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Effects of a glyphosate-based herbicide and predation threat on the behaviour of agile frog tadpoles

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ABSTRACT

The widespread application of pesticides emphasises the importance of understanding the impacts of these chemicals on natural communities. The most commonly applied broad-spectrum herbicides in the world are glyphosate-based herbicides, which have been suggested to induce significant behavioural changes in non-target organisms even at low environmental concentrations. To scrutinize the behavioural effects of herbicide-exposure we exposed agile frog (*Rana dalmatina*) tadpoles in an outdoor mesocosm experiment to three concentrations of a glyphosate-based herbicide (0, 2 and 6.5 mg acid equivalent (a.e.) / L). To assess whether anti-predator behaviour is affected by the pesticide, we combined all levels of herbicide-exposure with three predator treatments (no predator, caged *Aeshna cyanea* dragonfly larvae or *Lissotriton vulgaris* newt adults) in a full factorial design. We observed hiding, activity, proximity to the predator cage and vertical position of tadpoles. We found that at the higher herbicide concentration tadpoles decreased their activity and more tadpoles were hiding, and at least at the lower concentration their vertical position was closer to the water surface than in tadpoles of the control treatment. Tadpoles also decreased their activity in the presence of dragonfly larvae, but did not hide more in response to either predator, nor did tadpoles avoid predators spatially. Further, exposure to the herbicide did not significantly influence behavioural responses to predation threat. Our study documents a definite influence of glyphosate-based herbicides on the behaviour of agile frog tadpoles and indicates that some of these changes are similar to those induced by dangerous predators. This may suggest that the underlying physiological mechanisms or the adaptive value of behavioural changes may similar.

1. Introduction

Of the millions of tons of pesticides used each year worldwide (Pimentel, 2009) considerable amounts reach non-agricultural habitats by runoff, overspray, or aerial drift, endangering organisms living in these areas (Giesy et al., 2000; Lehman and Williams, 2010). Pesticides can affect behaviour, physiology, development, and, ultimately, survival and reproductive success of non-target organisms via direct toxicity, by disrupting endocrine functions and by exerting teratogenic and immunotoxic effects (e.g., Hoffman, 2003). The estimation of medial lethal concentrations or doses in acute toxicity tests is the conventional method of evaluating probable consequences of pesticide-exposure in non-target organisms, but measuring behavioural effects of pesticides is a promising alternative (Døving, 1991; Peakall, 1996). Changes in behaviour often appear first upon exposure to pesticides (Sparling et al., 2010), measuring behavioural alterations is relatively easy and does not require sacrificing experimental animals, while behaviour is an important life-history trait which directly influences fitness (Lind and Cresswell, 2005; Weis et al., 2001). Moreover, because concentrations that occur in the environment are usually lower than the LC/LD50 values of most chemical contaminants, examining behavioural changes at environmentally-relevant sublethal concentrations appears to be more relevant than the estimation of effects at concentrations that are never experienced under natural conditions (Bridges, 1997).

Aquatic pollutants can affect several aspects of animal behaviour, such as the preference / avoidance of areas with relatively high concentrations of the pollutant (Tierney et al., 2007; Yu et al., 2014), they can lead to altered foraging behaviour (Pavlov et al., 1992; Semlitsch et al., 1995) and predator avoidance (Bridges, 1999; Scholz et al., 2000), and can also cause abnormal motion (Denoël et al., 2013; Levin et al., 2004) and mating behaviour (De Silva and Samayawardhena, 2005). In aquatic toxicology, the most often used

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vertebrate model animals in behavioural investigations are fishes (Melvin and Wilson, 2013). Studying the effects of pesticides on the behaviour of amphibian larvae is similarly reasonable, because they have a highly permeable skin, are easy to maintain and observe under experimental conditions, and their behaviour can be easily quantified (Bridges, 1999; Denoël et al., 2013; Wojtaszek et al., 2004). Furthermore, because many amphibians use small puddles, temporary ponds and ditches which often occur adjacent to agricultural fields as breeding sites, they are especially likely to become exposed to high concentrations of agricultural contaminants (Bridges, 1997).

