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**1 Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic**  
**2 development in an amphibian species**

3 Marion Cheron<sup>1\*</sup>, David Costantini<sup>2</sup>, Frédéric Angelier<sup>1</sup>, Cécile Ribout<sup>1</sup>, François Brischoux<sup>1</sup>

4 <sup>1</sup> Centre d'Études Biologiques de Chizé (CEBC), UMR 7372 CNRS-La Rochelle Université,  
5 79360 Villiers en Bois, France

6 <sup>2</sup> Unité Physiologie Moléculaire et Adaptation (PhyMA), UMR 7221 Muséum National  
7 d'Histoire Naturelle, CNRS, CP32, 7 Rue Cuvier, Paris, France

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10 \*Correspondence: [cheron.marion@gmail.com](mailto:cheron.marion@gmail.com)

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## Abstract

Glyphosate's primary metabolite (aminomethylphosphonic acid, AMPA) is known to alter embryonic development at environmentally relevant concentrations in amphibians. However, we have limited understanding of the physiological mechanisms through which AMPA affects organisms. In this study, we tested whether alteration of the oxidative status is one mechanism through which AMPA affects organism performance. To this end, we analysed several oxidative status markers in hatchling tadpoles that were exposed to sublethal concentrations of AMPA during embryonic development (~16 days). We compared the influence of environmentally relevant concentrations of AMPA (from 0.07 to 3.57  $\mu\text{g}\cdot\text{l}^{-1}$ ) on the relation between developmental traits (i.e, embryonic development duration, embryonic mortality and hatchling size) and oxidative status markers known to alter homeostasis when unbalanced (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), thiols and ratios thereof). We included measures of telomere length as an indicator of physiological state. We found that AMPA concentrations induce non-monotonic effects on some oxidative status markers with hatchlings displaying elevated antioxidant responses (elevated thiols and unbalanced SOD/(GPx+CAT) ratio). The lack of effect of AMPA on the relation between developmental traits, oxidative status and telomere length suggests that selective mortality of embryos susceptible to oxidative stress may have occurred prior to hatching in individuals less resistant to AMPA which display lower hatching success. Future studies are required to disentangle whether oxidative unbalance is a cause or a consequence of AMPA exposition. This study highlights the need to investigate effects of the metabolites of contaminants at environmental concentrations to comprehensively assess impacts of anthropogenic contamination on wildlife.

Key-words: aminomethylphosphonic acid, *Bufo spinosus*, oxidative stress, telomere

## Introduction

Among the numerous sources of environmental contamination (e.g., fossil fuel combustion, industrial activities, (Kendall et al., 2016), modern agriculture is well-known for the massive release of toxic substances in the environments (Jayaraj et al., 2016; Solomon and Thompson, 2003). Indeed, pesticides are specifically designed to alter the functioning of diverse organisms (weeds, fungi, and insects) which are detrimental to crop productivity (Sharma et al., 2019). Although traditional toxicology studies have convincingly demonstrated the potential toxicity of such compounds on non-target organisms (Crump et al., 2002; de Brito Rodrigues et al., 2019; Hasenbein et al., 2017), they are not sufficient to assess their impact on wild animals for several reasons. First, they often rely on relatively high, environmentally irrelevant concentrations of agrochemicals in order to assess mortality thresholds (i.e., LD50, Relyea and Edwards, 2010). As a consequence, reliance on LD50 limits our ability to identify more subtle, sublethal effects occurring at lower concentrations (Beiras, 2018). However, such sublethal effects have been shown to occur at extremely low concentrations and to be responsible for the disruption of several organismal traits that can affect population persistence (Cauble and Wagner, 2005; Cheron and Brischoux, 2020; Lenkowski et al., 2010).

Importantly, field monitoring often reveals that environmental contamination occurs at concentrations that are well below LD50 (Awkerman and Raimondo, 2018; Egea-Serrano et al., 2012). Second, most toxicology studies use laboratory models which can differ from their wild counterparts in their response to environmental contamination (Hahn and Sadler, 2020; Patisaul et al., 2018). Indeed, laboratory conditions simplify the complexity that organisms face in natural environments and long-term selection of laboratory lineages are likely to influence the responses of these species to environmental constraints (Hahn and Sadler, 2020; Segner and Baumann, 2016). Overall, one of the prominent current challenges is to assess the

detrimental sublethal effects of contaminants at environmentally relevant concentrations on non-model organism's representative of exposure in the wild. This is particularly relevant for pesticides, because their breakdown after spread in agricultural fields can expose organisms to concentrations much lower than those originally used or tested in laboratory experiments (Bonansea et al., 2017; Wang et al., 2019).

Glyphosate remains one of the most widely used non-selective herbicide (Clapp, 2021; Helander et al., 2012). Accordingly, the effects of glyphosate on wildlife, including investigations of commercial formulations and/or of different surfactants have been relatively thoroughly studied (Berger et al., 2018; Bókonyi et al., 2017; Cauble and Wagner, 2005; de Brito Rodrigues et al., 2019; Giaquinto et al., 2017; Gill et al., 2018; Guilherme et al., 2010; Herek et al., 2020; Lenkowski et al., 2010; Matozzo et al., 2020; Muñoz et al., 2021; Solomon and Thompson, 2003). However, its primary metabolite, aminomethylphosphonic acid (AMPA) is arguably the most common compound found in surface and groundwater worldwide (Bonansea et al., 2017; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018). The greater occurrence and abundance of AMPA in natural environments is likely linked to the extensive use of glyphosate and also because AMPA is an organic phosphonate derived from detergents (Grandcoin et al., 2017). In North American wetland, AMPA occurred at 97% ranging from 0.25 to 10  $\mu\text{g}\cdot\text{l}^{-1}$  (Okada et al., 2020). Environmental concentrations found in aquatic environments in France range from 0.1  $\mu\text{g}\cdot\text{l}^{-1}$  to 6.6  $\mu\text{g}\cdot\text{l}^{-1}$  (data from Water Agencies, "Agence de l'eau Loire Bretagne" and "Agence de l'eau Adour-Garonne"). Despite its greater environmental occurrence and abundance than glyphosate, studies on the effects of AMPA at environmental concentrations on wildlife are comparatively fewer (Cheron and Brischoux, 2020; de Brito Rodrigues et al., 2019; Domínguez et al., 2016; Guilherme et al., 2014; Martinez and Al-Ahmad, 2019; Matozzo et al., 2019, 2018). Importantly, these studies have revealed sublethal effects on wild species, such as genotoxic effect (Battaglin et al., 2014;

Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018) and cellular and biochemical alterations (Matozzo et al., 2019, 2018). AMPA has been shown to alter embryonic development in amphibians, increasing embryonic mortality and altering hatchling phenotype through processes occurring at low concentrations and suggesting nonmonotonicity (Cheron and Brischoux, 2020).

Our goal was to identify potential physiological mechanisms that may be responsible for the alteration of embryonic development due to environmentally relevant concentrations of AMPA (Cheron and Brischoux, 2020). In this respect, we focused our investigations on oxidative stress, as such cellular biochemical process is known to reflect how environmental stressors affect organismal physiology (Costantini, 2014). The metabolic activity of cells is responsible for a continuous generation of reactive chemical species (e.g. reactive oxygen species ROS), which cause oxidation of numerous organic substrates (e.g. lipids, DNA). Organisms are equipped with a complex array of molecular defence mechanisms, generally referred to as antioxidants. The cellular oxidative status is, therefore, determined by a complex network of molecules, including oxidative damages to organic substates, levels of both enzymatic and non-enzymatic antioxidants, or ROS production (Costantini, 2019). Any changes in these molecular components of cellular oxidative status provide evidence for oxidative unbalance. There is growing consensus that oxidative status alteration is one relevant molecular mechanism that links environmental conditions (including contaminant exposure) to fitness, through effects on growth, fertility, reproduction and senescence (Alonso-Alvarez et al., 2004; Beaulieu and Costantini, 2014; Costantini et al., 2014; Stier et al., 2012). In addition to markers of oxidative status, we included in our investigations telomere length that may reveal complementary effects of AMPA on DNA damage during amphibian embryonic development (Burraco et al., 2017; Chatelain et al., 2020; Cheron et al., 2021). Indeed, telomeres are repetitive nucleotide sequences located in each chromosome to ensure

stability and viability, and telomere length is increasingly suggested to be an integrative biomarker of organismal, cellular, and molecular damages. Although telomere shortening occurs normally in cell division, this process can be magnified under stressful conditions especially when they are associated with oxidative stress (Reichert and Stier, 2017). Accordingly, stress-induced telomere attrition may be linked to an intense cellular oxidative stress (Chatelain et al., 2020) because guanine (the main nucleotide of telomeres) is particularly sensitive to oxidation (Bjelland, 2003) . Finally, telomere length might be seen as a proxy of individual quality although such relationship is not well establish in ectotherms species (Burraco et al., 2020a; Olsson et al., 2018)

Specifically, we hypothesized that environmentally relevant concentrations of AMPA, which have been shown to induce altered embryonic mortality, development duration and hatchling phenotype (Cheron and Brischoux, 2020), will induce significant changes in oxidative status. We expected that exposition to AMPA concentrations will induce increase in antioxidant enzymes such as SOD, GPx and CAT and a depletion of thiols levels. Moreover, we expected non monotonic relationships between oxidative markers and AMPA concentrations given that some studies showed nonlinear or hormetic effects of AMPA or glyphosate on organisms (Brito et al., 2018; Cheron and Brischoux, 2020; Domínguez et al., 2016). If exposure to AMPA leads to increased oxidative stress, we also predicted that exposure to AMPA should be associated with shorter telomeres.

## **Materials and methods**

### *Study species*

Spined toad (*Bufo spinosus*) is a widespread amphibian species that persists in agricultural landscapes (Gagné and Fahrig, 2007; Guillot et al., 2016). Breeding pond (46°09'N, 0°24'W, France) was monitored every day during the reproductive period. All eggs strings (N=10) were collected during a single day in January 2018 immediately after laying in order to avoid development in the natural environment. A subset of each string containing 120 eggs was randomly selected and kept for our experiment (see below), while the remaining eggs (i.e., 3000-5000) were released at their site of origin.

#### *Treatment concentrations and chemical solutions*

Environmental concentrations found in aquatic environments in France range from 0.1 µg l<sup>-1</sup> to 6.6 µg l<sup>-1</sup> (data from Water Agencies, “Agence de l’eau Loire Bretagne” and “Agence de l’eau Adour-Garonne”) and are similar to concentrations found in aquatic environments in Northern America and Europe (Bonansea et al., 2017; Coupe et al., 2012; Grandcoin et al., 2017). Because our goal was to mimic the range of concentrations found in the wild, we aimed at three nominal concentrations spanning from low to relatively high concentrations (0.1 µg.l<sup>-1</sup>; 0.30µg.l<sup>-1</sup> and 3.00 µg.l<sup>-1</sup>, Table 1). Stock solutions (0.1 g/L) of AMPA were obtained by dissolving commercial crystalline powder (aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) in dechlorinated tap water. These stock solutions were further diluted with dechlorinated tap water to reach the selected concentration for each treatment group (Table 1). We produced additional water samples for each treatment in order to perform analytical verifications of the concentrations. These analytical verifications of the actual concentrations were performed by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Determination of AMPA in water samples was assessed using liquid chromatography-mass spectrometry (LC/MS/MS) with 9-fluorenylmethyl chloroformate (FMOC-Cl) used as derivatization agent. The limit of quantification was 0.1 µg.l<sup>-1</sup> for the determination of AMPA in water samples. Overall, these



