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1 **Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative**
2 **status of hatchlings at environmental concentrations in an amphibian species.**

3 Marion Cheron^{1*}, David Costantini², François Brischoux¹

4 ¹ Centre d'Études Biologiques de Chizé, CEBC-CNRS UMR 7372, 79360 Villiers en Bois,
5 France

6 ² 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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9 **Running head:** Nicosulfuron altered embryonic development in tadpoles

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12 *Correspondence: cheron.marion@gmail.com

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Abstract

The widespread use of agrochemicals for controlling pests and diseases of crops is recognized as a main threat to biodiversity. Sulfonylurea herbicides are being increasingly used and display low levels of degradation in water which suggest that they might affect non-target organisms. In a common garden experiment, eggs of a widespread amphibian (*Bufo spinosus*) were exposed to sublethal environmentally relevant concentrations of a widely used sulfonylurea herbicide, nicosulfuron, during the whole embryonic development. We assessed development-related traits (i.e., development duration, hatching success, hatchling size and occurrence of malformation) as well as antioxidant markers in response to contamination (i.e., SOD, GPx, catalase, thiols and relevant ratios thereof). We found that sublethal concentrations of nicosulfuron increased embryonic development duration, increased hatchling size and tended to increase malformations. Embryos exposed to nicosulfuron displayed decreased thiols and increased catalase activity suggesting alteration of oxidative status. We did not find any effect of nicosulfuron on SOD and GPx levels. Interestingly, higher catalase activity was linked to higher proportion of malformed individuals, suggesting that exposure to nicosulfuron induced teratogenic effects. Our results suggest that alteration of antioxidant levels might be one physiological mechanism through which nicosulfuron might cause detrimental effects on amphibian embryos. Sublethal effects of pesticides at environmentally relevant concentrations have been overlooked and require further investigations, especially in non-target taxa occurring in agricultural landscapes.

Key-words: oxidative status, sulfonylurea, amphibian, herbicide toxicity

57 **Introduction**

58

59 Environmental contamination is one of the major threats to biodiversity since the beginning
60 of the century (Backhaus et al., 2012; Ceballos et al., 2015). The effects of contaminants on
61 wildlife range from consequences at the individual scale (i.e., genotoxicity, physiological
62 alterations, Beasley, 2020; Kendall et al., 2016; Lushchak, 2016; Relyea, 2009) to large scale
63 populational effects (Hamilton et al., 2016; McCarthy, 2018; Sievers et al., 2019). The majority
64 of environmental contamination comes from agricultural practices through the release of
65 agrochemical compounds (e.g. pesticides) in the wild (Prakash et al., 2018). Effects of
66 pesticides on wildlife are well studied but often focus on compounds that have been on the
67 market for long time and used massively (i.e., legacy pesticides such as glyphosate,
68 neonicotinoids, atrazine or DDT, Annett et al., 2014; Lenkowski et al., 2010; Pandey and
69 Mohanty, 2015).

70

71 Herbicides have been used to control weeds since decades (e.g., glyphosate, Prakash et al.,
72 2018) and new molecules are regularly developed and used, among which sulfonylurea
73 herbicides that are applied during the spring in a large variety of crops including wheat, oat,
74 soybean or maize (Eizenberg et al., 2003; Kearney, 1988). These herbicides are increasingly
75 being introduced into the environment through direct application and runoff from
76 agricultural activities (Battaglin et al., 2009, 2000; Cessna et al., 2015; de Lafontaine et al.,
77 2014). Among sulfonylurea herbicides, nicosulfuron is characterized by elevated DT50
78 values (75days) and slow photodegradation in water (Fenoll et al., 2012). This relative
79 stability in aquatic ecosystems suggests low levels of abiotic and microbial degradations
80 (Cessna et al., 2015). As a consequence, the persistence of nicosulfuron in aquatic ecosystems
81 further suggests that environmentally relevant concentrations may affect non target
82 organisms. For instance, nicosulfuron is frequently detected in surface waters (e.g., rivers

83 and ponds) and wetland sediments in northern America (Battaglin et al., 2009, 2000; de
84 Lafontaine et al., 2014; Degenhardt et al., 2009) and Europe (data from Agences de l'Eau).
85 Although detected concentrations are usually low (i.e., between 0.01 µg.l⁻¹ and 3.2 µg.l⁻¹,
86 Battaglin et al. 2009, de Lafontaine et al. 2014; as compared to a no observed effect
87 concentration [NOEC] of 5.2mg.l⁻¹ assessed on *Daphnia magna*, EFSA, 2007), nicosulfuron
88 causes increased oxidative stress and increased AChE activity in earthworms (Hackenberger
89 et al., 2018) as well as erratic behaviour and increased AChE in goldfish (Bretaud et al., 2000;
90 Saglio et al., 2001) at environmentally relevant concentrations. Conversely, it has been
91 suggested that nicosulfuron has small effects in some mammal, bird or amphibian laboratory
92 species (EFSA, 2007). Yet, these conflicting results are based on a limited number of studies
93 and species. It remains thus essential to assess the effects of environmentally relevant
94 concentrations of nicosulfuron on non-target organisms, and to identify the physiological
95 mechanisms mediating such effects.

96

97 Amphibians are particularly well-suited to assess the effects of agrochemicals on non-target
98 organisms (Beasley, 2020; Mann et al., 2009). Many amphibian species have complex
99 (biphasic) life cycles with terrestrial adults breeding in aquatic sites (ponds) where eggs and
100 larvae develop. Breeding and post metamorphosis migrations often coincide with
101 agrochemical application in agricultural lands, which makes amphibians particularly
102 vulnerable to pesticides (Berger et al., 2013). Their highly permeable skin, which allows for
103 the exchange of gases, water and ions, may also facilitate the diffusion of harmful
104 substances, such as agrochemicals, especially during their aquatic larval stage (Quaranta et
105 al., 2009). Many species of amphibians are particularly affected by environmental
106 contamination (Beasley, 2020), and they have been highlighted as relevant model organisms
107 to investigate the consequences of environmental contaminants (Hayes et al., 2006; Mann et
108 al., 2009). The use of oxidative status as a biomarker of contaminant exposure represents a

109 powerful tool for assessing the quality of an aquatic environment. Antioxidant enzyme
110 activity, the amount of thiol groups and markers of oxidative damage (ROS damage) to
111 biomolecules (lipids, vitamin, DNA) are often used to monitor the presence of pro-oxidant
112 substances in aquatic environments (Trevisan et al., 2013). For instance, in *Bufo spinosus*
113 embryos, exposure to a metabolite of glyphosate induced perturbation in antioxidant levels
114 at environmental concentrations (Cheron et al. 2022). Similarly, in *Pelobates cultripes* tadpoles,
115 exposure to sublethal concentrations of glyphosate decreased glutathione reductase activity
116 but did not affect SOD nor CAT levels (Burraco and Gomez-Mestre, 2016).

117

118 In this study, we experimentally investigated for the first time the effects of environmentally
119 relevant concentrations of nicosulfuron on embryonic development and physiology of the
120 spined toad (*Bufo spinosus*), a widespread amphibian species known to persist in agricultural
121 areas (Guillot et al., 2016) and presumably exposed to contaminants (Storrs Méndez et al.,
122 2009; Van Meter et al., 2015). We examined the influence of exposure to nicosulfuron on
123 metrics of embryonic development (embryonic survival, development duration and hatching
124 morphology, (Cheron et al., 2021a) in conjunction with markers of oxidative status that can
125 be influenced by nicosulfuron (e.g., Hackenberger et al., 2018). We tested low but
126 environmentally relevant concentrations (i.e $\sim 0.1 \mu\text{g.l}^{-1}$ and $\sim 0.8 \mu\text{g.l}^{-1}$, data from water
127 agencies) and we expected that exposure to nicosulfuron should alter embryonic
128 development (survival, duration and morphology) and affects oxidative status.

129

130 **Materials and methods**

131

132 *Study species*

133 We collected egg strings (N=20) of spined toad between the end of February and the
134 beginning of March in a pond near the present study site. Eggs were collected immediately

135 after laying and brought back to the laboratory. A subset of each string containing 90 eggs
136 was randomly selected and kept for our experiment, while the remaining eggs (i.e., 3000-
137 5000) were released at their site of origin. The 90 eggs from each clutch were separated into
138 segments containing ~30 eggs (ie. 90/3 treatments, see below) and placed in separate glass
139 tanks containing 2 L of dechlorinated tap water (changed once a week). We monitored the
140 eggs until hatching (total N= 2400 eggs). We determined the stage of development using
141 Gosner (1960). Hatching occurred at Gosner stage 22 (hereafter GS 22) after 12.97 ± 0.04 days
142 (see results). All experiments took place in a thermally controlled room with air and water
143 temperature set at 17°C. Photoperiod was controlled in a 12:12h cycle using LEDs placed
144 across the room.

