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Age-dependent changes in sensitivity to a pesticide in tadpoles of the common toad (*Bufo bufo*)

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ABSTRACT

The worldwide en masse application of pesticides and the frequently reported malign effects on several non-target organisms underpin the importance of ecotoxicological research on these anthropogenic pollutants. Previous studies showed that sensitivity to herbicides can vary widely depending on additional stress factors, on the species and even on the population investigated. However, there is little information about how sensitivity changes during ontogeny, and how the duration of exposure is linked to the magnitude of malign effects, even though this knowledge would be important for the interpretation of toxicity test results and for formulating recommendations regarding the timing of pesticide application. We exposed tadpoles of the common toad (*Bufo bufo*) to three concentrations (0, 2 and 4 mg a.e./L) of a glyphosate-based herbicide during the 1st, 2nd, 3rd, 4th, or 5th period of larval development or during the entire experiment, and measured survival, time until metamorphosis and body mass at metamorphosis to estimate fitness-consequences. Younger tadpoles were more sensitive to the herbicide in all measured traits than older ones, and this age-dependence was especially pronounced at the high herbicide concentration. Furthermore, tadpoles exposed to the herbicide during the entire experiment developed slower than tadpoles exposed only early on, but we did not observe a similar effect either on body mass or survival. The observed age-dependence of sensitivity to herbicides draws attention to the fact that results of toxicity tests obtained for one age-class are not necessarily generalizable across ontogeny. Also, the age of test animals has to be considered when planning ecotoxicological studies and interpreting their results. Finally, taking into account the temporal breeding habits of local amphibians when planning pesticide application would be highly favourable: if tadpoles would not get exposed to the herbicide during their most sensitive early development, they would sustain less anthropogenic damage from our efforts of controlling weeds.

1. Introduction

Pesticide application is a highly effective way of improving productivity in agriculture, but most pesticides can be harmful to non-target organisms, especially as a result of their overuse and unwary application (Pereira et al., 2009). Pesticides can damage endocrine functions and the immune system and exert cytotoxic and teratogenic effects in non-target organisms, leading to diminished reproductive success and survival, and, ultimately, impoverished biodiversity (Bianchi et al., 2006; Colborn et al., 1996; Gross et al., 2003; Sotherton and Holland, 2003; Suter, 2007). However, an important lesson learned from ecotoxicology studies is that the sensitivity to these chemicals is not a fixed, species specific value, but can vary widely depending on several extrinsic and intrinsic factors (Aguilar et al., 1999; Rattner and Heath, 2003).

Susceptibility to pesticides may depend on many properties of the physical and chemical environment: temperature (Talent, 2005), pH (Chen et al., 2004) or ultraviolet radiation (Beketov et al., 2011) can all modulate toxicity of pesticides. The presence of other biotic stress factors, such as predators (Qin et al., 2011), competitors (Foit et al., 2012) and parasites (Fauser-Misslin et al., 2014) can also alter the lethality of anthropogenic pollutants. Further, previous exposure of populations to pesticides can affect sensitivity as well (Hua et al., 2013; Poupardin et al., 2008). Finally, there is accumulating evidence that the internal state of organisms, such as body size, body composition, nutritive condition, the degree of infestation, sex or age (Aguilar et al., 1999; Christin et al., 2003; Medina et al., 2002; Pieters et al., 2006) can also influence effects of pesticides.

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Organism are generally able to process or inactivate harmful chemicals to some degree, while among-individual variation in sensitivity may prevail due to differences in the expression and activity of detoxification enzymes (Van Straalen, 1993). For example, cytochrome P450 enzymes and glutathione transferases are important detoxification enzymes, which can be found in a wide variety of organisms, and can metabolize many kinds of harmful substrates (Nelson, 1998; Pearson, 2005). However, the level of expression of these enzymes changes during development (Amicarelli et al., 2001; Stuart et al., 2001), which may contribute to altered sensitivity to pesticides. Whether such age-dependent changes in the cellular machinery of detoxification indeed translate to variation in susceptibility to toxic substances has been studied in detail in some model organisms, such as rats (Anand et al., 2006; Sheets, 2000; Timchalk et al., 2006), but much less is known about similar age-dependent patterns in other vertebrate groups, including amphibians.

