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

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**Running head:** Nicosulfuron altered embryonic development in tadpoles

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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

## Introduction

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

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

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

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

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

In this study, we experimentally investigated for the first time the effects of environmentally relevant concentrations of nicosulfuron on embryonic development and physiology of the spined toad (*Bufo spinosus*), a widespread amphibian species known to persist in agricultural areas (Guillot et al., 2016) and presumably exposed to contaminants (Storrs Méndez et al., 2009; Van Meter et al., 2015). We examined the influence of exposure to nicosulfuron on metrics of embryonic development (embryonic survival, development duration and hatching morphology, (Cheron et al., 2021a) in conjunction with markers of oxidative status that can be influenced by nicosulfuron (e.g., Hackenberger et al., 2018). We tested low but 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 agencies) and we expected that exposure to nicosulfuron should alter embryonic development (survival, duration and morphology) and affects oxidative status.

## **Materials and methods**

### *Study species*

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

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

#### *Treatment concentrations and chemical solutions*

In surface waters close to our study site, mean nicosulfuron concentrations range from 0.005  $\mu\text{g l}^{-1}$  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 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 Loire-Bretagne).

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

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

#### *Experimental design*

Eggs were exposed to two concentrations of nicosulfuron (Low 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 even though the half-life of nicosulfuron can range from 15 to 75 days (EFSA, 2007; Cessna et al., 2015). Egg jelly was maintained throughout the experiment.

#### *Measurements*

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

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



## *Oxidative status markers*

We randomly selected 60 hatchlings (20/experimental group), including two hatchlings per clutch. Because smaller portion did not provide enough tissue, we pooled together these two hatchlings for the analysis. We gave us 10 pools per experimental group (i.e. 30 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 catalase using the OxiSelect Catalase Activity Assay (Euromedex, France). We ran all analyses in duplicate and the CV of measurements was always < 10%. We standardized values of markers by 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.

Balanced or unbalanced ratio of markers activities are known to alter cells integrity (de Haan et al., 1996, 1992). Hence, in addition to markers alone we chose to display ratio of superoxide dismutase and glutathione peroxidase (SOD/GPx) and ratio ratio of superoxide dismutase on GPx and catalase activities (SOD/(GPx+CAT). Unbalanced ratio of (SOD/(GPx+CAT) can lead to a proliferation of deleterious radicals (de Haan et al., 1992) and unbalanced ratio of (SOD/GPx) can lead to a proliferation of H<sub>2</sub>O<sub>2</sub> heading to accelerated cellular senescence and altered morphology (de Haan et al., 1996).

## *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 residuals 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 5000, "fitdist" package). We scaled to mean of 0 and standard deviation of 1 (z\_score of oxidative status markers and developmental-related traits) to avoid multicollinearity between treatment when we tested interaction or relation with developmental-related traits. We checked for multicollinearity using the variance inflation factor (VIF, non-multicollinearity when below 2. "car" package). We kept clutch identity as a random factor to avoid significant pseudoreplication issues. We did not use tank as the replication unit in our analytical design because each tank represented a single treatment (either control, low or high) and contained only the eggs of one clutch.

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

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

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

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

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

## Results

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

### Effect of nicosulfuron treatment on embryonic traits

On the whole dataset, we found that nicosulfuron significantly influenced total length of hatchlings ( $F_{3,1234}=5.51$ ,  $p=0.004$ , Fig. 1). Post hoc tests showed that hatchlings from High treatment were longer than individuals from the Low treatment ( $p=0.003$ ,  $g=-0.22$ ). Nicosulfuron slowed down embryonic development duration ( $F_{3,1234}=19.185$ ,  $p<0.001$ , Fig. 1); post hoc tests showed that High groups differed from Control groups ( $p=0.001$ ,  $g=-0.08$ ) and Low groups ( $p=0.001$ ,  $g=-0.12$ ). We did not find any effect of nicosulfuron on hatching success ( $p=0.13$ , Fig. 1) and a marginal effect on the occurrence of malformed individuals ( $p=0.06$ , Fig. 1).

### Effect of nicosulfuron treatment on oxidative status

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

### Relationships between embryonic traits and oxidative status

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

levels ( $F_{2,24}=3.262$ ,  $p=0.055$ ), or their interaction (all  $p>0.089$ ) except for a marginal relation between development related traits, GPx and treatment ( $F_{2,20}=3.262$ ,  $p=0.059$ ).

