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Presence of an alien turtle accelerates hatching of common frog (*Rana temporaria*) tadpoles

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1 Presence of an alien turtle accelerates hatching of common frog (*Rana temporaria*) 2 tadpoles

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12 13 Abstract

14 The presence of a predator affects prey populations either by direct predation or by modifying
15 various parts of their life history. We investigated whether the hatching time, developmental
16 stage, and body size at hatching of common frog (*Rana temporaria*) embryos would alter in the
17 presence of a red-eared slider (*Trachemys scripta elegans*) as a predator. The presence of a
18 predator affected all factors examined. We found that in the absence of the slider, the embryos
19 hatched in 12 days, while hatching was accelerated by two days in slider treatment. At the same
20 time, the embryos hatched smaller and at a lower stage of development with the slider than
21 without it. Our study extends the range of predators studied, including the effect on different
22 phases of development of potential amphibian prey.

24 Introduction

25 The impacts of invasive species on native communities are still difficult to generalise due to the
 26 limited number of species and environments researched (Griesemer et al. 2018; Ramírez
 27 Albores et al. 2019; Rolim et al. 2015; Tricarico et al. 2016). However, inappropriate responses
 28 of individuals to invasive predators can strongly affect native populations (Mooney and Cleland
 29 2001). In amphibians, predation can account for a significant proportion of the total mortality
 30 of all their developmental stages (Gunzburger and Travis 2005; Chivers et al. 2001; Laurila et
 31 al. 2002; Nyström et al. 1997). The ability to detect, recognise, and respond to potential
 32 predators is, therefore, an important part of antipredatory behaviour (Bennett et al. 2013; Polo-
 33 Cavia and Gomez-Mestre 2014), and native populations can have especially serious problems
 34 facing the presence of new alien predators (Gomez-Mestre and Díaz-Paniagua 2011; Nunes et
 35 al. 2019; Polo-Cavia et al. 2010). In general, embryonic and early larval stages are the most
 36 vulnerable to predation (Laurila et al. 2002; Wells 2007), and the ability to respond to the
 37 presence of a predator can therefore significantly increase the fitness of an individual and thus
 38 the viability of the entire population (Vonesh and Bolker 2005; Warkentin 1995).

39 Whether intentionally or unintentionally introduced, the recent wide occurrence of the
 40 red-eared slider (*Trachemys scripta elegans*) in Europe (GISD 2021) presents a new
 41 opportunity to investigate the responses of naive native amphibian populations to a new
 42 predator. Although red-eared slider (hereafter referred to as slider) is not reproductively
 43 successful throughout Europe (Cadi et al. 2004; Ficetola et al. 2009; Mikátová and Šandera
 44 2015; Standfuss et al. 2016), even the mere presence of adults may pose a certain risk to native
 45 species. In previous studies, we found that the presence of the sliders affect several life history
 46 parameters of common frog (*Rana temporaria*) tadpoles, such as movement activity, trajectory
 47 of movement (Berec et al. 2016), time to metamorphosis, or size at metamorphosis (Vodrážková
 48 et al. 2020). Although sliders are usually still hibernating at the time of common frog breeding

(Gibbons et al. 1990; Speybroeck et al. 2016), which eliminates the risk of direct predation, kairomones released by sliders into the aquatic environment provide amphibians with information about their presence. Since the slider is an opportunistic predator and can consume frog eggs (Ernst and Lovich 2009), some response of common frog embryos is to be expected.

For frog embryos, there are two basic strategies for avoiding predation or significantly reducing its effects: the development of egg unpalatability and hatching plasticity (Wells 2007). The unpalatability of eggs is a passive strategy in which the embryo relies on the predator's inability or unwillingness to consume eggs, which imposes costs on its host even if the host never comes in contact with the predator; environmentally cued hatching is characterised by an embryo's active capability to alter the time of hatching according to the conditions it encounters during embryonic development. Hatching plasticity has been documented many times in amphibian embryos, and predator presence has been shown to trigger early hatching from eggs incubated in both air and water (Chivers et al. 2001; Warkentin 2011). In terrestrially laid eggs, hatching can be stimulated by vibrational cues during the direct physical attacks of predators, such as snakes (Jung et al. 2019; Warkentin 1995), frogs (Vonesh and Bolker 2005), katydid (Poo and Bickford 2014), wasps (Warkentin 2000), or egg-eating fly larvae (Vonesh and Bolker 2005). In aquatic environments, these responses are induced mainly by chemical cues from predators (kairomones) or by chemical cues that are released from injured prey during predation events (Dodson 1988; Laurila et al. 2002; Niecieza 1999; 2000; Petranka et al. 1987; Smith and Fortune 2009; Tollrian 1994).

