SPRINGER NATURE

Hydrobiologia

Acute Exposure to Roundup has Nonlinear Effects on Larval Stream Salamander Behavior and Recovery

Catherine Bratton · Kristen Cecala

Reprinted from Hydrobiologia
Vol. 850, No. 19, pp. 4283–4291, November 2023

Hydrobiologia (2023) 850:4283–4291
https://doi.org/10.1007/s10750-023-05304-5

PRIMARY RESEARCH PAPER

Acute exposure to Roundup has nonlinear effects on larval stream salamander behavior and recovery

Catherine Bratton · Kristen Cecala

Received: 30 August 2022 / Revised: 20 June 2023 / Accepted: 27 June 2023 / Published online: 2 August 2023

Abstract Glyphosate is a commonly used pesticide applied to agricultural fields, but runoff during rainfall introduces the chemical into surrounding waters. In stream systems, glyphosate has non-target effects and can negatively affect amphibian physiology, development, and behavior. These effects are previously documented from chronic exposure relative to the acute exposures more often experienced by organisms inhabiting streams. Previous studies indicate that glyphosate induces a dose-dependent effect on startle responses in stream salamanders, but it is unclear how long individuals need to recover from acute exposure. We described the behavioral recovery of stream salamander larvae, *Eurycea wilderae,* to short-term exposure of glyphosate. We observed that individuals recovered from glyphosate exposure between 2 and 4 h post-exposure. Length of exposure did not affect larval responses linearly, indicating a lack of accumulated effects and a non-monotonic response. Our data suggested that there was a maximum effect of glyphosate on stream salamander larvae that may be similar to exhaustion. We recommend researchers consider the frequency of testing when they explore this effect threshold. Because startle response is associated with swimming ability, impairment due to glyphosate could temporarily increase predation risk and contribute to downstream displacement of larvae inhabiting streams in agricultural and urban areas.

Keywords Glyphosate · Amphibian · Headwater · Chronic · Agriculture · Non-monotonic dose response · Disturbance

Handling editor: Lee B. Kats

C. Bratton · K. Cecala (✉)
Department of Biology, University of the South, Sewanee, TN 37383, USA
e-mail: kkcecala@sewanee.edu

Introduction

Glyphosate is a chemical compound found in many agricultural pesticides commonly used in the United States (Kolpin et al., 2006; Levis & Johnson, 2015) and with increasing use worldwide (Wagner et al., 2013). During rain events, many manufactured chemicals, such as glyphosate, run off into aquatic systems. This influx of contaminants can have detrimental and long-lasting effects on the wildlife present in these systems (Relyea, 2009, 2011). Glyphosate negatively impacts the rate of carbohydrate and lipid catabolism forcing vertebrates to reduce energy production as the chemical clears from their system (Wang et al., 2019). The compound also causes hepatocyte damage inhibiting the ability of the liver to remove glyphosate from the body. Because of these impacts, even low doses of glyphosate can have negative effects on wildlife and put them at risk for predation during and after exposure (Bridges, 1997). The cumulative effects of these individual-level responses include slowed growth and development (Howe et al., 2004; Cauble & Wagner, 2005), physical deformities (Howe et al., 2004; Wagner et al., 2013), delayed predator response (Bridges, 1997), increased susceptibility to disease (Rohr et al., 2008), and direct mortality (Relyea, 2009). All these reactions can accumulate to affect the health of the population and overall ecosystem.

Previous work on aquatic contaminants has focused on the impacts observed in lentic systems that have slower water turnover and flushing rates than lotic systems (Relyea & Hoverman, 2006). Although chronic exposure experiments are crucial for lentic systems, these results are not applicable to lotic systems, which make up over 3.5 million miles of habitat in the US alone (Lowe & Likens, 2005). Because of the velocity, flood magnitude, flashiness, gradient, and hydraulic regimes of a stream, pesticide exposure in streams is highly variable and likely occurs for short time periods (Annett et al., 2014). Past studies found that glyphosate concentrations are highest in urban streams relative to agricultural streams, and that peak concentrations are brief but consistent with spring flush following rainfall (Kolpin et al., 2006; Mahler et al., 2017). These results indicate that glyphosate runoff from residential areas can also be sizable, making glyphosate exposure in streams a widespread phenomenon. Despite acute exposure being most common in lotic systems, most studies have focused on chronic, life-long exposures and their effects on demographic rates like survival (Dinehart et al., 2009), and less work has focused on the effects of acute exposure more common in streams where short-term exposure may have minimal impacts on population demographic rates (Wagner et al., 2013).

