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Standardize or Diversify Experimental Conditions in Ecotoxicology? A Case Study on Herbicide Toxicity to Larvae of Two Anuran Amphibians

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Abstract Despite a steeply increasing number of ecotoxicological studies on the effects of pesticides on nontarget organisms, studies assessing the adequacy and reliability of different experimental approaches have remained scarce. We scrutinized effects of a glyphosate-based herbicide on larvae of two European anuran amphibians by estimating species-specific LC50 values, assessing how an additional stress factor may influence outcomes, and investigating whether replicate experiments yielded qualitatively the same results. We exposed *Rana dalmatina* and *Bufo bufo* tadpoles to two predator treatments (no predator vs. predator chemical cues) combined with varying herbicide concentrations, repeated the experiment with a subset of the experimental treatments and partly with slight modifications 1 week later and assessed survival. Our results indicated that the herbicide was moderately toxic to tadpoles. The presence of predator chemical cues did not affect the lethality of the herbicide in either species. The estimated sensitivity of *R. dalmatina* tadpoles varied considerably across experiments, whereas in case of *B. bufo* LC50 values remained very similar. Our results suggest that differences in the experimental setup may often have no influence on the measured effects of pesticides, whereas replicated experiments can deliver widely differing results in other cases, perhaps depending on the studied species, the population origin of the tested individuals, or the test conditions. This draws attention to the suggestion that strict standardization may not deliver widely applicable insights into the toxicity of contaminants and, instead, intentionally introducing variation into the design of ecotoxicological experiments and replicating entire experiments may prove highly beneficial.

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The application of pesticides is an effective and widespread way of improving productivity in agriculture, but these chemicals not only affect pest species, but may harm nontarget organisms as well (Ratcliffe 1967; Gross et al. 2003; Mann et al. 2009). Understanding consequences of pesticide use for non-target organisms is of fundamental importance for averting unwanted adverse effects and, ultimately, biodiversity loss. However, the reliable assessment of toxic effects requires methodologies that allow for conclusions also holding under natural conditions. To what extent currently applied protocols of ecotoxicological studies meet the requirement to precisely predict toxic effects in natural settings has remained controversial (Rowe and Dunson 1994; Versteeg et al. 1999; Johnson et al. 2013; Mikó et al. 2015).

To provide quantitative information about direct impacts of pesticides, the classic toxicological method of determining LC50 or LD50 values (medial lethal concentration/dose; the concentrations or doses of chemicals that kill half the members of a tested population after a specified test duration) under controlled laboratory conditions has remained the norm. Test results are then used to determine safe concentrations of pollutants (Sih et al. 2004). Laboratory toxicity tests also have played a valuable role in

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characterizing the sensitivity of different animal groups to chemical stressors (Gross et al. 2003). This laboratory-based approach can provide high throughput, precision, and control of potentially confounding variables, enabling the establishment of cause-and-effect relationships between contaminants and responses of organisms (Versteeg et al. 1999; Chalcraft et al. 2005). However, these tests usually do not take into account additive or synergistic effects of multiple biotic and abiotic stress factors (Sih et al. 2004), and studies explicitly testing among-experiment reproducibility of results are lacking.

One classic tenet of empirical research, including experimental ecotoxicology, is the need for standardization. Standardization is the process of creating and applying technical and methodological standards that strictly regulate the circumstances of the experiments and often even the strains of laboratory animals used to minimize background variation and to control for unintended effects, which could swamp or otherwise influence results. Standardization increases test accuracy and precision (Paylor 2009; Richter et al. 2009; van der Staay et al. 2010), aids in pinpointing cause-and-effect relationships (Bailoo et al. 2014), facilitates comparison of results among experiments (Beynen et al. 2001), and allows for a reduction in the number of individuals used (Festing 2004a, b; Howard et al. 2009). Nevertheless, in the past decade doubts emerged over the usefulness of environmental standardization in animal experiments (Wahlsten et al. 2003; Paylor 2009; Richter et al. 2009, 2010). One of the main concerns is that strict standardization can vastly reduce the generality of results, and conclusions will only be applicable to the exact study conditions (Richter et al. 2009). Over-standardization may pose an especially serious problem in toxicity testing, where false-negative or hardly generalizable results are not just counterproductive, but also may have direct catastrophic consequences.

