

RESEARCH ARTICLE

Contextual behavioural plasticity in Italian agile frog (*Rana latastei*) tadpoles exposed to native and alien predator cues

Andrea Gazzola¹, Alessandro Balestrieri¹, Giovanni Scribano¹, Andrea Fontana² and Daniele Pellitteri-Rosa^{1,*}

ABSTRACT

Predation is a strong driver for the evolution of prey behaviour. To properly assess the actual risk of predation, anuran tadpoles mostly rely on water-borne chemical cues, and their ability to evaluate environmental information is even more crucial when potential predators consist of unknown alien species. Behavioural plasticity – that is, the capacity to express changes in behaviour in response to different environmental stimuli – is crucial to cope with predation risk. We explored the defensive behaviour of Italian agile frog (*Rana latastei*) tadpoles when exposed to the chemical cues of two predator species, one native (dragonfly larvae) and one alien (red swamp crayfish). Firstly, we observed whether a plastic life history trait (i.e. hatching time) might be affected by native predatory cues. Secondly, we recorded a suite of behavioural responses (activity level, lateralization and sinuosity) to each cue. For assessing lateralization and sinuosity, we developed a C++ code for the automatic analysis of digitally recorded tadpole tracks. Hatching time seemed not to be affected by the potential risk of predation, while both predator species and diet affected tadpoles' defensive behaviour. Tadpoles responded to a predator threat by two main defensive strategies: freezing and 'zig-zagging'. While the first behaviour had previously been reported, the analysis of individual trajectories indicated that tadpoles can also increase path complexity, probably to prevent predators from anticipating their location. We also recorded a decrease in lateralization intensity, which suggests that under predation risk, tadpoles tend to scrutinize the surrounding environment equally on both sides.

KEY WORDS: Alien species, Chemical cues, Defensive behaviour, Lateralization, Sinuosity, Tadpoles

INTRODUCTION

Behavioural plasticity – the ability to detect and respond to environmental signals – is a necessary requirement for survival and reproduction in all organisms, and in the last three decades it has come to include virtually all behavioural traits that may show some kind of variation in response to environmental conditions (West-Eberhard, 1989; DeWitt and Scheiner, 2004). 'Contextual plasticity', as distinct from 'developmental plasticity', indicates an individual's response to a stimulus to which it has been exposed immediately before (Stamps, 2016).

A change in behaviour is the promptest reaction that animals can adopt to respond to external changes and can potentially improve individual fitness and enhance a species' long-term survival by preventing drastic population declines (Wong and Candolin, 2015). However, irrespective of its contribution to fitness, plasticity stands as an important benchmark to explore how organisms respond to environmental changes at the individual, population and community level (Stearns, 1989; Ghalambor et al., 2007).

Currently, human activity is the main agent of environmental change. Human-induced rapid environmental changes (HIREC; Sih et al., 2011) are considered the greatest threat to biodiversity (Tilman et al., 1994; Pimm and Raven, 2000), forcing species to face conditions never encountered previously (Wong and Candolin, 2015; Sih et al., 2016). Behavioural plasticity can play a major role in allowing species to cope with anthropogenic environmental changes: variation in each species' ability to express adaptive behaviours may explain why some species survive or even benefit from the new conditions while others decline, sometimes irreversibly (Sih et al., 2011; Tuomainen and Candolin, 2011; Van Buskirk, 2012).

The invasion of non-native species is a major contribution to HIREC, and can strongly affect the distribution, abundance, use of resources and habitats, reproduction, interspecific interactions and evolution of many native species (Strauss et al., 2006). Currently, freshwater ecosystems are amongst the most invaded ecosystems and are particularly vulnerable to introduced predators, which are considered to be one of the most important causes of biodiversity loss around the globe (Vitousek et al., 1997; Cox and Lima, 2006). Introduced predators can drive amphibian populations to extinction (Bradford et al., 1994; Gamradt and Kats, 1996; Matthews et al., 2001), amphibian eggs and larvae being particularly vulnerable to alien aquatic predators (Kats and Ferrer, 2003). Invasive predators are the major cause of the decline of rare or endemic prey species (Dick and Platvoet, 2000; Fukasawa et al., 2013), and predicting the impact of non-native predators is essential for their conservation, especially in human-altered environments (Hobbs and Huenneke, 1992; Chytrý et al., 2012).

In the intensively cultivated and urbanized lowlands of northern Italy, the red swamp crayfish (*Procambarus clarkii*) is by far the most widespread of four alien crayfish species (Morpurgo et al., 2010). Since the early 1990s, it has probably been introduced several times in ponds and streams (Gherardi, 2006), from which it has rapidly spread out over the extensive network of canals that crosses the whole lower catchment of the River Po (Gherardi et al., 1999; Fea et al., 2006).

This highly invasive (Gherardi, 2006; Aquiloni et al., 2008), and voracious predator preys on a wide range of freshwater species, including anuran larvae (Gherardi et al., 2001; Cruz et al., 2006; Ficetola et al., 2011), and can have dramatic impacts on freshwater fauna and alter aquatic ecosystems through trophic cascades (Souty-Grosset et al., 2016). Its introduction has been related to the local

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decline or extinction of several amphibian species (Cruz et al., 2008), including the Italian agile frog (*Rana latastei*), an endemic threatened species of northern Italy (Ficetola et al., 2012).

The Italian agile frog is a monotypical, small brown frog which occurs in the floodplains of northern Italy and small areas of the adjacent Swiss canton Ticino (Grossenbacher, 1997), western Slovenia and north-western Croatia (Burlin and Dolce, 1986; Barbieri and Mazzotti, 2006). *Rana latastei* is threatened by multiple factors, including loss of habitat due to agricultural intensification, increased isolation of populations and loss of genetic diversity (Ficetola and De Bernardi, 2004; Pearman and Garner, 2005; Ficetola et al., 2007; Canova and Balestrieri, 2018). It is considered globally vulnerable by the IUCN (<http://www.iucn.it/scheda.php?id=-1527036578>), and the Action Plan for its conservation in Europe includes the eradication of alien crayfish as an urgent priority action (Edgar and Bird, 2006).

In the current study, we tested the defensive behaviour of Italian agile frog tadpoles. Firstly, we investigated whether the presence of water-borne kairomones of a native predator (dragonfly larvae) alters the timing of hatching. The ability of anurans to modify the timing of hatching may represent an effective way to cope with the upcoming risk of falling prey both before and after hatching (Warkentin, 2011). For example, the presence of predators preying on hatchlings may lengthen the permanence time inside the protective jelly. Alternatively, the occurrence of predators that prefer feeding on eggs may induce an early exit from the egg mass (Warkentin, 2011). External conditions set the stage for a potential trade-off; that is, tadpoles need to make the most advantageous choice, relying on available information in the surrounding environment (Warkentin, 1995; Ireland et al., 2007).

