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1 **Exposure to low concentrations of AMPA influences morphology and**
2 **decreases survival during larval development in a widespread**
3 **amphibian species.**

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13

14 **Abstract**

15

16 Glyphosate's primary metabolite, AMPA (aminomethylphosphonic acid), is one of
17 the most widely detected anthropogenic substance in surface waters worldwide.
18 However, ecotoxicological studies on the potential effects of this metabolite at
19 environmental concentrations on wildlife are scarce. Yet, due to its chemical
20 properties, AMPA is likely to affect non-target species. In this study we investigated
21 sublethal effects of environmental concentrations of AMPA on the larval
22 development of a widespread amphibian species, the spined toad *Bufo spinosus*. We
23 performed a factorial experiment to study the effect of concentration and the timing
24 of exposure (during embryonic development, larval development or both) to AMPA
25 on the morphology, rate of development and survival of tadpoles. AMPA and
26 timing of exposure interactively affected tadpole size (individuals exposed to AMPA
27 after hatching were transiently smaller, while individuals exposed to AMPA before
28 hatching were longer), but not duration of development. Most of these effects were
29 linked to exposure during embryonic development. Such effects in individuals
30 exposed during embryonic development solely were long-lasting and persisted until
31 the latest larval stages. Finally, we found that exposure to AMPA after hatching
32 (during the larval stage) increased mortality. Exposure to low environmental
33 concentrations of AMPA could have long lasting consequences on fitness and
34 population persistence. These findings are especially important to take into account
35 at a time when multiple threats can interact to affect wildlife.

36

37 **Key-words** : Aminomethylphosphonic acid – Tadpoles - Development - Sublethal
38 effects – Survival

39

40 **Introduction**

41

42 Modern agricultural practices have intensified in recent years despite international
43 concerns on potential consequences to biodiversity (Brooks et al. 2002; Stoate et al.
44 2009). For instance, intensive agriculture can affect biodiversity through the
45 widespread use of agrochemicals, notably through subtle sublethal effects (Agostini
46 et al. 2020; Garcês et al. 2020). Although designed to be toxic for specific taxa,
47 pesticides can also affect non-target species (Isidori et al. 2005; Relyea 2009;
48 Hasenbein et al. 2017; de Brito Rodrigues et al. 2019).

49

50 The effects of agrochemicals on non-target species are being increasingly investigated
51 (Baier et al. 2016a; Hasenbein et al. 2017; Thiour-Mauprivez et al. 2019), and can
52 influence wildlife through both acute and chronic exposures (Bernabò et al. 2008;
53 Bókony et al. 2017; Hackenberger et al. 2018). Environmental concentrations are
54 often very low as compared to the values tested under laboratory conditions on
55 model species which define standards of use (e.g., based on LD50, Beiras, 2018;
56 Kramer et al., 2009). As a consequence, although these low, environmental
57 concentrations are usually disregarded by regulatory toxicity studies, an increasing
58 body of literature has highlighted their negative effects through several mechanisms
59 affecting DNA structure (Guilherme et al. 2010; Matozzo et al. 2019), oxidative status
60 (Isaksson 2010; Pašková et al. 2011; Lushchak 2016), physiology (Hayes et al. 2006;

61 Leemans et al. 2019; Muñoz et al. 2021), development (Relyea 2009; Lenkowski et al.
62 2010; Wang et al. 2019), morphology (Baier et al. 2016b; Cheron and Brischoux 2020)
63 or behaviour (Brunelli et al. 2009; Hellou 2011; Browne and Moore 2014), all of which
64 can ultimately influence individual survival (Baker et al. 2013; Herek et al. 2020)
65 and/or reproduction (Costantini et al. 2014; Hackenberger et al. 2018; Adams et al.
66 2021) and thus population persistence (Blaustein et al. 2011; Hamilton et al. 2016).

67

68 Despite the growing interest of the scientific community to assess the effects of
69 agrochemicals on wildlife, most studies have focused on the active ingredients
70 (Mesnage and Antoniou 2018). Yet, in natural conditions, most pesticides degrade
71 due to contact with air or water (Kamrin 1997), exposure to sunlight (Fenoll et al.
72 2012) and/or because of the activity of microbiota (Sviridov et al. 2015). As a
73 consequence, our knowledge is often limited to the effects of the parent compounds
74 (i.e., the listed active substances), and the potential effects of their metabolites has
75 been overlooked to date (de Brito Rodrigues et al., 2019; EFSA, 2012; Lee et al., 2017).

76

77 Such bias is exemplified by one of the most famous herbicides, glyphosate, and its
78 primary metabolite, aminomethylphosphonic acid (AMPA). Glyphosate remains one
79 of the most widely used non-selective herbicides despite the intense scientific and
80 societal debates regarding its toxicity (Helander et al. 2012; Clapp 2021).

81 Accordingly, the overall effects of glyphosate on wildlife, including investigations of
82 commercial formulations and/or of different surfactants have been relatively
83 thoroughly studied (Solomon and Thompson 2003; Cauble and Wagner 2005;

84 Lenkowski et al. 2010; Guilherme et al. 2010; Giaquinto et al. 2017; Bókony et al. 2017;
85 Berger et al. 2018; Gill et al. 2018; de Brito Rodrigues et al. 2019; Matozzo et al. 2019;
86 Herek et al. 2020; Muñoz et al. 2021). Yet, as mentioned above, its primary
87 metabolite, AMPA is arguably the most common compound found in surface and
88 groundwater worldwide (Grandcoin et al. 2017; Bonansea et al. 2017; Okada et al.
89 2020; Medalie et al. 2020). The greater occurrence and abundance of AMPA in
90 natural environments is likely linked to the extensive use of glyphosate and also
91 because AMPA is an organic phosphonate derived from water treatment facilities,
92 textile industries, and industrial or household detergents (Grandcoin et al. 2017).
93 Because AMPA has a longer half-life than glyphosate in water (Battaglin et al. 2014;
94 Bonansea et al. 2017; Silva et al. 2018), it tends to accumulate in aquatic habitats from
95 contaminated environments via runoff or soil erosion (Solomon and Thompson
96 2003). Despite these multiple sources of AMPA, studies on the effects of AMPA at
97 environmental concentrations on wildlife are comparatively fewer (Guilherme et al.
98 2014; Domínguez et al. 2016; Martinez and Al-Ahmad 2019; de Brito Rodrigues et al.
99 2019; Matozzo et al. 2019; Cheron and Brischoux 2020). Because of such a
100 discrepancy, it is crucial to collect data on the effects of AMPA on wildlife if we are
101 to provide a thorough assessment of the potential impacts of this global contaminant
102 on biodiversity (Hahn and Sadler 2020).

