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Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: Agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions

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ABSTRACT

Glyphosate-based herbicides are currently the most commonly used herbicides in the world. They have been shown to affect survival, growth, development and sexual differentiation of tadpoles under chronic laboratory exposures but this has not been investigated under more environmentally realistic conditions. The purpose of this study is (1) to determine if an agriculturally relevant exposure to Roundup WeatherMax®, a relatively new and understudied formulation, influences the development of wood frog tadpoles (*Lithobates sylvaticus*) through effects on the mRNA levels of genes involved in the control of metamorphosis; (2) to compare results to the well-studied Vision® formulation (containing the isopropylamine salt of glyphosate [IPA] and polyethoxylated tallowamine [POEA] surfactant) and to determine which ingredient(s) in the formulations are responsible for potential effects on development; and (3) to compare results to recent field studies that used a similar experimental design. In the present laboratory study, wood frog tadpoles were exposed to an agriculturally relevant application (i.e., two pulses) of Roundup WeatherMax® and Vision® herbicides as well as the active ingredient (IPA) and the POEA surfactant of Vision®. Survival, development, growth, sex ratios and mRNA levels of genes involved in tadpole metamorphosis were measured. Results show that Roundup WeatherMax® (2.89 mg acid equivalent (a.e.)/L) caused 100% mortality after the first pulse. Tadpoles treated with a lower concentration of Roundup WeatherMax® (0.21 mg a.e./L) as well as Vision® (2.89 mg a.e./L), IPA and POEA had an increased condition factor (based on length and weight measures in the tadpoles) relative to controls at Gosner stage (Gs) 36/38. At Gs42, tadpoles treated with IPA and POEA had a decreased condition factor. Also at Gs42, the effect on condition factor was dependent on the sex of tadpoles and significant treatment effects were only detected in males. In most cases, treatment reduced the normal mRNA increase of key genes controlling development in tadpoles between Gs37 and Gs42, such as genes encoding thyroid hormone receptor beta in brain, glucocorticoid receptor in tail and deiodinase enzyme in brain and tail. We conclude that glyphosate-based herbicides have the potential to alter mRNA profiles during metamorphosis. However, studies in natural systems have yet to replicate these negative effects, which highlight the need for more ecologically relevant studies for risk assessment.

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1. Introduction

Amphibian larvae develop in aquatic environments, making them extremely vulnerable to the absorption of contaminants through their permeable skin and gills. Chemical contaminants released to aquatic environments have been listed as the second most important threat to amphibians after habitat loss (Vié et al., 2009), so increased use of herbicides over the past several decades may represent a contributing factor to the global decline of amphibian populations.

Commercial herbicide formulations containing glyphosate as the active ingredient are currently dominating the agriculture and silviculture sectors worldwide (Thompson and Pitt, 2003; Woodburn, 2000), and are being used in over 130 countries on over 100 crops (Monsanto, 2009). The number of glyphosate-based products released to the market has increased enormously since the removal of patent protection for glyphosate in the early 1990s. The new formulations differ slightly based on the specific chemistry of the active herbicidal ingredient, and the properties of their so-called 'inert' ingredients including surfactants, dyes and foaming agents. In these formulations, the glyphosate acid is converted to a salt in order to increase water solubility and, for this reason glyphosate concentrations are reported as acid equivalents (a.e.) per liter. Glyphosate in the form of the isopropylamine (IPA) salt is present in most formulations including Roundup Original®, however, some newer formulations including Roundup WeatherMax® contain a potassium salt which increases the amount of active ingredient per volume (Monsanto, 2009). Glyphosate-based formulations typically contain, or require the addition of a surfactant which helps spread glyphosate across the plant surface and allows more efficient penetration of the active ingredient through the leaf cuticle. The chemical formulations of the inert ingredients are not often disclosed to the public because of proprietary considerations. As a result, these chemicals are often not considered during toxicity assessment and/or establishing usage guidelines for glyphosate-based herbicides. Roundup Original® (equivalent to Vision®) was one of the first marketed glyphosate-based herbicides and is one of the most studied to date. The surfactant used in this formulation is polyethoxylated tallowamine (POEA; CAS #61791-26-2). POEA is one of the few identified surfactants in these herbicide mixtures and is thought to be a principal surfactant used in many glyphosate-based herbicides (Giesy et al., 2000).