Amphibians are considered the most threatened vertebrate group today (Stuart et al., 2004; Wake and Vredenburg, 2008), with more than 40% of the species being at risk of extinction (IUCN, 2016). The extensive use of pesticides has been proposed to be one of the major drivers of these declines (Davidson et al., 2002; Relyea, 2005). It is their thin, highly permeable skin, unshelled eggs, and complex life-cycle that make amphibians especially vulnerable to pollutants both in the aquatic and the terrestrial environment. Also, amphibians use practically all types of water bodies for reproduction, which exposes eggs and larvae to the full spectrum of pesticides that reach natural surface waters.

Studies investigating ontogenetic changes in the sensitivity of amphibians in relation to pesticides have remained scarce. This is surprising, because if such variation is significant and widespread, sensitivities measured in one age-class could not be generalized to others. Hence, taking age-dependence of sensitivity into consideration when planning ecotoxicological studies and when interpreting results would be of fundamental importance. The consensus is that early life stages are more susceptible to contaminants, which is also one reason why embryos and larvae are most often used in toxicological investigations (Linder et al., 2010). Several experimental studies indeed found that tadpoles are more sensitive to pesticides than eggs and adults (Greulich and Pflugmacher, 2003; Harris et al., 2000). However, the pattern of age-dependent changes in susceptibility during larval development, and the effects of the duration of exposure to herbicides remained largely unknown (Harris et al., 2000; Jones et al., 2010). Because of the low number of studies on the age-dependency of sensitivity, but also because the existing tests delivered contradictory results (Fort et al., 2004; Howe et al., 1998), it has remained impossible to draw general conclusions regarding this important phenomenon, and further experimental investigations are urgently needed.

In this study our aim was to investigate age-dependent changes in the sensitivity of common toad (*Bufo bufo*) tadpoles to a glyphosate-based herbicide. We also tested if exposure beyond the sensitive period further enhances the effects of the herbicide. To achieve these goals, we exposed tadpoles to three concentrations of an herbicide containing glyphosate as the main ingredient and polyethoxylated tallowamines (POEA) as the surfactant, during the 1., 2., 3., 4., or 5. part of larval development, or continuously during the entire experiment. To estimate the effect of the herbicide, we measured survival, time until metamorphosis and body mass at metamorphosis. Based on previous findings, we predicted that younger tadpoles will be more sensitive to the herbicide (Jones et al., 2010), sensitivity will decrease during ontogeny (Howe et al., 2004; Jones et al., 2010) and tadpoles exposed to the herbicide during their entire larval period will be the most severely affected group (Bridges, 2000).

2. Material and methods

2.1. The applied pesticide

Glyphosate (*N*-(phosphonomethyl)glycine) is one of the most often applied pesticide agents in the world (Grube et al., 2011). It inhibits the key enzyme of the shikimic acid pathway in plants (Giesy et al., 2000). Because uptake of glyphosate takes place through the cuticle of leaves, it is usually administered along with surfactants, most commonly POEA, to enhance absorption efficiency. Previous studies found that glyphosate-based herbicides are toxic to amphibians (Lajmanovich et al., 2003; Mann and Bidwell, 1999; Mikó et al., 2015; Relyea, 2005) and their toxicity is likely mainly due to the POEA and not to the glyphosate itself (Perkins et al., 2000; Tsui and Chu, 2003). At sublethal concentrations, glyphosate-based herbicides (with POEA as ingredients) have been shown to influence behaviour, morphology, growth and development of tadpoles (Cauble and Wagner, 2005; Howe et al., 2004; Mikó et al., 2017; Relyea, 2012; Wojtaszek et al., 2004), and to cause developmental malformations, intersexuality and symptoms of oxidative stress (Costa et al., 2008; Güngördü, 2013; Howe et al., 2004; Jayawardena et al., 2010; Lajmanovich et al., 2003).

