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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.

23

## 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

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

53 For frog embryos, there are two basic strategies for avoiding predation or significantly  
54 reducing its effects: the development of egg unpalatability and hatching plasticity (Wells 2007).  
55 The unpalatability of eggs is a passive strategy in which the embryo relies on the predator's  
56 inability or unwillingness to consume eggs, which imposes costs on its host even if the host  
57 never comes in contact with the predator; environmentally cued hatching is characterised by an  
58 embryo's active capability to alter the time of hatching according to the conditions it encounters  
59 during embryonic development. Hatching plasticity has been documented many times in  
60 amphibian embryos, and predator presence has been shown to trigger early hatching from eggs  
61 incubated in both air and water (Chivers et al. 2001; Warkentin 2011). In terrestrially laid eggs,  
62 hatching can be stimulated by vibrational cues during the direct physical attacks of predators,  
63 such as snakes (Jung et al. 2019; Warkentin 1995), frogs (Vonesh and Bolker 2005), katydids  
64 (Poo and Bickford 2014), wasps (Warkentin 2000), or egg-eating fly larvae (Vonesh and Bolker  
65 2005). In aquatic environments, these responses are induced mainly by chemical cues from  
66 predators (kairomones) or by chemical cues that are released from injured prey during predation  
67 events (Dodson 1988; Laurila et al. 2002; Niecieza 1999; 2000; Petranka et al. 1987; Smith and  
68 Fortune 2009; Tollrian 1994).

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

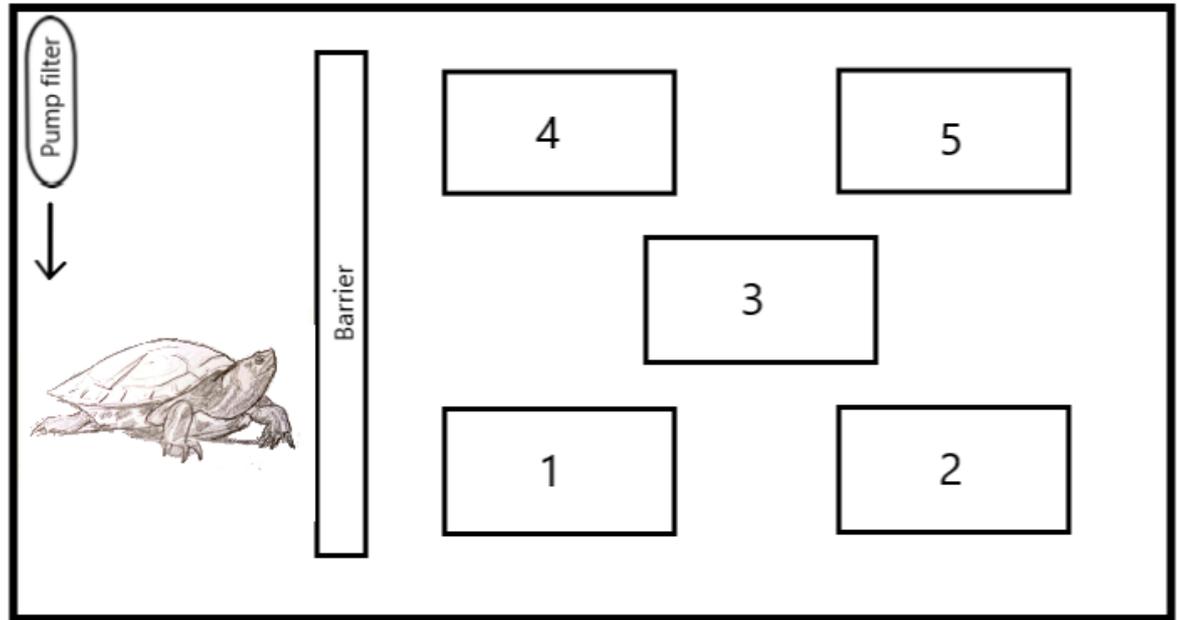
74 of a stage-nonspecific predator, which is virtually absent in the literature. At the same time, it  
75 is an alien predator from a taxonomic group to which the prey has no common history.