verifications showed that actual concentrations were similar to concentrations found in aquatic environments (see above) and that the differences between “low” ( $0.07 \pm 0.01 \mu\text{g.l}^{-1}$ ), “medium” ( $0.32 \pm 0.052 \mu\text{g.l}^{-1}$ ) and “high” ( $3.57 \pm 0.153 \mu\text{g.l}^{-1}$ ) treatments were significant (Table 1).

For clarity, we will refer to the treatments as Low, Medium and High hereafter.

### *Experimental design*

Each subset of each clutch (containing 120 eggs) was further separated into segments containing 30 eggs (i.e., 120/4 treatments) that were randomly placed in different glass-molded aquariums containing 2 L of water and one of our experimental treatments (i.e., Control, Low, Medium or High) and monitored until hatching (total N = 4 treatments \* 10 clutches representing 60 tanks containing 30 eggs each).

Eggs were subjected to different concentrations of AMPA (Low, Medium and High) and a control during the whole embryonic development until hatching. To maintain relatively constant exposure levels throughout the experiment but to avoid excessive mechanical disturbances to developing eggs, water was changed once a week according to the half-life of AMPA in water (Battaglin et al., 2014). Our procedures did not alter egg jelly matrix surrounding the developing embryos throughout the experiment. We determined the stage of development according to the developmental table of Gosner (1960). Eggs were exposed from laying to hatching and hatching occurred at Gosner stage 22 (hereafter GS 22) after  $16.10 \pm 0.16$  days (see Table 2). All experiments took place in a thermal-controlled room with air and water temperature set at 17°C and with a natural day/night photoperiod (9h/15h). We monitored egg segments and counted all the individuals that hatched (GS22) which we considered a metric of hatching success. Development duration was assessed as the time elapsed between the date of egg collection (egg laying) and the date of hatching. We took

pictures of all live hatchlings using a camera (Panasonic Lumix DC-TZ55) in order to assess the length of individuals (N=80). Morphological measurements were performed with the software ImageJ (Schneider et al., 2012).

Upon hatching, for each clutch and within each treatment, we randomly collected 2 hatchlings that were pooled to assess oxidative status (because a single individual did not yield enough tissue for these assays) and 2 hatchlings that were used for telomere analyses. Hence, we measured clutch levels of oxidative status parameters.

#### *Oxidative status markers*

We randomly selected 80 hatchlings (20/experimental group), including two hatchlings that hatched from each single clutch. We pooled together these two hatchlings because a smaller portion did not provide enough tissue for the analyses. Thus, we had 10 pools per experimental group (i.e. 40 samples in total). We homogenized hatchlings in Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France) as an inhibitor of proteases using a TissueLyser II (Qiagen) at 30 Hz for 4 minutes. Afterwards, we centrifuged tubes for 10 minutes at 4°C to obtain clean supernatants to be used for the assays. We measured the concentration of thiols using the -SHp test (Diacron International, Grosseto, Italy); the activity of the antioxidant enzyme superoxide dismutase (SOD) using the Ransod assay (RANDOX Laboratories, France); the activity of the antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (RANDOX Laboratories, France); the activity of CAT using the OxiSelect CAT Activity Assay (Euromedex, France). We ran all analyses in duplicate and an average value of each duplicate was used for the statistical analyses (coefficients of variation < 10%). We normalized values of markers to the amount of proteins as quantified using the Bradford protein assay with albumin as reference standard (Sigma-

Aldrich, France). All assays were run according to manufacturer's instructions and have been validated in amphibian species (Liparoto et al., 2020).

In addition to markers alone we chose to show balanced or unbalanced ratio of markers activities following de Haan et al. studies (1992, 1996) (de Haan et al., 1996, 1992). An unbalanced ratio of superoxide dismutase and glutathione peroxidase (SOD/GPx) can lead to a proliferation of  $H_2O_2$  leading to accelerated cellular senescence and altered morphology (de Haan et al., 1996). Moreover, since both GPx and catalase convert  $H_2O_2$  into  $H_2O$ , an unbalanced ratio of superoxide dismutase on GPx and catalase activities (SOD/(GPx+CAT) can lead to a proliferation of deleterious pro-oxidants (de Haan et al., 1992).

#### *Relative telomere length*

Similarly to sampling for oxidative stress markers, we randomly selected two hatchlings among the embryos that hatched of each clutch for telomere analysis (N=20 hatchlings from 10 clutches). Although, smaller portions of individuals (e.g., head, tail, muscle) can be used for telomere assays (Burraco et al., 2017; Sánchez-Montes et al., 2020), hatchling spined toads were too small to provide enough tissue for these analyses and we used whole individuals (Cheron et al. 2021). We used quantitative PCR (qPCR; BioRad CFX 96, Bio-Rad, USA) to determine telomere length using an established protocol (McLennan et al., 2019), which was adapted for spined toad (Cheron et al. 2021). Whole hatchlings were digested with proteinase K and DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel), following the manufacturer's instructions. DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). Universal telomere primers were used, and the control single-copy gene Recombination Activating Gene 1 (RAG-1) was selected and amplified using specific primers designed for the spined toad: RAG1-F 5'-GGGTCCTCTGATAGCCGAAA-3' and RAG1-R 5'-