Embryonic development duration was not related to treatment for the subsample of individuals for which we assessed oxidative status ( $F_{2,20}=0.439$ ,  $p=0.649$ ). Development duration was negatively linked to SOD ( $F_{2,20}=6.84$ ,  $p=0.015$ ) and thiols ( $F_{2,20}=4.62$ ,  $p=0.048$ ). We did not find any interaction between markers and treatment (all  $p>0.363$ ) except for a marginal interaction with SOD/(catalase+GPx) ( $F_{1,20}=6.88$ ,  $p=0.056$ ).

Hatching success was influenced by treatment in the individuals for which we assessed oxidative status ( $X^2=21.09$ ,  $p=0.001$ ). The interaction with treatment was significant for hatching success and SOD ( $X^2=10.86$ ,  $p=0.004$ ). Control individuals displayed a positive relationship between hatching success and SOD, which was marginally different from the relationships found in the High group ( $p=0.054$ ) and significantly different from Low group ( $p=0.03$ ) with both groups of treated individuals displaying negative relationships between hatching success and SOD. Catalase influenced hatching success between treatments ( $X^2=25.41$ ,  $p<0.01$ ). Control individuals displayed a positive relationship between hatching success and Catalase which was different from High group hatchlings which displayed negative relationship ( $p<0.01$ ) and Low group individuals which displayed positive relationship ( $p<0.01$ ). Hatching success was related to thiols ( $X^2=5.442$ ,  $p=0.019$ ) and the interaction with treatment was also significant ( $X^2=7.815$ ,  $p=0.020$ ). Control individuals displayed a positive relationship between hatching success and thiols that was significantly different from Low individuals that displayed a negative relationship ( $p=0.016$ ).

Finally, the number of malformed tadpoles did not differ among treatments ( $X^2=1.836$ ,  $p=0.340$ ). We found a negative relation between the occurrence of malformed hatchlings and

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

## Discussion

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

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

Székely et al., 2017). Conversely, increased body size may improve mobility and thus foraging ability while decreasing susceptibility to predation (Broomhall and Shine, 2003; Cheron et al., 2021b; Hoff and Wassersug, 2000). Such hypotheses will require thorough testing, especially as post-embryonic survival might be more critical to population persistence than egg survival (Touchon et al., 2013; Vonesh and De la Cruz, 2002). Surprisingly, these effects were detectable for our “high” treatment solely suggesting that the lower concentration we tested did not trigger visible effects on embryonic development, as expected from classical concentration-response relationships (Vandenberg et al., 2012). Indeed, a previous study on Goldfish showed an alteration of swimming behaviour due to exposure to nicosulfuron and further highlighted an increase in swimming alterations along with exposure to increasing nicosulfuron concentrations (Saglio et al., 2001). Because our “high” treatment is situated at the lower range of maximum concentrations assessed in surface water (see “Materials and methods section”), it is plausible that free ranging *Bufo spinosus* embryos are exposed to higher concentrations which could induce strong consequences on hatching success. Future studies including a larger range of tested concentrations are required to describe reaction norms of embryos exposed to nicosulfuron.

Regardless of the concentration tested, nicosulfuron influenced thiol levels with lower levels - hence higher oxidation of thiols - in hatchlings exposed during their embryonic development. Thiols are relevant indicators of the antioxidant capacity of an organism (Lushchak, 2011), so that their concentration decreases when there are strong perturbations to the oxidative status (Baba and Bhatnagar, 2018; Ulrich and Jakob, 2019). As a consequence, our results suggest that exposure to nicosulfuron altered oxidative status. We also found a trend for catalase to increase across treatments. The enzymatic antioxidant protection occurs through two major steps: first superoxide dismutase (SOD) breaks down  $O_2^-$  into  $H_2O_2$ , then  $H_2O_2$  is converted into  $H_2O$  by catalase and/or glutathione peroxidase

(GPx). However, when H<sub>2</sub>O<sub>2</sub> is produced at high concentrations, catalase is more efficient in the conversion of H<sub>2</sub>O<sub>2</sub> in H<sub>2</sub>O than GPx which can explain the trend we detected (Halliwell and Gutteridge, 2015). In keeping with the results on development duration (see above), it is noteworthy that a rapid development may increase oxidative damage, with possible long-lasting effects (Janssens and Stoks, 2018). In this context, slowing down embryonic development, as observed in individuals exposed to the highest nicosulfuron treatment might constitute a strategy to avoid a disproportional increase of oxidative damages linked to both exposure to nicosulfuron and rapid development.