2.2. Experimental setup

We collected 70 eggs from each of twelve freshly laid egg-clutches of the common toad (*Bufo bufo*) from a pond in Nagykovácsi, Hungary (47°34′35″N, 18°52′06″E), and transported them to the laboratory at the Experimental Station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences) in Juliannamajor, Budapest (47°32′52″N, 18°56′05″E). Until hatching, we kept clutches separately in 3 L containers, each holding 1 L reconstituted soft water (RSW; APHA, 1985), set the temperature to 20 °C and provided a 12: 12 h light: dark cycle. Two days after hatchlings reached the free swimming state (developmental stage 25; Gosner, 1960) we haphazardly selected 52 healthy-looking larvae from each clutch and started the experiment.

We reared tadpoles individually in 1.2 L containers filled with 0.7 L RSW at18 °C and a 12: 12 h light: dark cycle. We changed water every third day, while maintaining the initial pesticide concentrations. After each water change tadpoles were fed *ad libitum* with chopped and slightly boiled spinach. We applied a popular formulation of glyphosate-based herbicides (Glyphogan® Classic; Monsanto Europe S.A., Brussels, Belgium; containing 41.5 w/w% glyphosate and 15.5 w/w% POEA). At the start of the experiment and when changing water, we added 0, 1.11 or 2.22 mL of the herbicide to 200 L RSW, to attain concentrations of 0, 2 and 4 mg a.e./L of glyphosate (and 0, 0.00086 and 0.00172 mL POEA/L in parallel) and dispensed this water to rearing dishpans arranged on laboratory shelves in a randomized block design. According to ecotoxicological assessments, the observed worst case concentration of glyphosate-based herbicides falls within the range between 1.7–5.2 mg a.e./L glyphosate in shallow surface water bodies, depending on habitat characteristics, and on the distance to agricultural lands (Giesy et al., 2000; Relyea, 2012; Wagner et al., 2013). Based on the information available (Battaglin et al., 2005; Edwards et al., 1980; Thompson et al., 2004), the applied herbicide concentrations represent pristine, intermediately and heavily contaminated habitats. We exposed tadpoles to the two concentrations of the herbicide either during the entire duration of the experiment or only temporally, during the 1st, 2nd, 3rd, 4th, or 5th period of their larval development, each period lasting 9 days. Outside the period of herbicide exposure, we reared these tadpoles in RSW. In one more treatment we kept tadpoles in RSW throughout the experiment (control). We replicated each treatment by family combination 4 times, resulting in a total of 624 experimental units.

When tadpoles approached metamorphosis, we checked rearing containers twice daily. When a tadpole reached developmental stage 42

(Gosner, 1960), we noted the time until metamorphosis, measured body mass using a laboratory scale to the nearest mg and either released animals at the site of their collection or, in the case of 65 individuals, preserved them in methanol for later toxin analysis (data not shown). The Ethical Commission of the MTA ATK NÖVI and the Közép-Duna-Völgyi KTVF approved the investigation, all work was performed in accordance with the Ethical Codex of Animal Experimentation issued by the Hungarian Academy of Sciences (25/2010).

2.3. Statistical analyses

For the analysis of survival we used generalized linear mixed-effect models (GZLMM) with a binomial error distribution and a logit-link function. To handle computational problems, we used the 'bobyqa' optimizer function. We entered survival until metamorphosis as the dependent variable, herbicide concentration and the timing of exposure as fixed factors. Family nested within block was entered as random factors (Zuur et al., 2009). Inferences about treatment effects were based on likelihood ratio tests. To analyze variation in body mass of metamorphs and time to metamorphosis we used linear mixed effect models (LMM). We entered body mass at developmental stage 42 (the onset of metamorphosis; Gosner, 1960) or number of days to reach developmental stage 42 as the dependent variable, herbicide concentration and the timing of exposure as fixed factors, and family nested within block as a random factor. Because the design was not fully factorial (the control treatment exposing tadpoles to 0 mg a.e./L glyphosate could not be crossed with varying times of exposure, hence, there was just one level of the temporal treatments in this level of the herbicide treatment) we could not perform the analyses on the entire dataset at once. Instead, we performed planned comparisons using linear contrasts and controlled for repeated testing by correcting *P* values using the false discovery rate (FDR) method. We performed analyses using the 'glmer' and 'lme' functions of the 'lme4' and 'nlme' packages. In the post hoc analyses, we used the 'glht' function of the 'multcomp' package in 'R' (version 3.0.2; R Core Team, 2016).

