# When dangerous predators are ignored: antipredator responses in temporary-pond amphibians

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**Abstract**

Antipredator behaviour is recognised as a key factor of reintroduction success, yet it remains poorly considered in conservation practice. Despite their conservation relevance, little is known about antipredator behaviour in Pelobatidae tadpoles, among which the endangered Italian lineage of common spadefoot toad *Pelobates fuscus* has been the target of several captive breeding and reintroduction programs. We conducted four experiments to investigate the responses of spadefoot toad tadpoles to chemical cues associated with predation risk, focusing on ontogenetic variation, prior exposure, cue concentration, and predator identity. Contrary to expectations, tadpoles showed weak and inconsistent responses to odonate cues, despite dragonfly larvae eliciting strong antipredator responses in most previously tested anuran species. Prior exposure did not enhance responses, and variation in cue concentration produced only limited effects. Similarly, tadpoles did not modify activity level when exposed to alarm or disturbance cues. In contrast, when examining a set of eight different potential predators, clear antipredator responses emerged in response to those capable of exploiting temporary waterbodies. Overall, our results suggest that antipredator responses in *P. fuscus* tadpoles are shaped by the ecological conditions of the species' preferred breeding sites, with reduced responsiveness to predators that are unlikely to occur in temporary ponds. Tadpole-naiveté towards predators associated with permanent aquatic habitats may explain the previously recorded declines in permanent fish-free ponds managed as conservation sites. These findings confirm that predator-prey interactions and antipredator behaviour should not be overlooked in conservation programs and highlight the importance of incorporating behavioural processes into conservation strategies.

**Key words**: dragonfly larvae, chemical cues, predator-prey naiveté, temporary ponds, reintroduction

## Introduction

Amphibians are the most endangered class of vertebrates worldwide and, although conservation efforts have progressively increased since the 1980s, their global status continues to deteriorate. Major threats include habitat loss and degradation, diseases, and, in the last two decades, climate change (Luedtke et al., 2023; Stuart et al., 2004). To prevent extinctions, captive breeding is increasingly considered a key conservation tool (Tapley et al., 2024), although several projects failed to produce viable populations (Beck et al., 1994; Amphibian Ark, 2021), highlighting the need for further improvements in management strategies.

Out of the 93 native and naturalised species occurring in Europe, the common spadefoot toad, *Pelobates fuscus*, is listed as Least Concern by the IUCN, because of its wide distribution, ranging from central France to western Siberia and Kazakhstan (Agasyan et al., 2009; Crnobrnja-Isailović et al., 2025). However, the genetic status of many populations is concerning, especially in western Europe. The Italian population represents a relevant conservation unit, consisting of a genetically distinct lineage isolated from other populations (Mouton et al., 2025). The Italian spadefoot toad (previously known as *P. f. insubricus*) is listed in Annex II of the Habitats Directive (92/43/EEC) and the IUCN Red List of Italian Vertebrates as Endangered (Rondinini et al., 2022). To improve the conservation status of threatened populations and prevent their extinction, several action plans have been developed and conservation projects implemented (e.g., Struijk et al., 2016; Van Doorn et al., 2023; LIFE11 NAT/DE/000348; LIFE98 NAT/IT/005095, LIFE00 NAT/IT/007233), with mixed results. In northern Italy, 30 years of conservation efforts have not prevented the decline of the species (Crottini and Andreone, 2007; Giacoma et al., 2009).

Captive breeding and release are complex undertakings, requiring in-depth knowledge of animal ecology and genetics (Ewen et al., 2012; Linhoff et al., 2021). The behaviour of released animals can strongly affect their fitness and, since the late 1990s, has been recognised as a key factor in determining the success or failure of conservation programmes (Bessa Gomes and Sarrazin, 2016).

However, while most guidelines and action plans highlight the role played by habitat requirements and resource availability (Moore and Church, 2008; Bell, 2016; Germano et al., 2024), antipredator behaviour has received comparatively little attention (see Berger-Tal et al., 2011; West et al., 2025). Behavioural studies can provide information and tools to improve antipredator performances in captive-reared animals. However, research has mainly focused either on responses to novel predators (e.g., Cochran-Biederman et al., 2015; Blumstein et al., 2019; Kopack et al., 2023), or on the effects of captive environments on the development of antipredator skills (e.g., Olla et al., 1998; Jackson and Brown, 2011; Teixeira and Young, 2014). High post-release mortality, which often limits the success of reintroductions, is thought to be partly due to a lack of experience in responding to a range of novel predators (Ross et al., 2019). The most severe impacts of predator-prey naiveté (sensu Cox and Lima, 2006; Sih et al., 2010) occur when prey fail to recognise novel predators due to their long-term evolutionary isolation (Banks and Dickman, 2007). Otherwise, poor predator experience can be exhibited by prey occurring in habitats with a low diversity of predators (Moseby et al., 2015). Available data suggest that the spadefoot toads reproduce in deep, periodically dry, eutrophic ponds and rice fields (Andreone, 2006). The short aquatic phase of these waterbodies may limit the occurrence of fish and other aquatic predators, towards which toads may show weak or null antipredator responses.

To the best of our knowledge, little is known about the defensive behaviour of Pelobatidae tadpoles, which has only been investigated in Spain, using western spadefoot toad *Pelobates cultripes*. Tadpoles did not respond to the chemical cues of introduced turtles (Polo-Cavia et al., 2010), while showing inter-population variation in response to invasive crayfish (*Procambarus clarkii*) cues, associated with different levels of habitat connectivity and gene flow (Polo-Cavia et al., 2023).

Given the genetic distinctiveness and conservation status of the Italian spadefoot toad population, we conducted a series of behavioural experiments to characterise the defensive responses of tadpoles towards a range of potential invertebrate and vertebrate predators. We investigated the innate and experience-mediated antipredator responses of tadpoles to the chemical cues of odonate larvae, which

are known to elicit strong responses across a wide range of anuran species (e.g., Van Buskirk, 2001; Gallie et al., 2001; Gazzola et al., 2025a). Building on these results, we then exposed tadpoles to the cues of eight different predator species, predicting stronger responses towards those that thrive in temporary waterbodies. Based on previous research, we expected tadpoles to exhibit weak or negligible responses when exposed to cues from non-native crayfish and turtles.

## Methods

### Animal collection and maintenance

In March 2024, 20 egg string fragments from different breeding pairs of the Italian spadefoot toad (*Pelobates fuscus*), each consisting of approximately 50 eggs, were collected from Cascina Bellezza (Torino, Piedmont; 44°54 N, 7°47' E). They were transported to the laboratory and individually transferred into 20 L (38 × 28 × 20 cm) plastic tubs filled with 6 L of dechlorinated tap water. The same procedure was followed in March 2025, when 14 egg string fragments (~50 eggs each) were collected from Peverascia marsh (Arsago Seprio, Lombardy; 45°42′ N, 8°42′ E). All breeding pairs were part of a conservation programme within the framework of LIFE INSUBRICUS (LIFE19-NAT/IT/000883).

Experiments started one week after hatching, when tadpoles reached Gosner developmental stage 26–28. Throughout the study period, water temperature ranged between 17 and 22 °C. Approximately 50% of the water volume was replaced every three days. Tubs were kept under natural light conditions and, after hatching, tadpoles were fed daily ad libitum with dry grass pellets (rabbit food).

To prepare the chemical stimuli, in 2024 we collected 12 odonate larvae (*Anax imperator*) and an equal number of non-predatory aquatic beetles (*Hydrophilus piceus*). In 2025, we collected three adult green frogs (*Pelophylax* sp.), three grass snakes (*Natrix helvetica*), four adult red swamp crayfish (*Procambarus clarkii)*, 12 *A. imperator* larvae, six backswimmers (*Notonecta* sp.), two adult red-

eared sliders (*Trachemys scripta*), four Italian crested newts (*Triturus carnifex*), and three mosquitofish (*Gambusia holbrooki*). All animals were collected from different waterbodies across the provinces of Pavia and Milan (Lombardy).

Details on body mass and housing density of all predator species are summarised in the supplementary materials (Tables S1-S2). All animals were housed in dechlorinated water and hosted according to species-specific husbandry requirements. *Anax* larvae were kept individually in plastic tubs (15 × 10.5 × 5.0 cm), with a piece of mesh to allow grasping; *Procambarus*, *Notonecta* and *Gambusia* were housed into 38 × 28 ×20 cm tubs; *Pelophylax*, *Triturus* and juvenile *Natrix* were housed in 23 × 34 × 21 cm tubs, whereas adult *Natrix* and *Trachemys* specimens were kept in larger plastic containers (40 × 25 × 27 cm and 44 × 63 × 33 cm, respectively). All containers were provided with shelters to reduce animal stress and disturbance. Sliders were positioned to receive natural daylight, while snakes were provided with UV/heat lamps. Animals were fed according to taxon-specific diets: live gammarids for frogs, newts, crayfish, sliders, backswimmers and dragonfly larvae, goldfish food for mosquitofish and mealworms (*Tenebrio molitor*) for grass snakes.

**Experimental designs and procedures**

The four experiments were designed to progressively investigate different components of antipredator responses (Fig. 1). Experiment 1 tested responses to predator cues at two developmental stages and two cue concentrations. Experiment 2 examined the effects of prior exposure to predator cues and variation in cue concentration on subsequent responses. Experiment 3 compared responses to different types of risk-related chemical cues, including predator cues, alarm cues, and disturbance cues. Experiment 4 assessed responses to chemical cues from multiple predator species. In all experiments, tadpoles were recorded individually and each individual was tested only once. Experiments 1–3 were conducted in 2024, whereas Experiment 4 in 2025. All trials took place between 09:00 and 13:00 in the same laboratory under standardised environmental conditions.