76

## 77 **Materials and methods**

78 Five freshly laid clutches of common frogs were collected in a pool between Holubov and  
79 Vrábče, South Bohemia, the Czech Republic (48.9078633N, 14.3485608E), on 2 April 2021.  
80 Collection locality was monitored daily to collect egg clutches laid during the night before.  
81 Neither the slider nor any other species of turtle occurs at the collection locality, so the eggs  
82 and their parents are naive prey relative to the turtles. The experiment was performed in six  
83 glass tanks – three replications with the sliders and three replications of control. Glass tanks  
84 (size: 100 × 55 × 50 cm) filled with 20 cm of aged tap water were equipped with a Claro  
85 300 filter pump (300 L.h<sup>-1</sup>) and rinsed three times a week. The room temperature was set at  
86 15 °C and the datalogger (Dostman LOG200 PDF) recorded a mean air temperature of 14.8 ±  
87 0.4 °C (± S.D.; measured at hourly intervals) during the experiment. Fluorescent tubes (2 x  
88 36 W) with a light regime of 12 h/12 h were used. During the dark phase of the day, the glass  
89 tanks were illuminated with red light to allow permanent monitoring of egg hatching.

90 Three adult sliders (carapace length: 18 cm, 20 cm, and 21 cm) were used as predators.  
91 The slider was placed in each of three glass tanks three days to release kairomones into the  
92 water before the experiment was initiated and fed three times a week with ReptoMin Tetra turtle  
93 gammarus. To prevent physical but not chemical contact between the slider and frog eggs, a  
94 glass barrier was placed inside each glass tank with a 6 cm gap at both ends so that water could  
95 flow freely throughout the tank. On the other side of this barrier, five perforated opaque boxes  
96 (20 × 14 cm) with holes 1 mm in diameter were glued to the bottom of the glass tanks to contain  
97 the eggs (Fig. 1).



98

99 **Figure 1.**

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

104

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

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

117 The experiment involved a four factor design (slider: presence/absence, glass tank: 1-3  
 118 with slider, 4-6 controls, box: five in each glass tank, and clutch: six samples). The slider was  
 119 used as a fixed factor as both levels of this factor (presence/absence) were tested. All other three  
 120 factors (glass tank, clutch, and box) were random (Allen 2017) with the glass tank factor nested  
 121 in the slider presence/absence factor. According the experimental design, linear mixed model  
 122 was used for analysis (Quinn and Keough 2002). Three analyses were performed – for hatching  
 123 time, developmental stage, and the size at hatching. Adjusted R-squared was used as measure  
 124 of variability explained in the statistical model. Effect sizes were evaluated by partial eta-  
 125 squared (Richardson 2011). Given the number of eggs, statistical significance was assessed at  
 126 the 99.9% level (Steel et al. 2013). All calculations were done in Tibco Statistica (TIBCO  
 127 2017).

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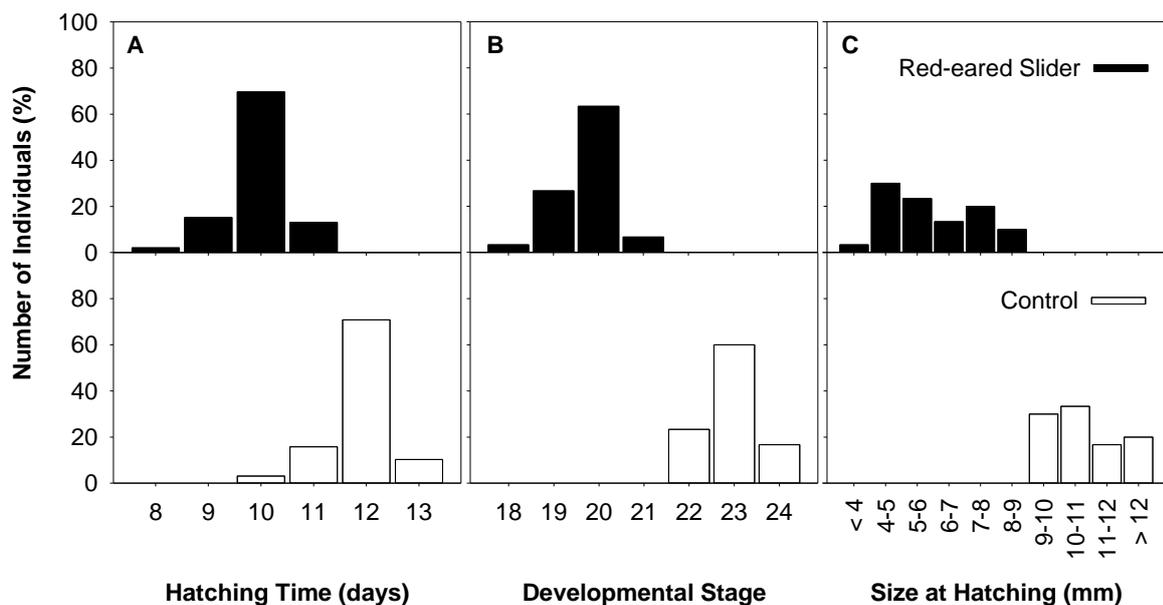
## 129 **Results**

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

135 We found a significant difference in hatching time between the presence and absence of  
 136 the slider ( $F(1,4)=915.0$ ;  $p<<0.001$ ). In the absence of the slider, embryos hatched in  
 137  $12 \pm 0.6$  days (mean  $\pm$  S.D.). The presence of the slider accelerated hatching by two days  
 138 ( $10 \pm 0.6$  days) (Fig. 2). Hatching time differed significantly also among glass tanks

139 (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),  
 140 but the effect sizes of these three factors were negligible in comparison to the effect of slider  
 141 presence (Supplementary file: partial eta-squared in Table 1).