Individual physiological state is closely connected to observed behaviors. Thus, impairment of key physiological processes like energy production can have observable impacts on energy-intensive behaviors like movement (Gandhi & Cecala, 2016; Pavan et al., 2021). Furthermore, outward behaviors are a key connection between physiological responses and demographic processes (e.g. survival) making it an easily observable metric with potential consequences on individual outcomes. For example, glyphosate-impaired glycogen catabolism could inhibit the ability of individuals to move in response to external stimuli (Wang et al., 2019). A startle response is a quick movement in response to a perceived threat like a predator attack or rapid displacement by fast flowing water (Barrett et al., 2010; Faria et al., 2019). This movement is a burst of maximum activity that will quickly exhaust glycogen stored within the muscles. With impaired glucose conversion, little energy should be available to individuals for sustained or repeated movements.

Determining whether individuals recover after exposure and how quickly they can respond is a key knowledge gap for understanding the magnitude of negative effects introduced by glyphosate runoff. As our knowledge of aquatic contaminants grow, researchers have concluded that the effects of contaminant exposure are unpredictable (Vandenberg et al., 2013). Glyphosate is one contaminant for which non-monotonic dose responses have been reported whereby the effects of a small dose cannot be extrapolated from larger doses (McMahon et al., 2011; Vandenberg et al., 2012; Hill et al., 2018; Cheron & Brischoux, 2020). Thus, the effects of chronic exposure may not predict the effects of acute exposure. Likewise, demographic responses to exposure requires long-term evaluation, and the effects of single acute exposures may be overwhelmed by other exposures, environmental changes, or individual variation limiting our ability to detect those impacts.

Although many living organisms could be impacted by chemical runoff in lotic systems (Little & Finger, 1990; Little et al., 1990; Doving, 1991; Dodson et al., 1995), amphibians, specifically, show severe responses to the introduction of pesticides into their habitats (Hall & Henry, 1992). This is partially due to the permeable nature of their skin (Quaranta et al., 2009), as well as the susceptibility of aquatic egg and larval stages (Petranka, 1984; Berrill et al., 1994; Duellmann & Trueb, 1994). Past studies have found that amphibian antipredator behaviors are consistently altered in the presence of human-introduced pollutants including heavy metals (Lefcort et al., 1998), UV-B radiation (Kats et al., 2000; Levis & Johnson, 2015), carbaryl (Relyea & Mills, 2001), and atrazine (Rohr & McCoy, 2010), all of which are common pollutants of lotic systems.

In this study, we sought to explore how acute exposure to glyphosate-based pesticides (e.g. Roundup) affects larval amphibian behavior and their recovery from exposure. Because most studies on contaminants examine outcomes like growth or survival, little information is available about the ways in which animals recover from exposure, particularly acute exposure. We assessed (1) the behavioral effects of

Roundup exposure on stream salamander behavior through time, (2) how the length of exposure impacted the rate of recovery from exposure, and (3) whether the frequency of observation impacted the recovery trajectory of tested individuals. We defined recovery as when exposed individuals exhibited similar behaviors at a timepoint as unexposed individuals. Previous research indicated that the antipredator behavior of a startle response was negatively associated with Roundup concentrations (Gandhi & Cecala, 2016), so we used this as our response variable. We hypothesized that longer exposure would result in more significant behavioral impairment and longer recovery periods.

Methods

We used *Eurycea wilderae* Dunn, 1920 (Blue Ridge Two-Lined Salamander) because of their widespread geographical distribution in streams of the eastern U.S., year-long larval phase, and their tolerance for multiple types of watershed disturbance (Petranka, 1998). Larval life stages were used because they are exposed to aquatic contamination until metamorphosis when they can escape poor aquatic conditions by moving into the terrestrial landscape. Specimens were collected from Tremlett Spring on the southern Cumberland Plateau using active surveys with a dipnet. Recent tests revealed below detection levels of glyphosate in Tremlett Spring (M. Knoll, Pers. Comm.). Collection occurred between August 2020 and May 2021. Larvae were kept individually in native stream water and placed in a dark and cool (10 ℃) environment while in captivity. Behavioral observations took place within one week of capture. We measured the snout-vent length and total length. Each individual was randomly assigned to a treatment. Because groups of individuals were captured weekly and randomly assigned as they were collected, that resulted in uneven sample sizes among treatments described below. We randomly assigned individuals to treatments until we achieved a minimum of 20 individuals per treatment.