103

104 In this study, we assessed the effects of exposure to environmental concentrations of
105 AMPA on the development of a widespread amphibian species (spined toad, *Bufo*
106 *spinosus*). Amphibians are particularly susceptible to the presence of pesticides in

107 surface waters (Mann et al. 2009; Quaranta et al. 2009; Hayes et al. 2010). First, in
108 temperate areas, breeding periods often coincide with the use of agrochemicals
109 (Berger et al. 2013; Lenhardt et al. 2015). Second, many amphibians have biphasic life
110 cycles, with terrestrial adults breeding in aquatic sites where eggs and larvae
111 develop (Reading et al. 1991). As a consequence, breeding occurs in lentic systems
112 where concentrations of pesticides can be relatively elevated due to stagnation of
113 water (Battaglin et al. 2009). Third, the skin of aquatic or semi-aquatic amphibian are
114 characterized by high permeability because it is actively involved in gas exchanges
115 and regulation of internal concentration of water and ions (Uchiyama and Konno
116 2006; Brischoux et al. 2021). Moreover, surface area to volume ratio is elevated in
117 amphibians compared to other species which influences the transcutaneous transfer
118 of xenobiotics (Quaranta et al. 2009). Fourth, tadpoles often forage on sediment and
119 associated biofilm where pesticides are known to accumulate (Degenhardt et al.
120 2009). Alteration of sediment load can result in reduced larval abundances or growth
121 and development rates (Gillespie 2002; Snodgrass et al. 2008; Wood and Richardson
122 2009). Early developmental (embryonic and larval) phases bear strong and long-
123 lasting influences on life-history traits such as body size, growth, survival, and
124 reproduction (Kashiwagi et al., 2009; Arrighi et al., 2013). As a consequence,
125 contamination of aquatic habitats might influence post-metamorphic development to
126 adulthood through early development alterations.

127

128 In this study, we investigated the effects of the timing of exposure to AMPA on larval
129 development until metamorphosis. Using a factorial design, we exposed eggs and

130 tadpoles to environmental concentrations of AMPA at different phases of their
131 development (embryonic development only, larval development only or embryo-
132 larval development). Such an approach allowed us to test not only for the effects of
133 exposure to AMPA but also to assess the relative susceptibility of different
134 developmental phases (embryonic *versus* larval). Previous studies showed that
135 embryonic development of *Bufo spinosus* is negatively affected by environmental
136 concentrations of AMPA which decrease embryonic survival, increase development
137 duration and influence hatchling morphology (Cheron and Brischoux 2020) through
138 mechanisms affecting oxidative status (Cheron et al. 2022a). Herein, we investigated
139 larval developmental alterations using relevant morphological (body size and shape)
140 and developmental (duration of developmental stages) characteristics and survival.

141

142

143 **Materials and Methods**

144

145 *Ethics approval*

146 All applicable institutional and national guidelines for the care and use of animals
147 were followed. This work was approved by the French authorities (COMETHEA
148 ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de
149 L'innovation) under permit #APAFIS#13477-2018032614077834.

150

151 *Eggs collection*

152 Spined toad (*Bufo spinosus*) eggs strings (number of egg strings=10) were collected
153 from 29/01/2018 to 28/02/2018 in ponds near the Centre d'Etudes Biologiques de

154 Chizé (46°09'N, 0°24'W) and returned to the laboratory. We monitored the ponds
155 twice a day to ensure that the eggs were collected quickly after oviposition to
156 minimize embryonic development in the natural environment. A subset of 120 eggs
157 from each clutch was collected for our experiment.

158

159 *Treatment concentrations and chemical solutions*

160 In this study, we aimed at reproducing the environmental concentrations found in
161 aquatic environments in France. AMPA concentrations range from 0.1 µg.l⁻¹ to 6.6
162 µg.l⁻¹ (data from Water Agencies, “Agence de l'eau Loire Bretagne” and “Agence de
163 l'eau Adour-Garonne”) which are similar to concentrations found in aquatic
164 environments in North America and Europe (Grandcoin et al, 2017; Coupe et al,
165 2012; Bonansea et al, 2017). Importantly, these data from French Water Agencies
166 showed that these concentrations were consistently found over time during a
167 temporal scale relevant to the larval development of *Bufo spinosus* tadpoles. We
168 produced three treatments: Low (0.07 ± 0.01 µg.l⁻¹), Medium (0.32 ± 0.052 µg.l⁻¹) and
169 High (3.57 ± 0.153 µg.l⁻¹) concentrations (values from analytical verification, see
170 below). We dissolved commercial crystalline powder (Aminomethylphosphonic
171 acid, 99% purity, ACROS ORGANICS™) in dechlorinated aged-tap water in order to
172 obtain stock solutions (0.1g/L) of AMPA which were further diluted to reach
173 concentrations close to those found in the environment (0.1, 0.5 and 5 µg.l⁻¹).

174

175 Analytical verifications of the actual concentrations in tanks were performed by an
176 independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-
177 Denis, France) on randomly (throughout the experiment) collected samples

178 (immediately after a water change) and in triplicates for each treatment.
179 Determination of AMPA in water samples was assessed using liquid
180 chromatography-mass spectrometry (LC/MS/MS) with 9-fluorenylmethyl
181 chloroformate (FMOC-Cl) used as derivatization agent. The limit of quantification
182 was $0.03 \mu\text{g l}^{-1}$ for the determination of AMPA in water samples. Overall, these
183 verifications showed that actual concentrations were within the range of
184 concentrations found in aquatic environments ($0.1 \mu\text{g.l}^{-1}$ to $6.6 \mu\text{g.l}^{-1}$, see above). For
185 clarity, we will refer to the treatments as Low, Medium and High hereafter.

186

187 Because all individuals were subjected to the same water except for the addition of
188 AMPA for exposed individuals, we did not measure additional water parameters
189 (e.g., pH, conductivity, hardness, alkalinity).

