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Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*

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HIGHLIGHTS

► Roundup WeatherMax has limited effects on juvenile amphibian survival.
► Roundup WeatherMax has limited effects on LSI, body condition and disease incidence.
► Laboratory studies do not replicate real-world exposures.

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ABSTRACT

Herbicides are commonly used in agriculture and silviculture to reduce interspecific competition among plants and thereby enhance crop growth, quality, and volume. Internationally, formulations of glyphosate-based herbicides are the most widely used herbicides in both these sectors. A large amount of work has focused on the effects of these herbicides on amphibians. Several laboratory and mesocosm studies have demonstrated that various formulations of glyphosate herbicides can be acutely toxic to larval and juvenile amphibians at concentrations at the upper end of environmental realism. However, to date there has been little work done investigating such effects in natural systems, limited work on juvenile amphibians, and only a few studies have investigated interactions with other stressors. We conducted a 16 day field experiment in which juveniles of two amphibian species (*Lithobates clamitans* and *Lithobates pipiens*) were exposed to the herbicide Roundup WeatherMax™ at four application rates (0, 2.16, 4.32 and 8.64 kg a.e./ha) to investigate effects on survival, liver somatic index (LSI), body condition, and incidence of disease caused by *Batrachochytrium dendrobatidis* (*Bd*). In a separate 16 day laboratory experiment, we exposed juvenile *L. clamitans* to both the herbicide and *Bd*. Results of our studies showed that this particular herbicide formulation had no effect on juvenile survival, LSI, body condition, or disease incidence, nor was there an interaction between exposure to herbicide and exposure to the disease in tests which closely mimic real world exposure scenarios. These experiments suggest that Roundup WeatherMax as typically used in agriculture is unlikely to cause significant deleterious effects on juvenile amphibians under real world exposure conditions.

1. Introduction

Herbicides are commonly used in both agriculture and silviculture to increase crop yields by reducing inter-species competition with other plants. There is concern about their widespread use because there is potential for them to contaminate sensitive aquatic environments and pose a threat to non-target species such as amphibians (Semlitsch, 2003). Commercial herbicide formulations containing the active ingredient glyphosate dominate both the agriculture and silviculture sectors worldwide (Woodburn, 2000; Duke and Powles, 2008; Thompson et al., 2010). Although a wide variety of formulations of glyphosate-based herbicides are available, all formulations contain three basic components, glyphosate in the form of a salt, a surfactant or surfactant mixture, and water (Giesy et al., 2000). These herbicides are applied to vegetation using a variety of different techniques, including backpack or handheld sprayers, rotary or fixed wing aircraft, and tractor mounted spray booms. One reason for their widespread use is that they are generally considered to pose negligible risk to wildlife; including fish, amphibians and other aquatic organisms (Giesy et al., 2000; Durkin, 2003; Solomon and Thompson, 2003; Thompson, 2011). Two fundamental reasons for this are, first, the plant-specific mode of action for glyphosate itself (Rubin et al., 1982; Williams et al., 2000), and, second, that both glyphosate and a commonly used surfactant (polyethoxylated

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tallowamine POEA) bind rapidly and strongly to sediments and are rapidly degraded by microbial decomposition in shallow freshwater systems (Goldsborough and Beck, 1989; Wojtaszek et al., 2004; Wang et al., 2005; Tsui and Chu, 2008; Degenhardt et al., 2012).

Although most formulations cannot be legally applied to water, the littoral region of small wetlands as well as the animals that inhabit these areas may be exposed through overspray, spray drift, runoff from adjacent fields, or through groundwater in cases with sandy soil with low organic content. Exposure to the herbicide could result in direct toxic effects on animals and/or effects on the immune system resulting in increased prevalence of disease (Carey, 1993; Carey et al., 1999; Forson and Storfor, 2006). Amphibians are ubiquitous in small shallow wetlands and there is increasing concern that amphibian species are declining around the world (Houlahan et al., 2000; Stuart et al., 2004). Pesticides and disease are among the many possible explanations for such declines of which also include climate change, increased UVB exposure, habitat loss, invasive species and interactions among these factors (Stuart et al., 2004). Chytridiomycosis is a disease caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Daszak et al., 1999; Kilpatrick et al., 2010), which attacks the keratinized integument of amphibians (Longcore et al., 2007) and has been linked to large scale population declines of amphibians around the world. Antimicrobial skin peptides may be a key factor in protecting post-metamorphic amphibians from chytrid infection (Rollins-Smith et al., 2002; Gibble and Baer, 2011). Thus, it is plausible that a herbicide applied directly to the skin of an amphibian could have an effect on skin peptide anti-microbial ability, or an effect on the disease organism itself (Edge et al., 2011; Gahl et al., 2011).

