NO EFFECT OF A GLYPHOSATE-BASED HERBICIDE ON LARVAL DRAGONFLIES (*AESHNA CYANEA*) AND ADULT NEWTS (*LISSOTRITON VULGARIS*) IN A LABORATORY-BASED EXPERIMENT*

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Pesticides can exert negative effects on aquatic organisms at very low concentrations. While several prey taxa are frequently used as models in ecotoxicology studies, there is little information about the pesticide-sensitivity of predators. We examined the effects of a frequently applied glyphosate-based herbicide on two common aquatic predators: larval *Aeshna cyanea* dragonflies and adult male *Lissotriton vulgaris* newts, which are top predators in ephemeral water bodies lacking fishes. We exposed predators to the herbicide for 18 days under laboratory conditions and measured potential effects on survival, activity, body mass and predatory activity. To maximize detectability of effects, we applied the herbicide at a concentration of 6.5 mg a. e. glyphosate/L, corresponding to the highest concentration expected in nature. Our results showed that the tested herbicide formulation did not have severe effects on any of the measured fitness-related traits. Results of the present study support the hypothesis that the tested species are insensitive to the herbicide and are able to fulfil their important ecological role of top-down regulation even in highly contaminated habitats. However, potential long-term or indirect effects of the herbicide on the fitness of aquatic predators remain unknown.

Key words: aquatic toxicology, aquatic community, aquatic predators, acute toxicity.

INTRODUCTION

Pesticides can have detrimental effects on non-target organisms and entire communities (Relyea *et al.* 2005, Relyea & Diecks 2008, Peters *et al.* 2013). Aquatic species are especially sensitive to pesticides, and already low concentrations can lead to mortalities and local extinctions (Pérez *et al.* 2011). However, sublethal concentrations are usually found in nature (e.g. Struger *et al.* 2008, Peruzzo *et al.* 2008, Battaglin *et al.* 2009). These can also have serious consequences, such as developmental malformations (Lajmanovich *et al.* 2003),

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* This article is dedicated to Prof. László Papp, in honor of his 70th birthday and his monumental contribution to the fields of dipteran taxonomy and ecology.

teratogenic effects (Paganelli *et al.* 2010), physiological and behavioural alterations (Bridges 1999, Lajmanovich *et al.* 2011), while exposure to pesticides may also escalate the impact of other biotic and abiotic stressors (Relyea & Mills 2001, Sih *et al.* 2004).

Glyphosate-based herbicides are among the most frequently applied pesticides worldwide (Pérez *et al.* 2011, Mörtl *et al.* 2013). Expected worst-case concentrations of these herbicides in shallow water bodies range from 1.4 to 7.6 mg acid equivalent (a.e.) / L (Mann & Bidwell 1999, Relyea 2012, Wagner *et al.* 2013), which is around or higher than the LC_{50} levels for most North-American and Australian amphibian larvae (Mann & Bidwell 1999, Relyea & Jones 2009, Relyea 2012). In Hungary more than 280 tons of glyphosate (only active ingredient) were sold in 2010 (Mörtl *et al.* 2013), so that severe contamination of aquatic habitats is likely to occur frequently in this part of the world, as well.

Temporary water bodies represent local biodiversity hotspots that harbour many rare and endangered species (Williams *et al.* 2003, Scheffer *et al.* 2006). As fishes are usually absent, spiders, larval dragonflies, heteropterans, water-beetles and caudate amphibians are the most important aquatic predators. These generalist predators regulate a wide variety of prey species spanning from zooplankton to amphibian larvae, thereby contributing to the maintenance of the ecological balance in these aquatic communities (Terborgh *et al.* 2001, Schmitz *et al.* 2004, Relyea 2005*a*). As ephemeral aquatic habitats are relatively small and shallow, and they are often immediately adjacent to cultivated fields, glyphosate concentrations can reach higher levels than measured or predicted for big water bodies with no-spray buffer zones (Mann & Bidwell 1999, Battaglin *et al.* 2009, Relyea 2012, Wagner *et al.* 2013).