190

191 *Experimental design*

192 Experimental design can be found in Figure 1. We subjected developing *Bufo spinosus*
193 to AMPA concentrations or control conditions (no AMPA) during embryonic
194 development only, larval development only or the entire development (see Figure 1
195 for details) in 2L tanks filled with dechlorinated tap water. We changed water once a
196 week according to the half-life of AMPA ranging from 7 to 14 days in water
197 (Battaglin et al. 2014).

198

199 For each clutch, the subset of 120 eggs was further divided in 4 segments of 30 eggs.
200 Each segment was subjected to one of the treatments (either one of the 3 AMPA
201 concentrations or control) during the whole embryonic development until hatching

202 (see Cheron and Brischoux 2020; Cheron et al. 2022a, b). This part of the experiment
203 is referred to as "Embryonic exposure" (Figure 1). Hatching occurred at Gosner stage
204 22 after 16.10 ± 0.02 days (Gosner 1960, hereafter GS 22).

205

206 Upon hatching, individuals of each segment were re-allocated to new experimental
207 groups (Figure 1). For each clutch, 4 individuals from the initial control groups were
208 randomly collected and individually placed in a 2L glass tank. One remained in
209 control conditions (no contaminant), one was assigned to low AMPA, one was
210 assigned to medium AMPA and one was allocated to high AMPA treatments until
211 metamorphosis (Figure 1).

212 We followed the same procedures for the individuals that were initially under low,
213 medium and high concentration treatments, but we randomly selected two tadpoles
214 from each clutch and reallocated them as follows: one tadpole was allocated to
215 control conditions and the other individual was maintained in their initial conditions
216 (either low, medium or high concentrations, Figure 1). This allowed to reach N=10
217 individuals (one per clutch and per experimental treatment) in each experimental
218 group.

219 Tadpoles were fed with boiled organic spinach that was added in aquaria weekly
220 (just after water renewal) ad libitum.

221 Maintaining the tadpoles individually in aquaria does not generate stressful
222 conditions (Melvin and Houlihan 2014; Bókony et al. 2021).

223 At the onset of metamorphosis (Gosner stage 42, Gosner 1960), tadpoles were
224 transferred to boxes (16x14x9 cm) with a small amount of water from their original

225 tank and a ramp which allowed them to climb out of water. When individuals were
226 observed perched on the ramp (Gosner stage 46, Gosner 1960), we removed the
227 water, and added a piece of damp paper to avoid desiccation. We fed the
228 metamorphic toadlets with pea aphids (*Acyrtosiphon pisum*) and springtails
229 (*Collembola sp.*) ad libitum and kept toadlets 130 days after metamorphosis in order to
230 test for long term survival as a proxy of fitness.

231 We checked for survival of tadpoles and toadlets every day.

232 All individuals (tadpoles and toadlets) were maintained in a thermally controlled
233 room with the temperature set at 17°C (both air and water) and under 12:12 hr
234 day/night photoperiod.

235

236 *Measurements*

237 We selected specific Gosner stages (GS 22, GS 25, GS 30, GS 41, GS 42) which
238 represent key developmental stages in our study species (Cheron et al. 2021). We
239 assessed the time elapsed between consecutive stages as well as morphological traits
240 at each stage Each tadpole was placed in a petri dish and photographed from above
241 and side-view using a camera (Panasonic Lumix DC-TZ55). We measured total
242 length (body + tail), body length, tail length, body width, tail height and body height
243 according to Watters et al. (2016) using with the software ImageJ (Schneider et al,
244 2012). All measurements were performed by the same person throughout the
245 experiment. Upon metamorphosis, toadlets were weighed and photographed to
246 measure their size (snout-vent length: SVL). Residuals of the linear regression
247 between SVL and body mass were used to calculate a body-condition index (BCI).

248

249 *Statistical Analyses*

250 All data were tested for homogeneity of variance and residuals normality with
251 Barlett's and Shapiro-Wilks tests, respectively and met the assumptions. We also
252 checked normality of the residuals using diagnostics plots. All statistical analyses
253 were conducted with R statistical software v.4.0.0 (R Core Team, 2020) and RStudio v
254 1.2.5042 (RStudio, Inc.).

255

256 First, to avoid multiple testing and as all morphological traits of tadpoles were
257 strongly correlated, we used Principal Component Analysis (PCA) of the 6
258 morphological variables to attribute to each tadpoles a "body size" score which was
259 used to assess whether the AMPA Concentration and Exposure influenced the size of
260 tadpoles at each Gosner stage. For each Gosner stage, PCA of the morphological
261 traits retained the first axis (eigenvalues greater than 1). Principal component 1
262 loaded strongly all morphological variables (minimum 0.57 and maximum 0.95).

263

264 We performed LMER (Linear mixed-effects models) on this principal component 1
265 (PC-1) to test for main effects and interactions between "Concentration" (Control,
266 Low, Medium or High), "Exposure" (embryonic, larval or embryo-larval) for each
267 stage. For each univariate model, we included Concentration, Exposure and their
268 interaction as fixed factor. Clutch identities were included as random effect. We
269 dropped all non-significant interactions for these models using marginal F tests (Stats
270 package, anova). We did pairwise comparisons using post hoc Tukey tests (emmeans
271 packages) to further investigate statistical difference.

272 Statistical analyses and figures on analyses focused on each morphological trait
273 separately (with body size as a covariate when necessary) are available in the
274 supplementary materials.

275

276 For tadpoles, we performed GLMER (Generalized linear mixed-effects models,
277 binomial family) to study whether larval mortality was influenced by AMPA
278 treatment. For toadlets, we performed survival analysis using log-rank method
279 ("survival" packages) to assess mortality rate until J+130. This method allowed us to
280 estimate the rate at which death occurs over time and whether mortality is more
281 likely to occur at a specific stage. We examined differences across treatment
282 (Concentration or Exposure) with a Cox model. The test for assumption of
283 proportional hazards for Cox regression showed that our model was appropriate.
284 Raw mortality data are available in supplementary materials (Table S2).

285

286

287 **Results**

288

289 *Tadpoles*

290 First, neither total development duration (Concentration * Exposure: $F_{4,114}=0.407$,
291 $p=0.803$) nor duration of each developmental stage (Concentration * Exposure*Stage:
292 $F_{16,538}=0.859$, $p=0.618$) were affected by our experimental treatment (concentration or
293 timing of exposure, Table S1, Figure 2).