147 *Experiment 1*

148 The first experiment aimed to test whether tadpoles exhibit antipredator responses to the chemical
149 cues of one of the most common predators of larval anurans, namely aeshnid odonate larvae. In
150 addition, we investigated whether tadpole sensitivity to predation risk varies across ontogeny (as
151 reported for other anuran species; e.g., Kurali et al., 2017; Gazzola et al., 2018; Gramapurohit et al.,
152 2025) by comparing the responses at two developmental time points, one and three weeks after
153 hatching (Gazzola et al., 2026).
154 Tadpoles were exposed to three types of stimuli: predator cues released by dragonfly larvae (predator
155 cue), control cues originating from non-predatory aquatic beetles, and aged tap water as a neutral
156 control. Predator cues were presented at two concentrations (undiluted and diluted 1:10) to assess
157 tadpoles' sensitivity to different levels of perceived predation risk. For each of the 20 egg clutches,
158 two tadpoles were randomly assigned to each stimulus at each developmental time point. Overall,
159 160 individuals were tested at each time point, with 40 replicates per stimulus.
160 Each recording session lasted two consecutive days. On each testing day, 30 mL of water was
161 collected from five randomly selected rearing tubs and pooled into a single container to obtain the
162 dragonfly cue solution. Aquatic coleopteran cues were collected from a separate tub containing 2.5 L
163 of aged tap water and five individuals. For both predator types, the water in the holding tubs was
164 replaced 24 hours prior to cue collection, and no food was provided during this period to ensure that
165 only predator-derived chemical cues (i.e., kairomones) were present in the stimulus solution.
166 The experiment followed a fully factorial design with stimulus type and developmental time point as
167 fixed factors.

168

169 *Experiment* 2

The second experiment aimed to test whether repeated exposure to predator cues from dragonfly larvae during a conditioning phase (i) affects subsequent antipredator responses in tadpoles, and (ii) tadpoles show graded, concentration-dependent responses.

On the day preceding the conditioning phase, 24 plastic tubs (17 × 13 × 8 cm) were filled with 1 L of aged tap water each. Twelve tadpoles were placed in each tub. Tadpoles originated from 12 different clutches; individuals from each clutch were equally distributed between control tubs (receiving aged tap water only) and predator-cue tubs. This resulted in a split-clutch design, ensuring that each clutch was represented in both conditioning treatments and controlling for potential clutch-specific effects. The conditioning phase lasted two days, during which 6 mL of either predator cues or control water were administered twice per day, at 11:00 a.m. and 5:00 p.m., using an 8 mL disposable syringe.

On the first day after the conditioning phase, tadpoles were individually exposed to either undiluted predator cue or control water in a fully factorial 2 × 2 design (two replicates per clutch). On the second day, tadpoles were exposed to one of four test stimuli in a fully factorial 2 × 4 design: aged tap water (control), undiluted predator cue, predator cue diluted to 10% (1:10), or predator cue diluted to 1% (1:100) (one replicate per clutch per treatment). The higher number of replicates per group (n = 24) of the first day was intended to increase the statistical power for detecting the overall effects of prior conditioning in a binary exposure context. On day 2, sample size was halved (n = 12) to accommodate a broader range of test stimuli (i.e., cue concentrations) while maintaining balanced and manageable group sizes. Odonate cues were produced using the same procedure as for the first experiment.

*Experiment 3*

The third experiment aimed to assess tadpole behavioural responses to different types of risk-related chemical information by comparing responses to (i) conspecific alarm cues, (ii) conspecific disturbance cues, and (iii) predator cues. This experiment was designed to highlight whether tadpoles

194 discriminate among cues that differ in their biological meaning, ranging from direct evidence of
195 conspecific injury to uncertain indicators of potential predation risk.
196 The experiment was conducted over a single day. Tadpoles were individually exposed to one of four
197 chemical stimuli: conspecific alarm cues, disturbance cues, predator cues from unfed odonate larvae,
198 and aged tap water as control. Each stimulus was replicated 24 times (three replicates for each of the
199 eight clutches) resulting in a balanced design across treatments.
200 Alarm cues were obtained from injured conspecifics following standard procedures (Gonzalo et al.,
201 2010; Gazzola et al., 2025b). Four tadpoles were randomly taken from the rearing tank and
202 anaesthetized by inducing deep hypothermia (−3 °C for 15 min; Wilson et al., 2009). They were
203 subsequently euthanized with a rapid cranial blow in accordance with current ethical
204 recommendations (Leary et al., 2020). Chemical anaesthetics were avoided, as they may alter the
205 chemical profile of prey-derived cues (Achtymichuk et al., 2022). The resulting cue solution was
206 filtered through absorbent paper to eliminate tissue fragments and then aliquoted into 10-mL portions,
207 which were frozen (–20 °C) until use. Disturbance cues were generated by exposing tadpoles to non-
208 injurious mechanical disturbance, assuming the release of chemical signals associated with stress or
209 fear but without tissue damage. Following Gonzalo et al. (2010), twenty tadpoles (two per clutch)
210 were placed in a 200-mL container and subjected to simulated predator attacks for 30 s using a
211 wooden stick. Care was taken to avoid direct contact with the animals. Predator cues were obtained
212 exclusively from unfed odonate larvae, following the same procedure of the previous two
213 experiments.
214
215 *Experiment 4*
216 This experiment aimed to investigate tadpole antipredator responses to olfactory cues from eight
217 different predator species: three invertebrate predators - the red swamp crayfish (*Procambarus*
218 *clarkii*), backswimmers (*Notonecta* sp.), and dragonfly larvae (*Anax imperator*) - and five vertebrate

predators, the grass snake (*Natrix helvetica*), mosquitofish (*Gambusia holbrooki*), red-eared slider (*Trachemys scripta*), Italian crested newt (*Triturus carnifex*) and green frogs (*Pelophylax* sp.). Eleven randomly selected clutches were included in the behavioural experiment, which consisted of nine treatments (eight predator cues and tap water as control), resulting in approximately two replicates per stimulus within each clutch and 20–22 replicates per chemical stimulus overall.

To obtain the chemical cues, predators were placed in separate containers filled with dechlorinated water, either individually (odonate, snake, turtle and crayfish) or in groups (backswimmer, frogs, fish and newt), and left undisturbed to allow the release of chemical cues. Whenever feasible, water volumes used for cue preparation were scaled to the total biomass of each predator species, with the aim of maintaining comparable biomass-to-water ratios. For *T. scripta*, cue concentrations were matched to those used in previous experiments (Gazzola et al., 2025a). For *N. helvetica*, water volume was adjusted based on body size and housing constraints to obtain detectable chemical cues. Full details are provided in Tables S1–S2. Water was replaced 24 h after the last feeding event to remove residual chemical cues and predators were then maintained in clean dechlorinated water for 24 h. After 24 hours, chemical cues were collected from the containers, transferred into 45 mL Falcon tubes, and stored at –20 °C until use.

*Behavioural trials*

The same procedure to collect behavioural data was adopted for all experiments. To quantify tadpole activity before and after exposure to predatory chemical cues, individuals were tested in opaque plastic containers (15 × 10.5 × 5 cm) filled with 250 mL of aged tap water. Each tadpole was left undisturbed in the testing arena for 15 min before starting the trial.

Experimental trials consisted of two consecutive phases: a 10-min baseline period and a 10-min observation period following cue injection. For experiment 4, the post-stimulus period was extended to 50 min. Predator cues were administered by gently injecting 2 mL of stimulus solution into the

container using a disposable syringe. During the trials, the final concentration of the undiluted stimulus in the arena was 1:125, consistent with concentrations used in previous studies (e.g., Gomez-Mestre and Diaz-Paniagua, 2011; Gazzola et al., 2018). For experiments 1-3, tadpoles were exposed to chemical cues within 1–4 h from collection, a time interval which ensures their efficacy (Peacor, 2006; Van Buskirk et al., 2014).

During each experimental session, 12 containers were arranged in a grid, allowing the simultaneous recording of as many individuals (Scribano et al., 2020; Guadin et al., 2021) using a digital video camera (Canon Legria; 1080-pixel resolution, 25 frames $s^{-1}$) positioned 1 m above the experimental setup. Trials were conducted indoors and the arenas were screened off with opaque panels and evenly illuminated by spotlights. Experimenters entered the room only to administer the stimulus.

Video recordings were analysed using ToxTrac, an open-source software for automated animal tracking (Rodriguez et al., 2018). Locomotor activity was quantified by extracting the x–y coordinates of each tadpole's centroid at 0.04-s intervals. To prevent bias, video analyses were conducted blind, with the operator unaware of the treatment assigned to each individual. Two main behavioural variables were recorded, namely the proportion of time spent inactive (freezing) and total distance swum (distance travelled) during each phase of the trial. Tadpoles were classified as "inactive" when they moved less than 5 mm within a 3-s interval (Gazzola et al., 2025a; Scharf et al., 2024).

This protocol was applied to all experiments except for the first, when tadpoles were too small to be reliably tracked using ToxTrac. In this case, activity was quantified by direct visual observation by comparing the position of each tadpole at 10-s intervals. Tadpoles were classified as active when a change in position ($\geq$ 5 mm) between consecutive observations was detected. This manual scoring approach provided a rough measure of activity comparable to that obtained through automated tracking. To validate this manual method, activity estimates obtained using the 10-s interval approach were compared with those derived from second-by-second tracking in a subset of individuals for which both measures were available (n = 39). Pearson's product–moment correlation tests revealed a

strong positive correlation between the two methods both before ($r = 0.89$, 95% CI = 0.80–0.94, $p < 0.001$) and after exposure to stimuli ($r = 0.91$, 95% CI = 0.83–0.95, $p < 0.001$).

