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NO OBSERVABLE EFFECT OF A GLYPHOSATE-BASED HERBICIDE ON TWO TOP PREDATORS OF TEMPORAL WATER BODIES

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Abstract: It has been implied that the application of pesticides is involved in the world-wide decline of biodiversity, but little is known about the influence of these chemicals on key predators of temporary wetlands. The direct impacts were examined of a frequently applied glyphosate-based herbicide on larval *Aeshna cyanea* (Müller, 1764; Odonata, Insecta) and adult male *Lissotriton vulgaris* (Linnaeus, 1758; Caudata, Amphibia), 2 top predators of Central European ephemeral ponds. The effects of herbicide exposure were measured on survival, behavior, body mass change, and predatory activity in an outdoor mesocosm experiment lasting for 17 d. No significant effects of exposure were observed in either predator species. The results suggest that the herbicide has no immediate effect on the predators studied at environmentally relevant concentrations and that these predators can also fulfill their top-down regulatory role in contaminated ecosystems. *Environ Toxicol Chem* 2015;34:307–313. © 2014 SETAC

Keywords: Aquatic toxicology *Aeshna cyanea* Aquatic invertebrates *Lissotriton vulgaris* Amphibians

INTRODUCTION

Studies on the potential consequences of contamination with pesticides for nontarget organisms are abundant and have demonstrated various effects on model organisms living in the aquatic environment [1–3]. At minute concentrations [3], agrochemicals can have lethal effects on aquatic organisms, and at sublethal concentrations, they can result in altered behavior [4], decreased growth and development rates [5], and increased frequency of developmental abnormalities [6]. Furthermore, pesticides can enhance the effects of other biotic and abiotic stress factors [7,8]. Consequently, pesticide contamination can have large effects on individuals, but also on ecological patterns and processes [2].

Glyphosate-based herbicides are the most frequently used herbicides worldwide, and glyphosate is 1 of the 3 most often detected anthropogenic chemicals in freshwater ecosystems [3]. According to ecotoxicological assessments, the expected worst-case concentration of glyphosate-based herbicides falls within the range of 1.4 acid equivalent (a.e.) to 7.6 mg a.e. glyphosate L^{-1} in shallow surface water bodies, depending on habitat characteristics, and on the distance to agricultural lands [3,9–11]. Glyphosate-based herbicides are also widely applied and frequently detected in freshwater ecosystems in Europe and Hungary [12–14], where the present study was conducted.

Glyphosate-based herbicides are generally composed of glyphosate, which inhibits protein synthesis in plants, and a surfactant (e.g., polyethoxylated tallow amine), which helps the glyphosate penetrate the cuticle and reach inner tissues [15]. Previous studies have indicated that although glyphosate itself can become toxic, the surfactants are the more harmful ingredients for aquatic animals [3,16–18] because they probably impair the functioning of the gills' respiratory surface, as suggested by Edginton et al. [17]. Nonetheless, the glyphosate contained in the herbicide can also reduce phytoplankton biomass and lead to lowered concentrations of dissolved oxygen [10], potentially further compromising the physiological performance of gill-breathing animals.

Most studies in the field of ecotoxicology have investigated model species belonging to a few taxa, such as cladocerans, fish, or larval anurans [8,16], the extent to which top predators of small water bodies are affected by pesticides has r emained largely unstudied, although top predators play a crucial regulatory role in such communities. They can prevent the overgrowth of prey populations, thus indirectly averting overgrazing of producers [19], and contribute to the maintenance of permanent coexistence among competing prey species [20]. In temporal water bodies lacking fish, top predators are often insects (especially odonates, hemipterans, and coleopterans) and caudate amphibians (salamanders and newts). These generalist predators consume a wide variety of prey species, ranging from zooplankton to larval amphibians [2,21,22]. Despite their important role, ecotoxicological studies focusing on potential effects of pesticides on these predators are scarce [11,23,24].