294

295 However, body size score (PCA first axis) was affected by AMPA Exposure
296 throughout ontogeny ($F_{4,397}=2.33$, $p=0.022$, $\eta^2=0.06$, Figure 3). PCA scores varied
297 depending on the stage considered. Indeed, we found a marginal effect of AMPA
298 exposure on the first axes score at GS25 (Concentration: $F_{3,130}=0.347$, $p=0.792$,
299 Exposure: $F_{2,130}=2.646$, $p=0.075$). We found no effect of AMPA at GS30 nor GS41
300 (respectively, Concentration: $F_{3,130}=0.426$, $p=0.734$, Exposure: $F_{2,130}=0.616$, $p=0.542$;
301 Concentration: $F_{3,130}=0.298$, $p=0.827$, Exposure: $F_{2,130}=0.313$, $p=0.732$). However, at the
302 onset of metamorphosis (GS42), we found that the interaction between AMPA
303 concentration and exposure had an effect on PC_1 (Concentration * Exposure:
304 $F_{4,126}=2.683$, $p=0.035$; Concentration: $F_{3,126}=0.439$, $p=0.725$, Exposure: $F_{2,126}=4.420$,
305 $p=0.014$). Post hoc analysis showed that tadpoles treated with high concentration
306 during the embryonic development solely had higher body size score than tadpoles
307 from the medium group (1.82 ± 0.678 , $p=0.04$). Moreover, tadpoles treated with high
308 concentration during the embryonic development solely had higher body size score
309 than those treated by the same concentration during larval development solely
310 (1.98 ± 0.568 , $p=0.01$).

311 Detailed results on each morphological trait analysed separately can be found in the
312 supplementary materials (Table S1).

313

314 *Post metamorphic toads*

315 We did not find any influence of AMPA concentration or Timing of exposure on the
316 body mass (Concentration * Exposure: $F_{4,114}=1.162$, $p=0.317$), SVL (Concentration *

317 Exposure: $F_{4,114}=0.339$, $p=0.851$) or BCI (Concentration * Exposure: $F_{4,114}=0.407$,
318 $p=0.804$) of metamorphs upon metamorphosis (Table S2, Figure 4).

319

320 *Survival*

321 We found an effect of the timing of exposure on larval mortality but no effect of the
322 concentration (Concentration $\chi^2_3=1.57$, $p=0.67$; Exposure: $\chi^2_2=6.48$, $p=0.039$, Figure 5
323 A). Mortality was higher in individuals exposed to AMPA during larval
324 development solely.

325 There were no differences in survival between groups after metamorphosis (log-rank
326 test, $\chi^2_9=10.9$, $p=0.3$, Figure 5B)

327

328 **Discussion**

329

330 Overall, our results suggest that low, environmental concentrations of AMPA
331 influence embryonic and larval development in *Bufo spinosus* tadpoles. AMPA and
332 timing of exposure interactively affected tadpole length, but not rate of development.
333 Most of these effects were linked to exposure during embryonic development, in
334 accordance with results from previous studies (Cheron and Brischoux 2020, Cheron
335 et al. 2022). Yet, in the current study, we show that these effects in individuals
336 exposed during embryonic development solely were long-lasting and occurred until
337 the latest larval stages at the onset of metamorphosis. Finally, we found that
338 exposure to AMPA after hatching during the larval stage increased mortality.

339

340 We found that exposure to AMPA during embryonic and larval development had
341 small, but significant effects on tadpole length. Specifically, we found that
342 individuals treated during a very short period of time during their larval
343 development (i.e., between GS22 and GS 25 which corresponds to ~5 days) displayed
344 smaller size compared to their counterparts from all other timing of exposure. Such
345 result indicates that exposure to AMPA just after hatching can rapidly affect growth
346 rates. Importantly, we found that these effects were transitory and were not
347 detectable at later developmental stages, suggesting that tadpoles may have
348 compensated for these altered early growth rates. Indeed, mechanisms of
349 compensatory growth has been shown in amphibians in response to different
350 environmental stressors such as desiccation (Altwegg & Reyer, 2003), density
351 (Jasienski, 2008) and predation (Vonesh & De la Cruz, 2002), presumably through
352 variations in feeding behaviour (Broomhall and Shine 2003). We can hypothesize
353 that similarly to these other environmental stressors, response to environmental
354 contamination may induce compensatory mechanisms which allow them to achieve
355 similar body size than control individuals; which remains to be tested. Alternatively,
356 but not exclusively, amphibians can display detoxification mechanisms which allows
357 them to cope with exposure to contamination (Pašková et al. 2011). In our context, it
358 is possible that exposure to AMPA during larval development induced such
359 mechanisms and such hypothesis will require thorough testing.

360

361 Whatever the mechanisms involved in these compensatory responses (i.e., increased
362 feeding behaviour, detoxification mechanisms or a combination thereof), they seem

363 to occur at a cost for developing tadpoles, as survival appeared jeopardized in this
364 group of individuals. Indeed, we found that survival was lower in individuals
365 exposed to AMPA after hatching, while no such effect was detectable for those
366 individuals exposed to AMPA during embryonic development. This result dovetails
367 relatively well with those from our previous studies (Cheron and Brischoux 2020;
368 Cheron et al. 2022a). In these studies, the embryos that hatched were those that
369 displayed elevated antioxidant defences, suggesting a selective mortality of
370 individuals less able to cope with AMPA exposure. Our current results further
371 support these findings and show that mortality was higher in individuals exposed to
372 AMPA after hatching presumably because the selective mortality processes linked to
373 AMPA exposure during embryonic development did not occur in this group.
374 Future studies are required to assess the survival costs of exposure to environmental
375 contaminants according to developmental stages in amphibians.