One of the most commonly applied herbicides in the world are glyphosate-based herbicides (Mörtl et al., 2013; Relyea, 2005). The formulations of this broad-spectrum pesticide contain two main components: glyphosate, which inhibits the production of essential aromatic amino acids in plant protein synthesis; and a surfactant, which facilitates the penetration of the cuticle layer (Giesy et al., 2000; Mann et al., 2009). While glyphosate can be harmful to non-target organisms as well, it seems that surfactants are more toxic, even though these surfactants are usually classified as inert ingredients (Moore et al., 2012). Previous studies showed that glyphosate-based herbicides can affect life history traits (Cauble and Wagner, 2005; Howe et al., 2004; Mikó et al., 2015; Relyea and Jones, 2009; Williams and Semlitsch, 2010) and body shape of tadpoles (Howe et al., 2004; Lajmanovich et al., 2003; Mikó et al., 2015; Relyea, 2012) and also cause symptoms of oxidative stress (Costa et al., 2008). Reports on the potential impacts of glyphosate-based herbicides on tadpole behaviour have, however, remained scarce (Katzenberger et al., 2014; Mikó et al., 2015; Moore et al., 2015; Wojtaszek et al., 2004).

Our aim was to scrutinize the impacts of a glyphosate-based herbicide in combination with predation threat on the behaviour of amphibian larvae, although the present experiment does not allow distinguishing between direct physiological and indirect behavioural effects. We combined herbicide treatments with the presence or absence of predator chemical cues to test if an additional stress factor enhanced the effects of the herbicide (Relyea, 2005, 2003; Sih et al., 2004), if exposure to the herbicide induced similar behavioural changes as can be observed in the presence of predators (see Bridges (1999), Semlitsch et al. (1995)), and if the herbicide inhibits the response to the presence of predators (see Mandrillon and Saglio (2007a)). To achieve this, we exposed agile frog tadpoles to three initial concentrations of a glyphosate-based herbicide (0, 2 and 6.5 mg a.e./L glyphosate), and to the presence or absence of caged predators (no predator, caged *Aeshna cyanea* dragonfly larvae or adult males of the newt *Lissotriton vulgaris*). Previous studies were mainly performed under highly simplified laboratory conditions, and little is known about the effects of the herbicide under more natural conditions. Also, they used Hence, we performed the experiment in outdoor mesocosms which are likely to more closely model the complex environment of natural habitats (Relyea and Hoverman, 2006, 2008; Winkler and Van Buskirk, 2012) and to provide the opportunity to examine several aspects of behavioural changes. In this study, we observed four behavioural traits: activity, hiding, spatial predator avoidance and vertical position of tadpoles. In a previous paper (Mikó et al., 2015) we presented an analysis on a part of the activity and vertical position data, but in that publication we were concerned with how pesticide-effects on several life history traits were influenced by the experimental venue, so that a detailed analysis of the behavioural data as presented here was technically impossible (because in the laboratory we had only two behavioural traits, and one predator type) and would have been out of focus there.

We predicted that in the presence of predators tadpoles would decrease their activity, hide more and avoid predator cages spatially, as generally reported by studies observing induced behavioural defences in anuran larvae (Laurila et al., 1997; Schoeppner and Relyea, 2008). In the presence of the herbicide we also expected to observe increased hiding and decreased activity (Bridges, 1999; Moore et al., 2015; Semlitsch et al., 1995), and anticipated that tadpoles would stay closer to the bottom of the mesocosms to avoid upper areas with high herbicide concentrations arising in parallel to temperature stratification (Jones et al., 2010). Moreover, we expected that the presence of predators would increase the behavioural effects of the herbicide at intermediate concentrations (Relyea, 2005), while at the high herbicide concentration we predicted that the response to predator cues would be inhibited (Moore et al., 2015).

2. Methods

We collected 350 eggs from each of ten freshly laid egg-clutches of the agile frog (*Rana dalmatina* Bonaparte, 1840) from a forest pond ca. 20 km to the north of Budapest, Hungary (47°44'20"N, 19°00'43"E) and transported them to the Julianna-major Experimental Station (Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences) in Budapest (47°32'52"N, 18°56'07"E). The sampled pond is located in the core area of a national park and has no history of contamination with herbicides. Until hatching, we kept clutches in the laboratory in 10-L containers holding 3 l of reconstituted soft water (RSW; APHA, 1985) at 20 °C and a 12: 12 h light: dark cycle. We started the experiment two days after tadpoles reached the free swimming stage.