A few days after tadpole hatching, we performed a second experiment to investigate how tadpoles alter their behaviour when briefly exposed to water-borne cues coming from either a native or alien predator, and how these changes are affected by predator's diet, i.e. the chemical cues actively or passively released by injured or preyed conspecifics. With this aim, we explored tadpole activity level (time spent active, time frozen, total distance covered) when exposed to different types of predatory cues (contextual behavioural plasticity). We included a further behavioural analysis by investigating lateralization (tadpole preference for a rotational direction) and sinuosity (the tortuosity of an animal's path; Benhamou, 2004).

By assessing behavioural and life history responses from different breeding sites, we explored the effect of genotype by environment interactions on the expression of defensive behaviour in the presence of different sources of information (i.e. predator species and diet). Consistently with current knowledge, we expected the strongest defensive responses from tadpoles exposed to both conspecific alarm cues and native predator odour, and the weakest towards fasted, alien predator cues.

MATERIALS AND METHODS

Sample collection

During March 2019, we collected 18 freshly laid Italian agile frog (*Rana latastei* Boulenger 1879) clutches from three different breeding sites (six clutches from each site) located in the Lombardy region (northern Italy). Permits were obtained from the Italian Ministry of Environment, Land and Sea (0006075–23/03/2018–PNM).

The first site, known as Sorgenti della Muzzetta (MZ: 45°27'N, 9°22'E), is a large pond located a few kilometres east of Milan, in a small protected natural area surrounded by agricultural land;

maximum water depth was >1 m, with high turbidity and aquatic vegetation cover. Bosco Castagnolo (BC: 45°15'N, 8°58'E) is a humid area, consisting of several small ponds connected by narrow canals, included in the riparian forest of the protected valley of the River Ticino. The main waterbody is an elongated elliptical pond fed by groundwater, with shallow water (80–100 cm) and low aquatic vegetation cover (<10%). Bosco Negri (BN: 45°10'N, 9°8'E) is a natural protected area close to the city of Pavia, consisting of a residual wetland forest intermingled with crops and rural and suburban areas. It includes some small ponds with a water depth <1 m and rich in submerged branches and leaves. In each site, egg clutches were collected along the banks of breeding ponds. During sampling, several juveniles and adults of *P. clarkii* were recorded in BN, both in the sampling pond and in nearby canals,

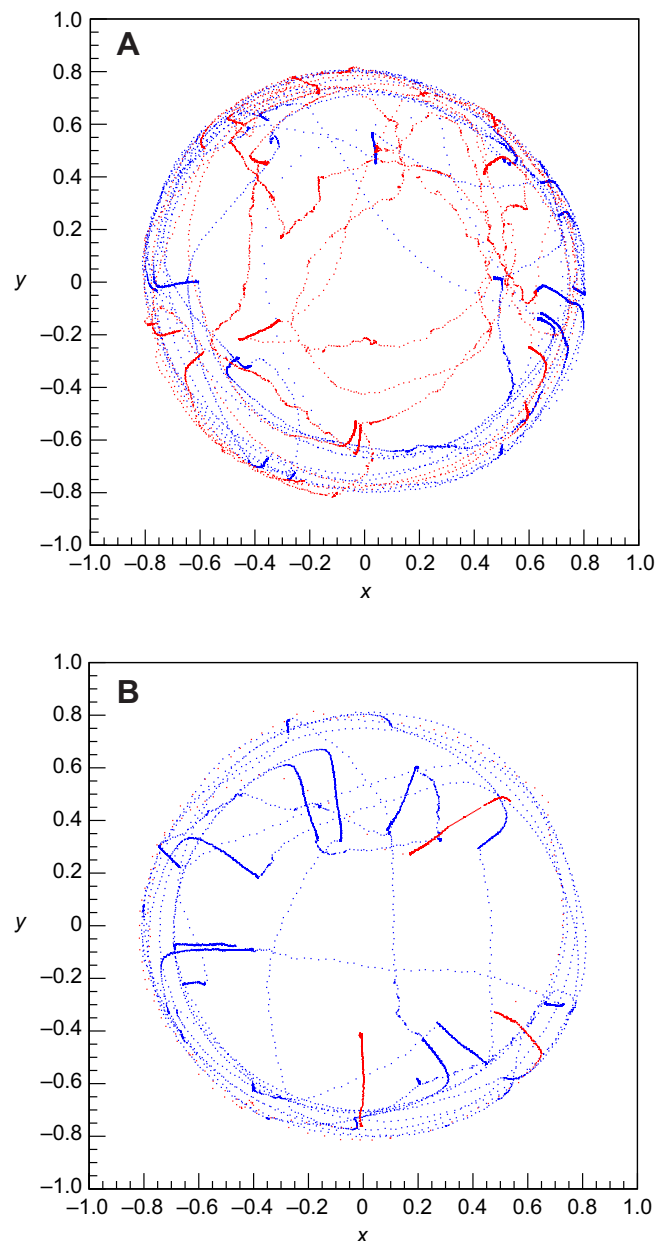


Fig. 1. *Rana latastei* tadpole responses to control and fed odonate cues. (A) Response to control cue, showing similar pre-stimulus (blue) and post-stimulus (red) mobility. (B) Response to fed odonate cue, showing lower mobility in the post-stimulus phase.

while only a few individuals were found in MZ, crayfish being more widespread in adjacent agricultural canals than in the pond where frogs reproduce. Finally, in BC, we never observed any crayfish (see also Gazzola et al., 2018), although *P. clarkii* has been reported to occur in the area since the start of the 21st century (Gherardi et al., 1999; Fea et al., 2006).

Egg masses were immediately transported into the laboratory and individually kept in 11 litre tanks filled with well water. A subsample of 10 eggs from each clutch was used to determine the Gosner level of development (mean \pm s.d.: MZ 9.7 \pm 0.3, BN 7.4 \pm 0.5, BC 9.7 \pm 0.3; Gosner, 1960). After hatching (one BC clutch did not hatch), tadpoles were kept in 50 l tanks in an unheated room, under natural light conditions, and fed *ad libitum* with rabbit chow. Throughout the study period, mean water temperature ranged between 15 and 17°C. Water was partially changed (ca. 50%) every 2 days.

Using dip-nets, 10 adult red swamp crayfish and 10 late instar dragonfly larvae (*Anax imperator*) were collected from a small canal near Pavia and an artificial pond located inside the botanic garden of the city, respectively. All predators were transferred to the laboratory and kept individually in plastic tubs containing, respectively, 0.5 and 2.0 l of aged tap water.

Preparation of odour cues

To assess predation risk, amphibian larvae generally rely on water-borne chemical cues (Kats and Dill, 1998). The chemicals to which prey respond may be predator-specific odours, cues released by conspecifics or, more frequently, a combination of the two (Chivers and Smith, 1998; Fraker, 2009; Schoeppner and Relyea, 2009; Hettyey et al., 2010). Several studies have shown that fed predators commonly elicit stronger antipredator defences than starved predators (Stirling, 1995; Ślusarczyk, 1999; Petranka and Hayes, 1998; Van Buskirk and Arioli, 2002; Schoeppner and Relyea, 2005, 2009). As a predator may become chemically 'labelled' by its diet via learning processes, recognition of a novel predator can be facilitated by its association with conspecific cues (reviewed in Ferrari et al., 2010).

