SUB-LETHAL EFFECTS OF ROUNDUP ON TADPOLE DEVELOPMENT AND PREDATOR AVOIDANCE

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Abstract

Roundup is a commonly used pesticide applied to agriculture and forest habitats. In Canada and parts of the North Eastern United States, these areas are generally optimal for amphibians due to the presence of small, ephemeral water bodies. While Roundup has been shown to have no adverse effects on a number of species, amphibians are one of the few groups who show high sensitivity to Roundup. My research aims to determine how an acute sub-lethal dose of Roundup affects several different facets of larvae development in wood frogs (*Lithobates sylvaticus*). In Chapter 2 I examined the effect of Roundup on amphibian development. Groups of tadpoles were treated with Roundup (0.5 mg a.e./L) for four days at three different times in their development (Gosner stage 26, 31-32 and 37-38), while a control group was maintained in similar conditions without Roundup. Pictures were taken every four days until tadpoles reached metamorphosis. Changes in development, body area or length were assessed, along with metamorphic endpoints, such as timing of metamorphosis and weight. Although there was no differential effect of Roundup on tadpole growth or weight, there was a slight, non-ecologically relevant, delay in development in tadpoles treated with Roundup at stage 26. The delay was not detectable in the second half of the experiment, indicating that compensatory mechanisms allowed those individuals to recover. Neither time to metamorphosis or weight at metamorphosis were affected by an environmentally relevant exposure to Roundup. In Chapter 3 I focused on the effect of Roundup on crucial behaviours related to the ability of larval amphibians to detect and avoid predation threats. I demonstrated that being exposed to Roundup for one hour eliminated the response of larval wood frogs to cues from injured conspecifics (i.e. cues known to elicit dramatic anti-predator responses in a wide variety of aquatic species). Tadpoles that were maintained in clean water and exposed to a combination of injured conspecific cues and Roundup, did not exhibit a decrease in movement, when compared to control tadpoles. This result indicates that Roundup deactivates the alarm function of the injured conspecific cues. However, it is possible that both the cues and the animal would be affected by Roundup. In Chapter 3 I also demonstrated that tadpoles that were exposed to Roundup as embryos had reduced basal movement rates. The results of this thesis illustrate that an environmentally relevant concentration of Roundup (0.5 mg a.e./L), does not negatively affect the development of tadpoles. The studies outlined in this thesis suggest that at this exposure concentration, behaviour acts as a more sensitive endpoint, than more traditional morphologic endpoints.

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Chapter 1: Introduction

1.1 Amphibian Decline

Many amphibians are currently experiencing a global decline due to anthropogenic changes primarily associated with habitat loss and fragmentation. However, there are also other contributing factors associated with population declines including increases in UV-B radiation and pathogens, a changing climate, the introduction of novel species and environmental pollution (Wake and Vredenburg 2008). Owing to their high site fidelity, permeable skin, need for both terrestrial and aquatic habitats, and complex life cycles, amphibians are more likely than most other taxa to be at increased risk to many stressors (Blaustein and Wake 1995, Daszak et al. 1999, Wake and Vredenburg 2008). Currently, amphibians may be going extinct at a rate up to 211 times the background extinction rate, when compared to extinction rates observed in the fossil record (McCallum 2007). This means that amphibians are often used as biomarkers of habitat quality (Pollet and Bendell-Young 2000). Many amphibians including salamanders, frogs and toads are numerous in ecosystems and are inexpensive to sample, making them excellent and convenient indicators of biodiversity (Welsh and Droege 2001).

1.2 Pesticide Use

The use of pesticides and herbicides both on agricultural and forested land is common practice. Worldwide, approximately 2.5 million tonnes of pesticides are used with the number of different pesticides in a given area being as high as 600 (Pimentel et al. 1992). In addition, many common chemicals used for commercial and household applications are applied to millions of hectares of land per year (Tome et al. 1991). Many of these products affect non-target species. Aquatic environments are often contaminated due to overspray from other applications and as the result of runoff.

In Canada, pesticide use is regulated by both federal and provincial governments. Companies that sell pesticides must submit detailed studies documenting three aspects of the chemical: health risks, environmental risks and merit of the product (Health Canada, Pesticide Management Regulatory Agency). For health risks, toxicological studies must investigate acute and chronic effects, carcinogenicity and teratogenicity, as well as how much exposure users could safely experience per day. Environmental risk includes the fate of the chemical in the environment and its overall toxicity. Environment Canada uses farm-raised rainbow trout (*Oncorhynchus mykiss*) and daphnia (*Daphnia magna*) as test organisms for aquatic habitats (Levine 2003). Most larval and adult amphibians are not taken into consideration when aquatic and terrestrial toxicity testing are required for pesticide usage by the government (Levine 2003). This creates a situation where amphibians are not directly protected from pesticides, the consequences of which are largely unknown.

Herbicides are commonly used throughout Canada, with the Prairie provinces of Manitoba, Saskatchewan and Alberta, being the heaviest users of these chemicals. In 2001, farmers in Saskatchewan alone applied 30.5 million acres of land with herbicides. This included over 71% of farmlands in the province. In total 57% of farmers used fertilizers with an additional 10% using fungicides and insecticides. While the use of fungicides is on the decline, insecticide use since 1996 has increased 1.7% and herbicide usage has increased almost 10% (Saskatchewan Agriculture, Food and Rural Revitalization, 2002).

Glyphosate (including Roundup) has been the most commonly used pesticide since 2001; 83 million kilograms were used worldwide in 2007 (Grube et al. 2011). Glyphosate resistant crops (i.e. Roundup Ready® canola, soybean, corn, wheat and others) are the most commonly used transgenic crops used in the world, representing 80% of the market (Duke and Powles 2009). These resistant crops allow farmers to spray crops a second time, killing weeds, while not damaging their harvest. Historically farmers would spray Roundup just before seeding, to “burn down” weeds prior to seeding. With Roundup resistant crops, Roundup is sprayed pre seed, during the emergence and flowering stages of the resistant crop, and once pre-harvest. This effectively kills weeds multiple times a growing season to increase production of crops. As a result, there has been a growing trend in the United States to use glyphosate-resistant plants, leading to inevitable increases in glyphosate use (Dill et al. 2008, Duke and Powles 2009).

Roundup is applied to fields at a rate between 0.20 and 2.8 L/acre depending on the concentration of the formulation and the target weed. The maximum concentration of Roundup in surface waters was found to be as high as 7.6 mg a.e./L and estimated to be as high as 2.7 mg a.e./L when sprayed over wetlands in a controlled direct overspray experiment (Edwards et al. 1980, Solomon and Thompson 2003). In Saskatchewan, for pre-seed application, Roundup can be applied aerially and requires a buffer zone of 30 metres for aquatic habitats and 70 meters for terrestrial habitats (Saskatchewan Ministry of Agriculture, 2011 Guide to Crop Protection).

1.3 Roundup: Mode of Action and General Toxicity

Roundup is a broad spectrum, post-emergent leaf and grass killing herbicide. The active ingredient in Roundup is glyphosate acid bound to one of five types of salts (Folmar et al. 1979). Isopropylamine salt (IPA) is the most commonly used salt bound to glyphosate. Potassium salt (K+), also commonly bound to glyphosate, allows for a higher loading of glyphosate in solution. The highest concentration available with IPA salt is 450 g a.e./L glyphosate, while potassium salt can achieve concentrations of 540 g a.e./L glyphosate. Roundup concentration is expressed in acid equivalents per liter (a.e./L) which is the amount of parent acid (the acid after being dissociated from its salt) present in the herbicide. This allows effectiveness to be compared between glyphosate formulations with different salt backbones. These different salts can increase the active ingredient per liter (due to added molecular weight), but will not increase effectiveness, as the salts do not bind to the plant causing death. Formulations of Roundup also contain a surfactant that allows the herbicide to penetrate through the waxy cuticle of the leaf. Each formulation of glyphosate will contain one of the five salt backbones; a concentration of glyphosate from 356-540 g a.e./L (for agricultural grade formulations) and an unknown surfactant. One commonly used surfactant is POEA (polyethoxylated tallowamine). Once in the leaf, the glyphosate disrupts aromatic amino acid synthesis of phenylalanine, tryptophan, and tyrosine, killing actively growing plants (Schulz et al. 1984). The half-life of glyphosate in surface water is estimated at four to 70 days depending on water conditions, such as pH, temperature and water flow. Roundup is highly water soluble therefore dissipates rapidly in ponds thus making the half-life between two and 11 days (Schuette 1998).

The popularity of Roundup is increasing with the generation of Roundup resistant crops, that are resistant to the effect of the herbicide. These crops are able to withstand the effect of Roundup, while the surrounding weeds are killed by the herbicide. With an increase in use, there is increased concern about human health. Roundup is generally non-toxic to mammalian species under normal circumstances. However, Roundup (glyphosate with a surfactant in formulation) has been shown to be lethal to humans, but only in very high doses (Stella and Ryan 2004). These high doses have only been observed in suicide attempts. Accidental and occupational related exposures have not been shown to cause mortality in humans. However, glyphosate formulations, when ingested, can cause gastrointestinal distress, ulceration, evidence of hepatic or renal damage and in more severe cases (suicide attempts), can result in cardiac arrest, coma and seizures. Historically Roundup was believed to be non-toxic to humans (Williams et al. 2000). This was confirmed by numerous health organizations (Williams et al. 2000). However, more recently, glyphosate paired with surfactants have been shown to cause mutagenic and toxic effects in human reproductive cell lines (Gasnier et al. 2009), but accidental exposure still shows no mortality (Stella and Ryan 2004, Gasnier et al. 2009).