We captured 24 dragonfly larvae (*Aeshna cyanea* Müller, 1764) and 24 adult male smooth newts (*Lissotriton vulgaris* Linnaeus, 1758) from two ponds close to the site from where we collected egg clutches (47°38'41"N, 18°36'42"E and 47°44'22"N, 19°00'42"E) and transported them to the Julianna-major Experimental Station. We kept dragonfly larvae individually in 300 ml cups holding 200 ml RSW and a wooden stick as a perching site, and newts in groups of 4 in 5-L boxes containing 1.5 l of RSW. Predators were fed with bloodworms (*Chironomus* sp.) every other day *ad libitum* until the start of the experiment.

Two weeks before the start of the experiment, we placed out 90-L opaque plastic tubs (42 cm wide, 72 cm long, 30 cm high) on an open outdoor area and filled them with 65 l of tap water. Two days later we added 40 g dried beech (*Fagus sylvatica*) leaves and 1 l pond water to each tub to provide nutrients and refuge for tadpoles, and to start up a self-sustaining ecosystem in the mesocosms (Hettyey et al., 2015; Mikó et al., 2015; Van Buskirk, 2012). We covered mesocosms with mosquito net lids to prevent colonization by additional predators. Each tub was equipped with a predator cage made of an opaque plastic tube (11 cm diameter, 21.5 cm long) and covered with mosquito nets on both ends, to allow focal tadpoles to sense the presence of predators both visually and chemically, while preventing predators from injuring focal tadpoles (for a similar set-up see Katzenberger et al. (2014), Nunes et al. (2014), Winkler and Van Buskirk (2012)). The cage was fixed to a short end of the tub. One day before the start of the experiment, we placed a larval dragonfly or an adult smooth newt into cages in accordance with the randomly distributed predator treatments, whereas in mesocosms assigned to no-predator treatments the cages remained empty. We fed predators with two naive agile frog tadpoles (~150 mg) three times a week. To equalize disturbance during feeding, we also lifted empty cages in mesocosms assigned to no-predator treatments. To set herbicide concentrations to 0, 2 and 6.5 mg a.e. glyphosate / litre, we added 0, 0.361 and 1.174 ml of the herbicide (Glyphogan® Classic, containing 41.5 w/w% glyphosate and 15.5 w/w% POEA) to mesocosms one day before the start of the experiment. We chose these concentrations on the basis of ecotoxicological assessments which reported between 0.1 µg/litre and 5.2 mg a.e. /L in natural surface waters (Battaglin et al., 2005; Edwards et al., 1980; Thompson et al., 2004). The expected worst case concentration of glyphosate is estimated to fall between 1.4 and 7.6 mg a.e./L (Mann and Bidwell, 1999; Relyea, 2012; Wagner et al., 2013). Also, ecotoxicological studies assessing the effects of glyphosate-based herbicides used similar concentrations (e.g., Jones et al., 2011: 0, 1, 2 and 3 mg a.e./L; Relyea, 2012: 0, 1, 2 and 3 mg a.e./L; Relyea and Jones, 2009: 0, 1, 2, 3, 4 and 5 mg a.e./L). We replicated the nine treatments (three herbicide concentrations × three predator treatments) eight times, resulting in a total of 72 experimental units, which we arranged in a randomized block design. We started the experiment by mixing tadpoles from different clutches and releasing 16 haphazardly selected healthy-looking individuals into each mesocosm.

We observed tadpole behaviour 21 days after start of the experiment, when tadpoles were in developmental stages 36–39 (according to Gosner, 1960), as determined based on photographs taken on eight tadpoles per mesocosm on the following day. We visited each experimental unit hourly for 8 h (between 10:25 a.m. and 6:25 p.m., along the same route) and noted the number of visible tadpoles (number of tadpoles above the leaves), number of active tadpoles (number of visible tadpoles that were swimming or feeding), number of tadpoles close to the predator cages (tadpoles in the third of the mesocosm that was closest to the predator cage) and their vertical position within the water column (number of tadpoles in the upper third of the mesocosm). The observer was blind in respect to the treatment combination the boxes belonged to. During behavioural observations, diurnal water temperature variation was between 15.6 °C and 23 °C. We raised tadpoles until metamorphosis, after which we released froglets at the site where egg-clutches had been collected from.

