	**.008**
	Control versus fish	**1,084.36 ± 190.81**	**123.29**	814.63, 1,354.09	**5.68**	**<.001**
	Newt versus dragonfly	47.76 ± 191.93	123.56	−223.55, 319.07	0.25	.804
	Newt versus fish	**590.23 ± 191.93**	**123.56**	318.92, 861.55	**3.08**	**.008**
	Dragonfly versus fish	**542.47 ± 190.81**	**123.29**	272.75, 812.20	**2.84**	**.008**

NBC: 0.37 ± 0.21 bufadienolide compounds, $t_{191.3} = 1.71$, $p = .09$; in TBQ: 293.06 ± 368.21 ng bufadienolides per tadpole, $t_{125.9} = 0.8$, $p = .43$; in mcTBQ: 0.05 ± 0.05 ng bufadienolides per mg tadpole mass, $t_{194.9} = 0.95$, $p = .34$).

3.3 | Plasticity in toxin production

Tadpoles exposed to predators responded with the production of increased numbers of bufadienolide compounds (Table 1; Figure 2). Linear contrasts revealed that NBC was significantly lower in predator-naive tadpoles (18.18 ± 0.13 compounds; mean ± *SE*) than in tadpoles exposed to cues of any species of predator and that the response was strongest to fish (19.55 ± 0.07), intermediate to dragonflies (19.28 ± 0.11) and weakest to newts (19.07 ± 0.12; Table 1, Figures 2 and 3). We detected significant variation among tadpoles according to population of origin in the intensity of predator-induced changes in NBC as indicated by non-overlapping 84% confidence intervals (Table S3, Figure S2). However, these differences were not attributable to pond permanence: tadpoles from both types of ponds produced significantly higher numbers of bufadienolide compounds in response to each of the three predator species, but this response did not differ between permanent and temporary ponds (Table 2, Figure 3).

Total bufadienolide quantity also responded to the predator treatments (Table 1, Figure 2). Tadpoles in the control treatment produced the lowest TBQ (4,155.72 ± 164.66 ng per tadpole; mean ± *SE*) and those reared in the presence of cues from fish the highest (5,240.08 ± 180.57), whereas tadpoles exposed to cues of newts and dragonflies contained intermediate toxin levels (newts: 4,658.7 ± 215.6; dragonflies: 4,697.6 ± 173.41; Table 1, Figures 2 and 3). Linear contrasts indicated that predator-naive tadpoles had significantly lower TBQ than tadpoles in any other treatment (Table 1, Figure 2). We did not detect significant among-population variation in the intensity of predator-induced changes in TBQ, except for a slight difference in response to fish cues between ponds P3 and T3 (Figure S2). When analysing antipredator responses in TBQ by pond permanence type, we found that tadpoles originating from temporary ponds responded to dragonflies with increased toxin production, and so did tadpoles originating from both types of water bodies exposed to chemical cues of fish (Table 2, Figure S2). On the other hand, the response to newts in either type of water body and the response to dragonflies in permanent ponds were marginally non-significant after FDR adjustment. However, linear contrasts did not reveal significant differences in the magnitude of responses between tadpoles originating from temporary and permanent ponds (Table 2; Figure 3).

We obtained qualitatively similar results when analysing variation in mass-corrected total bufadienolide quantity (mcTBQ); in some ponds, these responses were even stronger than the responses observed in TBQ (see Tables S2–S5; Figure S3).

4 | DISCUSSION

Our results demonstrate predator-induced changes in the chemical defence of *Bufo bufo* larvae. Tadpoles reared in the presence of chemical cues of predation risk produced a larger number of bufadienolide compounds and higher total bufadienolide quantity than did tadpoles that developed in a predator-free environment. There was a detectable increase in toxin production in the presence of three very different predator taxa. Furthermore, the strength of induced responses depended on the species of predator present in the larval environment, with fish causing the greatest response. Although plasticity in toxin production did vary significantly among populations, neither baseline toxin content in the absence of predators nor the magnitude of predator-induced responses differed significantly between tadpoles originating from permanent and temporary ponds.

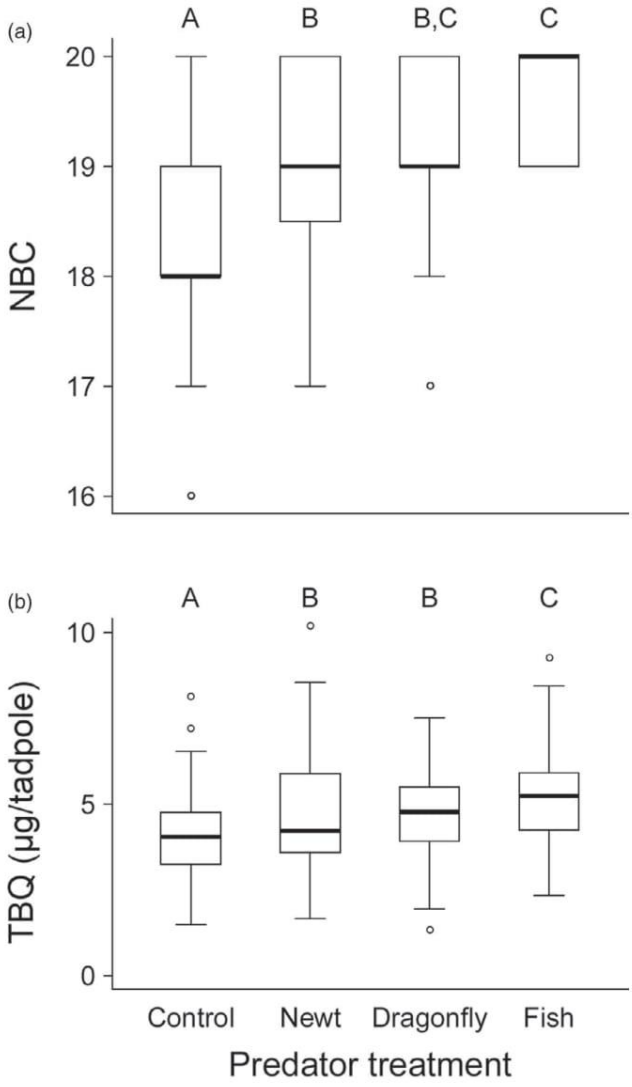

FIGURE 2 The number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in treatments differing in predation risk. Thick horizontal lines depict medians, boxes depict the interquartile ranges, and whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points. Letters above boxplots indicate homogeneous subsets according to pairwise comparisons based on linear contrasts corrected for false discovery rate

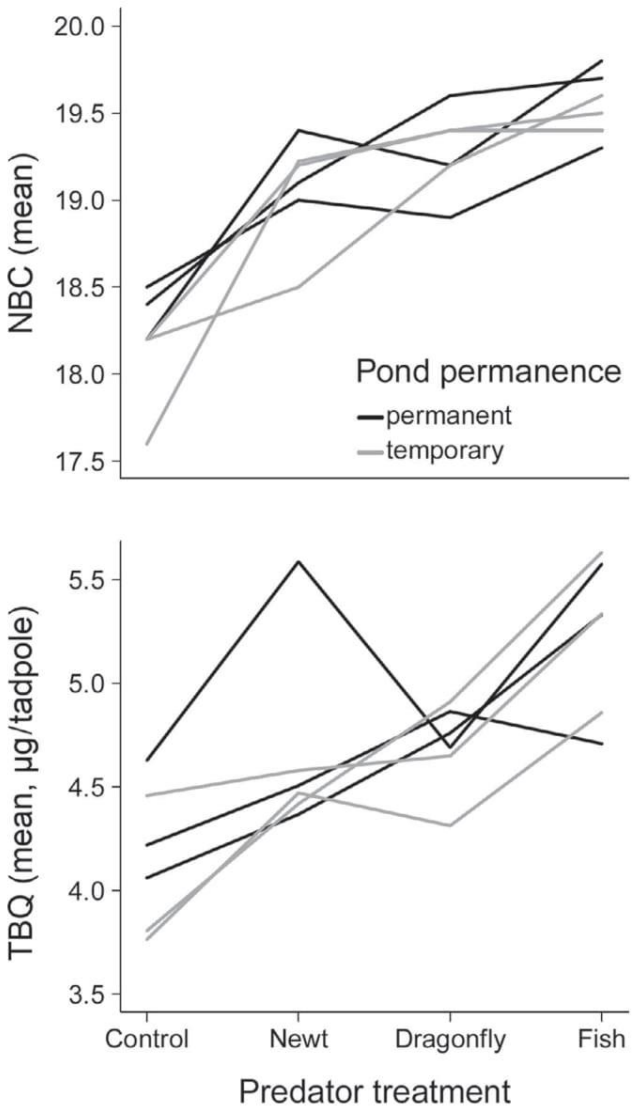

FIGURE 3 Mean values of each population for the number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in treatments differing in predation risk. Each line represents one population. The increase in bufadienolide production induced by predators was similar in tadpoles from permanent and temporary ponds. For standard errors of the mean values, see Figure S4

Our study is the first to deliver clear evidence for predator-induced changes in the chemical defence of a vertebrate that can be interpreted as adaptive phenotypic plasticity. Although it has been known for two decades that invertebrates can plastically adjust their toxin production to the presence of predators in ways that enhance survival (e.g. Ebel et al., 1997; Slattery et al., 2001; Thornton & Kerr, 2002), similar reports for vertebrates have so far provided only circumstantial evidence (Benard & Fordyce, 2003; Bucciarelli et al., 2017; Hagman et al., 2009). Results of the present study also indicate that induced changes in chemical defences can vary depending on the predator species present, much as they do for other defensive traits (Hettyey, Vincze, Zsarnóczai, Hoi, & Laurila, 2011; Kishida & Nishimura, 2005; Relyea, 2001; Sih, 1986; Van Buskirk, 2001). The question arises whether the responses to the different predators could be predicted based on the information available on their relationship with prey. The level of defence should depend on its benefits and costs. Fishes are the most voracious predators of tadpoles in general, followed by dragonfly larvae and newts. At the same time, vertebrate predators are more sensitive to the toxins produced by toad tadpoles than invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019). Thus, the highest benefit of toxin production is expected when the predator is potentially dangerous and highly voracious but also sensitive to the toxins (Üveges, Szederkényi, et al., 2019). Finally, costs

TABLE 2 Treatment effects on the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) in tadpole populations originating from temporary (T) and permanent (P) ponds. Estimates of linear contrasts compare tadpoles reared in the control treatment to those exposed to chemical cues of newts, dragonfly larvae or fish, within each population type, that is permanent (P) or temporary (T) ponds. *p*-values were corrected for false discovery rate. We also present comparisons of the effects of predator treatment (i.e. the difference between control and predator treatment) between permanent and temporary ponds (P vs. T) based on linear contrasts of the within-population contrasts. Significant differences are highlighted in bold

Trait	Contrasts	Pond type	Estimate ± *SE*	*df*	84% CI	t	*p*
NBC	Control versus newt	**T**	**0.979 ± 0.196**	**157.17**	**0.699, 1.258**	**4.95**	**<.001**
		P	**0.800 ± 0.196**	**156.01**	**0.524, 1.076**	**4.09**	**<.001**
		P versus T	−0.179 ± 0.278	156.60	−0.572, 0.214	−0.64	.522
	Control versus dragonfly	**T**	**1.333 ± 0.196**	**156.01**	**1.057, 1.610**	**6.81**	**<.001**
		P	**0.867 ± 0.196**	**156.01**	**0.590, 1.143**	**4.43**	**<.001**
		P versus T	−0.467 ± 0.277	156.01	−0.858, −0.076	−1.69	.094
	Control versus fish	**T**	**1.500 ± 0.196**	**156.01**	**1.224, 1.776**	**7.66**	**<.001**
		P	**1.233 ± 0.196**	**156.01**	**0.957, 1.510**	**6.30**	**<.001**
		P versus T	−0.267 ± 0.277	156.01	−0.658, 0.124	−0.96	.337
TBQ	Control versus newt	T	470.4 ± 273.0	123.82	84.4, 856.3	1.72	.087
		P	517.9 ± 269.8	123.29	136.4, 899.3	1.92	.087
		P versus T	47.5 ± 383.9	123.56	−495.1, 590.1	0.12	.902
	Control versus dragonfly	**T**	**614.8 ± 269.8**	**123.29**	**233.3, 996.2**	**2.28**	**.049**
		P	469.0 ± 269.8	123.29	87.5, 850.4	1.74	.085
		P versus T	−145.8 ± 381.6	123.29	−658.3, 393.6	−0.38	.703
	Control versus fish	**T**	**1,265.9 ± 269.8**	**123.29**	**884.5, 1647.4**	**4.69**	**<.001**
		P	**902.8 ± 269.8**	**123.29**	**521.3, 1,284.3**	**3.35**	**.001**
		P versus T	−363.1 ± 381.6	123.29	−902.6, 176.3	−0.95	.343

of enhanced bufadienolide production are expected to be substantial (Hettyey et al., 2014), and this was recently demonstrated in subadult and adult toads (Blennerhassett, Bell-Anderson, Shine, & Brown, 2019), although clear evidence for such costs in tadpoles has remained elusive (Benard & Fordyce, 2003; Hagman et al., 2009; Kurali, Pásztor, Hettyey, & Tóth, 2016; Üveges et al., 2017). Consequently, our observation that tadpoles produced the highest number and quantity of bufadienolide compounds in the presence of fish, the lowest in response to adult newts and intermediate levels in response to dragonfly larvae corresponds well to what is expected of an adaptive inducible defence (see also Üveges, Szederkényi, et al., 2019).

It is theoretically possible that predators could influence toxin production indirectly by affecting tadpole body size and development rate. Indeed, *B. bufo* larvae modify their growth and development rates under predation risk (e.g. Lardner, 2000; Laurila, Kujasalo, & Ranta, 1998; Van Buskirk, 2000), and toxin content is known to change during development (Ujszegi et al., 2017; Üveges et al., 2017). However, the details of our findings cannot be explained by simple developmental scaling of toxin production. Both NBC and TBQ consistently increased in response to all tested predators, while development rate and growth responded to different predators in different directions. At the same time, we observed that treatments inducing the largest increase in toxin production also caused the greatest decline in tadpole mass. While this may suggest that increasing toxin production is costly, that interpretation would be premature because the experiment was not designed to properly separate treatment-induced changes in individual traits from trade-offs among these traits.

Our finding that predation risk can induce changes in the toxin production of common toad tadpoles contradicts the results of two previous studies with this species (Üveges et al., 2017, Üveges, Szederkényi, et al.,2019). There are three possible explanations for this discrepancy. First, earlier experiments included tadpoles from only one population each, which may have by chance exhibited little plasticity in chemical defence. Indeed, populations can vary in their responses to environmental cues (Åbjörnsson et al., 2004; Crispo, 2007; Hettyey et al., 2016; Magurran, 1990; Pfennig et al., 2010; West-Eberhard, 2003), and the present study shows that this is also true for the strength of antipredator responses in toxin production. Second, the sample size per treatment was about five times higher in the present experiment than in the previous studies, and this may have resulted in a decisive improvement in statistical power. Finally, previous experiments raised tadpoles in groups (three tadpoles in 1.5 L and 60 tadpoles in 130 L), whereas in the present study we reared tadpoles individually. It is known that the presence of conspecifics in the environment can affect the expression of inducible defences due to prey risk assessment taking into account risk dilution and group vigilance (Peacor, 2003; Tollrian, Duggen, Weiss, Laforsch, & Kopp, 2015; Van Buskirk, Ferrari, Kueng, Näpflin, & Ritter, 2011). Moreover, we recently discovered that *B. bufo* tadpoles adjust their toxin production to the density of conspecifics even in the absence of

predators (Bókony et al., 2018), and the toxin content induced by high densities may be high enough for effective protection from various predators (Üveges, Szederkényi et al., 2019). All three of these explanations seem possible, and together they suggest that the contradiction between this study and previous findings may have been caused by chance effects and differences in methodology.

We found no evidence that chemical defences of toad tadpole populations are locally adapted to pond permanence. Although populations varied in their induced antipredator responses, those originating from temporary or permanent ponds did not show the strongest responses to predator taxa that predominate in their pond type. The hypothesis of local adaptation predicts that tadpoles from permanent ponds should show the greatest response to fish, whereas tadpoles from temporary ponds should respond more strongly to dragonflies or newts. These predictions were not upheld (Figure S2). For other kinds of inducible defence—for example involving behaviour, morphology and life history—populations exposed to continuously high predation risk sometimes exhibit more defended phenotypes and more intense antipredator responses than populations originating from low-risk habitats (Åbjörnsson et al., 2004; Herczeg et al., 2010; Hettyey et al., 2016; Kishida et al., 2007; Magurran, 1990). The absence of local adaptation in our study could reflect the swamping effect of gene flow (Blanquart, Gandon, & Nuismer, 2012; Kawecki & Ebert, 2004; Yeaman & Otto, 2011) between permanent ponds and temporary puddles, which are frequently situated immediately adjacent to one another in our study area. Also, selection favouring adaptation to either type of pond could be weakened by microhabitat heterogeneity within ponds. For example, permanent wetlands with fish often have shallow areas that are inaccessible to fish, and these provide safe refugia for tadpoles. Finally, chemical defences of toads are in general more effective against vertebrate than invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002), and even low quantities of bufadienolides can provide efficient defences against fishes (Üveges, Szederkényi, et al., 2019). Consequently, ecological factors other than fish presence, such as the density of other predators or conspecifics, may be more important in determining the strength of selection on chemical defences and on plasticity therein.

In conclusion, this study provides clear evidence for inducible responses to predators in chemical defences of a vertebrate. Four arguments suggest that these responses could reflect an adaptive outcome of natural selection imposed by predators: (a) the inducible changes in toxin synthesis occurred in the same environment in which animals encountered cues indicating risk, (b) the observed changes were induced by predators that coexist with *B. bufo* tadpoles in natural populations, (c) the direction of the response (i.e. an increase in both NBC and TBQ induced by predators) indicates that the response is likely to be beneficial to a tadpole under predation risk, and (d) the magnitude of the response varied among predators as predicted by the theory of adaptive phenotypic plasticity; that is, the strongest response was elicited when it was most beneficial because the predator species was potentially highly dangerous and at the same time also highly sensitive to the toxins. Nonetheless, it remains an open question whether antipredator responses in toxin synthesis of toad tadpoles are indeed adaptive, and how frequently predator-induced changes in chemical defences occur in the animal kingdom.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

A.H., B.Ü., J.V.B., R.J.C., Z.T. and V.B. conceived the study; A.H., B.Ü. and V.B. designed the study and conducted statistical analyses; B.Ü. conducted the experiment and prepared samples for chemical analysis; Á.M.M. and L.D. analysed toxin samples; and A.H., B.Ü., Á.M.M., J.V.B. and V.B. wrote the manuscript, and all other authors contributed to its final version.

DATA AVAILABILITY STATEMENT

The dataset analysed in the current study is available on figshare: https://doi.org/10.6084/m9.figshare.8256620.v1 (Üveges, Hettyey, et al. 2019)

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SUPPORTING INFORMATION

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RESEARCH ARTICLE

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Competition induces increased toxin production in toad larvae without allelopathic effects on heterospecific tadpoles

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Abstract

1. Inducible defences are a form of phenotypic plasticity by which organisms respond to and mitigate the threat posed by predators, parasites and competitors. While anti-predatory defences are often in trade-off with anti-competitor responses, chemicals that deter predators may have negative effects on competitors as well. Allelopathy is well known in plants and plant-like animals, but whether the toxins of mobile, behaviourally and morphologically complex animals are induced by and exert allelopathic effects on competitors is poorly known.
2. Common toads *Bufo bufo* synthesize bufadienolides which make them unpalatable or toxic to many predators. However, bufadienolide content of toad tadpoles correlates positively with the density of competitors in natural populations, suggesting that they may upregulate their toxin production to inhibit their competitors, such as heterospecific tadpoles that may be vulnerable to toad toxins.
3. We conducted a microcosm experiment with tadpoles of common toads and agile frogs *Rana dalmatina*, in which we manipulated the density of conspecific and heterospecific competitors. We measured the bufadienolide content of toad tadpoles to test for competitor-induced changes in toxin production, and we assessed the growth and development of agile frog tadpoles to test for allelopathy.
4. We found that toad tadpoles contained higher amounts of bufadienolides at higher densities; however, heterospecific competitors did not have a stronger effect than conspecifics. Furthermore, the presence or density of toad tadpoles had no effect on the body mass and development rate of agile frog tadpoles.
5. Our results demonstrate competitor-induced plasticity in toxin production, but we found no support for an allelopathic function of bufadienolides. Instead, we suggest that inducible changes in bufadienolide production may serve to mitigate risks posed by competitors, including aggression, cannibalism or disease. Therefore, bufadienolides are intriguing candidates for multi-purpose defences that may provide protection not only against predators but also against competitors.