In this study, we used as a model system a glyphosate-based herbicide and larval anuran amphibians. Glyphosate-based herbicides are the most widely applied broad-spectrum herbicides in the world (Relyea 2005a; Mörtl et al. 2013). Glyphosate inhibits the production of essential aromatic amino acids in plant protein synthesis but is known to be toxic to animals belonging to several taxa as well (Székács and Darvas 2012). Because glyphosate cannot penetrate the plant cuticle by itself, its commercially available herbicide formulations contain surfactants (Mann et al. 2009), such as polyethoxylated tallowamines (POEA), which can be toxic to aquatic organisms (Giesy et al. 2000; Tsui and Chu 2003; Székács and Darvas 2012). Anuran amphibians may be exposed to them at all life-stages during their ontogeny as a consequence of overspray, aerial drift, or runoff (Giesy et al. 2000; Lehman and Williams 2010). Eggs and larvae can become exposed in their aquatic environment (Edginton et al. 2004; Howe et al. 2004; Relyea and Jones 2009; Henao Muñoz et al. 2015), whereas adults can during reproduction, but also during migration and in their summer habitat (Relyea 2005b; Brühl et al. 2013; Lenhardt et al. 2014). Because of their thin and permeable skin, unshelled eggs, and complex life-cycle, amphibians are considered especially vulnerable to contaminants, which has been suggested to contribute to their current steep decline observed worldwide (Linder et al. 2010). Many previous studies indicated that they are indeed sensitive to chemical contaminants (Mann et al. 2009; Egea-Serrano et al. 2012), and glyphosate-based herbicides specifically have been demonstrated to have both lethal and sublethal effects on them already at concentrations found in nature (Wojtaszek et al. 2004; Howe et al. 2004; Relyea and Jones 2009; Lanctôt et al. 2014). Consequently, amphibians are important bioindicators in ecotoxicological studies in general, and in relation to glyphosate-based herbicides as well (Blaustein and Kiesecker 2002).

Our purpose was to determine LC50 values for larvae of two European anuran species, the agile frog (*Rana dalmatina* Bonaparte 1840) and the common toad (*Bufo bufo* Linnaeus 1758), in relation to a widely used formulation of glyphosate-based herbicides. To assess how an additional stress factor may influence outcomes of a standard toxicity test, we investigated whether predation threat (chemical cues from predatory dragonfly larvae) exacerbated effects of the pesticide. Finally, to study how reliably and accurately LC50 values are estimated by single experiments, we repeated the experiments 1 week later on another set of animals. We predicted that the herbicide will decrease tadpole survival in both species, especially at higher concentrations (Relyea 2005a; Relyea and Jones 2009). The presence of predator cues will increase tadpole sensitivity to the herbicide (Relyea and Mills 2001), and differences in experimental setting will modify the toxicity of the herbicide (Mikó et al. 2015).

Materials and Methods

Experiments on Rana dalmatina

In 2013, we collected 20 eggs from each of 10 freshly laid egg-clutches of *R. dalmatina* from a pond in the Pilis-Mountains, Hungary (47°42′48″N, 19°02′25″E; 314 m a.s.l.) and transported them to the laboratory at Júliannamajor Experimental Station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences), Budapest. We kept egg-clutches 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. Two

days after hatchlings reached the free swimming state (developmental stage 25 according to Gosner 1960), we mixed tadpoles from different families and started experiments on the same day.

We haphazardly selected 112 healthy looking tadpoles and placed them individually into 2-L containers filled with 1.4 L of RSW or an equal amount of RSW containing the pesticide. Temperature was set to 16 °C, lighting was set to a 12:12-h light: dark cycle. We exposed tadpoles to Glyphogan® Classic (Monsanto Europe S.A., Brussels, Belgium), a popular formulation of glyphosate-based herbicides. This formulation contains 41.5 w/w% glyphosate and 15.5 w/w% surfactant (POEA). We applied the herbicide in concentrations of 0, 0.009, 0.03, 0.24, 1, 2, and 6.5 mg a.e. glyphosate/L, representing a geometric progression often used in ecotoxicology (Hoffman 2003). We changed water 3 days after start while maintaining initial herbicide concentrations. We exposed half of the tadpoles to chemical cues on predation threat crossed with the herbicide treatments in a full factorial design. We captured six *Aeshna cyanea* dragonfly larvae from nearby ponds and individually kept them in 3-L boxes filled with 2 L of RSW and fed predators with two *R. dalmatina* tadpoles (totalling ca. 150 mg) every other day. Every day we mixed the water from half of the predator boxes and added 13.5 mL of this mix to each experimental container assigned to a predator treatment. In control treatments, we added the same amount of RSW. We fed tadpoles with chopped and slightly boiled spinach ad libitum. We replicated each treatment combination 8 times, resulting in a total of 112 experimental units. Five days after start, we recorded survival of tadpoles and released animals at the site of their collection.