Experimental procedures

All research questions were addressed by using standard concentrations of Roundup herbicide, the same response variable—startle response, and the same test enclosures described below. Unexposed or control individuals were exposed to fresh native stream water while exposed individuals were placed in an environmentally relevant dose of Roundup (Wagner et al., 2013). To create the Roundup solution, we added 2 mL of Roundup Weed and Grass Killer, whose active pesticide ingredient is glyphosate, to 1 L of native spring water (as in Gandhi & Cecala, 2016). Salamanders were exposed to either native stream water or the Roundup solution for various lengths of time (e.g. individuals only experienced one treatment). Treatments were randomly assigned to test enclosures in each experiment.

The response variable, startle response, has a linear relationship with glyphosate concentrations in a prior study in a smaller enclosure and was used in this study (Gandhi & Cecala, 2016). To simulate a predator attack, we lightly pinched the tail of the larva with forceps and measured the distance the individual moved in response (Ducey & Brodie, 1991; Bakkegard, 2005; Bliss & Cecala, 2017). The total distance moved was calculated visually in real time by including changes in direction or backtracking until the individual ceased movement similar to the protocol in Gandhi & Cecala (2016). If individuals climbed out of the water, we tapped them back into the enclosure and excluded that test measurement from the analysis. All individuals were allowed to acclimate to room temperature prior to beginning trials and placed in the center of the test enclosures to initiate trials.

Test enclosures were constructed from aluminum gutters that were 10 cm in width and 150 cm in length. No substrate was added to the enclosure to avoid interactions between Roundup and the substrate and to facilitate cleaning. Depending on the design for each experiment, either native stream water or the Roundup mixture was added to the experimental gutters to a depth of 2 cm. Water was replaced in the enclosures after every trial. Observations were performed by remaining a distance of 1 m from the enclosure. The enclosures had 1 cm markings on the side to facilitate taking observations without interfering with the response of the tested individuals. The observer of each trial also implemented the treatments due to a limitation in resources, this created a nonblind study due to logistical constraints on the availability of other personnel, but all visual cues of treatment (e.g. experimental unit labels or animal ID tags) were removed from the observer's field of view during behavioral observations, and random assignment to experimental enclosures minimized the risk of biased observations.

Experimental design

Objective 1: exposure length

To assess whether length of exposure to Roundup affected salamander behavior, we observed startle responses at 30 min increments in native stream water or the Roundup mixture for 6 h. Unexposed individuals were housed in native stream water both before the testing period and in the testing enclosure. Exposed individuals were housed in stream water prior to the treatment period before being placed into the Roundup mixture in the experimental enclosure for the duration of the testing period. Thus, the first observation of this study occurred at first exposure to Roundup for exposed treatments. We used 71 individuals for this experiment (Exposed $N=26$; Unexposed $N=45$).

Objective 2: recovery length

To evaluate if the length of Roundup exposure affected the time until exposed individuals exhibited similar behavior to unexposed individuals, we randomly assigned individuals to Roundup exposure lengths of 0, 30, 60, or 90 min prior to the testing period. Salamanders acclimated to room temperature in native stream water before being placed into a new enclosure with native stream water or the Roundup mixture. Individuals were left in those enclosures for the designated length of time before being placed in experimental enclosures with native stream water. Their startle response was measured every 2 h for a length of 6 h. We used 106 individuals for this experiment ($N=20$–34 per treatment).

Objective 3: observation frequency

Because glyphosate may affect individuals through exhaustion, we sought to evaluate if sampling frequency could affect our ability to detect recovery from glyphosate exposure. Therefore, we allowed individuals to acclimate to room temperature in stream water and randomly assigned them to a control or glyphosate treatment. They were exposed to fresh stream water or the glyphosate mixture for 90 min prior to introduction to the test enclosure filled with fresh stream water. Individuals in each treatment were randomly assigned to have an observation interval of either every 30 m or every 2 h. Observations were performed for 6 h resulting in startle response being tested either 13 or 4 times respectively. To minimize the effect of increased observation frequency in our statistical analysis, we only compared measurements taken at 0, 2, 4, and 6 h for all individuals. We used 129 individuals for this experiment ($N=20$–35 individuals per treatment).