Our aim was to test the sensitivity of 2 common top predator species of temporal water bodies to the glyphosate-based herbicide Glyphogan Classic (Monsanto ME), a formulation that is widely used in Hungary and, more generally, in Europe. We tested larvae of the southern hawker dragonfly (*Aeshna cyanea*, Müller, 1764; Odonata, Insecta), an invertebrate, gill-breathing, sit-and-wait predator, and adult males of the smooth newt (*Lissotriton vulgaris*, Linnaeus, 1758; Caudata, Amphibia), a vertebrate, lung-breathing, active forager. We picked these species, which represent 2 taxonomically, physiologically, and ecologically very distinct taxa, to extend previous knowledge on the potential consequences of glyphosate-based herbicides on different types of top predators and aquatic communities. We applied the chemical at an initial concentration of 6.5 mg a.e. glyphosate L^{-1}, which is close to the maximum expected worst-case concentration for temporal water bodies [9], to maximize the likelihood of detecting impacts of glyphosate-based

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herbicides. To scrutinize the potential effects of chronic and acute exposure to the herbicide under nature-like conditions [25], we tested in an outdoor mesocosm experiment whether the glyphosate-based herbicide affected survival, body mass change, activity, and predatory activity of the 2 predators.

MATERIALS AND METHODS

Experimental procedures

We collected 36 larvae of *A. cyanea* (instars F-1 and F-2) from a small pond near Bajna, Hungary (47°38′41″N, 18°36′41″E) using dip-nets and 36 adult males of *L. vulgaris* from 2 water bodies in the Pilis Mountains, Hungary (6 individuals from 47°44′20″N, 19°0′42″E; 8 individuals from 47°44′21″N, 19°0′42″E), and from a pond near Pilisjászfalu, Hungary (22 individuals, 47°38′40″N, 18°46′31″E) using plastic funnel traps. We transported captured animals to the Júliannamajor Experimental Station of the Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences. Until the start of the experiment 5 d later, we kept dragonfly larvae individually in cups containing 300 mL water and a wooden stick as a perching site, whereas newts were kept in groups of 5 in plastic boxes (23 cm × 19 cm × 12 cm) containing 1.5 L water. We also collected 10 freshly laid egg clutches of the agile frog (*Rana dalmatina*) from a pond in the Pilis Mountains, Hungary (47°44′21″N, 19°0′42″E), and used the tadpoles that hatched for feeding predators and for testing predatory activity (see below). Tadpoles were thus naïve in respect to predators and the herbicide. We maintained eggs in the laboratory at 19 °C and tadpoles at 15 °C to slow their growth. We fed tadpoles with slightly boiled spinach and changed the water every 3rd day.

We conducted the experiment in 72 plastic containers of 90-L volume placed outdoors. Three weeks before the start of the experiment, we filled containers with 65 L tap water and covered them with mosquito net lids immediately after filling to prevent colonization by macroinvertebrates. One day later, we added 1 L of pond water and 40 g of dried beech (*Fagus sylvatica*) leaves to enhance the growth of bacteria, phytoplankton and zooplankton, and to provide shelter for experimental animals. Similar mesocosms were successfully used previously in studies involving amphibian and invertebrate predators [26,27], and experiments using outdoors mesocosms are thought to better model natural conditions than laboratory-based studies [25]. We placed automatic temperature loggers (HOBO) into 3 randomly chosen experimental containers to record water temperatures during the experiment. To provide perching sites, we placed small plastic ladders into the mesocosms positioned vertically and reaching just below the water surface. Finally, to prevent excessive periphyton growth, we added 3 large snails (*Lymnaea stagnalis*) to each mesocosm. Snails had been hand collected from an artificial canal close to Bugyi, Hungary (47°12′46″N, 19°8′56″E).