2.1. Statistical analyses

We excluded one outdoor mesocosm from the analyses, because the predator (a newt) died before we performed the behavioural observations. Tadpole mortality was low: only 29 out of 1152 tadpoles died during the experiment. Nonetheless, for the analysis of hiding (calculated as the number of surviving tadpoles minus the number of visible tadpoles) and vertical position, we calculated means for each experimental unit from the eight observations and divided the obtained values with the number of surviving tadpoles. We assessed tadpole mortality by catching, removing and counting all survivors in each tub on the day following observations. In the case of activity and spatial predator avoidance, we calculated means for each experimental unit from the eight observations and divided the obtained values with the number of visible tadpoles found in each unit. To enhance normality of model residuals and homogeneity of variances, we arcsine-square root transformed all four response variables (Sokal and Rohlf, 1995). We analysed the effects of herbicide concentration and predator treatment using linear mixed-effect models (LMM), where activity, hiding, spatial predator avoidance and vertical position were the dependent variables, herbicide concentration and predator treatment were entered as fixed factors and block was a random factor. We entered the two-way interactions into initial models and applied backward stepwise removal (terms removed when $P > 0.05$; Grafen and Hails, 2002), to avoid 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. We performed Tukey's HSD (honest significant difference) tests as post hoc analyses. Statistical tests were performed with the 'lme' function of the 'nlme' package and with the 'glht' function of the 'multcomp' package in 'R' (version 3.0.2; R Core Team, 2016).

3. Results

The ratio of active tadpoles was affected by both the herbicide and the predator treatments, while their interaction was not significant (Table 1). Tadpoles exhibited a significantly decreased activity at the higher herbicide concentration compared to the experimental groups not exposed to the herbicide (Tukey's HSD test: $P < 0.001$), but compared with the control group and the experimental group at the lower herbicide concentration, we did not find a significant effect ($P = 0.41$; Fig. 1). Also, fewer tadpoles were active in the presence of dragonfly larvae than in the absence of predators ($P < 0.001$), while the presence of newts did not lead to decreased activity in tadpoles relative to controls ($P = 0.82$; Fig. 1).

Table 1
Results of the analyses of tadpole behaviour. Significant *P*-values are highlighted in bold, final models only contained the significant effects. Statistics for removed non-significant variables were obtained by re-entering them one by one to the final model.

Dependent variable	Effect	*df*	*F*	*P*
Activity	Herbicide	2, 60	8.53	**< 0.001**
	Predator	2, 60	14.49	**< 0.001**
	Herbicide × Predator	4, 56	1.21	0.320
Hiding	Herbicide	2, 60	5.37	**0.007**
	Predator	2, 60	3.96	**0.024**
	Herbicide × Predator	4, 56	1.86	0.130
Spatial predator avoidance	Herbicide	2, 62	1.37	0.260
	Predator	2, 62	1.56	0.220
	Herbicide × Predator	4, 56	1.18	0.330
Vertical position	Herbicide	2, 62	3.62	**0.033**
	Predator	2, 60	0.51	0.600
	Herbicide × Predator	4, 56	0.66	0.620

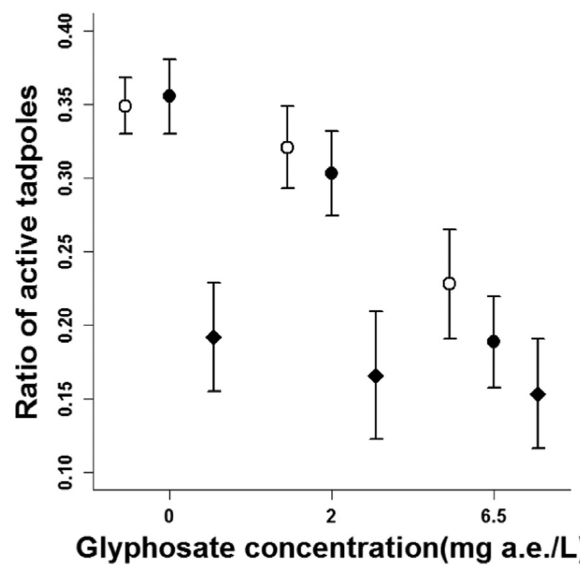

Fig. 1. The influence of the three herbicide concentrations and predator presence (○: no predator, •: *Lissotriton vulgaris*, ◆: *Aeshna cyanea*) on tadpole activity. Arcsine-square root transformed data are shown. Means and SE are depicted.