In experiment 4, which involved a longer post-stimulus tracking period (50 min), behavioural variables for the post-stimulus phase were derived directly from time series of X and Y coordinates recorded at 25 frames per second for each individual. Two different behavioural metrics were calculated to investigate tadpole movements over time: total distance swum and activity (freezing). To quantify tadpole activity over this long period, we implemented a binary classification of movement based on displacement over short temporal windows. The observation period was divided into consecutive, non-overlapping 2-min intervals. Each interval was then partitioned into 3-s blocks (corresponding to ca. 75 frames), and each block was assigned a binary value indicating whether the tadpole was active or not. The tadpole was classified as active when the Euclidean distance between its position at the start and end of the period exceeded 5 mm. The response variable was then defined as the proportion of 3-second blocks classified as active within each 2-minute interval.

**Statistical analysis**

*Experiment 1*

We analysed the effects of chemical stimuli and developmental time point on tadpole activity using generalized linear mixed models (GLMMs) with a beta distribution and a logit link function, implemented in the glmmTMB package (Brooks et al., 2017) in R. The response variable was the proportion of observations scored as active after stimulus presentation. The model included stimulus (control, coleopteran, odonate, odonate 1:10), session (7 days, 21 days), and their interaction as fixed effects, with pre-stimulus activity as a covariate to control for baseline individual differences in activity. Egg clutch was included as a random intercept to account for non-independence among individuals from the same clutch.

294 Planned comparisons between each stimulus and the control were conducted using estimated
295 marginal means, applying Dunnett-adjusted tests.

296

297 *Experiment 2*

298 GLMMs were used to test whether prior exposure to predator cues (conditioning) modulated tadpole
299 antipredator responses. For freezing behaviour, we fitted a beta regression model with a logit link.
300 For distance travelled we assumed a Gaussian error distribution. In both models, fixed effects
301 included the test stimulus (control and odonate cues at different concentrations), conditioning
302 treatment (control or conditioned), and their interaction. Pre-stimulus behaviour was included as a
303 covariate to control for baseline differences. To account for the split-clutch design and shared rearing
304 environment, clutch and rearing tub nested within clutch were included as random intercepts. Because
305 the experimental design differed between test days, models were fitted separately for day 1 and day
306 2 following conditioning. On day one, models included two test stimuli (control and undiluted
307 odonate cues) and conditioning treatment in a fully factorial 2 × 2 design. On day two, models
308 included four test stimuli (control, undiluted odonate cues, 10% dilution, and 1% dilution) and
309 conditioning treatment in a fully factorial 2 × 4 design.

310

311 *Experiment 3*

312 To evaluate whether tadpoles responded differently to the tested chemical stimuli, we fitted different
313 GLMMs for each behavioural response variable.
314 Freezing and distance travelled were modelled using, respectively, either a beta regression with logit
315 link function or a Gaussian error distribution. In both models, stimulus type (alarm, disturbance,
316 predator, control) was included as a fixed effect. To control for baseline variation in individual

behaviour, the corresponding pre-stimulus measure was included as a covariate. Clutch was modelled as a random intercept.

*Experiment 4*

For the fourth experiment, the same modelling framework described for Experiment 3 was used. For both models, stimulus type (eight predator species and a control) was included as a fixed effect, the corresponding pre-stimulus behavioural measure was included as a covariate to control for baseline differences, and clutch was included as a random intercept. For the distance travelled model, an interaction between stimulus and baseline distance was included, as it improved model fit and residual diagnostics.

Comparisons between each predator treatment and the control group were performed using Dunnett-adjusted contrasts on estimated marginal means.

Plots display estimated marginal means (or response proportions, where appropriate) with 95% confidence intervals for each stimulus condition, while planned comparisons versus the control treatment are shown as odds ratios (or mean differences) along with their 95% confidence intervals. These visualizations allow the interpretation of effect sizes and uncertainty.

To assess how tadpole activity varied over time across predator treatments, we fitted a Generalized Additive Mixed Model (GAMM) using the *bam* function from mgcv package. The response variable was the proportion of active 3-s blocks within each 2-min interval. We used a quasibinomial family with a logit link to account for overdispersion, and weighted each observation based on the number of blocks used to compute the proportion.

We modelled nonlinear temporal trends separately for each treatment using treatment-specific smooth functions of time since stimulus exposure (2-min intervals). Random smooth terms were included for individual identity and egg clutch to account for repeated measures and potential shared variance.

The model included a set of smooth functions describing the effect of time since stimulus exposure (in 2-min bins) separately for each chemical cue, allowing for treatment-specific, non-linear temporal trends.

Model diagnostics confirmed the absence of problematic concurvity (< 0.5), appropriate smoothness (k-index > 0.99 for all terms), and negligible residual autocorrelation, as confirmed by the visual inspection of the autocorrelation function (ACF) of model residuals (S1).

For all experiments, planned contrasts comparing each stimulus treatment with the control were performed using Dunnett-adjusted tests. For freezing models, effect sizes are reported as odds ratios (OR), obtained by back-transforming model estimates from the logit scale. All statistical analyses were performed in R (version 4.3.2; R Core Team, 2023).

## Results

*Experiment 1 – Effect of development on antipredator response*

A significant interaction between stimulus type and developmental time point was detected ($\chi^2$ = 11.45, df = 3, p = 0.0095), indicating that tadpole responses to chemical cues varied across ontogeny. At 7 days post-hatching, activity did not differ significantly for either the coleopteran (odds ratio (OR) = 1.63, p = 0.090) and odonate stimuli (OR = 0.62, p = 0.098; Fig. 2) with respect to controls. The diluted odonate treatment also did not differ from the control (OR = 1.01, p = 0.999).

At 21 days, individuals exposed to the odonate stimulus showed significantly lowered activity relative to controls (OR = 0.56, p = 0.022), while activity did not differ significantly for both the coleopteran (OR = 0.60, p = 0.057), and diluted odonate treatments (OR = 0.75, p = 0.414; Fig. 2). Using ToxTrac, neither the proportion of time spent frozen (Coleopteran vs Control: OR = 1.30, p = 0.195; Odonate vs Control: OR = 1.40, p = 0.067; diluted Odonate vs Control: OR = 1.27, p = 0.261), nor total

distance travelled (Coleopteran vs Control: estimate = −1574, p = 0.063; Odonate vs Control: p = 0.692; diluted Odonate vs Control: p = 0.995) differed significantly among treatments.

*Experiment 2 - Day 1: Effect of conditioning on antipredator response*

For both freezing (beta regression) and distance travelled (Gaussian model), the interaction between test stimulus and conditioning treatment was not significant (LRT: $\chi^2 = 0.29$, df = 1, p = 0.59 and $\chi^2 = 2.26$, df = 1, p = 0.322 respectively), indicating that conditioning did not affect behavioural responses. Comparisons revealed no significant difference between odonate-exposed tadpoles and controls in either group. In unconditioned tadpoles, exposure to odonate cues neither increased freezing relative to controls (OR = 1.17, p = 0.81; Fig. 3, left panel), nor reduced the total distance swum (mean difference = -662 mm, p = 0.866). Similarly, in conditioned tadpoles, freezing remained unchanged (OR = 1.00, p = 0.99; Fig. 3, right panel), and movement did not significantly differ (mean difference = 1427 mm, p = 0.41).

*Experiment 2 - Day 2: Effect of conditioning on sensitivity to graded predator cue concentrations*

To assess whether prior conditioning influenced tadpole responses to different concentrations of predator chemical cues, we analysed behaviour following exposure to three odonate cue concentrations (undiluted, 1:10, and 1:100) or a control stimulus.

For freezing behaviour, a beta regression model including the interaction between stimulus and conditioning treatment revealed no significant interaction effect ($\chi^2 = 2.44$, df = 3, p = 0.486), indicating that conditioning did not significantly modulate responses across cue concentrations. However, a main effect of stimulus was detected ($\chi^2 = 8.99$, df = 3, p = 0.03): unconditioned tadpoles exposed to undiluted odonate cues showed significantly increased freezing compared to controls (OR

388 = 2.35, p = 0.042; Fig. 4), while the 1:10 and 1:100 dilutions did not elicit significant changes (both
389 p > 0.05; Fig. 4). No significant effect of conditioning was detected ($\chi^2$ = 0.12, p = 0.725).
390 A Gaussian model was used to test whether prior conditioning influenced the distance travelled by
391 tadpoles in response to different concentrations of odonate chemical cues. Although the overall
392 interaction between stimulus and conditioning was not significant ($\chi^2$ = 4.88, df = 3, p = 0.18), there
393 was a significant main effect of stimulus ($\chi^2$ = 8.98, df = 3, p = 0.030). However, no individual contrast
394 with the control remained significant after Dunnett adjustment. Comparisons with the control within
395 each conditioning group indicated that, in unconditioned tadpoles, exposure to undiluted odonate cues
396 did not result in a significant reduction in distance travelled (mean difference = −3390 mm, p = 0.25;
397 Fig. 5), although the estimated difference was negative. No significant differences were observed for
398 the 1:10 and 1:100 dilutions (both p > 0.05), and no significant effects were detected among
399 conditioned individuals (all p > 0.05).

400

401 *Experiment 3*

402 Tadpole behavioural responses did not differ significantly among chemical stimuli. Alarm- (OR =
403 1.09, p = 0.94), disturbance- (OR = 1.08, p = 0.95) and predator cues (OR = 1.15, p = 0.83) did not
404 elicit stronger freezing responses than control water (Fig. 6). Consistently, no significant differences
405 were detected in distance travelled. Tadpoles exposed to alarm cues showed a slight, non-significant
406 reduction in distance travelled (mean difference = −571 mm, p = 0.84), whereas individuals exposed
407 to disturbance (mean difference = +652 mm, p = 0.80) or predator cues (mean difference = +298 mm,
408 p = 0.96) did not differ from controls (Fig. 6).