We added 1.174 mL Glyphogan Classic to the mesocosms assigned to the herbicide treatment to obtain an initial concentration of 6.5 mg a.e. glyphosate L^{-1} and 0.0028 mL polyethoxylated tallow amine L^{-1}. Mesocosms in the control treatment received an equal amount of aged tap water. We replicated predator × treatment combinations 18 times, which resulted in 72 experimental units. Mesocosms were arranged into spatial blocks, each containing 1 replicate from each predator × treatment combination; the locations of these replicates were assigned randomly within blocks. Two days after the addition of the herbicide, we weighed predators (to the nearest mg) using an analytical balance and started the experiment by placing predators into the mesocosms in a random order. Every other day we fed predators by adding to experimental containers 2 small naïve *R. dalmatina* tadpoles (~150 mg) and approximately 200 mg *Tubifex tubifex* worms. Thirteen and 16 d after beginning the experiment we siphoned water samples of 1 L from 3 depths (1 cm, 12 cm, and 23 cm) from 3 mesocosms set up identically as the experimental containers, but not containing a predator. To obtain analyzable amounts, we pooled samples across mesocosms and the 2 sampling occasions, keeping the samples from different water depths apart. To test for stratification [28] and decomposition of glyphosate [29] the resulting 3 samples were later analyzed using liquid chromatography mass spectrometry by the Herbicide Residue Analytical Laboratory, Directorate of Plant Protection and Soil Conservation, National Food Chain Safety Office, Miskolc, Hungary.

Sixteen days after start, we monitored predators and observed their behavior once per hour between 9:00 AM and 6:00 PM. We started the first observation 30 min after removing lids and left containers uncovered until the end of the 9-h observation period. We recorded 3 behavioral variables during 30-s scan observations: visibility, activity, and vertical position. We considered an animal visible if leaf litter did not cover its head. Animals were scored as active if any part of the body was moving. Finally, we noted whether the animal was in the bottom, middle, or top third of the water column. If the animal was not visible, we considered it to be inactive and to be in the bottom third of the water column. On the day of behavioral observations, water temperature varied between 18 °C and 22 °C, and pH ranged between 7.2 and 7.3; both variables were measured in 10 mesocosms using a Mettler Toledo MX300 X-mate^{Pro}.

Eighteen days after starting the experiment, we captured predators, measured their body mass, and assigned them from both chronic treatments to 2 subgroups, which were subsequently exposed to either clear water or water containing 6.5 mg

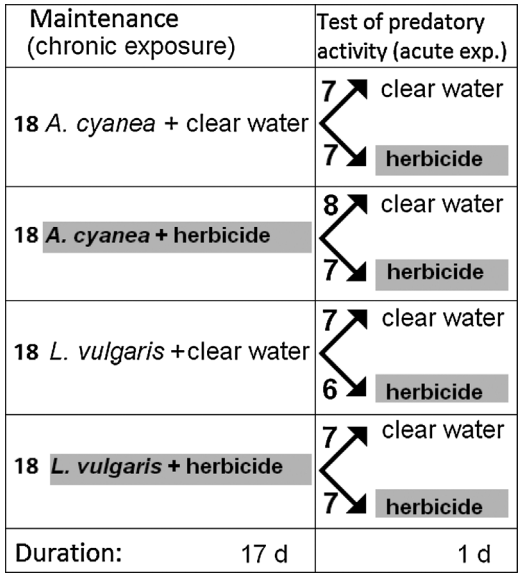

Figure 1. The experimental setup and process with the number of animals in each group. Numbers in the tests of predatory activity are lower because some *Aeshna cyanea* died and some *Lissotriton vulgaris* escaped during the experimental period. Predatory activity tests were accomplished applying a full factorial design. Gray backgrounds highlight herbicide treatments.

a.e. glyphosate L^{-1} (for a schematic delineation, see Figure 1). Individuals were roughly similar in size among the subgroups (based on Kruskal–Wallis tests; *Aeshna cyanea*: $n = 29$, $Chi^2 = 2.914$, $df = 3$, $p = 0.41$ and *Lissotriton vulgaris*: $n = 27$, $Chi^2 = 0.464$, $df = 3$, $p = 0.93$). We transferred predators into newly established mesocosms prepared the same way as described above. After 1 d of acclimation in the test containers, we provided predators with 8 small *R. dalmatina* tadpoles (~80 mg body mass). Twenty-four h later, we removed predators and counted surviving tadpoles. We tested predatory activity both in clear water and in water containing the herbicide, because glyphosate-based herbicides may induce phenotypic changes in tadpoles as well [10,30]. In this way we could not only filter out the effect of the herbicide on tadpoles, but could also investigate the effect of acute exposure on predators' foraging activity. After the experiment, we released the predators and remaining tadpoles at the sites of collection.