145

146 *Treatment concentrations and chemical solutions*

147 In surface waters close to our study site, mean nicosulfuron concentrations range from 0.005
148 $\mu\text{g l}^{-1}$ to 0.920 $\mu\text{g l}^{-1}$ (mean: $0.043 \pm 0.076 \mu\text{g l}^{-1}$) and maximum values range from 0.005 $\mu\text{g l}^{-1}$ to
149 10.72 $\mu\text{g l}^{-1}$ (mean of maximum concentrations: $0.142 \pm 0.076 \mu\text{g l}^{-1}$, data from Agence de l'Eau
150 Loire-Bretagne).

151 In order to mimic the range of concentrations found in the wild, we produced three
152 experimental concentrations of nicosulfuron: control treatment (0 $\mu\text{g.l}^{-1}$), low concentration
153 (0.15 $\mu\text{g.l}^{-1}$) and high concentration (0.83 $\mu\text{g.l}^{-1}$, Table 1). We obtained stock solutions (0.1 g.l^{-1})
154 of nicosulfuron by dissolving commercial crystalline powder (PESTANAL, Sigma-Aldrich)
155 in dechlorinated tap water. These stock solutions were further diluted with dechlorinated
156 tap water to reach the concentration required for each treatment (Table 1). Analytical
157 verifications of the concentration were performed in triplicate by an independent accredited
158 analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Determination of
159 nicosulfuron in water samples was assessed using liquid chromatography-mass
160 spectrometry (LC/MS/MS) by direct injection with a limit of quantification of 0.1 $\mu\text{g.l}^{-1}$.

161 Overall, these verifications showed that actual concentrations were similar to concentrations
162 found in aquatic environments and that the differences between “low” and “high”
163 treatments were significant (Table 1). For clarity, we will refer to the treatments as Low and
164 High hereafter.

165

166 *Experimental design*

167 Eggs were exposed to two concentrations of nicosulfuron (Low and High) and a control
168 during the whole embryonic development until hatching. To maintain relatively constant
169 exposure levels throughout the experiment but to avoid excessive mechanical disturbances
170 to developing eggs, water was changed once a week even though the half-life of
171 nicosulfuron can range from 15 to 75 days (EFSA, 2007; Cessna et al., 2015). Egg jelly was
172 maintained throughout the experiment.

173

174 *Measurements*

175 We monitored egg segments and counted all the individuals that hatched (GS22) which we
176 considered a metric of hatching success. Undeveloped embryos and embryos that did not
177 achieve total development were individually counted. Development duration was assessed
178 as the time elapsed between the date of egg collection (egg laying) and the date of hatching
179 (Cheron et al., 2021a). Development duration did not differ within egg segment of each
180 clutch. Malformed tadpoles (axial malformation, oedema, scoliosis of the tail, Cooke, 1981;
181 Wagner et al., 2014) were also individually counted.

182 We took pictures of all live and undeformed hatchlings using a camera in order to assess the
183 standardized length (body and tail, according to Watters et al., 2016) of individuals
184 (N=1252). Morphological measurements were performed with the software ImageJ
185 (Schneider et al., 2012).

186

187 *Oxidative status markers*

188 We randomly selected 60 hatchlings (20/experimental group), including two hatchlings per
189 clutch. Because smaller portion did not provide enough tissue, we pooled together these two
190 hatchlings for the analysis. We gave us 10 pools per experimental group (i.e. 30 samples in
191 total). We homogenized hatchlings in Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich,
192 France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France)
193 as an inhibitor of proteases using a TissueLyser II (Qiagen) at 30 Hz for 4 minutes.
194 Afterwards, we centrifuged tubes for 10 minutes at 4°C to obtain clean supernatants to be
195 used for the assays. We measured the concentration of thiols using the -SHp test (Diacron
196 International, Grosseto, Italy); the activity of the antioxidant enzyme superoxide dismutase
197 (SOD) using the Ransod assay (RANDOX Laboratories, France); the activity of the
198 antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (RANDOX
199 Laboratories, France); the activity of catalase using the OxiSelect Catalase Activity Assay
200 (Euromedex, France). We ran all analyses in duplicate and the CV of measurements was
201 always < 10%. We standardized values of markers by the amount of proteins as quantified
202 using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich,
203 France). All assays were run according to manufacturer's instructions.

204 Balanced or unbalanced ratio of markers activities are known to alter cells integrity (de Haan
205 et al., 1996, 1992). Hence, in addition to markers alone we chose to display ratio of
206 superoxide dismutase and glutathione peroxidase (SOD/GPx) and ratio ratio of superoxide
207 dismutase on GPx and catalase activities (SOD/(GPx+CAT). Unbalanced ratio of
208 (SOD/(GPx+CAT) can lead to a proliferation of deleterious radicals (de Haan et al., 1992)
209 and unbalanced ratio of (SOD/GPx) can lead to a proliferation of H₂O₂ heading to
210 accelerated cellular senescence and altered morphology (de Haan et al., 1996).

211

212 *Statistical analysis*

213 All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020)
214 and RStudio v 1.2.5042 (RStudio, Inc.).

215 All residuals were tested for homogeneity of variance and normality with the Barlett's test
216 and the Shapiro-Wilks test, respectively. We also checked the normality of the residuals
217 using diagnostic plots. We checked which distribution fitted the best the model using Cullen
218 & Frey plot (bootstrap at 5000, "fitdist" package). We scaled to mean of 0 and standard
219 deviation of 1 (z_score of oxidative status markers and developmental-related traits) to avoid
220 multicollinearity between treatment when we tested interaction or relation with
221 developmental-related traits. We checked for multicollinearity using the variance inflation
222 factor (VIF, non-multicollinearity when below 2. "car" package). We kept clutch identity as a
223 random factor to avoid significant pseudoreplication issues. We did not use tank as the
224 replication unit in our analytical design because each tank represented a single treatment
225 (either control, low or high) and contained only the eggs of one clutch.

226 Conversely, sampling date never influenced our results, and it was excluded from our final
227 analyses. Sample sizes were balanced across clutches and treatments (e.g., each clutch was
228 represented by 30 eggs in each treatment).

229 We performed effect size tests with statistical power analysis to assess the magnitude of the
230 difference between treatments (Table S1, Cohen, 2013). When $p < 0.05$, the magnitude of the
231 main effect was measured using η^2 and the magnitude between two groups was measured
232 using Hedge's g (package "effectsize", Supplementary materials table S1). DABEST package
233 was used to provide Gardner-Altman estimation plot as well as between- and within-group
234 effect sizes as well as bias-corrected, bootstrapped, 95% confidence intervals around these
235 estimates (Supplementary material, Figure S1, S2). All confidence intervals were produced
236 via bootstrap with 5 000 resamples. Effect sizes were considered to be small when $g = 0.2$ (1%
237 of the variance), intermediate when $g = 0.5$ (9% of the variance), or large when $g = 0.8$ (25%
238 of the variance) as suggested by Cohen (2013).

239 First, on the whole dataset, to test whether nicosulfuron treatment affected developmental
240 traits, we fitted two linear mixed models (LMERs, package *lme4*) with "Treatment" as fixed
241 and "Total length of hatchling" and "Embryonic development duration" as dependent
242 variables. Then, we fitted two generalized linear models (GLMERs, (package *lme4*)) with a
243 log-link function for variables following binomial distribution (hatching success and
244 occurrence of malformed tadpoles).

245 Second, to assess whether our nicosulfuron treatment affected oxidative status we fitted six
246 linear mixed-effect models (LMERs, package *lme4* (Bates et al., 2015)) with "Treatment" as
247 fixed effect and "SOD", "GPx", "catalase", "thiols", "SOD/GPX" "SOD/GPxCAT" as dependent
248 variables. We used "emmeans" package for pair-wise comparison.

