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Sublethal Effects of the Herbicide Glyphosate on Amphibian Metamorphosis and Development

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Amphibian populations have experienced dramatic declines attributed to habitat destruction, introduced species, pathogens, acid rain, global climate change, increased UV-B radiation, fertilizers, pesticides, herbicides, as well as synergistic interactions among different factors (Blaustein et al. 2003). Additionally, several studies have demonstrated sensitivity of amphibians to herbicides (Mann and Bidwell 1999; Smith 2001). Glyphosate is suggested to be a highly effective herbicide in weed eradication with a relatively short environmental persistence time (Giesy et al. 2000). As a consequence, glyphosate is used widely to clear roadside vegetation in rural areas, national parks, and is often applied to sites in preparation for native plant restoration (Tyser et al. 1998). In addition, many crops have been genetically engineered and are continuing to be developed with glyphosate resistance to provide an efficient system for weed eradication (Reddy 2001). Consequently, glyphosate based herbicide usage is expected to increase and may pose risks for amphibian biodiversity in a variety of habitats.

In addition, amphibian larvae may be particularly vulnerable to chemical exposure during metamorphosis. Xenobiotic chemicals may mimic hormones or interfere with the radical cellular rearrangements that take place during metamorphosis (Hayes et al. 2002). Additionally, accumulated pollutants may effect metamorphosis and lead to an increased risk of predation (Marco and Blaustein 1999; Lefcort et al. 1998). Most studies of glyphosate have focused on acute effects or the effects of different formulations on larval growth and development but not metamorphosis (Smith 2001; Lajmanovich et al. 2003; Howe et al. 2004). Thus, we investigated the effects of chronic exposure to Roundup® (50.2%) at non-acute levels on *Rana cascadae* larval metamorphosis and development.

MATERIALS AND METHODS

Portions of different clutches of *R. cascadae* egg masses were collected from Table Mountain in Kittitas County, Washington (USA). In the laboratory, larvae were hatched from eggs housed in 10-L glass tanks at 18°C under incandescent light with a 12-hour photoperiod. Larvae were fed *ad libitum* with certified

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organic lettuce boiled for one minute in distilled de-ionized water. Two weeks after hatching, larvae were transferred to treatment containers. Experimental treatments were conducted in pre-rinsed plastic containers (30 cm x 20 cm x 10 cm). Larvae were randomized into treatments (seven individuals per treatment) consisting of a control (0 ppm), 1 ppm, and 2 ppm for nominal concentrations of glyphosate. There were five replicates of each treatment for a total of 15 containers. Container placement was randomized in the laboratory and each was tilted at an angle of approximately 30 degrees so the upper portion was dry and lower filled with 1 liter of treatment solution.

Amphibian larvae have varying sensitivities to different glyphosate formulations composed of the active ingredient (glyphosate) and surfactants, which are important for plant adsorption (Mann and Bidwell 1999; Lajmanovich et al. 2003). Surfactant compositions are generally unknown since they are proprietary trade secrets for the manufacturer. Treatment solutions (1 ppm and 2 ppm) were diluted from a 250 ml stock (50ppm) mixed from a widely commercially available glyphosate formulation (Roundup®, active ingredient 50.2 % glyphosate isopropylamine salt, Monsanto Company). Dilutions were made from spring water treated to remove chlorine, heavy metals and buffer pH. All five treatment replicates were mixed together in a 5000 ml beaker and then 1000 ml was dispensed into treatment replicates. Previous experiments with *R. cascadae* suggested the 48 hour LC_{50} to be 3.2 ppm (King and Wagner *submitted*). Therefore, dilution concentrations (1ppm and 2pmm) of the glyphosate formulation were chosen to be lower than acute concentrations in order to examine the chronic effects on metamorphosis.

Initial dilutions were based upon nominal values for glyphosate concentration, however, starting treatment concentrations of glyphosate (N-phosphonomethyl glycine) were determined by analytical analyses. Glyphosate concentrations were determined by Columbia Analytical Services (Kelso, WA) using EPA method 547 (Winfield et al. 1990). For glyphosate determination, a 200 µl sample was analyzed using cation exchange HPLC with separation by isocratic elution at 65°C. Then the analyte was oxidized with calcium hypochlorite to yield glycine and coupled with o-phtalaldehyde-2-mercaptoethanol at 38°C. Flourimetry detected the flourophor complex with excitation at 340 nm and emission above 455 nm. Analytical concentrations of 0.96 ± 0.13 (SE) and 1.94 ± 0.13 (SE) ppm were not significantly different from the estimated nominal dilutions of 1.0 and 2.0 ppm, respectively.