CATCATAACCTGTACCCCGGA-3'. This single-copy gene has previously been successfully used in multiple species (birds (Sebastiano et al., 2020); fish (Dupoué et al., 2020; McLennan et al., 2019; Petitjean et al., 2020)) including amphibians (Canestrelli et al., 2020). All qPCRs were performed on three plates for each gene (RAG-1 and telomere) using 7.5 ng of DNA per reaction. The telomere and single-copy gene primers were respectively used at a concentration of 800 nM and 300 nM. To generate a six-point standard curve (from 50.0 ng to 1.5 ng) for controlling the amplifying efficiency of the reactions, serial dilutions of DNA from a pooled sample of 10 tadpoles were included on the plate (in triplicates). For both telomere and RAG-1 amplification, the melt curves displayed single sharp curves, validating amplification specificity of these primers. A reference toad sample was run in triplicate in all plates to account for inter-plate variation. All samples were run in duplicates and were randomly distributed across the PCR plates. Amplification efficiencies reached Mean  $\pm$  SE: RAG-1,  $87.48 \pm 5.92$ ; TEL,  $92.60 \pm 6.00$  and all  $R^2$  were always very high ( $>0.99$ ). The relative telomere length (expressed as T/S ratio) was calculated as the telomere copy number (T) relative to single-copy gene (S; RAG1), according to Cawthon (2002). Intra-plate variation for telomere and RAG1 were 2.07% and 2.53% respectively. Intra-plate variation for the T/S ratio was 3.46%. Inter-plate variations for telomere and RAG-1 were 2.07% and 1.89%, respectively. Inter-plate variation for the T/S ratio was 5.62%.

#### *Statistical analysis*

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.).

All data were tested for homogeneity of variance and normality with the Barlett's test and the Shapiro-Wilks test, respectively. We also checked the normality of the residuals using diagnostic plots. We checked which distribution fitted the best the model using Cullen & Frey plot (bootstrap at 500, "fitdistplus" package (Delignette-Muller and Dutang, 2015)). We

computed z-scores in all predictor variables (scaled to mean of 0 and standard deviation of 1) to avoid multicollinearity between treatment when we tested interaction or relation with developmental-related traits but also to avoid large differences in scale in the model. We checked for multicollinearity using the variance inflation factor (VIF, non-multicollinearity when below 2. "car" package). Clutch identity was used as random effect in all our models. Each clutch was assigned with the value of average telomere length ratio of the two tadpoles sampled. Because we did not measure oxidative status markers and telomere length on the same individual instead, we pooled individuals of the same family or used average values for telomere length, correlations between the two parameters were at clutch levels. We used  $\eta^2$  to measure the magnitude of the main effect (package "effect size").  $\eta^2$ : <0.01 considered negligible, between 0.01 and 0.06 considered small, between 0.06 and 0.14 considered medium and >0.14 considered large effect.

First to assess whether our AMPA treatment had an effect on oxidative status markers we fitted six linear mixed-effect models (LMERs, package *lme4* (Bates et al., 2015) with "Treatment" as fixed effect and "SOD", "GPx", "CAT", thiols", "SOD/GPX" "SOD/(GPx+CAT)" as dependent variables. Four individuals displayed disproportionately elevated CAT (tested with "rosnertest" and "EnvStats" packages) and were excluded from the analyses. We calculated 95% confidence intervals, p value and coefficient for each levels of fixed effect factor from 1000 bootstraps iterations ("*parameters*", packages, Appendix A, table S1).). When  $p < 0.05$ , the magnitude of the main effect was measured using  $\eta^2$  (package "effectsize").

Second, to test whether developmental-related traits differed between AMPA treatment groups, we fitted three linear mixed-effect models ("*lmer*" function, "*lme4*" package (Bates et

al., 2015)) with "Treatment" as fixed effect and "embryonic development duration", "total length" and "telomere length" as dependent variables. Because hatching success followed binomial distribution, we fitted one generalized linear mixed models ("glmer", "lme4" package(Bates et al., 2015) with "hatching success" as dependent variable.

Finally, to test if there was a relation between developmental-related traits and oxidative status and if this relation differed between treatment, we fitted LMER models with development-related trait ("embryonic development duration", "total length" and "telomere length") as dependent variables and z\_scored oxidative status markers ("SOD", "GPx", "CAT", thiols, "SOD/GPx" "SOD/(GPx+CAT)") as fixed effect in interaction with "Treatment". We ran GLMER for binomial data ("hatching success"). To compare the trends and regression slopes we used "emtrends" function in "emmeans" package. Due to high correlation between oxidative status markers (SOD, GPx, CAT) and ratios, we performed statistical models separately (Appendix A, table S2).

## Results

All oxidative status and developmental-related traits at each AMPA concentrations are summarized in Table 2.

### Effect of AMPA treatment on oxidative status markers

CAT, SOD and GPx were not influenced by AMPA treatment (respectively:  $F_{3,25}=2.05$ ,  $p=0.125$ ;  $F_{3,27}=0.92$   $p=0.439$ ;  $F_{3,27}=0.24$ ,  $p=0.869$ , Table 3). When including four outlier values for CAT, AMPA did not influence enzymatic activities ( $F_{3,27}=0.70$ ,  $p=0.560$ ). AMPA treatment

327 influenced concentration of thiols ( $F_{3,26}=3.66$ ,  $p=0.021$ ,  $\eta^2=0.21$ , Table 3). Hatchlings from low  
328 AMPA group displayed higher thiols than all other treatment groups (all  $p<0.028$ , Tukey  
329 HSD, Fig.1).