409

410 *Experiment 4*

411 Tadpoles exhibited differential behavioural responses to the various chemical stimuli. In terms of
412 freezing behaviour, several predator stimuli elicited stronger freezing responses than control cues.
413 Specifically, exposure to frog and turtle cues led to significantly higher immobility (odds ratio = 1.91,
414 $p = 0.015$; and 2.08, $p = 0.0025$, respectively), while the strongest response was observed following
415 exposure to newt cues (odds ratio = 2.86, $p < 0.0001$). Responses to snake and backswimmer cues
416 approached significance (odds ratios = 1.74 and 1.69; $p = 0.0502$ and 0.0540, respectively). No
417 significant differences were detected for crayfish, fish and odonate cues relative to the control (Fig.
418 7).
419 In terms of distance travelled, the Gaussian model revealed a significant reduction in movement in
420 response to several predator stimuli. Tadpoles exposed to turtle, newt, snake, and frog cues showed
421 significantly reduced distance swum compared to controls (mean difference = -3393 mm, $p < 0.0001$;
422 -2772 mm, $p = 0.0013$; -2711 mm, $p = 0.0031$; and -2588 mm, $p = 0.0046$, respectively). The response
423 to backswimmer cue approached significance ($p = 0.0756$), while cues from crayfish, fish and odonate
424 did not significantly affect distance travelled (Fig. 8).

425

426 *Temporal dynamics of activity in response to predator chemical cues*

427 The final model accounted for 51.6% of the deviance in tadpole activity levels (adjusted $R^2 = 0.548$).
428 Smooth terms for all predator stimuli showed significant temporal structure (all $p < 0.001$), while the
429 control group did not exhibit any systematic time-dependent change ($F = 0.20$, $p = 0.65$). The response
430 to snake and newt cues was particularly strong, with higher estimated degrees of freedom (edf = 3.23
431 and 4.73, respectively) and large F-values ($F = 14.88$ and 13.23). Other predators - including
432 backswimmer, odonate, frog, turtle, crayfish, and fish - also elicited significant nonlinear trends,
433 albeit with differences in timing and magnitude of suppression (Fig. 8). The lowest peaks in activity
434 were observed in response to frog, turtle, and newt cues, occurring approximately around the tenth
435 interval (i.e. 20 min after stimulus exposure; Fig. 8). In contrast, activity levels of control tadpoles

remained low and stable throughout the observation period. Both random effects - individual identity and clutch - accounted for substantial variation in baseline activity ($p < 0.001$ and $p = 0.045$, respectively).

These results indicate that predator cues triggered distinct temporal patterns in tadpole defensive behaviour, with rapid, time-dependent reductions in activity that varied in intensity across treatments.

## Discussion

Odonate larvae are key predators for tadpoles (Wilbur, 1980; Van Buskirk, 1988; Gazzola et al., 2025a), being able to prey on a wide range of size classes (Brodie and Formanowicz, 1983; Smith, 1983). Accordingly, odonate chemical cues have been reported to elicit either morphological or behavioural antipredator responses in most tested anuran species (e.g., *Rana lessonae*, Van Buskirk and Arioli, 2002; *Rhinella spinulosa*, Jara and Perotti, 2009; *Rana temporalis*, Mogali et al., 2012; *Alytes cisternasii*, *Discoglossus galganoi*, *Bufo bufo*, *Hyla meridionalis*, *Pelodytes ibericus, Pelobates cultripes*, *Hyla arborea* and P*elophylax perezi,* Nunes et al., 2013; *Rana temporaria*, Hettyey et al., 2015). Within them, all the species occurring in northern Italy tested to date - that is *Rana dalmatina* (Gazzola et al., 2018), *Pelophylax esculentus* (Balestrieri et al., 2019), *Rana latastei* (Scribano et al., 2020), *Bufotes balearicus* (Gazzola et al., 2022) and *Rana temporaria* (Gazzola et al., 2025a) - responded to the chemical cues of either tadpole-fed or unfed dragonfly larvae by reducing their activity rates. In *Rana dalmatina* tadpoles, embryonic exposure to odonate cues has been shown to tune the defensive responses of the larval stage (Gazzola et al., 2015, 2023), and information on predation risk has been demonstrated by in vivo patch-clamp recordings to be recorded at neuronal level even when cues do not elicit any visible response (Gazzola et al., 2024). More generally, available data suggest that previous experience can strengthen antipredator responses to predators that already elicit responses in naïve individuals, even when animals are exposed only to predator-derived chemical cues. For instance, Mogali (2025) reported that *Duttaphrynus*

461 *melanostictus* tadpoles previously exposed to predator kairomones showed stronger antipredator
462 behaviour than predator-naïve individuals, with response intensity increasing along an experience
463 gradient.
464 Based on this evidential background, the slight response shown by spadefoot toad tadpoles toward
465 odonate cues in our first experiment was unexpected and deemed worthy of in-depth exploration.
466 Experiments 2 and 3 confirmed the unconventional behaviour of these tadpoles, which showed
467 negligible and inconsistent responses, probably partially affected by slight variation in exterior
468 conditions (e.g. air temperature or lighting) or developmental stage. Finally, the fourth experiment
469 shed some light on the possible explanation of tadpole indifference to odonate cues, suggesting that
470 the marked preference of spadefoot toad for temporary ponds as breeding sites, where water persists
471 only for the duration of tadpole ontogenetic development, may tune their responses to a range of
472 potential predators.
473 Out of the eight tested predator cues, the species that most consistently elicited defensive responses
474 in tadpoles - namely backswimmers, grass snakes, red-eared sliders, Italian crested newts and green
475 frogs - are all capable of exploiting temporary waterbodies and moving away from drying ponds. In
476 contrast, dragonfly larvae and mosquitofish are less likely to persist in short-hydroperiod temporary
477 ponds. Although cue production likely differs among predator taxa, this pattern was not associated
478 with predator body size or presumed cue intensity, suggesting that tadpole responses were shaped
479 more by the ecological characteristics of predators than by purely quantitative differences in chemical
480 stimuli.
481 The duration of dragonfly larvae development in temperate zones ranges from 1 to 5 years, including
482 8-15 instars (Corbet, 1980; Goretti et al., 2001). Intra- and interspecific variation in hatching times
483 entails that from spring to late autumn temporary ponds can host a wide diversity of predatory larvae,
484 which can attack any prey small enough to subdue (Wissinger, 1988). Hence, temporary ponds, as
485 those selected by spadefoot toad for laying their eggs, can be expected to be unsuitable to most
486 Anisoptera larvae, as well as strictly aquatic predators such as fish.

Previous studies have shown that anuran larvae that coexist with fish usually display strong antipredator responses when exposed to their chemical cues (Kats et al., 1988; Stauffer and Semlitsch, 1993; Richardson, 2001; Smith et al., 2008a, b). Responses to non-native fish are more varied; in the case of mosquitofish, *Rana* species seem to recognise non-native cues (e.g.: *Rana aurora draytoni,* Lawler et al., 1999; *Rana sylvatica,* Burgett et al., 2007), as well as several south Chinese species (Xiao-Li and Zhi-Hua, 2017), while golden bell frog *Litoria aurea* (Hamer et al., 2002), wood frog *Lithobates sylvaticus* (Buttermore et al., 2011), and American toad *Bufo americanus* tadpoles (Smith et al., 2008a) did not reduce activity when exposed to mosquitofish cues.

However, the choice of mosquitofish as a fish predator, which was driven by the scarcity of native ichthyophagous fish in spadefoot toad's range, does not allow us to disentangle whether the apparent indifference of spadefoot toad tadpoles toward mosquitofish cues depended on fish naiveté or tadpoles' preference for temporary ponds, that is their lack of coexistence over evolutionary time with predatory fish. Time since introduction of mosquitofish in northern Italy, about one century (Di Tizio and Mojetta, 2020), may support the second hypothesis.

In turn, ontogenetic and evolutionary naiveté (Cox and Lima, 2006) may explain why crayfish cues did not elicit any reduction in the activity of tested tadpoles. The first records of *P. clarkii* in the study area date back to 2012 (Bergò et al., 2019), a time interval probably too short to allow the onset of effective antipredator responses. Consistently, in some reintroduction sites the entry of non-native crayfish caused a sharp decline in spadefoot toad populations (-89% in SCI IT1110021; Seglie, 2019). Native, white-clawed crayfish *Austropotamobius pallipes*, which may share both predatory adaptations and chemical signals with *P. clarkii* (predator archetype *sensu* Cox and Lima, 2006), prefer hilly, wooded habitats, preventing the chance of co-evolution with spadefoot toads.

In contrast, the third tested non-native species, the red-eared slider, has been reported for northern Italy since the 1980s (Ficetola and Scali, 2010), where introduced turtles contributed to the decline of native European pond turtle *Emys orbicularis* (Cadi and Joli, 2004). The recorded responses of

tadpoles to this species' cues may then depend on either the relatively long time of coexistence in introduction areas (ca. three decades) or cue similarity (Sih et al., 2010).

Among native predators, the species eliciting the strongest defensive responses were *Triturus carnifex* and *Pelophylax* sp., both widespread throughout spadefoot toad's range. The Italian crested newt selects temporary ponds as breeding sites (Bernini et al., 2004), while frogs are particularly abundant in lowland areas. Both species are known to predate other amphibians' larvae (Cooke,1974; Paunović et al., 2010). Long-term monitoring revealed that antipredator responses to these two native predators and pond sliders were the strongest shortly after cue exposure and gradually relaxed over time, suggesting that tadpoles continuously adjust their activity rates based on perceived predation risk, balancing the costs of defensive responses (e.g., loss of foraging opportunities).