KEYWORDS
allelopathy, amphibian toxins, chemical defence, chemical interference, growth inhibition, growth-defence trade-off, inducible defences, phenotypic plasticity

1 | INTRODUCTION

In response to the risk posed by natural enemies, many organisms including animals and plants produce altered phenotypes that provide protection against those enemies; this form of phenotypic plasticity is referred to as inducible defence (Adler & Harvell, 1990; Tollrian & Harvell, 1999). It occurs in diverse taxa in many forms, including changes in body shape that reduce palatability or enhance escape ability, behavioural responses that reduce the encounter rate with or detectability to predators, and accumulation of repellent or toxic chemicals (Adler & Harvell, 1990; Hettyey, Tóth, & Van Buskirk, 2014; Tollrian & Harvell, 1999). So far, the majority of research on inducible defences has focused on the effects of predators (not counting the extensive research on immune responses to pathogens), demonstrating that predator-induced phenotypic changes are ubiquitous and effective means of enhancing the survival of prey (Adler & Harvell, 1990; Hettyey, Vincze, Zsarnóczai, Hoi, & Laurila, 2011; Relyea & Auld, 2005; Tollrian & Harvell, 1999; Van Buskirk, 2002). However, predators are not the only kind of enemies that organisms need to fend off; competitors can also have large effects (Connell, 1983; Gurevitch, Morrow, Wallace, & Walsh, 1992). The adaptive responses against competitors are often in trade-off with the adaptive responses against predators: for example, behavioural and morphological changes that are beneficial in competition, such as elevated foraging activity and larger intestines which facilitate growth, expose individuals to higher predation risk (Relyea, 2002; Relyea & Auld, 2004, 2005; Tollrian & Harvell, 1999). Chemical defences are particularly intriguing in this respect because they may be multi-functional in the sense that a single phenotype may provide protection against several types of enemies (Hettyey et al., 2014). For example, in plants and soft corals, the defensive chemicals can have both anti-predatory and anti-competitor effects (Kubanek et al., 2002; Siemens, Garner, Mitchell-Olds, & Callaway, 2002). Understanding such responses whose effectiveness against predators and competitors is not traded off against each other (Ramamonjisoa & Natuhara, 2017; Siemens et al., 2002) should provide valuable insights into the ecology and evolution of phenotypic plasticity (Hettyey et al., 2014).

In competitive interactions, organisms can use chemical substances that provide advantage by harming their competitors; such substances have been variably termed defensive or offensive chemicals, allelochemicals or allomones (Berenbaum, 1995). Chemical interference or allelopathy can be an effective way of overcoming competitors, especially in sessile organisms like plants, fungi and benthic marine invertebrates (Reigosa, Pedrol, & González, 2006). The role of allelochemicals in competitive interactions is much less known in mobile animals that can employ a wide diversity of behavioural responses against their foes, although toxins can be found in many of such organisms (Brodie, 2009; Casewell, Wüster, Vonk, Harrison, & Fry, 2013). Defensive toxins of such animals are thought to function mainly as anti-predatory adaptations, and there is some evidence that they can be induced in prey animals by predation threat (Benard & Fordyce, 2003; Hagman, Hayes, Capon, & Shine, 2009) similar to the herbivore-induced chemical responses of primary producers (Tollrian & Harvell, 1999). However, we know very little about the phenotypic plasticity of toxin production in animals in response to competitors (Adler & Harvell, 1990; Hettyey et al., 2014).

In this study, we investigated the effect of competition on the toxin production of amphibian larvae, and the allelopathic potential of competitor-induced toxin production. At high densities, amphibian larvae compete for food by both exploitation and interference (Wells, 2007), and chemical interference has long been suspected as a mechanism by which tadpoles can inhibit the growth of their competitors (Crossland & Shine, 2012; Licht, 1967; Wells, 2007). Despite considerable research effort, however, it is still unclear whether this interference is mediated by specific growth-inhibitor substances, metabolic waste products, or facultative gut parasites such as yeasts or algae (Bardsley & Beebee, 2001; Griffiths, Denton, & Wong, 1993; Wells, 2007). Furthermore, it is not clear how tadpoles could inhibit the growth of conspecifics by such substances without suffering from autotoxicity themselves (Wells, 2007), suggesting that chemical interference is more likely to function in interspecific competition, similar to allelopathy among plants (Reigosa et al., 2006) and to the chemical repellents used by ants for deterring heterospecific competitors from food sources (Adams & Traniello, 1981).

We examined common toads *Bufo bufo*, which contain toxins that make them distasteful or even lethal upon ingestion or contact (Crossland, Brown, & Shine, 2011; Henrikson, 1990) or via indirect, waterborne interactions (Crossland & Shine, 2012; Crossland et al., 2011). Their main toxins are steroid compounds called bufadienolides, which they start to synthesize early during larval development (Üveges et al., 2017). Our earlier studies showed that in common toad larvae, the diversity and quantity of bufadienolides were higher in natural populations with higher competitor density (Bókony et al., 2016) and increased when tadpoles were food-restricted in the laboratory (Üveges et al., 2017); both findings suggested that competition induced toxin production. Toad tadpoles often develop in the same water bodies and live on similar diets as tadpoles of other, non-toxic species, such as agile frogs *Rana dalmatina* (Bókony et al., 2016; McDiarmid & Altig, 1999). Because agile frogs usually start to spawn several weeks before toads in Hungary (Hettyey, Török, & Kovács, 2003) and the tadpoles of the former species grow to larger sizes (Lardner, 2000), toad tadpoles would benefit from inhibiting the growth and development of agile frog tadpoles. Whether such inhibition occurs and whether it is associated with toad toxin levels has not been investigated yet, although other bufonid species were observed to have strong negative effects on other ranid species during larval competition (Alford & Wilbur, 1985; Licht, 1967). Using the common toad–agile frog system, we investigated competitor-induced toxicity and allelopathy by testing the following predictions: (1) stronger competition induces increased toxin production, (2) heterospecific competitors have a larger effect on toxin production than do conspecific competitors and (3) toxin-producing tadpoles inhibit the growth and development of non-toxic heterospecific tadpoles. We experimentally manipulated the strength of competition and the ratio of conspecific and heterospecific competitors in microcosm communities, mimicking natural conditions of small

ponds that are the typical larval habitats of these amphibians (Vági, Kovács, Băncilă, Hartel, & Anthony, 2013).

2 | MATERIALS AND METHODS

2.1 | Experimental design

We raised common toad (henceforth Bufo) and agile frog (henceforth Rana) tadpoles in eight density treatments (Figure 1a) following a response surface design (Inouye, 2001). The densities were chosen to reflect low, medium and high levels of competition based on our previous experience with mesocosm experiments with the two study species (Bókony, Mikó, Móricz, Krüzselyi, & Hettyey, 2017; Hettyey et al., 2011; Mikó, Ujszegi, Gál, Imrei, & Hettyey, 2015). Three treatment groups (6B, 12B and 24B) contained only Bufo tadpoles (Figure 1a) to test if the production of bufadienolides is adjusted to the density of conspecific competitors. Three treatment groups contained tadpoles of both species (Figure 1a) to compare the effects of conspecific competitors to the effects of heterospecific competitors on the production of bufadienolides, while keeping the total biomass constant. The relative numbers of the two species in these treatments were designed based on our observation that Rana tadpoles grow up to twice as large as Bufo tadpoles in outdoor mesocosms. Thus, we expected six Bufo larvae plus three Rana larvae (treatment 6B3R) to have similar total biomass as 12 Bufo larvae (treatment 12B). Similarly, we expected six Bufo larvae combined with nine Rana larvae (treatment 6B9R) to have a total biomass similar to that of 12 Bufo larvae combined with six Rana larvae (treatment 12B6R) or 24 Bufo larvae (treatment 24B). The expected ratio of the two species' biomass was 1:1 in treatments 6B3R and 12B6R, while in treatment 6B9R, it was 1:3 (Bufo:Rana). This latter treatment was added for double purpose: to address not only competition-induced toxicity but also allelopathy, because we

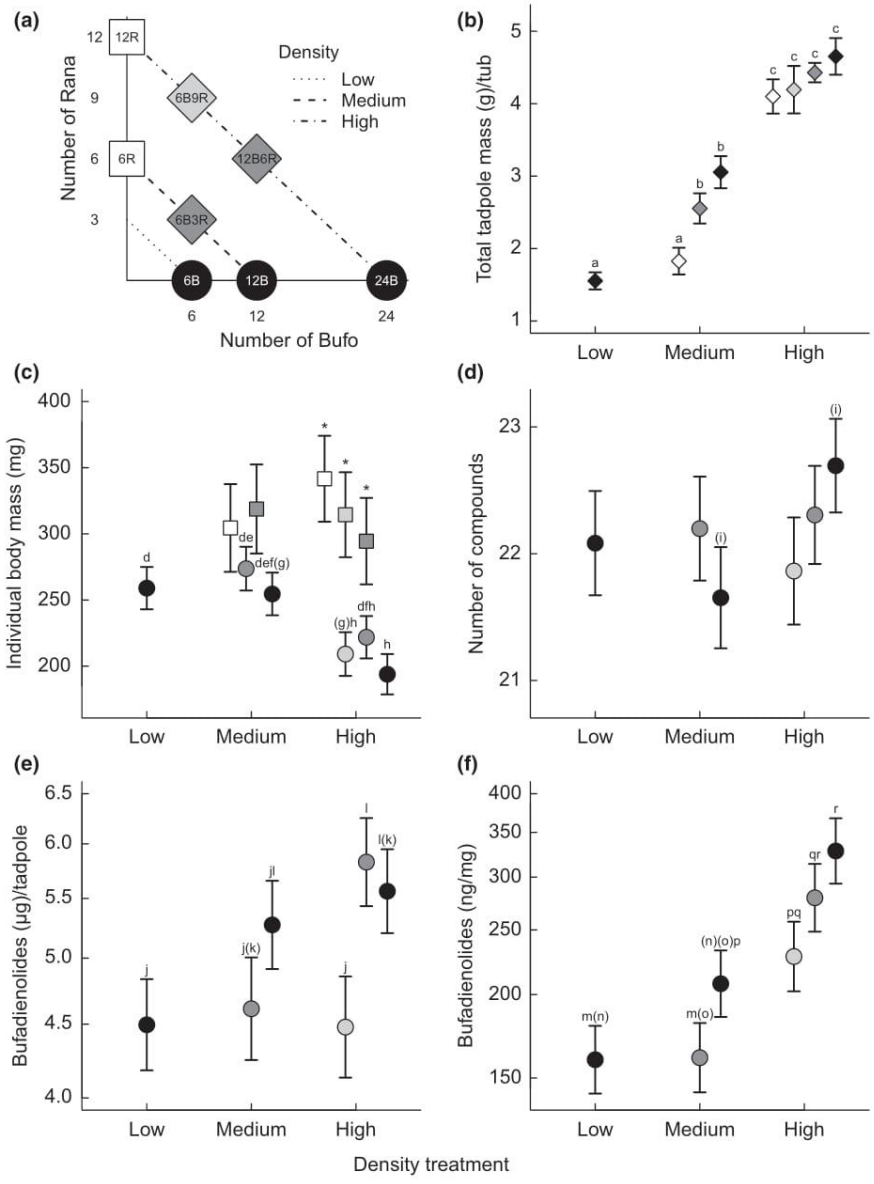

FIGURE 1 Schematics of the experimental design (a), and the effects of density treatments on the *M* ± *SE* of tadpole body mass (b, c) and bufadienolides (d–f). Letters above the error bars indicate homogenous subsets after correction for multiple comparisons, i.e. groups marked by different letters differ significantly from each other ($p < .05$), and letters in brackets indicate marginally non-significant differences (g: $p = .068$, i: $p = .085$, k: $p = .079$, n: $p = .070$, o: $p = .078$). Asterisks above error bars denote significant differences of Rana from Bufo at the same total number of tadpoles. Note the logarithmic scale on the *Y* axis in e and f. Symbol colour denotes the tubs' species composition (black: Bufo only, white: Rana only; dark grey: both species, more Bufo than Rana; light grey: both species, fewer Bufo than Rana); symbol shape denotes the species in which the dependent variable was measured (circles: Bufo, squares: Rana, diamonds: all tadpoles)

expected that 6 Bufo in treatment 6B9R could produce half as much toxin as 12 Bufo in treatment 12B6R, so the Rana tadpoles in these two treatments would experience the same total biomass (high density) but different exposure to toxins. Finally, two treatment groups (6R, 12R) contained Rana tadpoles only (Figure 1a), serving as controls for testing whether Bufo tadpoles inhibit the growth and development of Rana tadpoles. There was only one Bufo tadpole missing at the termination of the experiment possibly due to mortality (in treatment 6B3R). Due to an error, nine instead of six Bufo tadpoles were placed in one tub in treatment 6B9R; however, this tub was not an extreme data point in any of the examined variables (in the analyses we treated this tub as if there had been six Bufo in it, to avoid having a treatment group with $n = 1$). All treatments were started with the same amount of food (see below); we expected the *per capita* food availability to decrease more in treatments with higher density due to exploitation competition, reducing growth.

2.2 | Experimental procedures

In early spring 2016, we collected 60 eggs from each of nine freshly laid Bufo clutches and 30 eggs from each of nine freshly laid Rana clutches from a natural pond in Hungary (47°44′4.12″N, 18°49′7.04″E). We transported the eggs to the experimental station of the Plant Protection Institute in Budapest, where we kept Bufo eggs in 0.5 L and Rana eggs in 1 L reconstituted soft water (RSW; 48 mg $NaHCO_3$, 30 mg $CaSO_4 \times 2\ H_2O$, 61 mg $MgSO_4 \times 7\ H_2O$, 2 mg KCl added to 1 L reverse osmosis-filtered water). Room temperature was 21°C and lighting was set to mimic the natural photoperiod. Right before hatching we transferred embryos in groups of 60 (Bufo) or 30 (Rana) to containers with 5 L RSW to ensure constant density upon hatching.

Seven weeks before the start of the experiment, we placed 45-L plastic tubs (56 × 39 × 28 cm) in an open outdoor area and filled them with 40 L tap water. To each tub, we added 0.5 L pond water (containing phytoplankton and zooplankton) and 20 g dried beech (*Fagus sylvatica*) leaves to set up a self-sustaining ecosystem that provides shelter and nutrients for tadpoles. To prevent colonization by predators, we covered the tubs with mosquito net lids. Two days after hatching, we started the experiment by randomly selecting 44 healthy Bufo tadpoles and 24 Rana tadpoles from each family, and placing them into the tubs as follows. For each species, the nine families were divided into three groups of three families each, such that the first Bufo family group was paired up with the first Rana family group and so on. From each family group, we randomly distributed the tadpoles across the eight treatment groups (Figure 1a), with two replicates per family group × treatment combination, so there were six tubs in each treatment group (two from each family group). In total, we had 48 tubs arranged in six blocks, each block consisting of all treatments of a given family group. This design ensured that each tub contained siblings as well as non-kin tadpoles.

We terminated the experiment after 3 weeks because bufadienolide levels of Bufo tadpoles are highest and most sensitive to environmental conditions around the middle of larval development (Üveges et al., 2017). We weighed all tadpoles to the nearest 0.1 mg, and we preserved the Bufo tadpoles ($n = 398$) in HPLC-grade absolute methanol for chemical analysis of bufadienolides. We preserved the Rana tadpoles ($n = 216$) in 50% ethanol. We identified the developmental stage of all tadpoles according to Gosner (1960) by stereomicroscopic examination (we could not identify the developmental stage of one Rana tadpole because it was deformed).

All experimental procedures were carried out in accordance with Good Scientific Practice guidelines and national legislation. The Ethical Commission of the MTA ATK NÖVI approved the experiment, and the necessary permits were issued by the Government Agency of Pest County, Hungary (PE/KTF/3596-6/2016, PE/KTF/3596-7/2016 and PE/KTF/3596-8/2016).

2.3 | Chemical analysis

Each tadpole was homogenized and dried in vacuum to measure dry mass (±0.1 mg); then the samples were re-dissolved in 1 ml HPLC-grade absolute methanol and filtered using nylon syringe filters. Quantitative measurement of bufadienolide compounds was carried out by a single-quadrupole HPLC-MS system (Model LC-MS-2020, Shimadzu, Kyoto, Japan) equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector and a mass analyser with electrospray ionization (ESI/MS). From each sample, 10 μL were injected and analysed at 35°C on a Kinetex C18 2.6 μm column (100 × 3 mm i.d.) in series with an octadecyl C18 guard column (4 × 3 mm i.d.). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.6 ml/min and the gradient was as follows: 0–2 min, 10%–20% B; 2–15 min, 20%–32% B; 15–21 min, 32%–60% B; 21–21.5 min, 60%–100% B; 21.5–26 min 100% B; and 26–30 min 10% B. ESI conditions were as follows: interface temperature, 350°C; desolvation line (DL) temperature, 250°C; heat block temperature, 400°C; drying N_2 gas flow, 15 L/min; nebulizer N_2 gas flow, 1.5 L/min; positive ionization mode. Full scan spectra in the range of m/z (mass-to-charge ratio) values 350–800 were recorded, and selected-ion monitoring acquisition detecting the base peak of the bufadienolides we previously found in common toads (Bókony et al., 2016; Üveges et al., 2017) was performed as well. Bufadienolides were recognized by their characteristic UV spectrum, and identified by comparing their peak retention time and m/z to those of commercially purchased standards and to the peaks present in a toxin sample obtained from juvenile common toads (for more details, see Bókony et al., 2016; Üveges et al., 2017). The data were acquired and processed using LabSolutions 5.42v (Shimadzu).

We detected 24 bufadienolide compounds (Table S1). We used the calibration curve of the bufotalin standard to express the bufotalin-equivalent mass of each bufadienolide compound per sample (Benard & Fordyce, 2003; Hagman et al., 2009); then we summed the values of all compounds to estimate the total amount of bufadienolides per individual. This variable was then divided by tadpole dry mass to obtain the total amount of bufadienolides per body mass (mass-corrected amount of bufadienolides henceforward). We analysed both variables because they quantify two

different aspects of toxicity: the mass-corrected amount is more likely to express individual investment (i.e. proportion of resources allocated to toxin production) while the total amount is more likely to be relevant in inter-individual interactions (i.e. total toxin quantity available for allelopathy).

2.4 | Statistical analysis

All statistical analyses were run with R 3.3.1, using the packages "nlme" and "lsmeans". We used two alternative approaches as follows. First, we employed the concept of response surface analysis (Inouye, 2001) to assess how the tadpoles' mass, developmental stage and chemical defence varied with the density of both species. In these models, we assumed linear relationships, entering the number of Bufo and the number of Rana as covariates (numerical predictor variables) along with their interaction. Second, to be able to address potentially non-monotonous or cumulative effects of density, in another set of analyses we used the eight treatments as a fixed factor (categorical predictor variable). In these models, the proportion of variance explained by the treatments was tested using analysis of variance tables (i.e. *F*-tests) with type-III sums of squares; then, pairwise comparisons among treatment groups were tested by calculating linear contrasts and correcting the *p*-values for multiple testing with the FDR (false discovery rate) method (Pike, 2011).

All analyses were performed with linear mixed-effects (LME) models, in which we allowed for heteroscedasticity across treatment groups (Zuur, Ieno, Walker, Saveliev, & Smith, 2009) using the "varIdent" function in "lme" models. When the dependent variable was the total mass of tadpoles per tub, we used family group as a random factor. When the dependent variable was the body mass or developmental stage of individual tadpoles, number of bufadienolide compounds per tadpole, total or mass-corrected amount of bufadienolides, we used tub identity nested in family group as hierarchical random factors. We checked the requirements of LME analysis by inspecting residual plots; we $\log_{10}$-transformed the amount of bufadienolides (both total and mass-corrected) to improve the models' fit. All tests were two-tailed with 95% confidence level. Our analyses can be reproduced from Bókony, Üveges, Móricz, and Hettyey (2017).

3 | RESULTS

3.1 | Competitor biomass

The total mass of tadpoles per tub varied significantly among treatments ($F_{7,38} = 88.98$, $p < .001$, Figure 1b). The four high-density treatment groups did not differ among each other but had significantly larger total mass than the four treatment groups with medium or low density (Figure 1b). Also, the low-density group had significantly less total mass than two out of the three medium-density groups (Figure 1b). These differences agree well with our planned grouping of density treatments based on total mass (Figure 1a), except that total mass was smaller than we expected in tubs containing six Rana tadpoles (Figure 1b). This deviation from the planned densities arose because individual body mass did not differ significantly between the two species in the lower density treatments (Figure 1c), whereas at high densities Rana tadpoles had significantly (*c.* 1.5 times) larger body mass than Bufo tadpoles (Figure 1c).

3.2 | Effects on Bufo

The body mass of Bufo tadpoles was significantly reduced by high-density treatments ($F_{5,28} = 5.25$, $p = .002$; Figure 1c) and decreased with increasing numbers of both conspecific and heterospecific competitors (Table 1). The addition of one Rana was estimated to have about twice as large an effect as the addition of one Bufo (Table 1), suggesting that the effect of competitor biomass per species was similar; however, the effect of Rana was marginally non-significant, whereas the effect of conspecifics was highly significant (Table 1).