One week after start of the first experiment, we initiated a replicate experiment on a new set of animals. We collected 35 eggs from each of ten freshly laid egg-clutches of *R. dalmatina* from another pond in the Pilis-Mountains, Hungary (47°44′20″N, 19°00′43″E; 483 m a.s.l.). We collected eggs from another, somewhat cooler, habitat located at higher elevation for the second round of replicate experiments to be able to start out with a large enough number of freshly laid clutches also in this experiment. We held experimental conditions identical to those in the first experiment but only applied three herbicide concentrations (0, 2, and 6.5 mg a.e. glyphosate/L; selected based on the results of the first experiment) in combination with the predator treatments. Two days after hatching, we mixed families and exposed 40 tadpoles to each of the 6 treatment combinations, resulting in a total of 240 experimental units. We again evaluated survival after 5 days and released survivors at the site of their collection.

Experiments on Bufo bufo

In 2014, we collected 125 eggs from each of ten freshly laid egg-strings of *B. bufo* from a pond close to Piliscsaba, Hungary (47°37′25″N, 18°48′27″E; 244 m a.s.l.) and transported them to Júliannamajor Experimental Station. We kept eggs in 3-L containers holding 1 L of RSW in the laboratory until hatching at 20 °C and a 12:12-h light: dark cycle. Two days after hatchlings reached the free swimming state, we mixed families and started the first experiment by placing 14 haphazardly selected healthy tadpoles into 11-L containers filled with 10 L of RSW. We set the temperature to 18 °C and applied a 12:12-h light: dark cycle. We used the same formulation of glyphosate-based herbicide as described above and exposed tadpoles to concentrations of 0, 1, 2, 3, 4, 5, 6, 7, and 8 mg a.e. glyphosate/L. We applied a more evenly distributed set of herbicide concentrations, which differed from that used in the experiments involving *R. dalmatina*, but it was not our aim to compare statistically the sensitivity of the two species. We applied two predator treatments (presence/absence of chemical cues on predation threat). We prepared chemical cues on predation threat by keeping *Aeshna cyanea* dragonfly larvae individually in six 3-L boxes containing 2 L of RSW and feeding them with two *B. bufo* tadpoles (totalling ca. 100 mg). We mixed the water from three predator boxes and added 20 mL of this mix to each container assigned to a predator treatment. In treatments receiving no cues on predation, we added the same amount of RSW. We replicated each treatment combination 4 times, which resulted in a total of 72 experimental units. We did not change water during the experiment but fed tadpoles with spinach ad libitum at the start of the experiment. After 5 days, we counted live tadpoles and transported them back to the site of collection.

To replicate the experiment, we collected 20 eggs from each of 12 freshly laid common toad egg-strings from a cooler pond in Nagykovácsi, Hungary (47°34′35″N, 18°52′06″E; 344 m a.s.l.) and transported them to Júliannamajor Experimental Station. We incubated clutches under the same circumstances as in the first test. Two days after hatching, we haphazardly selected 12 healthy larvae from each clutch, mixed them, and placed them individually into 1.2-L containers filled with 0.7 L of RSW, resulting in a total of 144 experimental units. Temperature and lighting was the same as in the first test. We exposed tadpoles to herbicide concentrations of 0, 2, and 4 mg a.e./L of glyphosate, and because we did not find a significant effect of chemical cues on predation threat on tadpole

survival in the first experiment, we excluded this treatment from this replicate experiment. We changed water after 3 days, while maintaining initial pesticide concentrations, and fed tadpoles with chopped and slightly boiled spinach ad libitum. Five days after start, we counted survivors and released tadpoles at the site of collection.

To be able to test for actual concentrations resulting from the addition of calculated herbicide quantities into the experimental containers, we collected eight 125-mL water samples from a parallel experiment, with identical setting as the second *R. dalmatina* experiment, 15 days after start. As in the second *R. dalmatina* experiment, because all tadpoles died at concentration 6.5 mg a.e./L, we could not sample containers with that concentration. Samples collected from tubs with a concentration of 2 mg a.e./L were pooled and stored at 2 °C to prevent the breakdown of glyphosate (Tomlin 2006). The concentration was determined using LC–MS at the Herbicide Residue Analytical Laboratory, Directorate of Plant Protection and Soil Conservation, National Food Chain Safety Office, Miskolc, Hungary. Because the concentration did not differ considerably from the nominal concentration (actual concentration was 1.73 mg a.e./L), and we added the herbicide following the same procedure in all experiments, we could assume that herbicide treatments closely mirrored their nominal concentrations.