To obtain the odour stimuli of fed predators, dragonfly larvae and crayfish were fed every day at 13:30 h with Italian agile frog tadpoles (total mass 100–150 mg) from an early-laid clutch. Prey was always consumed within an hour and 100 ml of water were collected from each predator tub at 14:30 h. Aliquots from the same treatment were poured into the same container and 50 ml of the resulting mixture was used as an odour stimulus. Each time, predator tubs were refilled to keep the water volume constant. The same procedure was used to obtain chemical cues from fasted predators.

For both experiments, the final concentration of the odour stimulus was consistent with previous studies (e.g. Gomez-Mestre and Díaz-Paniagua, 2011; Gazzola et al., 2015, 2018). Cues were collected the same day as the trials. As predator cues have been observed to still trigger strong behavioural responses after 36–48 h of ageing (Peacor, 2006; Van Buskirk et al., 2014), we were confident in their effectiveness in stimulating behavioural responses.

Effect of predator cues on hatching time

The first experiment was performed to assess the influence of the risk of predation by dragonfly larvae on the hatching time of frog embryos. From the day of collection, two subsamples (50 eggs each) were taken from each clutch and placed into separated plastic containers (30 \times 20 \times 20 cm), filled with 6 l of aged tap water ($n=36$, 18 for each treatment). Embryos were then randomly exposed to two different odour treatments, one for each egg clutch subsample: (i) 50 ml of well water (control group), (ii) 50 ml of tadpole-fed dragonfly cue. Treatments were provided daily until the first egg hatched. Time of hatching for each experimental container was defined as the Julian date when 50% of tadpoles were detached from the jelly and 5 cm away from the egg mass (Ireland et al., 2007; Gazzola et al., 2015), and was recorded, as precisely as possible, by checking all containers 3 times per day (07:00 h, 13:00 h, 21:00 h). Throughout the experiment, water temperature ranged between 10 and 15°C, and random daily measures of temperature from different containers differed $\leq 0.7^\circ\text{C}$.

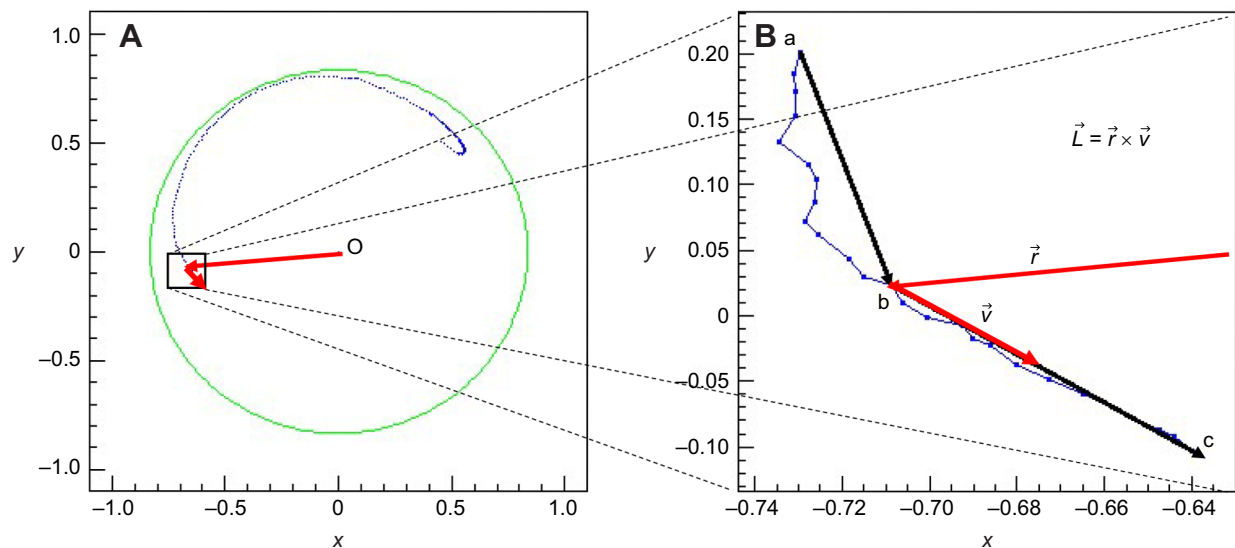


Fig. 2. Assessment of tadpole lateralization using the angular momentum of their motion. (A) Snapshot of the track reconstruction code (green, cup; blue, tadpole track). Red vectors represent tadpole position and velocity. (B) Expanded view of the boxed area in A, representing a 1 s time frame with 25 sampled tadpole positions on the path between a and c. The mid-point b is taken as a reference to evaluate tadpole position r and velocity v and calculate the angular momentum L as a vectorial quantity: being the motion planar, L points upwards or downwards depending on the motion direction and defines whether the motion is anti-clockwise or clockwise.