Data analysis

Each experiment was evaluated using a linear mixed model (with a normal error distribution) evaluating the effects of Roundup exposure on startle response. For objective 1, we also included a fixed factor of time in the experimental enclosure. Objective 2 analysis also included effects of Roundup exposure length (0, 30, 60, or 90 min exposure) and time in the experimental enclosure, and Objective 3 also included time in the experimental enclosures and measurement frequency. All time variables were included as categorical variables. Repeated observations per individual were accounted for with a variable intercept for each individual (random effect), and all models were evaluated in R with an analysis of deviance (R Core Team, 2021). We also performed pairwise, post hoc analyses of treatment at each time point using package emmeans with a Tukey correction for multiple comparisons for assessment of recovery for objectives 1 and 2.

Results

Overall, we observed 306 *E. wilderae* larvae. Our study animals were 23.72 ± 0.227 mm in snout-vent length and 45.89 ± 0.532 mm in total length. All models exhibited balanced residuals.

Objective 1: exposure length

We observed a non-significant interaction between exposure and length of exposure on the startle response ($X^2=19.41$, d$f=12$, $P=0.079$) and significant independent effects of each (Table 1). The startle response of exposed individuals was always less than that of control individuals, but the difference between them was minimized through time. Post hoc results revealed that treatment yielded statistically significant differences in startle response for observations between 60 and 120 min post Roundup exposure ($t \geq 4.23$, $P \leq 0.008$; Fig. 1). Control animals began to exhibit declining startle responses after 2 h and again after 4 h (Fig. 1).

Objective 2: recovery length

We observed a significant interaction between the length of exposure and time since exposure ($X^2 = 27.10$, d$f = 9$, $P = 0.001$; Table 1). Glyphosate exposed individuals all had lower startle responses at the beginning of recovery. Visually, all treatments had similar responses after 6 h except for those exposed to glyphosate for 30 min that had higher startle responses at 6 h (Fig. 2). Post hoc comparisons revealed no significant pairwise differences in startle responses.

Table 1 Significance of linear mixed models assessing the effects of glyphosate exposure (treatment), time since implementation of the treatment (time), and the frequency of observation (measurement frequency) on the startle response of larval *Eurycea wilderae*

Variable	X^2	d*f*	*P*-value
Exposure length			
Treatment	25.46	1	< 0.001
Time	48.60	12	< 0.001
Treatment × Time	19.41	12	0.079
Recovery length			
Exposure length	5.08	3	0.166
Time	9.41	3	0.024
Exposure length × Time	27.10	9	0.001
Observation frequency			
Measurement frequency	0.84	1	0.360
Treatment	9.11	1	0.003
Time	5.53	3	0.137
Measurement frequency × Treatment	0.47	1	0.495
Measurement frequency × Time	5.87	3	0.118
Treatment × Time	4.35	3	0.226
Measurement frequency × Treatment × Time	0.86	3	0.835

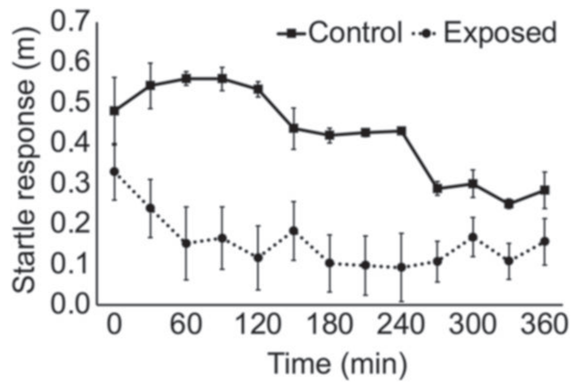

Fig. 1 Exposure to Roundup (active ingredient: glyphosate) decreases larval *Eurycea wilderae* mean startle responses (± 1 SE; $N = 71$) to a minimum level after approximately 1 h relative to unexposed individuals. Startle responses to a repeated tail pinch decline through time in unexposed individuals

Objective 3: observation frequency

We did not observe any significant interactions among exposure, time since exposure, and sampling frequency. Limited sample sizes and high variability may have contributed to non-significant interaction between time and sample frequency ($X^2 = 5.87$, d$f = 3$, $P = 0.118$; Table 1). Individuals not exposed to Roundup had startle distances 1.97 times higher than exposed individuals with no difference between sampling frequency (Fig. 3). By the end of the experiment, individuals tested every 2 h had startle