Although relatively innocuous in mammalian species, Roundup has been found to be lethal in some aquatic species. The lethality of Roundup to aquatic organisms, and most toxicants, is measured in amount of chemical needed to kill 50% of a population, or LC50 (lethal concentration). A study conducted by Tsui and Chu (2003) showed that Roundup can be lethal to specific microbes and crustaceans. The effect in some species is due to the active ingredient (glyphosate) but in most cases, a result of the surfactant (POEA). POEA was toxic to all organisms tested (bacteria: *Vibrio fischeri*, algae: *Selenastrum capricornutum* and *Skeletonema costatum*, ciliates: *Tetrahymena pyriformis* and *Euplotes vannus*, cadocerans: *C. dubia*, and copepods: *Acartia tonso*). Growth endpoints in all non-bacterial species had IC50's, half maximal inhibitory concentration (tested between 40 and 96 hours) of 10.2 mg a.e./L to 3.35 mg a.e./L and *C. dubia* and *A. tonso* having LC50's (96h) ranging from 0.57 and 1.15 mg a.e./L (Tsui and Chu 2003). Glyphosate alone was not toxic to all organisms.

Roundup is moderately toxic to some fish species, with fathead minnows having a LC50 of 2.3 mg a.e./L (Folmar et al. 1979). This is more lethal than the LC50 value in both rainbow trout (8.3 mg a.e./L) and channel catfish (13 mg a.e./L) (Folmar et al. 1979). Overall, the toxicity of Roundup to some aquatic species (example: *C. dubia*) depends considerably on the pH of the water, with lethality being lowest around seven and increasing as pH is increased and decreased to nine and six respectively (Tsui and Chu 2003). Due to the high exposure concentration needed, the LC50 generally equals or exceeds the maximum environmentally relevant concentration of 2.7 mg a.e./L.

1.4 Amphibian Toxicity to Roundup

The lethal impact of Roundup on amphibians and their communities is well documented (Relyea 2003, 2005c, Jones et al. 2010). Studies not only show that Roundup can cause mortality on its own (LC50 of 1.32 mg a.e./L on a stage 25 wood frog, and LC50 of 1.4 mg a.e./L on green frog larva) but when mixed with another commonly used chemical, malathion (an organophosphate insecticide), its lethality increases significantly (Relyea 2003, Edginton et al. 2004). The LC50 values of Roundup alone make it a moderately to highly toxic compound to amphibians. These LC50 values are also well under the accepted environmentally relevant concentration (estimates of a worst case scenario from a direct overspray of a wetland) of 2.7 mg a.e./L, meaning amphibians may be exposed to lethal concentrations of Roundup (Solomon and Thompson 2003).

Synergistic effects of Roundup with other non-agricultural chemicals are also present. A study by Relyea (2003) showed that when agricultural chemicals and predator odours (odors elicited by a predator that can warn a prey species) are combined, they can collectively quadruple the lethality of the pesticide to grey tree frogs (*Hyla versicolor*), green frogs (*Lithobates clamitans*) and bull frogs (*Lithobates catesbeiana*). These lethal concentrations fall within environmentally relevant exposure concentrations of 0.1 – 2.7 mg a.e./L (Relyea 2004). Exposure periods of between four (acute) and 16 (sub-chronic) days are commonly used when testing the effect of Roundup and are considered environmentally relevant due to the half-life of Roundup and its surfactant POEA being two to 11 days and four to 70 days respectively (Howe et al. 2004). Both pesticides and predator cues are expected to be present in nature at any given time suggesting the actual lethality of Roundup could be much higher than estimated.

1.5 Study Species: *Lithobates sylvaticus*

Wood frogs (*Lithobates sylvaticus*) are a wide ranging and very common member of the Ranidae family of true frogs. They occupy a diverse spectrum of habitats ranging from peat bogs, upland habitats, forests and many ephemeral ponds. These frogs are explosive breeders and can lay between a few hundred to several thousand eggs per breeding season. Wood frogs breed in early spring, thus are usually the first frogs to breed. This adaptation allows them to utilize ephemeral ponds. After hatching, the tadpoles take anywhere from six to 15 weeks to reach metamorphosis depending on temperatures and drying conditions (Crump 1989).

During the tadpole phase, wood frogs forage on leaf litter, detritus and also cannibalize eggs and larvae of conspecifics and other amphibians (Jefferson et al. 2013). Wood frogs actively choose to lay their eggs in waters free of fish (Hopey and Petranka 1994). Although fish are not present, invertebrate predators, salamanders and other aquatic predators still pose a risk to tadpoles (Relyea 2002, Jefferson et al. 2013) . Because of their habitat choice (ephemeral ponds with many different predator types), wood frog tadpoles have evolved high phenotypic plasticity in their anti-predator morphology to combat ever changing predator risks (Relyea 2002).

Wood frogs are ideal subjects for toxicological and behavioural research due to their vast geographical range and their ability to occupy many habitat types. They are also able to recognize an attack on a conspecific from chemical cues (Ferrari et al. 2009). Wood frogs inhabit many habitats that are exposed to spraying of Roundup and other agricultural chemicals due to their preference to breed in ephemeral ponds. In Saskatchewan, these ephemeral ponds can be small sloughs, ditches and potholes that generally only last part of the season before drying up. Wood frogs are easy to collect because of their explosive breeding behaviour and high biomass. They also have a short larval period that corresponds to spraying times, making wood frogs excellent subjects for work of this nature.

1.6 Amphibian Development

Many amphibians, including wood frogs, have bi-phasic life cycles consisting of an aquatic tadpole phase and a semi-aquatic or terrestrial adult phase. A table proposed by Gosner (1960) has been developed to determine the developmental stage of North American peolobatids, bufonids, hylids and ranids up to metamorphosis. This staging system categorizes the tadpoles from stage one (newly fertilized egg) to 46 (metamorphosis). These 46 stages each have characteristics that are unique and can determine developmental progress. From stages one to 24, the animal is embryonic; stages one to 17 being egg stages. At stage 17-20 depending on the species, the embryo emerges from the egg and continues to feed off the yolk sac until stage 25. During these stages, gills are reabsorbed and mouthparts develop. The tadpole is now independently feeding and swimming. During stages 23-25, the tadpole begins to develop body pigment. This also varies by species. From stages 25-30 limb buds are formed and length/diameter ratios of these limb buds are the determining characters for identification. Pre-metamorphosis are the early stages of development from stage one to 30. From stages 30-40, toe formation on these limb buds occurs. Toes will differentiate from paddle-like limbs to individual toes. During stages 38-40, the individual lengths and proportions of each toe are identifying features. This period is known as pro-metamorphosis. From stage 40 to metamorphosis, the tadpole begins to resemble a frog and gradually reabsorbs its tail; mouth and head features change dramatically. Changes that occur between stages 40-46 are the most dramatic. The total length of the animal decreases due to reabsorption of the tail; the cloacal tail piece disappears and larval mouth parts break down. Stages 42, 43 and 44 track the change in mouthparts and the length of the mouth in relation to the eye. At stage 45, the tadpole is basically a frog with a small tail attached. At stage 46, the froglet no longer has any resemblance to a tadpole and is considered terrestrial (Gosner 1960). This rapid change from a tadpole to a frog (stage 40-46) is known as the metamorphic climax.

1.7 The Effects of Roundup on Development

The effect of Roundup on development of amphibians is relatively undocumented, and many studies rely on Gosner stage 25 larvae (first free feeding stage) for use in toxicological testing (Edginton et al. 2004, Relyea 2004). Stage 25 tadpoles are easy to care for, plentiful, and little mortality occurs between hatching and stage 25. They require less time to raise compared to their older counterparts. Although using stage 25 tadpoles is common practice when studying larval amphibians, the developmental effects of many common herbicides and pesticides are mainly unknown. A study by Howe et al. (2004) showed that Gosner stage 20 (free- living, feeding off yolk sac) tadpoles of four species of North American frogs (green frogs, bull frogs, wood frogs and northern leopard frogs) were more resistant to Roundup than the same species at stage 25. Other studies have shown similar results hypothesizing that the effect is caused by lack of gill development (Edginton et al. 2004). This may suggest that younger, non-feeding stages are slightly more resistant to pesticides (Edginton et al. 2004). This study also showed an increase in time to metamorphosis, necrosis of the tail tip resulting in decreased tail length and decreased snout-vent length in exposed tadpoles. Decreased tail length, deformities and mutilation by other tadpoles can cause reduced survival rates due to inability to effectively avoid predators. Although these studies give valuable information on the developmental effect of Roundup exposure at stages 20 and 25, the effects exposure to this chemical at different stages of development are unknown.

1.8 Amphibian Anti-Predator Responses

An organism's ability to detect predators is crucial for survival and we know that many organisms have the ability to smell predators and odours associated with predation (Flowers and Graves 1997, Chivers et al. 1999, Belden et al. 2000). Kairomones (chemicals released from predators), disturbance cues (chemicals released from startled individuals), and alarm cues (chemicals released by individuals injured by a predator) are all chemical information sources on which aquatic species may rely. Amphibians, such as larval wood frogs, live in environments where predator composition is constantly changing due to the variable ecological composition of ephemeral ponds. Many aquatic prey, including larval amphibians, use the presence of injured conspecific cues as a sign of imminent danger, and display strong anti-predator behaviour upon detection of those cues (Ferrari et al. 2010). There are many factors affecting the efficiency of use of injured conspecific cues including concentration of the cue, distance from the cue, and association of the cue with other threats. Overall, concentration is likely the most important factor, as many organisms react to these chemical signals in a concentration dependant factor; the higher the concentration the stronger the behavioural response (Ferrari et al. 2009). Larval amphibians reduce their activity level and habitat usage (i.e. foraging) when faced with these generalized injured conspecific cues (Bridges 1999). Thus, the ability of prey to properly detect risk via these cues in their environment is of utmost importance to optimize foraging while avoiding predation.

1.9 Effects of Roundup on Predator Avoidance

Roundup has been shown to impact olfaction in some aquatic species. For instance, Roundup blocks olfaction in Coho salmon (Tierney et al. 2006). In that study, 1 mg a.e./L glyphosate acid causes significant reduction in electro-olfactogram (EOG) reading (within 10 minutes) in Coho salmon. This raises the possibility that glyphosate, in sub-lethal concentrations could inhibit anti-predator responses in larval amphibians due to alteration of some olfactory functions. Despite what is known about the effects of Roundup on amphibian survival and morphology, much work is still needed to understand how sub-lethal exposures to Roundup would affect crucial behaviours, such as predator avoidance.