Statistical Analyses

We estimated LC50 values for both species separately for experiments and predator treatments using generalized linear models (GZLM) with a binomial error distribution and probit link function. Because in two cases data showed almost perfect separation (fitted probabilities reached 0 or 1), we used Bayesian generalized linear models (Gelman et al. 2008). For calculating *P* values, we used the "Anova" function of the "car" package. To obtain LC50 values, we applied the "dose.p" function of the "MASS" package. We calculated 95% confidence intervals with the following method: exp (LC50 value ± 1.96 × standard error of the LC50 value; Hackshaw 2009). Statistical tests were performed with the "glm" and "bayesglm" function of the "arm" package in "R" (version 3.0.2; R Core Team 2016).

Results

In the first experiment on *R. dalmatina*, almost all tadpoles survived; of the 112 individuals only 7 tadpoles died in the entire experiment: 1 at a glyphosate concentration of 0.009 mg a.e./L (in the absence of predator cues) and 6 at a glyphosate concentration of 6.5 mg a.e./L, 4 of which were exposed to predator cues and 2 were not. In the absence of predator cues, the LC50 value could only be calculated with extremely high uncertainty, because it lied beyond the tested range of herbicide concentrations (7.35 ± 81.69, mean ± SE; 95% CI: 2.13×10^{-69}, 2.54×10^{70}), while in predator-exposed tadpoles it was close to the high end (6.5 ± 0.08; 95% CI: 5.55, 7.62; Fig. 1). In the second experiment, 99% of tadpoles died within the first 3 days at a glyphosate concentration of 6.5 mg a.e./L. The estimated LC50 for *R. dalmatina* in this experiment was 4.00 ± 0.11 (95% CI: 3.26, 4.92) mg a.e./L without predator cues and 3.54 ± 0.11 (95% CI: 2.86, 4.39) mg a.e./L with predator cues (Fig. 1). Because predator presence did not affect tadpole survival significantly in either experiment (first experiment: GZLM; $\chi^2 = 1.08$, $P = 0.3$, second experiment: Bayesian GZLM; $\chi^2 = 0.94$, $P = 0.33$), we calculated overall LC50 values for each test (6.92 ± 18.1 mg a.e./L; 95% CI: 2.76×10^{-15}, 1.74×10^{16} and 3.96 ± 0.1 mg a.e./L; 95% CI: 3.24, 4.83, respectively).

In the first experiment on *B. bufo*, all tadpoles died above 5 mg a.e. glyphosate/L without predator cues and at 8 mg a.e./L in the presence of predator cues. We did not observe death at 1 and 2 mg a.e. glyphosate/L in the predator control group. The estimated LC50 values were 4.25 ± 0.03 (95% CI: 4.03, 4.48) mg a.e./L without predator cues and 4.46 ± 0.03 (95% CI: 4.24, 4.69) mg a.e./L with predator cues (Fig. 2). The presence of predator cues did not affect tadpole survival (GZLM; $\chi^2 = 1.72$, $P = 0.19$); the overall LC50 value was 4.36 ± 0.02 (95% CI: 4.19, 4.52) mg a.e./L. In the second experiment, where we did not add predator cues, the LC50 value for the herbicide was 4.41 ± 0.07 (95% CI: 3.84, 5.07) mg a.e. glyphosate/L (Fig. 2).

Discussion

The LC50 estimates for *R. dalmatina* and *B. bufo* ranged from 3.54 to 7.35 mg a.e./L. This means that this herbicide is moderately toxic to these amphibians (LC50 values between 1 and 10 mg active ingredients/L, equalling 0.75–7.5 mg a.e./L; Giesy et al. 2000; Relyea 2011). This result is in agreement with previous studies which found glyphosate-based and POEA-containing herbicides to be moderately to highly toxic to amphibians (e.g., $LC50_{48\text{-h}} = 2.93$–$11.63$ mg a.e./L, Mann and Bidwell 1999; $LC50_{48\text{-h}} = 1.31$ mg a.e./L, Lajmanovich et al. 2003; $LC50_{16\text{-d}} = 0.41$–$1.89$ mg a.e./L, Relyea 2005a), where differences in the study species, in exposure time, and in the herbicide formulation used are all likely contributors to the variation among studies, but different experimental environments and chance effects also may have played an important role (Mann and Bidwell 1999; Howe et al. 2004; Moore et al. 2012; Edge et al. 2014; Mikó et al. 2015).