This study aimed to shift our previous focus (Berec et al. 2016; Vodrážková et al. 2020; in review) to a different developmental stage, namely, embryos in eggs. We investigated whether the presence of a slider can alter the hatching time of common frog embryos. We hypothesised that the presence of a slider would accelerate the hatching time, so the ontogenetic stage and body size at hatching were also measured. The uniqueness of this study lies in the use

of a stage-nonspecific predator, which is virtually absent in the literature. At the same time, it is an alien predator from a taxonomic group to which the prey has no common history.

Materials and methods

Five freshly laid clutches of common frogs were collected in a pool between Holubov and Vrábče, South Bohemia, the Czech Republic (48.9078633N, 14.3485608E), on 2 April 2021. Collection locality was monitored daily to collect egg clutches laid during the night before. Neither the slider nor any other species of turtle occurs at the collection locality, so the eggs and their parents are naive prey relative to the turtles. The experiment was performed in six glass tanks – three replications with the sliders and three replications of control. Glass tanks (size: 100 × 55 × 50 cm) filled with 20 cm of aged tap water were equipped with a Claro 300 filter pump (300 L.h⁻¹) and rinsed three times a week. The room temperature was set at 15 °C and the datalogger (Dostman LOG200 PDF) recorded a mean air temperature of 14.8 ± 0.4 °C (± S.D.; measured at hourly intervals) during the experiment. Fluorescent tubes (2 x 36 W) with a light regime of 12 h/12 h were used. During the dark phase of the day, the glass tanks were illuminated with red light to allow permanent monitoring of egg hatching.

Three adult sliders (carapace length: 18 cm, 20 cm, and 21 cm) were used as predators. The slider was placed in each of three glass tanks three days to release kairomones into the water before the experiment was initiated and fed three times a week with ReptoMin Tetra turtle gammarus. To prevent physical but not chemical contact between the slider and frog eggs, a glass barrier was placed inside each glass tank with a 6 cm gap at both ends so that water could flow freely throughout the tank. On the other side of this barrier, five perforated opaque boxes (20 × 14 cm) with holes 1 mm in diameter were glued to the bottom of the glass tanks to contain the eggs (Fig. 1).

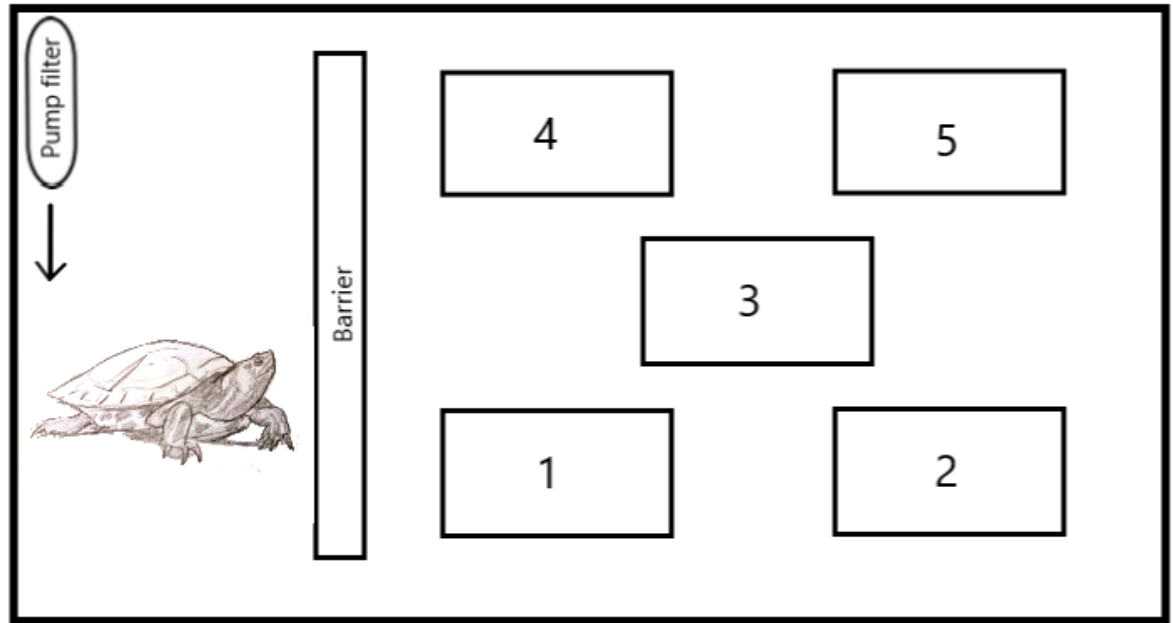


Figure 1.