249 Finally, on the subset of the individuals for which we assessed oxidative status markers to
250 test whether there is a relation between developmental-related traits and oxidative status
251 markers and whether such relation differed between treatments, we fitted LMER models
252 with development-related traits as dependent variable and *z_scored* oxidative status
253 markers in interaction with treatment in fixed effect. We fitted GLMER for binomial data
254 with "hatching success", "malformed" as dependent variable and *z_scored* oxidative status
255 markers in interaction with treatment as explanatory variables. To compare the trends and
256 regression slopes we used "emtrends" function in "emmeans" package. P-value ≥ 0.05 were
257 considered not significant.

258

259

260

261 **Results**

262 All data on embryonic traits and oxidative status markers are summarized in Table 2.

263

264 **Effect of nicosulfuron treatment on embryonic traits**

265 On the whole dataset, we found that nicosulfuron significantly influenced total length of
266 hatchlings ($F_{3,1234}=5.51$, $p=0.004$, Fig. 1). Post hoc tests showed that hatchlings from High
267 treatment were longer than individuals from the Low treatment ($p=0.003$, $g=-0.22$).

268 Nicosulfuron slowed down embryonic development duration ($F_{3,1234}=19.185$, $p<0.001$, Fig. 1);
269 post hoc tests showed that High groups differed from Control groups ($p=0.001$, $g=-0.08$) and
270 Low groups ($p=0.001$, $g=-0.12$). We did not find any effect of nicosulfuron on hatching
271 success ($p=0.13$, Fig. 1) and a marginal effect on the occurrence of malformed individuals
272 ($p=0.06$, Fig. 1).

273

274 **Effect of nicosulfuron treatment on oxidative status**

275 Neither SOD, GPx nor SOD/GPx and SOD/(GPx+CAT) ratios were affected by nicosulfuron
276 (all $p>0.214$, Fig.2). Nicosulfuron influenced concentration of thiols ($F_{2,28}=5.14$, $p=0.017$,
277 Fig.2). Hatchlings from Control group displayed higher thiols than all other treatment
278 groups (all $p<0.007$, all $g>0.94$, Fig.1). Catalase activity was marginally influenced by
279 nicosulfuron treatment ($F_{2,28}=3.41$, $p=0.055$, Fig.2), a result that was supported by effect size
280 estimates ($\eta^2 =0.15$).

281

282 **Relationships between embryonic traits and oxidative status**

283 In the subset of individuals in which we quantified oxidative status, total length of
284 hatchlings was influenced by the treatment ($F_{2,20}=3.721$, $p=0.044$, same pattern than above)
285 but not by oxidative status markers (all $p>0.085$) except for a marginal relation with thiols

286 levels ($F_{2,24}=3.262$, $p=0.055$), or their interaction (all $p>0.089$) except for a marginal relation
287 between development related traits, GPx and treatment ($F_{2,20}=3.262$, $p=0.059$).
288
289 Embryonic development duration was not related to treatment for the subsample of
290 individuals for which we assessed oxidative status ($F_{2,20}=0.439$, $p=0.649$). Development
291 duration was negatively linked to SOD ($F_{2,20}=6.84$, $p=0.015$) and thiols ($F_{2,20}=4.62$, $p=0.048$).
292 We did not find any interaction between markers and treatment (all $p>0.363$) except for a
293 marginal interaction with SOD/(catalase+GPx) ($F_{1,20}=6.88$, $p=0.056$).
294
295 Hatching success was influenced by treatment in the individuals for which we assessed
296 oxidative status ($X^2=21.09$, $p=0.001$). The interaction with treatment was significant for
297 hatching success and SOD ($X^2=10.86$, $p=0.004$). Control individuals displayed a positive
298 relationship between hatching success and SOD, which was marginally different from the
299 relationships found in the High group ($p=0.054$) and significantly different from Low group
300 ($p=0.03$) with both groups of treated individuals displaying negative relationships between
301 hatching success and SOD. Catalase influenced hatching success between treatments
302 ($X^2=25.41$, $p<0.01$). Control individuals displayed a positive relationship between hatching
303 success and Catalase which was different from High group hatchlings which displayed
304 negative relationship ($p<0.01$) and Low group individuals which displayed positive
305 relationship ($p<0.01$). Hatching success was related to thiols ($X^2=5.442$, $p=0.019$) and the
306 interaction with treatment was also significant ($X^2=7.815$, $p=0.020$). Control individuals
307 displayed a positive relationship between hatching success and thiols that was significantly
308 different from Low individuals that displayed a negative relationship ($p=0.016$).
309
310 Finally, the number of malformed tadpoles did not differ among treatments ($X^2=1.836$,
311 $p=0.340$). We found a negative relation between the occurrence of malformed hatchlings and

312 thiols ($X^2=12.012$, $p<0.005$, Fig.3) and a positive relation between the occurrence of
313 malformed hatchlings and catalase levels ($X^2=20.313$, $p<0.001$, Fig.3) with no interactions
314 with the treatment (all $p>0.07$).

315

316 **Discussion**

317

318 Overall, we found that environmentally relevant concentrations of nicosulfuron altered
319 embryonic development of *Bufo spinosus*, a species that occurs in agricultural areas. Most
320 notably, exposure to nicosulfuron increased embryonic development duration and
321 influenced hatchling morphology. In addition, exposure to nicosulfuron caused lower levels
322 of thiols and marginally higher levels of catalase activity. Interestingly, lower levels of thiols
323 were linked to higher embryonic mortality, higher proportion of malformed individuals and
324 longer embryonic development duration; and higher catalase activity were also linked to
325 higher proportion of malformed individuals, suggesting that the effects of exposure to
326 nicosulfuron induced teratogenic effects.

327

328 The highest concentration of nicosulfuron we tested ($0.83\mu\text{g.l}^{-1}$) is very similar to
329 concentrations found in surface waters (Battaglin et al., 2009). Although sublethal, this
330 concentration influenced embryonic development of *Bufo spinosus*, which is exemplified by
331 increased development durations and larger body size. Interestingly, the pattern of
332 covariation between these two traits was expected given the widespread relationship
333 between embryonic development time and body size (Gillooly and Dodson, 2000). Whether
334 these responses can have consequences for tadpoles developing under natural conditions is
335 complicated to assess. Delayed hatching may potentially increase the vulnerability of
336 immobile embryos to predators (Zamudio et al., 2016) or increase the vulnerability of
337 tadpoles to desiccation if spawning occurs in temporary water bodies (Lindgren et al., 2018;

338 Székely et al., 2017). Conversely, increased body size may improve mobility and thus
339 foraging ability while decreasing susceptibility to predation (Broomhall and Shine, 2003;
340 Cheron et al., 2021b; Hoff and Wassersug, 2000). Such hypotheses will require thorough
341 testing, especially as post-embryonic survival might be more critical to population
342 persistence than egg survival (Touchon et al., 2013; Vonesh and De la Cruz, 2002).
343 Surprisingly, these effects were detectable for our “high” treatment solely suggesting that the
344 lower concentration we tested did not trigger visible effects on embryonic development, as
345 expected from classical concentration-response relationships (Vandenberg et al., 2012).
346 Indeed, a previous study on Goldfish showed an alteration of swimming behaviour due to
347 exposure to nicosulfuron and further highlighted an increase in swimming alterations along
348 with exposure to increasing nicosulfuron concentrations (Saglio et al., 2001). Because our
349 “high” treatment is situated at the lower range of maximum concentrations assessed in
350 surface water (see “Materials and methods section”), it is plausible that free ranging *Bufo*
351 *spinosus* embryos are exposed to higher concentrations which could induce strong
352 consequences on hatching success. Future studies including a larger range of tested
353 concentrations are required to describe reaction norms of embryos exposed to nicosulfuron.
354
355 Regardless of the concentration tested, nicosulfuron influenced thiol levels with lower levels
356 - hence higher oxidation of thiols - in hatchlings exposed during their embryonic
357 development. Thiols are relevant indicators of the antioxidant capacity of an organism
358 (Lushchak, 2011), so that their concentration decreases when there are strong perturbations
359 to the oxidative status (Baba and Bhatnagar, 2018; Ulrich and Jakob, 2019). As a
360 consequence, our results suggest that exposure to nicosulfuron altered oxidative status. We
361 also found a trend for catalase to increase across treatments. The enzymatic antioxidant
362 protection occurs through two major steps: first superoxide dismutase (SOD) breaks down
363 O_2^- into H_2O_2 , then H_2O_2 is converted into H_2O by catalase and/or glutathione peroxidase

364 (GPx). However, when H₂O₂ is produced at high concentrations, catalase is more efficient in
365 the conversion of H₂O₂ in H₂O than GPx which can explain the trend we detected (Halliwell
366 and Gutteridge, 2015). In keeping with the results on development duration (see above), it is
367 noteworthy that a rapid development may increase oxidative damage, with possible long-
368 lasting effects (Janssens and Stoks, 2018). In this context, slowing down embryonic
369 development, as observed in individuals exposed to the highest nicosulfuron treatment
370 might constitute a strategy to avoid a disproportional increase of oxidative damages linked
371 to both exposure to nicosulfuron and rapid development.