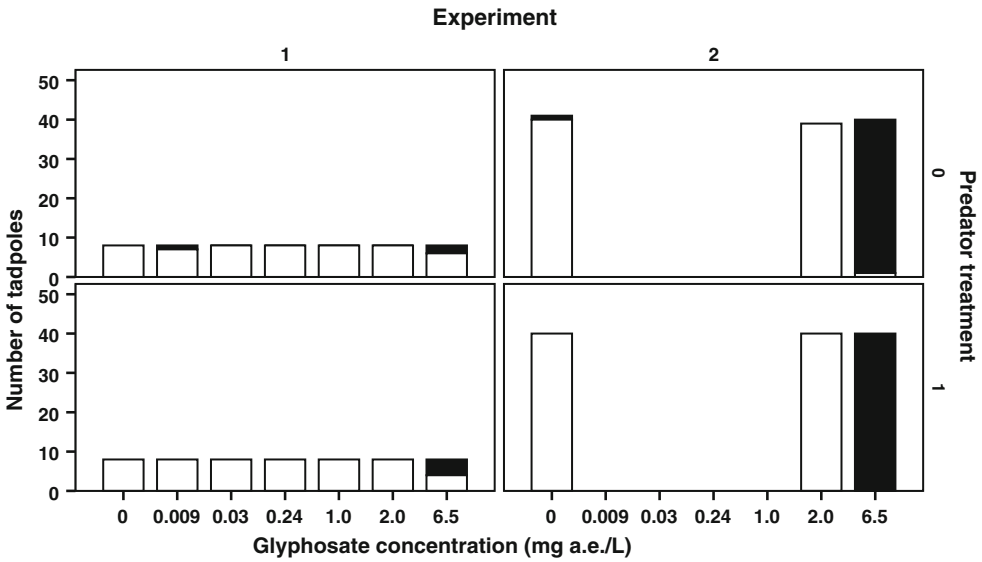

Fig. 1 Number of surviving (*open square*) and dead (*filled square*) *R. dalmatina* tadpoles in the first and second test in the absence and presence of predator cues. One week elapsed between the first and the second test. Concentrations applied in the second experiment were chosen based on results of the first experiment

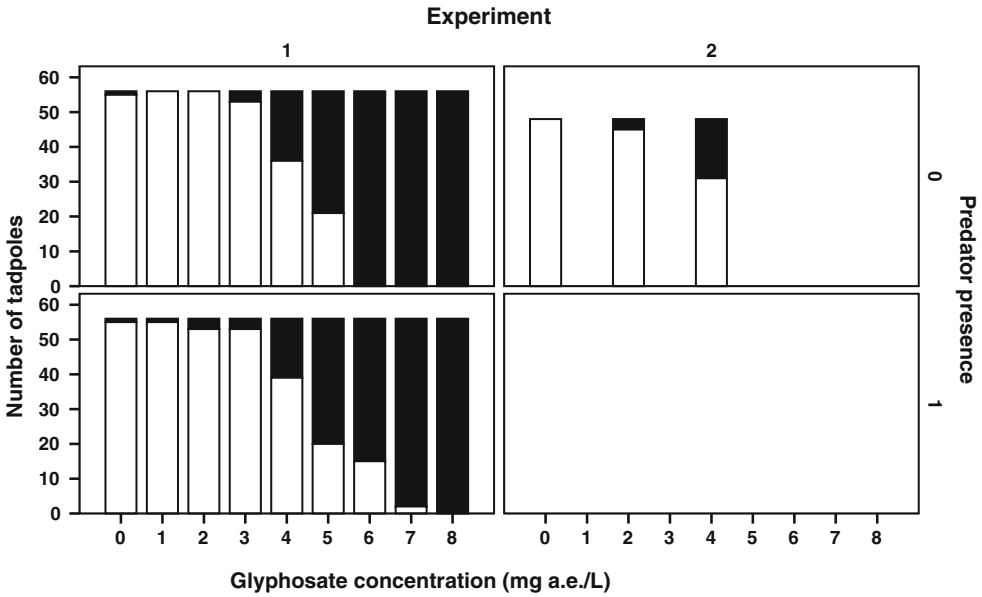

Fig. 2 Number of surviving (*open square*) and dead (*filled square*) *B. bufo* tadpoles in the first and second test in the absence and presence of predator cues. One week elapsed between the first and the second test, and we did not apply predator cues in the second experiment, hence the empty panel on the lower right. Herbicide concentrations in the second experiment were chosen based on results of the first experiment