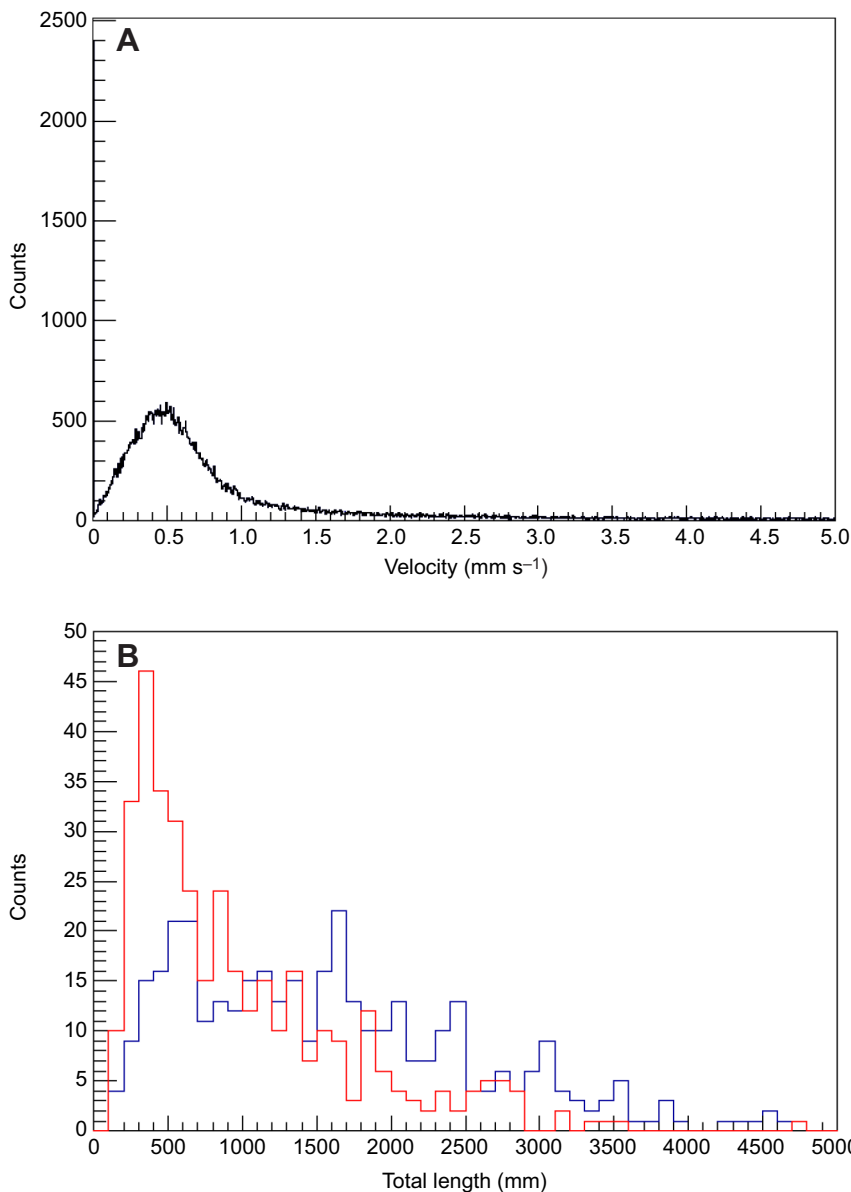


Fig. 3. Velocity and track length distribution. (A) Post-stimulus velocity distribution for one individual. (B) Track length distribution for all tadpoles (blue, pre-stimulus; red, post-stimulus).

Tadpole behavioural responses to predator cues

After hatching, when tadpoles had reached Gosner developmental stage 26–28, we recorded tadpole behavioural responses to a suite of different signals: fasted dragonfly cue, tadpole-fed dragonfly cue, fasted crayfish cue, tadpole-fed crayfish cue, control (tap water). Five predator specimens were used to obtain each type of predator chemical cue (for a total of 20 predators). To assess the activity of the larvae before and after cue infusion, 10 min individual trials were conducted, testing tadpoles that had not previously been exposed to predator cues (at least after clutch collection). Tadpoles were put into white, opaque, circular cups (12 cm in diameter) filled with 200 ml of aged tap water, and left to acclimate for 15 min. The trials consisted of a 5 min pre-stimulus recording period (i.e. before cue infusion), and a 5 min post-stimulus recording period (after cue infusion). To minimize disturbance, the odour stimulus (2 ml) was injected slowly (ca. 30 s) by a 5 ml syringe on one side of the cup.

Tadpoles were video recorded over the whole trial by a Canon Legria digital video camera. Each tadpole was tested once (375 tadpoles in total, 125 per site, 25 for each cue–site combination). All video clips were analysed using a source executable software for

image-based tracking (ToxTrac; Rodriguez et al., 2018), which tracks the position of the centre of the animal's detected shape.

Statistical analyses

Hatching time was explored by linear mixed models (LMMs), with the total time embryos took to hatch as the response variable. Models included embryonic treatment (presence or absence of predator odour) and site as fixed factors, and developmental stage at collection as a covariate; clutch identity, nested within the site of origin, was included as a random effect (intercepts varying among sites and among clutches within sites). The final model, obtained after AIC exploration, included treatment×stage at collection and treatment×site interactions.

Behavioural responses were based on three variables provided by the tracking software: mobility rate, time frozen and total distance. Mobility rate was calculated as the rate of instant speed (v) above a certain threshold ($v > 1 \text{ mm s}^{-1}$). Total time frozen was calculated as the total time the animal remained still during the recording period, while total distance was measured as the total length of the trajectory covered by the animal during the trial.

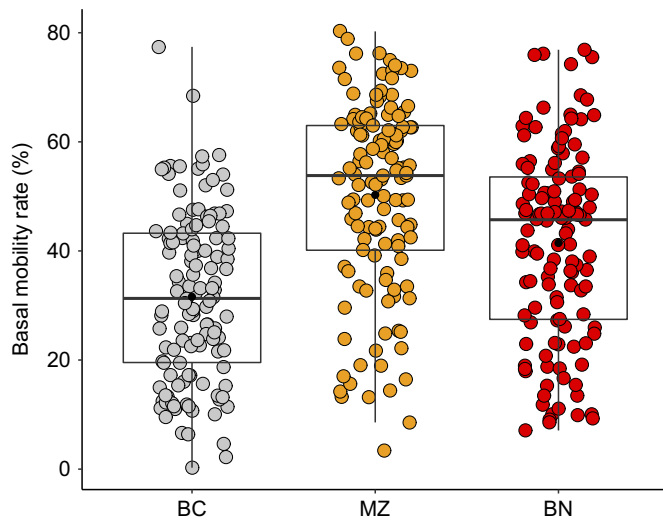


Fig. 4. Mean (\pm s.e.m.) basal mobility rate error for each breeding site. BC, Bosco Castagnolo; MZ, Sorgenti della Muzzetta; BN, Bosco Negri. Box plots indicate median values (horizontal line), upper and lower quartiles (box) and 1.5 \times interquartile range (whiskers).

As tadpole activity was observed both before (i.e. basal mobility rate) and after exposure to cues, we could assess individual behavioural reaction norms (Dingemanse et al., 2010; Stein and Bell, 2019) expressed by each tadpole group in response to each chemical stimulus. In addition, by analysing the trajectory of each individual, we assessed both sinuosity and lateralization in the pre- and post-stimulus phases. To this purpose, we developed a C++ code based on ROOT (Brun and Rademakers, 1997) for the automatic analysis of tadpole tracks recorded by the digital video camera (Fig. 1).

As testing cups were placed in different positions with respect to the video camera, raw coordinates were first normalized. To this purpose, the centre (X_c , Y_c) and maximum and minimum values of both X and Y were recorded for each cup, and coordinates were normalized to the interval $[-1, 1]$ by means of the following transformations:

$$x = \frac{X - X_c}{R}, \quad (1)$$

$$y = \frac{Y - Y_c}{R}, \quad (2)$$

where R is the cup radius (6 cm). Both x and y are dimensionless. Velocity and acceleration vectors were evaluated numerically, both step by step and as an average for each 1 s frame, for which 25 tadpole locations were recorded.

Sinuosity was defined as the ratio between the curvilinear length (actual tadpole trajectory) and Euclidean distance (straight line)

between the end points of the curve: this dimensionless quantity ranges from 1 to ∞ . Sinuosity was calculated for each time frame (1 s) and averaged over the total observation period (300 s pre-stimulus and 300 s post-stimulus). An index of sinuosity was calculated as: (sinuosity post-stimulus – sinuosity pre-stimulus) / sinuosity pre-stimulus.

The time spent by each tadpole moving clockwise and anti-clockwise was assessed for each time frame based on the angular momentum, i.e. (i) by identifying the frame mid-point and (ii) by measuring tadpole position (r) and the velocity (v) in the mid-point: as the motion is planar, the z -component of the angular momentum ($\vec{L} = \vec{r} \times \vec{v}$) points up or down depending on the rotation direction and can be used to distinguish between anti-clockwise and clockwise motion (i.e. the sign of L modulus is, respectively, positive or negative; Fig. 2). Two lateralization indices were then calculated following Lucon-Xiccato et al. (2017): $LR = [(\text{clockwise swimming time} - \text{anticlockwise swimming time}) / (\text{clockwise swimming time} + \text{anticlockwise swimming time})] \times 100$; and $LA = |LR|$, which assesses the intensity of lateralization. These procedures were applied to all 375 tadpoles, each recorded for 300 s before and after the stimulus for a total of about 15,000 locations for each tadpole. For both responses, we imposed $v > 0.1 \text{ mm s}^{-1}$ to exclude all frames when tadpoles remained still. Subsequently, after inspecting the velocity distribution (Fig. 3), all tadpoles moving less than a minimum threshold length (377 mm, i.e. the perimeter of the testing cup) over the sampling time (300 s) were removed from the analysis to prevent bias.