330 AMPA treatment significantly influenced SOD/(GPx+CAT) ratio ( $F_{3,25}=3.93$ ,  $p=0.017$ ,  
331  $\eta^2=0.27$ , Table 3). When including four outlier values for CAT, we found a marginal effect of  
332 AMPA in SOD/(GPx+CAT) ratio ( $F_{3,27}=2.92$ ,  $p=0.052$ ). Ratios at Low and Medium  
333 concentrations were significantly lower compared to High concentration (respectively: -  
334  $0.16[-0.30, -0.01]$ ,  $p=0.029$ ;  $-0.21[-0.35, -0.06]$ ,  $p=0.006$ , Fig.1). In Medium treatment,  
335 SOD/(GPx+CAT) ratio decreased compared to Control treatment ( $-0.13[-0.24, -0.02]$ ,  
336  $p=0.026$ ).

337 Finally, treatment did not affect SOD/GPx ratio ( $F_{3,36}=2.02$ ,  $p=0.127$ , Table 3) nor telomere  
338 length ( $F_{3,36}=0.51$ ,  $p=0.673$ )

339

#### 340 **Effect of AMPA treatment on developmental-related traits**

341 Hatching success varied among treatments ( $\chi^2_3=25.168$ ,  $p<0.001$ ); Low and Medium  
342 treatment yielded lower hatching success than Control or High treatment of AMPA (all  
343  $p>0.003$ ).

344 AMPA treatment did not influence embryonic development duration ( $F_{3,67}=1.15$ ,  $p=0.33$ ).

345 However, total length of hatchlings differed between treatments ( $F_{3,67}=5.57$ ,  $p=0.002$ ).

346 Hatchlings were larger at Low treatment compared to individuals from Control and High  
347 treatments (respectively,  $p=0.004$ ,  $p=0.002$ ).

348

#### 349 **Relationships among oxidative status markers and developmental-related traits**

350 We did not find any relationships among any oxidative status marker and telomere length

351 (all interaction  $p>0.26$ ). Total length of hatchlings was not related to any oxidative status

352 markers ( $p>0.25$ ). Relation between total length of hatchlings and oxidative status markers in

interaction with treatment was never significant (all  $p > 0.201$ ). We did not find any relation between oxidative status markers and embryonic development duration across or among groups (all  $p > 0.186$ ). Finally, although we found a significant interaction between SOD/GPx and SOD/(CAT+GPx) ratios, and hatching success, after close examination of the plots and removals of outliers, such interactions were no longer supported.

## Discussion

Our study shows that the primary metabolite of glyphosate – AMPA – alters oxidative status, but not telomere length, during embryonic development in an amphibian species that occurs in agricultural landscapes. Interestingly, these physiological effects occurred at environmentally relevant concentrations representative of actual concentrations of this contaminant in the wild. Furthermore, our results suggest non-monotonicity for some markers of oxidative status such as thiols and SOD/(CAT+GPx) ratio as shown for developmental-related traits in a related study (Cheron and Brischoux, 2020). SOD/(CAT+GPx) ratio is likely driven by CAT levels considering the trend for CAT to increase at Low and Medium level while GPx remained steady.

Exposure to low concentrations of AMPA influenced the levels of thiols but did not influence CAT levels, although both markers showed similar trends. For these markers, levels were higher at the lowest or the intermediate AMPA concentrations tested which suggest low concentration responses and non-monotonicity (Hill et al., 2018; Shi et al., 2016; Vandenberg et al., 2012). Interestingly, although we did not detect any influence of AMPA concentrations on SOD and GPx, we found that relevant ratios of these antioxidant enzymes (i.e., SOD/(GPx+CAT)) were influenced by AMPA treatments. Moreover, since both GPx and CAT convert  $H_2O_2$  into  $H_2O$ , an unbalanced ratio of superoxide dismutase on GPx and CAT



activities (SOD/(GPx+CAT) can lead to a proliferation of deleterious pro-oxidants (de Haan et al., 1992). SOD/(GPx+CAT) ratios showed low concentration responses and non-monotonicity, with the lowest values obtained for the lower and intermediate AMPA concentrations. These results suggest a possible low-concentration stimulatory effect of AMPA with upregulation of both CAT and thiols. One important feature of the regulation of oxidative status involves, first the dismutation by superoxide dismutase (SOD) of  $O_2^-$  into  $H_2O_2$  and then the conversion of  $H_2O_2$  to  $H_2O$  catalysed by CAT and/or GPx. However, when  $H_2O_2$  is produced at high concentrations, CAT is more efficient in the conversion of  $H_2O_2$  in  $H_2O$  than GPx, which might explain why we observed upregulation of CAT but not of GPx (Halliwell and Gutteridge, 2015). It is important to stress that antioxidants play an important role in protecting early development and, accordingly, their levels are known to increase during embryonic development in amphibians (Dandapat et al., 2003; Anguiano et al., 2001). Moreover, during early development, CAT levels are high while GPx levels are low which show the important role of CAT in removing toxic hydrogen peroxide (Aceto et al., 1994), and GPx activities tend to gradually increase during growth (Pašková et al., 2011).