Statistical analysis

We used linear mixed modeling procedures to analyze body mass data. Because of an erroneously noted mass value, we had to exclude 1 newt from the analysis of body mass change. To analyze behavioral data, we first calculated mean values for the 3 variables for each experimental container across the 8 observations. Data on vertical position were square-root transformed to enhance normality of error distributions and avoid problems as a result of heterogeneity of variances. Visibility and activity of predators correlated with each other (Spearman rank correlation; $r = 0.34$, $n = 56$, $p = 0.01$), so we excluded activity from the analyses. We analyzed visibility and vertical position using separate general linear models. Results of the predatory activity tests were analyzed with generalized linear models with a Poisson distribution and a log-link function. We entered all two-way interactions into initial models and applied a backward stepwise removal procedure (terms were removed when $p > 0.05$) to avoid problems potentially arising from the inclusion of nonsignificant terms. We obtained statistics for removed variables by reentering them 1 by 1 into the final model. All tests were 2-tailed. Statistics were calculated using SPSS Statistics 17.0 for Windows.

RESULTS AND DISCUSSION

Exposure to the herbicide had no effect on any life-history trait we measured either in larval *A. cyanea* or in adult male *L. vulgaris*: survival, body mass, and behavior did not differ between animals in the control and in the herbicide treatment at the end of the 17-d experimental period. Predatory activity appeared to be unaffected by both chronic and acute exposure to the herbicide as well. For details, see below.

By the end of the experimental period of 17 d, 7 *A. cyanea* larvae died out of 36 individuals, 4 from the control treatment and the other 3 from the chronic exposure treatment. We did not observe mortality in *L. vulgaris*, but 7 individuals escaped from the experimental containers, 4 from mesocosms holding clear water and 3 from mesocosms containing the herbicide. We may thus conclude that the presence of the herbicide did not result in elevated mortality rates in either species, nor did it induce escaping behavior in newts. Most previous studies have also not found effects of pesticides on the survival of larval dragonflies [2,31,32], suggesting that larvae of anisopteran dragonflies are relatively insensitive to these chemicals in terms of survival. In the case of newts, previous results are contradictory [21,33,34]. In the present study we did not observe an effect on adult newts, despite relatively high concentrations of the herbicide (6.5 mg a.e. glyphosate L^{-1} initial nominal concentration, decreasing to ~3.75 mg a.e. L^{-1} 2 wk later, because of degradation and precipitation [29]). Cutaneous breathing is known to be important for adult newts [35]; the functioning of such breathing did not seem to be severely impaired by the herbicide, or else lung breathing could have compensated for any detrimental effects on respiration. The observation that the herbicide did not induce newts to leave the water and escape also suggests that there were no severe physiological effects.

The presence of the herbicide did not affect body mass change in either predator species during the 17 d of the experiment. Body mass of dragonfly larvae doubled, but this change in mass was independent of exposure to the herbicide. At the same time, body mass did not change detectably in *L. vulgaris* during the experimental period, and exposure to the herbicide had no effect on it. Neither interaction was significant (Figure 2; for statistics see Table 1). We know of no similar study directly investigating changes in body mass of predatory insects related to glyphosate-based herbicides. In the case of adult caudate amphibians, the only existing study reported that another formulation of glyphosate-based herbicides did not affect growth of a North American newt species, *Notophthalmus viridescens* [21]. The absence of an effect on body mass implies that detoxification induced by the herbicide does not inflict severe costs in the species studied, at least not after