## Conclusions

Our study provides the first assessment of antipredator behaviour in tadpoles of the endangered Italian lineage of the common spadefoot toad. Across four experiments, tadpoles exhibited weak or null responses to the cues of well-known tadpole predators such as dragonfly larvae and fish, while clear reductions in activity emerged in response to aquatic and semi-aquatic predators sharing the capacity of thriving in temporary waterbodies. Overall, these results suggest that antipredator responses in this species appear to be shaped by the ecological characteristics and predator communities of its breeding sites and are consistent with the need for lowering the impact of non-consumptive effects of the fear of predation (Preisser and Bolnick, 2008; Allen et al., 2022) on spadefoot toad population dynamics. Although the behavioural plasticity of amphibians has been argued to be lower than that of higher vertebrates, making the former less demanding in terms of reintroduction protocols (Griffiths and Pavajeau, 2008), our results confirm that predator-prey relationships and antipredator behaviour should not be disregarded in captive breeding and reintroduction programs. Prey-naiveté towards predators associated with permanent aquatic habitats, such as dragonfly larvae, may explain

previously reported population declines in permanent, fish-free ponds managed as conservation sites (Sindaco et al., 2013).

## Declarations

**Funding** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflict of interest** The authors declare no conflict of interest.

**Ethics approval** Permits to perform this study were obtained from the Italian Ministry of the Environment and Energy Security (prot. 0021745-06/02/2024). All experimental procedures were approved by the ethical committee of the University of Pavia.

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## Figure legends

**Fig.1** Overview of the experimental design used to assess antipredator responses in *Pelobates fuscus* tadpoles. Exp 1 tested ontogenetic variation in responses to odonate cues; Exp 2 examined the effects of prior conditioning and cue concentration; Exp 3 compared responses to different cue types (alarm, disturbance, and predator cues); and Exp 4 evaluated responses to chemical cues released by different predator taxa. Behavioural responses were quantified from video recordings before and after stimulus administration.

**Fig. 2** Experiment 1. Post-stimulus activity responses across stimulus types and sessions (7 and 21 days post-hatching). Bottom panel: Estimated mean proportional activity following exposure to each stimulus (control water, coleopteran, undiluted odonate cue, and 1:10 diluted odonate cue), with 95% confidence intervals (on the response scale). Top panel: Estimated effects (odds ratios) for each stimulus relative to the control within each session, with 95% confidence intervals (on the log-odds scale). Significant differences from the control are indicated where the confidence interval does not include 1 ($p < 0.05$). Estimates are back-transformed from the logit scale.

**Fig. 3** Experiment 2. Post-stimulus activity responses across stimulus types and conditioning (control or odonate exposed) recorded the day after the conditioning period. Bottom panel: Estimated mean proportions of time spent frozen (left panel) and mean distance travelled (right panel) following exposure to each stimulus (control water, undiluted odonate cue), with 95% confidence intervals (on the response scale). Top panel: Estimated effects (odds ratios or differences) for each stimulus relative to the control within each conditioning treatment, with 95% confidence intervals (on the log-odds scale; left panel). Significant differences from the control are indicated where the confidence interval

841 does not include 1 or 0 ($p < 0.05$). Estimates are back-transformed from the logit scale for the time
842 spent frozen.

843

844 **Fig. 4** Experiment 2. Post-stimulus activity responses across stimulus types and conditioning (control
845 or odonate exposed) recorded two days after the conditioning period. Bottom panel: Estimated mean
846 proportions of time spent frozen following exposure to each stimulus (control water, odonate pure
847 cue, 1:10 and 1:100 diluted odonate cue), with 95% confidence intervals (on the response scale). Top
848 panel: Estimated effects (odds ratios) for each stimulus relative to the control within each conditioning
849 treatment, with 95% confidence intervals (on the log-odds scale). Significant differences from the
850 control are indicated where the confidence interval does not include 1 ($p < 0.05$). Estimates are back-
851 transformed from the logit scale for the time spent frozen.

852

853 **Fig. 5** Experiment 2. Post-stimulus activity responses across stimulus types and conditioning (control
854 or odonate exposed) recorded two days after the conditioning period. Bottom panel: Estimated mean
855 distance travelled following exposure to each stimulus (control water, undiluted odonate cue, 1:10
856 diluted odonate cue, and 1:100 diluted odonate cue), with 95% confidence intervals (on the response
857 scale). Top panel: Estimated effects (differences) for each stimulus relative to the control within each
858 conditioning treatment, with 95% confidence intervals. Significant differences from the control are
859 indicated where the confidence interval does not include 0 ($p < 0.05$).

860

861 **Fig. 6** Experiment 3. Post-stimulus activity responses across stimulus types (control, alarm cues,
862 disturbance cues and odonate). Bottom panel: Estimated mean proportions of time spent frozen (left
863 panel) and distance travelled (right panel) following exposure to each stimulus, with 95% confidence
864 intervals (on the response scale). Top panel: Estimated effects shown as odds ratios for time spent

865 frozen (left panel) and mean differences for distance travelled (right panel), relative to the control
866 group, with 95% confidence intervals. Significant differences from the control are indicated where
867 the confidence interval does not include 1 or 0 ($p < 0.05$). Estimates are back-transformed from the
868 logit scale for the time spent frozen (n = 96).

869

870 **Fig. 7** Experiment 4. Post-stimulus activity responses across stimulus types from vertebrate and
871 invertebrate predators. Bottom panel: Estimated mean proportions of time spent frozen (left panel)
872 and mean distance travelled (right panel) following exposure to each stimulus, with 95% confidence
873 intervals (on the response scale). Top panel: Estimated effects shown as odds ratios for time spent
874 frozen (left panel; on the log-odds scale) and mean differences for distance travelled (right panel),
875 with 95% confidence intervals, relative to the control group. Significant differences from the control
876 are indicated where the confidence interval does not include 1 or 0 ($p < 0.05$). Estimates are back-
877 transformed from the logit scale for the time spent frozen (n = 192).

878

879 **Fig. 8** Experiment 4 (temporal dynamics). Predicted tadpole activity over time, expressed as the
880 proportion of active 3-s blocks, for each chemical cue treatment. Lines represent estimated smooths
881 from a generalized additive model (GAMM), and shaded areas indicate 95% confidence intervals.
882 The dashed horizontal line at y = 0.18 represents the baseline level of activity (control condition).
883 Each panel corresponds to a different chemical cue, with a fixed y-axis scale to allow direct visual
884 comparison.

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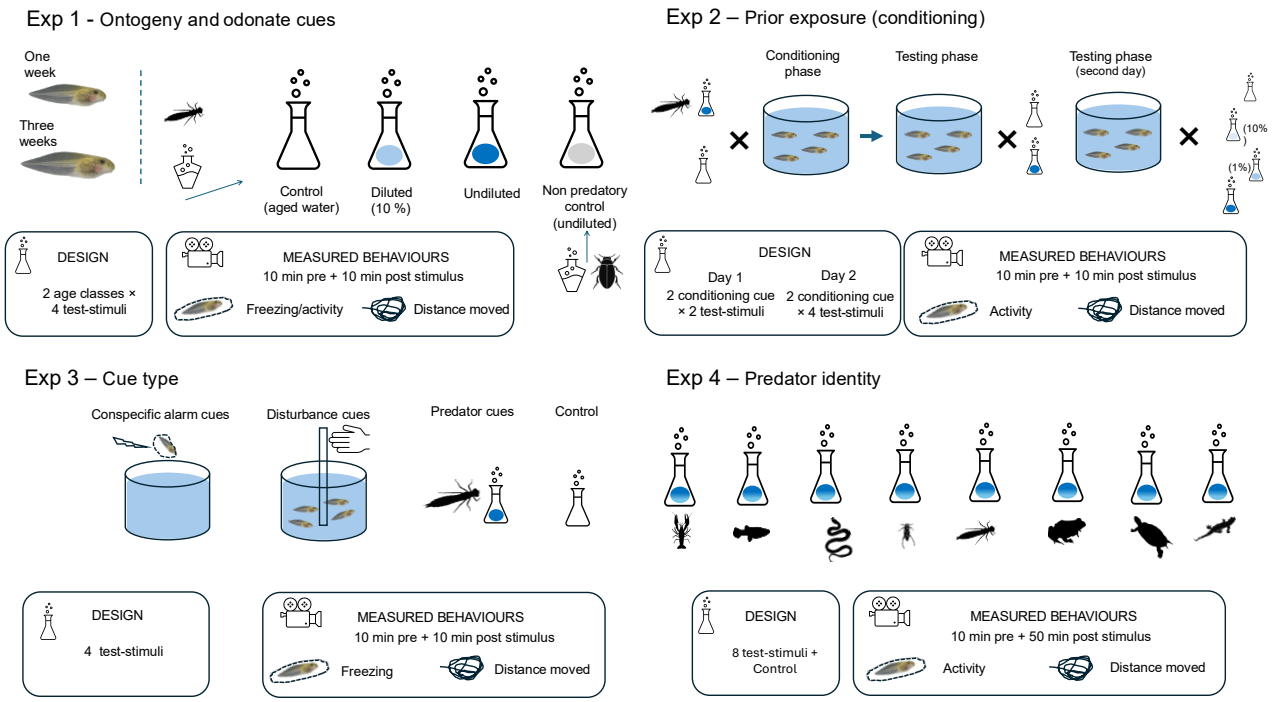
888 **Fig. 1**