We detected 17–24 (most often 21–23) bufadienolide compounds in individual tadpoles (Table S1). While the number of compounds per tadpole showed a marginally non-significant tendency to increase with the number of conspecifics (Table 1), the number of Rana had no significant effect (Table 1) and none of the pairwise differences among treatment groups were significant after correction for multiple testing ($F_{5,28} = 2.15$, $p = .089$; Figure 1d).

In contrast, treatments had highly significant effects on the amount of bufadienolides (total amount per tadpole: $F_{5,28} = 4.24$, $p = .005$; mass-corrected amount: $F_{5,28} = 10.65$, $p < .001$). The total amount of bufadienolides per tadpole was not reduced at high density (Figure 1e), despite the smaller body mass of these tadpoles (Figure 1c). Instead, total bufadienolide amount was explained by a significant interaction between the numbers of Bufo and Rana tadpoles (Table 1, Figure S1): conspecifics had a significant, consistently positive effect while the effect of Rana was marginally non-significant and negative when they were few and increased as their numbers grew (Table 1, Figure S1). As a result, total bufadienolide amount was higher in the two treatments with the largest total mass containing 12 or 24 Bufo than in the three treatments containing six Bufo tadpoles irrespective of total mass (Figure 1e).

The mass-corrected amount of bufadienolides increased gradually with total competitor density (Figure 1f) and increased significantly with the number of conspecifics, whereas the number of Rana had no significant effect (Table 1). These differences in bufadienolide content were not attributable to developmental stage, because there was no significant variation in the developmental stage of Bufo tadpoles among treatment groups ($F_{5,28} = 1.20$, $p = .334$; Figure 2) and it was not significantly related to the number of conspecific or heterospecific competitors (Table 1).

3.3 | Effects on Rana

The individual body mass of Rana tadpoles did not vary significantly among treatment groups ($F_{4,23} = 0.56$, $p = .691$; Figure 1c) and was not significantly explained by the number of conspecific or heterospecific competitors (Table 1). Notably, the body mass of six Rana tadpoles was essentially the same when they were raised in the presence

TABLE 1 Results of response surface analysis testing the effects of Bufo and Rana tadpoles and their interaction

Dependent variable	Parameters[a]	Coefficient ± *SE*	*df*	*t*	*p*
Bufo tadpoles[b]					
Body mass (mg)	Intercept	300.957 ± 17.373	362	17.32	<.001
	Number of Bufo	−4.387 ± 0.992	30	−4.42	<.001
	Number of Rana	−8.764 ± 4.334	30	−2.02	.052
	Bufo × Rana	0.363 ± 0.488	30	0.74	.463
Developmental stage	Intercept	33.341 ± 0.434	362	76.83	<.001
	Number of Bufo	−0.011 ± 0.022	30	−0.50	.619
	Number of Rana	−0.091 ± 0.097	30	−0.93	.357
	Bufo × Rana	0.005 ± 0.011	30	0.47	.644
Number of bufadienolide compounds	Intercept	21.685 ± 0.428	362	50.64	<.001
	Number of Bufo	0.037 ± 0.019	30	1.98	.057
	Number of Rana	−0.017 ± 0.088	30	−0.19	.850
	Bufo × Rana	0.004 ± 0.009	30	0.40	.691
Total bufadienolide amount (log_{10} μg)	Intercept	0.644 ± 0.033	362	19.30	<.001
	Number of Bufo	0.005 ± 0.002	30	2.76	.010
	Number of Rana	−0.016 ± 0.008	30	−1.99	.056
	Bufo × Rana	0.002 ± 0.001	30	2.67	.012
Mass-corrected bufadienolide amount (log_{10} ng/mg)	Intercept	2.082 ± 0.053	362	39.37	<.001
	Number of Bufo	0.018 ± 0.003	30	6.76	<.001
	Number of Rana	0.010 ± 0.012	30	0.85	.403
	Bufo × Rana	0.001 ± 0.001	30	0.87	.392
Rana tadpoles					
Body mass[c] (mg)	Intercept	284.873 ± 52.567	186	5.42	<.001
	Number of Bufo	4.328 ± 8.958	24	0.48	.633
	Number of Rana	4.731 ± 5.177	24	0.91	.370
	Bufo × Rana	−0.867 ± 1.254	24	−0.69	.496
Developmental stage[d]	Intercept	28.768 ± 0.346	185	83.24	<.001
	Number of Bufo	−0.006 ± 0.061	24	−0.10	.919
	Number of Rana	0.047 ± 0.035	24	1.33	.195
	Bufo × Rana	0.002 ± 0.009	24	0.20	.840

[a]Parameters are given as the number of tadpoles per tub. To express the effect of Rana in biomass units (assuming that Rana grow twice as large as Bufo), divide the parameters "Number of Rana" and "Bufo × Rana" by 2.
[b]398 tadpoles in 36 tubs.
[c]216 tadpoles in 30 tubs.
[d]215 tadpoles in 30 tubs.

or absence of 12 Bufo tadpoles (Figure 1c). Developmental stage showed very limited variation among Rana tadpoles (Figure 2); it did not vary significantly among treatment groups ($F_{4,23}$ = 0.90, p = .479; Figure 2), nor with the number of conspecific or heterospecific competitors (Table 1).

4 | DISCUSSION

Our study yielded two main results. On the one hand, we found that Bufo tadpoles contained increased quantities of bufadienolides at higher competitor densities, demonstrating competition-induced plasticity in toxin production. On the other hand, we did not find support for the hypothesis that bufadienolides function to suppress heterospecific competitors, because the growth and development of Rana tadpoles was not inhibited by the presence of Bufo tadpoles and also because Rana tadpoles did not induce higher toxin production in Bufo tadpoles than conspecifics did.

To our knowledge, this is the first unequivocal evidence for induced toxin synthesis in response to increased competition in free-moving animals, demonstrating that phenotypic plasticity of chemical defence (or offence) is not limited to predator–prey interactions and immune responses in behaviourally and morphologically complex organisms (Hettyey et al., 2014; Tollrian & Harvell, 1999). This experimental result

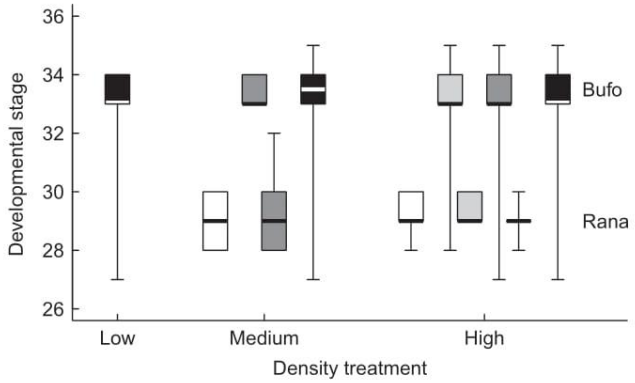

FIGURE 2 Developmental stage of Bufo (upper boxes) and Rana (lower boxes) tadpoles in the eight treatment groups. In each box plot, the thick middle line, box and whiskers represent the median, interquartile range and data range respectively. Box colour denotes the tubs' species composition as explained in Figure 1a (black: Bufo only, white: Rana only; dark grey: both species, more Bufo than Rana; light grey: both species, fewer Bufo than Rana)

corroborates our earlier finding that the toxin content of common toad tadpoles correlated positively with the density of competitors across natural ponds (Bókony et al., 2016). Such correlation may arise either by local adaptation in constitutive defences or *via* phenotypic plasticity (Bókony et al., 2016); our present results support the latter explanation. Furthermore, in another laboratory experiment, we found that the bufadienolide amount of common toad tadpoles increased when competition was simulated by decreasing food availability for small groups of tadpoles at a single density (Üveges et al., 2017). Although this might have been a stress response to hunger irrespective of competition, our present results clearly demonstrate that increased bufadienolide production is induced by competition even when food is relatively abundant (i.e. mortality was negligible). In both of our experiments, tadpoles reared in more competitive environments attained smaller body mass, but in spite of this inhibited growth, their total bufadienolide levels were at least as high or even higher compared to tadpoles reared in less competitive environments (Üveges et al., 2017; figure 1e,f in the present study). This suggests that competing tadpoles invested their resources into toxin production at the expense of growth; or alternatively, they may have been able to maintain or even increase their bufadienolide levels despite food limitation because the costs of bufadienolide synthesis may be low in terms of dietary resources (Kurali, Pásztor, Hettyey, & Tóth, 2016; Üveges et al., 2017). It is possible, however, that induced bufadienolide synthesis is traded off against long-term investment into critical life-history traits, as suggested by earlier studies (Benard & Fordyce, 2003; Hagman et al., 2009).

Although we found competition-induced changes in the bufadienolide content of Bufo tadpoles, the role of these chemicals in allelopathy remains unclear. We expected that bufadienolides would mainly be induced by, and effective against, heterospecific competitors because toxin-producing species should have evolved protection from autotoxicity; for example, consuming the bufadienolide-rich eggs or tissues of cane toads *Rhinella marina* has no ill effect on conspecific tadpoles but kills other species (Crossland & Shine, 2012; Crossland et al., 2011). However, in the common toad–agile frog system, we found no indication that interspecific competition would be the specific driver of toxin production. Bufo tadpoles' bufadienolide levels were not increased by the presence of Rana tadpoles more than by the same total mass of conspecific competitors, and the presence of Bufo larvae did not reduce the growth and development of Rana larvae. It is unlikely that the tadpoles could not discriminate between conspecific and heterospecific competitors (Relyea, 2002). Instead, a possible explanation for the lack of interspecific effects is that the encounter rate between the two species may have been relatively low, because Bufo larvae are more active and more gregarious than Rana larvae (our pers. obs.). If Bufo tadpoles use proximity or physical interaction (e.g. visual and tactile cues) for assessing competitor density (Rot-Nikcevic, Denver, & Wassersug, 2005) to adjust their toxin production, they will have perceived stronger competition by conspecifics than by Rana tadpoles. Low encounter rates might also explain the lack of allelopathic effects on Rana tadpoles, because bufadienolides are amphiphilic molecules so their highest concentrations are likely to occur at the interface of tadpole skin and water (Kubanek et al., 2002). In this case, allelopathy would become important only at very high interspecific encounter rates, e.g. when water depth is low due to desiccation (Cabrera-Guzmán, Crossland, & Shine, 2013), or at very low food availability which may increase the importance of scavenging on injured or dead toad tadpoles (Jefferson, Hobson, & Chivers, 2014; Jordan, Rombough, Pearl, & McCreary, 2004; Mahapatra, Dutta, & Sahoo, 2017; Wildy, Chivers, Kiesecker, & Blaustein, 2001).

Response surface analysis indicated that intraspecific competition had stronger effects on bufadienolide production than interspecific competition did, and high competitor biomass increased the total bufadienolide amount only when the majority of the competitors were conspecifics. This suggests that an important function of the inducibility of toxin production may be to mitigate some risk posed primarily by conspecifics; we propose two, mutually non-exclusive hypotheses. First, high densities and low per capita food levels are known to increase the incidence of intraspecific aggression and cannibalism in amphibian larvae (Jefferson et al., 2014; Jordan et al., 2004; Mahapatra et al., 2017; Wildy et al., 2001), and elevated bufadienolide levels might prevent or mitigate intraspecific biting by deterring conspecific attacks. Although toads are tolerant to the toxins of their own species (Crossland & Shine, 2011; Crossland et al., 2011), they still might find these substances distasteful as do many other species (Gunzburger & Travis, 2005). Alternatively, toad toxins may function in intraspecific chemical communication and species recognition (Crossland & Shine, 2011; Hagman & Shine, 2009), and thereby might help preventing cannibalistic attempts against kin in sibling schools which are characteristic of toad larvae (Blaustein, 1988).

The second possible function of competitor-induced chemical defence is the prevention of disease. Bufadienolides are known to have antimicrobial effects (Cunha Filho et al., 2005; Tempone et al., 2008), so they may be an important component of immune defence in toads which lack the antimicrobial skin peptides that are found in many other amphibians (Conlon, Iwamuro, & King, 2009). Infection risk can induce chemical defences, for example in leopard frog *Lithobates pipiens*

tadpoles, doubling the density of conspecifics caused more than 250% increase in skin peptides (Groner et al., 2014). Because the chances of transmitting parasites or pathogens are likely to be higher at high densities (Briggs, Knapp, & Vredenburg, 2010), and individuals are more likely to be susceptible to the diseases of conspecifics than other species (Freeland, 1983), our results are in concordance with the hypothesis that tadpoles produce more bufadienolides in response to elevated infection risk. It remains to be tested whether the upregulated bufadienolide production is effective in preventing disease transmission and/or cannibalistic interactions.

In sum, our results demonstrate that a form of chemical defence, considered to have evolved to provide protection against predators, can be induced by competitors. Although we found no indication of interspecific allelopathic effects, the potential of bufadienolides to mitigate infection risk and/or to prevent cannibalism makes them ideal candidates for multi-purpose allomones. So far, theoretical and empirical studies of inducible defences have, by far the most frequently, focused on the effects of predators (Tollrian & Harvell, 1999); the time is ripe for addressing the role of defensive and/or offensive chemicals against multiple enemies, and the consequences thereof for resource allocation trade-offs, life-history evolution and responses to anthropogenic change (Bókony, Mikó, et al., 2017; Hettyey et al., 2014).

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AUTHORS' CONTRIBUTIONS

A.H., B.Ü. and V.B. designed the experiment, B.Ü. performed the experiments, Á.M.M. performed the HPLC analyses; V.B. conducted the statistical analyses and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.q3g70 (Bókony, Üveges, et al., 2017).

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RESEARCH ARTICLE

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Chemical defence effective against multiple enemies: Does the response to conspecifics alleviate the response to predators?

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Abstract

1. Animals living in groups with high conspecific densities typically decrease their level of plastic anti-predatory defence because its benefits diminish with reduced per capita predation risk (a benefit of aggregation), whereas its costs increase due to intensifying competition and increased infection risk. Furthermore, phenotypic responses that provide protection from predators are also often disadvantageous against competitors and infections.
2. Such a trade-off may be absent when the same phenotype provides an effective defence against both predators and competitors, as is the case with some chemical defences. For such multifunctional defensive traits, both predation risk and high conspecific density are expected to increase defence expression while exposure to both predators and conspecifics may result in non-additive effects whereby the defence level induced by two enemies is lower than the sum of responses induced by either of them alone.
3. We tested this theoretical prediction by studying the effects of multiple enemies on chemical defence in a vertebrate animal. We investigated patterns of change in toxin production of common toad *Bufo bufo* tadpoles following exposure to different conspecific densities and the simultaneous presence or absence of chemical cues on predation risk.
4. We found that tadpoles significantly increased their production of bufadienolide toxins in response to high tadpole density, as well as to predation risk when tadpole density was low. Although the response in bufadienolide production to predation risk was not significant at high tadpole density, the magnitude of anti-predatory response did not differ significantly between low and high tadpole densities.
5. These results show that toad tadpoles adjust their chemical defence to conspecific density and to predation risk simultaneously, and these two effects are more likely additive than non-additive, at least within the range of densities and predation-risk levels studied here. Nevertheless, the trend we found suggests that toxin levels induced by very high conspecific density might weaken the chemical response to predators, which is relevant for the evolutionary ecology of chemical defences, as well as for the conservation of fauna impacted by toxic invaders.

KEYWORDS
anti-predator response, anuran amphibian, chemical defence, defensive synergy, inducible defence, multiple stressors, phenotypic plasticity, toxins

1 | INTRODUCTION

Predation is one of the most important selective forces driving evolutionary change; therefore, organisms must adapt their defences to the actual levels of predation pressure to maximize their fitness. This adaptation is possible in part due to prey's ability to respond to environmental variability by phenotypically plastic adjustment of traits such as behaviour, morphology and life history (DeWitt & Scheiner, 2004; West-Eberhard, 2003). To be able to respond quickly and efficiently to threats *via* such inducible defences, organisms need to continuously monitor their environment and assess predation risk. In case of animals forming groups either for foraging or to avoid predation, individuals also need to consider the size of the assemblage to correctly estimate predation risk and mount a cost-effective response. This is because the per capita predation risk may be inversely related to the size of the group, due to the decrease in chance of predation (dilution effect), increased vigilance (the 'many eyes' effect) or predator confusion (Elgar, 1989; Lima, 1995; Peacor, 2003; Pulliam, 1973; Roberts, 1996). Therefore, when the per capita predation risk is lower at higher conspecific densities, individuals should invest less in costly plastic anti-predator responses (Peacor, 2003). In line with this theory, empirical studies on several taxa demonstrated that prey individuals adjust their morphological and behavioural anti-predator defences to high conspecific density by producing less intense responses to predation risk (McCoy, 2007; Tollrian et al., 2015; Van Buskirk et al., 2011), although the effect of conspecific density on anti-predator responses may change during ontogeny, at least in part due to changes in predation risk (Davenport & Chalcraft, 2014).

Aggregations may provide protection against predation, but exposure to high densities of conspecifics can also entail costs, arising from increased resource competition (Amundsen et al., 2007; Hixon & Jones, 2005; Holbrook & Schmitt, 2002; Morin, 1986), cannibalism (DeVore et al., 2021; Jefferson et al., 2014; Jordan et al., 2004; Wildy et al., 2001), or facilitated spread of pathogens (Briggs et al., 2010; Eskew & Todd, 2013; Malagon et al., 2020; Sanchez & Hudgens, 2019; Smith et al., 2009) and parasites (Arneberg et al., 1998; Lindsey et al., 2009; Morand & Poulin, 1998). Limited resources allocated to preventing or combating these negative effects of group living may be traded-off against anti-predator responses. Furthermore, anti-predator responses may be also weakened when a phenotype beneficial against conspecifics or infections is disadvantageous against predators. For example, certain phenotypic changes in amphibian larvae, like higher foraging activity, a longer body and a shallower tail, benefit fitness in the presence of competitors, but the same changes are maladaptive in the presence of certain predators (Relyea, 2002, 2004; Relyea & Auld, 2005). Examples of such conflicts among responses induced by different enemies are abundant (e.g. DeWitt et al., 2000; Sih et al., 1998; Teplitsky et al., 2004).

Conflict between anti-predator and anti-competitor defences is, however, not inevitable because a single response may provide protection against both predators and the perils of aggregations. Chemical defence, that is, production of toxic or noxious compounds against enemies, often represents such a multifunctional response (Apponyi et al., 2004; Gasch et al., 2013; Holopainen, 2004; Izhaki, 2002; Núñez-Pons et al., 2012; Schierling et al., 2013; Thoms & Schupp, 2007). For example, toxins of toads (Bufonidae, Amphibia) deter several predators (e.g. Greenlees et al., 2010; Henrikson, 1990; Üveges et al., 2019), but they also have anti-bacterial (Cunha Filho et al., 2005), anti-fungal (Barnhart et al., 2017) and anti-parasitic properties (Tempone et al., 2008). Also, the cell type (giant cells; Riesenzellen) associated with toxin synthesis in toad tadpoles (Delfino et al., 1995; Regueira et al., 2016) was suggested to be the source of allelochemical agents that inhibit the growth of conspecifics (Clarke et al., 2015; Crossland & Shine, 2012). In line with this potential of toad toxins for providing protection from multiple enemies, it has also been shown that larvae and juveniles of toads increase their toxin production in response to predation risk (Benard & Fordyce, 2003; Hagman et al., 2009; Hettyey et al., 2019) and high conspecific density (Bókony et al., 2018).

When the same phenotype is beneficial against both predators and the negative effects of group living, investment in such a multifunctional defence is expected to respond differently to the interplay between predation risk and conspecific density than when the anti-predator and anti-group responses are in conflict (Figure 1; Poitrineau et al., 2003). When the anti-predator defence is in trade-off with the anti-group defences (Figure 1A), expression of the anti-predatory trait should increase with increasing predation risk and decrease with increasing conspecific density (McCoy, 2007; Peacor, 2003; Tollrian et al., 2015; Van Buskirk et al., 2011). These two effects were usually found to be additive (Tollrian et al., 2015; Van Buskirk et al., 2011). In contrast, when the same induced response is effective against both predators and competitors, exposure to both types of enemies should result in enhanced responses (Figure 1B). However, to alleviate costs due to physiological constraints or energetic trade-offs (Blennerhassett et al., 2019), the optimal strategy for animals with such defences should be to 'kill two birds with one stone'. That is, they should dampen their response to an enemy if their defence level is already so high, due to their response to other enemies, that a further induction of toxin synthesis by the enemy in question no longer provides additional fitness benefits (Poitrineau et al., 2003). Therefore, we expect the effects of the two enemies to be non-additive: a combination of high predation risk and high conspecific density may induce only slightly higher investment into defence than either one of these factors alone (Figure 1B).