Typical forestry and agricultural label application rates of glyphosate-based herbicides range from 0.9 to 4.27 kg acid equivalents (a.e.)/ha, and in the United States annual application may not exceed 6.73 kg a.e./ha annually for crop uses, and 8.92 kg a.e./ha for noncrop uses (Giesy et al., 2000). Directly overspraying a wetland 15 cm deep with no intercepting vegetation at these application rates would result in aqueous concentrations between 2.89 (at the maximum label rate) and 5.95 mg a.e./L (one time application of the maximum annual application rate). Recent laboratory and mesocosm studies have demonstrated that some commercial glyphosate-based herbicide formulations can be toxic to non-target amphibian larvae at concentrations between 1.0 and 4.5 mg a.e./L (Mann and Bidwell, 1999; Howe et al., 2004; Edginton et al., 2004; Relyea and Jones, 2009; Moore et al., 2011), with the lowest reported 96 h LC50 values for anuran species approximating 0.8 mg a.e./L (Edginton et al., 2004; Relyea and Jones, 2009). However, field studies have only detected detrimental effects on amphibian larvae at much higher exposure levels, such as 7.15 mg a.e./L (Thompson et al., 2004; Wojtaszek et al., 2004) or limited to no effect at concentrations that approximate worst-case exposures (2.89 mg a.e./L (Edge et al., 2012)).

The effect of glyphosate-based herbicides on amphibian larvae has been well studied, but there are only a few studies on terrestrial and semi-aquatic life stages. The juvenile life stage of many North American amphibian species is terrestrial or semi-aquatic and they inhabit the littoral region of wetlands for the periods after metamorphosis or in some cases throughout their lifespan. Understanding the impact of natural and anthropomorphic stressors on the post-metamorphic stage is extremely important because juvenile and adult amphibians contribute not only in terms of their reproductive potential to the viability of local populations, but also to the cycling of energy and nutrients in ecosystems (Travis, 1984; Beard et al., 2002; Verburg et al., 2007), by predating on a variety of invertebrate species, and by serving as a significant food source for other vertebrates and invertebrates (Beard et al., 2003; Greenless et al., 2006). In addition to potential direct acute effects, herbicide exposure could result in a variety of sublethal effects on juvenile amphibians, including effects on the incidence of disease, reduced energy stores or the activity of detoxifying organs (e.g., liver) directly, or through a variety of potential indirect pathways such as changes to vegetation or food sources.

The few laboratory and mesocosm studies that focused on terrestrial and semi-aquatic life stages have found that these herbicides can be toxic to juvenile amphibians at application rates between 4 and 10 kg a.e./ha (Relyea, 2005b; Bernal et al., 2009; Dinehart et al., 2009) and the lowest range of application rates reported to result in mortality of juvenile amphibians is between 1.85 and 3.69 kg a.e./ha (Bernal et al., 2009). Similar to studies on larvae, the one field study on juvenile amphibians did not detect negative effects on juvenile amphibians at application rates up to 4.27 kg a.e./ha (Edge et al., 2011). Differences between the results of laboratory/mesocosm studies and field studies are likely due to the fact that both the active ingredient (Glyphosate) and some surfactant (such as POEA) components of glyphosate-based herbicides have relatively short half-lives and do not persist in water or soils of natural systems (Goldsborough and Beck, 1989; Feng et al., 1990; Giesy et al., 2000; Thompson et al., 2000; Wojtaszek et al., 2004; Tsui and Chu, 2008). Moreover, both glyphosate and the POEA surfactant bind quickly and strongly to sediment and other organic matrices such as soils, biofilms and plant surfaces (Tsui and Chu, 2004; Wang et al., 2005) making them potentially less bioavailable. Nonetheless, these results have raised concern about the toxicity of commercial formulations of glyphosate-based herbicides to larval and juvenile amphibians (Mann and Bidwell, 1999; Giesy et al., 2000; Solomon and Thompson, 2003; Edginton et al., 2004; Howe et al., 2004; Relyea, 2005a; Relyea and Jones, 2009). In addition, our understanding of how these herbicides may interact with other stressors, such as disease, is poor. This is of importance because one leading hypothesis regarding amphibian declines is that anthropogenic stressors act synergistically with diseases, such as *Bd*, to increase mortality (Carey, 1993). However, this relationship has not been found for *Bd* and glyphosate-based herbicides, instead the herbicide may have a direct negative effect on the disease organism and reduce infections in both larvae (Gahl et al., 2011) and semi-aquatic juveniles (Edge et al., 2011).

The primary goal of this study is to assess whether exposure to a formulation of a glyphosate-based herbicide commonly used in agriculture (Roundup WeatherMax™) has negative effects on semi-aquatic juvenile amphibians. To meet this goal we exposed two species of amphibians to four application rates of the herbicide in a field experiment. In a separate study in the laboratory, we also exposed one species to four application rates of the herbicide in combination with exposure to the pathogenic chytrid fungus (*Bd*). The secondary objective was to investigate if the venue (lab or field enclosure) had an effect on the results of the experiments by comparing the results of the two parallel experiments.

2. Materials and methods

2.1. Site description

The field site was located in the Long Term Experimental Wetlands Area (LEWA) on Canadian Forces Base Gagetown (CFB Gagetown) in southeastern New Brunswick, Canada (45°40′ N, 66°29′ W). CFB Gagetown is a 1200 km^2 military installation primarily used for ground training. The study area is approximately 7 km^2 and was mechanically cleared of trees and the top soil was pushed into berms in 1997 and 1998. This created a landscape of elevated treed ridges and open areas, with hundreds of small wetlands ranging from <1 ha to several hectares in size, to which there has been no prior chemical application (Ollsen and Knopper, 2006).

2.2. Field experiment

For the field portion of this study, we chose two permanent wetlands that had not been used for any other experiment. In June of 2010, 24 enclosures (0.8 m × 2 m, 1.6 m^2) were constructed from aluminum flashing and 3.2 mm high density polyethylene (HDPE) mesh and 12