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



## 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 (Bretaud 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.

## **Declaration of interests**

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

## **Data availability**

Data are available upon request

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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

#### **Author contributions**

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

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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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**Table 2:** Mean values of markers of oxidative status and developmental-related traits amongst treatments of nicosulfuron

	Treatment		
	Control	Low	High
<b>Developmental-related traits</b>			
<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
<b>Oxidative status markers</b>			
<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

Values are Mean±SE

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

\*: (units/mg protein)

**Table 3:** Comparisons of outputs of linear mixed models (LMER, "lmerTest" package) of markers of oxidative status amongst treatment of nicosulfuron. 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	120.50	<b>&lt;0.001</b>	0.433	2.913
	Treatment	3,38	0.17	0.843		
<i>Thiols</i>	Intercept	1,9	298.73	<b>&lt;0.001</b>	<0.001	<0.001
	Treatment	3,38	5.14	<b>0.017</b>		
<i>Catalase</i> *	Intercept	1,9	36.48	<b>&lt;0.001</b>	28.92	68.17
	Treatment	3,38	3.41	0.055		
<i>GPx</i> *	Intercept	1,9	18.03	<b>&lt;0.001</b>	<0.001	<0.001
	Treatment	3,38	0.422	0.661		
<i>SOD/(GPX+CAT)</i>	Intercept	1,9	68.50	<b>&lt;0.001</b>	<0.001	0.020
	Treatment	3,38	1.68	0.214		
<i>SOD/GPx</i>	Intercept	1,9	47.88	<b>&lt;0.001</b>	<0.001	1989.76
	Treatment	3,37	0.970	0.399		

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

Values in bold are considered significant P<0.05

\*: (units/mg protein)

**Caption to figures**

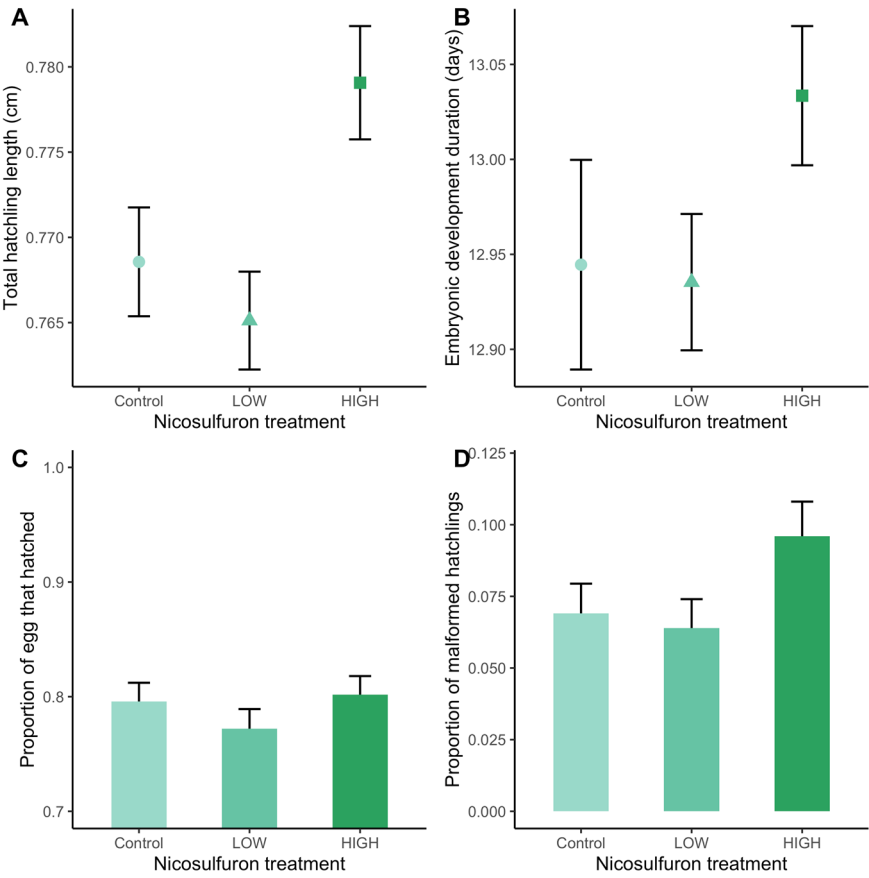
**Figure 1:** **A/** Hatchlings total length at Gosner 22 (cm, upper left), **B/** Embryonic development duration (days, upper right) **C/** Proportion of eggs that hatched (lower left) **D/** Proportion of malformed hatchlings (lower right) relative to nicosulfuron treatments in *Bufo spinosus*. Mean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