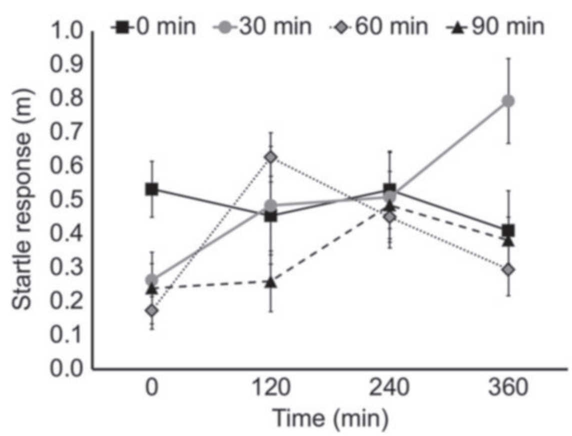

Fig. 2 Recovery of mean startle responses (± 1 SE; $N = 106$) of larval *Eurycea wilderae* varied in a non-linear way relative to the length of Roundup (active ingredient: glyphosate) exposure prior to initiating trials

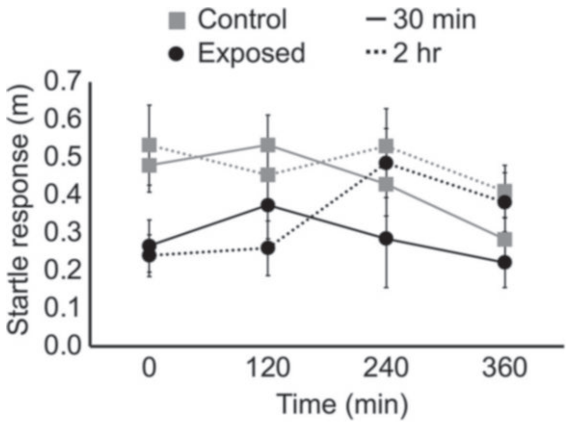

Fig. 3 High frequency observation frequencies and Roundup (active ingredient: glyphosate) exposure negatively affect the mean startle response (±1 SE; $N = 129$) of larval *Eurycea wilderae* relative to less frequently observed and unexposed individuals

responses that were 1.47 times and 1.42 times higher on average than those tested every 30 min in unexposed and exposed treatments.

Discussion

Overall, Roundup exposure consistently impacts a key anti-predator behavior in larval salamanders. This study demonstrated that acute exposure (< 2 h) was sufficient to induce a behavioral effect but that the length of exposure had a non-linear effect. Longer exposures to Roundup did not induce significantly greater impacts on behavior. Recovery from acute exposure occurred quickly suggesting that salamanders were able to metabolize and remove glyphosate quickly. Impaired movement following exposure to Roundup could make them more susceptible to predation and downstream displacement during rain events, but fast behavioral recovery may mean that the effects of Roundup are not long-lasting. Lastly, we were concerned that frequent testing could either exhaust larval salamanders or allow them to learn to reduce their responses to a repeated predator-like stimuli. Our data consistently revealed patterns of behavioral habituation or exhaustion, but we were unable to statistically confirm a pattern of interaction between testing frequency and time since exposure. We recommend that researchers carefully consider the need for frequent observations using this response variable.

Previous studies indicate that glyphosate inhibits carbohydrate and lipid catabolism which in turn reduces protein synthesis and energy production (Wagner et al., 2013; Mesnage et al., 2015a,b; Wang et al., 2019; Riaño et al., 2020; da Silva et al., 2021). Impaired glycolysis intensifies a trade-off for the exposed individual between metabolic needs and anti-predator behaviors. Differences in the startle response could be explained by exposed larvae not being able to produce as much energy. However, the exposed individuals did recover and exhibit similar startle response to unexposed individuals. We hypothesize that this recovery in startle response could be indicative of the reinitiation of lipid production following glyphosate exposure. When all the glyphosate has been removed from the amphibian's system, they are able to synthesize proteins and produce energy, thus exhibiting the mean unexposed startle response.