Only a few studies have dealt with potential consequences of glyphosate-based herbicides on predatory species of temporary aquatic communities. Sensitivity of predatory arthropods has mainly been tested towards heavy metallic ions and insecticides (Tollett *et al.* 2009, Relyea & Edwards 2010), and also very little is known about the sensitivity of adult caudate amphibians towards any pesticide (Wagner *et al.* 2013). In the sole previous experimental study that investigated effects of exposure to a glyphosate-based herbicide on larval *Aeshna cyanea* dragonflies and adult male *Lissotriton vulgaris* newts we did not find any effects (Ujszegi *et al.* 2015). However, in that experiment the initially high glyphosate concentration diminished to half of the starting concentration by the end of the experimental period, presumably due to degradation, precipitation and adsorption (Katagi 2006). Also, Ujszegi *et al.* (2015) was performed in outdoor mesocosms, and the choice of experimental venue can strongly influence the outcome of ecotoxicological studies (Mikó *et al.* 2015). Consequently, further investigations are necessary to ascertain the

insensitivity of predators inhabiting temporary wetlands to glyphosate-based herbicides.

In this study, we examined the effects of exposure to a permanently high concentration of a glyphosate-based herbicide on dragonfly larvae and adult newts under laboratory conditions. We applied Glyphogan® Classic (Monsanto ME, Brussels, Belgium) at a concentration of 6.5 mg a.e. glyphosate/L. We selected this concentration to be close to the maximum expected worst-case value predicted for shallow water bodies (Mann & Bidwell 1999, Relyea 2012, Wagner *et al.* 2013), thereby maximizing the detectability of potential impacts. As model species we used two common predators of central European temporary wetlands: larvae of the southern hawker (*Aeshna cyanea*), an invertebrate sit and wait predator, and adults of the smooth newt (*Lissotriton vulgaris*), a vertebrate active forager. We assessed if the herbicide affected survival, changes in body mass, activity and predatory activity of animals.

MATERIAL AND METHODS

We collected 32 larvae of *A. cyanea* (mainly F-2 instars) and 32 adult males of *L. vulgaris* from four forest ponds in the Pilis Mountains, Hungary on 22 March 2014. (*A. cyanea* larvae: all originated from 47°44′21″N, 19°0′42″E; *L. vulgaris* males: 11 individuals originated from 47°42′27″N, 19°2′24″E, 17 individuals from 47°42′47″N, 19°2′25″E and 4 individuals from 47°42′40″N, 19°2′44″E). We captured dragonfly larvae using dip-nets and newts using plastic funnel traps, and transported animals to the laboratory at the Experimental Station Júliannamajor (Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences). We kept dragonfly larvae individually in plastic cups containing 300 mL reconstituted soft water (RSW; APHA, 1985) and a wooden perching site. Newts were placed in groups of four individuals into plastic boxes (23 × 19 × 12 cm) containing 1.5 L RSW. We kept predators at 19 °C and a 12 : 12 h light : dark cycle and fed them *ad libitum* with tubicid worms (*Tubifex* sp.) until the start of the experiment two days later.

On 16th March we collected 200 eggs from each of 10 clutches of the agile frog (*Rana dalmatina*) from a pond near Pilisjászfalu, Hungary. We kept eggs separated by family in 10 L buckets filled with 5 L RSW, at 19 °C and a 12 : 12 h light : dark cycle, and hatching tadpoles in large plastic boxes (37 × 27 × 15.5 cm) filled with 10 L RSW, at 15 °C and a 12 : 12 h light : dark cycle. We maintained tadpoles at low temperatures and at high densities to slow their growth. We fed tadpoles with slightly boiled spinach and changed water twice a week. Tadpoles raised this way were, thus, not exposed to either predator cues or the herbicide before their use as food for predators and for testing predatory activity. We used these tadpoles for feeding predators during the experiment and for testing predatory activity.