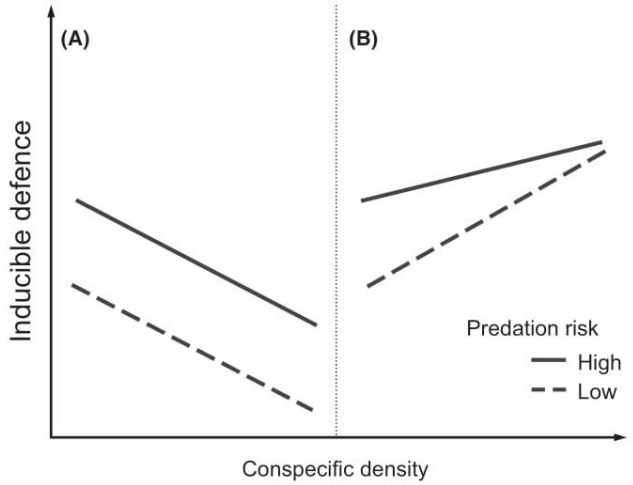

FIGURE 1 Schematic illustration of density-dependent adjustments of inducible defences. Both defensive traits (A, B) provide anti-predatory protection, and their expression level increases in response to increased predation risk. For simplicity, we assume a linear (type 1) functional response of predators to prey density. For an anti-predatory defence that is disadvantageous in competition (A), expression level decreases in response to increased conspecific density due decreased pay-off. For a defence which is effective against both predators and conspecifics (B), expression level is increased by higher conspecific density due to its benefits against competitors and infections, diminishing the need for further increases in expression in response to predation risk

However, in the case of animals, we know of no study that has investigated the interaction between the effects of predation risk and conspecific density on inducible chemical defences that are effective against both predators and the dangers posed by conspecifics.

We investigated the combined effects of conspecific density and predation risk on inducible chemical defence by conducting a mesocosm experiment in which we reared common toad *Bufo bufo* tadpoles at three conspecific densities in the presence or absence of chemical cues indicating predation risk. Tadpoles of this species regularly form aggregations (Griffiths & Foster, 1998; Watt et al., 1997) and synthesize toxins from an early age (Üveges et al., 2017). The main defensive compounds of toads are cardiotoxic steroids called bufadienolides (Gao et al., 2010; Krenn & Kopp, 1998; Toledo & Jared, 1995) that are distasteful, poisonous or even deadly to predators (Chen & Huang, 2013; Greenlees et al., 2010; Somaweera et al., 2011). A previous experiment showed that common toad tadpoles increase their bufadienolide synthesis in response to high conspecific density in the absence of predators (Bókony et al., 2018). Also, tadpoles raised in groups did not change their toxin production in response to chemical cues indicating predation risk in two other studies (Üveges et al., 2017, 2019). However, when tadpoles were kept individually in a fourth experiment, toxin synthesis was enhanced upon exposure to chemical cues indicating predation risk (Hettyey et al., 2019). Together, these results suggest that the effect of predators on toxin production of common toad tadpoles may depend on conspecific density. Therefore, we predicted a non-additive effect when tadpole density and predation risk are manipulated simultaneously (Figure 1B), that is, the difference in bufadienolide content between tadpoles raised with and without cues indicating predation risk should diminish with increasing conspecific density.

2 | MATERIALS AND METHODS

2.1 | Experimental procedures

In spring 2018, we collected 140 eggs from each of six freshly laid common toad clutches from a pond in the Pilis Mountains, Hungary (Szárazfarkas-belső; 47°44′4.12″N, 18°49′7.04″E). We also collected 120 eggs from each of 10 clutches of agile frogs *Rana dalmatina* from the same pond to be later used as food for predators (see below). We transported eggs to the laboratory of the Plant Protection Institute, Centre for Agricultural Research (Budapest, Hungary), and kept each family until hatching in 1 L reconstituted soft water (RSW, 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \times 2\ H_2O$, 61 mg/L $MgSO_4 \times 7\ H_2O$, 2 mg/L KCl added to reverse-osmosis filtered, UV-sterilized tap water). After hatching, we kept each family of tadpoles in 5 L RSW in the laboratory until they reached the free-swimming stage (developmental stage 25, Gosner, 1960). During this part of the experiment, tadpoles developed at 21°C ambient temperature and a 13:11 hr light:dark cycle.

Three weeks before the start of the experiment, we set up 48 outdoor mesocosms by filling plastic containers (57 × 39 × 28 cm, length × width × height) with 40 L aged tap water, inoculating them with 0.6 L pond water containing algae and zooplankton, and adding 20 g dried beech *Fagus sylvatica* leaves into each container. This ensured food availability due to algal growth, and provided refugia for tadpoles. During the course of the study, overflow holes in the wall of plastic containers kept the water levels from rising. To prevent colonization of mesocosms by invertebrate predators, we covered containers with mosquito net lids. When toad tadpoles reached the free-swimming stage, we introduced them into the mesocosms and raised them for the treatment period. We kept remaining toad tadpoles and all agile frog tadpoles in additional mesocosms (82 × 58 × 30 cm) filled with 130 L aged tap water.

To test the effects of conspecific density and predation risk on induced chemical defences, we applied a factorial experimental design. We transferred one, two or four haphazardly selected toad tadpoles from each family into each mesocosm, resulting in 6, 12 or 24 tadpoles per mesocosm, which represented low, medium and high tadpole densities, respectively (Bókony et al., 2018), and we assigned each mesocosm to one of two predator treatments: cues present or absent. We replicated each of the six treatment combinations eight times, resulting in 48 experimental units (three densities of conspecifics × two predator treatments × eight replicates). We arranged treatments in a randomized block design, where each of the eight blocks contained one mesocosm from each treatment combination.

Chemical cues indicating predation risk were provided by eight adult European perch *Perca fluviatilis* which were kept together in a tank (82 × 58 × 30 cm) containing 130 L aerated aged tap water.

Fishes are widely regarded as the most dangerous predators to tadpoles in general (Wells, 2007) and, compared to newts and dragonfly larvae, perch elicited the strongest response in the chemical defence of toad tadpoles in a previous experiment (Hettyey et al., 2019). Fish weighed in total 336.46 g at the beginning and 290.77 g at the termination of the experiment. Fish were fed daily with 6.05 ± 0.04 g (mean ± *SD*) agile frog larvae and 0.61 ± 0.11 g common toad tadpoles. Fish always ate all agile frog tadpoles and killed 40.09 ± 27.02% of toad tadpoles that were offered as food (mean ± *SD*; of all offered toad tadpoles 35.17 ± 25.05% were consumed and 4.92 ± 7.19% were killed but not consumed).

We created stimulus water by homogenizing 1,885.67 ± 6.92 mg toad tadpoles with a blender in *c*. 50 ml RSW and adding this homogenate to 25 L water taken from the fish tank (Benard & Fordyce, 2003; Hettyey et al., 2019). We repeated this process daily 2–3 hr after feeding the fish and subsequently refilled the fish tank to the original volume using aged tap water. The addition of the tadpole homogenate was necessary to ensure that experimental tadpoles were exposed to sufficiently high concentrations of prey-borne cues of predation even when fish did not eat all toad tadpoles, because conspecific alarm cues are required for eliciting strong anti-predator responses (Hettyey et al., 2015; Laurila et al., 1997; Schoeppner & Relyea, 2005).

After thoroughly mixing the stimulus water, we poured 800 ml of the mixture into each mesocosm assigned to the predator treatment, and 800 ml of aged tap water into each mesocosm holding control tadpoles (i.e. those assigned to the treatment groups without cues indicating predation risk). As a result, experimental tadpoles were exposed to chemical cues corresponding to 48.25 ± 4.97 mg/L fish (kairomones, mean ± *SD*), 0.86 ± 0.14 mg/L heterospecifics and a maximum of 0.09 ± 0.01 mg/L conspecifics (alarm pheromones ['Schreckstoff', von Frisch, 1942], and chemical cues released via the digestion of tadpoles), as well as to 1.51 ± 0.01 mg/L homogenized conspecifics (cues released by mechanical damage). Similar cue concentrations elicited clear anti-predator responses in chemical defences of common toad tadpoles in a previous study (Hettyey et al., 2019).

We terminated the experiment after 2 weeks of treatment, when most of the experimental tadpoles reached developmental stage 36 (Gosner, 1960). We chose this time frame because bufadienolide content of common toads peaks around this stage during their larval development (Ujszegi et al., 2017; Üveges et al., 2017). We haphazardly selected six tadpoles from each mesocosm and preserved them in HPLC-grade absolute methanol for chemical analysis (n = 288). We randomly selected three methanol-preserved tadpoles from each tub (n = 144) and assessed their developmental stage according to Gosner (1960) using a stereomicroscope. Developmental stage of tadpoles was highly uniform (stage 35: n = 10, stage 36: n = 134) and similarly distributed across all six treatment combinations (Fisher's exact test, p = 0.229). No experimental animals died before the termination of treatments, and after the experiment we released all remaining tadpoles into their pond of origin.

2.2 | Chemical analysis

We prepared samples by homogenizing preserved tadpoles using a VWR VDI 12 homogenizer with an IKA S12N-7S dispersing tool. Subsequently, we dried homogenates in vacuo at 45°C using a Büchi Rotavapor R-134 rotary evaporator and measured dry mass to the nearest 0.1 mg with an analytical balance (Sartorius Entris 224i-1S). Samples were re-dissolved in 1 ml HPLC-grade absolute methanol, facilitated by brief exposure to ultrasound in a Tesla UC005AJ1 bath sonicator. Finally, we filtered samples using FilterBio nylon syringe filters (pore size = 0.22 μm).

We analysed samples using high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS). Bufadienolides were identified based on their characteristic peaks in the UV spectrum (Benard & Fordyce, 2003; Bókony et al., 2018; Hagman et al., 2009; Hettyey et al., 2019; Üveges et al., 2017, 2019) and by co-injection with standards of the following bufadienolides: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals), cinobufagin (Chembest), cinobufotalin (Quality Phytochemicals), digitoxigenin (Santa Cruz Biotechnology) and marinobufotoxin (courtesy of Dr Rob Capon, University of Queensland, Brisbane, Australia). Furthermore, to help identify bufadienolide compounds present in low quantities, we analysed a bulk sample obtained from 49 juvenile common toads by manually applying pressure to their parotoid glands.

We quantified bufadienolide compounds using a single-quadrupole HPLC-MS system (Model LC-MS-2020) equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector and a mass analyser with electrospray ionization (ESI/MS). Ten microliters of samples were injected at 35°C on a Kinetex C18 2.6 μm column (100 mm × 3 mm i.d.) in series with an octadecyl C_{18} guard column (4 mm × 3 mm i.d.). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid, eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.6 ml/min and the gradient was as follows: 0–1 min: 10%–20% B; 1–11 min: 20%–29% B; 11–13 min: 29%–58% B; 13.1–16 min: 100% B; 16.1–20 min: 10% B. ESI conditions were set as follows: interface temperature: 350°C; desolvation line (DL) temperature: 250°C; heat block temperature: 400°C; drying N_2 gas flow: 15 L/min; nebulizer N_2 gas flow: 1.5 L/min; positive ionization mode. Full scan spectra were recorded in the range of 350–800 m/z and we also performed selected-ion monitoring (SIM) detecting the base peaks of bufadienolides we previously found in common toads (Bókony et al., 2018; Hettyey et al., 2019; Üveges et al., 2017). Data were processed using the LabSolutions 5.42v software.

2.3 | Statistical analysis

We used total bufadienolide quantity (TBQ), mass-corrected total bufadienolide quantity (mcTBQ) and the number of bufadienolide compounds (NBC) to analyse toxin content of toad tadpoles. We calculated TBQ and NBC from MS chromatogram peaks. We considered a specific bufadienolide to be present if its signal to noise ratio

was at least three in the chromatogram (Hettyey et al., 2019; Üveges et al., 2019). We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the marinobufotoxin standard. This approach results in rough estimates of bufadienolide content, but because commercially available standards are lacking for most bufadienolide compounds, this is currently the best quantification method available, and was used also in former studies (e.g. Benard & Fordyce, 2003; Bókony et al., 2018; Hagman et al., 2009; Hettyey et al., 2019; Üveges et al., 2017, 2019). We subsequently summed these values to obtain estimates of TBQ for each individual. We divided TBQ by the dry mass of samples to get mass-corrected total bufadienolide quantity (mcTBQ). TBQ measures the total toxin quantity of tadpoles, which is likely to be relevant for the efficacy of the chemical defence, whereas mcTBQ reflects the relative amount of resources allocated to chemical defence. Although the bufadienolide quantity of the skin is the most relevant for predator deterrence, we did not estimate body surface area because it is strongly correlated with body mass in wood frog *Rana sylvatica* tadpoles (Davis et al., 2008) and we did not expect body shape differences between treatment groups due to the low morphological plasticity of common toad larvae (Lardner, 2000; Üveges et al., 2019; Van Buskirk, 2002, 2009). Finally, NBC is a measure of diversity of the toxin cocktail produced by individual tadpoles, which may be relevant for protection from multiple threats (i.e. different toxin compounds may be effective against different enemies).

Statistical analyses were run in R 3.4.0 (R Development Core Team, 2017). We used linear mixed-effects models (LMM), implemented with the 'lme' function in 'NLME' (Pinheiro et al., 2017), entering TBQ, mcTBQ, NBC or dry mass as the dependent variable, predator treatment, conspecific density, and their interaction as fixed factors, and mesocosm as random factor. Preliminary likelihood-ratio tests indicated that block as random factor had no effect; therefore, it was omitted from the analyses. In the models of mcTBQ and dry mass, we included the 'weights' argument with the 'varIdent' function to account for differences in variances between the six treatment combinations and to improve model fit. We obtained *p* values for each model term (the two main effects and their interaction) from type-2 analysis-of-deviance tables using the 'ANOVA' function of the CAR package (Fox & Weisberg, 2019). To test our predictions, we conducted planned comparisons (Chen et al., 2018; Ruxton & Beauchamp, 2008) using linear contrasts for each of our dependent variables calculated from our LMM models, similarly to Hettyey et al. (2019). First, we tested whether density of conspecifics affected the dependent variables by comparing the estimated marginal means pairwise between the three density treatments. We performed these tests as averaged for the two predator treatment groups (controls and tadpoles exposed to cues indicating predation risk) and also within each predator treatment group. Second, we also tested whether the predator treatment affected the dependent variables within each density group. Finally, we tested whether the effect of cues indicating predation risk varied with tadpole density, by comparing the anti-predator response (i.e. the estimated difference between the tadpoles reared in the presence and absence of cues indicating predation risk at each tadpole density) pairwise between the three density treatments, and also between the two lowest densities versus the highest density of conspecifics. We calculated linear contrasts with the EMMEANS package (Lenth et al., 2019), and applied the FDR (false discovery rate) method to adjust *p* values for multiple comparisons (Benjamini & Hochberg, 1995; Pike, 2011). For the annotated R script of the statistical analysis, see the Supporting Information.

3 | RESULTS

Tadpoles in the high-density treatment exhibited significantly decreased body mass compared to the two lower densities of tadpoles, and exposure to chemical cues indicating predation risk resulted in significantly decreased tadpole body mass compared to control tadpoles at medium and low densities (Tables S1–S4; Figure S1). Despite these differences, toxin content did not decrease either with high conspecific density or under predation risk. Total bufadienolide quantity (TBQ) of tadpoles reared at high density was significantly higher than at medium density, whereas TBQ did not differ between high and low density and between the two lower densities (Table 1; Tables S2 and S5; Figure 2; note however

TABLE 1 The effect of conspecific density and predator treatments and their interaction on chemical defence of toad tadpoles, shown as type-2 analysis-of-deviance tables

Response	Effect	χ^2	*df*	*p*
TBQ	**Conspecific density**	**7.840**	**2**	**0.020**
	Predator treatment	3.590	1	0.058
	Conspecific density × predator treatment	0.124	2	0.940
mcTBQ	**Conspecific density**	**66.551**	**2**	**<0.001**
	Predator treatment	**23.801**	**1**	**<0.001**
	Conspecific density × predator treatment	0.400	2	0.819
NBC	Conspecific density	3.269	2	0.195
	Predator treatment	0.000	1	1.000
	Conspecific density × predator treatment	2.373	2	0.305

Note: Significant terms are highlighted in bold.

Abbreviations: mcTBQ, mass-corrected total bufadienolide quantity; NBC, number of bufadienolide compounds; TBQ, total bufadienolide quantity.

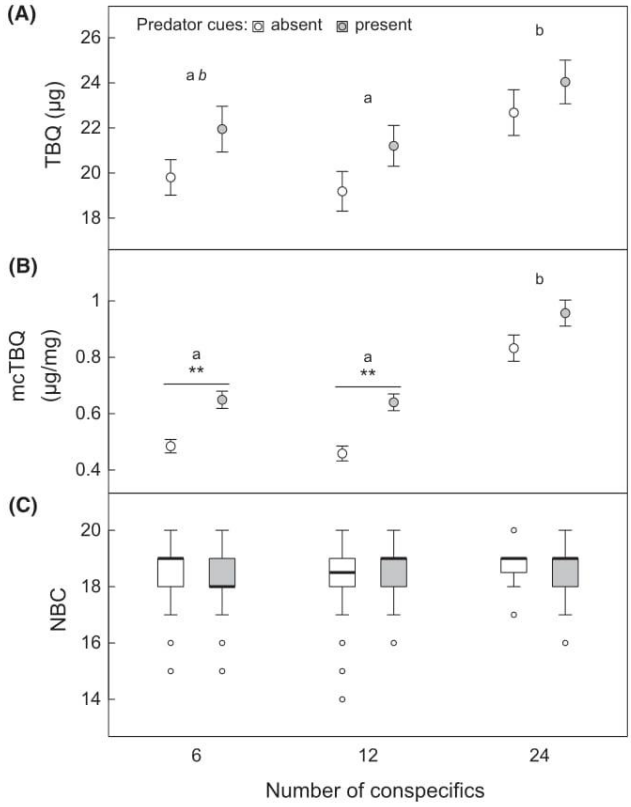

FIGURE 2 Effects of predation risk and number of conspecifics on chemical defence of toad tadpoles. For total bufadienolide quantity (TBQ) and mass-corrected total bufadienolide quantity (mcTBQ), means ± *SE* are shown (panels A, B). Significant differences between groups, based on linear contrasts corrected for false discovery rate, are indicated by lower case letters (between density treatments; groups indicated by different plain letters differ significantly at $p < 0.05$, whereas a marginally non-significant difference ($p = 0.064$) is italicized) and asterisks (between predator treatments within each density treatment; $^{**}p < 0.01$). For the number of bufadienolide compounds (NBC), boxplots are shown (panel C), and differences between groups are not indicated because all were non-significant ($p > 0.05$). In each boxplot, the thick horizontal line and the box represent the median and the interquartile range, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range, and open circles represent extreme data points

the marginally non-significant difference between the high and low densities). When analysed within density treatments, TBQ of tadpoles did not differ significantly between predator treatments (Table S3; Figure 2; Figure S2) despite an overall tendency for higher TBQ in response to cues indicating predation risk (Table 1; Table S3; Figure 2). This slight response to cues indicating predation risk on TBQ did not vary significantly with tadpole density (Table 1; Table S4; Figure 2; Figure S2).

Similarly to TBQ, mass-corrected total bufadienolide quantity (mcTBQ) of tadpoles was also significantly higher at high conspecific density than at medium and low densities, and did not differ between the two lower densities (Table 1; Table S2; Figure 2). In contrast to TBQ, however, tadpoles that received chemical cues indicating predation risk had significantly higher mcTBQ compared to their control conspecifics at both low and medium densities, and there was a similar but non-significant tendency when density was high (Table S3; Figure 2; Figure S2). The response to predation risk in mcTBQ did not vary significantly with tadpole density (Table 1; Table S4; Figure S2).

The number of bufadienolide compounds (NBC) was not affected either by different levels of conspecific density or by the presence or absence of chemical cues indicating predation risk (Table 1; Tables S1–S4; Figure 2; Figure S2).

4 | DISCUSSION

Both high conspecific density and exposure to chemical cues indicating predation risk can induce a plastic increase in the toxin synthesis of toad tadpoles, as can be expected of a defence effective against multiple enemies (Bókony et al., 2018; Hettyey et al., 2019). The present study shows that plastic responses in chemical defence induced by conspecific density and by cues indicating predation risk are expressed simultaneously. That is, high conspecific density increases toxin content not only in a predator-free environment (Bókony et al., 2018) but also in the presence of predators (Table S2), and similarly, the presence of chemical cues of predation risk increases investment into toxin production not only in isolated tadpoles (as shown by Hettyey et al., 2019), but also in groups, at least at low and medium densities of conspecifics.