376

377 Interestingly, we found that individuals exposed to the highest concentration of
378 AMPA during embryonic development solely displayed altered size at the latest
379 larval stages (e.g., GS 42 corresponding to the onset of actual metamorphosis, see
380 also Supplementary materials Fig.S1-S6). Such results raise important questions
381 regarding the possible long-lasting effects of an early and temporary exposure to
382 contamination in developing vertebrates. Indeed, although these individuals have
383 been exposed to AMPA during embryonic development (i.e., ~16 days), they were
384 raised in control conditions during the whole larval development (i.e., ~74 days).
385 The latest larval stages at which the effects of earlier exposure to AMPA were

386 detectable correspond to strong morphological, physiological and behavioural
387 changes associated to metamorphosis (feeding and digestive apparatus, respiratory
388 system, tail resorption to fuel metamorphosis; Beck and Congdon 2003; Brown and
389 Cai 2007; Wright et al. 2011) All of these changes could be affected by an early
390 exposure to contamination, and presumably associated mechanisms that induce
391 lower survival in a contaminated environment (e.g., detoxification mechanisms,
392 altered oxidative status, selective mortality, Cheron et al. 2022). Clearly, future
393 studies are required to decipher the mechanisms that underlie such long-lasting
394 effects of early contamination in developing amphibians.

395

396 Despite these different effects of exposure to AMPA according to different timing of
397 exposure and developmental stages, we did not find any significant effects of
398 exposure to AMPA on toadlets after metamorphosis. Such lack of effect may support
399 the hypothesis that compensatory mechanisms may be involved and continue to
400 occur throughout metamorphosis (see above) as well as selective mortality which
401 may have favoured individuals less susceptible to AMPA exposure (Cheron et al.
402 2022). Despite this lack of direct influence of our treatment on young toads, and
403 given the long-lasting effects we found for individuals exposed during embryonic
404 development solely (see above), it is plausible that long-term effects could be
405 detectable later in life (Awkerman and Raimondo 2018). Such long-term effects
406 should usefully be investigated by raising experimental toadlets under similar
407 conditions (i.e., common garden) until adulthood.

408

409 We do not know if the alterations we detected can have consequences for developing
410 embryos and tadpoles in natural ponds contaminated with AMPA. Indeed, the
411 magnitude of the effects we highlight appears relatively small. Although we cannot
412 entirely rule out possible ecological significance of these effects, we emphasize that
413 this hypothesis needs to be taken with caution and will require thorough testing.
414 Actually, it is also noteworthy that experimental tadpoles were raised under optimal
415 conditions with food available *ad libitum*, constant thermal conditions and absence of
416 predators and competitors. Such optimal conditions are clearly different to what is
417 observed *in natura*, where competition, predation, and/or food shortage can occur
418 (Goater 1994; Broomhall and Shine 2003; Jones et al. 2011). It is possible that the
419 conditions we offered to tadpoles during their growth were optimal enough for them
420 to compensate for putative effects of AMPA (Capellán and Nicieza 2007). In keeping
421 with this idea, it is important to highlight that the toadlets from our experiment were
422 ~1.5 times larger and ~4 times heavier than wild toadlets captured at the same stage
423 (SVL=1.15±0.08 cm; Mass=0.19±0.03 g for experimental metamorphs *versus*
424 SVL=0.78±0.07 cm; Mass=0.05±0.01 g for n=16 metamorphic toadlets captured in the
425 field). These strong differences suggest that tadpoles raised under natural conditions
426 produce smaller and lighter toadlets. Although the magnitude of the effects we
427 found seems relatively low and question whether AMPA actually poses a risk for
428 amphibians, we can further hypothesize that under relatively harsher natural
429 conditions, the influence of anthropogenic contaminants such as AMPA may be
430 much more pronounced than under optimal experimental conditions. Future studies
431 should aim at assessing the influence of environmental contamination on developing

432 tadpoles under constraining environmental conditions by manipulating food
433 availability, competition, predation and a combination thereof (Relyea 2001; Hua et
434 al. 2017).

435

436 We believe that our results complement previous studies focused on the effects of
437 glyphosate (parent compound) or glyphosate-based commercial formulations (i.e.,
438 containing surfactants) on amphibians (e.g., Cauble and Wagner 2005; Lenkowski et
439 al. 2010; Jones et al. 2011; Baier et al. 2016a,b; Bokony et al. 2017; Berger et al. 2018;
440 Wang et al. 2019; Herek et al. 2020). Yet, direct straightforward comparisons (e.g., to
441 test the relative toxicity of glyphosate *versus* AMPA) remain precluded because most
442 of these study investigated different response variables on different species in
443 different settings (laboratory *versus* field). Indeed, in order to draw valid
444 comparisons between studies, one would need to compare the same response
445 variables on the same study species in the same conditions and ideally with
446 comparable AMPA or glyphosate concentrations. Such comparative data are not
447 available to date and future studies should usefully aim at performing specific
448 experiments in order to comprehensively compare similar response variables
449 (development, morphology, physiology and/or behaviour) to AMPA, glyphosate
450 and commercial glyphosate-based formulation containing surfactants in order to
451 answer to this question.

452

453 **Conclusion**

454 Overall, our results show that minute concentrations of AMPA (100 to 6000 times
455 lower than the official “Predicted No Effect Concentration”, Ineris, 2013) can affect

456 development and survival of spined toads. It seems particularly urgent to
457 investigate such effects in the context of the multiple threats that are known to affect
458 amphibian populations worldwide (IUCN 2020).

459

460

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771 **Statements & Declarations**

772

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778

779 **Competing interests**

780 The authors have no relevant financial or non-financial interests to disclose.

781

782 **Author Contributions**

783 Both authors conceived and designed the study. Marion Cheron performed the
784 experiment and collected the data. Both authors participated during data curation.
785 Both authors interpreted the data, designed the figures and wrote the initial draft.
786 Both authors contributed to manuscript revision. Both authors read and approved
787 the final manuscript.

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789 **Data availability**

790 Data is available upon requests

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795 **Captions to figures**

796

797 **Figure 1:** Experimental design used to study the effects of both AMPA concentrations
798 and timing of exposure on *Bufo spinosus* tadpoles. Colours represent concentration
799 (Grey = Control, light blue = Low, blue = Medium, dark blue =High). Line shape
800 represent Exposure phase (Solid : Embryo-larval development, long dashed : Larval
801 exposure only, dashed : Embryonic exposure only).

802

803 **Figure 2:** Mean Δ development duration days between treatment and control
804 throughout ontogeny (GS25, GS30, GS41, GS42, GS46, according to the timing of
805 exposure to AMPA (embryonic development solely [embryo], larval development
806 solely [larval] or embryo-larval [total] exposure) and AMPA concentrations (Grey =
807 Control, light blue = Low, blue = Medium, dark blue =High) in *Bufo spinosus*
808 tadpoles. Data represent mean \pm SE.).