3. Results

Tadpole survival was not lowered by the lower herbicide concentration, but at the higher concentration tadpoles exposed to the herbicide during the first or second exposure period or during the entire experiment had lower survival than tadpoles in the control group (control: 87.5% surviving; high conc. during 1st exposure period: 22.9% surviving; high conc. during 2nd exposure period: 50% surviving; high conc. during entire experiment: 12.5% surviving; Table 1 and 2; Fig. 1). Furthermore, tadpoles exposed to the higher concentration for the duration of the entire experiment had significantly lower survival compared to tadpoles treated any time after the initial exposure period, while tadpoles exposed to the herbicide during the first nine days had similarly low survival (Table 3; Fig. 1).

Body mass at metamorphosis was lower in animals exposed to either concentration of the herbicide during the entire experiment than in the control (control: 288.29 mg; low conc. during entire experiment: 250.14 mg; high conc. during entire experiment: 228.17 mg; Table 1; Fig. 2). Body mass was also lowered compared to controls in tadpoles that were exposed to the higher herbicide concentration during the first, fourth and fifth exposure period (high conc. during 1st exposure period: 252.2 mg; high conc. during 4th exposure period: 264.25 mg; high conc. during 5th exposure period: 264.45 mg; Table 2; Fig. 2). Further, body mass of tadpoles that were exposed to the herbicide during the entire experiment did not differ from that of tadpoles exposed to the herbicide only during the first exposure period, but it was significantly lower than in tadpoles that were exposed to the herbicide only later (Table 3, Fig. 2).

Time to metamorphosis was longer in tadpoles that were exposed to the herbicide during the entire experiment than in control tadpoles by ca. 6 days (14%) at the lower herbicide concentration and by ca. 14 days (33%) at the higher herbicide concentration (Table 1; Fig. 3). From among the tadpoles that were exposed to the herbicide for a limited period of time, at the low herbicide concentration only individuals in the first exposure period group developed slower than the controls, whereas at the higher herbicide concentration only the tadpoles in the latest exposure period group did not develop slower than controls (Table 2; Fig. 3). Tadpoles exposed to the herbicide during the entire experiment developed slower at both herbicide concentrations than individuals in any experimental group exposed to the

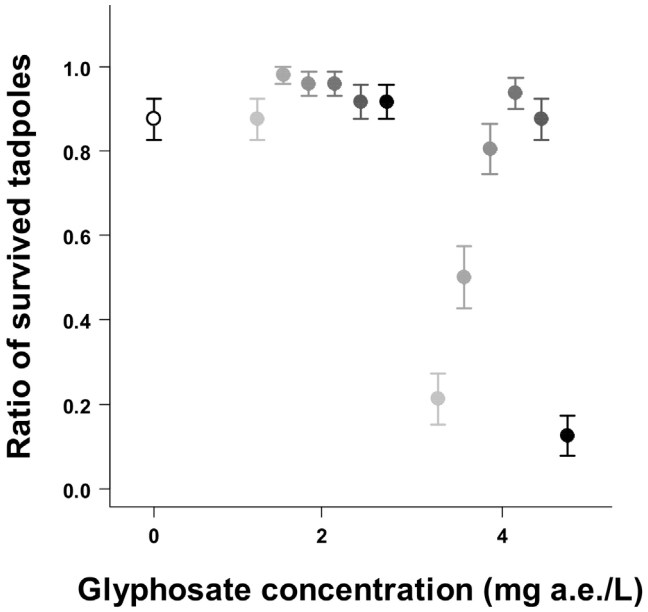

Fig 1. The influence of the timing of herbicide exposure on survival at glyphosate concentrations of 0 mg a.e./L, 2 mg a.e./L and 4 mg a.e./L (○: no exposure, ●: exposure during the 1st, 2nd, 3rd, 4th, or 5th period of larval development, ●: exposure during the entire experiment). Means ± SE are depicted.