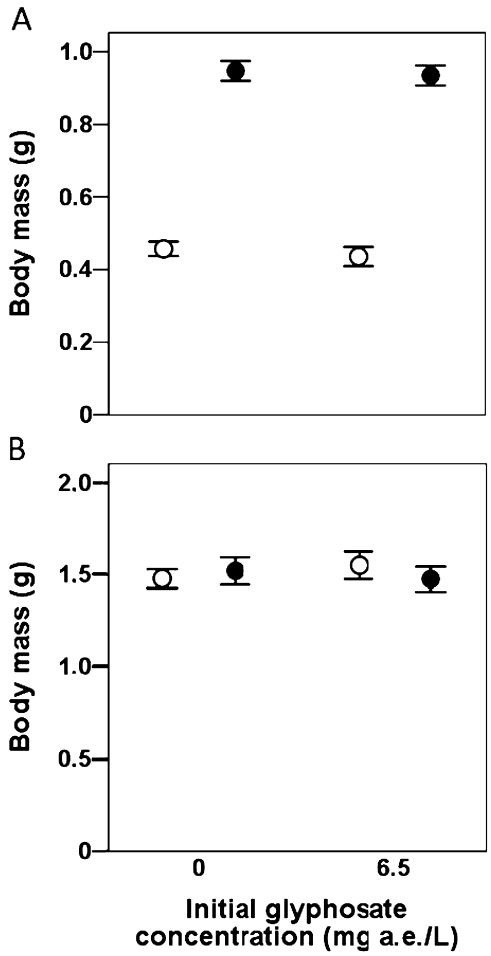

Figure 2. Body mass (mean ± 1 standard error [SE]) of *Aeshna cyanea* larvae (**A**) and *Lissotriton vulgaris* males (**B**) before (◯) and after (●) chronic exposure to clear water or water containing the herbicide.

Table 1. Results of statistical analyses[a]

Species	Variable	Effect	*F*	*df*	η^2 (CI)[b]	*p*	LR Chi^2	*df*	Exp(B) (CI)[c]	*p*
Aeshna cyanea	Body mass	Chronic exposure	0.485	1, 54	0.0090 (0–0.112)	0.49				
		BA	386.520	1, 55	0.8760 (0.808–0.909)	<0.001				
		Chronic exposure × BA	0.029	1, 53	0.0005 (0–0.022)	0.87				
	Visibility	Chronic exposure	0.008	1, 27	0.0003 (0–0.012)	0.93				
		Body mass	0.059	1, 27	0.0022 (0–0.080)	0.81				
		Chronic exposure × body mass	0.090	1, 25	0.0024 (0–0.086)	0.77				
	Vertical position	Chronic exposure	0.586	1, 27	0.0214 (0–0.204)	0.45				
		Body mass	0.223	1, 27	0.0081 (0–0.165)	0.64				
		Chronic exposure × body mass	0.714	1, 25	0.0276 (0–0.226)	0.41				
	Predatory activity	Chronic exposure					1.046	1	1.21 (0.839–1.747)	0.31
		Acute exposure					1.046	1	1.21 (0.839–1.747)	0.31
		Body mass					0.127	1	1.40 (0.218–8.988)	0.72
		Chronic exposure × acute exposure					1.863	1	1.67 (0.801–3.474)	0.17
		Acute exposure × body mass					0.238	1	0.38 (0.008–18.393)	0.63
		Chronic exposure × body mass					0.909	1	6.02 (0.151–239.574)[d]	0.34
Lissotriton vulgaris										
	Body mass	Chronic exposure	0.082	1, 52	0.0016 (0–0.619)	0.78				
		BA	0.078	1, 52	0.0015 (0–0.059)	0.78				
		Chronic exposure × BA	0.727	1, 50	0.0145 (0–0.133)	0.40				
	Visibility	Chronic exposure	2.226	1, 25	0.0819 (0–0.310)	0.15				
		Body mass	0.002	1, 25	0.0001 (0–0.003)	0.97				
		Chronic exposure × body mass	0.064	1, 23	0.0028 (0–0.099)	0.80				
	Vertical position	Chronic exposure	<0.001	1, 25	0.0000 (0–0)	0.99				
		Body mass	3.333	1, 25	0.1175 (0–0.352)	0.08				
		Chronic exposure × body mass	0.178	1, 23	0.0077 (0–0.178)	0.68				
	Predatory activity	Chronic exposure					2.798	1	0.73 (0.501–1.059)	0.09
		Acute exposure					0.593	1	0.87 (0.599–1.250)	0.44
		Body mass					1.152	1	1.49 (0.722–3.053)	0.28
		Chronic exposure × acute exp.					1.542	1	0.62 (0.293–1.319)	0.21
		Acute exposure × body mass					2.909	1	3.72 (0.804–17.181)	0.09
		Chronic exposure × body mass					0.879	1	0.49 (0.112–2.173)	0.35

[a]Body mass was analyzed using linear mixed models, behavior using general linear models, and predatory activity using generalized linear models.
[b]Effect size (confidence interval).
[c]Odds ratio (confidence interval).
[d]The high odds ratio and wide confidence interval were caused by 1 individual refusing to eat during the predatory activity tests due to ecdysis.
BA = change with time from before to after chronic exposure.

exposure to the applied concentration of the tested herbicide for a time period of 2.5 wk.