Hiding behaviour was also influenced by exposure to the herbicide and by the predator treatments but not by their interaction (Table 1). At the higher herbicide-concentration more tadpoles were hiding than in the absence of the herbicide (Tukey's HSD test: $P = 0.005$) while the lower herbicide-concentrations did not have such an effect ($P = 0.75$; Fig. 2). In the presence of newts tadpoles tended to hide less than in the presence of dragonfly larvae ($P = 0.014$), but the ratio of hiding tadpoles was intermediate in the absence of predators, not differing significantly from either treatment containing predators (no predator vs. newts: $P = 0.25$; no predator vs. dragonfly larvae: $P = 0.48$; Fig. 2).

We did not observe spatial avoidance of predators irrespective of which predator was present in them and at either herbicide concentration, also the interaction between the effects of herbicide and predator treatments were neither significant (Table 1, Fig. 3). However, exposure to the herbicide significantly affected vertical position (Table 1): compared to the tadpoles not exposed to the herbicide, tadpoles moved upwards in the water column at the lower (Tukey's HSD test: $P = 0.02$), but not significantly so at the higher herbicide concentration (Tukey's post hoc: compared to control: $P = 0.26$; Fig. 4). Predator presence did not affect vertical position of tadpoles either alone or in interaction with exposure to the herbicide (Table 1, Fig. 4).

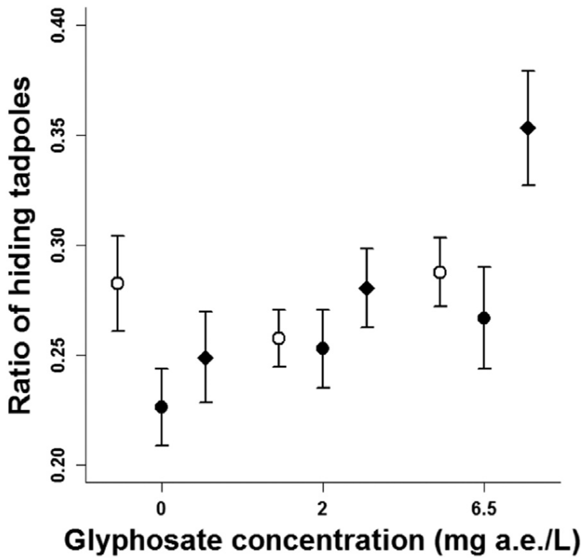

Fig. 2. The influence of the three herbicide concentrations and predator presence (○: no predator, •: *Lissotriton vulgaris*, ◆: *Aeshna cyanea*) on hiding. Arcsine-square root transformed data are shown. Means and SE are depicted.

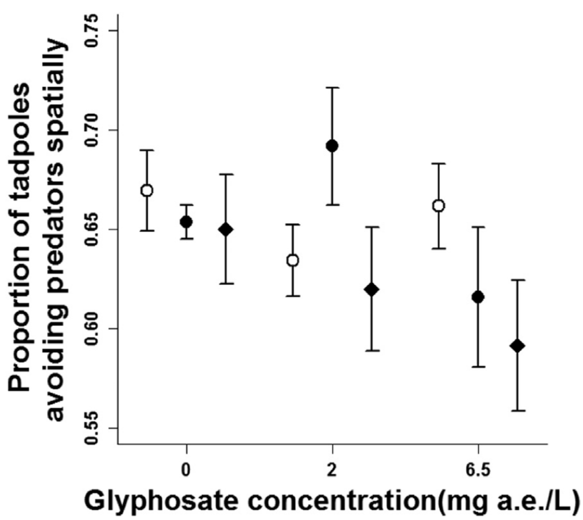

Fig. 3. The influence of the three herbicide concentrations and predator presence (○: no predator, •: *Lissotriton vulgaris*, ◆: *Aeshna cyanea*) on spatial predator avoidance of tadpoles. Arcsine-square root transformed data are shown. Means and SE are depicted.

4. Discussion

Our results demonstrate that glyphosate-based herbicides can affect several aspects of the behaviour of agile frog tadpoles. At the higher herbicide concentration, tadpoles reduced their activity, they hid more, and at the lower concentration they moved upward in the water column. Some of these behavioural changes resembled those that anuran larvae generally display under the threat of predation. In our experiment, we also observed decreased activity in the presence of dragonfly larvae, while increased hiding and spatial avoidance remained elusive. Tadpoles did not respond to newts with significant behavioural alterations. Finally, exposure to the herbicide did not affect tadpoles' behavioural responses to predators.

