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Exposure to Low Concentrations of AMPA Influences Morphology and Decreases Survival During Larval Development in a Widespread Amphibian Species

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

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

The effects of agrochemicals on non-target species are being increasingly investigated (Baier et al. 2016a; Hasenbein et al. 2017; Thiour-Mauprivez et al. 2019) and can influence wildlife through both acute and chronic exposures (Bernabò et al. 2008; Bókony et al. 2017; Hackenberger et al. 2018). Environmental concentrations are often very low as compared to the values tested under laboratory conditions on model species which define standards of use (e.g. based on LD50, Beiras 2018; Kramer et al. 2009). As a consequence, although these low, environmental concentrations are usually disregarded by regulatory toxicity studies, an increasing body of literature has highlighted their negative effects through several mechanisms affecting DNA structure (Guilherme et al. 2010; Matozzo et al. 2019), oxidative status (Isaksson 2010; Pašková et al. 2011; Lushchak 2016), physiology (Hayes et al. 2006; Leemans et al. 2019; Muñoz et al. 2021), development (Relyea 2009; Lenkowski et al. 2010; Wang et al. 2019), morphology (Baier et al. 2016b; Cheron and Brischoux 2020) or behaviour (Brunelli et al. 2009; Hellou 2011; Browne and Moore 2014), all of which can ultimately influence individual survival (Baker et al. 2013; Herek et al. 2020) and/or reproduction (Costantini et al. 2014; Hackenberger et al. 2018; Adams et al. 2021) and thus population persistence (Blaustein et al. 2011; Hamilton et al. 2016).

Despite the growing interest of the scientific community to assess the effects of agrochemicals on wildlife, most studies have focused on the active ingredients (Mesnage and

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Antoniou 2018). Yet, in natural conditions, most pesticides degrade due to contact with air or water (Kamrin 1997), exposure to sunlight (Fenoll et al. 2012) and/or the activity of microbiota (Sviridov et al. 2015). As a consequence, our knowledge is often limited to the effects of the parent compounds (i.e. the listed active substances), and the potential effects of their metabolites have been overlooked to date (de Brito Rodrigues et al., 2019; EFSA, 2012; Lee et al. 2017).

Such bias is exemplified by one of the most famous herbicides, glyphosate, and its primary metabolite, aminomethylphosphonic acid (AMPA). Glyphosate remains one of the most widely used non-selective herbicides despite the intense scientific and societal debates regarding its toxicity (Helander et al. 2012; Clapp 2021). Accordingly, the overall effects of glyphosate on wildlife including investigations of commercial formulations and/or of different surfactants have been relatively thoroughly studied (Solomon and Thompson 2003; Cauble and Wagner 2005; Lenkowski et al. 2010; Guilherme et al. 2010; Giaquinto et al. 2017; Bókony et al. 2017; Berger et al. 2018; Gill et al. 2018; de Brito Rodrigues et al. 2019; Matozzo et al. 2019; Herek et al. 2020; Muñoz et al. 2021). Yet, as mentioned above, its primary metabolite, AMPA is arguably the most common compound found in surface and groundwater worldwide (Grandcoin et al. 2017; Bonansea et al. 2017; Okada et al. 2020; Medalie et al. 2020). The greater occurrence and abundance of AMPA in natural environments are likely linked to the extensive use of glyphosate because AMPA is an organic phosphonate derived from water treatment facilities, textile industries, and industrial or household detergents (Grandcoin et al. 2017). Because AMPA has a longer half-life than glyphosate in water (Battaglin et al. 2014; Bonansea et al. 2017; Silva et al. 2018), it tends to accumulate in aquatic habitats from contaminated environments via runoff or soil erosion (Solomon and Thompson 2003). Despite these multiple sources of AMPA, studies on the effects of AMPA at environmental concentrations on wildlife are comparatively fewer (Guilherme et al. 2014; Domínguez et al. 2016; Martinez and Al-Ahmad 2019; de Brito Rodrigues et al. 2019; Matozzo et al. 2019; Cheron and Brischoux 2020). Because of such a discrepancy, it is crucial to collect data on the effects of AMPA on wildlife if we are to provide a thorough assessment of the potential impacts of this global contaminant on biodiversity (Hahn and Sadler 2020) (Table 1).