In agreement with our prediction (Figure 1B), we found that the effect of predation risk on toxin content was no longer significant at the highest conspecific density, suggesting that the effects of predators and conspecifics may become non-additive with increasing conspecific densities. At the same time, however, the interaction between predator treatment and conspecific density was not significant, that is, the intensity of the anti-predator response in toxin synthesis did not differ significantly between density treatments. This latter result does not support non-additive effects, suggesting instead that the effects of predation risk and conspecific density on toxin synthesis may simply be additive. This complexity of our results is apparently due to relatively small effect sizes coupled with relatively high variability (Figure 2). The anti-predatory response in mcTBQ at high conspecific density was only 72% of the average response seen at the two lower densities (Table S4), which seems a biologically relevant difference. However, there was high variance between responses of tadpoles especially at high density, resulting in largely overlapping ranges of anti-predatory responses at all densities (Figure S2). Altogether, these findings suggest that the high toxin levels induced by high conspecific density might lead to reduced further increases in toxin investment in response to predation risk, but this reduction was very small in our study. It is possible that the effects of predators and conspecifics on chemical defence are additive at certain levels and non-additive at other levels of predation risk and conspecific density. Exploring this possibility in future

studies could yield valuable insights into the functional ecology of inducible defences that are effective against multiple enemies.

The higher bufadienolide content of tadpoles at high conspecific density aligns well with the notion that this chemical defence is likely effective against multiple enemies. Enhanced bufadienolide content may benefit toad tadpoles exposed to high densities of conspecifics in several ways. Theoretically, toxins may be utilized against competitors as allelochemicals to reduce their growth (Clarke et al., 2015; Crossland & Shine, 2012) or as a defence against cannibalistic attempts (Jefferson et al., 2014; Jordan et al., 2004; Wildy et al., 2001). However, toads appear to be resistant to the toxic effects of bufadienolides (Crossland et al., 2011; Crossland & Shine, 2011; DeVore et al., 2021; Moore et al., 2009). For example, in cane toad *Rhinella marina* tadpoles, bufadienolides do not deter, but rather attract cannibalistic conspecifics (Crossland et al., 2012) so that cannibals can devour up to 99.9% of hatchling conspecifics (DeVore et al., 2021). Therefore, bufadienolides may not provide an effective defence against attacks from conspecific tadpoles (DeVore et al., 2021). It is more likely that toad toxin production is induced in response to high conspecific densities because bufadienolides may mitigate infection risk by inhibiting the growth of pathogenic bacteria (Cunha Filho et al., 2005), the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Barnhart et al., 2017) and endoparasitic protozoans (Tempone et al., 2008).

The other major function of bufadienolides is anti-predatory protection (Greenlees et al., 2010; Llewelyn et al., 2012; Toledo & Jared, 1995; Üveges et al., 2019). For anti-predatory defences, the pay-off of investment is expected to decrease with increasing conspecific density (Figure 1A) because the benefits diminish as a consequence of reduced per capita predation risk (Peacor, 2003; Van Buskirk et al., 2011), whereas the costs increase due to intensifying competition for resources, and/or because physiology may set an upper limit to defence expression. This has been supported by several empirical studies on density dependence of behavioural and morphological anti-predator responses of different animal species (Davenport & Chalcraft, 2014; McCoy, 2007; Relyea, 2004; Relyea & Hoverman, 2003; Tollrian et al., 2015; Van Buskirk et al., 2011; Wiackowski & Starońska, 1999). However, for defences that provide protection against multiple types of enemies, the effects of high conspecific density on anti-predator defences may be different (Figure 1B), similar to the synergy proposed between defensive traits that provide cross-resistance against multiple enemies (Poitrineau et al., 2003). Since toad tadpoles exposed to cues indicating predation risk increased rather than decreased their bufadienolide content with increasing conspecific density, our findings suggest that the density dependence of toxin production was more strongly affected by the need for protection against the negative effects of high conspecific density than by the positive effects of group size on anti-predatory protection (such as risk dilution). Notably, the high-density treatment in our study was not extreme compared to naturally occurring densities of toad tadpoles (Arnold & Wassersug, 1978; Bókony et al., 2016; B. Üveges, pers. obs.). Consequently, it is possible that the per capita predation risk perceived by tadpoles in our experiment was not low enough to make a decrease in anti-predator chemical defence pay off, nor to make a further increase in response to predation risk impossible due to physiological limits. Thus, it remains to be tested if tadpole densities higher than those applied in this study would result in greatly reduced anti-predatory responses in terms of bufadienolide synthesis.

The number of bufadienolide compounds present in tadpoles was not affected either by conspecific density or by predation risk (similar to Bókony et al., 2018; Üveges et al., 2017). Although an earlier study found inducible changes in bufadienolide compound diversity as a response to predators (Hettyey et al., 2019), the magnitude of that change was small. It is possible that toxin cocktail diversity is a less plastic trait than toxin amount, or the plastic response in compound diversity may be relatively difficult to detect, perhaps because cocktail diversity may be confounded by bacterial transformation of bufadienolide compounds (Hayes et al., 2009; Kamalakkannan et al., 2017). Currently, the relative effects of each bufadienolide compound on natural enemies are barely known (Barnhart et al., 2017; Chen & Chen, 1933; Crossland et al., 2012; Cunha Filho et al., 2005; Tempone et al., 2008), despite the possibility that different enemies might be sensitive to different compounds. Thus, the functional importance of toxin diversity in chemical defences remains to be tested.

The observed decrease in body mass in response to increasing conspecific density and to the presence of cues indicating predation risk (Figure S1) aligns well with previous studies and is likely a consequence of competition for food and reduced activity in response to predation risk (e.g. Laurila et al., 1998; Skelly & Werner, 1990; Werner & Anholt, 1993). This decrease in body mass may have arisen, at least in part, as a cost of higher investment into toxin production at high densities and under predation risk because such investment can interfere with energy metabolism and growth (Blennerhassett et al., 2019). However, this scenario seems unlikely in our case because there seems to be no systematic relationship between body mass and total bufadienolide quantity within treatment groups in our experiment (Figure S3) and previous studies also did not find considerable costs of toxin synthesis in common toad tadpoles (Kurali et al., 2016; Üveges et al., 2017).

Lastly, our results may also have implications for conservation biology. Invasive toad species, such as the cane toad in Australia (Shine, 2010), and the Asian common toad *Duttaphrynus melanostictus* in Madagascar (Licata et al., 2019) pose serious threats to the native fauna, mainly due to their toxicity. If the results of our study are applicable to these toad species, removal efforts focusing on early-stage tadpoles may be beneficial not only by decreasing the number of toads in invaded regions but also by decreasing the toxin content of their tadpoles (which might also have long-lasting effects on their toxicity after metamorphosis; see Benard & Fordyce, 2003; Hagman et al., 2009). Lower toxicity of toads may prevent mortality of native predators due to poisoning and may allow them to learn to avoid toxic invaders, thereby facilitating adaptation of the local predator fauna (Caller & Brown, 2013; Greenlees et al., 2010; Phillips & Shine, 2006). Therefore, information on how chemically defended invaders adjust their toxin production to environmental conditions

may help efforts focusing on their management and the protection of native species.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

B.Ü., V.B. and A.H. conceived and designed the study; B.Ü. and A.C.B. conducted the experiment, collected data and prepared samples for chemical analysis which were run by Á.M.M.; B.Ü., A.C.B. and V.B. analysed the data; B.Ü., V.B. and A.H. wrote the manuscript, and all authors gave approval for publication.

DATA AVAILABILITY STATEMENT

Dataset of the study available from the figshare digital repository at https://figshare.com/s/c43acbb01d493a7f10e5 (https://doi.org/10.6084/m9.figshare.11353637; Üveges et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Exposure to *Batrachochytrium dendrobatidis* affects chemical defences in two anuran amphibians, *Rana dalmatina* and *Bufo bufo*

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Abstract

Background: *Batrachochytrium dendrobatidis* (*Bd*) is the causative agent of chytridiomycosis, one of the major causes of worldwide amphibian biodiversity loss. Many amphibians exhibit skin-based chemical defences, which may play an important role against invading pathogens, but whether the synthesis of these chemical compounds is enhanced or suppressed in the presence of pathogens is largely unknown. Here we investigated direct and indirect effects of larval exposure to the globally distributed and highly virulent *Bd-GPL* strain on skin secreted chemical defences and life history traits during early ontogeny of agile frogs (*Rana dalmatina*) and common toads (*Bufo bufo*).

Results: Exposure to *Bd* during the larval stage did not result in enhanced synthesis of the antimicrobial peptide Brevinin-1 Da in *R. dalmatina* tadpoles or in increased production of bufadienolides in *B. bufo* tadpoles. However, exposure to *Bd* during the larval stage had a carry-over effect reaching beyond metamorphosis: both *R. dalmatina* and *B. bufo* froglets contained smaller quantities of defensive chemicals than their *Bd*-naïve conspecifics in the control treatment. Prevalence of *Bd* and infection intensities were very low in both larvae and metamorphs of *R. dalmatina*, while in *B. bufo* we observed high *Bd* prevalence and infection intensities, especially in metamorphs. At the same time, we did not find a significant effect of *Bd*-exposure on body mass or development rate in larvae or metamorphs in either species.

Conclusions: The lack of detrimental effect of *Bd*-exposure on life history traits, even parallel with high infection intensities in the case of *B. bufo* individuals, is surprising and suggests high tolerance of local populations of these two species against *Bd*. However, the lowered quantity of defensive chemicals may compromise antimicrobial and antipredatory defences of froglets, which may ultimately contribute to population declines also in the absence of conspicuous mass-mortality events.

Keywords: Antimicrobial peptide, Bufadienolide, Indirect effect, Infectious diseases, Innate immunity

Background

Amphibians are among the most threatened vertebrate groups, with their populations declining worldwide [1–3]. Although there may be no single main cause of declines [3], diseases caused by infection with viral, bacterial or fungal agents are clearly among the most devastating factors [4–6]. Studies aiming at uncovering and better understanding the processes that take place during interactions between amphibian hosts and their

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pathogens are therefore in the focus of current conservation-oriented research [7–9].

Chytridiomycosis, a disease affecting amphibians, is caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) [10]. Because *Bsal* has only been discovered recently [11], we know comparatively little about it [12], so that here we concentrate on chytridiomycosis caused by *Bd*. Chytridiomycosis has already led to the decline or extinction of several hundred species [13] and continues to cause mass mortality events on five continents due to repeated introductions arising from human activities [14]. *Bd* infects keratinous epidermal layers of the skin [15] and impairs its osmoregulatory function. This effect can cause shifts in electrolyte balance leading to cardiac asystolic death in juveniles and adults [16]. Tadpoles exhibit keratinous elements only in their mouthparts, so that they are less susceptible to *Bd* infection than later life-stages [17, 18], and can act as reservoirs in natural habitats [19–21].

As a part of innate immune defences, amphibians de novo synthesise numerous chemical compounds in their skin, serving as the first line of defence against pathogens and parasites [22]. These compounds can be cytolytic peptides, steroids, alkaloids or biogenic amines [23–27]. The most widespread defences against pathogens are cytolytic antimicrobial peptides (AMPs) which have been reported for species across eleven anuran families [27]. These AMPs are active against viruses, bacteria and microscopic fungi, including *Bd* [27–31]. The susceptibility of amphibian species and populations to chytridiomycosis is related to differences in AMP profiles [32, 33]. Bufonid toads lack skin-secreted AMPs [22], but may instead produce bufadienolides from early larval development on [34–36]. These steroid compounds exhibit antimicrobial, antiprotozoal activity, and may protect toads also against *Bd* [37, 38].

In this study, our aim was to experimentally investigate whether exposure to *Bd* resulted in increased production of skin-borne chemical defences as expressions of phenotypic plasticity, or if it caused lowered synthesis of defensive chemicals due to costs of infection or because of immune suppression. Therefore we exposed agile frog (*Rana dalmatina*; Fitzinger 1838) and common toad (*Bufo bufo*; Linnaeus, 1758) larvae to a highly virulent *Bd* isolate and monitored consequences for chemical defences and life history traits in well-developed tadpoles. Potential effects of larval infection reaching beyond metamorphosis on the abovementioned parameters were also tested. Adult *R. dalmatina* individuals produce at least one skin secreted AMP, Brevinin-1 Da [39], and *B. bufo* secrete several bufadienolide compounds in the skin [35, 36, 40]. Therefore, these species are suitable for the investigation of interactions between *Bd* infection and chemical defences.

Results

Rana dalmatina

Control individuals all remained uninfected. The low *Bd* dose treatment resulted in very low infection prevalence and intensity in both tadpoles and froglets, while the high *Bd* dose treatment resulted in higher prevalence and intensity values in tadpoles and especially so in froglets (Table 1; Fig. 1A).

Treatment had no effect on the relative amount of Brevinin-1 Da in pooled samples of tadpoles (GLM: $F_{2,15}=2.31$, $P=0.13$; Fig. 2A). However, older tadpoles tended to exhibit larger amounts of the peptide than younger conspecifics ($F_{1,16}=4.51$, $P=0.05$). Infection intensity and body mass were not related to Brevinin-1 Da quantity (infection intensity: $F_{1,16}=1.14$, $P=0.30$; body mass: $F_{1,16}=0.67$, $P=0.43$). In case of froglets, however, exposure to *Bd* resulted in significantly reduced relative amounts of Brevinin-1 Da in both *Bd* treatments as compared to the controls (GLM: $F_{2,13}=30.60$, $P<0.001$; Fig. 2B). The other measured variables had no detectable effect on Brevinin-1 Da quantity (length of larval development: $F_{1,12}=0.37$, $P=0.55$; infection intensity: $F_{1,12}=0.35$, $P=0.57$; body mass: $F_{1,12}=3.31$, $P=0.09$).

Body mass of tadpoles was positively affected by development stage, but treatment had no significant effect on it, either alone, or in interaction with development stage. In case of froglets, treatment had a marginally non-significant effect on body mass, where individuals exposed to the high *Bd* dose treatment tended to be heavier than others. Length of larval development had no effect on body mass, either alone or in interaction with treatment (For details see Table 2). Treatment had no significant

Table 1 Prevalence of *Bd* infection in the studied species after experimental exposure to *Bd*. N_{tot} is the total number of *Bd*-exposed individuals surviving until sampling, N_{inf} is the number of infected individuals at sampling. Control individuals are not shown because all remained uninfected

Species	Life stage	Treatment	N_{tot}	N_{inf}	Prevalence (%)
Rana dalmatina	Tadpoles	Low *Bd* dose	18	1	6
		High *Bd* dose	18	3	17
	Froglets	Low *Bd* dose	16	1	6
		High *Bd* dose	17	8	47
Bufo bufo	Tadpoles	Low *Bd* dose	18	6	33
		High *Bd* dose	17	17	100
	Toadlets	Low *Bd* dose	12	6	50
		High *Bd* dose	12	12	100

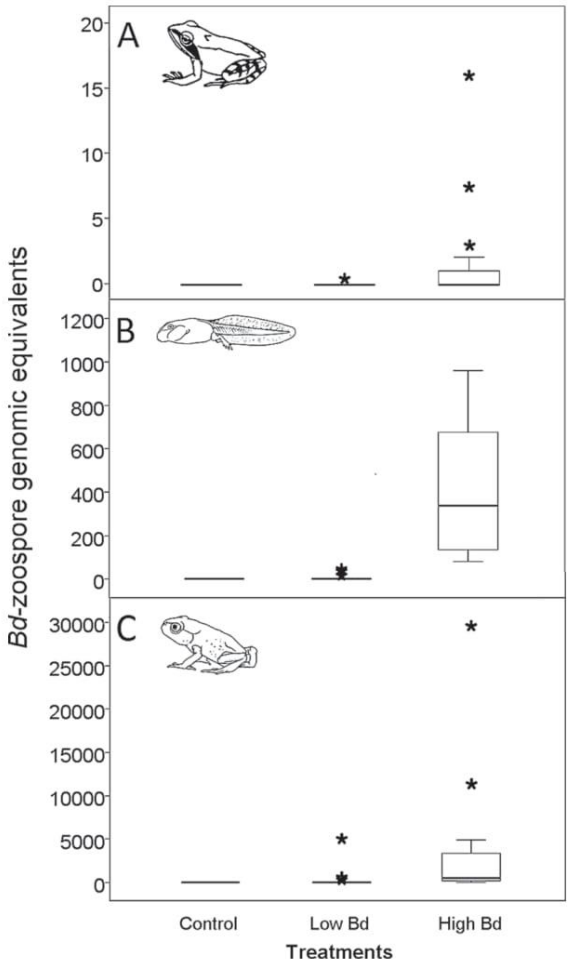

Fig. 1 *Bd* Load as zoospore genomic equivalents in case of *Rana dalmatina* froglets (**A**) *Bufo bufo* tadpoles (**B**) and *B. bufo* toadlets (**C**). Tadpoles of *R. dalmatina* are not depicted because of extremely low *Bd* prevalence (one tadpole infected in the low *Bd* treatment and three tadpoles infected in the high *Bd* treatment). Note that scales are different. Horizontal lines represent medians, boxes represent interquartiles, bars represent ranges, asterisks indicate outliers (deviating from the boundary of the interquartile range (IQR) by more than 1.5 × IQR)

effect on development, either measured as tadpoles' development stage at sampling or as the length of larval development in case of froglets (Table 2).

Bufo bufo

None of the control individuals were infected, but we obtained relatively high infection prevalence and intensities in toadlets exposed to the low *Bd* dose treatment and in both life stages exposed to the high *Bd* dose treatment (Table 1; Fig. 1B and C).

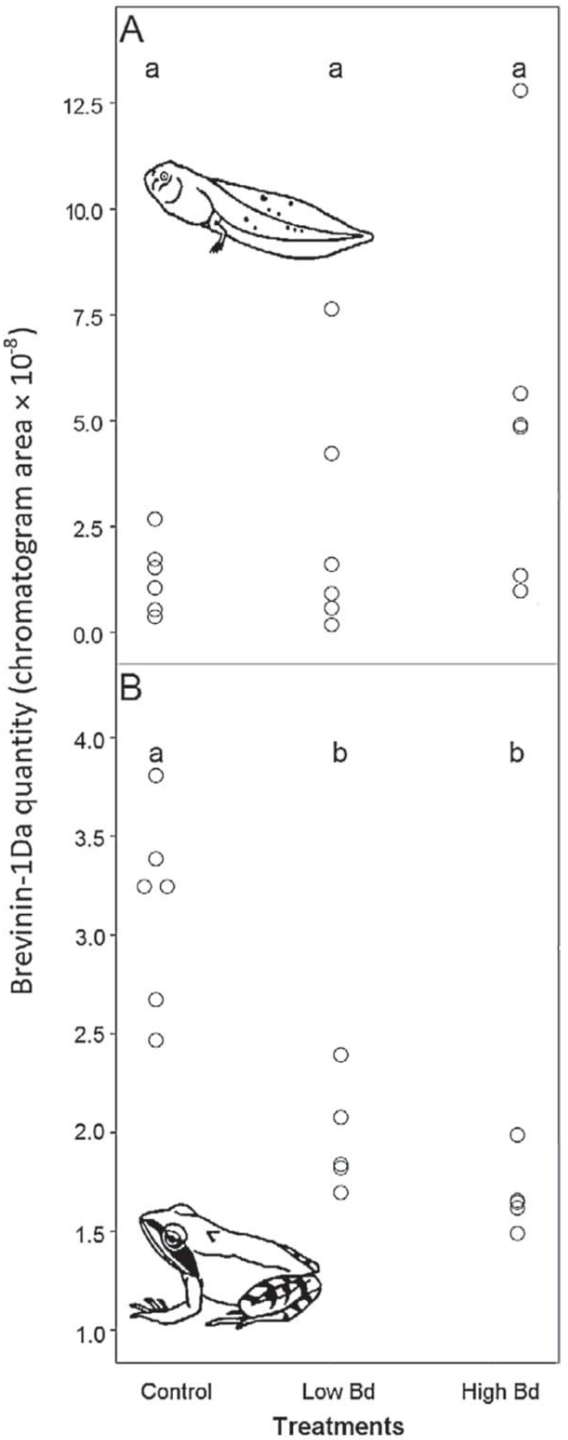

Fig. 2 Quantity of the antimicrobial peptide Brevinin-1 Da in pooled samples of *R. dalmatina* tadpoles (**A**) and froglets (**B**). To obtain detectable quantities of AMP, we had to pool groups of three samples during sample preparation preceding chemical analysis. Letters in lower case indicate homogeneous subsets according to Tukey HSD post-hoc tests. Two overlapping data points are depicted next to each other in panel B in the control treatment. Note that in froglets the low *Bd* dose and the high *Bd* dose treatment contained only 5 replicates as opposed to 6 replicates in the other groups

Table 2 The effect of treatments on development and body mass of individuals at two life stages in the two studied species. Results are based on General Linear Models. Significant differences are highlighted in bold

Species	Life stage	Dependent variables	Explanatory variables	*B*	*SE*	*df*	*F*	*P*
Rana dalmatina	Tadpoles	Development stage	Treatment			2, 50	0.36	0.70
		Body mass	**Development stage**	**0.06**	**0.01**	**1, 51**	**44.51**	**< 0.001**
			Treatment			2, 49	0.84	0.44
			Treatment × development stage			2, 47	1.49	0.24
	Froglets	Length of larval development	Treatment					0.95*
		Body mass	Length of larval development	− 1.63	1.61	1, 49	1.02	0.31
			Treatment			2, 48	3.13	0.05
			Treatment × length of larval development			2, 45	0.89	0.42
Bufo bufo	Tadpoles	Development stage	Treatment			2, 50	1.07	0.35
		Body mass	**Development stage**	**26.02**	**4.06**	**1, 51**	**41.11**	**< 0.001**
			Treatment			2, 49	0.25	0.78
			Treatment × development stage			2, 47	0.88	0.42
	Toadlets	Length of larval development	Treatment			2, 35	0.03	0.97
		Body mass	**Length of larval development**	**− 0.19**	**0.01**	**1, 36**	**9.87**	**0.003**
			Treatment			2, 34	0.95	0.40
			Treatment × length of larval development			2, 32	0.92	0.41

*Result based on Kruskal–Wallis test

We detected 22 different bufadienolide compounds in *B. bufo* extracts, three of which we identified with the help of the standards as arenobufagin, telocinobufagin and bufotalin (Table 3). The presence of individual compounds showed varied age-dependent patterns: some bufadienolides were present at both life stages in all (e.g., compound 7) or nearly all individuals (e.g., compound 13), while others occurred in a high proportion of individuals only after metamorphosis (e.g., compounds 10–12; Table 3). After metamorphosis, toadlets produced more bufadienolide compounds and also experienced a two to three fold increase in TBQ compared to tadpoles (Fig. 3).

