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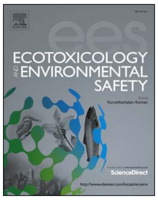

Effects of a glyphosate-based herbicide and predation threat on the behaviour of agile frog tadpoles

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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