To our knowledge, only few studies investigated relevant concentration of AMPA on biochemical parameters (Guilherme et al., 2014; Matozzo et al., 2019, 2018) and most studies have investigated its parent compound, glyphosate (de Brito Rodrigues et al., 2019). Guilherme et al. (2014) found DNA and chromosomal damage when *Anguilla Anguilla* were exposed for 1 or 3 days at sublethal concentration (11.8 and 23.6  $\mu g.l^{-1}$ ). In marine invertebrates, AMPA exposure altered oxidative status and enzymatic activities in *Mytilus galloprovincialis* when exposed for 7, 14 or 21 days to 1, 10 and 100  $\mu g.l^{-1}$  (Matozzo et al., 2019, 2018). In tadpole of another amphibian species (*Pelobates cultripipes*), exposure to sublethal concentration of glyphosate induced altered antioxidant activity by decreasing glutathione reductase activity but did not affect SOD nor CAT levels (Burraco and Gomez-Mestre, 2016).

In *Oncorhynchus mykiss*, glyphosate-based formulations altered energy metabolism (Le Du-Carrée et al., 2021) when exposed to 1 µg.l<sup>-1</sup> and in *Anguilla Anguilla* increased CAT levels in gills when exposed for 1 or 3 days at 58 and 116 µg.l<sup>-1</sup> (Guilherme et al., 2012). Focusing on glyphosate's primary metabolite, our study reinforces the potential of this widely used herbicide and of its metabolite AMPA (the main contaminants detected in surface waters worldwide, Battaglin et al., 2014; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018) to alter oxidative status in wildlife.

Interestingly, given the lower hatching success in Low and Medium groups, selective mortality of embryos susceptible to oxidative stress may have occurred in these two groups prior to hatching. According to this hypothesis, surviving embryos may be the individuals that are more resistant to both AMPA and AMPA-mediated oxidative stress. Such hypothesis dovetails with the lack of relationship between developmental traits and oxidative status markers. Indeed, embryonic development can be constrained by oxidative stress through oxidation of the thiol group of proteins (Goto, 1992), and accelerated development might enhance metabolism and induced cellular oxidative stress (Burraco et al., 2020b; Smith et al., 2016).

This process might also explain why we did not detect any correlation between telomere length and oxidative status parameters at clutch level. Telomere dynamics are influenced by environmental conditions and telomere length generally shortens under environmental stress (Chatelain et al., 2020). We expected that a reduction in telomere length because prior work showed that exposure to environmentally relevant concentrations of AMPA may damage DNA (de Brito Rodrigues et al., 2019; Guilherme et al., 2014). In our context, telomere length may be influenced by increased production of pro-oxidants or decreased of antioxidant defences (Chatelain et al., 2020; Reichert and Stier, 2017). However, if surviving embryos were more able to cope with oxidative stress, damages to their DNA may be

reduced (Coluzzi et al., 2014). Since the proliferation of H<sub>2</sub>O<sub>2</sub> induces cellular senescence (de Haan et al., 1996) or telomere attrition in human primary fibroblasts (Coluzzi et al., 2014), selective mortality might select coping individuals with preserved telomere length. Early development constitutes a phase where oxidative stress levels are high because of the presumed high metabolic needs. However, our results on AMPA exposure suggest an alteration of the trade-off between oxidative stress and developmental traits, thus the potential for AMPA to affect expression of life-history traits.

## Conclusions

Our results provide rare evidence for physiological effects of environmentally-relevant AMPA concentrations on amphibian embryos (Cheron and Brischoux, 2020). Interestingly, the integration of markers of oxidative status in our investigations allows to suggest potential physiological mechanisms responsible for the effects on developmental related traits detected in a previous study (Cheron and Brischoux, 2020). Our results also strengthen the idea that AMPA may also induce non-monotonic effects. These effects might indicate stimulation of the antioxidant machinery to buffer any pro-oxidant effect of AMPA, but also selected embryos that were more resistant to oxidative stress, two hypotheses which are not mutually exclusive. Further experiments using non-lethal concentration of AMPA are required in order to disentangle whether AMPA acts directly on the antioxidant machinery of tadpoles or if surviving individuals have higher ability to cope with oxidative stress. If our hypothesis on selective mortality holds true, it is possible that the long-term use of glyphosate may have acted as a potent selective pressure, thereby explaining why our study species can persist in agricultural landscapes (Guillot et al. 2016, but see Renoirt et al. 2021). In light of our findings, we urge for regulatory toxicology to investigate effects of parent compounds but, more importantly, also of their metabolites at environmentally relevant

456 concentrations on non-model species in order to comprehensively assess impacts of  
457 anthropogenic contaminants on the environment.

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## **Abbreviations**

AMPA, aminomethylphosphonic acid; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; SODGPx, ratio between SOD and GPx; SODCAT, ratio between SOD and CAT + GPx. GS: Gosner stage

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## **Ethics approval**

All applicable institutional and/or 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 Supérieur, de la Recherche et de L'innovation) under permit #APAFIS#13477-2018032614077834 v7

## **Competing interests**

We declare we have no competing interests.

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487 **Authors' contributions**

488 F.B. & M.C. conceived and designed the study, M.C. performed the experiment and collected  
489 the data, F.A., C.R. & M.C. performed telomeres assays, D.C performed oxidative stress  
490 markers assays, M.C, D.C., F.A & F.B. interpreted the data, M.C. & F.B. designed the figures  
491 and wrote the initial draft, M.C, D.C., F.A & F.B. revised the paper. All authors approved the  
492 final version of the manuscript and agreed to be accountable for all aspects of the work in  
493 ensuring that questions related to the accuracy or integrity of any part of the work are  
494 appropriately investigated and resolved.