Studies that investigated effects of pesticides on amphibian behaviour mostly examined insecticides (Bridges, 1999, 1997; Brunelli et al., 2009; Denoël et al., 2013; Relyea and Edwards, 2010), whereas reports on behavioural changes to glyphosate-based herbicides have remained scarce. Wojtaszek et al. (2004) observed that some *Rana clamitans* and *R. pipiens* tadpoles showed no or delayed predator avoidance responses at glyphosate concentrations of 7.15 and 14.3 mg a.e./L a few hours after herbicide exposure, but this effect was not statistically significant. In a previous paper (Mikó et al., 2015), we documented that the herbicide had a venue-dependent effect on *R. dalmatina* tadpole behaviour; it made the tadpoles more active in the laboratory and they stayed at the bottom of the boxes, whereas it had the opposite effect in mesocosms. Moore et al. (2015) reported that wood frog (*Lithobates sylvatica*) tadpoles decreased their activity under chronic exposure to a glyphosate-based herbicide (at a concentration of 0.5 mg a.e. glyphosate/L), while they did not observe altered activity levels after an exposure of only 12 h. In our study we observed lowered activity and increased hiding when exposed to the herbicide, which is corresponds to previous findings.

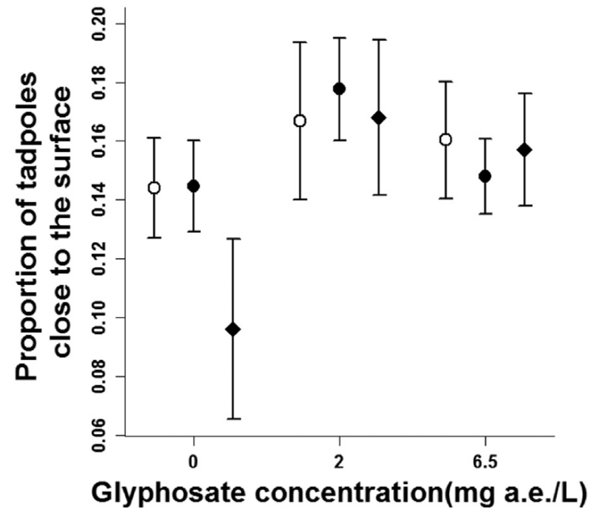

Fig. 4. The influence of the three herbicide concentrations and predator presence (○: no predator, •: *Lissotriton vulgaris*, ◆: *Aeshna cyanea*) on the proportion of tadpoles close to the surface. Arcsine-square root transformed data are shown. Means and SE are depicted.

Reduced activity and increased hiding seems to be part of a general stress-response in tadpoles, because it occurs in the presence of predators (Laurila et al., 1997; Schoeppner and Relyea, 2008), and upon exposure to herbicides, insecticides and fungicides as well (e.g., Mandrillon and Saglio, 2007b; Relyea and Mills, 2001; Teplitsky et al., 2005). This suggests that the physiological mechanisms behind the pesticide-caused and the predator-induced responses, or the adaptive value of these changes are similar. Predator mediated stress responses in amphibian larvae are associated with changes in corticosterone levels (Fraker et al., 2009; Middlemis Maher et al., 2013). It is possible that the reaction to pesticides involves the same hormonal mediator, but the physiological mechanism behind behavioural alterations caused by the herbicide has as yet remained unknown. Lowered activity caused by pesticide-exposure, may at the same time elevate fitness of responsive individuals by reducing active intake via feeding and respiration, and by allowing more time for detoxification, but also for the dissipation and degradation of the pesticide in the environment. These hypotheses are, however, speculative and will require further investigations.

Previous studies showed that the effects of glyphosate-based herbicides can be modulated by several biotic and abiotic factors (Chen et al., 2004; Edginton et al., 2004; Jones et al., 2011; Mikó et al., 2015). Studies on the effects of predation threat and herbicide presence delivered contradictory results. For example, under laboratory conditions Relyea (2005) found that simultaneous exposure to predator cues strengthened the toxicity of glyphosate-based herbicides, while in outdoor mesocosms, it made the herbicide less lethal to tadpoles (Relyea, 2012). Concerning studies on behaviour, Katzenberger et al. (2014) observed that predator cues enhanced the effects of a glyphosate-based herbicide on tadpole burst speed. Moore et al. (2015) found that tadpoles decreased their activity when exposed to chemical predator cues, but when tadpoles were also exposed to a glyphosate-based herbicide, their activity returned to normal levels. In contrast, we did not find significant interactive effects of cues on predation threat and a glyphosate-based herbicide. However, it is possible that behavioural changes to the herbicide can altered by developmental stage; in another experiment we observed slight changes with age in behaviour of tadpoles exposed to the herbicide, but only when temperature was low. Thus, further investigations are needed to elucidate how predator presence influences the effects of the herbicide.