In this study, we assessed the effects of exposure to environmental concentrations of AMPA on the development of a widespread amphibian species (spined toad, *Bufo spinosus*). Amphibians are particularly susceptible to the presence of pesticides in surface waters (Mann et al. 2009; Quaranta et al. 2009; Hayes et al. 2010). First, in temperate areas, breeding periods often coincide with the use of agrochemicals (Berger et al. 2013; Lenhardt et al. 2015). Second, many amphibians have biphasic life cycles, with terrestrial adults breeding in aquatic sites where eggs and larvae develop (Reading et al. 1991). As a consequence, breeding occurs in lentic systems where concentrations of pesticides can be relatively elevated due to stagnation of water (Battaglin et al. 2009). Third, the skin of aquatic or semi-aquatic amphibian is characterized by high permeability because it is actively involved in gas exchanges and regulation of internal concentration of water and ions (Uchiyama and Konno 2006; Brischoux et al. 2021). Moreover, surface area-to-volume ratio is elevated in amphibians compared with other species, which influences the transcutaneous transfer of xenobiotics (Quaranta et al. 2009). Fourth, tadpoles often forage on sediment and associated biofilm where pesticides are known to accumulate (Degenhardt et al. 2009). Alteration of sediment load can result in reduced larval abundances or growth and development rates (Gillespie 2002; Snodgrass et al. 2008; Wood and Richardson 2009). Early developmental

Table 1 Principal component analysis (PCA) of six morphological traits at four stages of development of *Bufo spinosus* tadpoles

	Principal component											
	25			30			41			42		
PCA results	**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												
Total length	**0.94**	−0.09	0.15	**0.95**	0.10	−0.28	**0.90**	−0.35	−0.24	**0.93**	−0.10	−0.35
Body length	**0.70**	−0.40	0.58	**0.85**	−0.22	−0.33	**0.71**	0.28	−0.63	**0.57**	0.72	−0.38
Body width	**0.60**	0.71	0.24	**0.75**	0.43	0.43	**0.69**	0.19	0.37	**0.63**	0.18	0.31
Tail length	**0.82**	0.18	−0.24	**0.85**	0.37	−0.18	**0.70**	−0.70	0.14	**0.80**	−0.53	−0.22
Tail height	**0.71**	−0.24	−0.46	**0.79**	−0.40	0.26	**0.77**	0.30	0.14	**0.85**	−0.19	0.20
Body height	**0.81**	−0.04	−0.21	**0.85**	−0.27	0.20	**0.80**	0.29	0.25	**0.71**	0.21	0.49

PC Principal component. PC in bold represents axis we kept for analysis (eigenvalue > 1)

(embryonic and larval) phases bear strong and long-lasting influences on life-history traits such as body size, growth, survival, and reproduction (Kashiwagi et al. 2009; Arrighi et al. 2013). As a consequence, contamination of aquatic habitats might influence post-metamorphic development to adulthood through early development alterations.

In this study, we investigated the effects of the timing of exposure to AMPA on larval development until metamorphosis. Using a factorial design, we exposed eggs and tadpoles to environmental concentrations of AMPA at different phases of their development (embryonic development only, larval development only or embryo-larval development). Such an approach allowed us not only to test for the effects of exposure to AMPA but also to assess the relative susceptibility of different developmental phases (embryonic *versus* larval). Previous studies showed that embryonic development of *Bufo spinosus* is negatively affected by environmental concentrations of AMPA which decrease embryonic survival, increase development duration and influence hatchling morphology (Cheron and Brischoux 2020) through mechanisms affecting oxidative status (Cheron et al. 2022a). Herein, we investigated larval developmental alterations using relevant morphological (body size and shape) and developmental (duration of developmental stages) characteristics and survival.