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770 **Tables**

771

772 Table 1. Concentrations ( $\mu\text{g.l}^{-1}$ ) measured in the experimental tanks (Mean $\pm$  SD)

<b>Treatment</b>	<b>Actual AMPA concentrations</b>
Control	0.00 $\pm$ 0.00
Low	0.07 $\pm$ 0.01
Medium	0.32 $\pm$ 0.052
High	3.57 $\pm$ 0.153

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**Table 2:** Mean values of oxidative status markers and developmental-related traits amongst treatment groups of aminomethylphosphonic acid (AMPA)

	Treatment			
	Control (n=10)	Low (n=10)	Medium (n=10)	High (n=10)
<b>Oxidative stress markers</b>				
<i>SOD*</i>	3.822±0.447	3.139±0.360	2.835±0.386	3.277±0.509
<i>Thiols*</i>	0.075±0.002	0.082±0.002	0.072±0.002	0.070±0.002
<i>CAT*</i>	13.813±1.436	16.943±2.347	18.728±2.612	11.212±2.702
<i>GPx*</i>	0.110±0.013	0.106±0.009	0.114±0.008	0.118±0.012
<i>SOD/(GPx+CAT)</i>	0.307±0.048	0.204±0.039	0.175±0.035	0.352±0.067
<i>SOD/GPx</i>	39.274±6.419	30.239±2.896	25.145±3.355	28.077±3.514
<b>Developmental-related traits</b>				
<i>Total length (cm)</i>	0.842±0.016	0.896±0.016	0.864±0.020	0.837±0.013
<i>Hatching success</i>	0.997±0.002	0.963±0.020	0.953±0.032	0.997±0.003
<i>Telomere length (T/S ratio)</i>	0.951±0.041	0.942±0.020	0.928±0.042	0.972±0.044
<i>Development duration (days)</i>	16.10±0.233	16.30±0.213	16.30±0.163	16.45±0.221

Values are Mean±SE

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: CAT

\*: (units/mg protein)

**Table 3:** Comparisons of outputs of linear mixed models (LMER, "lmerTest" package) of oxidative stress markers amongst treatment of aminomethylphosphonic acid (AMPA). Clutch identity was used as random factor in all our models

Variable	Effect	df	F-value	p-value	Clutch identity	
					Variance	Residuals variance
<i>SOD</i> *	Intercept	1,9	79.21	<b>&lt;0.001</b>	0.365	1.401
	Treatment	3,36	0.92	0.439		
<i>Thiols</i> *	Intercept	1,9	727.65	<b>&lt;0.001</b>	<0.001	<0.001
	Treatment	3,36	3.66	<b>0.02</b>		
<i>CAT</i> *	Intercept	1,9	37.38	<b>&lt;0.001</b>	4.11	44.35
	Treatment	3,36	2.05	0.125		
<i>GPx</i> *	Intercept	1,9	103.99	<b>&lt;0.001</b>	<0.001	<0.001
	Treatment	3,36	0.24	0.869		
<i>SOD/(GPX+CAT)</i>	Intercept	1,9	44.85	<b>&lt;0.001</b>	0.0012	0.019
	Treatment	3,36	3.93	<b>0.017</b>		
<i>SOD/GPx</i>	Intercept	1,9	84.28	<b>&lt;0.001</b>	23.08	157.44
	Treatment	3,36	2.03	0.127		

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: CAT  
Values in bold are considered significant P<0.05