1.10 Research Objectives

My overall objective was to determine the sub-lethal effects of Roundup on developing tadpoles. In my thesis, I present two distinct data chapters showing the effects of Roundup on tadpole development and the effects of Roundup on the ability to detect injured conspecific cues in the presence of this commonly used herbicide. I used wood frog tadpoles to answer the following questions:

Which larval stages are most susceptible to a single exposure of Roundup? Many agricultural chemicals are known to have an adverse effect on amphibian development and physiology. I tested the ability of Roundup at an environmentally relevant concentration of 0.5 mg a.e./L to alter the developmental processes of wood frog tadpoles at different stages in their development (null hypothesis- Roundup has no differential effects on development).

Does having Roundup in the environment alter a tadpole's ability to respond to injured conspecific cues? To date, no studies have tested the effect of this herbicide on threat recognition in an amphibian species. My objective was to determine if Roundup has the ability to alter a tadpole's ability to detect general threats through injured conspecific cues (null hypothesis- Roundup has no effect on the response to injured conspecific cue). If there is an effect, I plan to determine if Roundup influences the organism's ability to detect the cue or if it alters the chemical cue itself.

Chapter 2: Effects of an Acute Environmentally Relevant Exposure to Roundup on the Development of Wood Frog Tadpoles

2.1 Introduction

The larval stages of amphibian development represent a relatively short, but critical, portion of their life span. Many different factors affect how amphibians develop during their larval stage. For example the rate of pond drying, predator activity, density of conspecifics, temperature all contribute to how these species develop, with implications for survival later in life (Wilbur 1987). Added stressors during the larval period can result in reduced weight at metamorphosis and increased time to metamorphosis, both of which can be costly to the organism due to reduced overwintering success and desiccation due to pond drying respectively (Skelly 1996). Tadpoles experience size-based predation, meaning growth lags can increase predation, as the tadpole cannot outgrow its predators (Wilbur 1980). If an introduced chemical causes delays in growth, tadpoles may experience increased predation (Carey and Bryant 1995).

The effect of Roundup on tadpole development is not well studied. Many studies use tadpoles that are stage 25 for toxicology testing, due to ease of use and identification. Roundup has been shown to increase mortality and the time needed to reach metamorphosis (Cauble and Wagner 2005). In most studies, the concentration of Roundup is the factor that is manipulated experimentally, with developmental stage being a fixed variable. Few studies have tested the effects of Roundup amongst different developmental stages. To my knowledge, no studies have looked at the effect of Roundup on later stage tadpoles (30-46). A study by Edginton et al (2004) showed that embryonic tadpoles (stage eight to 25) are more resistant than stage 25 tadpoles, but tadpoles past stage 25 were not included. A recent study by Jones et al. (2010) had similar results to Edginton et al. (2004), showing that tadpoles later in ontogeny were slightly more resistant to the lethal effects of a single exposure of Roundup. The effect in this study was hypothesised to occur because of one of two mechanisms: either wood frog tadpoles are more sensitive early in development or the group exposed early being exposed longer, had an increase in mortality due to increased exposure time (Jones et al. 2010). Since most of the mortality in this study occurred between one and three days in both treatment groups (early and late), it is possible earlier stages are more resistant to the lethal effects of Roundup exposure.

Although the effect of Roundup on different developmental periods of tadpoles is mostly unknown, there are a few studies that focus on this effect with other chemicals. A common agricultural chemical, carbaryl, has been shown to stimulate metamorphosis in green frog tadpoles (Boone and Bridges 2003). This increased time to metamorphosis, which is deleterious to green frog tadpoles as they generally overwinter, has been shown to occur to a greater extent when exposed early in development rather than later. As carbaryl has also been shown to reduce mass and survivability over time in tadpoles, this effect is magnified the earlier a tadpole is exposed (Bridges 2000). These results are not observed with alachlor and atrazine. These chemicals both become less lethal when American toads and leopard frogs are exposed later in development (Howe et al. 1998). A similar result can be observed in LC50 values for copper sulfate in *Epidalea calamite* tadpoles at Gosner stage 3, 19 and 25, with stage three tadpoles more resistant than their more developed counterparts (García-Muñoz et al. 2009). The susceptibility to chemicals throughout amphibian development is poorly understood and no general consensus has been reached. Knowing how chemicals affect an animal's development and which areas of development are most susceptible, provides crucial information on application times and the effect on animals. This fact is crucial, especially when considering chemicals can be applied multiple times per season. Therefore, this information regarding Roundup is extremely important and has the potential for changing application practise worldwide.

The objective of this study was to determine the sensitivity of tadpoles beginning with stage 25 (free feeding) while including their later developmental stages, up to metamorphosis. To achieve this, tadpoles were exposed to Roundup at a concentration of 0.5 mg a.e./L at three different stages in their development. Tadpoles were exposed as they reached stages 25, 31, and 37-38. These stages all mark important developmental periods including free feeding, limb bud formation and toe formation. This study will help determine which stages of tadpole development are more resistant, and also include tadpoles post stage 30. This is important as Roundup use in North America is on the rise. Also, the advent of Roundup resistant crops, means spraying is now occurring more than once per season. This study will test for the possible effects of both the first and subsequent applications of Roundup on individual wood frog tadpoles.

2.2 Methodology

2.2.1 Collection and Care

Wood frog egg masses were collected from multiple ephemeral ponds north east of Saskatoon (30 to 50km) in early May. They were collected in a block of land bordered by township road 410 (north) and Neuhorst road (south) and highway 12 (west) and highway 11 (east). The egg masses were gently collected by hand and put in 1 L containers and kept cool. Egg masses were collected in roadside ponds during the first week of May. These ponds would not have been sprayed by agricultural chemicals during the collection season, as planting/weed burn-off had yet to occur. The egg masses were then brought back to the University of Saskatchewan where they were assigned to four outdoor 65 L stock tanks. Densities of eggs masses per container were roughly six egg masses per 65 L. Water changes were performed on the stock tanks every two days with half the water being renewed. All water used was from the municipal water supply of the City of Saskatoon but was dechlorinated with activated charcoal. Tadpoles in these tanks were fed ad libitum with commercial rabbit food after hatching. Tadpoles were raised until stage 25 and were then used in the experiment and kept for additional experiments.

2.2.2 Roundup Preparation

Roundup Weathermax (Monsanto, Winnipeg, Canada) was purchased from an agricultural chemical supply store near Saskatoon in 2011. Roundup Weathermax has a base concentration of glyphosate of 540 g a.e./L. This base solution was diluted to a stock solution of 5000 mg a.e./L (pH of 4.7) and was aliquoted into 0.675 ml allotments (allowing for ease of dilution in a set container) which were stored in air tight micro-centrifuge tubes at room temperature in a dark cabinet. These micro-centrifuge allotments were also stored in airtight containers. These aliquots were later diluted down to a concentration of 0.5 mg a.e./L for use in the following experiments. This exposure concentration was chosen to be a sub-lethal concentration based on multiple studies testing the toxicity of Roundup to wood frogs (Relyea 2003, Edginton et al. 2004, Relyea 2005c, b). All concentrations listed are nominal concentrations as actual concentration was not analysed.

2.2.3 Developmental Effects of a Single Acute Exposure of Roundup

Tadpoles were removed from the outdoor stock tanks and staged. Tadpoles were then placed in a weigh boat with a ruler beside it for scale (millimeter to pixel ratio) and were photographed with a digital camera on a small tri-pod at a set distance of 12.5 cm, for baseline morphometrics. The photographs were later analysed using ImageJ (U.S. National Institutes of Health, Bathesda, U.S.A.) in a non-invasive methodology outlined by Davis et al. (2008). Tadpoles were then randomly assigned to individual plastic containers (13.5 cm x 13.5 cm x 10 cm) with transparent lids. Each tadpole had its own plastic container and was considered a single replicate. The containers were then put on a single uniformly lit shelving system with a conformation of four tanks deep by 20 tanks wide. The position of each tank was randomly determined. The photoperiod of the room was 16:8 (light: dark) to correspond with the local photoperiod, and tanks were kept at just below ambient temperature, which for June and July ranged from 17-20 °C, water temperature. During testing, tadpoles were fed *ad libitum* with rabbit food (Great Choice®, Pheonix, U.S.A.) and complete water changes (with dechlorinated water) were performed every two days with food being renewed at this time to avoid water fouling. During water changes, tadpoles are removed into a fine mesh net, the water was changed completely and the tadpole was quickly placed back into the newly changed water (Roundup water or clean water). Food was then added to insure that tadpoles had continuous access to food during the experiment.

Treatment groups consisted of a control (no Roundup), a group exposed at stage 26, a group exposed at stage 31-32 and a group exposed at stage 37-38. There were 20 replicates per treatment. All dosed tadpoles received exposure to 0.5 mg a.e./L of Roundup for four days. Treatment groups were exposed when >80% of the tadpoles within that treatment group fell within the given stages. This treatment coincided with a water change and the clean water was removed and replaced with the solution of Roundup. This exposure was renewed with new Roundup after two days (at the same time as the regular water changes of the other tadpoles). Every fourth day, photos were taken (at the same time as the water change to avoid unnecessary stress) of all tadpoles for later morphometric analyses. Any mortality was recorded and any deformities were noted. As the tadpoles reached stage 42, one side of the container was elevated (approximately 30°), feeding ceased and the containers were lined with paper towel. Water level was also lowered. This provided the metamorphs with a half-aquatic/half-terrestrial habitat. After metamorphosis was complete, the tadpoles were euthanized with an overdose of MS-222, photographs were taken and the metamorphs were weighed to the nearest milligram.

On day 0 (May 24th 2013) the first treatment group of tadpoles were exposed at stage 26. Treatment group two (stages 31-32) was exposed on day 16 (June 9th), and treatment group three was exposed on day 24 (June 17) when they reached stage 37 or 38. The experiment finished on July 11th.