On 24th March we started the experiment by weighing predators (to the nearest mg) and randomly assigned them to herbicide or control treatments, placing them into transparent plastic boxes (23 × 19 × 12 cm), arranged in randomized spatial blocks on a laboratory shelf system. Boxes contained a small plastic pot and a wooden stick as hiding and perching sites. At the start, and subsequent water changes we applied the herbicide by adding 1.19 ml pure Glyphogan® Classic (360 g a.e. glyphosate/L) to 66 L RSW which resulted in 6.5 mg a.e. glyphosate/L final concentration. We dispensed 2 L of this solution into boxes

assigned to the herbicide treatment. Boxes assigned to the control treatment received 2 L of RSW. We replicated predator × treatment combinations 16 times, resulting in a total of 64 experimental units. The average temperature was 17.6 °C in the laboratory (min.–max.: 16.5–18.6 °C; as measured using Onset HOBO automatic temperature loggers). We applied a 12 : 12 h light : dark cycle during the experiment. Predators were fed every other day with a mixture of two naïve *R. dalmatina* tadpoles (totalling 230 mg) and 150 mg *Tubifex* sp. worms. We changed water twice a week, where we used separate nets for each treatment and species to avoid contamination with the herbicide and transmission of species-specific chemical cues.

Sixteen days after start, we observed activity of predators hourly between 10:00 and 18:00. We scored animals active if any part of the body was moving and inactive if it was sitting completely still.

To test the effects of chronic and acute exposure to the herbicide on predatory activity, nineteen days after start we captured predators, weighed them, and randomly assigned them to one of two treatment groups: they were either placed into boxes holding RSW or RSW containing the herbicide (Table 1). Experimental boxes used in this test were similar to those used in the first part of the experiment. Body mass of predators did not differ significantly between the four treatment groups (ANOVA; *A. cyanea*: $F_{3,28}$ = 0.92, *P* = 0.45; *L. vulgaris:* $F_{3,26}$ = 1.237, *P* = 0.32; Tables 1 & 2). After one day of acclimation, we tested the predatory activity of animals with small naïve *R. dalmatina* tadpoles (~100 mg each). We placed 10 tadpoles into each boxes containing a dragonfly larva and 5 tadpoles into each box containing a newt. Four hours later, we counted surviving tadpoles and terminated the experiment. We tested predatory activity both in clear water, and in water containing the herbicide to be able to investigate the effects of chronic and acute exposure on the predators' foraging activity. One newt in the control treatment left the water before the predator activity test, so we excluded this individual from the analyses on behaviour. One larval dragonfly was also excluded due to ecdysis. After the experiment, predators and remaining tadpoles were released at the sites of their collection.

Table 1. Design of the experiment with sample sizes in each group. Note: one newt accidentally died, and another one was excluded, hence the lower numbers in the tests of predatory activity in the case of "*L. vulgaris* + clear water" treatment group. Also one larval dragonfly was excluded because of ecdysis in the case of "*A. cyanea* + clear water" treatment group.

Maintenance (chronic exposure)	Test of predatory acitivity (acute exposure)	No. specimens
Aeshna cyanea + clear water	clear water	7
	herbicide	8
Aeshna cyanea + herbicide	clear water	8
	herbicide	8
Lissotriton vulgaris + clear water	clear water	7
	herbicide	7
Lissotriton vulgaris + herbicide	clear water	8
	herbicide	8
Duration: 18 days	4 hours	

Table 2. Average weights of predators with associated standard deviations in each treatment groups. The four treatments are: 1. no chronic herbicide exposure & no acute exposure, 2. no chronic exposure & acute exposure, 3. chronic exposure & no acute exposure, 4. chronic exposure & acute exposure.