Diagram of the glass tank showing the position of the slider (if present) and the boxes for clutch samples. These were placed randomly in the boxes in each glass tank (see Materials and methods). Three replications with the sliders and three replications without them (control) were used. Slider drawing by Jakub Berec.

Six samples of approximately 150 eggs each were taken from the collected clutch and randomly placed in six boxes, one in each glass tank. This procedure was repeated for all five clutches, so that there were five boxes in each tank with sample from each clutch. Each glass tank was continuously monitored using a camera (Niceboy Stream Pro). Hatched tadpoles were counted every 24 h. Hatching was defined as the moment at which the whole hatchling had left the protective jelly of the eggs. To maintain a good processing of the camera recordings (the large number of hatched tadpoles in a small box makes it difficult to count them), hatched tadpoles were transferred every six hours to a depot tank. At the time when half of the eggs in each box had hatched, two tadpoles were taken from the group of tadpoles hatched in the last

six hours. These tadpoles were photographed under a stereomicroscope (Olympus SZX 7) and measured (to the nearest 0.01 mm) using QuickPHOTO MICRO 3.2 software. Their developmental phase was determined according to Gosner (1960).

The experiment involved a four factor design (slider: presence/absence, glass tank: 1-3 with slider, 4-6 controls, box: five in each glass tank, and clutch: six samples). The slider was used as a fixed factor as both levels of this factor (presence/absence) were tested. All other three factors (glass tank, clutch, and box) were random (Allen 2017) with the glass tank factor nested in the slider presence/absence factor. According the experimental design, linear mixed model was used for analysis (Quinn and Keough 2002). Three analyses were performed – for hatching time, developmental stage, and the size at hatching. Adjusted R-squared was used as measure of variability explained in the statistical model. Effect sizes were evaluated by partial eta-squared (Richardson 2011). Given the number of eggs, statistical significance was assessed at the 99.9% level (Steel et al. 2013). All calculations were done in Tibco Statistica (TIBCO 2017).

Results

All three models for life history parameters measured were statistically significant (hatching time: $F=688.7$, $p<<0.001$; adjusted $R^2=0.749$; developmental stage: $F=27.1$, $p<<0.001$; adjusted $R^2=0.852$; size at hatching: $F=23.6$, $p<<0.001$; adjusted $R^2=0.833$). For all these parameters, the presence of the slider was the only significant or far most important factor in each model (Supplementary file: partial eta-squared in Tables 1-3).

We found a significant difference in hatching time between the presence and absence of the slider ($F(1,4)=915.0$; $p<<0.001$). In the absence of the slider, embryos hatched in 12 ± 0.6 days (mean \pm S.D.). The presence of the slider accelerated hatching by two days (10 ± 0.6 days) (Fig. 2). Hatching time differed significantly also among glass tanks

(F(1,4)=9.5; $p<0.001$), boxes (F(1,4)=7.6; $p<0.001$) and clutches (F(1,4)=44.3; $p<0.001$), but the effect sizes of these three factors were negligible in comparison to the effect of slider presence (Supplementary file: partial eta-squared in Table 1).

Similarly, significant differences were found between the developmental stage (F(1,4)=4608.0; $p<0.01$) and size (F(1,4)=75.1; $p<0.001$) of freshly hatched embryos in the presence of the slider and without it. In the presence of a slider, embryos hatched at developmental stage 20 ± 1.5 (mean \pm S.D.) with an average size of 5.92 ± 1.460 mm, while in the control, freshly hatched embryos had developed to stage 23 ± 1.0 , with an average size of 10.77 ± 1.042 mm (Fig. 2). Neither developmental stage nor hatching size was significantly affected by glass tank, clutch and boxes (Supplementary file: Tables 2 and 3).