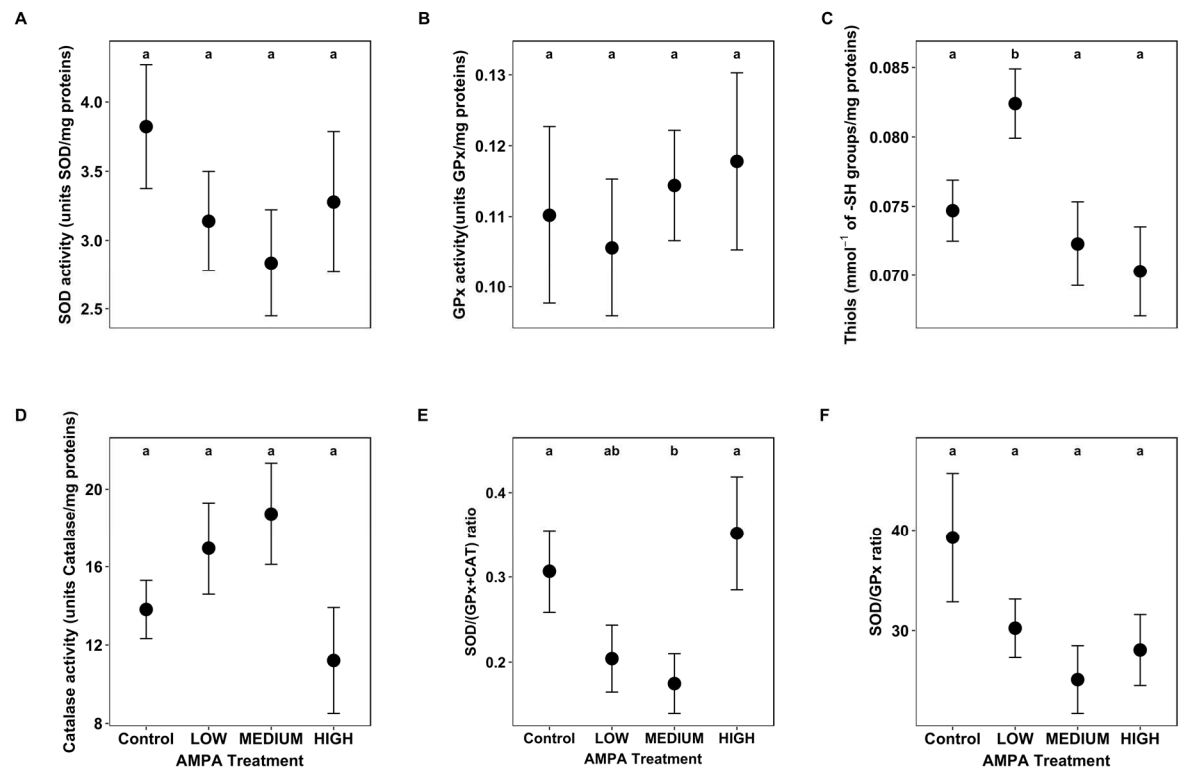
**Caption to figures**

**Figure 1:** **A/** Superoxide dismutase activity (units SOD/ mg proteins), **B/** Glutathione peroxidase activity (units GPx/ mg proteins) **C/** Thiols ( $\text{mmol}^{-1}$  of -SH groups/ mg proteins,) **D/** CAT activity (units CAT/ mg proteins) **E/** SOD/ (GPx+CAT) ratio **F/** SOD/ GPx ratio relative to AMPA treatments in *Bufo spinosus* tadpoles. Data represent mean  $\pm$  SE. Letters indicate significant differences.

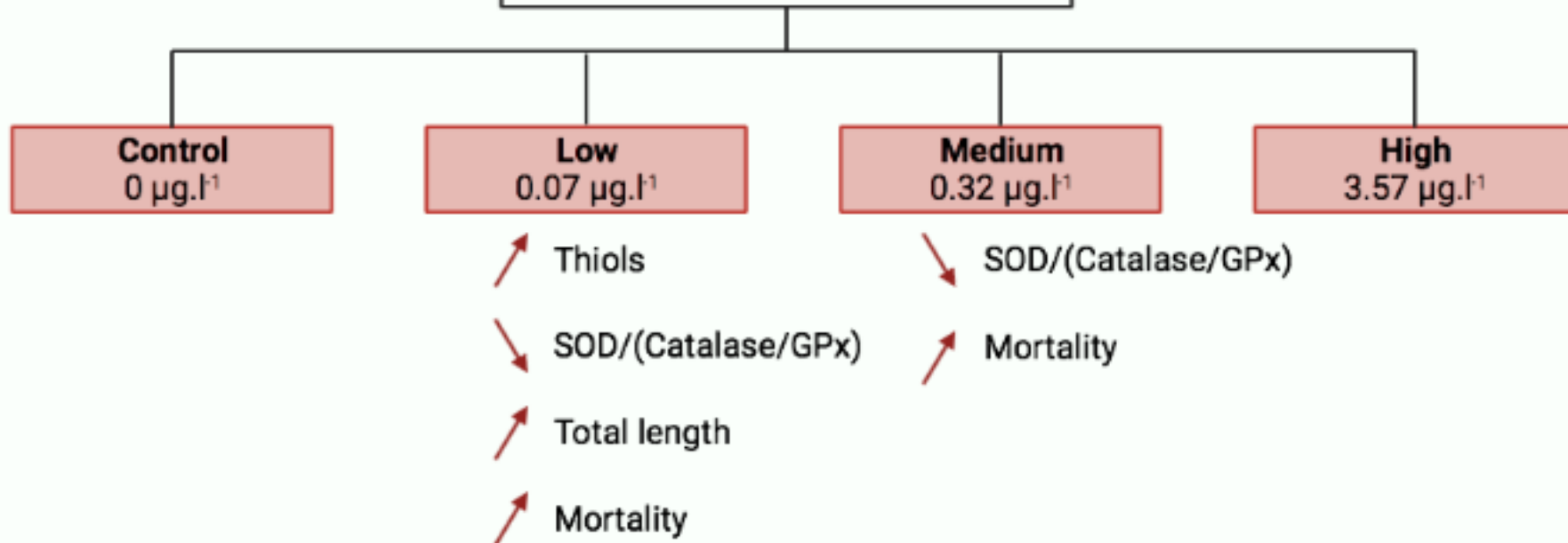
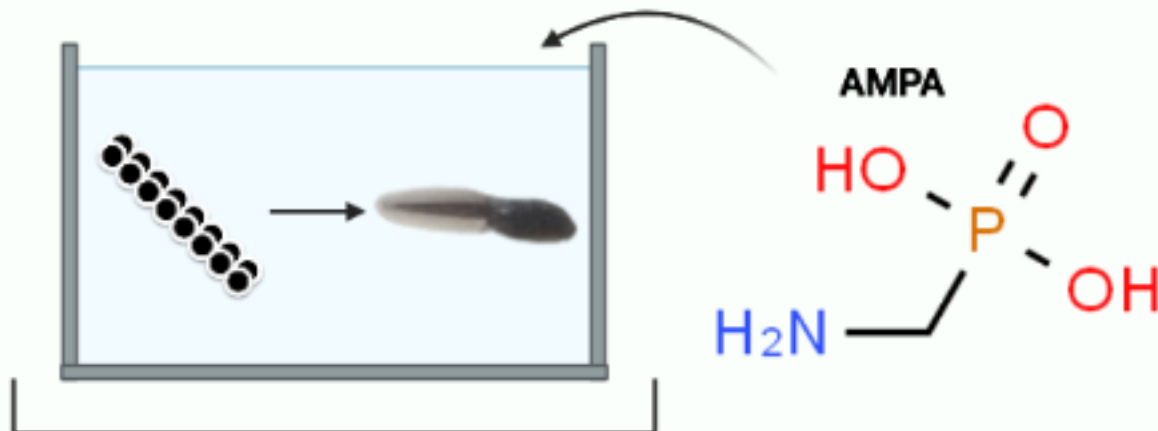


Figures

Figure 1:



*Bufo spinosus*



**Non monotonic dose response at sublethal concentrations**