Mobility rate, time frozen and total distance were explored by LMMs. We investigated behavioural plasticity by including the effects of chemical predation cue (i.e. environmental effect), site of origin (genetic background) and their interaction (see also Carter et al., 2015) as main predictors in the models for each behavioural response (i.e. mobility rate, total distance, frozen time) after odour infusion. The corresponding behavioural response before the infusion of the odour was included as a covariate and clutch nested within site as a random effect (intercepts varying among sites and among clutches within sites). Basal level of activity, considered to be the mobility rate expressed before cue infusion, was explored with a LMM with site as the main factor (pairwise comparison between sites were tested using Tukey adjustment); paired Wilcoxon signed rank test (V) was used to compare behavioural variables after and before water infusion (controls) between sites. All planned comparisons with the control treatment were obtained from LMMs by the emmeans package in R (<https://github.com/rvnlenth/emmeans>).

As sinuosity and lateralization violated the assumptions of LMMs, these variables were tested by a non-parametric Kruskal–Wallis test, using Mann–Whitney test for *post hoc* comparisons. All statistical analyses were conducted in R version 3.6.0 (<http://www.R-project.org/>).

Table 1. Fixed effects of linear mixed models for tadpole ($n=375$) behavioural responses after cue infusion

Variable	Mobility rate		Total distance		Time frozen		d.f.
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	
Treatment	25.9	<0.001	12.2	<0.001	30.1	<0.001	4
Site	6.11	0.013	5.22	0.021	4.23	0.037	2
Basal mobility rate	53.6	<0.001	144.7	<0.001	72.9	<0.001	1
Treatment \times site	1.13	0.344	1.48	0.161	1.74	0.087	8
Pre-mobility rate \times site	2.53	0.08	0.21	0.816	1.59	0.205	2

Bold indicates significance.

Table 2. Comparison among different chemical cues and control treatment (water) for mobility rate post-cue infusion as a response variable

Contrasts (difference)	BC			MZ			BN		
	Est.	<i>t</i>	<i>P</i>	Est.	<i>t</i>	<i>P</i>	Est.	<i>t</i>	<i>P</i>
Fasted crayfish – control	6.88	0.59	0.90	–5.8	–0.50	0.93	–30.5	–2.55	0.04
Fed crayfish – control	–14.6	–1.24	0.53	–35.3	–3.11	0.007	–53.3	–4.60	<0.001
Fasted odonate – control	–25.8	–2.20	0.10	–47.2	–4.16	<0.001	–66.6	–5.74	<0.001
Fed odonate – control	–38.3	–3.24	0.005	–56.4	–4.97	<0.001	–75.0	–6.46	<0.001

BC, Bosco Castagnolo; MZ, Sorgenti della Muzzetta; BN, Bosco Negri. *P*-value adjustment was performed with Dunnett method for four tests for each population. Bold indicates significance.

RESULTS

Hatching time

The chemical treatment provided during embryonic development did not significantly affect the time of tadpole emergence from the jelly in any breeding site (Table S1), despite all groups showing a weak effect of hatching time when predator cues were injected (Fig. S1). Developmental stage at collection was highly significant ($P=0.002$), with hatching time inversely related to developmental stage, but no significant effect of either treatment×site or treatment×stage interactions was detected (Table S1).

Behavioural responses

Tadpole basal mobility rate differed among breeding sites ($F=38.06$, d.f.=2, $P<0.0001$), with BC and MZ showing the lowest and highest level of activity, respectively (BC: 31.6 ± 1.4 ; MZ: 50.3 ± 1.6 ; BN: 41.5 ± 1.6 ; pairwise comparisons, BN–BC: t -ratio=4.60, $P<0.0001$; BN–MZ: t -ratio=–4.12, $P=0.0001$; BC–MZ: t -ratio=–8.72, $P<0.0001$; Fig. 4).

After infusion of the control cue (water), tadpoles did not significantly modify their level of activity for either BC or BN sites

($V=137$, $P=0.507$ and $V=119$, $P=0.252$, respectively), while MZ tadpoles showed a significant decrease ($V=77$, $P=0.02$). Total distance was not affected by water infusion in all the sites examined, while time frozen revealed a significant increase for MZ ($V=257$, $P=0.01$).

For all variables, after infusion of the cues, the degree of behavioural activity showed significant effects of treatment, site and basal mobility rate (i.e. pre-infusion activity). No significant interaction was detected for treatment×site or basal mobility rate×site (Table 1).

The comparison with control treatments indicated a marked decrease in mobility rate after infusion of the tadpole-fed odonate cue in all groups (Table 2). The fasted odonate cue and tadpole-fed crayfish cue clearly lowered the activity of both BN and MZ tadpoles but had no significant effect on BC tadpoles (Table 2, Fig. 5). The fasted crayfish cue had a weak effect ($P=0.04$) only on BN tadpoles (Table 2).

The examination of behavioural reaction norms showed that more than 80% (range: 84–100% in BC and BN) of tadpoles lowered their mobility rate when exposed to the fed odonate cue. The same pattern