Table 1
A summarizing table of the results of planned comparisons performed on life-history traits of tadpoles in the control group (not exposed to the herbicide) and the tadpoles exposed to the herbicide during the entire duration of the experiment. Presented *P*-values were FDR corrected.

	Survival to metamorphosis				Mass at metamorphosis				Days to metamorphosis			
	ß	SE	z-value	*P*	ß	SE	z-value	*P*	ß	SE	z-value	*P*
Control vs:												
Exposure during the entire experiment:												
Low concentration	0.48	0.69	0.69	0.49	−37.26	10.27	−3.63	< **0.001**	6.75	0.71	9.57	< **0.001**
High concentration	−4.37	0.67	−6.57	< **0.001**	−68.62	21.03	−3.26	**0.002**	14.68	1.45	10.14	< **0.001**
Exposure during the entire experiment, low concentration vs:												
High concentration:	−4.85	0.73	−6.69	< **0.001**	−31.36	20.93	−1.49	0.13	7.95	1.44	5.5	< **0.001**

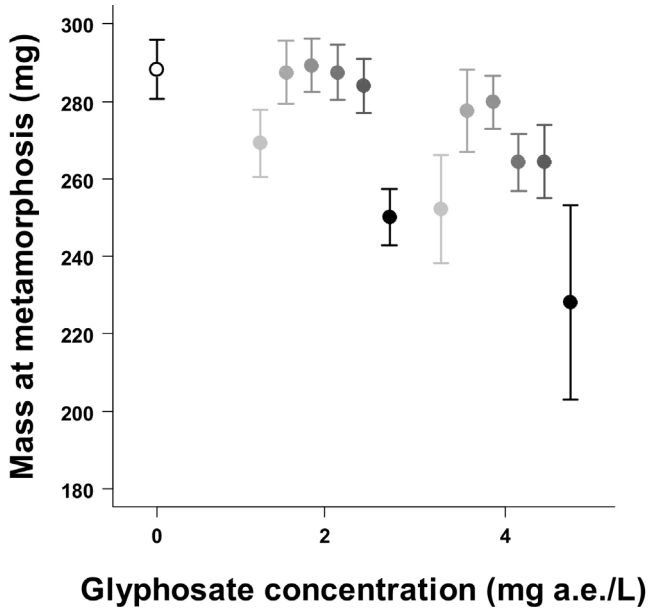

Fig. 2. The influence of the timing of herbicide exposure on body mass at metamorphosis in at glyphosate concentrations of 0 mg a.e./L, 2 mg a.e./L and 4 mg a.e./L (○: no exposure, ●: exposure during the 1st, 2nd, 3rd, 4th, or 5th period of larval development, ●: exposure during the entire experiment). Means ± SE are depicted.

herbicide only for a limited period of time (Table 3; Fig. 3).

4. Discussion

Our results demonstrate that younger *B. bufo* tadpoles are more sensitive to the tested herbicide than older ones, which was clearly visible in all measured traits at the higher concentration, and partly also at the lower concentration. Furthermore, tadpoles that were exposed to the herbicide during the majority of their larval period developed slower than tadpoles that were exposed only early on, but we did not observe a similarly enhanced effect of enduring exposure to the herbicide beyond the first exposure period either in body mass or in survival.