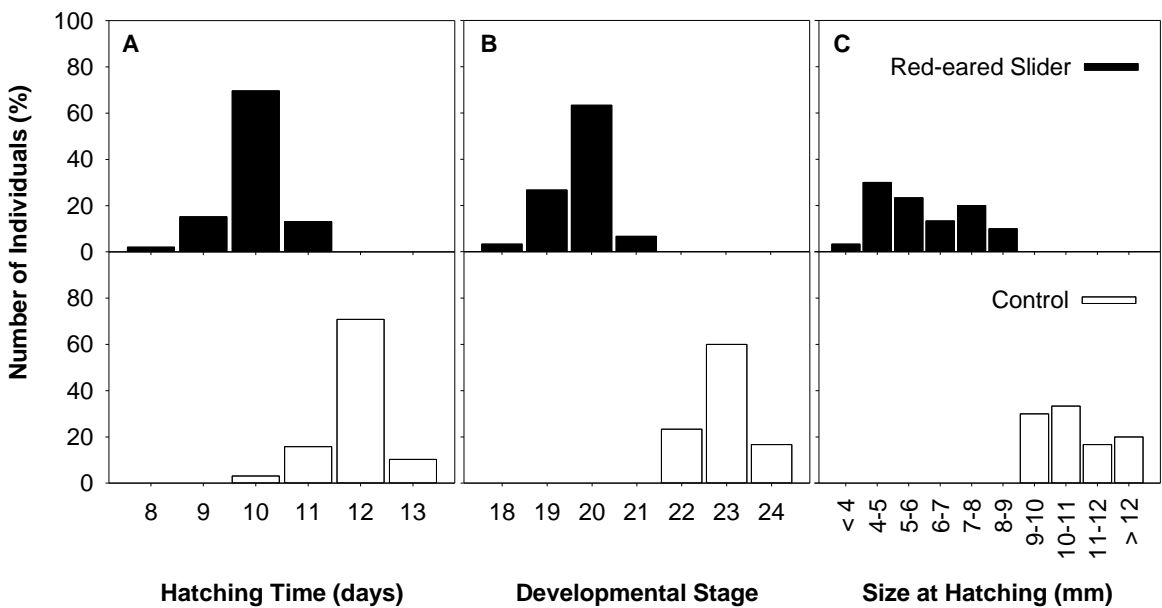


Figure 2.

Histogram of **A** hatching time, **B** Gosner (developmental) stage, and **C** size at hatching of the embryos of common frogs in the presence of red-eared slider and control.

Discussion

Developmental plasticity is an adaptive response of anuran embryos and larvae to the risk of predation (Altig and McDiarmid 1999; Benard 2004; Warkentin 2011). Here, we present evidence for the developmental plasticity of common frog embryos in the presence of a red-eared slider and, in addition to a previous study (Vodrážková et al. 2020), provide a comprehensive insight of the influence of this alien predator on the early phases of the common frog life cycle. We have previously shown (Vodrážková et al. 2020) that, in the slider presence, tadpoles of common frogs are able to modify the duration of larval development. In the present study, we confirmed a similar response in common frog embryos, which hatched earlier in the presence of a slider. At the same time, the embryos were smaller and less developed when exposed to the chemical signals of a predator. We also found the effect of glass tank, clutch and box on hatching time, which was nevertheless negligible in comparison with the effect of predator presence.

In the presence of stage-specific predators, amphibians can modify the duration of the relevant developmental stage (Chivers et al. 2001; Ireland et al. 2007; Mitchell et al. 2017). In anuran embryos, specifically, the presence of egg predators has mostly been shown to induce early hatching of embryos (Chivers et al. 2001; Johnson et al. 2003; Laurila et al. 2001; Segev et al. 2015; Warkentin 1995; 2000), while tadpole predators induce delayed hatching (Laurila et al. 2002; Mitchell et al. 2017; Schalk et al. 2002; Sih and Moore 1993), thus increasing their chance of survival by escaping possible attacks. However, the slider is not a stage-specific predator, as it is capable of consuming both amphibian eggs and larvae (Brown et al. 1995; Ernst and Lovich 2009; Chen 2006); thus, the allocation of risk between developmental stages of the frog may be more complex in this case (Warkentin 2011). Studies examining predator effects on the developmental rates of both eggs and larvae are rare because few predators consume both eggs and larvae simultaneously. Muraro et al. (2021) used a stage-nonspecific predator (*Procambarus clarkii*) and found, in concordance with our results, a reduction in

hatching time in *Rana latastei* embryos. However, they did not study larval development. Ireland et al. (2007) solved the problem of predator stage specificity by simultaneously exposing frog eggs to stage-specific predators of eggs (leech: *Nephelopsis obscura*) and larvae (dragonfly: *Aeshna canadensis* nymphs), which resulted in no change in hatching time, whereas tests with separately acting predators produced the expected response of a reduction in hatching time in the egg predator treatment and an increase in hatching time in the larval predator treatment. This study on embryos and a previous study on tadpoles (Vodrážková et al. 2020) jointly clarify that the embryos/tadpoles of the common frog responded to the presence of a predator by shortening the stage of development during which the embryo/tadpole would be exposed to the predator. It would be interesting to analyse how common frog tadpoles react to the presence of a slider if the entire development from eggs to metamorphosis was taking place with this predator present.