Materials and Methods

Ethics Approval

All applicable institutional and national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de L'Enseignement Superieur, de la Recherche et de L'innovation) under permit #APA FIS#13,477–2,018,032,614,077,834.

Eggs Collection

Spined toad (*Bufo spinosus*) eggs strings (number of egg strings = 10) were collected from 29 January 2018 to 28 February 2018 in ponds near the Centre d'Etudes Biologiques de Chizé (46°090N, 0°240W) and returned to the laboratory. We monitored the ponds twice a day to ensure that the eggs were collected quickly after oviposition to minimize embryonic development in the natural environment. A subset of 120 eggs from each clutch was collected for our experiment.

Treatment Concentrations and Chemical Solutions

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

Analytical verifications of the actual concentrations in tanks were performed by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France) on randomly (throughout the experiment) collected samples (immediately after a water change) and in triplicates for each treatment. Determination of AMPA in water samples was assessed using liquid chromatography–mass spectrometry (LC–MS) with 9-fluorenylmethyl chloroformate (FMOC-Cl) used as derivatization agent. The limit of quantification was 0.03 µg l^{-1} for the determination of AMPA in water samples. Overall, these verifications showed that actual concentrations were within the range of concentrations found in aquatic environments (0.1–6.6 µg.l^{-1}, see above). For clarity, we will refer to the treatments as low, medium and high hereafter.

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

Experimental Design

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

For each clutch, the subset of 120 eggs was further divided into four segments of 30 eggs. Each segment was subjected to one of the treatments (either one of the three AMPA concentrations or control) during the whole embryonic development until hatching (see Cheron and Brischoux

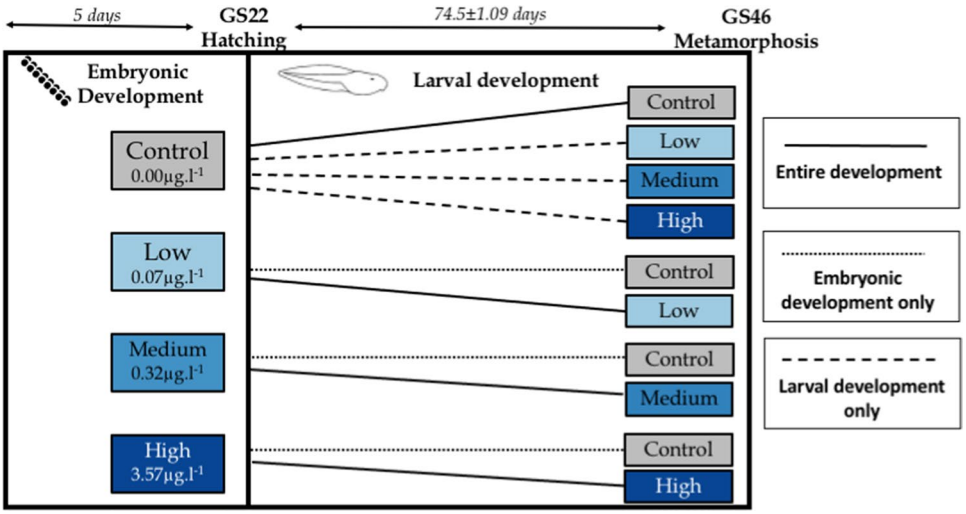

Fig. 1 Experimental design used to study the effects of both AMPA concentrations and timing of exposure on *Bufo spinosus* tadpoles. Colours represent concentration (grey = control, light blue = low, blue = medium, dark blue = high). Line shape represents exposure phase (solid: embryo–larval development, long dashed: larval exposure only, dashed: embryonic exposure only)

2020; Cheron et al. 2022a, b). This part of the experiment is referred to as "embryonic exposure" (Fig. 1). Hatching occurred at Gosner stage 22 after 16.10 ± 0.02 days (Gosner 1960, hereafter GS 22).