We know relatively little about ontogenetic changes of pesticide tolerance in amphibians (Boone et al., 2013; Boone and Bridges, 2003; Bridges, 2000; Greulich and Pflugmacher, 2003; Harris et al., 2000) and even less is known in relation to glyphosate-based herbicides, while a comprehensive study following changes in sensitivity throughout larval development has been lacking completely. Edginton et al. (2004) compared the mortality of embryos and early stage larvae of four anuran species (*Xenopus laevis*, *Anaxyrus americanus*, *Rana clamitans* and *R. pipiens*) to a glyphosate-based herbicide and found that young larvae were more sensitive to the herbicide than embryos. Howe et al. (2004) investigated the sensitivity of *A. americanus*, *R. clamitans* and *R. pipiens* tadpoles to a glyphosate-based herbicide at two developmental stages, and reported that young tadpoles suffered higher mortality rates upon exposure to the herbicide compared to hatchlings. Jones et al. (2010) studied age-dependent effects of a glyphosate-based herbicide at three larval stages of *R. sylvatica* and *A. americanus* tadpoles and reported highest mortality early on during larval ontogeny. Finally, Hanlon and Parris (2014) exposed *Hyla versicolor* tadpoles to two pesticides at three time-points during larval development and detected reduced survival in tadpoles exposed to the insecticide early on during larval development, while a similar age-dependent effect of the glyphosate-based herbicide was not observed, perhaps because the applied concentration was too low (2 mg a.e./L). In our study we found that sensitivity to the herbicide was highest in young tadpoles and that mortality gradually decreased with age. Hence, based on the literature and our own experimental results, it appears that sensitivity to glyphosate-based herbicides is low during embryonic stages, most likely due to the protection provided by the semi-permeable egg capsules, it increases after hatching, peaks in young tadpoles and decreases again in more developed larval stages.

Apart from age-dependent effects on survival, we also found that tadpoles had reduced body mass if they were exposed to the herbicide during the entire experiment irrespective of herbicide concentration, or during the first, fourth or fifth exposure period at the high herbicide concentration. At the same time the herbicide also had a negative effect on developmental time: tadpoles that were exposed to the herbicide early on developed slower than tadpoles exposed later during development. In addition, tadpoles exposed to the herbicide during the entire experiment had the lowest mass at metamorphosis and took longest to metamorphose. A negative effect of the herbicide on body mass was also observed by several previous studies (Cauble and Wagner, 2005; Mikó et al., 2015; Relyea, 2004) and a decreasing effect of postponed exposure on body mass was reported by Jones et al. (Jones et al., 2010). However, the present study is the first to provide evidence for the age-

Table 2
A summarizing table of the results of planned comparisons performed on life-history traits of tadpoles in the control group (not exposed to the herbicide) and the tadpoles exposed to the herbicide for a limited time period. Presented *P*-values were FDR corrected.

	Survival to metamorphosis				Mass at metamorphosis				Days to metamorphosis			
	ß	SE	z-value	*P*	ß	SE	z-value	*P*	ß	SE	z-value	*P*
Control vs:												
Exposure during 1st period:												
Low concentration	-2.76×10^{-7}	0.63	< −0.001	> 0.99	−18.09	10.38	−1.74	0.41	5.03	0.71	7.06	< **0.001**
High concentration	−3.67	0.61	−6.04	< **0.001**	−37.72	16.86	−2.24	**0.04**	9.29	1.16	8.02	< **0.001**
Exposure during 2nd period:												
Low concentration	1.97	1.1	1.78	0.24	−0.75	10.09	−0.07	0.94	1.57	0.69	2.26	0.06
High concentration	−2.18	0.55	−3.95	< **0.001**	−15.8	12.25	−1.29	0.25	6.66	0.84	7.92	< **0.001**
Exposure during 3rd period:												
Low concentration	1.29	0.85	1.51	0.24	1.22	10.16	0.12	0.94	0.58	0.69	0.83	0.41
High concentration	−0.59	0.59	−0.99	0.4	−10.11	10.71	−0.94	0.35	2.73	0.73	3.71	< **0.001**
Exposure during 4th period:												
Low concentration	1.24	0.85	1.46	0.24	−1.58	10.16	−0.16	0.94	0.99	0.69	1.43	0.26
High concentration	0.77	0.75	1.03	0.4	−24.84	10.25	−2.42	**0.04**	1.54	0.7	2.19	**0.04**
Exposure during 5th period:												
Low concentration	0.43	0.69	0.62	0.67	−4.05	10.27	−0.39	0.94	0.66	0.71	0.94	0.41
High concentration	-2.97×10^{-7}	0.63	< −0.001	> 0.99	−24.56	10.38	−2.37	**0.04**	0.28	0.71	0.39	0.69