However, some studies have shown that frog embryos, including the common frog, do not always respond specifically to stage-specific predators by shortening hatching time (Capellán and Nicieza 2010; Laurila et al. 2001; Laurila et al. 2002; Saglio and Mandrillon 2006; Schalk et al. 2002; Touchon et al. 2006; Touchon and Wojdak 2014). The published differences in embryo responses may correspond to different signal intensities of the presence of a specific predator, and thus, the responses to indirect waterborne cues might be weaker than those to the direct, mechanical cues of a predator attack (Warkentin 2011). An evident response to water-borne cues of sliders may be related to a markedly stronger signal of a much larger-sized predator in our experiment compared to commonly tested invertebrate predators. The ability to scale predator danger and adjust hatching time accordingly has been found, for example, in embryos of southern leopard frogs (*Lithobates sphenoccephalus*) (Johnson et al. 2003). Moreover, a possible absence of a change in hatching time does not necessarily imply a complete lack of response to the presence of a predator. It may be manifested by other types of

responses, such as changes in the body shape of tadpoles (Laurila et al. 2001; Mandrillon and Saglio 2007; Saglio and Mandrillon 2006; Touchon and Wojdak 2014) or their behaviour (Saglio and Mandrillon 2006; Touchon and Wojdak 2014).

Native and naive prey may fail to detect the novel predator adequately as a dangerous threat, resulting in no (Cox and Lima 2006; Sih et al. 2010) or inefficient antipredator responses to counter the predator's attack strategies (Sih et al. 2010; Strauss et al. 2006). However, when responses in hatching time in naive prey are detected, they are often explained by the presence of syntopic, taxonomically related predators (Melotto et al. 2021; Muraro et al. 2021; Sih et al. 2010), although the time since invasion may also play an important role (Gomez-Mestre and Díaz-Paniagua 2011; Nunes et al. 2013). Our results suggested that a common evolutionary history is not necessary for a detectable response. Such a result has already been published for tadpole development time (Stav et al. 2007; Vodrážková et al. 2020), but as far as we know, it has not yet been published for hatching time in frog embryos. An explanation for embryo response to an alien slider may be in the ability of embryos to detect a kind of general "smell of fear" that is elicited by most predators, regardless of taxonomic classification (Sih et al. 2010).

Finding a general tendency in the phenotypic plasticity responses of prey across a broad range of animal predators (different taxa and feeding spectra), environmental and experimental conditions is a challenge even in anurans themselves (Relyea et al. 2018). However, in frogs, the earlier hatching time was generally associated with smaller size at hatching (Capellán and Nicieza 2007; Chivers et al. 2001; Ireland et al. 2007; Laurila et al. 2002) and lower developmental stage (Capellán and Nicieza 2007; Chivers et al. 2001; Ireland et al. 2007; Laurila et al. 2002; Muraro 2021), and our results confirm this relationship. In some cases, earlier hatched tadpoles performed higher growth rate and reached the size of later hatched tadpoles at metamorphosis (Capellán and Nicieza 2007). However, if tadpoles are unable to

compensate for their smaller size at hatching, this can impose significant costs in later developmental phases. These costs have been demonstrated in increased mortality during the larval stage (Smith 1987; Warkentin 1995; but see Vonesh and Bolker (2005) where early hatchlings survived better), reduced size at metamorphosis (Vonesh and Bolker 2005; Vodrážková et al. 2020), lower post-metamorphic survival (Altwegg and Reyer 2003; Berven 1990), change of behaviour (Buckley et al. 2005, Capellán and Nicieza 2007), delayed maturity (Smith 1987) and lower reproductive success (Smith 1987).

Our work added a slider as an additional predator inducing changes in the embryonic developmental rate in Ranidae. Since the impact of earlier embryo hatching (lower body size and lower stage of development) on fitness has been confirmed in several frog species (Laurila et al. 2002; Touchon et al. 2013; Vonesh and Bolker 2005; Warkentin 1995), the same impact can be expected for the common frog. The existence of defensive responses in slider-exposed embryos may reduce the threat that poses the spreading of this invasive species in Europe. On the other hand, the reduced size at hatching and developmental stage of common frog hatchlings represents additional risks of negative fitness impacts, and at the very least, the presence of sliders in non-native areas should receive increased attention.

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All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Czech Ministry of Agriculture, Department of Animal Welfare according to article No. 15, section 2 of the act registered under number 9103/2009-17210.

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