Toxicity of herbicides can be influenced by biotic and abiotic environmental factors (Sparling 2003). For example, lethality of glyphosate-based herbicides may be enhanced by high pH (Chen et al. 2004; Edginton et al. 2004; Wojtaszek et al. 2004) or competitive situations (Jones et al. 2011) but may be strongly affected by the experimental venue (Mikó et al. 2015). Stress posed by predation threat clearly has the potential to influence the lethality of environmental contaminants, but the few existing empirical studies delivered contradictory results. Under laboratory conditions, Relyea (2005a) found that predator cues strengthen the toxicity of glyphosate-based herbicides; however, in outdoor mesocosms, predator presence made the herbicide less lethal to amphibian tadpoles (Relyea 2012). In a previous study, we did not find a significant influence of predator presence on the survival of tadpoles (Mikó et al. 2015). In the present study, we also did not observe significant differences in tadpole survival according to the presence or absence of predator chemical cues. Theoretically, the observed absence of an effect of predation risk on susceptibility to the herbicide may have been due to too low concentrations of chemical cues of predation risk. However, chemical cues have been shown to elicit antipredator responses at similar concentrations (this study: 10.63 and 10.5 mg tadpoles L^{-1} $week^{-1}$; Relyea 2012: 7.36 mg tadpoles L^{-1} $week^{-1}$), so that tadpoles in this experiment are likely to have recognized exposure to predation threat. Further investigations will be necessary to conclude on the circumstances when predator presence enhances or lowers toxicity of herbicides.

Our results support the hypothesis that larvae of different amphibian species may largely vary in their susceptibility to glyphosate-based herbicides (Mann and Bidwell 1999; Relyea 2005a; Bernal et al. 2009; Relyea and Jones 2009; Moore et al. 2012). Moreover, differences in pesticide tolerance may not only occur among species, but among populations of a given species as well (Semlitsch et al. 2000; Cothran et al. 2013). The observed difference between results of the replicate experiments in the case of *R. dalmatina* may be attributed to such interpopulation differences in sensitivity (see Brausch and Smith 2009; Coors et al. 2009; Hua et al. 2015). Because intraspecific variation in susceptibility is of fundamental importance for the interpretation and design of ecotoxicological studies, further rigorous investigations targeting the evaluation of among-population differences in herbicide sensitivity are urgently needed.

The need for highest-possible levels of standardization and its omnipotency has been questioned recently by rodent behavioural scientists (Paylor 2009; Richter et al. 2009). The debate had been fuelled by several studies finding differences in the behaviour of rodent model organisms both within and between laboratories despite highly standardized housing and testing conditions (Chesler et al. 2002; Wahlsten et al. 2003; Kafkafi et al. 2005; Lewejohann et al. 2006). Allowing some level of variation in environmental parameters of experiments instead of rigid standardization has been put forward as part of the solution. In our study, the estimated LC50 values appeared to be robust in the case of *B. bufo*, because the two experiments yielded roughly the same value despite considerable differences in experimental design. While we only replicated the experiment once, this strengthens our confidence in the general validity of the toxicity value estimated for this species. In *R. dalmatina*, however, LC50 estimates differed significantly between the experiments despite almost identical experimental conditions. Hence, in this case a high level of standardization did not eradicate variation among results, and this variation may alert to the uncertainty of estimated LC50 values. Although the interspecific differences in the reproducibility of our results cannot be explained by discrepancies in the level of standardization, these results nicely illustrate the idea that some level of variation deliberately introduced into the experimental methodology may strengthen our confidence in the results, while also enhancing the generality of conclusions.

The other important conclusion that our results draw attention to is that firm conclusions regarding toxicity, and in fact any experimental result, can only be drawn from replicated experiments. Replicability problems of scientific results have surfaced recently within various fields, such as cancer biology, drug discovery, or psychology (Prinz et al. 2011; Begley and Ellis 2012; Open Science Collaboration 2015). These all together suggest that replication of experiments should not be regarded as pointless and unoriginal copying of previous studies but should be looked at as a prerequisite of scientific progress and should be valued accordingly (Open Science Collaboration 2015).

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical Approval The Közép-Duna-Völgyi KTVF issued the permissions to conduct the study (KTF: 5192-7/2013, 603-3/2014) and the Ethical Commission of the MTA ATK NÖVI approved the investigation.

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