The number of bufadienolide compounds (NBC) did not differ among treatments in either life stage (GLM; tadpoles: $F_{2,49}=0.49$, $P=0.62$; toadlets: N = 38, $F_{2,35}=1.6$, $P=0.22$; Fig. 3 A and B). NBC was positively related to dry mass in tadpoles ($B=0.16$, $SE=0.08$, $F_{1,51}=4.56$, $P=0.038$), but not in toadlets ($F_{1,36}=1.78$, $P=0.19$). Infection intensity and development had no significant effect on NBC either in tadpoles (infection intensity: $F_{1,50}=1.82$, $P=0.18$; development stage: $F_{1,50}=0.002$, $P=0.97$) or in toadlets (infection intensity: $F_{1,36}=2.31$, $P=0.13$; length of larval development: $F_{1,36}=0.02$, $P=0.88$).

Total bufadienolide quantity (TBQ) was not affected by treatment in *B. bufo* tadpoles (GLM; $F_{2,50}=0.31$, $P=0.74$; Fig. 3C), but differed significantly among treatments in case of toadlets ($F_{2,34}=4.08$, $P=0.026$; Fig. 3D). TBQ was not related to dry mass ($F_{1,51}=10.68$, $P=0.20$), but this relationship was positive in case of toadlets ($B=2.21$, $SE=0.69$, $F_{1,34}=10.39$, $P=0.003$). According to Tukey HSD post hoc tests on the residuals of the regression of TBQ on toadlet dry mass ($R=0.47$, $F_{1,37}=10.06$, $P=0.003$), relative TBQ was lower by 23% in the high *Bd* dose treatment than in the low *Bd* dose treatment (Mean difference = − 33.31, $SE=11.93$, $P=0.022$), but these two treatment groups did not differ from the control (high *Bd* dose: Mean difference = − 23.99, $SE=11.49$, $P=0.11$; (low *Bd* dose: Mean difference = 9.32, $SE=11.49$, $P=0.7$; Fig. 3D). Infection intensity and development stage did not have an effect on NBC either in tadpoles (infection intensity: $F_{1,51}=0.69$, $P=0.41$; development stage: $F_{1,51}=0.04$, $P=0.85$) or in toadlets (infection intensity: $F_{1,33}=0.038$, $P=0.85$; length of larval development: $F_{1,33}=1.46$, $P=0.24$).

Body mass was affected by development stage in case of tadpoles: individuals that were less developed also had a lower body mass. Treatment and its interaction with development stage had no significant effect on body mass (Table 2). Within the high *Bd* dose treatment, where *Bd* prevalence was sufficiently high to be analyzed, tadpole body mass was again positively related to development stage ($F_{1,15}=29.6$, $P<0.001$), while infection intensity had no effect on mass (GLM: $F_{1,14}=0.003$, $P=0.96$). Fourteen days after completion of metamorphosis, body mass of toadlets was in a negative relationship with the length of larval development (Table 2). Treatment and its interaction with the length of larval development had no significant effect on toadlet body mass (Table 2).

Table 3 Percentages of *Bufo bufo* individuals that contained various bufadienolide compounds in the two development stages. We could unambiguously identify three compounds based on the standards and detected another 19 compounds as unknown bufadienolides based on their characteristic UV spectra. Analytical properties of the detected compounds are provided. (N: sample size (number of individuals), m/z: mass/charge)

Bufadienolide compounds	Percentage of individuals containing the compound		Analytical properties	
	Tadpoles (N = 53) (%)	Toadlets (N = 38) (%)	Retention time (minute)	m/z ($M+H^+$)
Arenobufagin	59	74	4.8	417
Telocinobufagin	34	100	8.8	403
Bufotalin	66	100	9.8	445
Compound 1	9	53	4.1	615
Compound 2	17	100	4.7	699
Compound 3	81	100	5.0	417
Compound 4	26	95	5.8	713
Compound 5	0	58	6.0	601
Compound 6	51	100	6.1	415
Compound 7	100	100	7.0	729
Compound 8	49	100	7.5	701
Compound 9	100	100	8.0	727
Compound 10	0	100	9.3	715
Compound 11	0	100	10.6	713
Compound 12	19	100	11.1	401
Compound 13	96	95	11.3	715
Compound 14	0	100	14.9	701
compound 15	100	95	17.5	757
Compound 16	98	84	19.3	573
Compound 17	100	100	20.5	571
Compound 18	100	97	22.1	367
Compound 19	100	100	23.3	365

Within the low *Bd* dose treatment group, neither infection intensity, nor the length of larval development had an effect on body mass of toadlets (GLM: infection intensity: $F_{1,10}=2.01$, $P=0.19$; length of larval development: $F_{1,10}=0.38$, $P=0.55$). However, in the high *Bd* dose treatment, infection intensity had a significant negative effect (GLM: $B=-4.38$, $SE=1.73$, $F_{1,10}=6.39$, $P=0.03$), and the length of larval development a marginally significant positive effect ($B=-5.01$, $SE=2.28$, $F_{1,9}=5.01$, $P=0.052$) on toadlet body mass. Development was not affected by treatment at either life stage (Table 2), and it was not related to infection intensity in either one of the assessed treatment groups (Spearman correlation in case of tadpoles in the high *Bd* dose treatment: $R=0.025$, $N=17$, $P=0.93$; Pearson correlations in case of toadlets: low *Bd* dose: $R=-0.301$, $N=12$, $P=0.34$; high *Bd* dose: $R=0.157$, $N=12$, $P=0.63$).

Discussion

In the present study experimental exposure to *Bd* did not result in significantly increased production of the antimicrobial peptide Brevinin-1 Da in *R. dalmatina* tadpoles, nor did it influence bufadienolide toxin synthesis in *B. bufo* tadpoles. However, *Bd*-exposure during the larval stage negatively affected chemical defences in metamorphosed individuals of both species. These results suggest that neither larvae nor freshly metamorphosed individuals respond to *Bd*-exposure with enhanced synthesis of antimicrobial chemicals, and that infection during the larval stage may rather carries costs that manifest in decreased quantities of chemical defences in metamorphs. Our results further indicate that larvae of the agile frog (*R. dalmatina*) were resistant to infection with a highly virulent *Bd* isolate, as indicated by low prevalence and infection intensities. At the same time, tadpoles of the common toad (*B. bufo*) were not resistant, but tolerant, as suggested by high *Bd* prevalence and high infection intensities, but no malign effects on

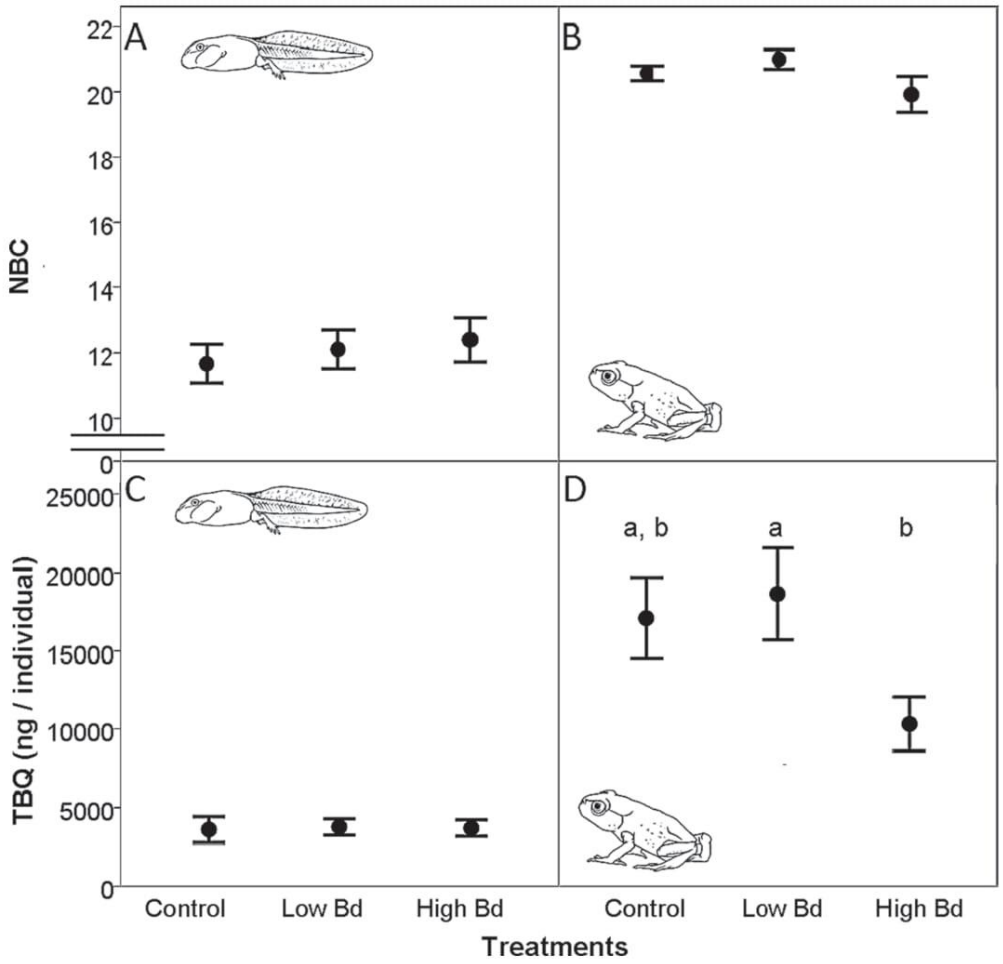

Fig. 3 Toxin content of *B. bufo* larvae and toadlets in the control and *Bd*-exposure treatments: Number of bufadienolide compounds (NBC; mean ± SE) in tadpoles (**A**) and toadlets (**B**), and total bufadienolide quantity (TBQ; mean ± SE) in tadpoles (**C**) and toadlets (**D**) after exposure to zero (control), low or high *Bd* zoospore concentrations. Note that the scale is not continuous in case of NBC. Letters in lower case indicate homogeneous subsets according to Tukey HSD post-hoc tests

life history traits: exposure to *Bd* did not influence body mass or development rate in larvae or metamorphs in either species.

The knowledge available regarding the occurrence of AMP synthesis in larval anurans is limited and controversial. Skin-associated granular glands and their ducts are mostly immature before metamorphosis in most species [41, 42], suggesting no, or limited AMP production (but see [43]). However, at the same time, gland products may also be secreted by a merocrine process, where the secretum reaches the skin surface via exocytosis directly or through the epidermal interstitium [41, 44, 45]. Furthermore, the adaptive immune system is suppressed during metamorphosis to prevent immune responses against newly emerging tissue types [46], suggesting a greater reliance on innate immune defences against invading pathogens during this susceptible period. Schadich et al. [47] found no evidence of AMP synthesis in tadpoles of *Litoria ewingii* despite intense AMP production after metamorphosis, while Wabnitz et al. [48] demonstrated efficient AMP synthesis in the skin of *Litoria splendida* at both larval and adult life stages. While Woodhams et al. [49] did not find AMPs in an early larval stage (development stage 25 according to Gosner, [50]) in two closely related species, *Rana arvalis* and *R. temporaria*, we detected that *R. dalmatina* tadpoles produce de novo the same AMP as do adults [39] at least during late larval development (development stage 37 according to Gosner). Whether this discrepancy among studies indicates species-specific differences in AMP synthesis, or if

AMP production starts in Ranids some time later during larval development remains to be determined.

Our expectation that AMP synthesis is boosted upon exposure to *Bd* in *R. dalmatina* was not met. Phenotypic plasticity in chemical defences is not well understood [51], but some studies suggest that environmental stressors such as competition [52] and pathogen presence [53, 54] can induce an increase in AMP synthesis in metamorphosed anuran amphibians. Furthermore, adult European water frogs (*Pelophylax lessonae* and *P. esculentus*) are capable of elevating AMP synthesis in response to *Bd*, if the skin microbiota is suppressed [55]. Although during the tadpole stage we also observed a slight increase in the relative amount of Brevinin-1 Da upon *Bd* exposure, this was not significant, and we detected a sharp reduction in AMP quantity in both *Bd*-exposed groups in froglets. Wild caught, infected adults of the frog *Litoria serrata* exhibited similarly reduced quantities of AMPs compared to uninfected conspecifics [56], but whether this was a cause or consequence of infection remained unknown. Our results suggest that reduced AMP expression can be a consequence of exposure to *Bd*. A reduced synthesis of AMPs may result from immunosuppression by *Bd*, as demonstrated in case of the adaptive immune system [57–59]. Lowered AMP production may also be a direct cost of infection, or may be indirectly caused by elevated corticosterone levels resulting from *Bd*-infection [60, 61] because elevated corticosterone levels can cause reduced AMP synthesis [62, 63]. Whether the reduced quantity of Brevinin-1 Da is still large enough to be effective against *Bd* in metamorphs, or other agents of the immune system can take over the role of Brevinin-1 Da in preventing severe infection requires further investigation.

Interestingly, AMP synthesis was reduced in metamorphs arising from the low *Bd* dose treatment without detectable amounts of *Bd* on all but one froglet. There are, however, precedents for significant effects of *Bd* exposure on various traits in the absence of confirmed infection. For example, Garner et al. [64] experienced significant mortality in *B. bufo* tadpoles and freshly metamorphosed individuals due to *Bd* exposure without detectable infection loads. Also, *Bd* exposure reduced growth in European treefrogs (*Hyla arborea*) without detectable levels of infection [65]. Individuals are probably able to prevent initial infections or naturally clear *Bd* infections acquired but may suffer the costs of the mounted immune response [66]. Alternatively, *Bd* presence in the surrounding aquatic environment may be sufficient to induce pathology or responses (e.g., via waterborne chemicals) even if infection does not occur, as shown in case of crayfishes (*Procambarus* spp. and *Orconectes virilis*) which are alternative hosts of *Bd* [67].

Exposure to *Bd* had no detectable effect either on the number of bufadienolide compounds or on total bufadienolide quantity in case of *B. bufo* tadpoles. Similarly, NBC of toadlets was also not affected by *Bd* presence. However, TBQ in the high *Bd* dose treatment was significantly lower compared to the low *Bd* dose treatment in toadlets. These results clearly indicate that *Bd*-prevalence and higher infection loads did not induce enhanced toxin synthesis. Besides their role in the chemical defence system, bufadienolides also contribute to the osmotic homeostasis of toads [68, 69]. The decreased TBQ in the high *Bd* dose may have resulted from a compensatory response to the altered electrolyte balance (reduced sodium and potassium concentrations) due to *Bd* infection [16], but this speculation needs experimental confirmation. Alternatively, *Bd* infection can lead to structural damage in the skin and its glands [10], which may have contributed to the lowered toxin production in the high *Bd* dose treatment. Whatever the cause is, heavily infected toadlets unable to produce the increase in toxin content after metamorphosis, may suffer from detrimental consequences because skin secreted bufadienolides can act as repellents against vertebrate predators [70, 71], they play a role in immune defence [37, 38, 72] and are important for osmotic homeostasis [68, 69]. Lowered TBQ in the high *Bd* dose treatment, thus, indicates a possible indirect negative effect of *Bd* infection, similarly to what we found in regard to the chemical defence of *R. dalmatina*.

Exposure to *Bd* did not affect the measured life history traits in either species at the tadpole stage. In larval anurans only the mouthparts are keratinized structures [17], thus, mortalities due to chytridiomycosis are rare and susceptibility varies among species [10, 18, 73, 74]. However, sublethal negative effects of *Bd* infection can also occur due to mouthpart damage [75, 76], lethargy and poor swimming performance, resulting in lowered body mass and growth [18, 74, 76, 77]. In the present study only a very few tadpoles of *R. dalmatina* became infected and in these individuals infection intensity was very low in both *Bd* treatments. In case of *B. bufo* tadpoles, prevalence of infection and infection intensities were high, especially in the high *Bd* dose treatment, so that the lack of fitness consequences was somewhat surprising. Two out of the three identified bufadienolide compounds (arenobufagin and telocinobufagin) were previously documented to moderately inhibit the growth of *Bd* [38]. These and some of the other unidentified bufadienolide compounds may have contributed to the high *Bd*-tolerance of *B. bufo* individuals, which lack AMPs. These results suggest that the studied populations exhibit low susceptibility to *Bd* infection during larval development:

tadpoles of *R. dalmatina* appear to be highly resistant, while *B. bufo* larvae may be highly tolerant.

During metamorphic climax, *Bd* starts to colonise newly keratinized skin surfaces and spreads out on the entire animal [17, 78]. In the present study, both the prevalence of *Bd* (GPL, IA042) and infection intensities were high in case of *B. bufo*, which is consistent with previous studies that used the same isolate and resulted in significant negative effects on body mass and mortality of *B. bufo* originated from the United Kingdom [64, 79]. Mortalities due to chytridiomycosis in the closely related *B. spinosus* (formerly a subspecies of *B. bufo*) were also observed in Spain [64, 80]. Contrary to this, the length of larval development was not affected by *Bd* exposure in our experiment, and we did not observe significant negative effects on body mass of toadlets. Only in the high *Bd* dose treatment, infection intensity was negatively related to body mass: lighter individuals had higher infection intensities, than heavier ones. Whether this pattern was a cause or consequence of infection remains unclear. All in all, these results suggest that *B. bufo* individuals of the studied population in Central Europe may be more tolerant to *Bd* than those in Western European populations. Alternatively to this spatial hypothesis, the pattern may also arise due to temporal differences; toads may have adapted to the presence of *Bd* since the earlier studies [64, 79, 80]. Furthermore, populations may respond differently to *Bd* infection from year to year due to phenotypic plasticity or epigenetic changes. These processes could also have contributed to the observed differences between the present and former studies in toad susceptibility. Regarding *R. dalmatina*, we detected low prevalence of infection in the low *Bd* dose treatment and moderate prevalence with low infection intensities in the high *Bd* dose treatment, and no adverse effects of *Bd*-exposure on life history traits of froglets. These results are in line with those of previous field studies suggesting that *R. dalmatina* is resistant to chytridiomycosis [21, 81, 82]. The less keratinized skin as well as the presence of Brevinin-1 Da may make the skin of *R. dalmatina* less suitable for *Bd* growth, hence the lower probability and intensity of infection as compared to *B. bufo*, which speculations need further investigations in the future.

Conclusions

Our results provide evidence that exposure to *Bd* can have negative effects on two different types of chemical defences of phylogenetically distant species in a later life-stage, even in the absence of obvious immediate effects on life-history traits, or, indeed, actual *Bd* infection. Because the investigated chemical defences are widespread among amphibians, the results of this study are likely to be applicable to many species with similar chemical defences, and this hidden effect of *Bd* presence in aquatic habitats should be considered in the future. Whether weakened chemical defences lead to lowered fitness in affected individuals remains an open question that will need further investigation.

Methods

Experimental procedures

In March 2016, we collected 40 eggs from each of nine freshly laid egg clutches of *R. dalmatina* from a pond in the Pilis-Visegrádi-Hills, Hungary (47.767058 N, 18.981325 E). We transported them to the Experimental Station Júliannamajor of the Plant Protection Institute, Centre for Agricultural Research. The Közép-Duna-Völgyi KTVF issued the permission to conduct the study (PE/KTF:3596-6-8/2016) and the Ethical Commission of the ATK NÖVI approved the investigation in accordance with Good Scientific Practice guidelines and national legislation. We placed eggs from each clutch separately into plastic boxes ($24 \times 16 \times 13$ cm) holding 1 L of reconstituted soft water (RSW; [83]) at a constant temperature of 19 °C and a 12:12 h light:dark cycle. Eggs were disinfected by bathing them for 3 days in 10 mg/L chloramphenicol in order to prevent accidental *Bd* infection [84] because *Bd* is reportedly present in the study area [21], even if with low prevalence [82]. Although chloramphenicol is an antibiotic agent, it is also effective against *Bd* [84, 85] and can be safely used for the disinfection of amphibian eggs [86].