Photographs were analysed using ImageJ via methodology proposed by Davis et al. (2008). Due to the invasiveness of traditional weight and length measurements, this non-invasive methodology was used to help keep handling stress and associated mortality to a minimum. Constant handling and traditional measuring of tadpole weight: remove tadpole from water, blot dry and weigh with a scale, exposes tadpoles to undue stress. This design includes taking measurements every four days, and this additional stress would severely alter the desired outcome. Tail length, body length and body area were all calculated, using ImageJ, to the nearest millimeter for lengths and square millimeter for area. Tail length was deemed too inaccurate to use in analysis due to the tadpole moving the tip of the tail. As the tadpole steadied itself in the weigh boat, the tip of the tail moves in a non-uniform matter, causing it to blur in the photograph, thus rendering the measurement inaccurate.

2.2.4 Statistical Methodology

All parametric assumptions were met for this dataset. To test the effects of the four different Roundup treatments on growth (developmental stage, body area and body length), a repeated measures ANOVA was used, with treatment as a fixed factor and time as the repeated measure. Multiple repeated measures were run on each parameter (stage, body area and body weight) testing all time points (13 total), an early time series (days 0, 8, 16, 24) and a late time series (days 28, 32, 44, 48). A one-way ANOVA was used to test the differences in treatments in time to metamorphosis and weight at metamorphosis. SPSS (IBM Corp. Chicago, U.S.A.) was used for all statistics performed in Chapter 2.

2.3 Results

A 2-way repeated measures ANOVA revealed a significant effect of time ($F_{12,735}$=0.612 $P \leq 0.001$), no effect of treatment ($F_{3,68}$=1.304 P=0.280) and no interaction ($F_{36,735}$=4.818 P=0.965) on the developmental stage of tadpoles (Figure 1). The effect of time simply indicates tadpoles were developing during the experiment. This analysis used all 13 time points and has the effect of reducing power; consequently the analysis was split into an early (days 0-24) and late (days 28-48) period for further analysis. This time the results of the 2-way repeated measures ANOVA showed a significant effect of time ($F_{3,208}$=0.482 P≤0.001), a significant effect of treatment ($F_{15,123}$=3.014 P=0.036) but no interaction ($F_{9,208}$=0.807 P=0.610) on the developmental stage of tadpoles early in their development (Figure 2). Tukey post hoc tests show that treatment group one (exposed at stage 26) differed from all the other groups (P=0.020). For late stage development, a 2-way ANOVA showed a significant effect of time ($F_{3,184}$=550.482 P≤0.001), no effect of treatment ($F_{4,578}$=0.145 P=0.932) and no interaction ($F_{6,251}$=7.623 P=0.853). This indicates that tadpoles exposed early in development were slightly delayed in their development up to day 28, but were able to catch up with the growth of the other tadpoles.

A 2-way repeated measures ANOVA revealed a significant effect of time ($F_{12,695}$=387.342 P≤0.001), no effect of treatment ($F_{3,70}$=1.279 P=0.288) and no interaction ($F_{36,696}$=0.530 P=0.990) on the body area of tadpoles (Figure 4). The effect of time again indicates tadpoles were growing during the experiment. This analysis was again split into an early (days 0-24) and late (days 28-48) period for further analysis. A significant effect of time was found ($F_{3,202}$=663.935 P≤0.001), but no effect of treatment ($F_{3,70}$=1.608 P=0.195) and no interaction ($F_{9,203}$=0.336 P=0.962) on the body area of tadpoles early in their development. For late stage body area, a 2-way ANOVA showed a significant effect of time ($F_{3,136}$=21.591 P≤0.001), no effect of treatment ($F_{3,51}$=0.312 P=0.817) and no interaction ($F_{9,137}$=0.553 P=0.833).

A 2-way repeated measures ANOVA revealed a significant effect of time ($F_{12,653}$=105.596 P≤0.001), no effect of treatment ($F_{3,60}$=2.146 P=0.104) and no interaction ($F_{36,654}$=0.781 P=0.819) on the body length of tadpoles. The effect of time indicates tadpoles were growing during the experiment. Again the analysis was split into early (days 0-24) and late (days 28-48) groups for further analysis. The results of the 2-way repeated measures ANOVA showed a significant effect of time ($F_{3,213}$=1289.739 P≤0.001), no effect of treatment ($F_{3,71}$=1.975 P=0.125) and no interaction ($F_{9,214}$=0.375 P=0.946) on the body length of tadpoles early in their development. For late stage body length, a 2-way ANOVA showed no effect of time ($F_{3,102}$=0.736 P=0.533) no effect of treatment ($F_{3,35}$=1.048 P=0.383) and no interaction ($F_{9,103}$=0.736 P=0.922).

There was also no effect of treatment on weight at metamorphosis ($F_{3,37}$= 0.931 P=0.435) or time to metamorphosis ($F_{3,40}$= 0.799 P=0.502) (Figure 3).

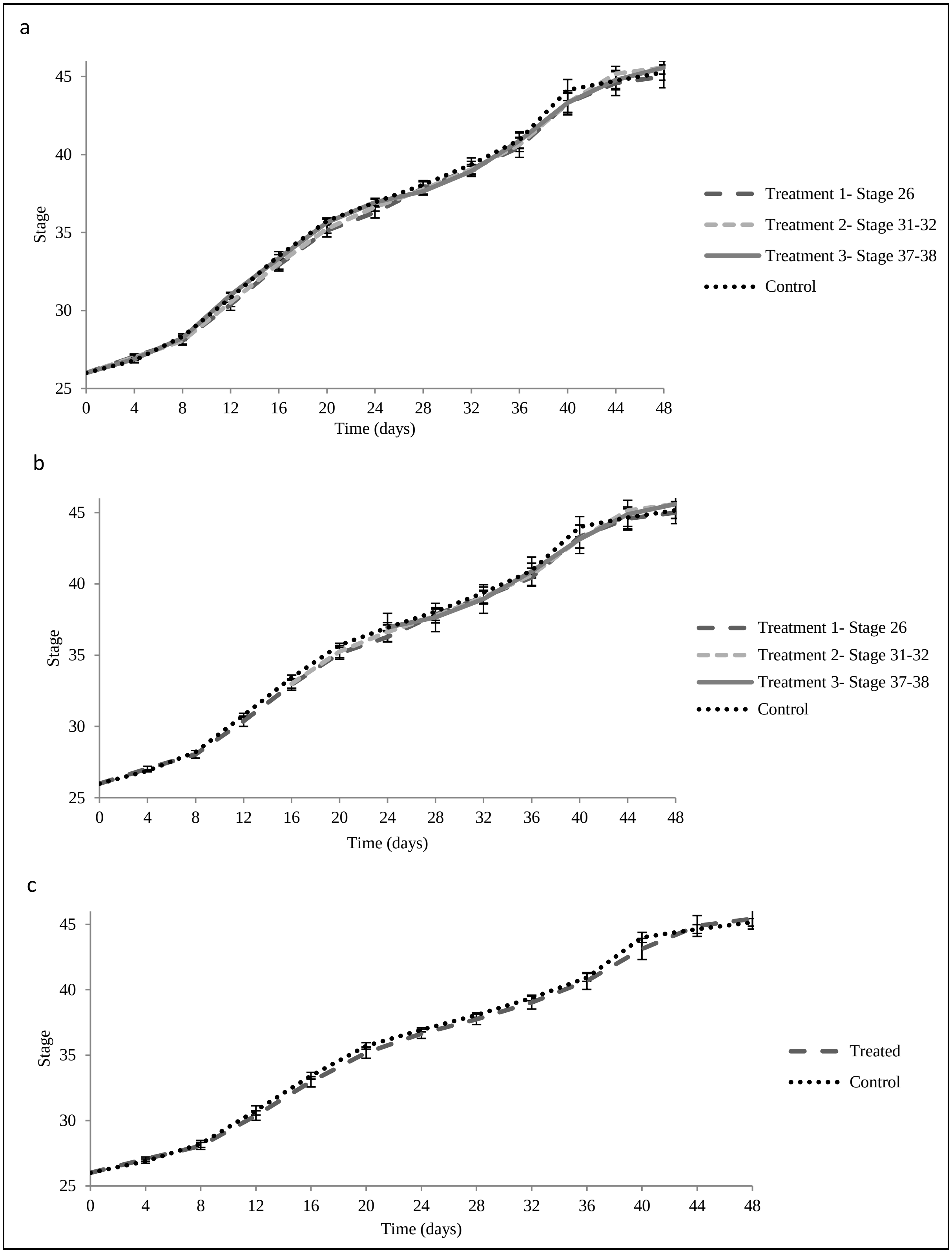

Figure 1: *(a) Development of tadpoles over time with three possible Roundup treatments (0.5 mg a.e./L) at different stages of development (mean ± SE). (b) Development of tadpoles over time with three possible Roundup treatments (0.5 mg a.e./L) at different stages of development (mean ± SE) with all non-exposed tadpoles treated as controls. (c) Development of tadpoles over time with all treatment groups (solid line-exposed with 0.5 mg a.e./L Roundup) combined vs. control (dotted line)(mean ± SE).*