372

373 Finally, we found that development-related traits (i.e., embryonic development duration,
374 number of malformed hatchlings, hatching success) were correlated to some markers of
375 oxidative status (SOD, thiols and catalase). The negative relationship between embryonic
376 development duration and SOD or thiols is consistent with other studies that suggested that
377 enhanced metabolism linked to accelerated development can increase cellular oxidative
378 stress (Burraco et al., 2020; Smith et al., 2016). Thiol levels were negatively correlated with
379 proportion of malformed hatchlings, and catalase activity was positively correlated to higher
380 proportion of malformed individuals. Such results strongly suggest that disruption in
381 oxidative status might be related to teratogenic effects (Wu et al., 2017; Xie et al., 2016).
382 Indeed, occurrence of malformations is known to be related to pesticide-induced oxidative
383 stress (Liendro et al., 2015; Rutkoski et al., 2020; Wells et al., 2005), suggesting that, in our
384 study, these teratogenic effects may be linked to nicosulfuron-induced oxidative unbalance.
385 Interestingly, occurrence of physical malformations in anuran tadpoles is known to be
386 frequent in agricultural habitats (Lajmanovich et al., 2003; Mann et al., 2009; Taylor et al.,
387 2005), and it has also been shown that such malformations are detrimental for mobility and
388 performance (Sotomayor et al., 2012), growth and survival (Herek et al., 2020; Ma et al., 2019;
389 Ruiz et al., 2010; Schuytema and Nebeker, 1998).

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Conclusions

Our results reinforce the fact that pesticides constitute a major threat to amphibians and point out to the importance of testing the effects of novel pesticides on wildlife. Studies on the impact of nicosulfuron on wildlife are lacking in the literature, although the substance is known to alter behaviour and have noticeable effect on AchE activity in fish at sublethal concentrations (Breteau et al., 2000; Saglio et al., 2001). To our knowledge, this is the first study to show the effects of nicosulfuron on biochemical markers and development-related traits in a non-target aquatic species. Given the fact that post metamorphic fitness is mostly explained by quality of larval development in amphibians (Bekhet et al., 2014; Boonekamp et al., 2018; Bredeweg et al., 2019), future studies should usefully investigate potential effects of nicosulfuron on larval development and metamorphosis. Finally, we emphasize that the sublethal effects of nicosulfuron have been overlooked to date (but see Hackenberger et al., 2018) and require further investigations on other non-target taxa occurring in agricultural landscapes.

408 **Declaration of interests**

409 The authors declare that they have no known competing financial interests or personal
410 relationships that could have appeared to influence the work reported in this paper.

411

412 **Data availability**

413 Data are available upon request

414

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426

427 **Ethics approval**

428 All applicable institutional and/or national guidelines for the care and use of animals were
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433

434 **Author contributions**

435 F.B. & M.C. conceived and designed the study, M.C. performed the experiment and collected
436 the data, D.C performed oxidative stress markers assays, M.C, D.C & F.B. interpreted the
437 data, M.C. & F.B. designed the figures and wrote the initial draft, M.C, D.C & F.B. revised the
438 paper. All authors approved the final version of the manuscript and agreed to be
439 accountable for all aspects of the work in ensuring that questions related to the accuracy or
440 integrity of any part of the work are appropriately investigated and resolved.

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444 **References**

445

- 446 Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based
 447 herbicides on the freshwater environment: Impact of glyphosate-based herbicides.
 448 *Journal of Applied Toxicology* 34, 458–479. <https://doi.org/10.1002/jat.2997>
 449 Baba, S.P., Bhatnagar, A., 2018. Role of thiols in oxidative stress. *Current Opinion in*
 450 *Toxicology* 7, 133–139. <https://doi.org/10.1016/j.cotox.2018.03.005>
 451 Backhaus, T., Snape, J., Lazorchak, J., 2012. The impact of chemical pollution on
 452 biodiversity and ecosystem services: the need for an improved understanding. *Integr*
 453 *Environ Assess Manag* 8, 575–576. <https://doi.org/10.1002/ieam.1353>
 454 Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models
 455 Using lme4. *Journal of Statistical Software* 67, 1–48.
 456 <https://doi.org/10.18637/jss.v067.i01>
 457 Battaglin, W.A., Furlong, E.T., Burkhardt, M.R., Peter, C.J., 2000. Occurrence of
 458 sulfonyleurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs
 459 and ground water in the Midwestern United States, 1998. *Science of The Total*
 460 *Environment* 248, 123–133. [https://doi.org/10.1016/S0048-9697\(99\)00536-7](https://doi.org/10.1016/S0048-9697(99)00536-7)
 461 Battaglin, W.A., Rice, K.C., Focazio, M.J., Salmons, S., Barry, R.X., 2009. The occurrence of
 462 glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in
 463 Washington, DC, Maryland, Iowa, and Wyoming, 2005-2006. *Environ Monit Assess*
 464 155, 281–307. <https://doi.org/10.1007/s10661-008-0435-y>
 465 Beasley, V.R., 2020. Direct and Indirect Effects of Environmental Contaminants on
 466 Amphibians, in: *Reference Module in Earth Systems and Environmental Sciences*.
 467 Elsevier. <https://doi.org/10.1016/B978-0-12-409548-9.11274-6>
 468 Bekhet, G.A., Abdou, H.A., Dekinesh, S.A., Hussein, H.A., Sebiae, S.S., 2014. Biological
 469 factors controlling developmental duration, growth and metamorphosis of the larval
 470 green toad, *Bufo viridis viridis*. *The Journal of Basic & Applied Zoology* 67, 67–82.
 471 <https://doi.org/10.1016/j.jobaz.2014.09.005>
 472 Berger, G., Graef, F., Pfeiffer, H., 2013. Glyphosate applications on arable fields considerably
 473 coincide with migrating amphibians. *Scientific Reports* 3.
 474 <https://doi.org/10.1038/srep02622>
 475 Boonekamp, J.J., Mulder, E., Verhulst, S., 2018. Canalisation in the wild: effects of
 476 developmental conditions on physiological traits are inversely linked to their
 477 association with fitness. *Ecology Letters* 21, 857–864.
 478 <https://doi.org/10.1111/ele.12953>
 479 Bredeweg, E.M., Urbina, J., Morzillo, A.T., Garcia, T.S., 2019. Starting on the Right Foot:
 480 Carryover Effects of Larval Hydroperiod and Terrain Moisture on Post-metamorphic
 481 Frog Movement Behavior. *Front. Ecol. Evol.* 7, 97.
 482 <https://doi.org/10.3389/fevo.2019.00097>
 483 Breaud, S., Toutant, J.-P., Saglio, P., 2000. Effects of Carbofuran, Diuron, and Nicosulfuron
 484 on Acetylcholinesterase Activity in Goldfish (*Carassius auratus*). *Ecotoxicology and*
 485 *Environmental Safety* 47, 117–124. <https://doi.org/10.1006/eesa.2000.1954>
 486 Broomhall, S., Shine, R., 2003. Effects of the Insecticide Endosulfan and Presence of
 487 Congeneric Tadpoles on Australian Treefrog (*Litoria freycineti*) Tadpoles. *Archives*
 488 *of Environmental Contamination and Toxicology* 45, 221–226.
 489 <https://doi.org/10.1007/s00244-003-0172-8>
 490 Burraco, P., Gomez-Mestre, I., 2016. Physiological Stress Responses in Amphibian Larvae to
 491 Multiple Stressors Reveal Marked Anthropogenic Effects even below Lethal Levels.
 492 *Physiological and Biochemical Zoology* 89, 462–472. <https://doi.org/10.1086/688737>

493 Burraco, P., Valdés, A.E., Orizaola, G., 2020. Metabolic costs of altered growth trajectories
494 across life transitions in amphibians. *Journal of Animal Ecology* 89, 855–866.
495 <https://doi.org/10.1111/1365-2656.13138>