Table 3
A summarizing table of the results of planned comparisons performed on life-history traits of tadpoles exposed to the herbicide during the entire duration of the experiment and the tadpoles exposed to the herbicide for a limited time period. Presented *P*-values were FDR corrected.

	Survival to metamorphosis				Mass at metamorphosis				Days to metamorphosis			
	ß	SE	z-value	*P*	ß	SE	z-value	*P*	ß	SE	z-value	*P*
Exposure during the entire experiment vs:												
Exposure during 1st period:												
Low concentration	0.47	0.69	0.69	0.62	−19.17	10.26	−1.87	0.06	1.72	0.7	2.45	**0.01**
High concentration	−0.7	0.59	−1.18	0.24	−30.9	24.8	−1.25	0.21	5.39	1.71	3.16	**0.002**
Exposure during 2nd period:												
Low concentration	−1.49	1.14	−1.31	0.62	−36.51	9.97	−3.66	< **0.001**	5.19	0.68	7.59	< **0.001**
High concentration	−2.19	0.56	−3.91	< **0.001**	−52.82	21.91	−2.41	**0.04**	8.01	1.51	5.32	< **0.001**
Exposure during 3rd period:												
Low concentration	−0.81	0.89	−0.89	0.62	−38.48	10.02	−3.84	< **0.001**	6.17	0.69	8.99	< **0.001**
High concentration	−3.79	0.62	−6.09	< **0.001**	−58.51	21.22	−2.76	**0.03**	11.95	1.46	8.18	< **0.001**
Exposure during 4th period:												
Low concentration	−0.76	0.89	−0.85	0.62	−35.69	10.02	−3.56	< **0.001**	5.76	0.69	8.38	< **0.001**
High concentration	−5.15	0.78	−6.59	< **0.001**	−43.78	20.89	−2.09	**0.04**	13.14	1.44	9.15	< **0.001**
Exposure during 5th period:												
Low concentration	0.05	0.75	0.06	0.95	−33.21	10.12	−3.28	**0.001**	6.09	0.69	8.77	< **0.001**
High concentration	−4.38	0.67	−6.57	< **0.001**	−44.06	20.97	−2.1	**0.04**	14.39	1.44	9.98	< **0.001**

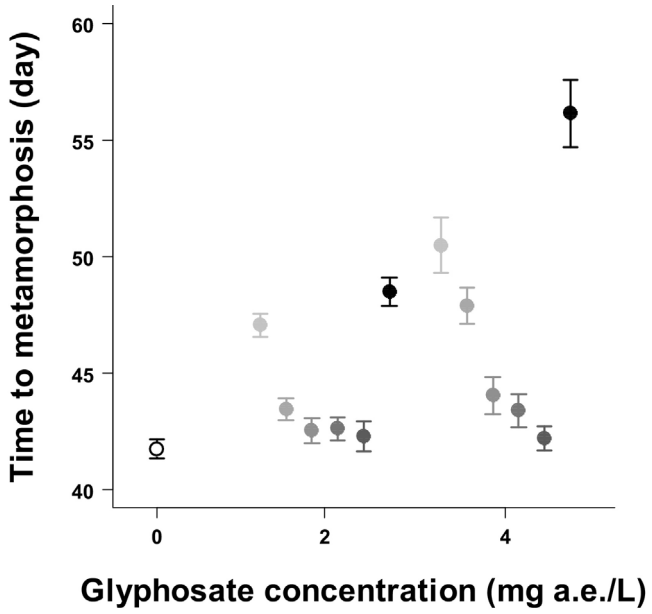

Fig. 3. The influence of the timing of herbicide exposure on time to metamorphosis at glyphosate concentrations of 0 mg a.e./L, 2 mg a.e./L and 4 mg a.e./L (○: no exposure, ●: exposure during the 1st, 2nd, 3rd, 4th, or 5th period of larval development, ●: exposure during the entire experiment). Means ± SE are depicted.