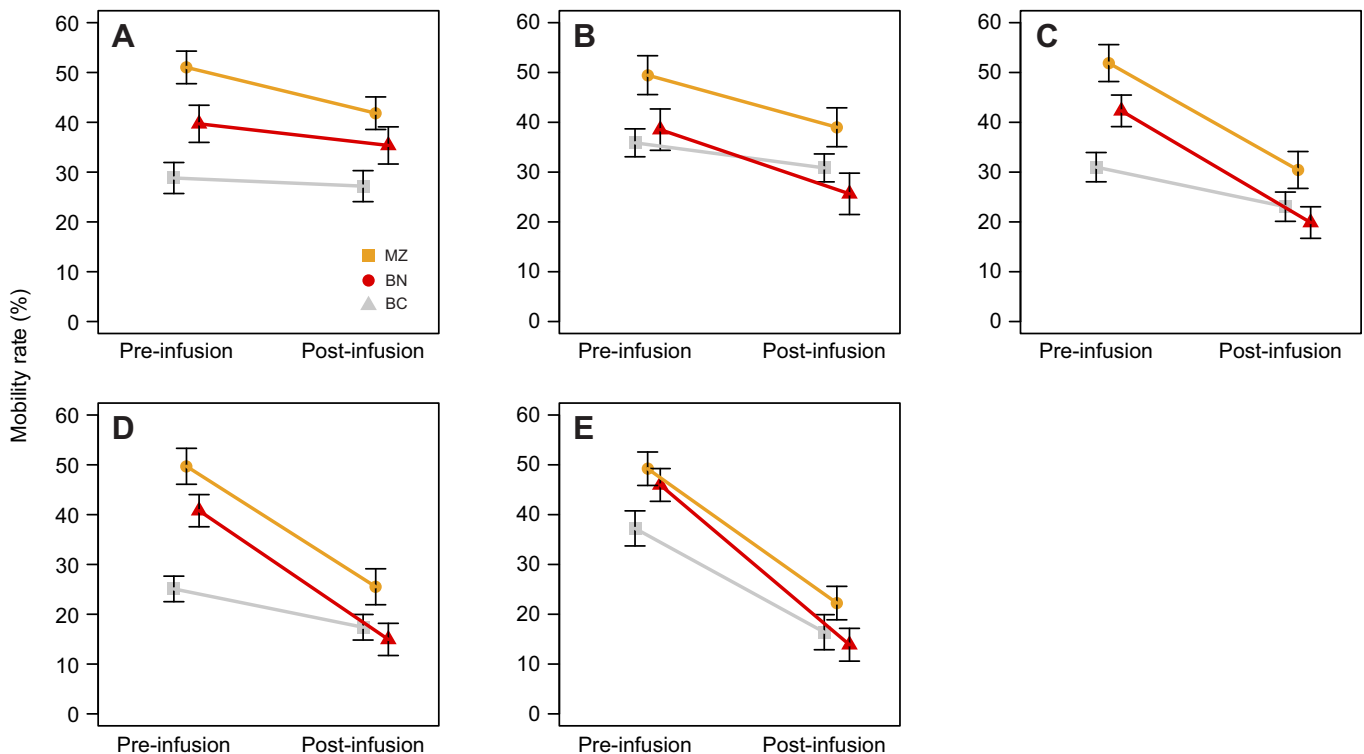


Fig. 5. Mean (\pm s.e.m.) mobility rate for each breeding site. (A) Control cue, (B) fasted crayfish cue, (C) fed crayfish cue, (D) fasted odonate cue and (E) fed odonate cue. Each plot shows the effect of predation risk (pre- and post-infusion of predatory cues) and genetic background (site) on tadpole mobility rate ($n=75$ for each plot).

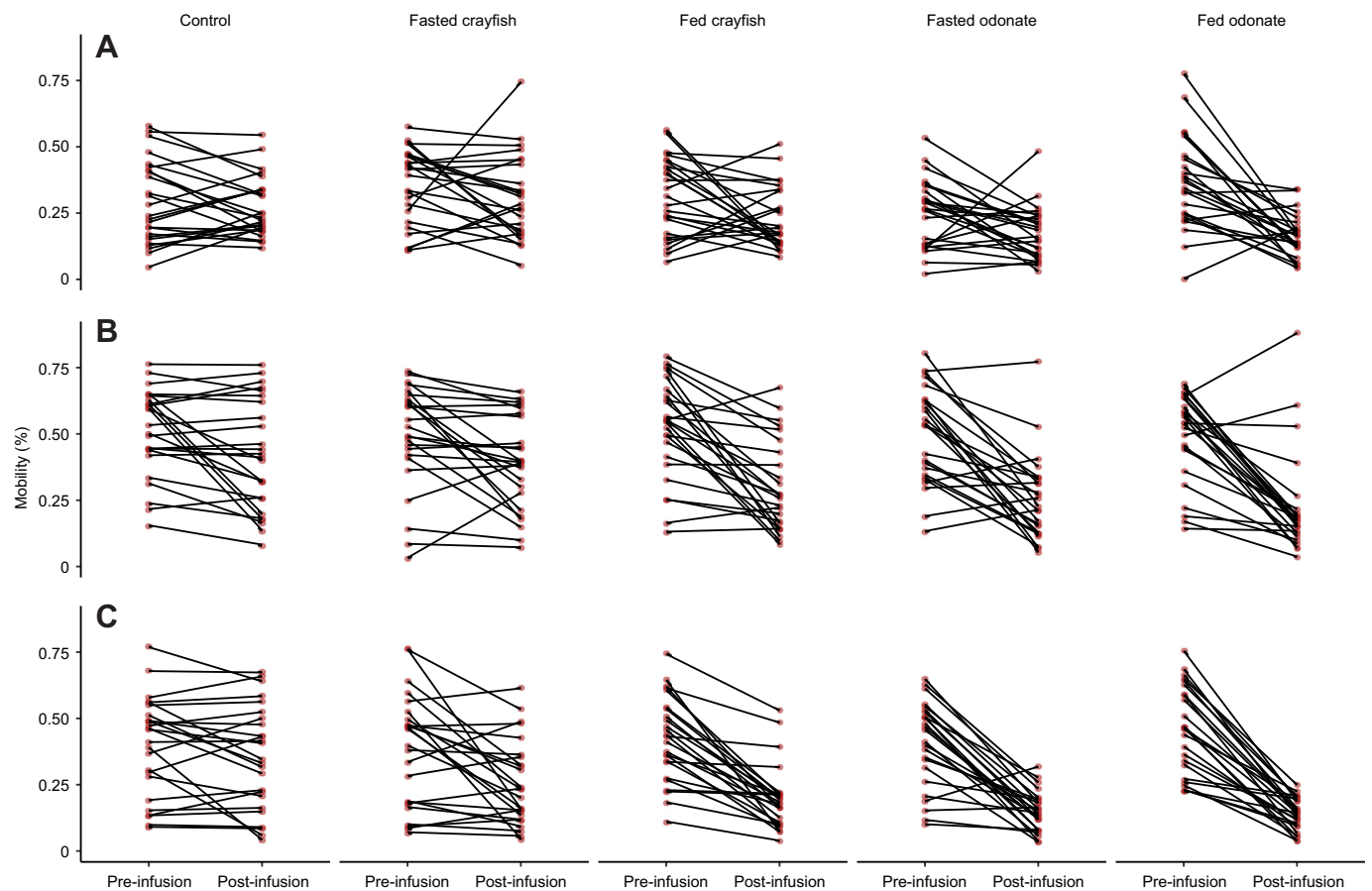


Fig. 6. Individual reaction norms showing mobility rate before and after the infusion of chemical cues. Mobility rate is shown at each site (A, BC; B, MZ; and C, BN) pre- and post-infusion of the five different cues, as indicated ($n=375$; $n=25$ for each subplot).

was recorded for tadpoles exposed to either fed crayfish or fasted odonate cue, except for BC tadpoles, which were less consistent in their responses (64% and 72%, respectively; Fig. 6).

Total distance varied in all sites but BC (Table S2). The highest reduction in path length was obtained using the tadpole-fed odonate cue for both BN and MZ. The fasted odonate cue did not induce any significant reduction in the overall path length of MZ tadpoles, while BN tadpoles strongly decreased the distance covered. The tadpole-fed crayfish cue induced a strong distance reduction for both MZ and BN tadpoles, while exposure to the fasted crayfish cue only weakly affected the response of BN tadpoles (Table S2). Behavioural reaction norms were highly consistent (>80%) for both MZ and BN tadpoles exposed to fed predators (Fig. S2).