Five days after hatching, when larvae were at development stage 25 (Gosner), we started the experiment with 12 healthy-looking tadpoles from each family. We maintained tadpoles individually in plastic boxes ($15 \times 12 \times 12$ cm) filled with 1 L of RSW, and fed tadpoles with slightly boiled and smashed spinach complemented with *Spirulina* powder (1 m/m%) ad libitum. We changed water twice a week using different dip nets for each treatment to prevent contamination across treatments. Temperature was 19.4 ± 0.7 °C (mean ± SD) during the experiment. The light:dark cycle was adjusted weekly to outdoor conditions, starting with 12:12 h light:dark in late March which we gradually changed to 14:10 h by the end of April. We exposed tadpoles during the entire larval development to sterile culture broth (control), or to a low or high zoospore concentration in liquid *Bd* culture (low *Bd* dose and high *Bd* dose treatments hereafter; for details, see below). We assigned tadpoles to the treatments using stratified randomization, and arranged rearing boxes into randomized spatial blocks, each containing one replicate from each treatment. We exposed individuals of nine families to the

three treatments in four replicates, which resulted in a total of 108 experimental units. Hatchlings that were not used in the experiment were released at the site of origin.

Thirty days after start of the experiment, we randomly selected half of the tadpoles, sampled their skin secretions (for details see below) then gently blotted them dry and weighed them to the nearest mg using an OHAUS-PA213 analytical balance. Thereafter, we euthanized and preserved tadpoles in 70% ethanol and stored samples at 4 °C until further analysis.

We monitored development of remaining tadpoles daily. When an individual reached development stage 42 (emergence of forelimbs; according to Gosner) we poured the water off, placed back the tadpole into the same box covered with a transparent and perforated lid, added 100 ml RSW and lifted one side of the container by ca. 2 cm to provide metamorphs with both a body of water and a dry surface. Once metamorphs reached stage 46 (complete tail resorption; according to Gosner) we placed individuals into new, covered boxes of the same size as before, equipped with wet paper towels and a piece of cardboard egg-holder as a shelter. We fed the froglets with small crickets (*Acheta domestica*, instar stage 1–2) ad libitum. Dates of metamorphosis and of completion of tail resorption were registered daily. Fourteen days after completion of tail resorption we weighed animals and humanely euthanized them using the "cooling then freezing" method [87], preserved individuals in 70% ethanol and kept samples at 4 °C until further processing.

We conducted the same experiment using individuals of *B. bufo*. However, we observed high mortality independently from treatments (15 out of 36 tadpoles died both in the control and in the low *Bd*-dose treatment, and 10 out of 36 tadpoles died in the high *Bd*-dose treatment), presumably due to a bacterial bloom caused by the simultaneous presence of *Spirulina* and culture broth (an effect we did not observe in case of *R. dalmatina* tadpoles in this experiment, or when tadpoles were fed solely with spinach in previous experiments). Consequently, 17 days after start of the first experiment, we re-started this part of the study and conducted the same experiment as described above with 108 randomly chosen *B. bufo* tadpoles from the same location with the following additional differences: (1) Eggs originating from 14 different clutches were kept in mixed groups in outdoor mesocosms containing 130 L of aged tap water, 40 g beech leaves and 0.5 L pond water with no prior chloramphenicol treatment. (2) Once brought into the laboratory after hatching, we fed tadpoles with smashed and slightly boiled spinach only (no *Spirulina*). (3) Light exposure adjustment was different because of the delayed start (4) Because of their smaller size, we fed toadlets with springtails (*Folsomia* sp.) after metamorphosis.

Maintenance of *Bd* culture and experimental exposure

We experimentally infected tadpoles with the global pandemic lineage (GPL) of *Bd*. This isolate originated from a dead *Alytes obstetricans* (IA042) collected in 2004 from a mass mortality event in Spanish Pyrenees. Cultures were maintained in mTGhL broth (8 g tryptone, 2 g gelatine-hydrolysate and 4 g lactose in 1000 ml distilled water) in 25 cm^2 cell culture flasks at 4 °C and passed every three months into sterile mTGhL. One week before use, we inoculated 100 ml mTGhL broth with 1–2 ml of these cultures in 175 cm^2 cell culture flasks and incubated them for seven days at 22 °C. We assessed the concentration of intact zoospores using a Bürker chamber at × 400 magnification. During inoculation of tadpoles' rearing boxes, the mean initial concentrations were ~ 1.8×10^6 (used to infect *R. dalmatina*) and ~ 2×10^6 (used to infect *B. bufo*) zoospores (zsp)/ml in the flasks. These cultures were used for the high *Bd* dose treatment and we prepared a 100-fold dilution with sterile mTGhL broth for the low *Bd* dose treatment. After each water change, we inoculated 1 ml of these cultures into the tadpoles' rearing boxes holding 1 L RSW, resulting in 18–20 zsp/ml in the low *Bd* dose treatment and 1800–2000 zsp/ml in the high *Bd* dose treatment. Similar zoospore concentrations have been used widely and successfully in studies involving experimental infection [18, 75, 88]. We inoculated controls with the same quantity of sterile mTGhL broth. Contaminated water and equipment were disinfected overnight with VirkonS before disposal [89].

Assessment of infection intensity using qPCR

We assessed infection intensity from dissected mouthparts in case of preserved tadpoles and from toe clips in case of metamorphs. We homogenized tissue samples, extracted DNA using PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to previous recommendations [90], and stored extracted DNA at − 20 °C until further analyses. We assessed infection intensity using real-time quantitative real-time polymerase chain reaction (qPCR) following a standard amplification methodology targeting the ITS-1/5.8S rDNA region [90] on a BioRad CFX96 Touch Real-Time PCR System. To avoid PCR inhibition by ingredients of PrepMan, samples were diluted ten-fold with double-distilled water. We ran samples in duplicate. In case the result was equivocal, we repeated reactions in duplicate. If it again returned an equivocal result, we considered that sample to be *Bd* positive [91]. Genomic equivalent (GE) values were estimated from standard curves based on four dilutions of a standard (100, 10, 1 and 0.1 zoospore genomic equivalents; provided by J. Bosch; Museo Nacional de Ciencias Naturales, Madrid, Spain).

Skin secretion sampling in *R. dalmatina* and analysis of Brevinin-1 Da

We analysed skin secretions in two ontogenetic stages: 30 days after start of the experiment, when larvae were in development stage 36.9 ± 1.4 (mean ± SD; according to Gosner) and 14 days after completion of metamorphosis. Following the procedure described in [92] we collected skin secretions non-invasively by bathing tadpoles or froglets individually in 5 ml polypropylene tubes containing 5 ml collection buffer with 0.1 mM norepinephrine (NE) bitartrate for 15 min. After removing animals, we acidified the NE solution by adding 50 µL of 99% trifluoracetic acid (TFA), to reach a final concentration of 1 V/V%. Samples were stored at − 20 °C until further analyses. As indicated by preliminary assessments of Brevinin-1 Da concentrations, we had to pool groups of three samples within treatments to obtain detectable quantities of the targeted AMP. As a pre-step of purification, we activated each reverse-phase Sep-Pak cartridges (200 mg, LiChrolut RP-18, Merck-Millipore) with 2 ml acetonitrile and subsequently rinsed with 2 ml solvent A (HPLC-grade water with 0.12 V/V% TFA; according to [93]). Next, we loaded the skin extracts onto the cartridges, saved the solution, rinsed cartridges with 2 ml solvent A, and loaded the saved solution onto the cartridges again. Finally, we rinsed cartridges with 4 ml solvent A. We eluted the purified skin secretion with 2 ml solvent B (70:30 acetonitrile:HPLC grade water, acidified with TFA to a final concentration of 0.1 V/V%) into 2 ml polypropylene tubes and dried samples using a vacuum centrifuge (Savant, Integrated Speed Vac System, ISS 100).

We accomplished peptide identification and quantification using nano-UHPLC-MS/MS liquid chromatography-mass spectrometry, with a Maxis II ETD QqTOF (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 nanoRSLC system (Dionex, Sunnyvale, CA, USA) under the control of Hystar v.3.2 (Bruker Daltonics, Bremen, Germany). We dissolved samples in 2% acetonitrile and 0.1% formic acid in water, out of which 5 µl were injected onto an Acclaim PepMap100 C-18 trap column (100 µm × 20 mm, Thermo Scientific, Sunnyvale, CA, USA). We performed sample desalting and preconcentration with 0.1% TFA for 8 min with a flow rate of 5 µl/min. Peptides were separated on an ACQUITY UPLC M-Class Peptide BEH C18 column (130 Å, 1.7 µm, 75 µm × 250 mm, Waters, Milford, MA, USA) at 48 °C, using a flow rate of 300 nl/min. We used the following HPLC solvents: solvent A containing 0.1% formic acid in water and solvent B containing 0.1% formic acid in acetonitrile, with the gradient: 4% B from 0 to 11 min, followed by a 120 min gradient to 50% B, then elevated the concentration of solvent B to 90% in 1 min and kept it there for 10 min. After each sample a blank was run to avoid carry-over. Sample ionization was achieved in the positive electrospray ionization mode via a CaptiveSpray nanoBooster ion source with capillary voltage set to 1300 V, at 0.2 Bar nanoBooster pressure. The drying gas was heated to 150 °C with 3 L/min flow rate. For external mass calibration, we used the low concentration tuning mix from Agilent technologies via direct infusion. Internal mass calibration was performed via lock mass for each run using sodium formate with the following ion transfer parameters: prepulse storage 10 µs, collision transfer 10 µs, quadrupole ion energy 5 eV, Funnel 1 RF 400 Vpp, Multipole RF 400 Vpp. The collision RF was set to 1200 Vpp with 120 µs ion transfer time. We identified the chromatographic peaks as Brevinin-1 Da by comparing retention time and mass spectrum of the 453.3551^{4+} fragment to a commercially purchased molecular standard (Biocenter Kft., Szeged, Hungary). We analysed chromatograms using the software Compass Data Analysis (version 4.0, Bruker daltonics Inc., Billerica, USA) to obtain chromatogram area values as quantity estimates for statistical analysis.

Skin toxin sampling in *B. bufo* and analysis of bufadienolides

We collected bufadienolide samples from tadpoles preserved at development stage 36.3 ± 0.9 (mean ± SD; according to Gosner) and from 14 day old toadlets (that finished metamorphosis 14 days earlier). We homogenized whole bodies with a homogenizer (VWR VDI 12) equipped with a dispersing tool (IKA S12N-7S), dried samples under vacuum at 45 °C using a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland), weighed dry mass (dry body mass henceforth) and re-dissolved samples in 1 ml absolute HPLC-grade methanol, aided by brief exposure to ultrasound in a bath sonicator (Tesla UC005AJ1). As the last step of sample preparation, we filtered samples through FilterBio nylon syringe filters (pore size = 0.22 µm) and stored them at − 20 °C until further analyses.

We analysed bufadienolide compounds by means of high-performance liquid chromatography coupled with diode-array detector and electrospray ionization mass spectrometry (HPLC–DAD-ESI–MS). We identified the chromatographic peaks as bufadienolides based on the UV spectrum [34] and by comparing their retention time and mass spectrum to those of the following commercially available standards: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals, New Jersey, USA) and digitoxigenin (Santa Cruz Biotechnology, Dallas, TX, USA)

or to compounds established by analysing a large sample obtained from an adult male common toad by gently massaging the parotoid glands.

We performed HPLC–MS measurements on a Shimadzu LC–MS 2020 instrument (Shimadzu, Kyoto, Japan) that consisted of a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a diode array detector and a single-quadrupole mass analyser with electrospray ionization (ESI–MS). Chromatographic separations were carried out at 35 °C on a Kinetex C18 2.6 µm column (100 mm × 3 mm i.d., Phenomenex) in series with a C18 guard column (4 mm × 3 mm i.d.) using 10 µL injections. Eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.8 mL/min and the gradient was as follows: 0–2 min: 10.5–21.1% B; 2–15 min: 21.1–26.3% B; 15–24 min: 26.3–47.4% B; 24–25 min: 47.4–100% B; 25–30 min: 100% B; 30–31 min: 100–10.5% B; 31–35 min: 10.5% B. ESI conditions were as follows: desolvation line (DL) temperature: 250 °C; heat block temperature: 400 °C; drying N_2 gas flow: 15 L/min; nebulizer N_2 gas flow: 1.5 L/min; positive ionization mode. Data were acquired and processed using the LabSolutions 5.42v software (Shimadzu).

Statistical analyses

We analysed the data on the two species and life stages separately. We excluded two individuals due to extremely slow development (development stage was lower than the mean by more than 3 SD), and accidentally lost three individuals. This resulted in the following sample sizes used in the analyses: *R. dalmatina*; control: 35, low *Bd* dose: 36, high *Bd* dose: 35; *B. bufo*: control: 34, low *Bd* dose: 36, high *Bd* dose: 35.

We averaged GE values obtained from qPCR runs for each sample and subsequently rank-transformed means, because GE values that fall outside the standard interpolation curve are not estimated reliably.

In *R. dalmatina* we compared Brevinin-1 Da quantity among treatments using general linear models (GLM) entering log-transformed values of the chromatogram areas as the dependent variable. We used log-transformation to enhance normality of model residuals and homogeneity of variances. In case of 14 days old froglets, we only had 5–5 replicates in *Bd*-exposed treatments because of mortality. For each triplet, we calculated mean values of body mass, development stage and infection intensity. The initial model included treatment as a fixed factor, and body mass, development stage and infection intensity as covariates.

To calculate the number of bufadienolide compounds (NBC) present in each *B. bufo* individual, we assumed a compound to be present if the signal-to-noise ratio (S/N) of its peak was at least three. We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the bufotalin standard, and summed up these values to obtain an estimate of total bufadienolide quantity (TBQ) for each individual (for a similar approach see [34, 94]). We analysed effects of treatment on both toxin variables separated by life stages using GLMs. In case of TBQ, we entered as the dependent variable log-transformed values in case of tadpoles and square-root transformed values in case of metamorphs to enhance normality of model residuals and homogeneity of variances. Initial models included treatment as a fixed factor and dry mass, development stage and infection intensity as covariates. In case of a significant treatment effect, we used Tukey HSD post-hoc tests to reveal significant differences between treatment groups. When a covariate also had a significant effect, we ran post-hoc tests on residuals extracted from the regression of the dependent variable on the covariate.

We analysed body mass data using GLMs. Initial models included treatment as a fixed factor and an estimate of the speed of development (Gosner stage in case of tadpoles and length of larval development in case of metamorphs) as a covariate, as well as their interaction. To enhance normality of model residuals and homogeneity of variances, we entered log-transformed values of body mass in case of *R. dalmatina* tadpoles and *B. bufo* metamorphs. In case of the high *Bd* dose treatment in *B. bufo* tadpoles, and both *Bd* treatments in metamorphs, where *Bd* prevalence was high, we also investigated the effect of infection intensity on body mass in the *Bd*-exposed treatment groups with GLMs, including infection intensity and development stage as covariates. We analysed variation in development stage using GLMs with treatment as a fixed factor, except for *R. dalmatina* metamorphs where we used Kruskal–Wallis tests due to the non-normal distribution of model residuals and inhomogeneity of variances. In case of *B. bufo* tadpoles in the high *Bd* dose treatment we analysed the relationship between infection intensity and development stage using Spearman rank correlation and Pearson correlations in both *Bd* treatments of metamorphs.

We verified normal distribution of model residuals using Shapiro–Wilk tests and by inspecting diagnostic plots, and homogeneity of variances using Levene's tests. We applied a backward stepwise model simplification procedure [95] to avoid potential problems due to the inclusion of non-significant terms [96]. We obtained statistics for removed variables by re-entering them one by one to the final model. All tests were two-tailed. Statistics were calculated using SPSS Statistics 20.0 for Windows.

Abbreviations
AMP: Antimicrobial peptide; Bd: *Batrachochytrium dendrobatidis*; Bsal: *Batrachochytrium salamandrivorans*; GE: Genomic equivalent; GLM: General linear model; GPL: Global pandemic lineage; HPLC: High performance liquid chromatography; MS: Mass spectrometry; NBC: Number of bufadienolide compounds; NE: Norepinephrine; qPCR: Quantitative polymerase chain reaction; RSW: Reconstituted soft water; SD: Standard deviation; TBQ: Total bufadienolide quantity; TFA: Trifluoracetic acid.

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Authors' contributions
JU, JV, TWJG and AH conceived the ideas and designed methodology; JU, TD and AH accomplished the experiment; KL, ÁMM, DK and LD performed and evaluated chemical analytical measurements. JU, MZN and JV accomplished qPCR analyses. JU and AH analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. All authors read and approved the final manuscript.

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Data availability
All data used in the analyses will be available from Figshare Repository. https://doi.org/10.6084/m9.figshare.12098115 (Ujszegi et al., 2021).

Declarations

Ethics approval and consent to participate
The Közép-Duna-Völgyi KTVF issued the permission to conduct the study (PE/KTF:3596-6-8/2016) and the Ethical Commission of the ATK NÖVI approved the investigation in accordance with Good Scientific Practice guidelines and national legislation.

Consent for publication
Not applicable.

Competing interest
The authors have no conflict of interest to declare.

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4. General discussion

4.1. Summary of results

Phenotypic plasticity is a central concept in evolutionary ecology due to the diversity and importance of the roles it plays in shaping ecological patterns and processes. Here I aimed to compile studies in which we investigated two aspects of inducible defences. First, we wanted to deliver insights about the origin of cues used by anuran larvae to assess predation risk and the information these may convey to tadpoles when adjusting their phenotypic responses to predators. Second, we were looking for evidence supporting the hypothesis that chemical defences can be inducible in vertebrates, just as in plants and lower animals. The conclusions that may be drawn from the results of the research presented in this dissertation may be summarized as follows:

(1) When investigating how the dangerousness of predators affected the strength of phenotypic responses and how these translated into benefits and costs of induced defences, the intensities of induced behavioural and morphological defences of tadpoles clearly mirrored predator dangerousness. Tadpole survival was lower and larval development took longer in the nonlethal presence of the most dangerous predator than in all other treatments, but we did not find further costs of induced defences at or after metamorphosis. Tadpoles exhibiting an induced phenotype enjoyed elevated survival in the presence of free-ranging predators, but individuals exhibiting more extreme phenotypes were not better defended, and survival was not higher in the presence of the type of predator tadpoles had been raised with. The beneficial effect of an induced phenotype was not apparent in large tadpoles. In summary, we found that **the intensity of predator-induced defences in tadpoles can mirror differences in the dangerousness of predators, but the arising costs and benefits are only loosely related to the magnitude of the induced plastic responses**.

(2) When testing how predator species, acute predation risk, the types of chemical cues available as well as their interactions influenced induced defences, we showed that the presence of predator kairomones together with digestion-released cues were sufficient to result in strong antipredator responses in *R. dalmatina* larvae. Further, it seemed that tadpoles used predator kairomones and predator-specific digestion-released cues to adjust the type of responses and prey-borne cues to adjust the intensity of responses according to the actual predation risk. Small tadpoles reacted more intensely to dragonfly larvae than to newts irrespective of their acute dangerousness, probably because the former is inherently a more voracious predator of anuran larvae. Further, large tadpoles only responded to the gape-limited newts when these appeared to be feeding heavily on tadpoles. We also observed stronger responses in small tadpoles than in large ones, which was most likely due to size-dependent vulnerability to predators. In summary, these results support the hypothesis that **tadpoles integrate multiple cues about the risk of predation to fine-tune their induced defences while also taking into account their internal state and the resulting vulnerability to the predators in their environment.**

(3) When examining what sources of information anuran larvae use for predator detection besides chemical cues, we observed the largest reduction in *Rana temporaria* tadpole activity when all cues were available. Tadpoles did not respond

to the combination of acoustic and hydraulic cues, but they clearly reduced their activity when only visual cues were available. We did not observe spatial avoidance of predators, but this was presumably due to the small size of experimental containers. Our results provide support for the hypothesis that **besides chemical cues, visual cues can also elicit antipredator behaviour in tadpoles, at least when the predator is up-close, while acoustic and hydraulic cues may be of little importance for predator detection**.