To determine herbicide formulation effects on development and metamorphosis of *R. cascadae*, larvae were evaluated on a daily basis for 43 days. Larvae were checked daily for mortality (time to death), feeding (feeding or not feeding), swimming activity (high, medium and slow), abnormalities (edema, lesions, bent tail), head out of water, erupted forelimbs, erupted hind limbs, and emersion from water into the dry part of the container. Mortality was determined by probing

individual larvae with a plastic pipette for responsiveness and swimming ability for 15 seconds. If individuals were not responsive they were assumed dead and removed from the container. Container treatments were replaced every seven days. Glyphosate concentration was analyzed for 1.0 ppm and 2.0 ppm treatments after seven days using EPA method 547 (Winfield et al. 1990). After 43 days, dry mass of surviving metamorphs was recorded to the nearest 0.001 grams.

Differences among replicates and treatments (0ppm, 1ppm, and 2ppm) for each factor were compared using one-way ANOVA and Tukey-Kramer Multiple-Comparison tests with NCSS (Hintze 2001). Mortality was examined as time to death (TTD) for each individual to allow for comparisons among replicates and treatments. Mean dry masses of surviving metamorphs for 0 ppm and 1 ppm were compared using a student's t-test with alpha set to 0.05.

RESULTS AND DISCUSSION

Low concentrations of Roundup® significantly effect *R. cascadae* larval development and metamorphosis in static renewal tests. Major effects on larvae include survivability, rate of metamorphosis, and post-metamorphosis mass. Survivability analyzed as time to death (TTD) demonstrated the greatest difference among treatments (Figure 1). In fact, over the course of the experiment (43 days) no individuals survived until metamorphosis when exposed to the highest concentration treatment (1.94 ppm) with a mean time to death of 7.5 ± 1.6 days. Tukey-Kramer comparison tests indicated a significant difference in time to death among all treatment groups (alpha = 0.5, CV 3.45) with 8.6% and 51.4 % mortality for 0.96 ppm and 1.94 ppm treatments, respectively. Mortality primarily occurred before metamorphosis events, head out of water and erupted forelimbs, with the dominant abnormalities of bent tails or slow swimming ability present before death. Further, no increase or decrease in rate of mortality was observed when treatment solutions were replaced every seven days. This suggests mortality occurred primarily due to chronic exposure and not acute conditions created by treatment evaporation, replacement or stress of transfer. In fact, glyphosate concentration in treatment solutions after seven days was not significantly differently (0.94 ± 0.13 and 1.92 ± 0.13 ppm) from initial dilutions 0.96 ± 0.13 (SE) and 1.94 ± 0.13 (SE) ppm, respectively. Additionally, other studies have observed no significant increase or decrease in glyphosate concentration in static renewal type experiments (Howe et al. 2004).

Timing of metamorphosis was significantly different in comparisons among the control and 1ppm treatment group (Figure 2). Treated (1ppm) individuals had earlier times for head out of water, erupted frontlimbs, erupted backlimbs, and emergence from water compared to controls (0ppm). The mean differences among treatments ranged from 3.95 days for emergence from water to 8.54 days for head out of water. In addition to earlier metamorphosis, mass after metamorphosis was significantly different among control (0ppm) and treated

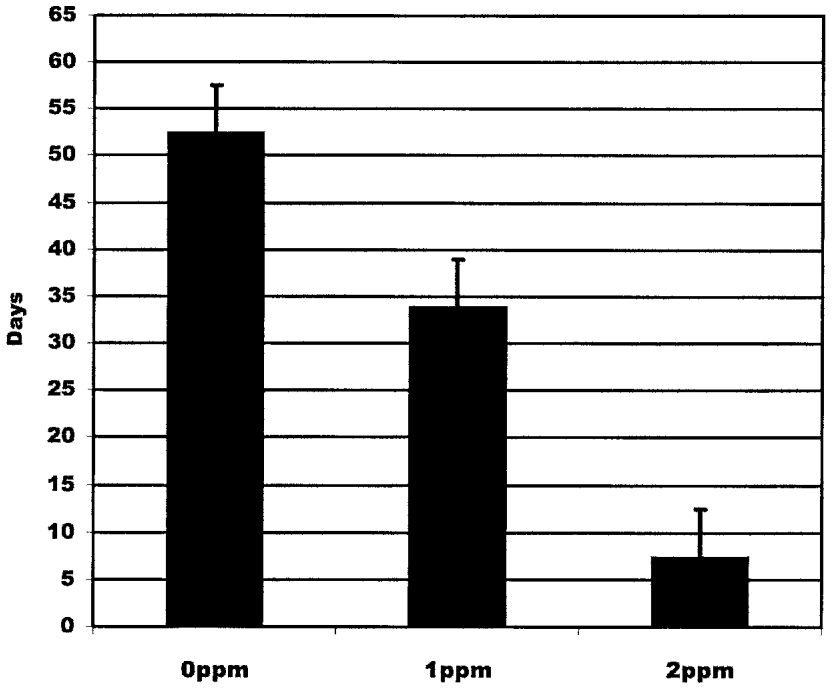

Figure 1. Effects of glyphosate on time to death (days) among *R. cascadae* larvae for three different treatments. There was a significant difference in time to death among all treatments (ANOVA, $F= 119.31$, $p< 0.01$). The standard error of the estimate is represented by error bars.