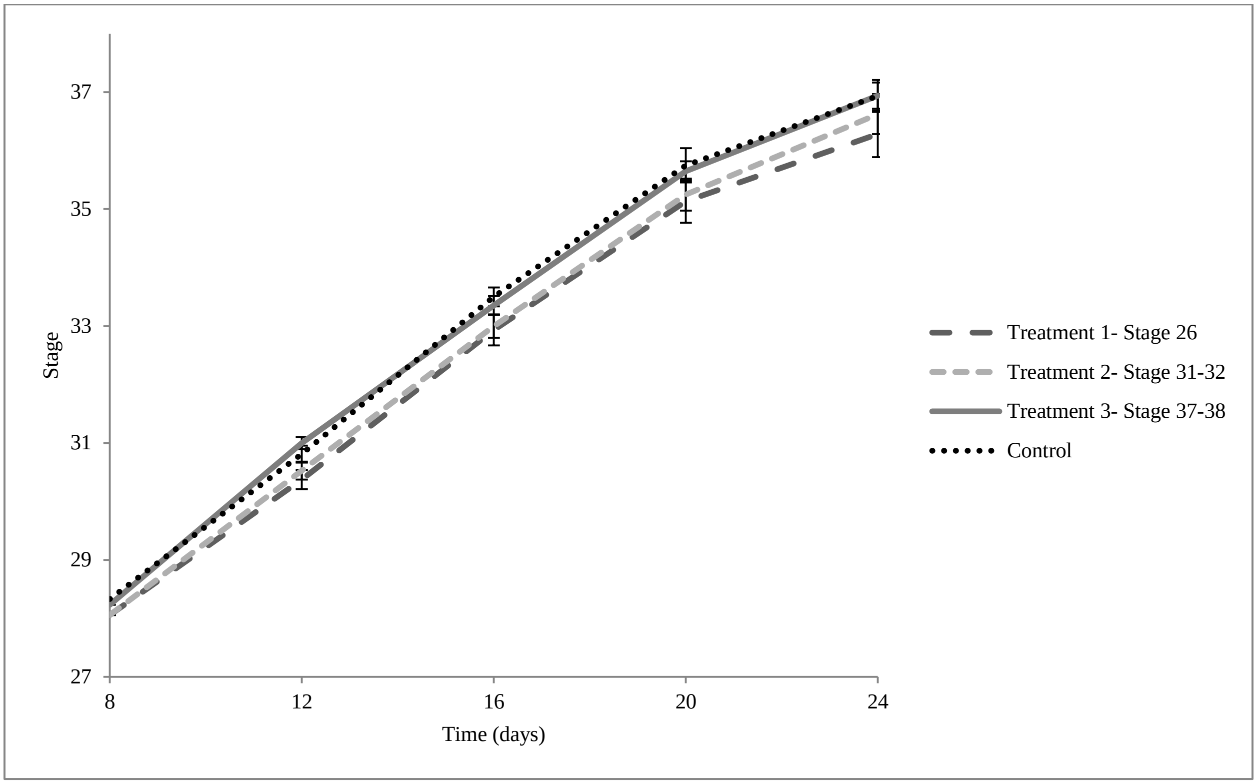

Figure 2: *Development of tadpoles over time with three possible Roundup treatments (0.5 mg a.e./L), treated at different stages of development (mean ± SE). Days 8-24 are magnified to show significant effect of treatment one on tadpoles exposed at stage 26*

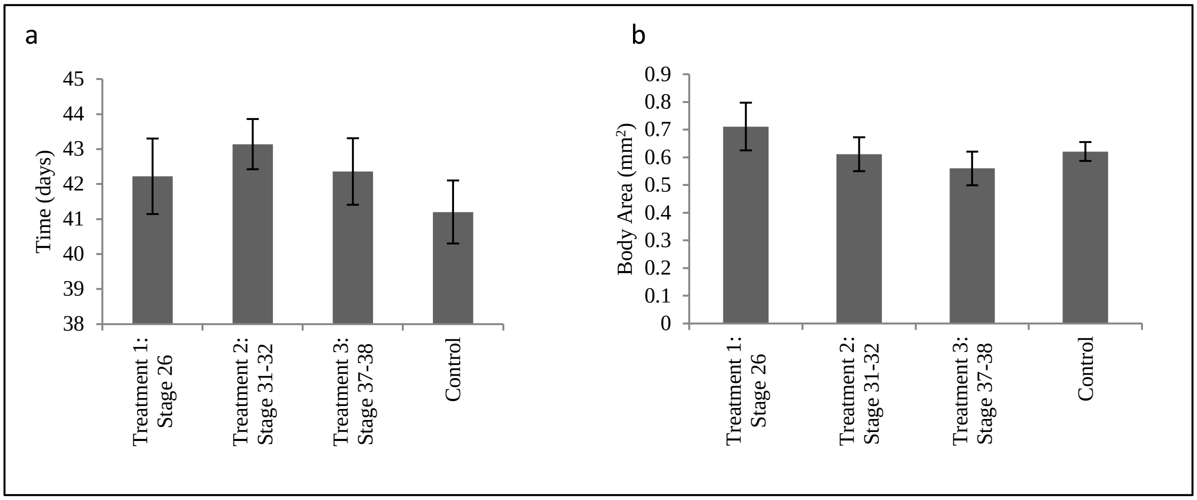

Figure 3: *(a) Time to metamorphosis of tadpoles treated at different times, with 0.5 mg a.e./L of Roundup, during development measured in days (mean ± SE). (b) Body area at metamorphosis of tadpoles treated at different times, with 0.5 mg a.e./L of Roundup, during development measured in mm^2 (mean ± SE).No effect of treatment on body area or time.*

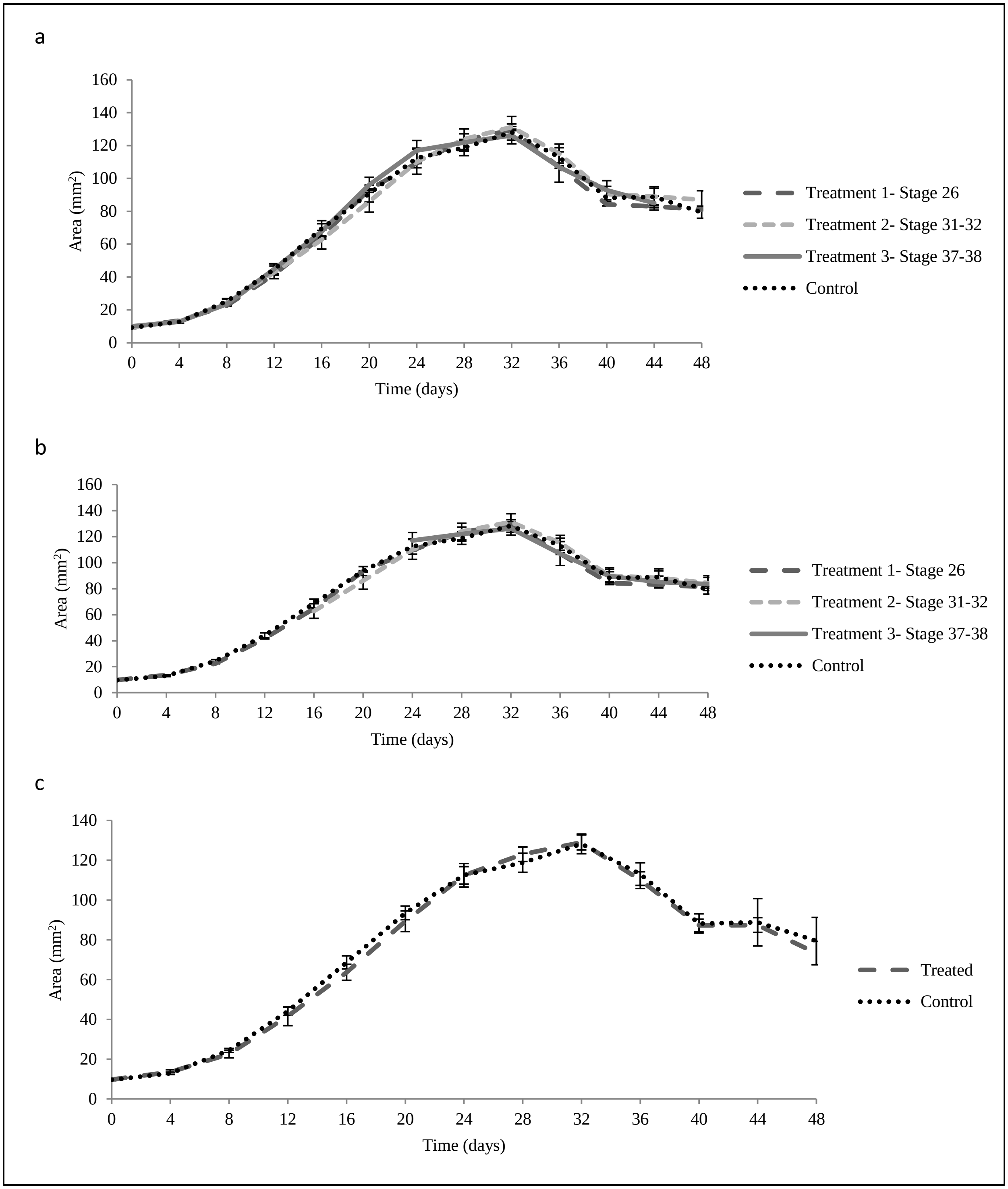

Figure 4*: (a) Tadpole growth measured in body area (mm^2) over time with three identical Roundup treatments (0.5 mg a.e./L) at different stages in development and a control (mean ± SE). (b) Tadpole growth measured in body area (mm^2) over time with three identical Roundup treatments (0.5 mg a.e./L) at different stages in development and a control (mean ± SE) with all non-treated tadpoles combined with control group. (c) Tadpole growth measured in body area (mm^2) over time with three identical Roundup treatments (0.5 mg a.e./L) at different stages in development and a control (mean ± SE) with all non-treated tadpoles grouped with control group (solid line) and all treated tadpoles grouped (hashed line).*

2.4 Discussion

The results of this laboratory experiment show little effect of an environmentally relevant exposure of Roundup on any of the developmental endpoints measured. There is little evidence to suggest that, a four day exposure to this concentration of Roundup resulted in negative effects on development. Tadpoles exposed in the first treatment group (exposed at stage 26) showed a slight lag in developmental progression when compared to the other groups; however this was not observed in any of the other morphometric variables. The tadpoles in this group were able to catch up to the other groups in terms of developmental stage by day 28. Since no change was observed in body area (weight), this slight delay in stage would likely have little ecological relevance due to its reversible nature. This delay in development suggests that, at higher exposure concentrations or in a situation with multiple stressors, the developmental period of stage 26 may be more susceptible than at later stages (31+). In amphibians there is currently no clear indicator of which stages is most susceptible to pesticides, including Roundup. The sensitivity of amphibians during development to different chemicals is not well understood, as some chemicals are more lethal early on, while others are not (Howe et al. 1998, Boone and Bridges 2003). For Roundup, tadpoles at stage 17 are more resistant when compared with tadpoles at stage 25 (based on lethality), but no studies have focused on tadpoles after stage 30 (Edginton et al. 2004). Further studies, focusing on tadpoles post stage 30 should be completed to determine if these later stages are less, equally or more susceptible than the traditionally tested stage 25 tadpoles. Comparing early to late stage tadpoles and using a series of different chemicals can also provide insight into chemical specificity regarding their effects at different developmental stages. These studies would help clarify which stages are most susceptible, if there is a most susceptible stage, and which chemicals affects development most drastically.