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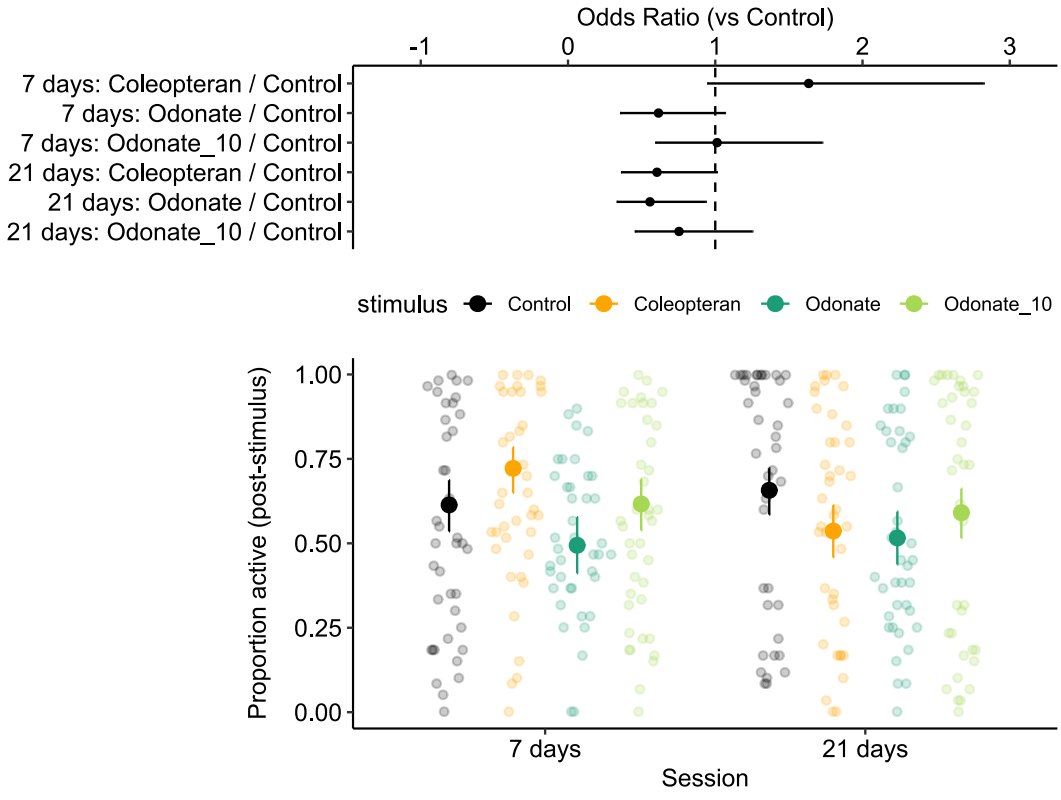


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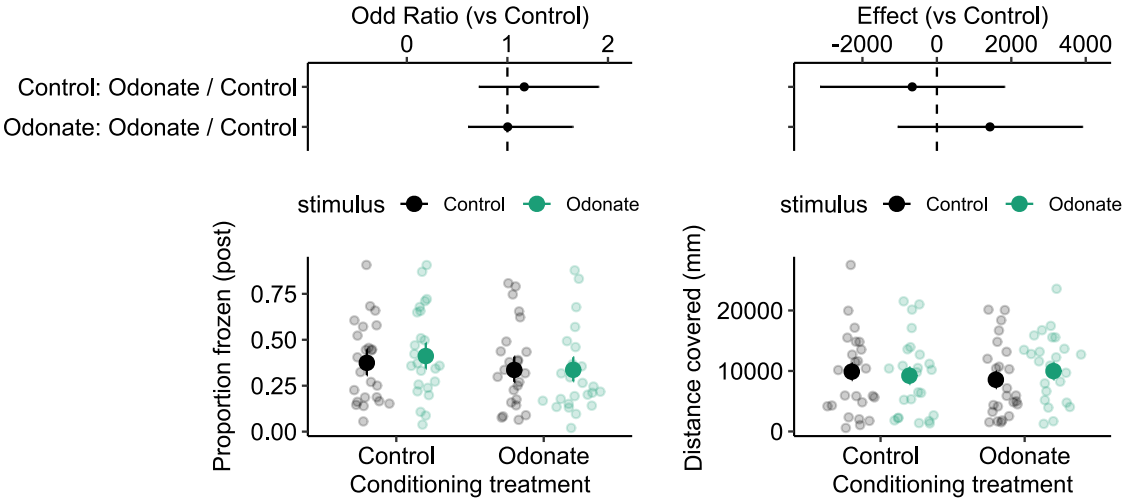
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2
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Effect (vs Control)
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2000
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stimulus
Control
Odonate
Proportion frozen (post)
0.75
0.50
0.25
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Conditioning treatment
Distance covered (mm)
20000
10000
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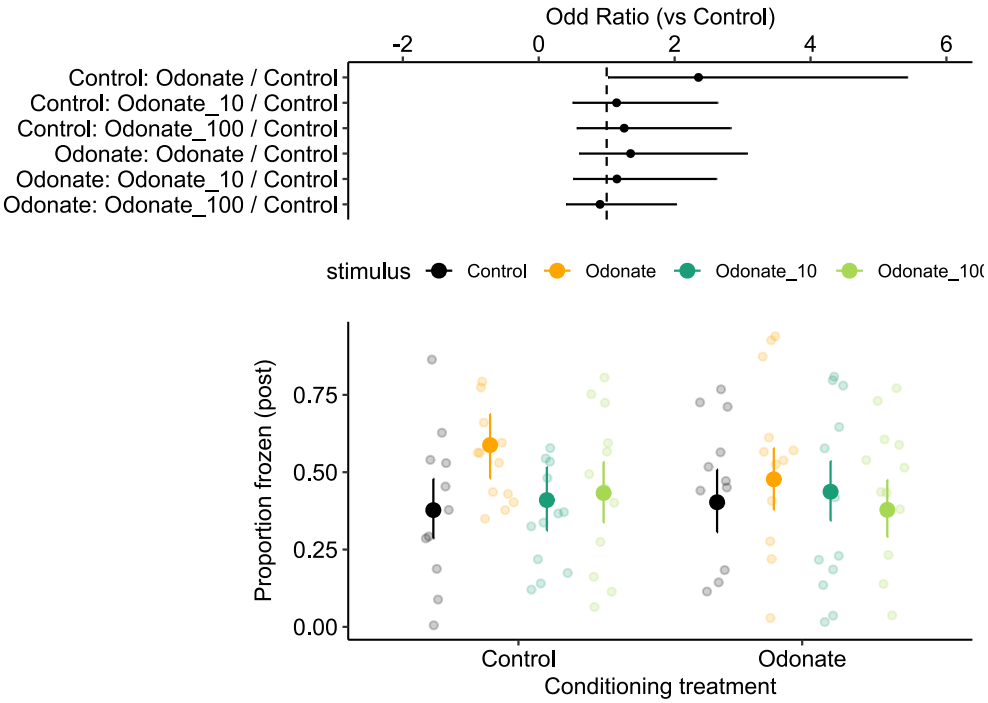
Control
Odonate
Conditioning treatment

**Fig. 4**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Control: Odonate_100 / Control
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Odonate: Odonate_10 / Control
Odonate: Odonate_100 / Control
stimulus
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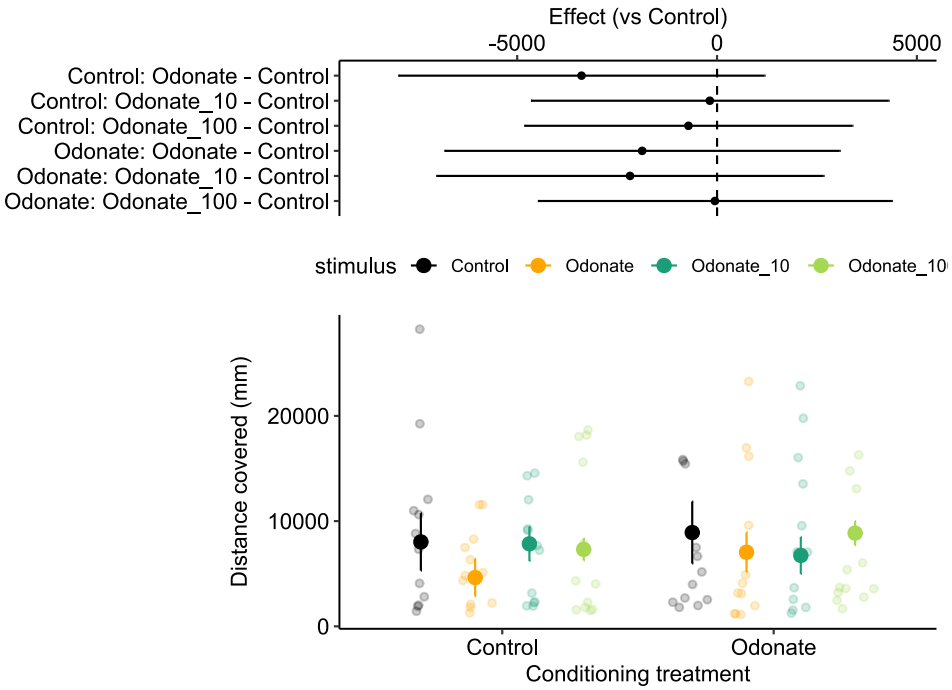
Conditioning treatment

903

904 **Fig. 5**

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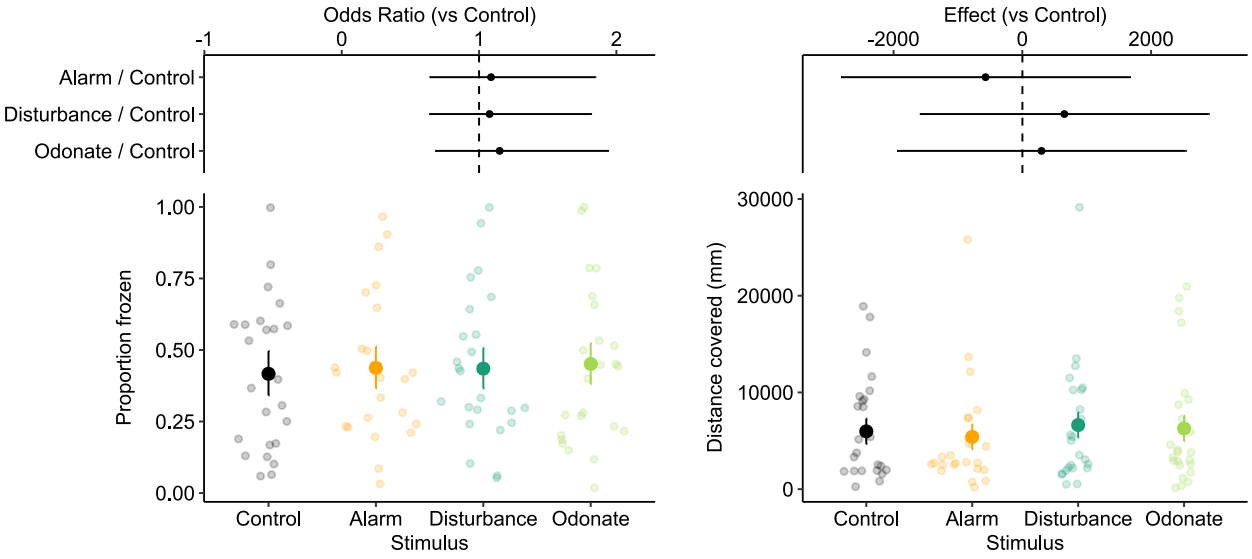
908 **Fig. 6**