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

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

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

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

At the onset of metamorphosis (Gosner stage 42, Gosner 1960), tadpoles were transferred to boxes (16 × 14 × 9 cm) with a small amount of water from their original tank and a ramp which allowed them to climb out of water. When individuals were observed perched on the ramp (Gosner stage 46, Gosner 1960), we removed the water and added a piece of damp paper to avoid desiccation. We fed the metamorphic toadlets with pea aphids (*Acyrthosiphon pisum)* and springtails (*Collembola sp.)* ad libitum and kept toadlets 130 days after metamorphosis in order to test for long-term survival as a proxy of fitness.

We checked for survival of tadpoles and toadlets every day.

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

Measurements

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

Statistical Analyses

All data were tested for homogeneity of variance and residuals normality with Bartlett's and Shapiro–Wilks tests, respectively, and met the assumptions. We also checked normality of the residuals using diagnostics plots. All statistical analyses were conducted with R statistical software

v.4.0.0 (R Core Team, 2020) and RStudio v 1.2.5042 (RStudio, Inc.).

First, to avoid multiple testing and as all morphological traits of tadpoles were strongly correlated, we used principal component analysis (PCA) of the six morphological variables to attribute to each tadpole a "body size" score which was used to assess whether the AMPA concentration and exposure influenced the size of tadpoles at each Gosner stage. For each Gosner stage, PCA of the morphological traits retained the first axis (eigenvalues greater than 1). Principal component 1 loaded strongly all morphological variables (minimum 0.57 and maximum 0.95).

We performed LMER (linear mixed-effects models) on this principal component 1 (PC-1) to test for main effects and interactions between "concentration" (control, low, medium or high) and "exposure" (embryonic, larval or embryo-larval) for each stage. For each univariate model, we included concentration, exposure and their interaction as fixed factor. Clutch identities were included as random effect. We dropped all non-significant interactions for these models using marginal F tests (Stats package, ANOVA). We did pairwise comparisons using post hoc Tukey tests (emmeans packages) to further investigate statistical difference.

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

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

Results

Tadpoles

First, neither total development duration (concentration * exposure: $F_{4,114} = 0.407$, p = 0.803) nor duration of each developmental stage (concentration * exposure*stage: $F_{16,538} = 0.859$, $p = 0.618$) was affected by our experimental treatment (concentration or timing of exposure, Table S1, Fig. 2).

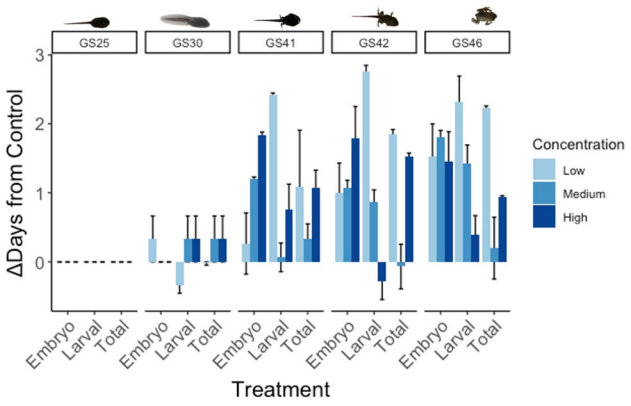

Fig. 2 Mean Δ development duration days between treatment and control throughout ontogeny (GS25, GS30, GS41, GS42, GS46, according to the timing of exposure to AMPA (embryonic development solely [embryo], larval development solely [larval] or embryo-larval [total] exposure) and AMPA concentrations (grey = control, light blue = low, blue = medium, dark blue = high) in *Bufo spinosus* tadpoles. Data represent mean ± SE)

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

Detailed results on each morphological trait analysed separately are given in supplementary materials (Table S1).