496 Ceballos, G., Ehrlich, P.R., Barnosky, A.D., García, A., Pringle, R.M., Palmer, T.M., 2015.
497 Accelerated modern human-induced species losses: Entering the sixth mass
498 extinction. *Science Advances* 1, e1400253. <https://doi.org/10.1126/sciadv.1400253>

499 Cessna, A.J., Donald, D.B., Bailey, J., Waiser, M., 2015. Persistence of the Sulfonylurea
500 Herbicides Sulfosulfuron, Rimsulfuron, and Nicosulfuron in Farm Dugouts (Ponds). *J*
501 *Environ Qual* 44, 1948–1955. <https://doi.org/10.2134/jeq2014.11.0503>

502 Cheron, M., Angelier, F., Ribout, C., Brischoux, F., 2021a. Clutch quality is related to
503 embryonic development duration, hatchling body size and telomere length in the
504 spined toad (*Bufo spinosus*). *Biological Journal of the Linnean Society* 133, 135–142.
505 <https://doi.org/10.1093/biolinnean/blab035>

506 Cheron, M., Costantini, D., Angelier, F., Ribout, C., Brischoux, F., 2022.
507 Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic
508 development in an amphibian species. *Chemosphere* 131882.
509 <https://doi.org/10.1016/j.chemosphere.2021.131882>

510 Cheron, M., Raelison, L., Kato, A., Ropert-Coudert, Y., Meyer, X., MacIntosh, A.J.J.,
511 Brischoux, F., 2021b. Ontogenetic changes in activity, locomotion and behavioural
512 complexity in tadpoles. *Biological Journal of the Linnean Society* blab077.
513 <https://doi.org/10.1093/biolinnean/blab077>

514 Cohen, J., 2013. *Statistical power analysis for the behavioral sciences*, Routledge. ed.

515 Cooke, A.S., 1981. Tadpoles as indicators of harmful levels of pollution in the field.
516 *Environmental Pollution Series A, Ecological and Biological* 25, 123–133.
517 [https://doi.org/10.1016/0143-1471\(81\)90012-X](https://doi.org/10.1016/0143-1471(81)90012-X)

518 de Haan, J.B., Cristiano, F., Iannello, R., Bladier, C., Kelner, M.J., Kola, I., 1996. Elevation in
519 the Ratio of Cu/Zn-Superoxide Dismutase to Glutathione Peroxidase Activity Induces
520 Features of Cellular Senescence and This Effect Is Mediated by Hydrogen Peroxide.
521 *Hum. Mol. Genet.* 5, 283–292. <https://doi.org/10.1093/hmg/5.2.283>

522 de Haan, J.B., Newman, J.D., Kola, I., 1992. Cu/Zn superoxide dismutase mRNA and enzyme
523 activity, and susceptibility to lipid peroxidation, increases with aging in murine brains.
524 *Molecular Brain Research* 13, 179–187. [https://doi.org/10.1016/0169-328X\(92\)90025-](https://doi.org/10.1016/0169-328X(92)90025-7)
525 [7](https://doi.org/10.1016/0169-328X(92)90025-7)

526 de Lafontaine, Y., Beauvais, C., Cessna, A.J., Gagnon, P., Hudon, C., Poissant, L., 2014.
527 Sulfonylurea herbicides in an agricultural catchment basin and its adjacent wetland in
528 the St. Lawrence River basin. *Science of The Total Environment* 479–480, 1–10.
529 <https://doi.org/10.1016/j.scitotenv.2014.01.094>

530 Degenhardt, D., Cessna, A.J., Raina, R., Pennock, D.J., Farenhorst, A., 2009. Trace level
531 determination of selected sulfonylurea herbicides in wetland sediment by liquid
532 chromatography electrospray tandem mass spectrometry. *Journal of Environmental*
533 *Science and Health, Part B* 45, 11–24. <https://doi.org/10.1080/03601230903404291>

534 EFSA, 2007. Conclusion regarding the peer review of the pesticide risk assessment of the
535 active substance nicosulfuron. *EFSA Journal*. <https://doi.org/10.2903/j.efsa.2008.120r>

536 Eizenberg, H., Goldwasser, Y., Achdary, G., Hershenhorn, J., 2003. The Potential of
537 Sulfosulfuron to Control Troublesome Weeds in Tomato ¹. *Weed Technology* 17,
538 133–137. [https://doi.org/10.1614/0890-037X\(2003\)017\[0133:TPOSTC\]2.0.CO;2](https://doi.org/10.1614/0890-037X(2003)017[0133:TPOSTC]2.0.CO;2)

539 Fenoll, J., Hellín, P., Flores, P., Martínez, C.M., Navarro, S., 2012. Photocatalytic degradation
540 of five sulfonylurea herbicides in aqueous semiconductor suspensions under natural
541 sunlight. *Chemosphere* 87, 954–961.
542 <https://doi.org/10.1016/j.chemosphere.2012.01.056>

- 543 Gillooly, J.F., Dodson, S.I., 2000. The relationship of neonate mass and incubation
544 temperature to embryonic development time in a range of animal taxa. *Journal of*
545 *Zoology* 251, 369–375. <https://doi.org/10.1111/j.1469-7998.2000.tb01087.x>
- 546 Guillot, H., Boissinot, A., Angelier, F., Lourdais, O., Bonnet, X., Brischoux, F., 2016.
547 Landscape influences the morphology of male common toads (*Bufo bufo*).
548 *Agriculture, Ecosystems & Environment* 233, 106–110.
549 <https://doi.org/10.1016/j.agee.2016.08.032>
- 550 Hackenberger, D.K., Stjepanović, N., Lončarić, Ž., Hackenberger, B.K., 2018. Acute and
551 subchronic effects of three herbicides on biomarkers and reproduction in earthworm
552 *Dendrobaena veneta*. *Chemosphere* 208, 722–730.
553 <https://doi.org/10.1016/j.chemosphere.2018.06.047>
- 554 Halliwell, B., Gutteridge, J.M.C., 2015. *Free Radicals in Biology and Medicine*. Oxford
555 University Press. <https://doi.org/10.1093/acprof:oso/9780198717478.001.0001>
- 556 Hamilton, P.B., Cowx, I.G., Oleksiak, M.F., Griffiths, A.M., Grahn, M., Stevens, J.R.,
557 Carvalho, G.R., Nicol, E., Tyler, C.R., 2016. Population-level consequences for wild
558 fish exposed to sublethal concentrations of chemicals - a critical review. *Fish Fish* 17,
559 545–566. <https://doi.org/10.1111/faf.12125>
- 560 Hayes, T.B., Case, P., Chui, S., Chung, D., Haefele, C., Haston, K., Lee, M., Mai, V.P.,
561 Marjuoa, Y., Parker, J., Tsui, M., 2006. Pesticide Mixtures, Endocrine Disruption, and
562 Amphibian Declines: Are We Underestimating the Impact? *Environmental Health*
563 *Perspectives* 114, 40–50. <https://doi.org/10.1289/ehp.8051>
- 564 Herek, J.S., Vargas, L., Trindade, S.A.R., Rutkoski, C.F., Macagnan, N., Hartmann, P.A.,
565 Hartmann, M.T., 2020. Can environmental concentrations of glyphosate affect
566 survival and cause malformation in amphibians? Effects from a glyphosate-based
567 herbicide on *Physalaemus cuvieri* and *P. gracilis* (Anura: Leptodactylidae). *Environ*
568 *Sci Pollut Res* 27, 22619–22630. <https://doi.org/10.1007/s11356-020-08869-z>
- 569 Hoff, K. vS, Wassersug, R.J., 2000. Tadpole Locomotion: Axial Movement and Tail
570 Functions in a Largely Vertebraeless Vertebrate. *Integr Comp Biol* 40, 62–076.
571 <https://doi.org/10.1093/icb/40.1.62>
- 572 Janssens, L., Stoks, R., 2018. Rapid larval development under time stress reduces adult life
573 span through increasing oxidative damage. *Funct Ecol* 32, 1036–1045.
574 <https://doi.org/10.1111/1365-2435.13068>
- 575 Kearney, P.C. (Ed.), 1988. *Herbicides: chemistry, degradation and mode of action*. Vol. 3: ...
576 Dekker, New York, NY.
- 577 Kendall, R.J., Lacher, T.E., Cobb, G.C., Cox, S.B., Lacher, T.E., Cobb, G.C., Cox, S.B.,
578 2016. *Wildlife Toxicology : Emerging Contaminant and Biodiversity Issues*. CRC
579 Press. <https://doi.org/10.1201/9781439817957>
- 580 Lajmanovich, R.C., Sandoval, M.T., Peltzer, P.M., 2003. Induction of mortality and
581 malformation in *Scinax nasicus* tadpoles exposed to glyphosate formulations. *Bull*
582 *Environ Contam Toxicol* 70, 612–618. <https://doi.org/10.1007/s00128-003-0029-x>
- 583 Lenkowski, J.R., Sanchez-Bravo, G., McLaughlin, K.A., 2010. Low concentrations of
584 atrazine, glyphosate, 2, 4-dichlorophenoxyacetic acid, and triadimefon exposures have
585 diverse effects on *Xenopus laevis* organ morphogenesis. *Journal of Environmental*
586 *Sciences* 22, 1305–1308. [https://doi.org/10.1016/S1001-0742\(09\)60254-0](https://doi.org/10.1016/S1001-0742(09)60254-0)
- 587 Liendro, N., Ferrari, A., Mardirosian, M., Lascano, C.I., Venturino, A., 2015. Toxicity of the
588 insecticide chlorpyrifos to the South American toad *Rhinella arenarum* at larval
589 developmental stage. *Environmental Toxicology and Pharmacology* 39, 525–535.
590 <https://doi.org/10.1016/j.etap.2014.12.022>
- 591 Lindgren, B., Orizaola, G., Laurila, A., 2018. Interacting effects of predation risk and
592 resource level on escape speed of amphibian larvae along a latitudinal gradient.