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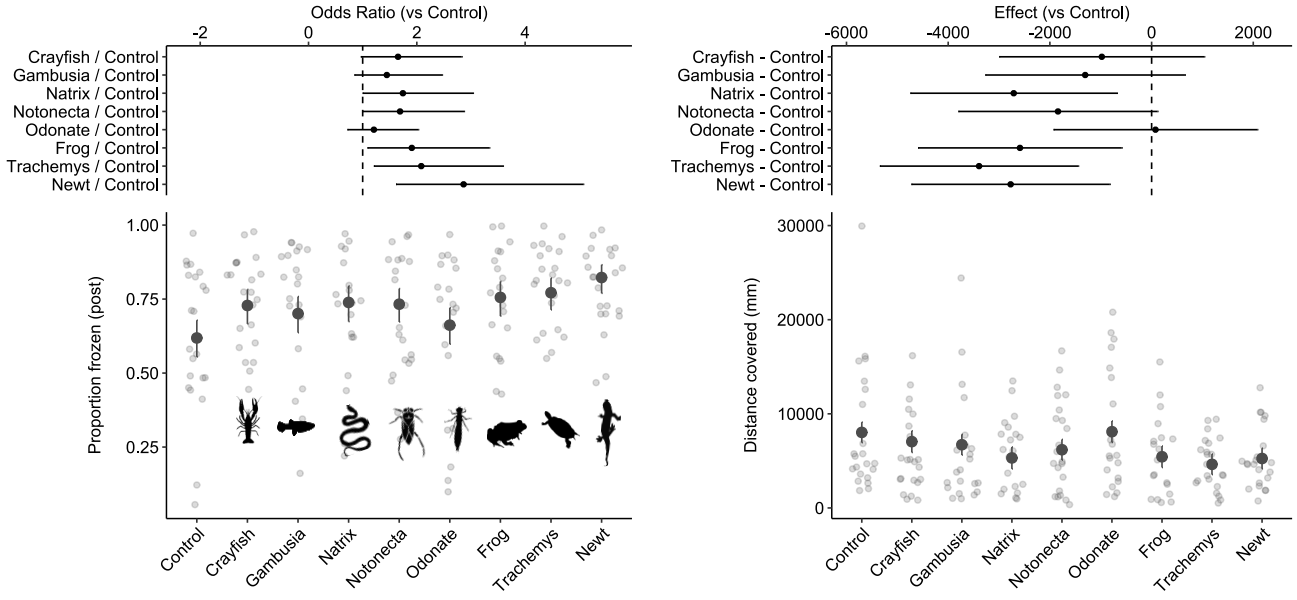


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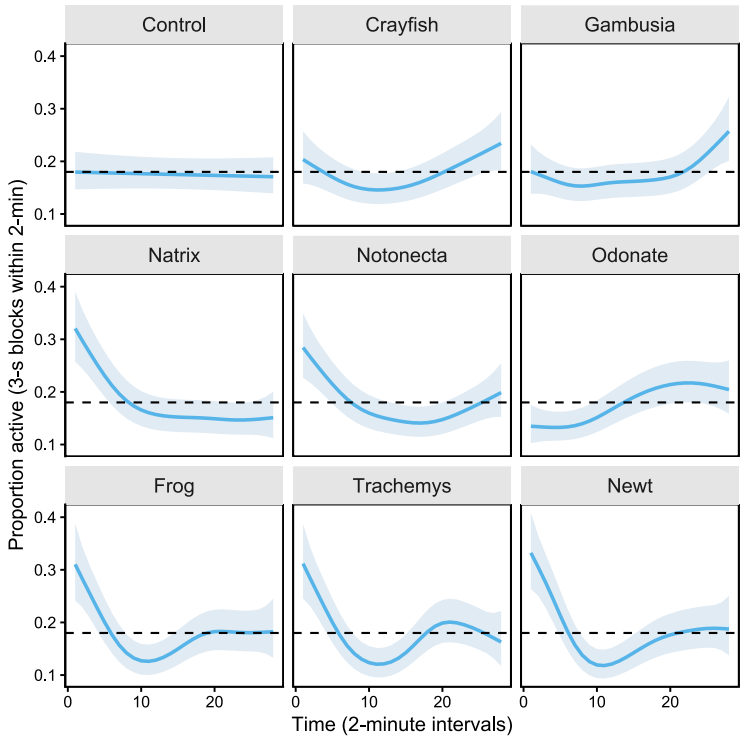
914 **Fig. 8**

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**Supplementary materials**

**Table S1**. Mean body mass (g) and density (number of individuals per container) of predator species used for the preparation of chemical cues. Predator species were maintained in separate containers under controlled conditions prior to cue collection.

| Species | Mean mass (g) | Density (ind./container) |
|---|---|---|
| *Pelophylax* sp. | 11.67 | 3 per tub |
| *Natrix helvetica* | 70.67 | 1 per tub |
| *Procambarus clarkii* | 19.75 | 1 per tub |
| *Anax imperator* | 1.78 | 1 per tub |
| *Trachemys scripta* | 1500 | 1 per tub |
| *Triturus carnifex* | 2.43 | 4 per tub |
| *Notonecta* sp. | 0.40 | 4 per tub |
| *Gambusia holbrooki* | 1.50 | 3 per tub |

**Table S2**. Total biomass (g), water volume (L), and resulting nominal cue concentration (g/L) for each predator species used to generate chemical stimuli. Cue concentrations were not standardized across species, as cue production depends on species-specific traits (e.g., body size, metabolism, and activity). Instead, cue preparation followed species-specific protocols based on previous experiments or established laboratory practices. Therefore, values represent nominal cue concentrations, rather than standardized stimulus intensities across predator types.

| Species | Total mass (g) | Water volume (L) | Cue concentration (g/L) |
|---|---|---|---|
| *Pelophylax* sp. | 35 | 4 | 8.75 |
| *Natrix helvetica* | 212 | 11 | 19.27 |
| *Procambarus clarkii* | 19.75 | 2.5 | 7.9 |
| *Anax imperator* | 1.78 | 0.25 | 7.15 |
| *Trachemys scripta* | 3000 | 20 | 150 |
| *Triturus carnifex* | 8.7 | 2 | 4.35 |
| *Notonecta* sp. | 1.6 | 2 | 0.8 |
| *Gambusia holbrooki* | 4.5 | 2 | 2.25 |