The presence of herbicides may impede predator recognition by inactivating chemical cues on predation threat, by negatively impacting the olfactory system itself, or by lowering the learning ability of tadpoles. Indeed, Mandrillon and Saglio (2007a, 2007b) found that the presence of sublethal concentrations of amitrole (a non-selective triazole herbicide) can impair the chemical recognition of predators. Also, Moore et al. (2015) observed that tadpoles did not react to cues from injured conspecifics when a glyphosate-based herbicide was present. We did not observe a similar effect of the herbicide. This is likely due to the fact that while previous studies exposed focal tadpoles only to bursts of chemical cues on predation threat by adding stimulus water to rearing tanks, in our case, predators were in the same water body as the tadpoles throughout the experiment. Chemical cues on predation threat may have been present for long enough and at a high enough concentration to allow for predator recognition in our experiment, even in the presence of the herbicide. Although visual cues on predators may also have contributed to predator recognition in tadpoles, their importance is generally limited (e.g., Hettyey et al., 2012; Kiesecker et al., 1996; Takahara et al., 2012) and, specifically, we used opaque predator cages equipped with a double-net bottom, highly limiting predator visibility. Hence, visual cues are unlikely to be responsible for the independence of tadpole antipredator responses from herbicide presence.

Our result that tadpoles altered their behaviour in response to dragonfly larvae but not to newts may partly be explained with the different foraging strategies of these predators (Hettyey et al., 2010): dragonfly larvae are sit and wait predators, against which lowered activity and increased hiding may be effective, whereas newts are active foragers which search for prey, rendering these behavioural defences useless. Also, the relatively large tadpoles we observed are no easy prey for the gape-limited newts (Urban, 2007; Wilbur et al., 1983). Consequently, large tadpoles may not consider newts a source of danger and may not respond to them anymore, while dragonfly larvae remain dangerous to tadpoles regardless of their size (Caldwell et al., 1980).

When the herbicide was present at the lower concentration, tadpoles moved closer to the water surface. It is known that the herbicide may stratify in the water column with concentrations increasing upwards (Jones et al., 2011, 2010), while the ground, leaves and other litter on the bottom of mesocosms and natural ponds may absorb the herbicide (Tsui and Chu, 2004), likely leading to locally high concentrations immediately on the bottom. Further, Rodriguez-Gil et al. (2016) found, that polyoxyethylene tallow amines (POEA), which are commonly used surfactants in glyphosate-based herbicides and can be more toxic than the main ingredient (Moore et al., 2012), can bind to the sediment and surfaces of microcosms. We did not experience clear stratification in our experiment (see Mikó et al. (2015)). Also, although adult frogs may avoid ovipositing in glyphosate-contaminated water (Takahashi, 2007), and, hence, appear to be able to sense the herbicide, we know of no report on a similar ability of tadpoles. Finally, if the herbicide was accumulated in the leaf litter, and if tadpoles were evading the herbicide in the low concentration treatment by moving upwards, why did the high concentration treatment not induce a similar vertical distribution? Clearly, further studies will be necessary to clarify the spatial distribution of herbicides in natural waters and the importance of vertical position in waters contaminated with herbicides.

In summary, our study showed that exposure to glyphosate-based herbicides may effect changes in tadpole behaviour, some of which resemble responses tadpoles express in the presence of predators. This result accords to the hypothesis that tadpoles may respond to stress with basic and universal behavioural alterations, possibly resulting from the same physiological mechanism, irrespective of the inducing environmental factor. However, it is equally likely that the observed behavioural changes in herbicide-exposed tadpoles were direct consequences of toxicity. Further, we did not find evidence for interactive effects of predation threat and herbicide exposure: predation threat, as an additional stress factor, did not enhance behavioural effects of the herbicide, and predator recognition remained intact even at the high herbicide concentration. Our study provides insights into the behavioural effects of glyphosate-based herbicides and supports the idea that the assessment of behavioural alterations effected by relatively low concentrations of contaminants may be a fruitful avenue within the field of ecotoxicology.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.02.032.

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