809

810 **Figure 3:** Principal component 1 of principal component analysis of morphological
811 traits (Total length, tail length, body length, body length, body width, tail height,
812 body height) throughout ontogeny (A : GS25, B : GS30, C : GS41, D : GS42) according
813 to the timing of exposure to AMPA (control, embryonic development solely
814 [embryo], larval development solely [larval] or embryo-larval [total] exposure) and
815 AMPA concentrations (Grey = Control, light blue = Low, blue = Medium, dark blue
816 =High) in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first
817 and last quartiles, the horizontal line within the box represents the median, the
818 whiskers represent the fifth and 95th percentiles, and the circles represent outliers.

819

820 **Figure 4:** Mass at metamorphosis (A) and snout-vent length at metamorphosis (B)
821 according to the timing of exposure to AMPA (control, embryonic development
822 solely [embryo], larval development solely [larval] or embryo-larval [total] exposure)
823 and AMPA concentrations (grey = Control, light blue = Low, blue = Medium, dark
824 blue =High) in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the
825 first and last quartiles, the horizontal line within the box represents the median, the
826 whiskers represent the fifth and 95th percentiles, and the circles represent outliers.

827

828 **Figure 5.** Larval mortality (A) relative to the timing of exposure to AMPA. Larval
829 exposure yielded significantly higher mortality. Colors represent different
830 concentrations (i.e Grey = Control, light blue = Low, blue = Medium, dark blue
831 =High). Survival during 130 days post metamorphosis according to the timing of
832 exposure to AMPA (control, embryonic development solely [embryo], larval
833 development solely [larval] or embryo-larval [total] exposure) and AMPA
834 concentrations (Grey = Control, light blue = Low, blue = Medium, dark blue =High)
835 in *Bufo spinosus* tadpoles.

836

837

838 **Table 1 : Principal component analysis (PCA) of 6 morphological traits at 4 stages**
 839 **of development of *Bufo spinosus* tadpoles. PC = principal component. PC in bold**
 840 **represent axis we kept for analysis (eigenvalue>1).**

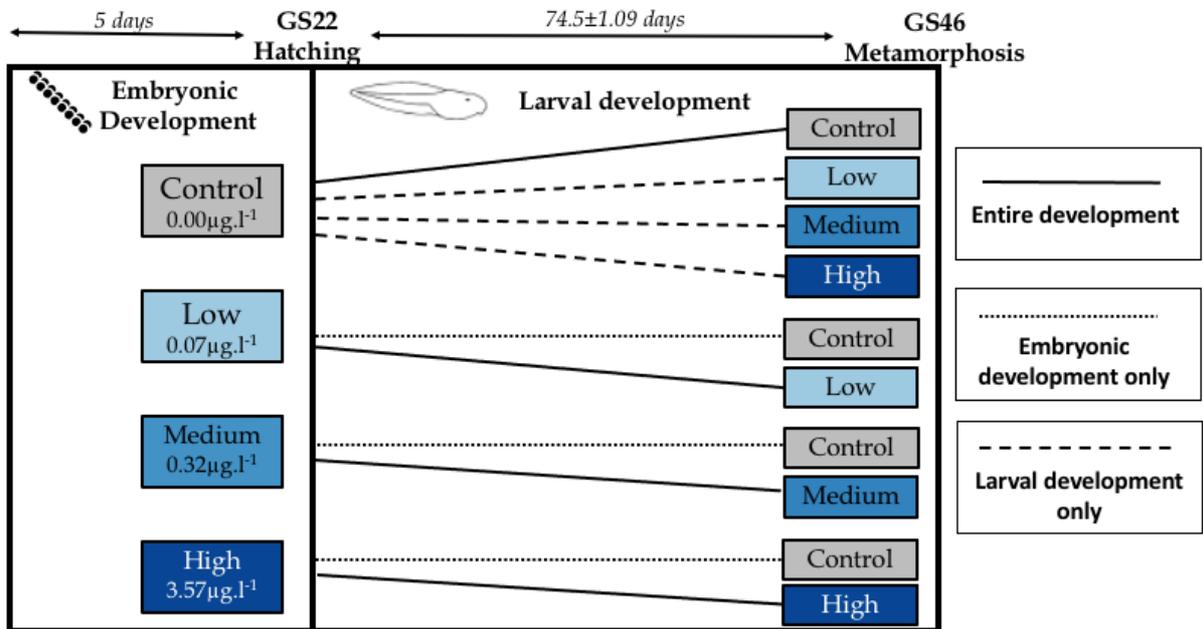
PCA results	Principal component											
	25			30			41			42		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
% of Variance	59.62	12.69	12.07	71.13	10.25	8.64	59.02	15.04	11.56	57.61	15.46	11.57
Eigenvalue	3.58	0.76	0.72	4.27	0.62	0.52	3.54	0.90	0.69	3.46	0.93	0.69
Factor loading												
<i>Total length</i>	0.94	-0.09	0.15	0.95	0.10	-0.28	0.90	-0.35	-0.24	0.93	-0.10	-0.35
<i>Body length</i>	0.70	-0.40	0.58	0.85	-0.22	-0.33	0.71	0.28	-0.63	0.57	0.72	-0.38
<i>Body width</i>	0.60	0.71	0.24	0.75	0.43	0.43	0.69	0.19	0.37	0.63	0.18	0.31
<i>Tail length</i>	0.82	0.18	-0.24	0.85	0.37	-0.18	0.70	-0.70	0.14	0.80	-0.53	-0.22
<i>Tail height</i>	0.71	-0.24	-0.46	0.79	-0.40	0.26	0.77	0.30	0.14	0.85	-0.19	0.20
<i>Body height</i>	0.81	-0.04	-0.21	0.85	-0.27	0.20	0.80	0.29	0.25	0.71	0.21	0.49