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

**Figure 3:** **A/** Linear regression between embryonic development duration (days) and Superoxide dismutase activity (units SOD/mg proteins) regardless of nicosulfuron treatment, **B/** Linear regression between embryonic development duration (days) and thiols (mmol<sup>-1</sup> of -SH groups/mg proteins)). **C/** Linear regression between proportion of malformed hatchlings and thiols (mmol<sup>-1</sup> of -SH groups/mg proteins) regardless of nicosulfuron treatment, **D/** Linear regression between proportion of malformed hatchlings and catalase activity (units catalase/mg proteins). Colour and shape represent nicosulfuron treatment (Control: light green-circle; LOW: green-triangle; HIGH: dark green-square).

Figures

Figure 1:



**Figure 2:**

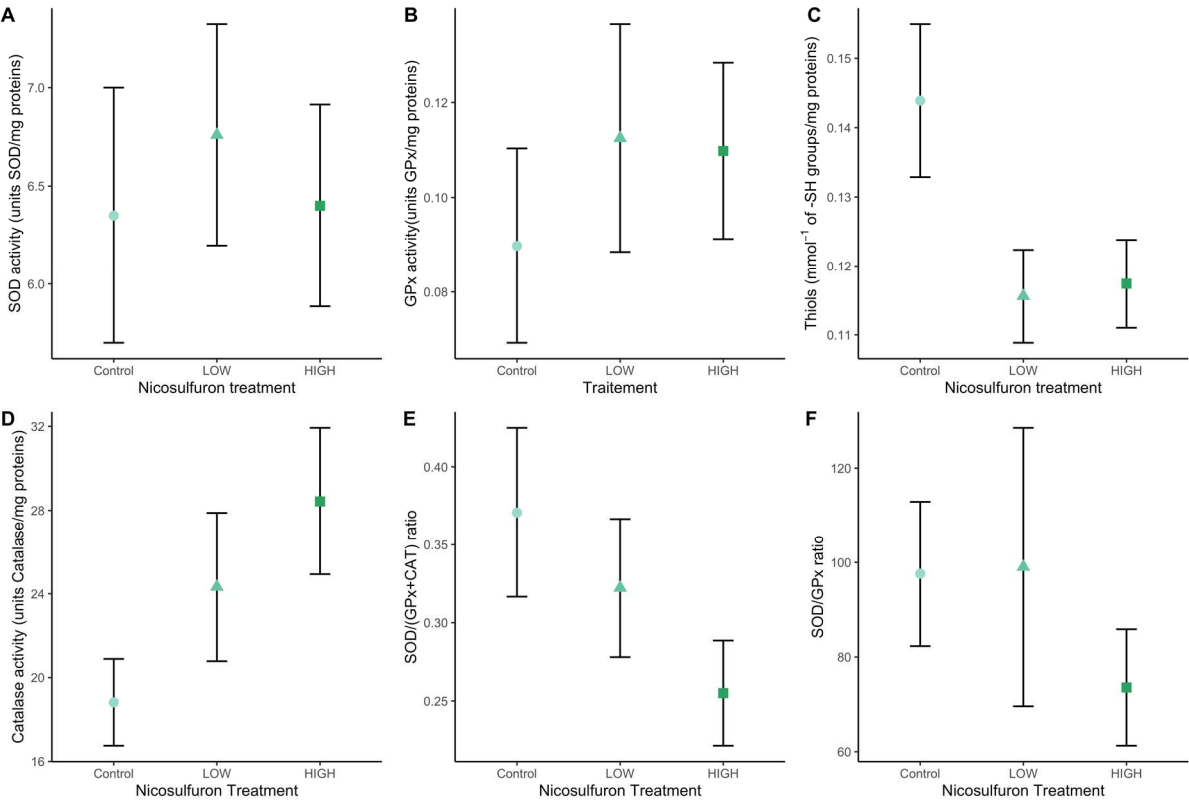
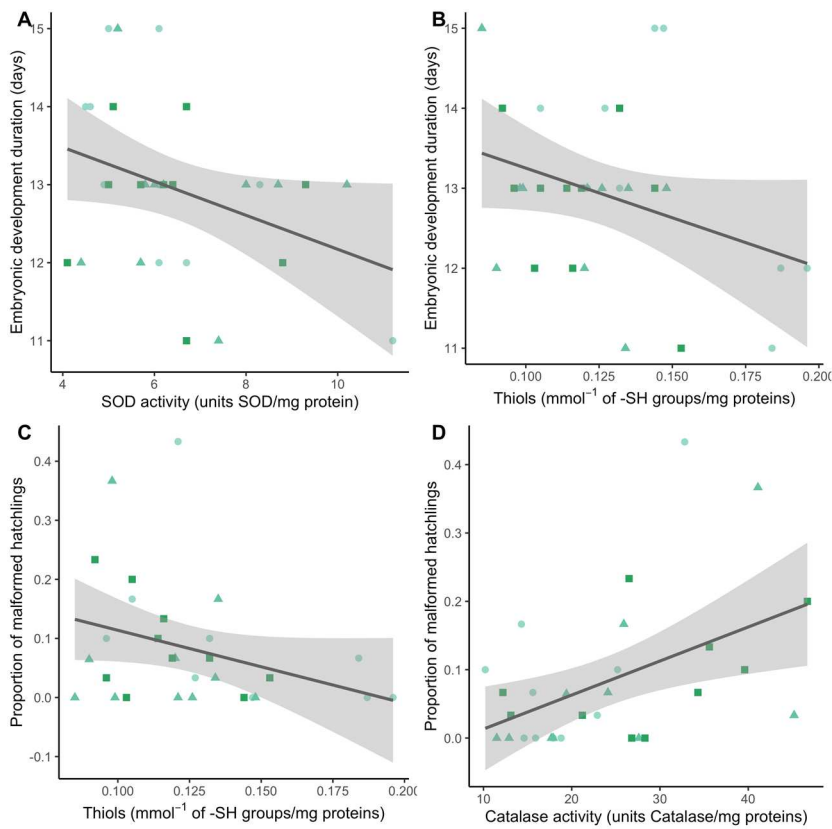