Species	Treatment group	Average weight (g)	Standard deviation
Aeshna cyanea	1	0.60	0.12
	2	0.59	0.12
	3	0.63	0.15
	4	0.70	0.20
Lissotriton vulgaris	1	1.24	0.17
	2	1.08	0.23
	3	1.26	0.24
	4	1.10	0.27

Statistical analyses

We analyzed data on the two species separately. We analyzed body mass data using linear mixed modelling procedures (LMM). To analyse activity, we first calculated mean values for the eight observations, and thereafter used general linear models (GLM). Results of the predatory activity tests were also analysed using GLM in the case of newts, whereas predatory activity of dragonfly larvae was analysed using generalized linear models (GZLM) with quasibinomial error distribution and a logit link function. GLM and GZLM models contained body mass of animals as a covariate. We checked for the homogeneity of variances of response variables using Levene-tests, and investigated the normal distribution of model residuals using diagnostic plots. We included all two-way interactions into initial models and applied backward stepwise removal (Grafen & Hails 2002) to avoid potential problems due to the inclusion of non-significant terms (Engqvist 2005). We obtained statistics for removed variables by re-entering them one by one to the final model. All tests were two-tailed. Statistics were calculated using SPSS Statistics 17.0 for Windows and R (version 3.2.2) for Windows.

RESULTS

All predators survived until the end of the experiment except for one newt in the control treatment, which entangled in the plastic shelter and died.

Presence of the herbicide had no influence on body mass change in either predator species (LMM; *A. cyanea*: $F_{1,49.9} = 1.54$, $P = 0.22$; *L. vulgaris*: $F_{1,57.0} = 0.15$, $P = 0.67$; Fig. 1). Independently from exposure to the herbicide, dragonfly larvae nearly doubled their body mass during the 18 days of the experiment ($F_{1,50.3} = 83.88$; $P < 0.001$), while body mass of newts slightly decreased by *cca* 8.6% by the end of the experiment ($F_{1,57.9} = 5.67$; $P = 0.021$). The interaction between the effect of time and exposure was non-significant (for both species $P > 0.39$).
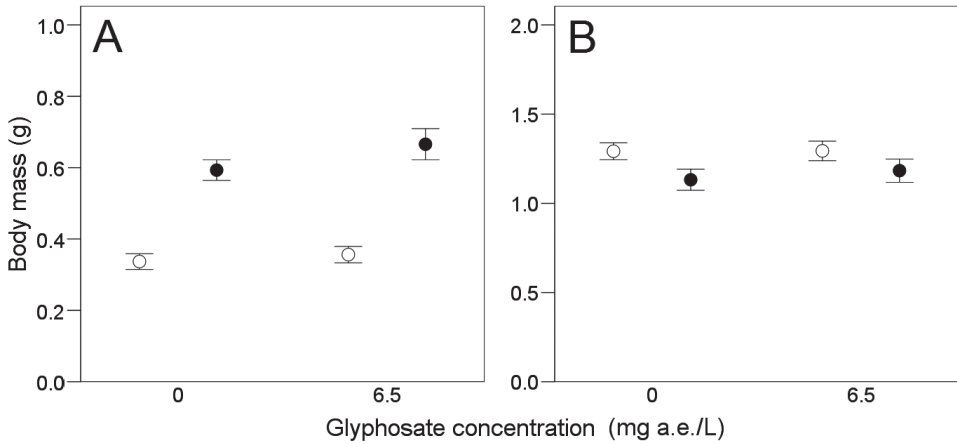
Fig. 1. Body mass (mean ± 1SE) of *A. cyanea* larvae (**A**) and *L. vulgaris* males (**B**) before (○) and after (●) chronic exposure to 0 or 6.5 mg a.e. glyphosate /L for 18 days.