A recent field study by Lanctôt et al. (2013) exposed wood frog tadpoles in natural wetlands to Roundup at a concentration of 0.21 mg a.e./L and found no negative effects. There was no effect on body length or development. Although the present study had a higher exposure duration and lower exposure concentration, similar results were observed to the study presented in this chapter. This suggests that at environmentally relevant exposures, Roundup has little effect on tadpole development. The study performed by Lanctôt et al. (2013) had a sample size of seven tadpoles per treatment, whereas this study had a sample size of 20 tadpoles per treatment. Power does not seem to be a limitation for lack of effect. Contrary to this study, another lab based study by Williams and Semlitsch (2010) used a similar exposure concentration (0.57 mg a.e./L) on individually housed American toads (*B. americanus*), Western chorus frogs (*P. triseriata*) and Gray tree frogs (*H. versicolor*). This work showed that Chorus frogs had 80% mortality and American toads took 14% longer to reach metamorphosis. The study by Williams and Semlitsch (2010) utilized a very similar methodology to that performed in this chapter. The major difference between the study presented here and the study performed by Williams and Semlitsch were the species examined. Mass at metamorphosis was not affected in the three species nor was survival affected in the American toads or Gray tree frogs. The results of the study presented here combined with those of Lanctôt et al. (2013) and Williams and Semlitsch (2010), suggest a difference in sensitivity amongst species.

Many studies identify differences in lethality of chemicals between species. Roundup has different lethality values for different species ranging from 0.8 to 2.0 mg a.e./L but intraspecific variation seems to be high. Indeed, different studies with the same species often found different LC50's (Relyea 2005c, Relyea and Jones 2009). Interspecific, intraspecific, population and geographical sensitivities all have been shown to be variable when different species have been exposed to carbaryl (Bridges and Semlitsch 2000). Currently, it is unknown whether the species specific sensitivity contributes more to overall chemical sensitivity than population, or geographical variation in sensitivities.

As the wood frog egg masses used in this study were collected in agricultural areas it is possible that the population could be somewhat resistant to Roundup. However, a pilot study performed in 2012, using 1 mg a.e./L, showed almost complete mortality to wood frogs. Given the high mortality rate observed in 2012 it seems unlikely the population I used was highly resistant to Roundup as the LC50 for wood frogs is close to 1.4 mg a.e./L.

The results of the experiment performed here, coupled with the results of similar experiments do not give a clear answer to the question "Does Roundup cause differential developmental effects based on stage of exposure"? A possible explanation for this variation amongst experiments is the proximity to agricultural land and chemical use. Wood frog tadpoles have been shown to be more sensitive to certain agricultural chemicals when collected over 800 meters from agricultural areas (Hua et al. 2013). These tadpoles also have increased plasticity in developing a resistance to agricultural chemicals than tadpoles in non-agricultural areas (Hua et al. 2013). Populations of tadpoles exposed to toxicants on a regular basis (year to year) are able to develop a resistance, which is not observed in unexposed tadpoles of the same species. Tadpoles taken from varying distances from agricultural land that have been exposed to chlorpyrifos (an organophosphate insecticide) had varying degrees of resistance to the chemical (Cothran et al. 2013). Frogs closer to agricultural areas were more resistant to chlorpyrifos. This is thought to be due to the conserved effects of many different insecticides (i.e. organophosphates) having a similar mode of action, therefore allowing populations to adapt to the mechanism of toxicity of organophosphates rather than to a single chemical. This would protect them from a variety of insecticides, which all rely on a similar mechanism of toxicity. This may not be the case for herbicides (including Roundup), which have varying modes of action (Cothran et al. 2013). Roundup is also used in home, forest and non-agricultural settings, thus exposing many different populations to its effect. Roundup is the most used chemical in North America and in the populations sampled for this thesis, habitat distance from agricultural land may account for results seen herein. Eggs were collected near or on croplands; in which adults may be sprayed each year with Roundup.

In the present study, the tadpoles experienced no additional stressors. Each tadpole was kept in a suitable amount of clean water and received as little disruption as possible (once every two days for 10 minutes during water changes). Tadpole densities were similar (one tadpole/L) to densities reported by others (Relyea 2005c). There was no food competition, starvation, disease, or cannibalism to cause additional stress. This is contrary to Lanctôt et al. (2013), where tadpoles were exposed to natural conditions, and many had died due to predation, disease and drying conditions. These stressors can all potentially lead to a compound effect that was not observed in the present study.

The importance of knowing the lethal and sub-lethal effects of chemicals during a tadpole's development can change current spraying practises. Spray times could be changed to occur during the 'least' sensitive stages. More recently, with the increasing popularity of Roundup resistant crops, amphibians are being exposed multiple times per season. Laboratory tests, like the one completed here, help determine where sensitivities lie in development. Although this experiment shows no differing sensitivities, the results do illustrate that at an environmentally relevant exposure of 0.5 mg a.e./L of Roundup at different developmental periods, should have little effect on growth and developmental endpoints. This finding may be due to a multitude of factors, including increased resistance due to proximity to agricultural land, an exposure that is too low or exposure duration that is too short. A species dependant factor could also contribute to these findings. This project helps to clarify the effect, in terms of growth and development endpoints, Roundup may have on native populations of wood frogs in Saskatchewan.

Chapter 3: The Effects of Roundup on Predator Recognition in Wood Frog Tadpoles

3.1 Introduction

The ability of aquatic animals to detect and appropriately avoid predators is critical for survival. One way prey species avoid predation is through the use of injured conspecific cues (Ferrari et al. 2010). These chemicals are released in the water column when an individual is injured or captured by a predator and play an important ecological role in mediating predator-prey interactions by serving as an early warning system for nearby danger. These cues elicit an immediate and overt anti-predator response when detected by nearby conspecifics (Ferrari et al. 2010). Alterations to these cues can result in the loss of response to these critical information sources and have potential to severely and negatively affect survival.

Many different chemicals, natural and anthropogenic, can negatively affect the ability of aquatic species to detect and respond to chemical cues. Salinity, acidity and agricultural chemicals have all been shown to alter predator-related chemical cues (Bridges 1999, Leduc et al. 2006, Hoover et al. 2012). For instance, when exposed to carbaryl, tadpoles stopped using refuge thus leaving them at risk for predator attack (Bridges 1999).

The effect of Roundup has not been studied thoroughly in terms of its effects on predator avoidance. Roundup has been shown to bind to olfactory receptors in Coho salmon, thus causing a possible lack of predator avoidance due to the loss of chemosensory ability (Tierney et al. 2006). Other studies have looked at the lethal impacts of Roundup contamination, paired with injured conspecific cues and predator odours on the survivability of tadpoles (Relyea 2003). To date, few studies have looked at the ability of Roundup to alter the anti-predator behaviour of the majority of species. A study by Relyea (2012) looked at the combined effects of Roundup and predator odours. Contrary to previous work by Relyea (2005), where predator cues increased Roundup's lethality, Roundup became less lethal to wood frog tadpoles exposed to predator odours collected from dragonfly larvae (Relyea 2012). This effect was thought to be due to Roundup stratifying in the water column of the mesocosms and predator odour causing tadpoles to retreat to a lower column of water. Effects of injured conspecific cues and Roundup are currently unknown. Understanding the chemical composition of Roundup, consisting of a salt bound to glyphosate acid, it may be possible for this commonly used chemical to alter the ability of tadpoles to sense predators for many reasons. Both salinity and acidity of a water body may be altered after a spraying of Roundup but at standard spraying concentrations, this effect would be negligible.

The goal of this chapter was to determine if acute and sub-chronic exposures to Roundup could cause alteration of anti-predator behaviour in tadpoles. In the first experiment, tadpoles were exposed to an environmentally relevant exposure of Roundup of 0.5 mg a.e./L for one hour and subsequently tested for the ability of the tadpoles to display anti-predator response to injured conspecific cues. Due to the significant loss of anti-predator response recorded, the cause of the lack of response was investigated. The objective was to determine if the lack of response was due to the inactivity of the cue (hypothesis A) or due to damage caused to the nasal epithelium of the tadpoles (hypothesis B). Note that these two alternatives need not be mutually exclusive. To test hypothesis A (experiment 2), injured conspecific cues were prepared in either clean water or Roundup water, and injected into cups containing tadpoles raised and maintained in clean water. It was predicted that if injured conspecific cues were inactivated by Roundup, the tadpoles would not respond to Roundup-treated injured conspecific cues. To test hypothesis B (experiment 3), tadpoles were raised with Roundup from early development (eggs to stage 25), and transferred to clean water, and exposed to injured conspecific cues or a water control. It was predicted that if Roundup was causing significant damage to chemosensory organs, tadpoles raised with Roundup would not be able to respond to injured conspecific cues in clean water.

3.2 Methodology

3.2.1 Collection and Care

All tadpoles used in behaviour trials were collected and cared for in the methodology outlined in section 2.2.1.

Egg masses were placed in 10 liter glass tanks and exposed to Roundup (0.5 mg a.e./L) during the egg stages and throughout development up to stage 25. These exposures were renewed every four days with a 100 % water change. There were four 10 liter treatment tanks with a density of one egg mass per tank and four 10 liter control tanks with the same density. Additional control tadpoles were kept in identical tanks and received the same water change but no Roundup exposure. These tadpoles were raised until stage 25 and immediately used in the experiment outlined in 3.2.4. Throughout development tadpole densities did not change in either the sub-chronically treated tadpoles and control tadpole.