dependency of the development-retarding effect of a glyphosate-based herbicide. This result, nonetheless, aligns to previous reports, where some studies documented that glyphosate-based herbicides increased time to metamorphosis if exposure occurred early on during development (Howe et al., 2004; Jayawardena et al., 2011; Navarro-Martín et al., 2014; Williams and Semlitsch, 2010), but not if tadpoles were exposed to the herbicide later on (Gahl et al., 2011). For tadpoles developing in ephemeral water bodies, prolonged larval development can lead to catastrophic losses in dry years. Also, smaller metamorph size can increase susceptibility to predation threat, reduce survival probabilities later on and lead to diminished reproductive success in adulthood (Altwegg and Reyer, 2003; Semlitsch et al., 1988; Smith, 1987; Vonesh, 2005). Consequently, the effects of early exposure to the herbicide can be serious even if the majority of individuals survive the acute phase and successfully metamorphose.

Ontogenetic changes in sensitivity to toxic substances can be caused by the development of the organs dedicated to detoxification (skin, gills, liver, urinary system) and of the immune system. For example, some studies suggest that the activity of glutathione transferases increases during larval development of amphibians, and, consequently, later developmental stages may be more able to counteract the toxic effects of reactive metabolites of xenobiotics (Aceto et al., 1993; Bucciarelli et al., 1999). The development of the amphibian immune system has been studied in *Xenopus laevis*, where the thymus and spleen first become lymphoid at about the same time that hind limbs begin to develop (thymus: ~stage 47–48, spleen: ~stage 49–50; Robert and Ohta, 2009; Rollins-Smith, 1998), suggesting that the immune system of young tadpoles is less effective compared with later larval stages. This presumably contributes to high susceptibility to diseases during early ontogeny (Hsu and Du Pasquier, 1984; Langhammer et al., 2014) and may also render detoxification processes less effective in young larvae. Nevertheless, pesticides can also negatively affect the activity of detoxification enzymes and impair immune function (Christin et al., 2004; Lajmanovich et al., 2011). Thus, further investigations are needed to reveal the underlying mechanisms of age-dependent herbicide-sensitivity of tadpoles.

In summary, our results have important implications for the interpretation of the results of past ecotoxicological studies, for the planning of future toxicity tests, as well as for the agricultural application of pesticides. Apparent contradictions among the results of previous studies my partly be explained by the age-dependency of sensitivity. Also, when planning and performing toxicity tests, the age of test animals has to be carefully considered, because it appears that exposure to a given pesticide can have no measurable effect at one time-point, while the same contaminant can have serious consequences at the very same concentration if exposure occurs just a few days earlier during ontogeny. Another conclusion that may be drawn from this study is that the timing of pesticide application may be critical for the persistence of amphibians in the vicinity of intensively managed agricultural fields and horticultural gardens. Because younger tadpoles appear to be highly susceptible to chemical contamination while later developmental stages seem to be more tolerant, considering the breeding habits of animals and thereby avoiding exposure of young tadpoles to pesticides may be highly beneficial from the perspective of amphibian conservation. Feasibility is always an important question and gathering information on various aspects of the area targeted with the pesticide, such as the current state of reproductive activities of

amphibian populations, clearly requires additional investment. However, if appliers of pesticides would briefly check the water bodies in and around the site of application, especially in the vicinity of conservation areas, and postpone spraying if the majority of amphibian eggs are close to hatching or if young tadpoles are present, that would not be a large effort, but would represent a great step forward in minimizing collateral damage caused by pesticides.

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