Post-Metamorphic Toads

We did not find any influence of AMPA concentration or timing of exposure on the body mass (concentration * exposure: $F_{4,114} = 1.162$, $p = 0.317$), SVL (concentration * exposure: $F_{4,114} = 0.339$, $p = 0.851$) or BCI (concentration

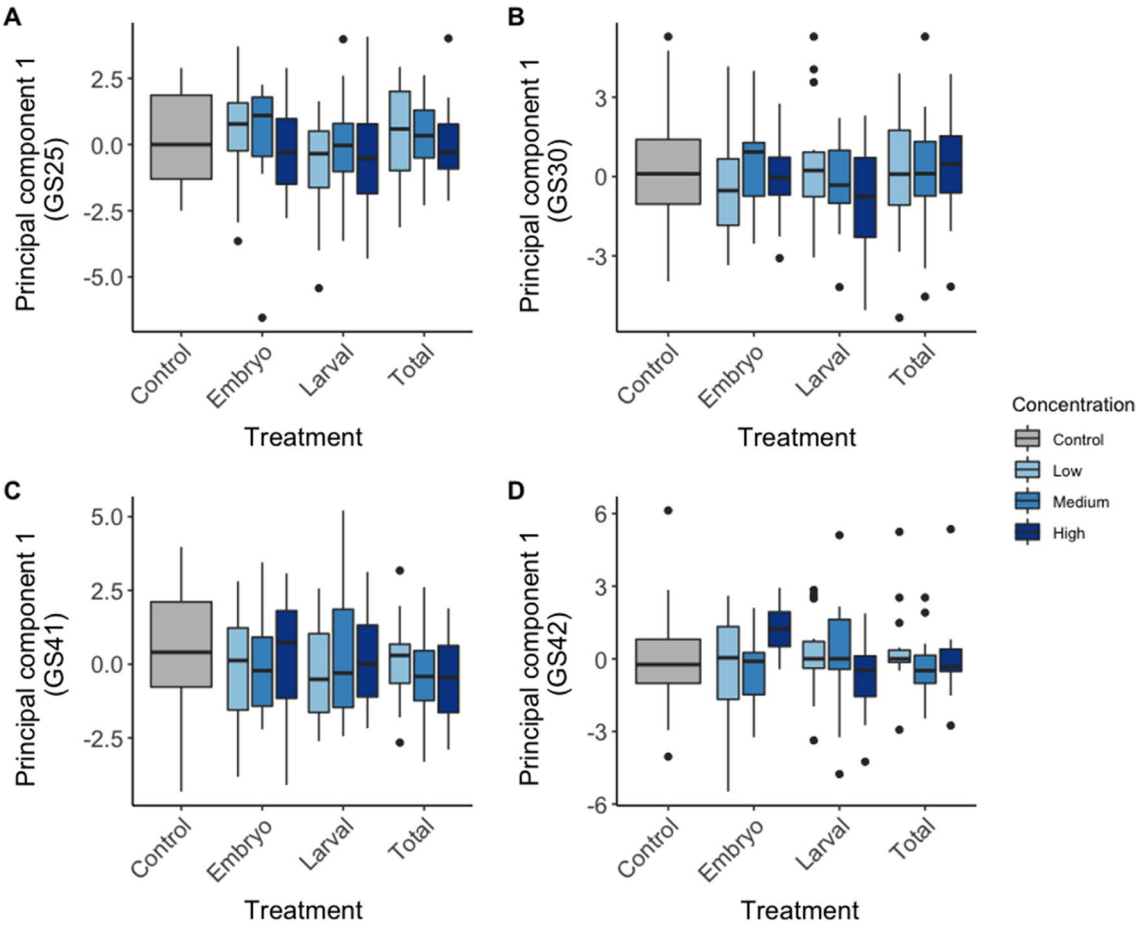

Fig. 3 Principal component 1 of principal component analysis of morphological traits (total length, tail length, body length, body length, body width, tail height, body height) throughout ontogeny (**A** GS25, **B** GS30, **C** GS41, **D** GS42) according to the timing of exposure to AMPA (control, embryonic development solely [embryo], larval development solely [larval] or embryo-larval [total] exposure) and AMPA concentrations (grey = control, light blue = low, blue = medium, dark blue = high) in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first and last quartiles, the horizontal line within the box represents the median, the whiskers represent the fifth and 95th percentiles, and the circles represent outliers

* exposure: $F_{4,114} = 0.407$, $p = 0.804$) of metamorphs upon metamorphosis (Table S2, Fig. 4).