(1ppm) larvae ($F1,4 = 111.26$, $p < 0.00001$). The mean mass of treated larvae (1ppm) was 1.22 ± 0.02 grams compared to 1.59 ± 0.03 for control larvae (0ppm).

Several non-exclusive hypotheses may account for our results of earlier metamorphosis and a smaller size at metamorphosis for Roundup® exposed *R. cascadae*. First, glyphosate and/or surfactants may cause slower growth (lower metabolic rate) of larvae, accounting for increased mortality and smaller size at metamorphosis. It has been suggested that larvae must reach a particular minimum size or they will fail to undergo metamorphosis, which might account for increased mortality at a high concentration (Collins 1979). Next, herbicide formulations may mimic specific hormones or disrupt endocrine systems to increase the rate of metamorphosis resulting in a lower mass at metamorphosis (Howe et al. 2003). Finally, larvae under stress or suboptimal conditions may metamorphose earlier and at a smaller size as an adaptive response to escape to

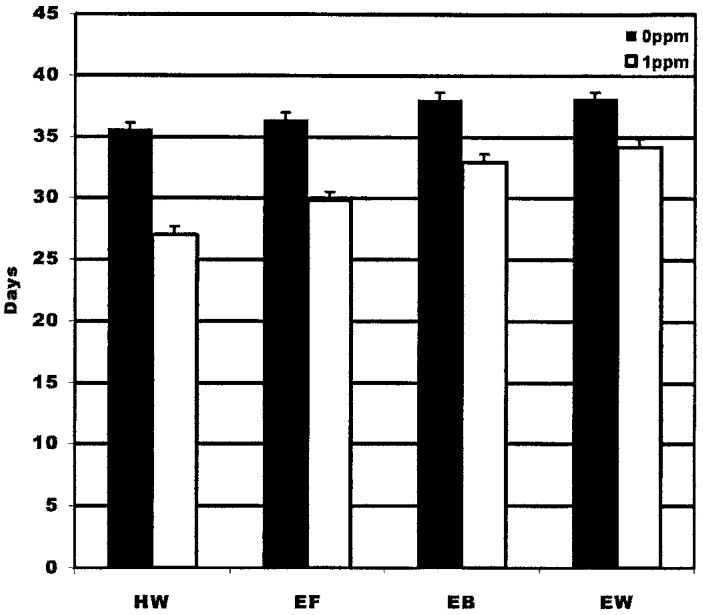

Figure 2. Time to major metamorphosis events measured in days. All metamorphosis events which included head out of water (HW), erupted forelimbs (EF), erupted backlimbs (EB), and emergence from water (EW) were significantly different among control (0ppm) and treated (1ppm) *R. cascadae* larvae ($F_{1,4} = 82.60$, $p < 0.00001$; $F_{1,4} = 67.33$, $p < 0.00001$; $F_{1,4} = 42.01$, $p < 0.00001$; $F_{1,4} = 24.36$, $p < 0.00001$; respectively). Bars represent standard error of the estimate.

better conditions (Smith-Gill and Berven 1979; Lefcort et al. 1997). However, the aforementioned hypotheses are non-exclusive with regards to our results because we cannot rule out a specific effect (potential hormonal mimic or disruption) from an overall global effect of stress or damage imposed by the herbicide. Thus, we suggest future studies investigate the potential hormonal effects of glyphosate formulations. In addition, future studies would benefit from examining the potential effects of glyphosate below 0.96 ppm to investigate the minimum effect concentration.

Indirect effects of chronic exposure to glyphosate formulations may include an increased risk of predation due to decreased size at metamorphosis. A previous study suggested sublethal exposure of *R. cascadae* to nitrates causes incomplete and earlier metamorphosis, potentially increasing their vulnerability to predation (Marco and Blaustein 1999). *R. cascadae* populations are in decline throughout

their range and other indirect effects may also include the interaction or synergistic effects of Roundup® with other stressors including nitrates, phthalic esters, pesticides, pathogenic fungus and UV-B radiation (Blaustein et al. 2003). Therefore, to help mitigate the potential effects of glyphosate applications we suggest its use in aquatic environments occurs at times that do not coincide with amphibian larval development and metamorphosis. It has been suggested later stage larvae may be less sensitive to glyphosate formulations (Smith 2001). Additionally, use of less toxic formulations of glyphosate such as Roundup® BiActive may help to mitigate risks to amphibian larvae (Mann and Bidwell 1999; Smith 2001). Therefore, it is suggested such formulations be used in areas where amphibian larvae are potentially sensitive.

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