The main effect of the herbicide on the activity of *A. cyanea* larvae was marginally non-significant (GLM; $F_{1,28} = 3.79$, $P = 0.06$; Fig. 2), and body mass alone did not have an effect on activity either ($F_{1,28} = 1.78$, $P = 0.19$), however, the interaction between herbicide exposure and body mass was significant ($F_{1,28} = 4.46$, $P = 0.04$): smaller individuals moved 5.7 times more in the control treatment than larger ones, but in the herbicide treatment this difference
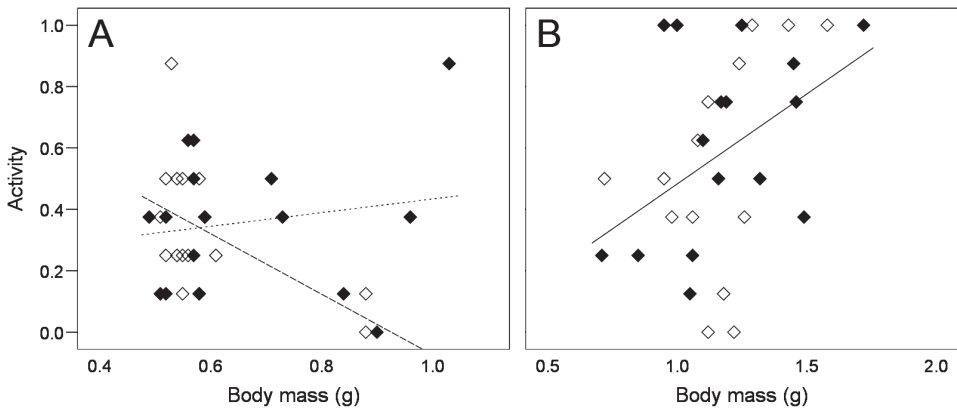

Fig. 2. Activity of *A. cyanea* larvae (**A**) and *L. vulgaris* males (**B**) on the 16th day of the experiment (◊ = control, ◆ = herbicide treatment). Activity corresponds to the proportion of the times when animals were observed active. Note that heavier dragonfly larvae were less active, than lighter ones in the control treatment (dashed line), but this relationship disappeared in the herbicide treatment (dotted line). Activity of heavier newts was always higher, than that of lighter ones, independently from exposure to the herbicide (solid line).

disappeared. Activity of newts was unaffected by exposure to the herbicide ($F_{1,27}$ = 0.45, P = 0.51; Fig. 2), but body mass had a significant effect: heavier individuals were 1.35-fold more active than lighter ones ($F_{1,28}$ = 5.94, P = 0.021). The interaction between exposure and body mass was non-significant in the case of newts ($F_{1,26}$ = 0.24, P = 0.63).

In the test on predatory activity, the number of consumed tadpoles was not significantly affected by either chronic or acute exposure to the herbicide (*A. cyanea*: GZLM; chronic exposure: t = –0.58, P = 0.57, acute exposure: t = 0.01, P = 0.99; *L. vulgaris*: GLM; chronic exposure: $F_{1,27}$ = 0.02, P = 0.88, acute exposure: $F_{1,27}$ = 1.60, P = 0.22; Fig. 3). The number of consumed tadpoles was affected by body mass in the case of newts (GLM; $F_{1,28}$ = 12.68, P = 0.001) independently from the treatments. Body mass had no effect on the predatory activity of dragonfly larvae (GZLM; t = 0.22, P = 0.83), and none of the two-way interactions were significant (all P > 0.40).
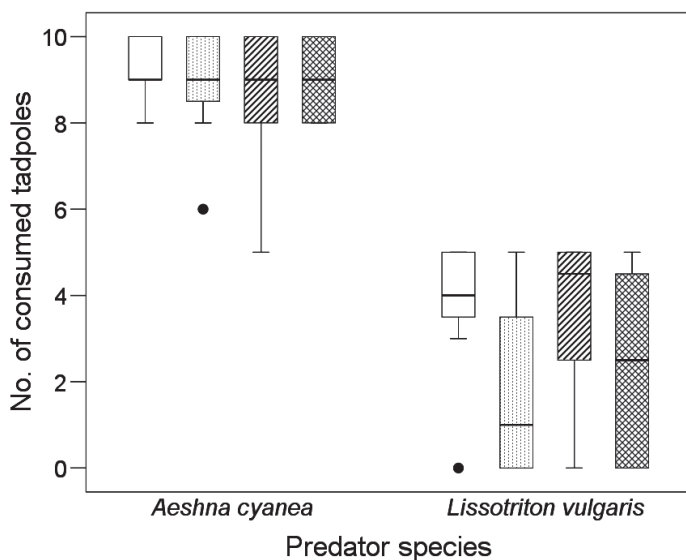