Survival

We found an effect of the timing of exposure on larval mortality but no effect of the concentration (concentration $X_3 = 1.57$, $p = 0.67$; exposure: $X_2 = 6.48$, $p = 0.039$, Fig. 5A). Mortality was higher in individuals exposed to AMPA during larval development solely.

There were no differences in survival between groups after metamorphosis (log-rank test, $X_9 = 10.9$, $p = 0.3$, Fig. 5B).

Discussion

Overall, our results suggest that low environmental concentrations of AMPA influence embryonic and larval development in *Bufo spinosus* tadpoles. AMPA and timing of exposure interactively affected tadpole length, but not rate of development. Most of these effects were linked to exposure during embryonic development, in accordance with results from previous studies (Cheron and Brischoux 2020; Cheron et al. 2022a, b). Yet, in the current study, we show that these effects in individuals exposed during embryonic development solely were long-lasting and occurred until the latest larval stages at the onset of

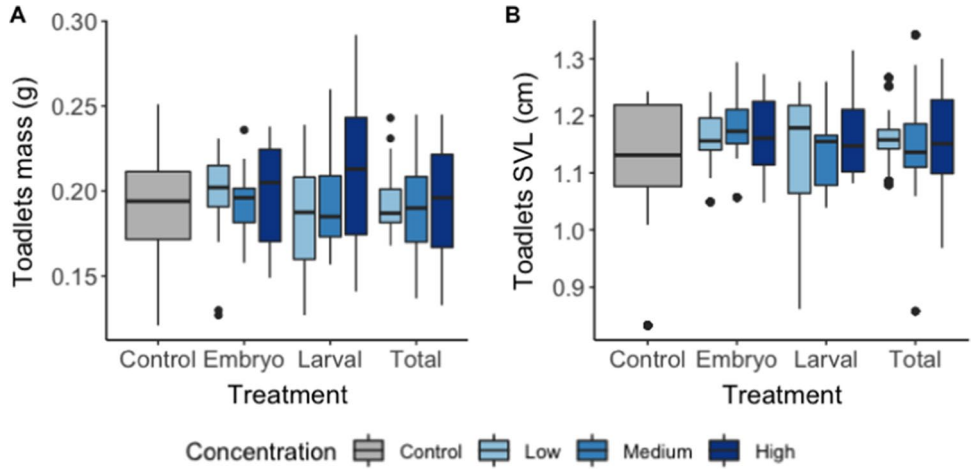

Fig. 4 Mass at metamorphosis (**A**) and snout–vent length at metamorphosis (**B**) according to the timing of exposure to AMPA (control, embryonic development solely [embryo], larval development solely [larval] or embryo–larval [total] exposure) and AMPA concentrations (grey = control, light blue = low, blue = medium, dark blue = high) in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first and last quartiles, the horizontal line within the box represents the median, the whiskers represent the fifth and 95th percentiles, and the circles represent outliers

metamorphosis. Finally, we found that exposure to AMPA after hatching during the larval stage increased mortality.

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

Whatever the mechanisms involved in these compensatory responses (i.e. increased feeding behaviour, detoxification mechanisms or a combination thereof), they seem to occur at a cost for developing tadpoles, as survival appeared jeopardized in this group of individuals. Indeed, we found that survival was lower in individuals exposed to AMPA after hatching, while no such effect was detectable for those individuals exposed to AMPA during embryonic development. This result dovetails relatively well with those from our previous studies (Cheron and Brischoux 2020; Cheron et al. 2022a). In these studies, the embryos that hatched were those that displayed elevated antioxidant defences, suggesting a selective mortality of individuals less able to cope with AMPA exposure. Our current results further support these findings and show that mortality was higher in individuals exposed to AMPA after hatching presumably because the selective mortality processes linked to AMPA exposure during embryonic development did not occur in this group. Future studies are required to assess the survival costs of exposure to environmental contaminants according to developmental stages in amphibians.