593 Journal of Evolutionary Biology 31, 1216–1226. <https://doi.org/10.1111/jeb.13298>

594 Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish: a mechanistic approach.

595 Fish Physiology and Biochemistry 42, 711–747. [https://doi.org/10.1007/s10695-015-](https://doi.org/10.1007/s10695-015-0171-5)

596 0171-5

597 Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic

598 Toxicology 101, 13–30. <https://doi.org/10.1016/j.aquatox.2010.10.006>

599 Ma, Y., Li, B., Ke, Y., Zhang, Y., 2019. Effects of low doses Trichlorfon exposure on *Rana*

600 *chensinensis* tadpoles. Environmental Toxicology 34, 30–36.

601 <https://doi.org/10.1002/tox.22654>

602 Mann, R.M., Hyne, R.V., Choung, C.B., Wilson, Scott.P., 2009. Amphibians and agricultural

603 chemicals: Review of the risks in a complex environment. Environmental Pollution

604 157, 2903–2927. <https://doi.org/10.1016/j.envpol.2009.05.015>

605 McCarthy, O., 2018. Biomarkers of environmental contamination. CRC Press, Milton.

606 Pandey, S.P., Mohanty, B., 2015. The neonicotinoid pesticide imidacloprid and the

607 dithiocarbamate fungicide mancozeb disrupt the pituitary-thyroid axis of a wildlife

608 bird. Chemosphere 122, 227–234. <https://doi.org/10.1016/j.chemosphere.2014.11.061>

609 Prakash, A., Rao, J., Rao, J., 2018. Botanical Pesticides in Agriculture. CRC Press.

610 <https://doi.org/10.1201/9781315138572>

611 Quaranta, A., Bellantuono, V., Cassano, G., Lippe, C., 2009. Why Amphibians Are More

612 Sensitive than Mammals to Xenobiotics. PLoS ONE 4, e7699.

613 <https://doi.org/10.1371/journal.pone.0007699>

614 R Core Team, 2020. R: A Language and Environment for Statistical Computing R Foundation

615 for Statistical Computing.

616 Relyea, R.A., 2009. A cocktail of contaminants: how mixtures of pesticides at low

617 concentrations affect aquatic communities. Oecologia 159, 363–376.

618 <https://doi.org/10.1007/s00442-008-1213-9>

619 Ruiz, A.M., Maerz, J.C., Davis, A.K., Keel, M.K., Ferreira, A.R., Conroy, M.J., Morris, L.A.,

620 Fisk, A.T., 2010. Patterns of Development and Abnormalities among Tadpoles in a

621 Constructed Wetland Receiving Treated Wastewater. Environ. Sci. Technol. 44,

622 4862–4868. <https://doi.org/10.1021/es903785x>

623 Rutkoski, C.F., Macagnan, N., Folador, A., Skovronski, V.J., do Amaral, A.M.B.,

624 Leitemperger, J., Costa, M.D., Hartmann, P.A., Müller, C., Loro, V.L., Hartmann,

625 M.T., 2020. Morphological and biochemical traits and mortality in *Physalaemus*

626 *gracilis* (Anura: Leptodactylidae) tadpoles exposed to the insecticide chlorpyrifos.

627 Chemosphere 250, 126162. <https://doi.org/10.1016/j.chemosphere.2020.126162>

628 Saglio, K.H., Olsén, S., Bre, P., 2001. Behavioral and Olfactory Responses to Prochloraz,

629 Bentazone, and Nicosulfuron-Contaminated Flows in Goldfish. Archives of

630 Environmental Contamination and Toxicology 41, 192–200.

631 <https://doi.org/10.1007/s002440010237>

632 Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of

633 Image Analysis. Nat Methods 9, 671–675.

634 Schuytema, G.S., Nebeker, A.V., 1998. Comparative Toxicity of Diuron on Survival and

635 Growth of Pacific Treefrog, Bullfrog, Red-Legged Frog, and African Clawed Frog

636 Embryos and Tadpoles. Archives of Environmental Contamination and Toxicology

637 34, 370–376. <https://doi.org/10.1007/s002449900332>

638 Sievers, M., Hale, R., Parris, K.M., Melvin, S.D., Lanctôt, C.M., Swearer, S.E., 2019.

639 Contaminant-induced behavioural changes in amphibians: A meta-analysis. Science of

640 The Total Environment 693, 133570. <https://doi.org/10.1016/j.scitotenv.2019.07.376>

641 Smith, S.M., Nager, R.G., Costantini, D., 2016. Meta-analysis indicates that oxidative stress is

642 both a constraint on and a cost of growth. Ecol Evol 6, 2833–2842.

643 <https://doi.org/10.1002/ece3.2080>

644 Sotomayor, V., Lascano, C., de D'Angelo, A.M.P., Venturino, A., 2012. Developmental and
645 polyamine metabolism alterations in *Rhinella arenarum* embryos exposed to the
646 organophosphate chlorpyrifos. *Environmental Toxicology and Chemistry* 31, 2052–
647 2058. <https://doi.org/10.1002/etc.1921>

648 Storrs Méndez, S.I., Tillitt, D.E., Rittenhouse, T. a. G., Semlitsch, R.D., 2009. Behavioral
649 response and kinetics of terrestrial atrazine exposure in American toads (*Bufo*
650 *americanus*). *Arch Environ Contam Toxicol* 57, 590–597.
651 <https://doi.org/10.1007/s00244-009-9292-0>

652 Székely, D., Denoël, M., Székely, P., Cogălniceanu, D., 2017. Pond drying cues and their
653 effects on growth and metamorphosis in a fast developing amphibian. *J Zool* 303,
654 129–135. <https://doi.org/10.1111/jzo.12468>

655 Taylor, B., Skelly, D., Demarchis, L.K., Slade, M.D., Galusha, D., Rabinowitz, P.M., 2005.
656 Proximity to Pollution Sources and Risk of Amphibian Limb Malformation. *Environ*
657 *Health Perspect* 113, 1497–1501. <https://doi.org/10.1289/ehp.7585>

658 Touchon, J.C., Jiménez, R.R., Abinette, S.H., Vonesh, J.R., Warkentin, K.M., 2013.
659 Behavioral plasticity mitigates risk across environments and predators during anuran
660 metamorphosis. *Oecologia* 173, 801–811. <https://doi.org/10.1007/s00442-013-2714-8>

661 Ulrich, K., Jakob, U., 2019. The role of thiols in antioxidant systems. *Free Radical Biology*
662 *and Medicine* 140, 14–27. <https://doi.org/10.1016/j.freeradbiomed.2019.05.035>

663 Van Meter, R.J., Glinski, D.A., Henderson, W.M., Garrison, A.W., Cyterski, M., Purucker,
664 S.T., 2015. Pesticide Uptake Across the Amphibian Dermis Through Soil and
665 Overspray Exposures. *Arch Environ Contam Toxicol* 69, 545–556.
666 <https://doi.org/10.1007/s00244-015-0183-2>