Fig. 3. Numbers of consumed tadpoles in the case of *A. cyanea* larvae and adult *L. vulgaris* males in the four treatments of the experiment on predation activity. Explanation of shading types representing the four treatments: 1 (white): no chronic herbicide exposure & no acute exposure, 2 (dotted): no chronic exposure & acute exposure, 3 (striated): chronic exposure & no acute exposure, 4 (reticulated): chronic exposure & acute exposure (Vertical lines depict medians, boxes represent interquartiles, bars represent ranges, dots indicate outliers). Predatory activity was not affected by exposure to the herbicide in either predator. The apparent treatment effect in newts was caused by size dependent predatory foraging activity.

DISCUSSION

Our laboratory experiment indicated that chronic exposure to relatively high concentrations (6.5 mg a.e. / L) of the herbicide formulation Glyphogan® Classic do not have severe impacts either on *A. cyanea* larvae or on adult *L. vulgaris* males. These results corroborate previous findings delivered by an outdoor mesocosm experiment about the insensitivity of these predator species to glyphosate-based herbicides (Ujszegi *et al.* 2015). This confirmation of earlier results was vital, because the outcome of ecotoxicological experiments can greatly depend on the experimental venue (Mikó *et al.* 2015).

The few existing reports on the effects of herbicides, insecticides and heavy metallic ions on survival of larval odonate predators came to a similar conclusion as our study, suggesting that dragonfly larvae are highly tolerant not only to glyphosate-based herbicides, but also to several other forms of anthropogenic contaminants (Rohr & Cumrine 2005, Relyea 2005*a*, Tollett *et al.* 2009, Ujszegi *et al.* 2015). During the 18 days of the present experiment, all dragonfly larvae survived and nearly doubled their body mass independently of exposure to the herbicide, suggesting low (if any) metabolic costs of detoxification (Fig. 1). Only activity was slightly affected by the interaction between herbicide exposure and body mass, where heavier individuals were less active compared to lighter ones, but only in the control treatment, and heavy individuals were more active in the herbicide treatment than in the control treatment. Janssens and Stoks (2012) found that activity and related predator-induced changes in behaviour of larval *Enallagma cyathigerum*, a zygopteran dragonfly, may be impaired by acute exposure to a glyphosate-based herbicide, but these changes were also relatively slight and did not translate into elevated mortality in the presence of free-ranging predators. It is possible that in our experiment the observed increased activity of large herbicide-exposed dragonfly larvae was a result of attempted escape from waters with high pesticide concentrations, but this hypothesis remains speculative and would need further investigation. The lack of strong effects of herbicide-exposure on *A. cyanea* behaviour is further strengthened by the observation that predatory activity was also not affected by either acute, or chronic exposure to the herbicide (Fig. 3).