Larvae exhibited a continual decrease in startle responses until 1 h of exposure at which point their responses stabilized (Fig. 1). These results indicate a potential maximum effect of glyphosate on larval salamander startle responses. This minimum level of response was also observed with high frequency testing in both exposed and unexposed individuals (Fig. 3). Therefore, it is likely that declining startle responses could be due to fatigue. Glyphosate exposure is known to affect glycolysis, reduce glucose and muscle glycogen availability, and decrease activity of the neurotransmitter acetylcholinesterase in the brain (Glusczak et al., 2007; Hasković et al., 2016). Low rates of glycolysis would minimize the ability of larval salamanders to recover following startle responses and maintain limited startle distances observed in this study in a similar manner to physical exhaustion through repeated testing.

Because glyphosate impairs liver function, it may potentially impact the ability of individuals to process and eliminate contaminants like glyphosate and could contribute to non-monotonic responses observed in this study (Hasković et al., 2016). The rate at which the larvae appeared to remove the glyphosate from their systems was not linearly associated with the length of exposure. We had hypothesized that the removal rate for glyphosate would be the same among experimental groups because all tests were conducted at the same water temperature and the larvae were similarly sized. However, we expected that longer exposures would increase the negative effects of glyphosate exposure and require longer periods for recovery. However, this is not what we observed, and we hypothesize that glyphosate exposure results in a non-monotonic response in larval salamanders regardless of whether the exposure is varied by concentration or length of exposure (Vandenberg et al., 2013; Gandhi & Cecala, 2016; Hill et al., 2018). This non-linear response causes different rates at which the glyphosate is removed from the amphibians' body (Vandenberg et al., 2012; Lagarde et al., 2015). We are unsure exactly why glyphosate causes a non-monotonic response in amphibians, so more detailed research is needed. In general, it appears to take individuals 2 to 4 h to recover from acute glyphosate exposure, but more exploration at finer time scales and at different concentrations would improve this estimate.

Overall, we found that acute exposure to Roundup has negative impacts on stream salamander larvae. Due to the nature of stream systems, these concentrations and exposure times are environmentally relevant and may occur multiple times before an individual's metamorphosis (Annett et al., 2014). These acute exposures can also occur at multiple times during the egg and larval development and during key developmental windows for stream salamanders, increasing the probability of lasting effects (Baier et al., 2016). Even though exposures in lotic systems are small and move out of the system quickly, as opposed to lentic systems where the herbicide can accumulate, they still negatively impact the anti-predator behavior of stream salamanders (Gandhi & Cecala, 2016). This altered behavior can lead to more frequent predation and downstream displacement due to glyphosate-induced fatigue. Our results indicate non-linear responses to exposure, which underscores the complex way in which glyphosate is metabolized. We suggest that more studies be conducted on the way in which glyphosate in Roundup is metabolized and if it has long-term effects. This key information could describe the extent to which stream salamanders are impacted both short- and long-term by acute exposures.

Acknowledgements J.L. Allen, E. Culp, C. Gramig, B. Hatfield, K. Kennedy, R. Middendorf, M. Pryor, T. Smartt, S. Sniatecki, and A. Walker assisted with field collection. J. Gandhi and V. Moss provided input into the study design. All procedures and protocols were approved by the University of the South Animal Care and Use Committee (Cecala-4-2019) and Tennessee Wildlife Resources Agency Scientific Collection Permit (#1296).

Author contributions CB and KC: conceptualization, investigation, formal analysis, data curation, visualization, writing—original draft, and writing—review and editing.

Data availability Data will be made available via Zenodo upon acceptance of the manuscript for publication.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

References

Annett, R., H. R. Habibi & A. Hontela, 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. Journal of Applied Toxicology 34: 458–479.

Baier, F., M. Jedinger, E. Gruber & J. G. Zaller, 2016. Temperature-dependence of glyphosate-based herbicide's effects on egg and tadpole growth of common toads. Frontiers in Environmental Science 24: 51.

Bakkegard, K. A., 2005. Antipredator behaviors of the Red Hills Salamander, *Phaegonathus hubrichti*. Southeastern Naturalist 4: 23–32.

Barrett, K., B. S. Helms, C. Guyer & J. E. Schoonover, 2010. Linking process to pattern: causes of stream-breeding salamander decline in urbanized watersheds. Biological Conservation 143: 1998–2005.

Berrill, M., S. Bertram, L. McGillvray, M. Holohon & B. Pauli, 1994. Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. Environmental Toxicology and Chemistry 13: 657–664.

Bliss, M. M. & K. K. Cecala, 2017. Terrestrial salamanders alter antipredator behavior thresholds following tail autotomy. Herpetologica 73: 94–99.