(4) When scrutinizing how important chemical cues of various origins are for the adjustment of anti-predator defences, we first proposed a precise and consistent binomial nomenclature indicating both the timing/mechanism of cue release (stress-, attack-, capture-, digestion- or continually released cues) and the origin of cues (prey-borne or predator-borne cues) to lessen the confusion stemming from inconsistently used terminology. The results of our study conducted on *R. temporaria* tadpoles supported previous observations that the phylogenetic relatedness between prey falling victim to predators and the prey sensing the predation event has a strong influence on antipredator responses. Most importantly, however, our study delivered the most compelling and detailed empirical evidence that **continually released predator-borne cues and digestion-released prey-borne cues are used by larvae of anuran amphibians to fine-tune their induced defences**.

(5) When assessing to what extent prey are capable of detecting invasive alien predators, our results indicated that *R. dalmatina* larvae responded to the simulated presence of a native and a long-established invasive perciform, but not to a recently arrived invader or individuals of an allopatric species. Interestingly, stimulus water transferred from any of the siluriforms did not induce behavioural responses in tadpoles, even not when predators were previously fed with conspecifics. Also, tadpoles did not respond to the simulated appearance of non-dangerous cypriniforms. Finally, tadpoles originating from fish-infested floodplain populations exhibited lower baseline activity and responded more intensely than their conspecifics originating from isolated hill-ponds. We concluded that **anuran larvae may be highly vulnerable to recently arrived invasive predatory fishes because of their inability to recognize them as dangerous, but, presumably due to intense selection and the existence of sufficient genetic variability, the ability to recognize these predators can evolve in less than 30 generations**.

(6) When summarizing what was documented in the literature about inducible chemical defences in animals, we proposed that when inducible chemical defences are detected, their study would provide unique opportunities for scrutinizing life-history trade-offs, especially if toxin synthesis of animals proves accessible to direct biochemical manipulation. We put forward the hypothesis that research on the inducibility of chemical defence would deliver a deeper understanding of interspecific interactions and life-histories of toxin-producing animals, and, ultimately, such studies would help uncover the evolutionary processes leading to the appearance and maintenance of plasticity in natural populations. Most importantly, we concluded that **phenotypic plasticity in chemical defences is very likely to occur much more frequently in many taxa of the animal kingdom than it is generally thought**.

(7) When testing for inducible changes in the chemical defence of larval *B. bufo* upon exposure to predators, our results provided clear evidence that tadpoles responded to chemical cues of predation risk by producing more bufadienolide compounds and larger total quantities of bufadienolides as compared to predator-naive conspecifics. Further, the intensities of induced responses mirrored predator dangerousness because they were strongest in the fish treatment, weakest in the newt treatment and intermediate in the dragonfly treatment. We did not perform predation trials to assess the survival benefits delivered by the observed changes in toxin content, and also did not scrutinize fitness costs of increased toxin production, so that we cannot conclude on the adaptive value of the observed antipredator responses. Nonetheless, this study provided the **first clear evidence for predator-induced changes in the chemical defence of a vertebrate**.

(8) When examining the possibility that competitors may also induce changes in the chemical defence of larval *B. bufo*, we showed that tadpoles contained larger quantities of bufadienolides at higher tadpole densities, where the density of heterospecifics was not more important than that of conspecifics. Also, mortality, growth and development rate of *R. dalmatina* tadpoles did not vary according to the density of *B. bufo* tadpoles. This negative result regarding allelopathy indicated that the observed changes in toxin synthesis may not serve to enhance the relative competitive ability of *B. bufo* tadpoles, but rather to lower risks arising from high densities of competitors: increased risks of cannibalism and of disease transmission. This study, therefore, delivered the **first proof that free-moving animals can adjust their toxin production also to the presence and density of competitors**.

(9) When investigating how inducible chemical defences are adjusted to the simultaneous presence of predators and high competitor densities, our results repeated the previous main findings that perceived predation risk and high conspecific density can both induce an increase in the toxin production of *B. bufo* tadpoles, at least when differences in body size are accounted for. However, at high conspecific densities the effect of predation risk was not significant, while the anti-predator response did not differ significantly between low and high tadpole densities. It, thus, appears that **tadpoles can adjust their toxin production to predation risk and conspecific density simultaneously, where at high tadpole densities the presence of predators does not induce an additional enhancement of chemical defences**.

(10) When assessing whether the synthesis of defensive chemicals in the skin is enhanced or suppressed upon exposure to obligate pathogens, the genetic analyses showed that the prevalence of the pathogenic fungus *Batrachochytrium dendrobatidis* and the infection intensities were very low in *R. dalmatina* tadpoles and froglets, whereas prevalence was high in *B. bufo* tadpoles and froglets, accompanied by high infection intensities, especially after metamorphosis. Exposure to the chytrid fungus did not induce an increase in the production of chemical defences in tadpoles of either species. However, metamorphosed individuals of both species that had been exposed to the fungus during the larval life-stage contained lower amounts of defensive chemicals as compared to non-exposed control individuals. Thus, we found **no evidence for pathogen-induced enhancement of chemical defences, but rather detected long-term negative effects of pathogen exposure, which may compromise defences against microbes, predators and competitors**.

4.2. Comprehensive discussion

The papers presented here provided novel insights on several aspects of predator detection and the subsequent appearance of induced defences in anuran larvae. We delivered clear evidence for the importance of continually released predator-borne cues and digestion-released prey-borne cues in shaping tadpoles' antipredator responses (Hettyey et al. 2015). We also showed that tadpoles integrate the information content delivered by various types of chemical cues while also taking into account their own body size to adjust their inducible defences (Hettyey et al. 2010). In addition to chemical cues, we documented that visual signals can also play a crucial role in eliciting antipredator behaviour, especially when predators are in close proximity to tadpoles, while acoustic and hydraulic cues appeared to have little impact on the tadpoles' defensive behaviour (Hettyey et al. 2012; also see Szabo et al. 2021; Fouilloux et al. 2023; Gazzola et al. 2022). The fine-tuning of antipredator responses based on external cues of various origins and types, as well as on the tadpoles' internal state draws attention to the complexity of the decision-tree underlying the phenotypic materialization of inducible defences. This complexity necessitates a precise and well-defined terminology if we are to avoid confusion. We therefore proposed a clear binomial nomenclature for categorizing chemical cues used in predator detection (Hettyey et al. 2015). This terminology states the timing of cue release (stress-, attack-, capture-, digestion- or continually-released cues) as well as the origin of cues (prey-borne vs. predator-borne cues). By facilitating the avoidance of ambiguities, this terminology also improves the among-study comparability of results.

Our results also supported some general concepts about how prey adjust their induced defenses to the presence of predators. Chemical cues again proved to be of fundamental importance to anuran larvae when it comes to predator recognition (all papers involving predators, but especially Hettyey et al. 2012, 2015). Also, prey appeared to use predator-borne cues to adjust the type of their antipredator response, and prey-borne cues to modulate its intensity (Hettyey et al. 2010). Different types of cues in isolation were capable of inducing responses in tadpoles, but only the simultaneous presence of various cues triggered the full magnitude of induced defences (Hettyey et al. 2015). Finally, the phylogenetic distance between the prey attacked by predators and the individuals eavesdropping on the predation event appeared to be decisively important for the strength of inducible defences (Hettyey et al. 2015; also see Ramamonjisoa and Mori 2019; Gazzola et al. 2025). These observations further strengthen the view that tadpoles evolved to be able to sense and use various types of information on the dangers present in their environment and to very carefully adjust their antipredatory responses.

One general observation recurring in several papers was that the strength and type of defensive responses can closely mirror the level of threat posed by predators. For example, tadpoles exposed to predators that are more dangerous exhibited stronger morphological, behavioural, and chemical defences (Hettyey et al. 2010, 2011, 2019). Also, large tadpoles that were less vulnerable to predation showed weaker behavioural responses to predators (Hettyey et al. 2010). More intense antipredator responses appeared to have some immediate costs in terms of lowered survival rates and slowed development in the presence of high-risk predators, but we did not observe clear long-term costs reaching beyond metamorphosis (Hettyey et al. 2011). Interestingly, while induced phenotypes provided a survival advantage, more extreme phenotypes did not necessarily offer additional protection, and the advantage of these phenotypes diminished as tadpoles grew larger (Hettyey et al. 2011).

One of the most striking results we obtained regards the tadpoles' difficulty in detecting newly introduced invasive predators. While tadpoles responded well to native and long-established invasive percids, they failed to recognize more recently arrived invaders as a threat (Hettyey et al. 2016; also see Méndez-Méndez et al. 2023;Wang et al. 2024). This inability to

detect new invasive species suggests a high vulnerability to these predators. However, based on our results and on those of the few existing similar studies we hypothesize that with intense natural selection and sufficient genetic variability, tadpoles could evolve the ability to recognize these invasive predators within as few as 30 generations (also see Nunes et al. 2013, 2014a, b). This highlights not only the potential risks posed by biological invasions but also the capacity for rapid evolutionary adaptation in response to new environmental challenges.

In order to maximize our ability to tell if tadpoles were capable of detecting the simulated threat posed by predators, in the first five papers we measured traits as our response variables that were well-known to be phenotypically plastic and to form parts of inducible defences. Our results on such induced changes in behaviour and morphology mostly aligned to our predictions and to what can be found in the relevant literature. However, beyond assessing antipredator responses in these ecologically important characteristics serving as gold standards when it comes to the study of inducible defences, in the second half of the presented studies we explored phenotypic plasticity in chemical defences of anuran larvae, a phenomenon that had previously remained practically unstudied.

In a review, we summarized what was known about inducible chemical defences in animals (Hettyey et al. 2014). We identified three contexts in which inducible chemical defences may play a crucial role: against predators, against parasites and pathogens, and in competitive interactions. In vertebrates, we found no study providing clear evidence of induced changes in toxin production in response to predators or competitors, and only a very few had reported it in response to pathogens. We suggested that by expanding research into different taxa and testing the fitness benefits of these defences, we could refine our understanding of the evolutionary trade-offs associated with plasticity. Such research would also have the potential to provide insights into broader ecological and evolutionary processes and may ultimately have applications in medicine, pharmacology, and agriculture. We concluded that inducible chemical defences represented a critical, yet underappreciated, component of phenotypic plasticity that deserved greater attention in research.

In the experimental studies, we provided the first compelling evidence of predator-induced changes in chemical defences of a vertebrate by showing that *Bufo bufo* tadpoles increased the production and diversity of bufadienolide compounds when exposed to chemical cues from multiple predator species (Hettyey et al. 2019). Notably, the predators that pose the highest risk and are also most sensitive to bufadienolides triggered the strongest responses. In subsequent studies, we expanded the focus to include the effect of competition in addition to predation and found that *B. bufo* tadpoles also exhibited competition-induced plasticity in chemical defences, with higher densities of conspecifics leading to increased bufadienolide production (Bókony et al. 2018; Üveges et al. 2021). This suggests that competition itself acts as a trigger for chemical defence enhancement, potentially as a response to the elevated risk of cannibalism or of increased disease transmission at high densities. However, we found no evidence that bufadienolides function as allelochemicals, as the presence of ranid tadpoles did not induce higher bufadienolide production in *B. bufo*, and the presence of *B. bufo* tadpoles did not inhibit the growth of heterospecific larvae. This indicates that bufadienolides are more likely to play a role in intraspecific rather than interspecific competitive interactions. When exploring pathogen-induced effects on chemical defences, we revealed that exposure to an obligate amphibian pathogen (*B. dendrobatidis*) did not result in significantly increased toxin production in tadpoles, but rather resulted in suppressed chemical defences in metamorphosed individuals (Ujszegi et al. 2017, 2021; Kásler et al. 2022; also see Le Sage et al. 2024). This suggests that infection during the larval stage results in immunosuppression or incurs a cost that manifests in reduced bufadienolide production after metamorphosis, which in turn has the potential to compromise the animals' long-term survival prospects.

It is worth mentioning that, beyond the ones described here, we performed several further studies on the plasticity of chemical defences in *B. bufo* tadpoles. We started off with two correlative investigations (Bókony et al. 2016; Ujszegi et al. 2020), both of which found considerable among-population variation in the toxin content of *B. bufo* tadpoles (for similar studies see Cao et al. 2019; De Meester et al. 2021; Hudson et al. 2021) and a positive correlation with tadpole density, but neither one found a relationship between bufadienolide quantities and predation risk. We also performed two experimental investigations where we tested for predator-induced changes in toxin synthesis, but we observed no effect of simulated predator presence (Üveges et al. 2017, 2019). The question arises, what may have caused these negative results regarding antipredator responses in toxin synthesis, and why one should believe the outcomes of the studies presented here in detail (Papers 7 & 9), which happen to support its existence? It is important to point out that in the first two experimental studies focal animals originated from permanent, fish-inhabited ponds and tadpoles were raised at rather high densities, whereas in the latter experiments we (also) used animals originating from temporary ponds lacking fish and tadpoles were raised alone or (also) at low densities. Consequently, one possible explanation for why tadpoles did not respond to predator exposure with altered toxin synthesis in the two previous experiments (Üveges et al. 2017, 2019) may be that they were locally adapted to permanently high predation risk with toxin production genetically fixed at a high level, leaving little space for plasticity to manifest. However, in Paper 7 we showed that tadpoles from both permanent and temporary ponds did not differ in their toxin production in the absence of predators and showed similar responses to predator cues, refuting the explanation relying on local adaptation to high predation pressure. Another possibility is that in earlier studies we may have sampled populations, which, just by chance, exhibited little plasticity in chemical defence, while in Papers 7 & 9 we happened to use specimens of populations with high plasticity. We cannot refute this hypothesis, as among-population variation in the level of phenotypic plasticity is a well-known phenomenon. However, we think that the best explanation for the discrepancy between the outcomes of the previous experiments and Papers 7 & 9 was delivered by Papers 8 and 9. In Paper 8 we showed that, in alignment to results of the correlative studies (Bókony et al. 2016; Ujszegi et al. 2020), *B. bufo* tadpoles adjusted their toxin synthesis to conspecific density, while in Paper 9 we found that tadpoles developing at high densities do not further increase their toxin production upon sensing the presence of predators. The possible explanations of decreasing antipredator responses with increasing conspecific densities include lower per capita predation risk at high densities, increasing costs of toxin production due to intensifying competition for resources, and the existence of an upper limit to defence expression as shown for behavioural and morphological antipredator responses. Not knowing this yet, we raised tadpoles in the two previous experiments (Üveges et al. 2017, 2019) at high densities (three tadpoles in 1.5 litres and 60 tadpoles in 130 litres, respectively), and observed no predator-induced changes in tadpole toxin content. In contrast, we did document inducible changes in toxin content in Paper 7, where we reared tadpoles individually, and in Paper 9, where we raised 6 or 12 (but not when raising 24!) tadpoles in 40 litres of water. It, thus, appears that the contradiction between the outcomes of our studies was shaped perhaps by chance effects and definitely by differences in tadpole densities.

It is important to note that in the presented studies on plasticity in chemical defences we did not demonstrate fitness benefits of increased toxin production in terms of elevated survival when exposed to free-ranging predators, although this would also be necessary for concluding on the adaptive value of the detected responses (DeWitt and Scheiner 2004). This is all the more an issue because we also demonstrated increased toxin production in toad tadpoles upon exposure to a pesticide (Bókony et al. 2017), raising the possibility that the upregulation of bufadienolide synthesis is a general, undirected stress response. We later refuted the hypothesis that increased toxin production was proximately driven directly by elevated stress hormone

levels (Üveges et al. 2023), but the exact regulatory pathway remains unknown. In a study not detailed here (Üveges et al. 2019) we did attempt to capture survival benefits of elevated bufadienolide synthesis when facing predators. We reared toad tadpoles in the presence of four types of caged predators and subsequently exposed predator-experienced and predator-naïve tadpoles to free-ranging predators. We observed that the two tested vertebrate predators avoided preying on toad tadpoles all together, while at least one of the invertebrate predators consumed more naïve than experienced tadpoles. However, there was no significant difference in the toxin content between predator-experienced and predator-naïve tadpoles, so that the elevated survival of experienced tadpoles could not be attributed to a protective effect of enhanced chemical defences. A truly elegant and convincing approach would be to manipulate the toxin production of tadpoles via biochemical manipulation while not inducing changes in other traits, but this requires the uncovering of expression pathways which remains to be done. Nonetheless, future studies comparing fitness of induced and non-induced phenotypes in different environments will likely prove fruitful and would largely enhance our understanding of the evolutionary emergence and maintenance of inducible chemical defences.

Besides describing changes in toxin production induced by the presence of predators, competitors and pathogens, we also assessed fitness correlates in induced phenotypes outside the inducing environment, thereby fulfilling an important requirement posed by theoretical considerations for a documentation of adaptive plasticity (DeWitt and Scheiner 2004). We could not conclude on the costs of plasticity itself, but, as opposed to results on *Rhinella marina* toads (Blennerhassett et al. 2019), the costs of producing elevated quantities of toxins were non-detectable or weak (Kurali et al. 2016; Üveges et al. 2017; Tóth et al. 2019). Costs of plasticity are generally found to be weak and to surface only under extreme conditions and depending on the context (Steiner 2007; Van Buskirk and Steiner 2009; Auld et al. 2010; Murren et al. 2015). Costs of expressing the induced phenotype, on the other hand, do not necessarily appear in the measured traits, in all environments and simultaneously with the induced defence (Scheiner and Berrigan 1998; Agrawal et al. 1999; Van Buskirk and Saxer 2001). Finally, costs would need to be assessed in terms of net fitness change, which is notoriously difficult to measure, and the surrogate measures taken may or may not provide reliable estimates of fitness-consequences. Consequently, the costs of induced defences have often remained elusive, and detecting them can turn out to be a difficult task (Tollrian and Harvell 1999b; Murren et al. 2015). However, it is important to note that costs may also disappear over evolutionary time, so that not finding a cost does not necessarily mean a contradiction between theory and empirical data (DeWitt et al. 1998).

From a methodological and conceptual point of view, it is interesting to re-visit how well our initial correlative studies on among-population variation in chemical defences (Bókony et al. 2016; Ujszegi et al. 2020) managed to detect relationships between environmental factors and toxin content of tadpoles, which were later demonstrated or refuted to be cause-and-effect relations by the relevant experimental studies (Papers 6-10). The correlative studies indicated a potential importance of tadpole density for toxin production, which we later confirmed in the experimental studies. However, the correlative studies failed to reveal the relationship between predator densities and tadpole toxin content, which was most likely due to relatively high tadpole densities in the sampled ponds. Finally, the correlative studies indicated a relationship between microbiota composition and toxin synthesis, but this was presumably not the result of a cause-and-effect relationship, but was likely caused by a third, non-measured factor influencing both (e.g., permanence of water body). Our studies altogether deliver a beautiful example that the correlative approach is vitally important for uncovering natural patterns and for obtaining a first impression on what may have shaped them, but the scrutiny of experimental studies is clearly necessary for an unambiguous determination of the underlying processes.

Overall, we demonstrated that tadpoles exhibit highly plastic responses to several threats, modulating behavior, morphology, and toxin production. This adaptability likely provides a survival advantage in dynamic and unpredictable natural environments, where tadpoles fine-tune their responses to maximize their chances of evading external threats while navigating the trade-offs between immediate and long-term fitness benefits and costs. Moreover, our findings stress that if we want to understand inducible defences, we cannot be content with studying the effect of just one environmental factor, but rather have to simultaneously consider multiple potentially important ones. Finding costs and demonstrating benefits of predator-induced changes in morphological, behavioural, and especially chemical defences remains a promising avenue that will shed light on the evolutionary appearance and maintenance of inducible defences. Such studies on adaptive plasticity continue to have important repercussions for evolutionary biology, chemical ecology, behavioural ecology and conservation biology, but, especially those on inducible chemical defences of animals, may also provide new impulses to agriculture, medicine, and pharmacology. Most importantly, however, expanding the knowledge regarding inducible defences contributes to our basic understanding of how animals cope with their environment – a question that has fascinated mankind for thousands of years, and which has been a real privilege to study.

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7. Papers forming the core of the thesis in their order of appearance

1) Hettyey A, Vincze K, Zsarnóczai S, Hoi H, Laurila A (2011): Costs and benefits of defences induced by predators differing in dangerousness.
Journal of Evolutionary Biology, 24: 1007–1019. DOI: 10.1111/j.1420-9101.2011.02233.x
JIF_{2011} = 3.28, D1

2) Hettyey A, Zsarnóczai S, Vincze K, Hoi H, Laurila A (2010): Interactions between the information content of different chemical cues affect induced defences in tadpoles.
Oikos, 119: 1814–1822. DOI: 10.1111/j.1600-0706.2010.18563.x
JIF_{2010} = 3.39, D1

3) Hettyey A, Rölli F, Thürlimann N, Zürcher A-C, Van Buskirk J (2012): Visual cues contribute to predator detection in anuran larvae.
Biological Journal of the Linnean Society, 106: 820–827. DOI: 10.1111/j.1095-8312.2012.01923.x
JIF_{2012} = 2.41, Q1

4) Hettyey A, Tóth Z, Thonhauser KE, Frommen JG, Penn DJ, Van Buskirk J (2015): The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles.
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8. Further publications related to the thesis

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