841

842 Figures

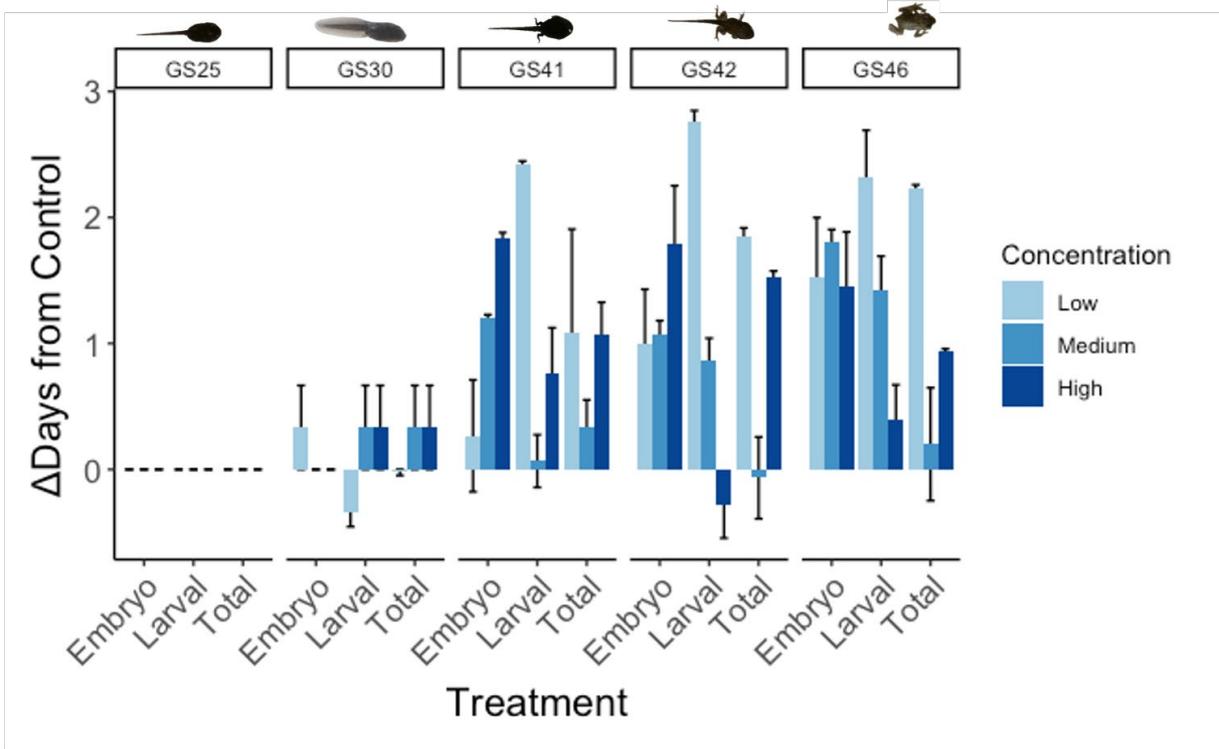
843

844 Figure 1



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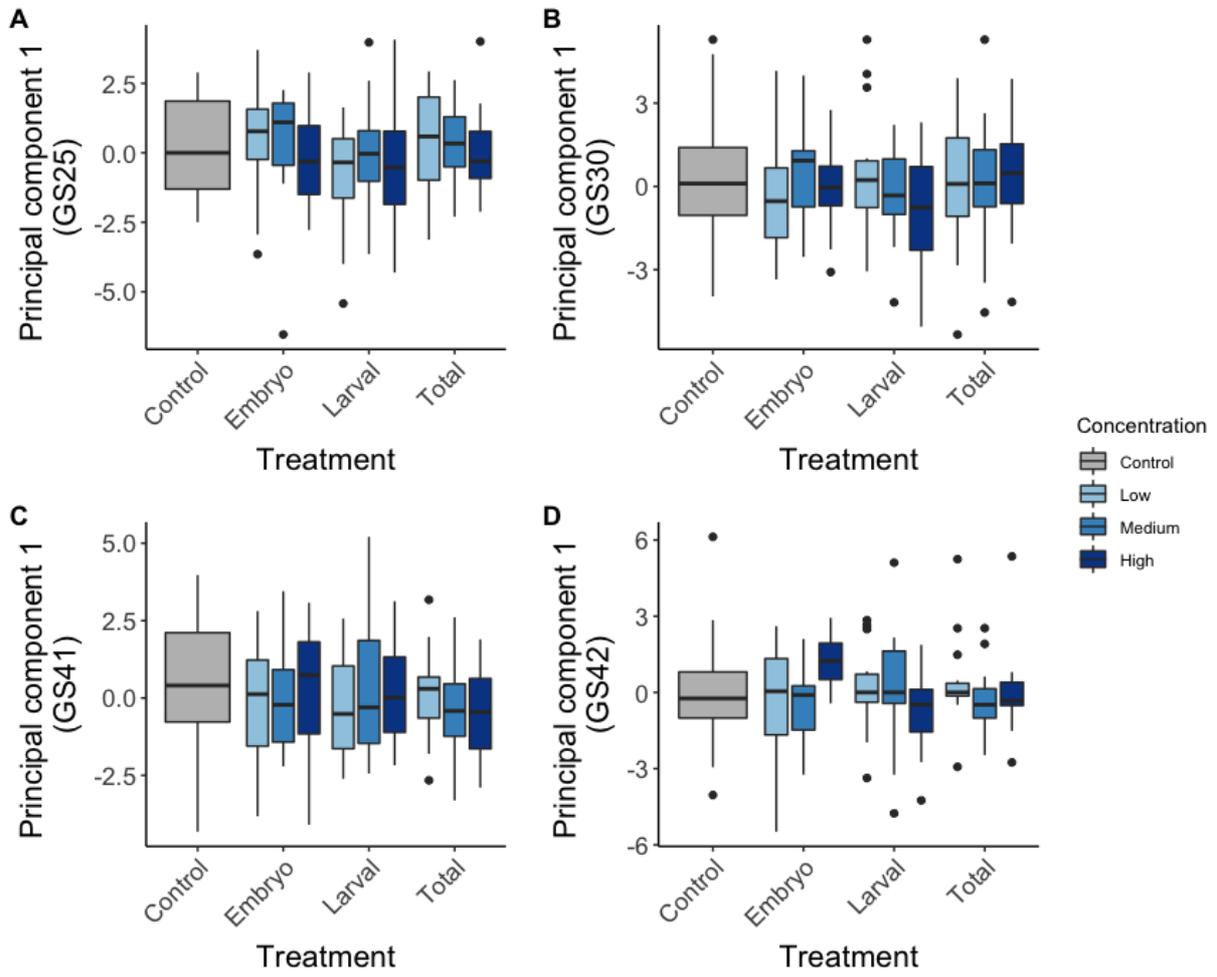
846 **Figure 2**