The presence of the herbicide did not influence the behavior of either dragonfly larvae or adult newts, neither visibility nor vertical position assumed by predators changed after exposure. Body mass also had no effect on behavior, and all two-way interactions were nonsignificant (Figure 3; Table 1). Glyphosate concentration can be higher in the upper, warmer water layers in natural water bodies and in large mesocosms [28], but the herbicide did not seem to stratify in our experimental units (3.81 mg a.e. glyphosate L^{-1}, 3.69 mg a.e. glyphosate L^{-1}, and 3.74 mg a.e. glyphosate L^{-1}, from the bottom to the top, respectively, measured 2 wk after start), which we attribute to the relatively small size of the containers. The lack of a behavioral response of predators to the herbicide may be because dragonfly larvae and adult newts are not affected by the herbicide directly and thus do not have to avoid it [2,21; present study]. However, it is also possible that these animals are unable to detect the presence of the herbicide [36], or, conversely, that they can sense and avoid locations with high concentrations [37] but did not experience sufficient variation of concentration within the experimental mesocosms. It remains unknown which of these mechanisms may have contributed to the lack of behavioral responses to the herbicide. Water temperature (which fluctuated between 12 °C and 28 °C, with a mean of 19 °C) had no permanent excessively high values, and thus presumably did not affect animal behavior.

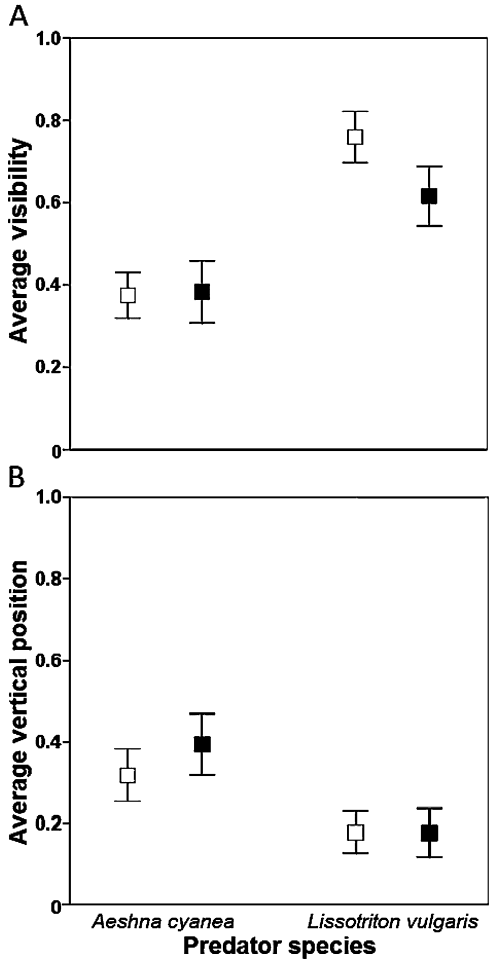

Figure 3. Between-treatment pattern of average visibility (**A**) and average vertical position (**B**) in the 2 predator species. We obtained average visibility and average vertical position by averaging scores on behavior noted during 8 observation periods. Means ± 1 standard error (SE) are depicted; □ = control, ■ = herbicide treatment.