667 Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.-H., Shioda,
668 T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012.
669 Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and
670 Nonmonotonic Dose Responses. *Endocrine Reviews* 33, 378–455.
671 <https://doi.org/10.1210/er.2011-1050>

672 Vonesh, J.R., De la Cruz, O., 2002. Complex life cycles and density dependence: assessing
673 the contribution of egg mortality to amphibian declines. *Oecologia* 133, 325–333.
674 <https://doi.org/10.1007/s00442-002-1039-9>

675 Wagner, N., Züghart, W., Mingo, V., Lötters, S., 2014. Are deformation rates of anuran
676 developmental stages suitable indicators for environmental pollution? Possibilities and
677 limitations. *Ecological Indicators* 45, 394–401.
678 <https://doi.org/10.1016/j.ecolind.2014.04.039>

679 Watters, J.L., Cummings, S.T., Flanagan, R.L., Siler, C.D., 2016. Review of morphometric
680 measurements used in anuran species descriptions and recommendations for a
681 standardized approach. *Zootaxa* 4072, 477. <https://doi.org/10.11646/zootaxa.4072.4.6>

682 Wells, P., Bhuller, Y., Chen, C., Jeng, W., Kasapinovic, S., Kennedy, J., Kim, P., Laposa, R.,
683 Mccallum, G., Nicol, C., 2005. Molecular and biochemical mechanisms in
684 teratogenesis involving reactive oxygen species. *Toxicology and Applied*
685 *Pharmacology* 207, 354–366. <https://doi.org/10.1016/j.taap.2005.01.061>

686 Wu, C., Zhang, Y., Chai, L., Wang, H., 2017. Oxidative stress, endocrine disruption, and
687 malformation of *Bufo gargarizans* embryo exposed to sub-lethal cadmium
688 concentrations. *Environmental Toxicology and Pharmacology* 49, 97–104.
689 <https://doi.org/10.1016/j.etap.2016.12.005>

690 Xie, H., Wang, J., Jiang, L., Geng, C., Li, Q., Mei, D., Zhao, L., Cao, J., 2016. ROS-
691 dependent HMG2 upregulation mediates Cd-induced proliferation in MRC-5 cells.
692 *Toxicology in Vitro* 34, 146–152. <https://doi.org/10.1016/j.tiv.2016.04.001>

693 Zamudio, K.R., Bell, R.C., Nali, R.C., Haddad, C.F.B., Prado, C.P.A., 2016. Polyandry,
694 Predation, and the Evolution of Frog Reproductive Modes. *Am. Nat.* 188 Suppl 1,
695 S41-61. <https://doi.org/10.1086/687547>
696

697 **Abbreviations**

698 SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase, GS: Gosner stage

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701 **Tables**

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

Treatment	Nicosulfuron concentrations
Control	0.00 \pm 0.00
Low	0.15 \pm 0.05
High	0.83 \pm 0.04

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705 **Table 2:** Mean values of markers of oxidative status and developmental-related traits
 706 amongst treatments of nicosulfuron
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	Treatment		
	Control	Low	High
Developmental-related traits			
<i>Total length (cm)</i>	0.77±0.00	0.77±0.00	0.78±0.00
<i>Hatching success</i>	0.80±0.02	0.77±0.02	0.80±0.02
<i>Malformation</i>	0.07±0.01	0.06±0.01	0.10±0.01
<i>Development duration (days)</i>	12.94±0.06	12.94±0.04	13.03±0.04
Oxidative status markers			
<i>SOD*</i>	6.35±0.65	6.76±0.56	6.40±0.52
<i>Thiols</i>	0.14±0.01	0.12±0.01	0.12±0.00
<i>Catalase*</i>	18.82±2.07	24.33±3.55	28.43±3.50
<i>GPx*</i>	0.09±0.01	0.11±0.02	0.11±0.01
<i>SOD/(GPx+CAT)</i>	0.37±0.05	0.32±0.04	0.26±0.03
<i>SOD/GPx</i>	97.60±15.31	99.04±29.44	73.57±12.29

708 Values are Mean±SE

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

710 *: (units/mg protein)

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714 **Table 3:** Comparisons of outputs of linear mixed models (LMER, "lmerTest" package) of
 715 markers of oxidative status amongst treatment of nicosulfuron. Clutch identity was used as
 716 random factor in all our models

Variable	Effect	df	F-value	p-value	Clutch identity	
					Variance	Residuals variance
<i>SOD</i> *	Intercept	1,9	120.50	<0.001	0.433	2.913
	Treatment	3,38	0.17	0.843		
<i>Thiols</i>	Intercept	1,9	298.73	<0.001	<0.001	<0.001
	Treatment	3,38	5.14	0.017		
<i>Catalase</i> *	Intercept	1,9	36.48	<0.001	28.92	68.17
	Treatment	3,38	3.41	0.055		
<i>GPx</i> *	Intercept	1,9	18.03	<0.001	<0.001	<0.001
	Treatment	3,38	0.422	0.661		
<i>SOD/(GPX+CAT)</i>	Intercept	1,9	68.50	<0.001	<0.001	0.020
	Treatment	3,38	1.68	0.214		
<i>SOD/GPx</i>	Intercept	1,9	47.88	<0.001	<0.001	1989.76
	Treatment	3,37	0.970	0.399		

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

718 Values in bold are considered significant P<0.05

719 *: (units/mg protein)

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724 **Caption to figures**

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726 **Figure 1:** **A/** Hatchlings total length at Gosner 22 (cm, upper left), **B/** Embryonic development
727 duration (days, upper right) **C/** Proportion of eggs that hatched (lower left) **D/** Proportion of
728 malformed hatchlings (lower right) relative to nicosulfuron treatments in *Bufo spinosus*.
729 Mean±SE are represented. Colour and shape represent nicosulfuron treatment (Control: light
730 green-circle; LOW: green-triangle; HIGH: dark green-square).

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733 **Figure 2:** **A/** Superoxide dismutase activity (units SOD/mg proteins, upper left), **B/**
734 Glutathione peroxidase activity (units GPx/mg proteins, upper right) **C/** Thiols (mmol^{-1} of -
735 SH groups/mg proteins, middle left) **D/** Catalase activity (units Catalase/mg proteins,
736 middle right) **E/** SOD/(GPx+CAT) ratio (lower left) **F/** SOD/GPx ratio (lower right) relative
737 to nicosulfuron treatments in *Bufo spinosus* hatchlings. Mean±SE are represented. Colour and
738 shape represent nicosulfuron treatment (Control: light green-circle; LOW: green-triangle;
739 HIGH: dark green-square).

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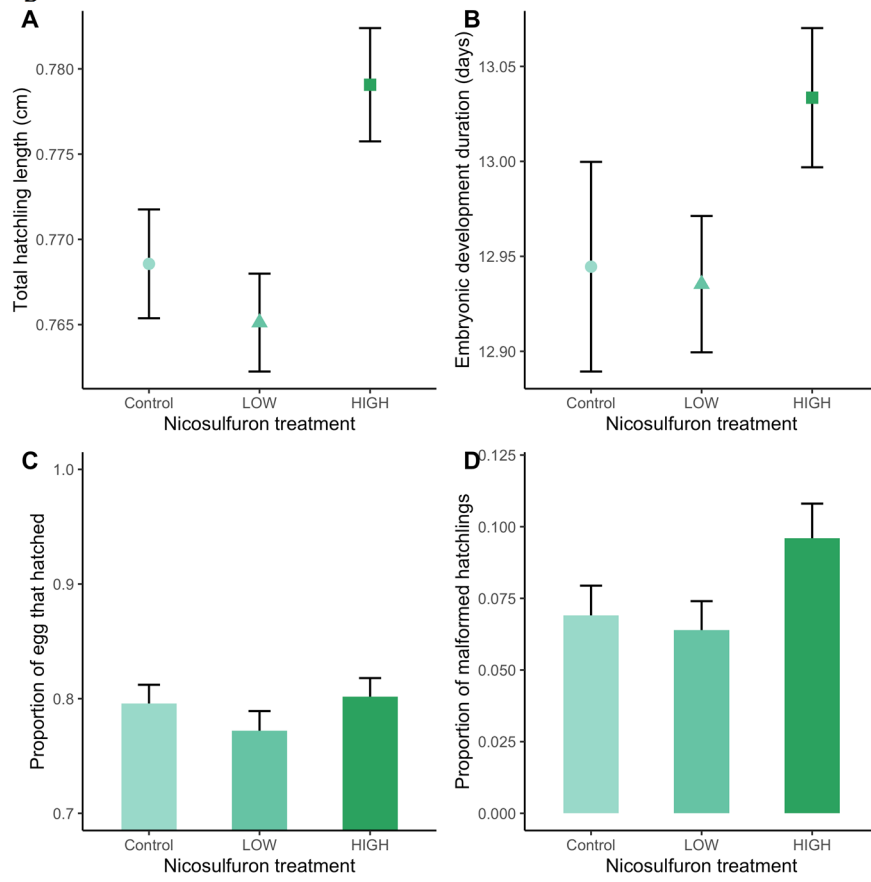
742 **Figure 3:** **A/** Linear regression between embryonic development duration (days) and
743 Superoxide dismutase activity (units SOD/mg proteins) regardless of nicosulfuron
744 treatment, **B/** Linear regression between embryonic development duration (days) and thiols
745 (mmol^{-1} of -SH groups/mg proteins)). **C/** Linear regression between proportion of
746 malformed hatchlings and thiols (mmol^{-1} of -SH groups/mg proteins) regardless of
747 nicosulfuron treatment, **D/** Linear regression between proportion of malformed hatchlings
748 and catalase activity (units catalase/mg proteins). Colour and shape represent nicosulfuron
749 treatment (Control: light green-circle; LOW: green-triangle; HIGH: dark green-square).