Time spent frozen provided a pattern similar to that obtained for mobility rate. Comparisons with controls showed that the tadpole-fed odonate cue induced the strongest response in all sites, with BC showing the weakest difference (Table S3). Both fasted odonate and tadpole-fed crayfish cues significantly increased time spent frozen in both BN and MZ tadpoles, while only the former treatment affected the behaviour of BC tadpoles (Table S3). Individual tadpoles from all sites showed consistent responses to both fed and fasted odonate cues, as well as to the fed crayfish cue, except for BC (Fig. S3).

After cue infusion, the mean length of tadpole paths did not differ among treatments ($\chi^2=9.2$, d.f.=4, $P=0.06$), ranging between 1048 mm (tadpole-fed odonate cue) and 1444 mm (control), while their sinuosity varied among treatments ($\chi^2=9.23$, d.f.=4, $P=0.05$). Overall, sinuosity increased for tadpoles exposed to the

cues of both fasted and fed odonates with respect to controls ($\chi^2=31.5$, d.f.=4, $P<0.001$; Fig. 7). *Post hoc* comparisons showed the same trend for each breeding site ($P<0.03$ for all comparisons), except for MZ, which responded only to the fasted odonate cue. For BN, a nearly significant response ($P=0.06$) was also recorded for tadpoles exposed to the tadpole-fed crayfish cue.

Before cue infusion, neither the directionality ($\chi^2=5.5$, d.f.=2, $P=0.07$) nor the intensity ($\chi^2=4.5$, d.f.=2, $P=0.1$) of lateralization differed among sites, while both fasted and fed odonate cues lowered the intensity of lateralization ($\chi^2=18.1$, d.f.=4, $P<0.001$; Fig. 8). At the site level, BC and BN responded to the tadpole-fed dragonfly larvae cue ($P=0.012$ and 0.002 , respectively), while MZ showed no statistically significant response.

DISCUSSION

Several studies have recently reported a certain degree of behavioural plasticity in the defensive responses of a wide range of anuran species when exposed to water-borne chemical stimuli, usually highlighting the synergistic role played by conspecific alarm cues in enhancing the response to predator kairomones (Schoeppner and Relyea, 2009; Sih et al., 2010; Gazzola et al., 2018). Differently from most *Rana* species, the Italian agile frog has been shown to display strong behavioural responses towards the cues of native dragonfly larvae in the absence of conspecific alarm cues (Scribano et al., 2020). Although this ability may allow Italian agile frogs to efficiently escape predation from native predators, weak sensitivity towards conspecific signals may increase the risk of misleading novel threats, such as introduced potential predators (Sih et al.,

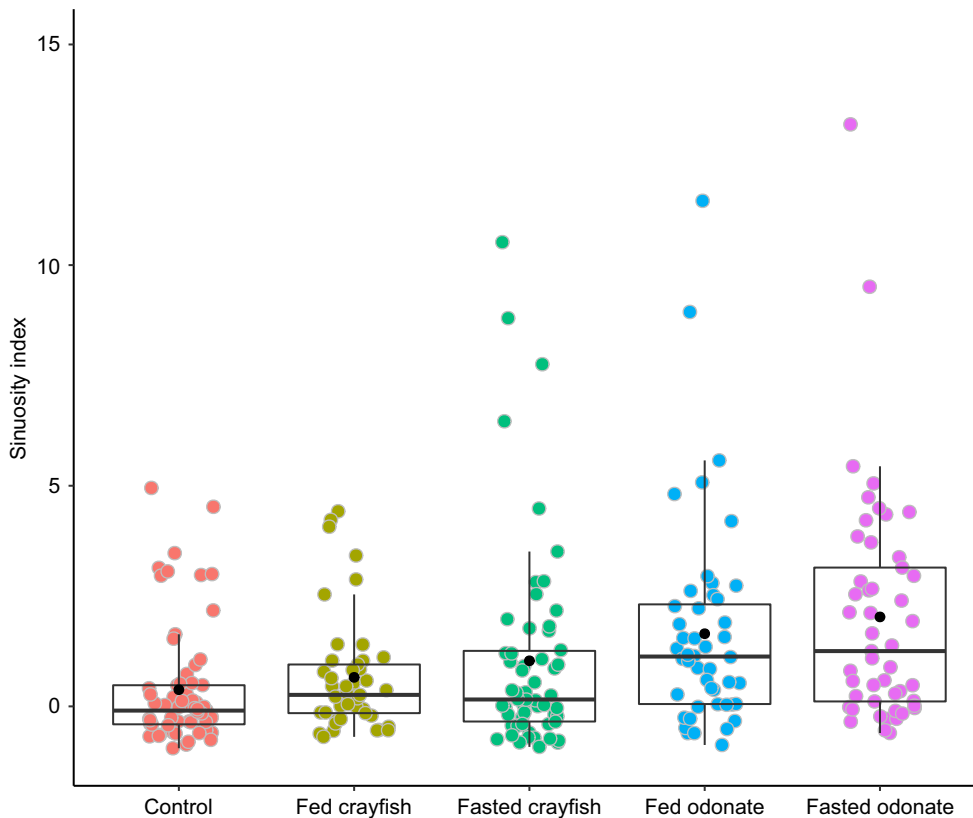


Fig. 7. Boxplot of the sinuosity index for each chemical cue. Black circles represent means.

2010). To test for this hypothesis, we exposed tadpoles to the cues of either native dragonfly larvae or alien crayfish and assessed their efficacy when infused both alone and with prey alarm cues. The use of ToxTrac allowed the precise recording of individual paths, from which we could assess several behavioural variables with greater accuracy than with traditional methods (Scribano et al., 2020).

Overall, when exposed to odonate cues, tadpoles showed a strong defensive response, particularly consistent among breeding sites, towards tadpole-fed dragonflies, suggesting that, although kairomones are sufficient to elicit a prompt reduction in tadpole activity, the synergistic effect of predator and prey cues may trigger a more shared and intense response (Schoeppner and Relyea, 2009; Hettyey et al., 2015).

Reduction of activity, to lower the probability of encountering or being detected by predators, is a common behavioural response shown by threatened tadpoles, usually measured as either the proportion of active individuals in a group or duration of activity over a standard period (Van Buskirk, 2001; Steiner, 2007; Gazzola et al., 2017). By measuring the variables ‘time frozen’ and ‘sinuosity’, we highlighted that tadpoles respond to a predator threat by two different, not mutually exclusive defensive strategies: freezing and zig-zagging. While the first behaviour had previously been roughly described by counting the number of tadpoles actively moving, individual recordings and analysis allowed the more precise assessment of the proportion of time that each tadpole spent still before and after being exposed to predator cues. The analysis of individual trajectories indicated that under predation threat, tadpoles incorporate protean elements into their movement, increasing path complexity. Protean behaviour (Humphries and Driver, 1970) prevents predators from anticipating the future position of their prey, lowering their targeting accuracy (Jones et al., 2011; Richardson et al., 2018), especially in small arenas, where the distance between opponents is short (Furuichi, 2002). Both strategies, and their

combination, are expected to be effective in the face of attacks by dragonfly larvae, which detect their prey at a distance by sight or vibration (freezing) and then move towards it until the prey is within range of their labium (zig-zagging) (Rowe, 1994).