Bridges, C. M., 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Environmental Toxicology and Chemistry 6: 1935–1939.

Cauble, K. & R. S. Wagner, 2005. Sublethal effects of the herbicide Glyphosate on amphibian metamorphosis and development. Bulletin of Environmental Contamination and Toxicology 75: 429–435.

Cheron, M. & F. Brischoux, 2020. Aminomethylphosphonic acid alters amphibian embryonic development at environmental concentrations. Environmental Research 190: 109944.

da Silva, P. R., M. Borges-Martins & G. T. Oliveira, 2021. *Melanophryniscus admirabilis* tadpoles' responses to sulfentrazone and glyphosate-based herbicides: an approach on metabolism and antioxidant defenses. Environmental Science and Pollution Research 28: 4156–4172.

Dinehart, S., L. Smith, S. McMurry, T. Anderson, P. Smith & D. Haukos, 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Science of the Total Environment 407: 1065–1071.

Dodson, S. I., T. Hanzato & P. R. Gorski, 1995. Behavioral responses of *Daphnia pulex* exposed to carbaryl and *Chaoborus* kairomone. Environmental Toxicology and Chemistry 14: 43–50.

Doving, K. B., 1991. Assessment of animal behaviour as a method to indicate environmental toxicity. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 100: 247–252.

Ducey, P. K. & E. D. Brodie Jr., 1991. Evolution of antipredator behavior: individual and population variation in a neotropical salamander. Herpetologica 47: 89–95.

Duellmann, W. E. & L. Trueb, 1994. Biology of Amphibians, Johns Hopkins University Press, New York:

Faria, M., E. Prats, K. Novoa-Luna, J. Bedrossiantz, C. Gómez-Canela, L. M. Gómez-Oliván & D. Raldúa, 2019. Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance. Science of the Total Environment 650: 87–96.

Gandhi, J. S. & K. K. Cecala, 2016. Interactive effects of temperature and glyphosate on the behavior of blue ridge two-lined salamanders (*Eurycea wilderae*). Environmental Toxicology and Chemistry 35: 2297–2303.

Glusczak, L., D. de Santos Miron, B. S. Moraes, R. R. Simões, M. R. C. Schetinger, V. M. Morsch & V. L. Loro, 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 146: 519–524.

Hall, R. J. & P. F. P. Henry, 1992. Assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetology Journal 2: 65–71.

Hasković, E., M. Pekić, M. Fočak, D. Suljević & L. Mešalić, 2016. Effects of glyphosate on enzyme activity and serum glucose in rats *Rattus norvegicus*. Acta Veterinaria-Beograd 66: 214–221.

Hill, C., J. P. Myers & L. N. Vandenberg, 2018. Non-monotonic dose–response curves occur in dose ranges that are relevant to regulatory decision-making. Dose-Response 16: 1559325818798282.

Howe, C. M., M. Berril, B. D. Pauli, C. C. Helbing, K. Werry & N. Velhoen, 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry 23: 1928–1938.

Kats, L. B., J. M. Kiesecker, D. P. Chivers & A. R. Blaustein, 2000. Effects of UV-B radiation on anti-predator behavior in three species of amphibians. Ethology 106: 921–931.

Kolpin, D. W., E. M. Thurman, E. A. Lee, M. T. Meyer, E. T. Furlong & S. T. Glassmeyer, 2006. Urban contributions of glyphosate and its degradate AMPA to streams in the United States. Science of the Total Environment 354: 191–197.

Lagarde, F., C. Beausoleil, S. M. Belcher, L. P. Belzunces, C. Emond, M. Guerbet & C. Rousselle, 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. Environmental Health 14: 13.

Lefcort, H., R. A. Meguire, L. H. Wilson & W. F. Ettinger, 1998. Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. Archives of Environmental Contamination and Toxicology 35: 447–456.

Levis, N. A. & J. R. Johnson, 2015. Level of UV-B radiation influences the effects of glyphosate-based herbicide on the spotted salamander. Ecotoxicology 24: 1073–1086.

Little, E. E. & S. E. Finger, 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology Chemistry 9: 13–19.

Little, E. E., R. D. Archeski, B. A. Flerov & V. I. Kozlovskaya, 1990. Behavioral indicators of sublethal toxicity in rainbow trout. Archives of Environmental Toxicology and Chemistry 19: 380–385.