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

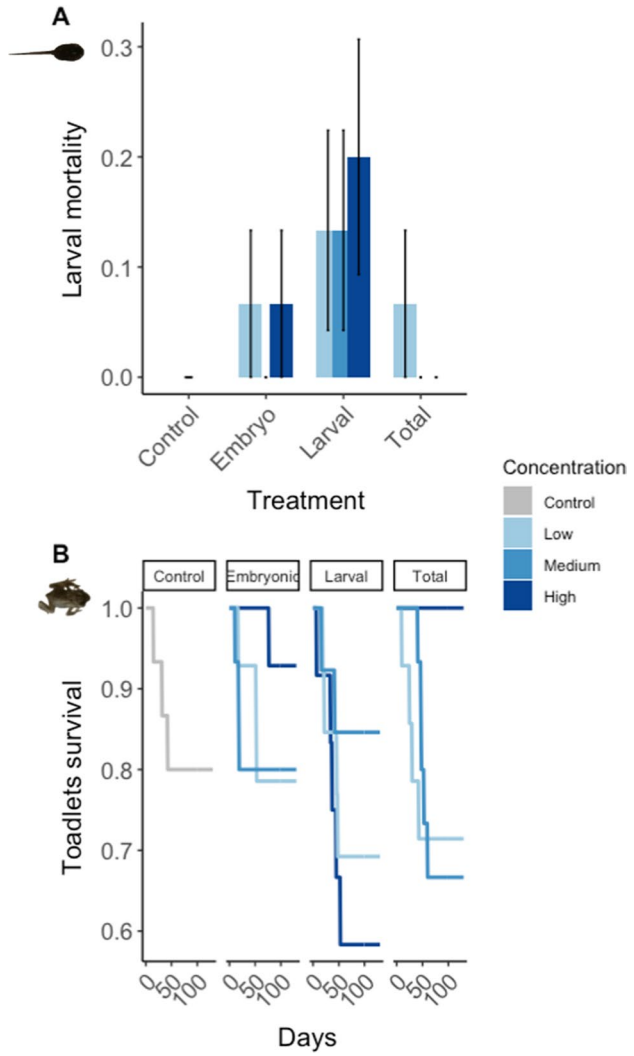

Fig. 5 **A** Larval mortality relative to the timing of exposure to AMPA. Larval exposure yielded significantly higher mortality. Colours represent different concentrations (grey = control, light blue = low, blue = medium, dark blue = high). **B** Survival during 130 days post-metamorphosis according to the timing of exposure to AMPA (control, embryonic development solely [embryo], larval development solely [larval] or embryo–larval [total] exposure) and AMPA concentrations (grey = control, light blue = low, blue = medium, dark blue = high) in *Bufo spinosus* tadpoles

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

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

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

manipulating food availability, competition, predation and a combination thereof (Relyea 2001; Hua et al. 2017).

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

Conclusion

Overall, our results show that minute concentrations of AMPA (100–6000 times lower than the official "Predicted No Effect Concentration," Ineris, 2013) can affect development and survival of spined toads. It seems particularly urgent to investigate such effects in the context of the multiple threats that are known to affect amphibian populations worldwide (IUCN 2020).

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Author Contributions Both authors conceived and designed the study. MC performed the experiment and collected the data. Both authors participated during data curation. Both authors interpreted the data, designed the figures and wrote the initial draft. Both authors contributed to manuscript revision. Both authors read and approved the final manuscript.

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Data Availability Data is available upon requests.

Declarations

Competing interest The authors declare that they have no competing interest.

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