3.2.1 Cue Preparation

Injured conspecific cues were prepared with a concentration of one tadpole per 20 ml of water (Ferrari et al. 2009). In total 40 tadpoles were homogenized in 800 ml of distilled water (average body area of tadpoles 9.655 mm^2, 0.1774 g/tadpole). After homogenization, the tadpole solution was filtered through filter paper (25 micron particle retention, VWR brand, West Chester, U.S.A.) divided into 30 ml aliquots, and then frozen for later use in experiments.

3.2.2 Effect of Roundup on Tadpole Anti-predator Response

Tadpoles (stage 25) were taken from the outdoor stock tanks and randomly assigned to 0.5 liter cups containing either clean water or Roundup (0.5 mg a.e./L). The tadpoles were then left outdoors in the sun to acclimate for an hour. To insure tadpoles were not stressed due to the observer (observation started after normal activity was resumed if altered by the observer) or the sun the time of day was controlled for (limited time exposed to sun during hottest times of day). Cascade frog tadpoles exposed to UVB+ and UVB- show no change in survivability or corticosterone after one week exposure, therefor an hour exposure should have no effect (Belden et al. 2003). After the hour, behavioural trials were conducted using a well-established methodology (Ferrari et al. 2009). Tadpoles were observed for four minutes (pre-stimulus) during which the number of times a tadpole crossed the median line of the cup was recorded. After the four minute pre-stimulus, a 30 second injection period occurred in which either water control (5 ml) or injured conspecific cues (5 ml) were added to the cup. Immediately after the injection period, line crosses were measured during the four minute post stimulus period. Each of the four treatment groups consisted of 15 individuals.

3.2.3 Acute Effects of Roundup on Injured Conspecific Cues

To test if the effect of Roundup on response to injured conspecific cues was due to the herbicide affecting the organism or the chemical cue, an experiment was conducted combining the injured conspecific cues with an equal volume of Roundup (0.5 mg a.e./L).

Tadpoles (stage 25) were taken from the outdoor stock tanks and put in 0.5 liter cups filled with clean water. Tadpoles were left to acclimate for one hour outdoors in the sun. Behavioural trials began in an identical fashion to the previous experiment outlined above. A four minute pre-stimulus was followed by a 30 second injection period which was then followed by a four minute post-stimulus. Line crosses were measured in both the pre- and post- stimulus periods. The injection treatments of this experiment were as follows: 1) 10 ml of water, 2) 5 ml injured conspecific cues mixed with 5 ml of water, and 3) 5 ml injured conspecific cues mixed with 5 ml of 0.5 mg a.e./L Roundup. Solutions were administered using 60 ml syringes and were left for at least 15 minutes, prior to being administered, to allow any possible interactions between the water, Roundup and injured conspecific cues to occur. Each treatment group consisted of 15 replicates.

3.2.4 Effect of Roundup on Basal Movement Rates of Tadpoles

The goal of this experiment was to test the effect of a sub-chronic exposure to Roundup on the chemosensory assessment of tadpoles. Initially two groups of tadpoles would be exposed to injured conspecific cues: (1) a control group consisting of a non-exposed tadpoles in clean water and (2) a group of tadpoles sub-chronically exposed (three weeks) to Roundup (concentration of 0.5 mg a.e./L) in clean water. However, the experiment was interrupted because the sub-chronically-exposed tadpoles did not show any of the regular behaviour seen prior to stimulus injection, although control tadpoles behaved as expected. Treated tadpoles were not moving, rendering the experiment impossible and suggesting an effect of Roundup on basal movement. In light of these observations, the objective of the experiment shifted slightly to focus on basal activity level, rather than chemosensory assessment. The goal became to investigate if a sub-chronic exposure to Roundup would cause marked changes to basal behaviours such as activity. For this experiment, the two tests groups outlined above were used and a group of a non-exposed tadpoles in a Roundup solution of 0.5 mg a.e./L was added (to test if the effect is due to the sub-chronic exposure rather than any exposure). Group one served as a positive control, with tadpoles displaying full, unaltered behavioural profiles, while the other two served as treatment groups. The treatment group would provide information on the effect of sub-chronic Roundup exposure on the basal activity of tadpoles, while group three investigated the immediate effects of Roundup exposure on baseline behaviour.

Each of these treatment groups had 20 replicates of one individual tadpole (stage 25) in a 0.5 L cup and left indoors overnight to acclimate. To measure the tadpole's activity, cups were organised in three lines of 20. Each group of three cups was watched for 15 seconds; if the tadpole moved during this time they received a score of one. A score of 0 was given to inactive tadpoles. Each tadpole was recorded once in a four minute period (each run through of the groups of three took 15 seconds for a total of four minutes for all 60 cups) for one hour. Each tadpole's activity was measured 15 times in total, within the hour. Tadpole activity was measured at 10:00 hours and again at 19:00 hours.

3.2.5 Water Parameters

Samples of water were taken from both the control water (dechlorinated water from facility) and from the Roundup solution of 0.5 mg a.e./L. Both the control and treated water had five samples taken and measured for pH using a pH probe and meter. There was no significant difference between the Roundup solution and control water as shown by a paired T-test ($P=0.805$).

3.2.6 Statistical Methodology

A two-way ANOVA was used to test the effects of pesticide exposure (Roundup vs. water) and test cue (injured conspecific cues vs. water) on the anti-predator response of tadpoles. These data were normally distributed and the variance was homogeneous among the treatments. Significant interactions were explored by splitting the analysis into individual one-way ANOVAs. Tukey post-hoc tests were used to determine where the significant differences ($P \leq 0.05$) among the three treatment groups.

A one-way ANOVA was used to compare the effects of cue (water, Roundup injured conspecific cues, untreated injured conspecific cues), followed by Tukey post-hoc comparisons.

To test the effect of a sub-chronic exposure of Roundup on tadpole basal activity, a two-way repeated-measures ANOVA was used. Treatment was the fixed factor and time was the repeated measure ion this analysis. This tested the effect of time (morning or evening recording) and treatment group on the activity of tadpoles. A Tukey post-hoc test was used to determine where the significant differences among the three groups.

3.3 Results

3.3.1. Acute Effects of Roundup Contaminated Water on Injured Conspecific Cues Detection

Homogeneity of variance, as well as normality assumptions were met and the result of the two way ANOVA revealed an interaction between Roundup and test cue on tadpole activity. ($F_{1,76}$=4.096, P=0.047) (Figure 5). In the absence of Roundup, activity decreased significantly in tadpoles exposed to injured conspecific cues when compared to water controls ($F_{1,38}$=15.9, P≤ 0.001). However, in the presence of Roundup there was no difference in how the tadpoles responded to water and injured conspecific cues ($F_{1,38}$=0.8, P=0.37). Tadpoles with no exposure to Roundup significantly lowered their movement when exposed to injured conspecific cues however; tadpoles exposed to Roundup did not exhibit a significant change in behaviour.

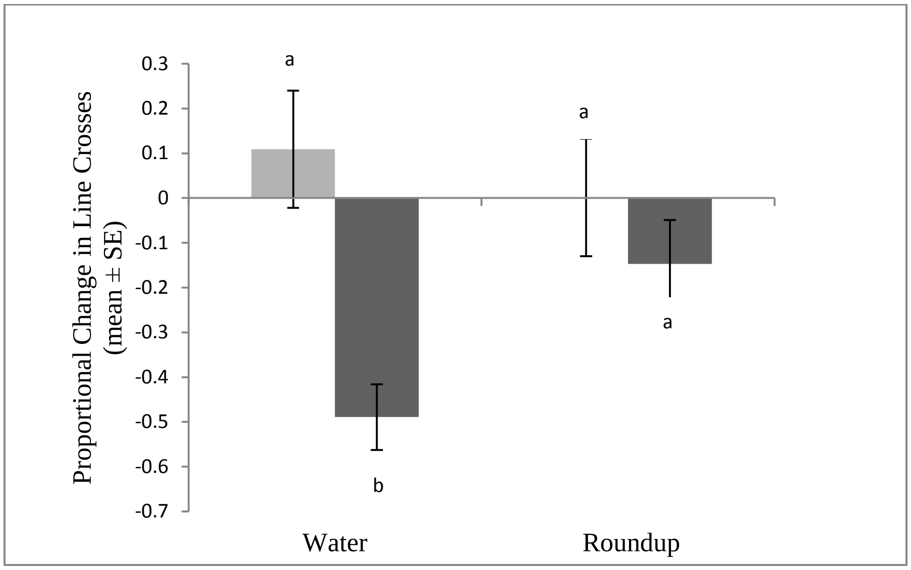

Figure 5*: Change in proportion of tadpole movement measured in proportion of change in line crosses from pre-stimulus to post stimulus. Each tadpole received a random treatment of a 2x2 factorial design with one factor being a Roundup solution (0.5 mg a.e./L) or water in the container and the other being an injected injured conspecific cues (1 tadpole/ 20ml) or water (mean ± SE). Each treatment group consisted of 15 replicates. Light bars exposed to water stimulus, dark bars exposed to injured conspecific cues stimulus.*

3.3.2 Acute Effects of Roundup on Injured Conspecific Cues

Homogeneity of variance as well as normality assumptions were met and the results of an ANOVA indicated a significant change in activity among tadpoles from the three treatment groups ($F_{2,47}$=8.960 P≤0.001) (Figure 6). Tukey post hoc tests revealed that tadpoles exposed to water and the Roundup + injured conspecific cues mixture did not alter their behavior (P=0.8).

Conversely, the tadpoles exposed to injured conspecific cues significantly decreased in movement after the injection as compared to both the control and the injured conspecific cues + Roundup mixture ($P \leq 0.001$ and $P=0.003$, respectively).