As, in principle, predators may reach their prey and attack on any side, lateralization, which is an asymmetrical perceptual system, is expected to be detrimental to prey, which would be more vulnerable on their deficient side (Corballis, 1998). Nonetheless, lateral bias seems to be ubiquitous in animals and it has been hypothesized that it may arise from the need to coordinate behaviours in asymmetrically organized groups (Vallortigara and Rogers, 2005). The decrease in lateralization intensity in tadpoles exposed to odonate cues may suggest that under predation risk, tadpoles tend to scrutinize the surrounding environment equally on both sides. This behaviour may be enhanced by the chemical nature of the signal, which rapidly disperses throughout the test arena, while an increase in lateralization intensity may be expected in tadpoles exposed to caged predators, a hypothesis that needs further testing.

Because of the absence of a common evolutionary history (Gamradt and Kats, 1996; Freeman and Byers, 2006; Banks and Dickman, 2007; Smith et al., 2008) and, consequently, the lack of defensive adaptations, native prey are likely to exhibit weak or inappropriate antipredator responses when facing novel predation threats. The degree of naivety, and thus the impact of the alien predator, may depend on its phylogenetic relatedness to native predators (Cox and Lima, 2006; Sih et al., 2010). As expected, tadpole responses towards alien crayfish were less sharp and, in general, were mostly elicited by tadpole-fed crayfish. The role played by conspecific cues in eliciting a defensive response in tadpoles exposed to alien predators has been shown for several species (Nunes et al., 2013), and our results confirm that alarm cues from damaged conspecifics are also able to elicit behavioural responses in Italian agile frog larvae, at least in laboratory

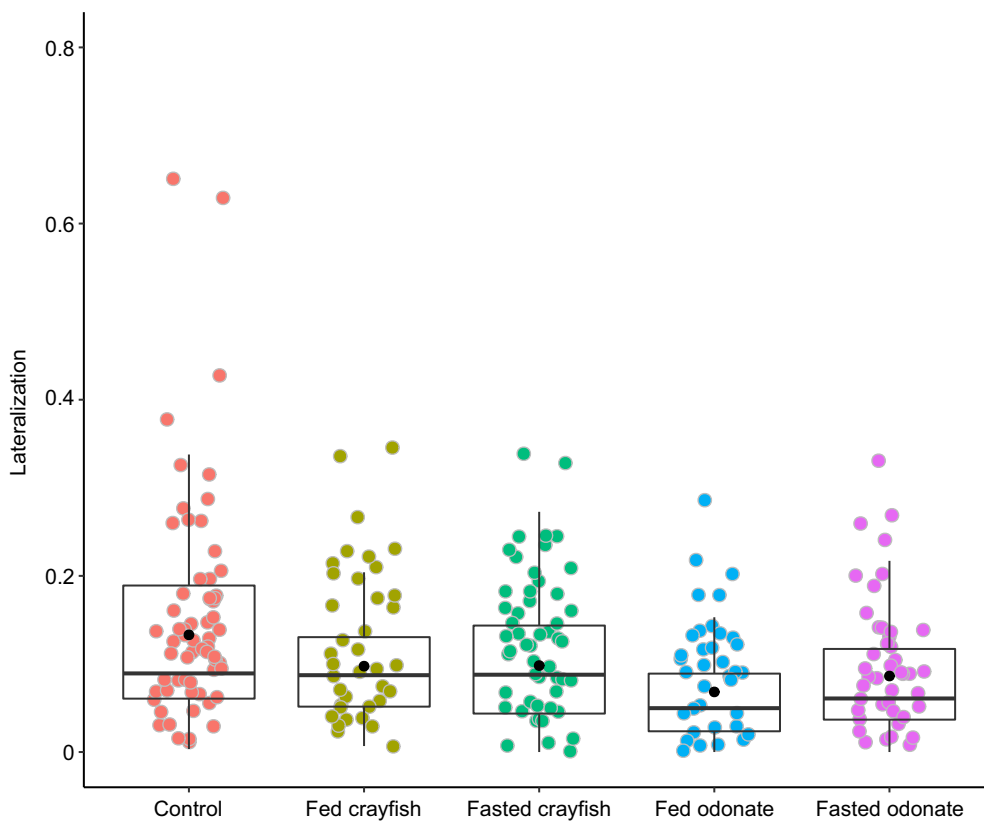


Fig. 8. Boxplot of the lateralization index for each chemical cue. Black circles represent means.

conditions. Interestingly, we recorded inter-site variability in tadpole responses to crayfish, as BC tadpoles did not react significantly to either fed or fasted alien predators, while BN tadpoles also showed a weak but significant response to fasted crayfish. This inter-site gradient (BN>MZ>BC) in the defensive response of tadpoles was consistent with the relative abundance of *P. clarkii* visually recorded at the sites during the sampling of egg clutches. These results suggest that coexistence may enhance behavioural adaptations to a novel predatory threat – that is, native species are able to learn to recognize cues from novel invasive predators (Strauss et al., 2006) – probably by associating conspecific alarm cues with predator kairomones (Ferrari et al., 2010). This association may occur during egg development (before collection) or be genetically based, given that enough time has been allowed for evolution (Strauss et al., 2006). The latter hypothesis seems improbable, as crayfish were introduced to northern Italy only recently (<30 years).

Despite all tadpoles being kept in standard conditions after hatching, basal mobility rates differed among populations, in agreement with previous studies (Nunes et al., 2013). Different environmental conditions and pressures are known to affect activity and motor behaviours (Richardson, 2001), and studies on the relationship between predator cues and hatching time have reported discordant results (Ireland et al., 2007; Gazzola et al., 2015, 2018). Consistent with the potential effect of environmental conditions (Vences et al., 2002), our results agree with those reported for syntopic *Rana dalmatina*, for which Gazzola et al. (2018) did not observe any effect of odonate cues on hatching time.

Anuran larvae are a well-studied system to test for predator–prey interactions, behavioural responses usually being used for analysing contextual plasticity (Relyea, 2003; Ferrari et al., 2010). By measuring sinuosity and lateralization in tadpoles

individually exposed to predation threat, we could identify details about tadpole escape strategies that are usually missed when employing activity-based behavioural variables. To gather this kind of information is pivotal for endemic, threatened species as, although prey may evolve the ability to recognize and respond to alien predators, time may be insufficient to prevent the extinction of several fragmented populations. Finally, we suggest that video-tracking techniques, which have seldom been used for assessing tadpole behaviour (Scribano et al., 2020), offer several opportunities to further investigate predator–prey relationships in aquatic habitats.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.G., D.P.-R.; Methodology: A.G., D.P.-R.; Software: G.S.; Formal analysis: A.G., A.B., A.F.; Investigation: A.G., A.B., A.F.; Data curation: A.B.; Writing - original draft: A.G., A.B., A.F.; Writing - review & editing: A.G., A.B., G.S., D.P.-R.; Supervision: A.G., D.P.-R.; Project administration: D.P.-R.

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Data availability

Data are available from the Dryad digital repository (Pellitteri-Rosa et al., 2021): <https://doi.org/10.5061/dryad.qfttdz0gb>

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