142 Similarly, significant differences were found between the developmental stage  
 143 (F(1,4)=4608.0; p<0.01) and size (F(1,4)=75.1; p<0.001) of freshly hatched embryos in the  
 144 presence of the slider and without it. In the presence of a slider, embryos hatched at  
 145 developmental stage  $20 \pm 1.5$  (mean  $\pm$  S.D.) with an average size of  $5.92 \pm 1.460$  mm, while in  
 146 the control, freshly hatched embryos had developed to stage  $23 \pm 1.0$ , with an average size of  
 147  $10.77 \pm 1.042$  mm (Fig. 2). Neither developmental stage nor hatching size was significantly  
 148 affected by glass tank, clutch and boxes (Supplementary file: Tables 2 and 3).



149 **Figure 2.**  
 150 Histogram of **A** hatching time, **B** Gosner (developmental) stage, and **C** size at hatching of the  
 151 embryos of common frogs in the presence of red-eared slider and control.  
 152

153

154 **Discussion**

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

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

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

192 However, some studies have shown that frog embryos, including the common frog, do  
 193 not always respond specifically to stage-specific predators by shortening hatching time  
 194 (Capellán and Nicieza 2010; Laurila et al. 2001; Laurila et al. 2002; Saglio and Mandrillon  
 195 2006; Schalk et al. 2002; Touchon et al. 2006; Touchon and Wojdak 2014). The published  
 196 differences in embryo responses may correspond to different signal intensities of the presence  
 197 of a specific predator, and thus, the responses to indirect waterborne cues might be weaker than  
 198 those to the direct, mechanical cues of a predator attack (Warkentin 2011). An evident response  
 199 to water-borne cues of sliders may be related to a markedly stronger signal of a much larger-  
 200 sized predator in our experiment compared to commonly tested invertebrate predators. The  
 201 ability to scale predator danger and adjust hatching time accordingly has been found, for  
 202 example, in embryos of southern leopard frogs (*Lithobates sphenoccephalus*) (Johnson et al.  
 203 2003). Moreover, a possible absence of a change in hatching time does not necessarily imply a  
 204 complete lack of response to the presence of a predator. It may be manifested by other types of

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

208 Native and naive prey may fail to detect the novel predator adequately as a dangerous  
209 threat, resulting in no (Cox and Lima 2006; Sih et al. 2010) or inefficient antipredator responses  
210 to counter the predator's attack strategies (Sih et al. 2010; Strauss et al. 2006). However, when  
211 responses in hatching time in naive prey are detected, they are often explained by the presence  
212 of syntopic, taxonomically related predators (Melotto et al. 2021; Muraro et al. 2021; Sih et al.  
213 2010), although the time since invasion may also play an important role (Gomez-Mestre and  
214 Díaz-Paniagua 2011; Nunes et al. 2013). Our results suggested that a common evolutionary  
215 history is not necessary for a detectable response. Such a result has already been published for  
216 tadpole development time (Stav et al. 2007; Vodrážková et al. 2020), but as far as we know, it  
217 has not yet been published for hatching time in frog embryos. An explanation for embryo  
218 response to an alien slider may be in the ability of embryos to detect a kind of general "smell  
219 of fear" that is elicited by most predators, regardless of taxonomic classification (Sih et al.  
220 2010).

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

230 compensate for their smaller size at hatching, this can impose significant costs in later  
231 developmental phases. These costs have been demonstrated in increased mortality during the  
232 larval stage (Smith 1987; Warkentin 1995; but see Vonesh and Bolker (2005) where early  
233 hatchlings survived better), reduced size at metamorphosis (Vonesh and Bolker 2005;  
234 Vodrážková et al. 2020), lower post-metamorphic survival (Altwegg and Reyer 2003; Berven  
235 1990), change of behaviour (Buckley et al. 2005, Capellán and Nicieza 2007), delayed maturity  
236 (Smith 1987) and lower reproductive success (Smith 1987).

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

246

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250 All methods were carried out in accordance with relevant guidelines and regulations. All  
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252 Animal Welfare according to article No. 15, section 2 of the act registered under number  
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