Although these formulations were thought to have few effects on vertebrates, recent studies suggest that glyphosate-based herbicides might have negative impacts on the environment (reviewed by Relyea (2011)). For example, multiple laboratory and mesocosm studies have now shown that exposures to glyphosate-based herbicides increases mortality and decreases size and developmental rates of tadpoles (Howe et al., 2004; Jones et al., 2010, 2011; Relyea, 2004, 2005a,b,c, 2012; Relyea et al., 2005; Relyea and Jones, 2009; Williams and Semlitsch, 2010). Previous studies have also demonstrated that Roundup® formulations with POEA and POEA alone each cause significantly higher mortality in amphibians than glyphosate active ingredient alone (Edginton et al., 2004; Howe et al., 2004; Moore et al., 2012; Perkins et al., 2000), indicating that, in at least some cases, the surfactant is a more harmful constituent than the active herbicidal ingredient. Despite evidence that chemical surfactants such as POEA may play a primary role in negative impacts associated with exposure of amphibians to glyphosate-based herbicides, there have been relatively few studies investigating this. At the same time, since chronic laboratory exposures do not reflect realistic, environmentally-relevant herbicide exposures, more work is required to assess the impacts of glyphosate-based herbicides on amphibians under realistic exposure scenarios. In agriculture, herbicides may be applied several times during the growing season (pulse applications), so experiments reflecting this will provide a more accurate indication of real world effects on exposed tadpoles.

The purpose of this study is to determine if an agriculturally realistic exposure to Roundup WeatherMax®, a relatively new and understudied formulation containing an unknown surfactant, influences the development of wood frog tadpoles (*Lithobates sylvaticus*), through effects on the mRNA levels of genes involved in the control of metamorphosis. Specific thyroid-related and stress-related genes were selected because they have been shown to play important roles in anuran developmental processes (reviewed by Brown and Cai (2007), Denver (2009), Fort et al. (2007), Galton (1992), Shi (2000) and Tata (2006)). These include genes encoding thyroid hormone receptor beta (*trβ*), deiodinase enzymes (*dio2*, and *dio3*), corticotropin-releasing factor (*crf*) and glucocorticoid receptor (*grII*). Despite their known importance for the orchestration of amphibian metamorphosis, and the knowledge that many environmental contaminants negatively influence metamorphosis, there have been few studies investigating the effects of exposure to glyphosate formulations on TH receptors, deiodinase enzymes and stress-related genes, or how these may influence developmental processes (Howe et al., 2004; Lanctôt et al., 2013).

This study allowed us to compare results to the well-studied Vision® formulation (containing the IPA salt of glyphosate and POEA surfactant), and to determine which ingredient(s) in the formulations are responsible for any potential effects on development we might observe. We also compare our laboratory-based results to data from field studies that used a similar experimental design (Edge and Houlahan, unpublished; Lanctôt et al., 2013). Increasing our understanding of mechanisms through which specific compounds exert effects on amphibian development and metamorphosis is critical, as it may lead to insights regarding the mitigation or elimination of adverse health effects of environmental contaminants on natural populations.

2. Materials and methods

2.1. Animals

Fertilized *L. sylvaticus* egg masses were collected from natural wetlands in the Long-term Experimental Wetlands Area (LEWA) located on Canadian Forces Base Gagetown in New Brunswick, Canada (45°40′N, 66°29′W). When tadpoles reached Gosner stage (Gs; Gosner, 1960) 25, tadpoles ($n = 50$) were placed into twenty-one 50-liter glass aquaria (1 tadpole/L) and divided into seven treatment groups (3 aquaria per treatment): control, Roundup WeatherMax® (0.21 and 2.89 mg a.e./L), Vision® (2.89 mg a.e./L), isopropylamine (IPA) salt of glyphosate (2.89 mg a.e./L, the active ingredient), polyethoxylated tallowamine (POEA, 1.43 mg/L, pulse exposure) and POEA Chronic (1.43 mg/L, continuous exposure until tadpoles reached Gs42). Tadpoles were fed a combination of boiled kale and Ward's dry tadpole food (4:1; kale:Wards) daily *ad libitum*, supplemented with algal pellets weekly.

2.2. Roundup WeatherMax®, Vision®, IPA and POEA exposure

Roundup WeatherMax® (540 g a.e./L as the potassium salt of glyphosate, Monsanto, Winnipeg, MB, CAN), a formulation used primarily for agricultural purposes, was chosen for this study due to its widespread use throughout North America and because of the lack of information pertaining to its potential effects on amphibians. Vision® formulation (356 g a.e./L present as the IPA salt of glyphosate, Monsanto, Winnipeg, MB, CAN), a formulation used for forestry, is equivalent to the Roundup Original® formulation, and was chosen for this study because it is one of the most studied glyphosate-based herbicides to date and contains one of the few identified surfactants, POEA.