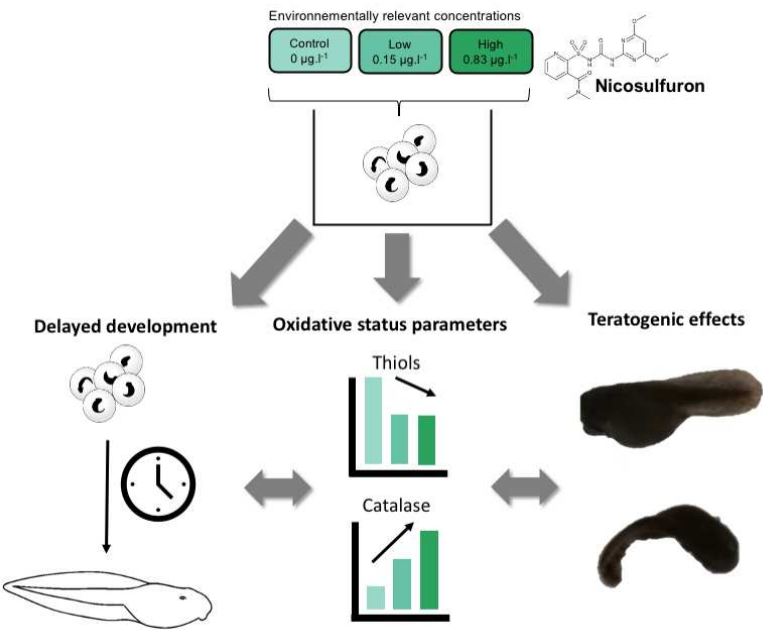
Figure 3





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769    **Graphical abstract**



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