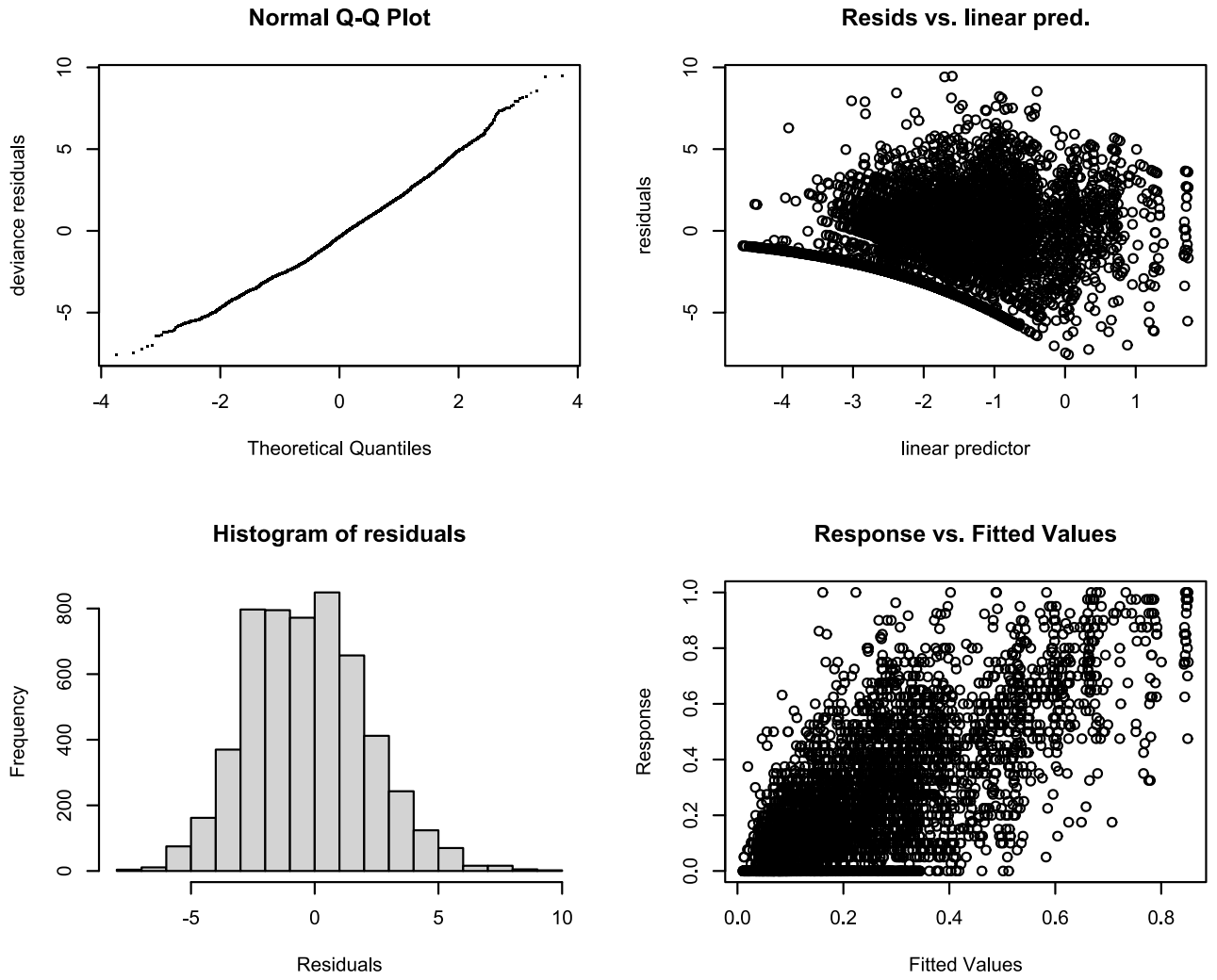
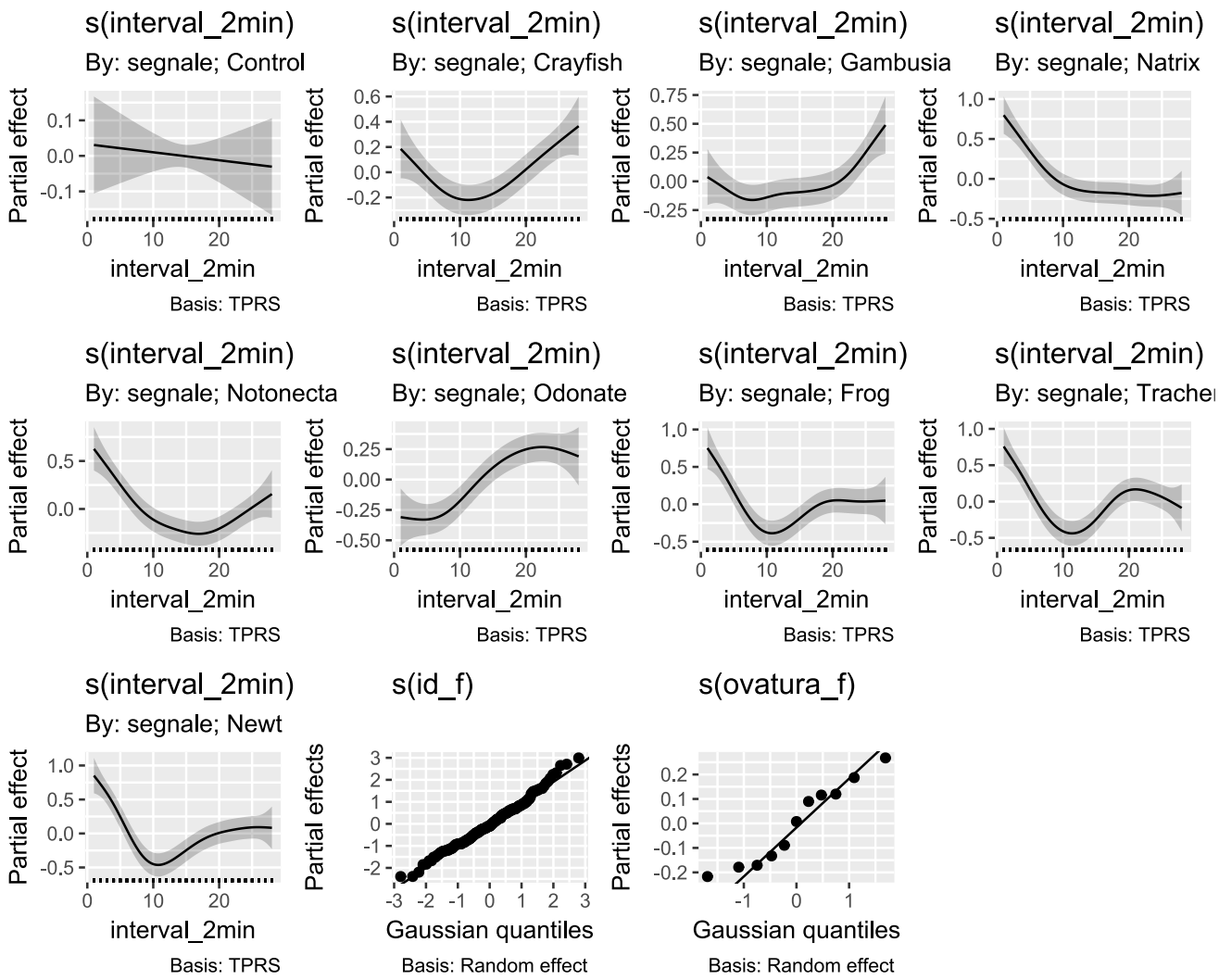



**Figure S1**. Diagnostic plots for the generalized additive model used to analyse tadpole activity over time across predator treatments. Panels show (A) residuals vs. fitted values, (B) QQ-plot of residuals, (C) histogram of residuals, and (D) response vs. fitted values. The model showed acceptable residual patterns with no major violations of assumptions. Smoothing parameter estimates indicated appropriate basis dimensions (all k-index ≈ 0.99, $p > 0.7$), and no signs of overfitting or underfitting were detected.




946

947 **Figure S2**. Estimated smooth terms from the generalized additive model predicting tadpole activity
948 over time across predator treatments. Each panel shows the time-varying effect (on the logit scale)
949 for a different stimulus condition, with shaded areas representing 95% confidence intervals. Curves
950 reflect the predicted change in the proportion of active tadpoles across consecutive 2-minute intervals.
951 Most predator cues elicited non-linear temporal responses, while no significant trend was observed
952 under control conditions.

953

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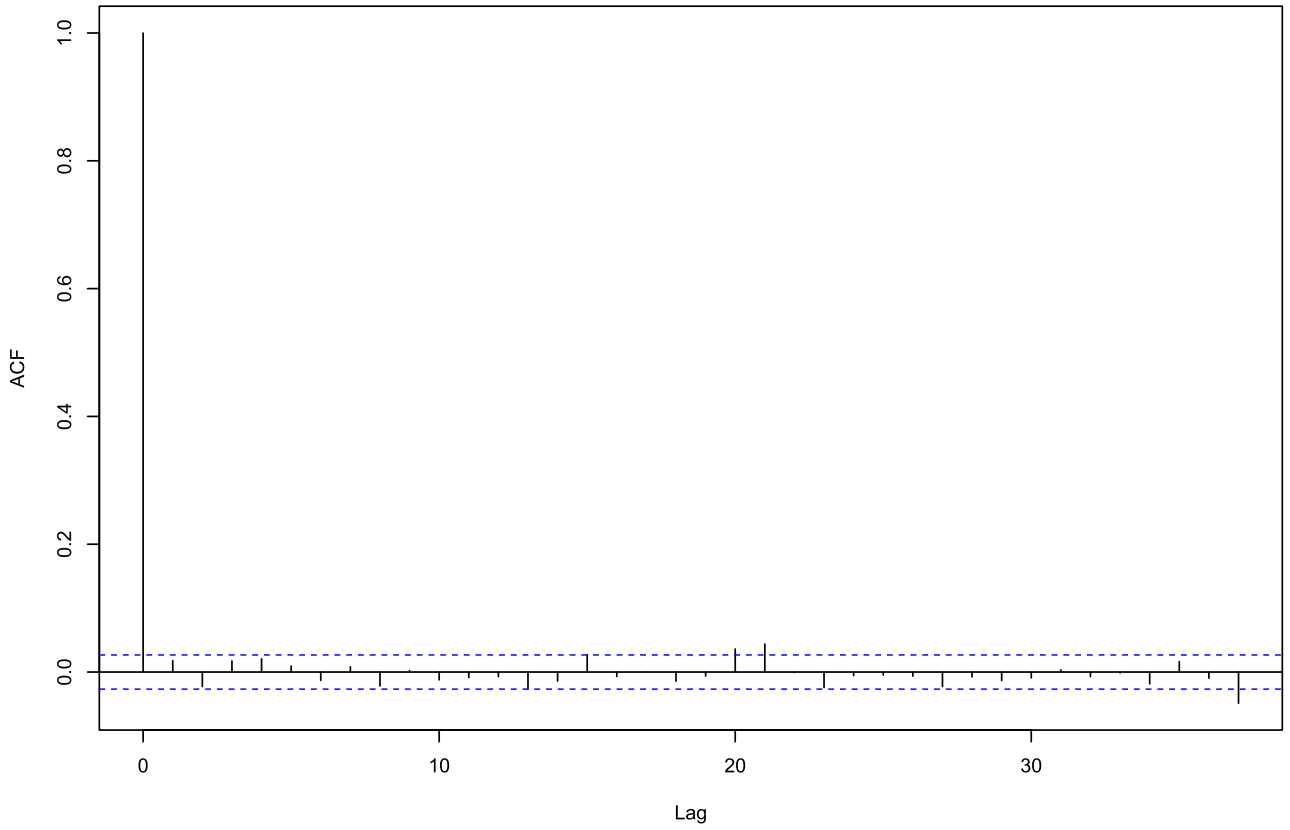


**Figure S3**. Autocorrelation function (ACF) plot of Pearson residuals from the generalized additive model for tadpole activity. No substantial residual autocorrelation is detected, supporting the assumption of independence across observations after accounting for smooth temporal effects and random factors.