Lowe, W. H. & G. E. Likens, 2005. Moving headwater streams to the head of the class. BioScience 55: 196–197.

Mahler, B. J., P. C. Van Metre, T. E. Burley, K. A. Loftin, M. T. Meyer & L. H. Nowell, 2017. Similarities and differences in occurrence and temporal fluctuations in glyphosate and atrazine in small Midwestern streams (USA) during the 2013 growing season. Science of the Total Environment 579: 149–158.

McMahon, T. A., N. T. Halstead, S. Johnson, T. R. Raffel, J. M. Romansic, P. W. Crumrine, R. K. Boughton, L. B. Martin & J. R. Rohr, 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environmental Health Perspectives 119: 1098–1103.

Mesnage, R., M. Arno, M. Costanzo, M. Malatesta, G. E. Séralini & M. N. Antoniou, 2015a. Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. Environmental Health 14: 70.

Mesnage, R., N. Defarge, J. Spiroux de Vendômois & G. E. Séralini, 2015b. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food and Chemical Toxicology 84: 133–153.

Pavan, F. A., C. G. Samojeden, C. F. Rutkoski, A. Folador, S. P. Da Fré, C. Müller, P. A. Hartmann & M. T. Hartmann, 2021. Morphological, behavioral, and genotoxic effects of glyphosate and 2,4-D mixture in tadpoles of two native species of South American amphibians. Environmental Toxicology and Pharmacology 85: 103637.

Petranka, J. W., 1984. Sources of interpopulational variation in growth responses of larval salamanders. Ecology 65: 1857–1865.

Petranka, J. W., 1998. Salamanders of the United States and Canada, Smithsonian Institution, Washington DC:

Quaranta, A., V. Bellantuono, G. Cassano & C. Lippe, 2009. Why amphibians are more sensitive than mammals to xenobiotics. PLoS ONE 4: e7699.

R Core Team, 2021. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna:

Relyea, R., 2009. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 19: 276–276.

Relyea, R., 2011. Amphibians Are Not Ready for Roundup®. In Elliot, J. E., C. Bishop & C. Morrisey (eds), Wildlife

Ecotoxicology: Forensic Approaches Springer, New York: 267–300.
Relyea, R. A. & J. Hoverman, 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology Letters 9: 1157–1171.
Relyea, R. A. & N. Mills, 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles. Proceedings of the National Academy of Sciences of the United States of America 98: 2491–2496.
Riaño, C., M. Ortiz-Ruiz, N. R. Pinto-Sánchez & E. Gómez-Ramírez, 2020. Effect of glyphosate (Roundup Active®) on liver of tadpoles of the Colombian endemic frog *Dendropsophus molitor* (Amphibia: Anura). Chemosphere 250: 126287.
Rohr, J. R. & K. A. McCoy, 2010. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environmental Health Perspectives 118: 20–32.
Rohr, J. R., T. R. Raffel, S. K. Sessions & P. J. Hudson, 2008. Understanding the net effects of pesticides on amphibian trematode infections. Ecological Applications 18: 1743–1753.
Vandenberg, L., T. Colborn, T. Hayes, J. Heindel, D. Jacobs Jr., D. Lee, T. Shioda, A. Soto, F. vom Saal, W. Welshons, R. T. Zoeller & J. P. Myers, 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrine Reviews 33: 378–455.
Vandenberg, L. N., T. Colborn, T. B. Hayes, J. J. Heindel, D. R. Jacobs Jr., D. H. Lee, J. P. Meyers, T. Shioda, A. M. Soto, F. S. vom Saal, W. V. Welshons & R. T. Zoeller, 2013. Regulatory decisions on endocrine disruptin chemicals should be based on the principles of endocrinology. Reproductive Toxicology 38: 1–15.
Wagner, N., W. Reichenbecher, H. Teichmann, B. Tappeser & S. Lötters, 2013. Questions concerning the potential impact of glyphosate-based herbicides on amphibians. Environmental Toxicology and Chemistry 32: 1688–1700.
Wang, X., L. Chang, T. Zhao, L. Liu, M. Zhang, C. Li, F. Xie, J. Jiang & W. Zhu, 2019. Metabolic switch in energy metabolism mediates the sublethal effects induced by glyphosate-based herbicide on tadpoles of a farmland frog *Microhyla fissipes*. Ecotoxicology and Environmental Safety 186: 109794.