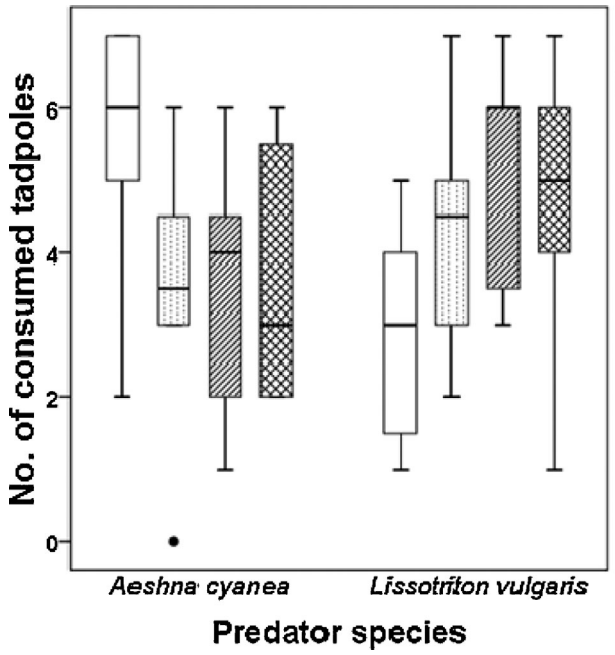

Figure 4. Number of tadpoles consumed in the case of *Aeshna cyanea* and *Lissotriton vulgaris* with different exposure types. Explanation of transitions: 1 (white), no chronic exposure and no acute exposure; 2 (dotted), no chronic exposure and acute exposure subsequently; 3 (striated), chronic exposure and no acute exposure; 4 (reticulated), chronic exposure and acute exposure subsequently. Vertical lines depict medians, boxes represent interquartiles, and bars represent range.

Tests on predatory activity revealed that neither chronic nor acute exposure to the herbicide significantly affected the number of tadpoles consumed. Body mass did not affect the number of consumed tadpoles, and none of the two-way interactions were significant (Figure 4; Table 1). The effect of pesticides on predatory activity has rarely been tested on dragonfly larvae or amphibians [2]. The only study we know of did not report an effect of acute exposure to pesticides (malathion and carbaryl insecticides) on predatory activity in the newt *N. viridescens* [24]. We also did not observe significant changes in predatory activity in either the chronic or the acute presence of the herbicide. The herbicide may have affected tadpoles in the trials performed in the presence of the herbicide [4,10], swamping effects on predators, but we consider this unlikely, because tadpoles seemed neither to enjoy a systematic benefit nor to suffer a disadvantage from acute exposure. Nonetheless, because sample sizes were rather low in this part of the experiment (6, 7, or 8 animals in acute tests; Figure 1), and, consequently, effects of interactions could only be estimated with low confidence (Table 1), we cannot rule out the possibility that exposure to the herbicide does have some effect on predatory activity (Table 1; Figure 4). Indeed, in the case of 2 interactions (chronic exposure × body mass for *A. cyanea*; acute exposure × body mass for *L. vulgaris*; Table 1), the odds ratio values appear to be high. However, any interpretation of these interactions remains highly speculative because the corresponding confidence intervals are wide and span values both below and above equity. Even if some effect exists, it is likely to be moderate to insignificant, especially as we did not observe any effect of chronic exposure on body mass change.

In conclusion, the tested formulation of glyphosate-based herbicides had no measurable effect on survival, body mass, behavior, or predatory activity in either larval *A. cyanea* or

adult male *L. vulgaris*. This result is surprising because predators were exposed to an initial nominal concentration of 6.5 mg a.e. glyphosate L^{-1}. Even though this concentration diminished over the course of the experiment, the concentration measured 2 wk later was ~3.75 mg a.e. glyphosate L^{-1}, which still represents a relatively high value in comparison with other studies [2,21,32,34], and is close to worst-case concentrations likely to occur in nature [10,11]. Lower concentrations of the surfactant in the herbicide formulation we used than in previous reports may have contributed to the absence of herbicide effects in the present study. Because information on the type and concentration of the surfactant in the previously tested formulations of the herbicide is unavailable, this hypothesis remains untested. However, our experiment only lasted for 17 d, and we have no information about potential long-term effects of the herbicide on the predators studied. Glyphosate has been documented to be genotoxic to certain fish [38], and can be teratogenic to amphibians [39]. Furthermore, newts may be more sensitive during the larval than the adult stage, and predators may also be exposed to herbicides not only in the water, but during their terrestrial life as well [40]. Pesticides may also affect predatory biomass through indirect trophic cascade effects [2]. Although our results suggest that the predator species studied are not affected directly by exposure to the herbicide and thus may also fulfill their top-down regulatory role in ponds contaminated with herbicides, further studies are needed to clarify potential effects of exposure to herbicides over longer time periods and to test whether malign effects of exposure may manifest later in life.

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