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751 **Figures**

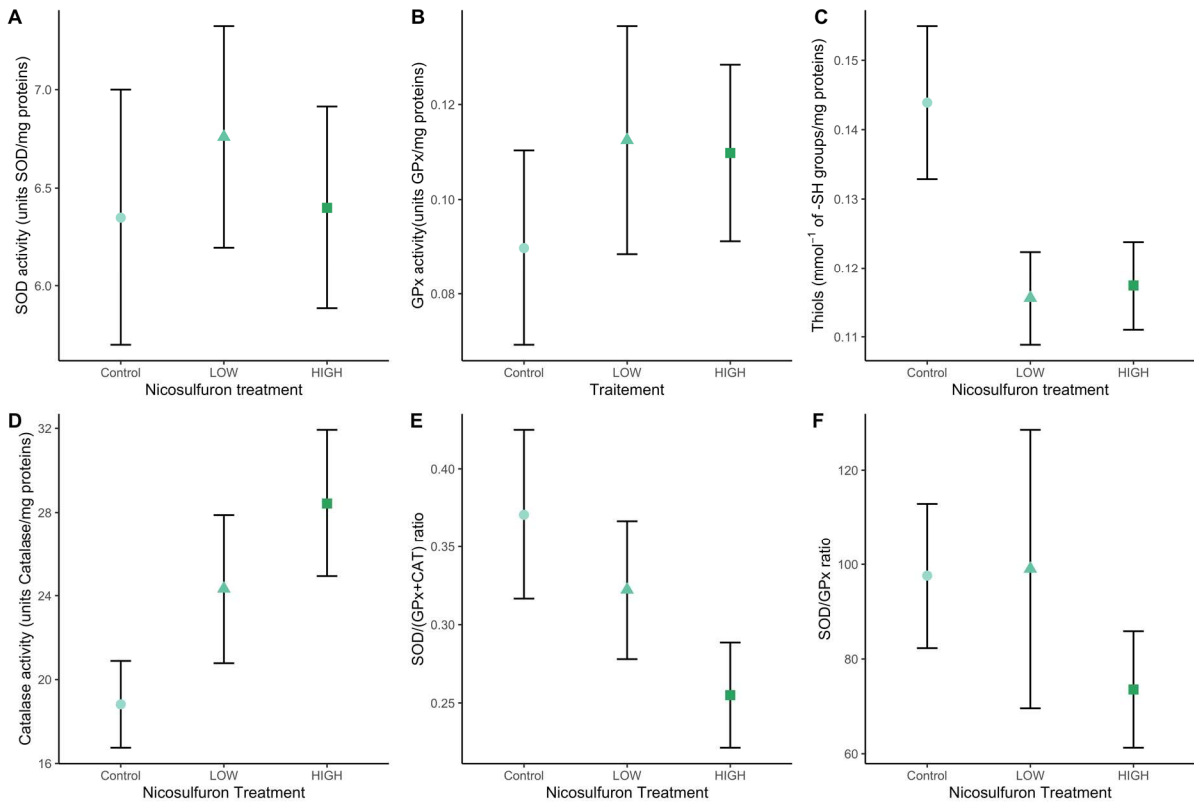
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753 **Figure 1:**



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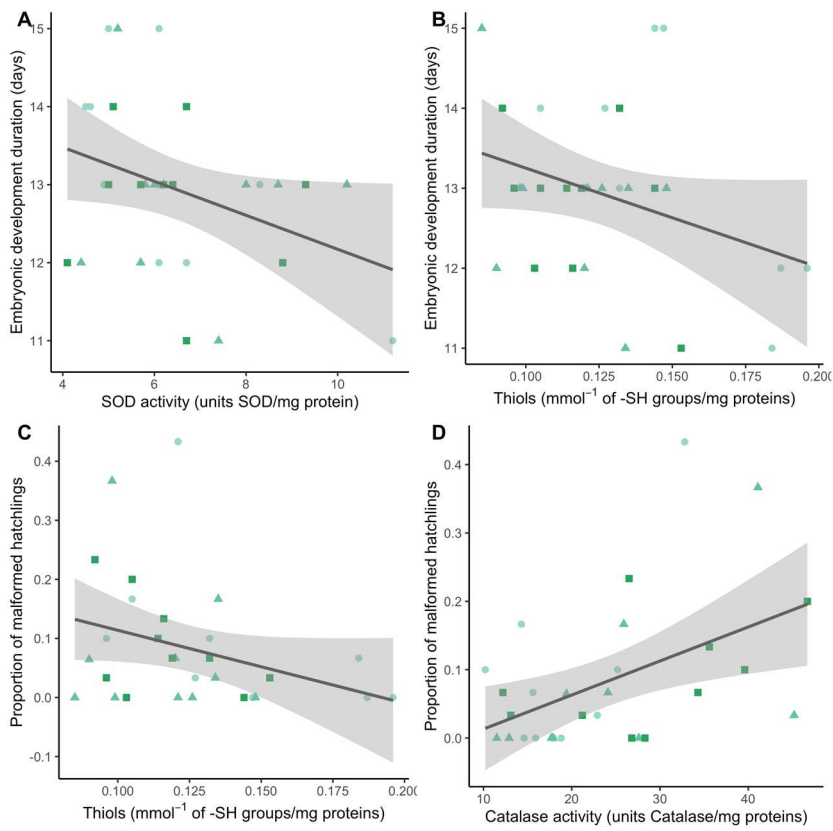
755 **Figure 2:**
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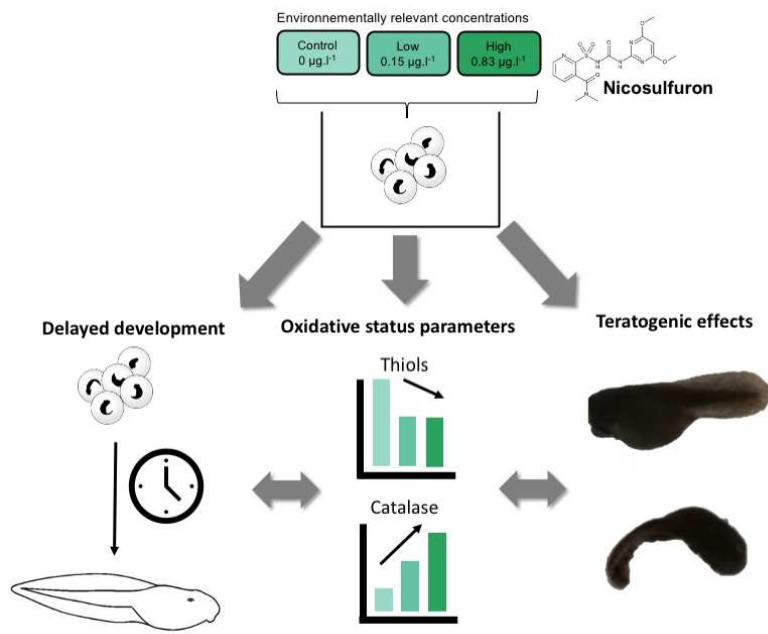
Figure 3



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769 **Graphical abstract**



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