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848 **Figure 3**

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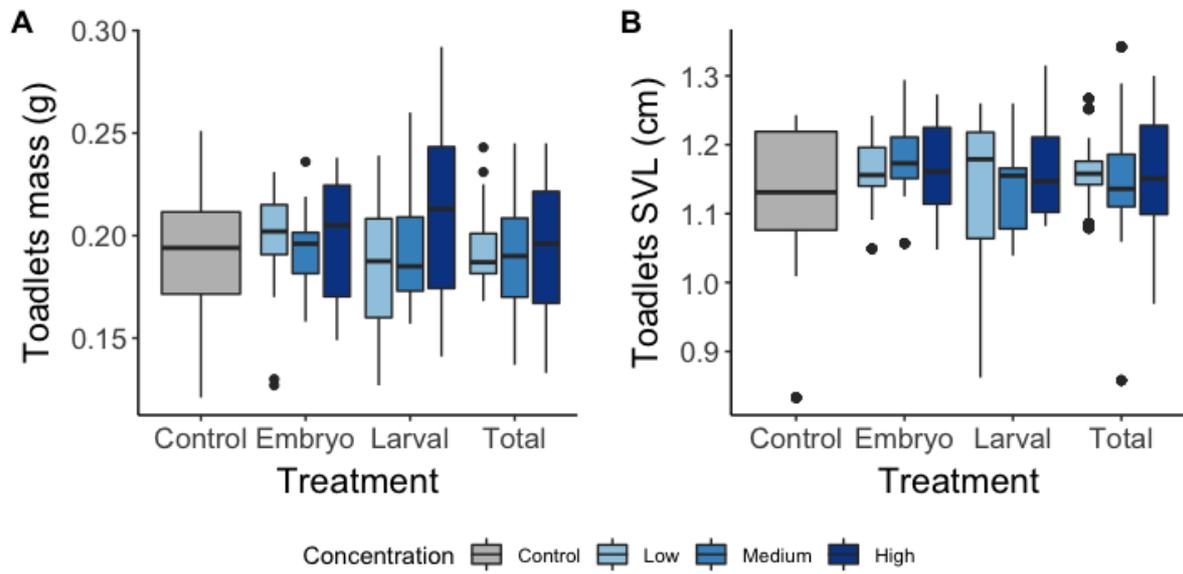
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857 **Figure 4**

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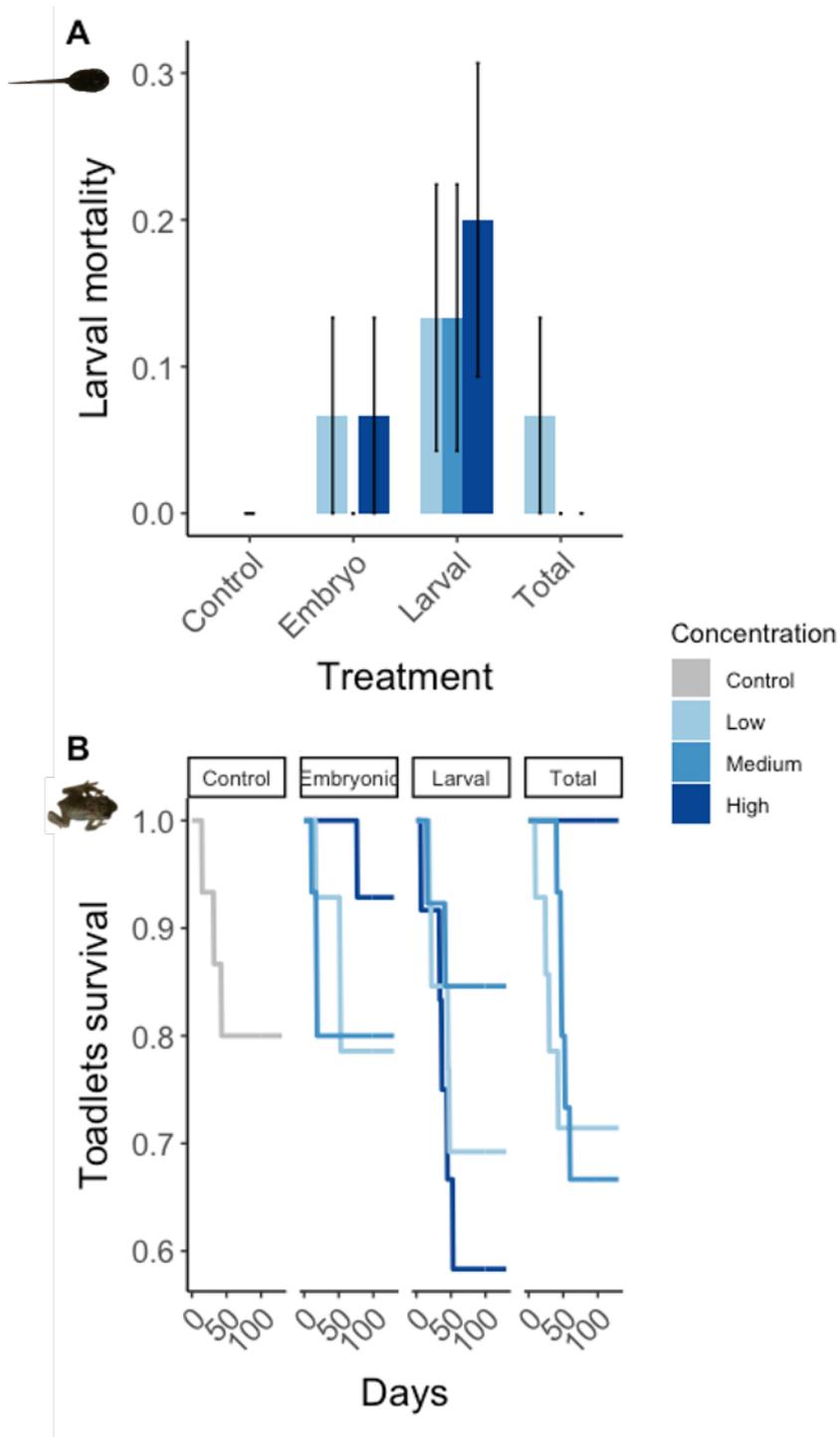
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863 **Figure 5**
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