Effects of the herbicide were similarly slight on adult smooth newts as on dragonfly larvae. Although another formulation of a glyphosate-based herbicide has been reported to be lethal already at relatively low concentrations (2–3 mg a.e. glyphosate/L) to tadpoles, larval newts and salamanders (Relyea & Jones 2009), we did not experience elevated mortality in adult newts even at a higher herbicide concentration. This is in line with previous findings on adult animals (Relyea *et al.* 2005, Wagner & Lötters 2013, Ujszegi *et al.* 2015). As

Edginton *et al.* (2004) suggested, surfactants in glyphosate based herbicides (e.g. polyethoxylated tallow amine; POEA) may impair the functioning of the gills of larval amphibians. At the same time it seems that these chemicals have no significant effect on lung-breathing adults, despite the importance of skin-breathing (Feder & Burggren 1985). Herbicide exposure also did not affect body mass change in adult newts, which is in line with our previous findings as well (Ujszegi *et al.* 2015). The observed slight reduction in body mass of newts by the end of the chronic exposure time (Fig. 1), which was independent from herbicide-exposure, was most likely caused by the stress due to unnatural laboratory conditions, and perhaps less food available, than in mesocosms, where zooplankton would provide further food supply. We did not experience changes in activity or predation efficiency due to herbicide-exposure in newts either. Former studies indicated that pesticides can alter activity and behaviour of anuran larvae, the most frequently used amphibian models in ecotoxicology (Bridges 1999, Moore *et al.* 2015). At the same time, Relyea and Edwards (2010) did not find any effect of insecticide exposure on predatory activity of adult *Notophthalmus viridescens* newts, similarly to our findings (Ujszegi *et al.* 2015, present experiment). Body mass, however, positively affected both activity and predatory activity. This may be due to larger individuals requiring more food, being more able to prey on large prey items, such as tadpoles, and being less vulnerable to other predators than their smaller conspecifics (Wilbur 1988, Urban 2007).

This insensitivity of predators to the herbicide in absolute terms and also relative to anuran larvae can have serious negative consequences for tadpoles in contaminated habitats: while the mortality rate of tadpoles is already elevated, their growth, development and overall physiological performance may also be constrained due to contamination (Lajmanovich *et al.* 2003, 2011), so that tadpoles may suffer extreme losses from predators that remain unaffected by the herbicide. This effect may be dampened once tadpole densities drop low and generalist predators, like newts and dragonfly larvae switch to other, more resistant prey taxa, such as scuds (Folmar *et al.* 1979) or zooplankton species (Relyea 2005a). Also, morphological and behavioural responses of tadpoles to the herbicide closely resemble those induced by predators (Relyea 2012), and are likely to provide some level of protection against predation. Nonetheless, tadpoles, and several other prey taxa, are likely to suffer additional losses from elevated predation pressure due to the relative insensitivity of predators, which may add up on direct effects of herbicides.

In conclusion, the studied glyphosate-based herbicide did not have an effect on survival, body mass change, and predatory activity of either larval *A. cyanea* dragonflies or adult male *L. vulgaris* individuals, even though the applied concentration was close to the expected worst-case values and exposure

lasted for 18 days. The only effect we observed was slightly elevated activity in large (but not small) dragonfly larvae, while activity remained unaffected in newts. These results confirmed previous findings of no severe effect of herbicide-exposure on the same species obtained in a mesocosm experiment (Ujszegi *et al.* 2015). In addition, here we demonstrated that the herbicide also does not have severe effects on the studied predators if it is present at a constantly high concentration and does not diminish due to degradation, precipitation or adsorption. These results are surprising, because glyphosate-based herbicides have been shown to have strong impacts at lower concentrations on other aquatic arthropods and amphibians even in larval (Relyea 2005*b*, Relyea & Jones 2009, Pérez *et al.* 2011) or adult stages (Brühl *et al.* 2011). Hence, our results stress the need for further studies uncovering differences between the sensitivity of different taxa and life stages. Furthermore, revealing long-term effects of exposure and assessing indirect effects via trophic cascades on these top predators will also be necessary for understanding consequences of herbicide-contamination in their entirety.

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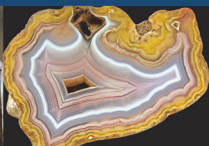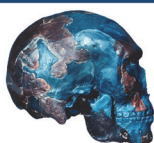
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