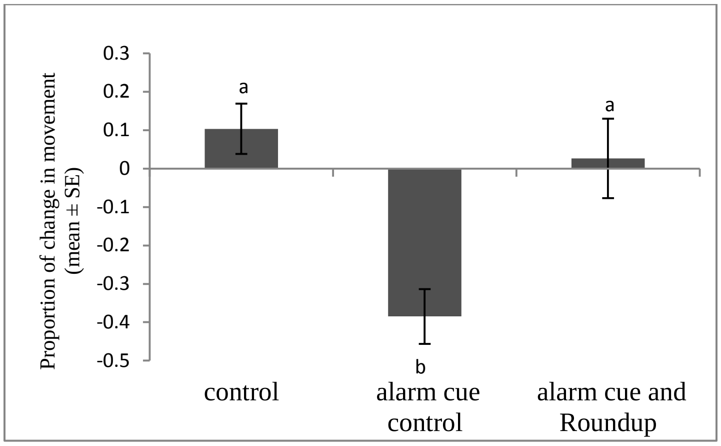

Figure 6: *Change (mean ± SE) in tadpole movement (measured in proportional change from pre-stimulus to post stimulus) after receiving one of three treatments: 10 ml water, injured conspecific cues (1 tadpole/ml) with 5 ml water, and 5 ml Roundup solution (0.5 mg a.e./L) with 5 ml injured conspecific cues. Each treatment group consisted of 15 replicates.*

3.3.3 Sub-Chronic and Acute Effects of Roundup on Tadpole Basal Movement Rates

Homogeneity of variance and normality assumptions were met and the results of a repeated measures ANOVA indicated a significant change in movement for tadpoles sub-chronically exposed to Roundup ($F_{2,87}=0.016$ $P=0.016$), with no significant effect in time tested (morning and night) or in the treatment by time interaction term ($F_{1,87}=0.20$ $P=0.656$ and $F_{2,87}=0.33$ $P=0.722$ respectively). A Tukey post-hoc test showed the group sub-chronically exposed was significantly different from both the control and acutely exposed group ($T=4.451$ $P \leq 0.001$ and $T=4.399$ $P \leq 0.001$) (see Figure 7).

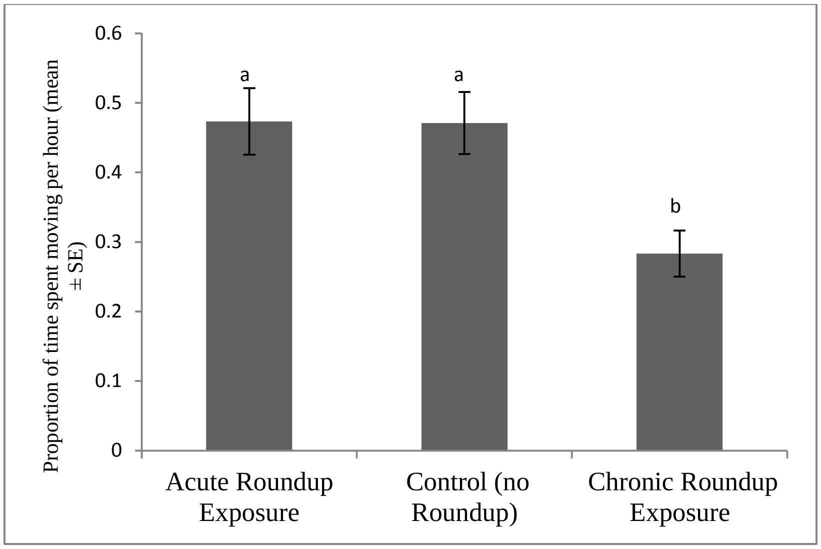

Figure 7: *Change in tadpole movement (measured in number of movement events every 4 minutes for 60 minutes, with two recording events) after receiving one of three Roundup treatments: An acute exposure of Roundup at a concentration of 0.5 mg a.e./L, a control never being exposure to Roundup and a sub-chronic exposure for 3 week at a concentration of 0.5 mg a.e./L renewed every two days (mean ± SE).Each treatment group consisted of 20 replicates.*

3.4 Discussion

Multiple effects of Roundup on tadpole movement and anti-predator response were observed in these three experiments. As expected, tadpoles in clean water, exposed to a water stimulus (negative control) did not exhibit an anti-predator response. Moreover, tadpoles in clean water exposed to injured conspecific cues (positive control) significantly reduced activity. In contrast, tadpoles exposed to Roundup did not show the typical anti-predator response when injured conspecific cues were added to their environment. Tadpoles in Roundup contaminated water exposed to a water stimulus did not alter their activity. This demonstrates that when exposed to an environmentally relevant exposure of Roundup, tadpoles do not normally respond to injured conspecific cues.

When injured conspecific cues were mixed with a low concentration Roundup solution (0.5 mg a.e./L) the tadpoles no longer responded to the stimuli. As seen in the previous experiment, the negative and positive controls behaved as expected, by not altering or lowering their activity, respectively. The tadpoles exposed to the injured conspecific cues mixed with Roundup also did not alter their activity. This suggests that Roundup altered the cue, rendering it unable to alert the tadpole appropriately.

In Teirney et al. (2004) an EOG response was observed at 1 mg a.e./L after 10 minutes in coho salmon. Due to the nominal concentration observed in 3.3.2 of 0.005 mg a.e./L and the maximum time of exposure of four minutes, this data suggest that the response is due to Roundup altering the injured conspecific cues. The tadpoles are still exposed to Roundup and injured conspecific cues at the same time, and therefore, it is still possible that Roundup is affecting the nasal epithelium at a very lower concentration and shorter time period than stated in the literature.

When sub-chronically exposed to Roundup, tadpoles significantly reduced their basal movement. This is in contrast to tadpoles that are unexposed, which have a higher basal movement rate. Tadpoles exposed to an acute exposure (0.5 mg a.e./L for four days) of Roundup did not significantly alter their activity when compared to the control tadpoles. Acutely exposed tadpoles show no difference in basal movement rates, and have a reduced anti-predator response to the same acute exposure. This suggests that activity does not change, even in the presence of injured conspecific cues, where activity should be actively reduced to avoid predation. Sub-chronically exposed tadpoles significantly reduce their basal movement suggesting the mechanism of action for loss of anti-predator response (seen in 3.3.1 and 3.3.2) is different than the mechanism for reduced basal activity.

These results support the hypothesis that Roundup has the ability to significantly alter tadpole anti-predator behaviour; yet, the mechanism was different than the mechanism observed in Tierney et al. (2004) with rainbow trout. Rather than causing a change in the olfactory systems, these results suggest that Roundup has the ability to alter the chemical cue that causes the anti-predator response in tadpoles, but this requires further study.

The loss of injured conspecific response in tadpoles exposed to Roundup could be due to one of two mechanisms. Roundup can either alter the cue itself, or the nasal epithelium of the tadpole causing a loss in olfactory ability. Results of the present study suggest that Roundup is able to alter the chemical cue released when a conspecific is injured. One possible explanation for this is that Roundup, being a weak acid, is able to render the cue ineffective at alarming tadpoles of nearby danger. This has been shown in a study by Leduc et al. (2004), where in mildly acidic conditions (pH of ~6), juvenile Atlantic salmon (*Salmo salar*) did not respond to injured conspecific cues whereas salmon in neutral water did (Leduc et al. 2006). Roundup is normally mixed in a moderately acidic solution (between pH of 4.0 and 5.0), to achieve minimal hydrolysis of the glyphosate. Once sprayed into an ephemeral body of water, Roundup is unlikely to lower the pH at spraying concentrations and cause a similar outcome observed in these studies. In the present study, the pH of the test water and control water did not differ, with both the control water and Roundup (0.5 mg a.e./L) having a pH of 7.6. This is most likely due to the extremely low concentration of Roundup added to the water (with only a concentration after dilution of 0.5 mg a.e./L) and the buffering capacity of the water. In nature, the buffering capacity of water may differ and Roundup can be applied at a higher concentration, potentially leading to a change in pH. This would only happen in a case of misuse of the chemical, a spill, or if an extremely small body of water being exposed.

Another possibility for the loss of alarm response in the Roundup treated water is the change in salinity due to the addition of the salt backbone in Roundup. Salinity has been shown to alter fathead minnow baseline activity and intensity of anti-predator response (Hoover et al. 2012). When exposed to varying levels of salinity (1000, 4000 and 8000 ppm) fathead minnows with the highest level of salinity exposure decreased their baseline movement and the intensity of response to alarm cue. These results mirror the results seen in these studies. When sub-chronically exposed to Roundup for three weeks, tadpoles had reduced basal movement. This reduction may be due in part to the physiological cost of maintaining homeostasis in unfavorable conditions (Luz et al. 2008, Hoover et al. 2012). Tadpoles were exposed to Roundup for three weeks prior to being observed for basal activity, whereas acutely exposed tadpoles only had exposure for 12 (morning sample) and 24 hours (evening sample). When acutely exposed to Roundup (one hour), response to injured conspecific cues were lost which is similar to those results seen in studies by Hoover et al. (2012) however, but is likely due to another mechanism. A change in salinity is unlikely to be observed in this study, because of the low concentration of Roundup used (presented in 3.3.3).

To further clarify the effect of Roundup on tadpole’s anti-predator response, sub-chronic exposure studies need to be conducted. The experiments completed in the basal movement section of this thesis demonstrate that Roundup has an effect on the basal movement of tadpoles which has the potential to cause of myriad of negative impacts. A study by Bridges (2002) looked at the effect of predator stress on activity and feeding behaviour and found that in the presence of predators, tadpoles reduced activity but fed normally. This suggests that tadpoles will attempt to maximize growth even when faced with a stressor (Bridges 2002). In contrast to a behavioural change caused by predators, the added stress of a toxicant can cause a homeostatic stress, which may lead to reduced foraging success and growth (Luz et al. 2008). Tadpoles under constant pollutant stress increase their immune system up regulation which is energetically costly. Studies examining the long term effects of this loss in activity, due to Roundup use, on foraging and growth endpoints would complement this work well. A sub-chronic exposure to Roundup may also cause permanent damage to the olfactory systems in these tadpoles, but further investigation is needed. Establishing a concentration threshold, such as how much exposure is required to cause an effect and how long the effect lasts, is a logical next step.

In conclusion, Roundup has the ability to significantly alter a tadpole's ability to detect injured conspecific cues. This cue is important in alerting a tadpole of a nearby attack on a conspecific from some unknown predator. The studies presented in Chapter 3 suggest that the cue itself is changed by Roundup exposure rather causing a lack of response. Conversely, previous studies indicate that Roundup affects the olfactory system in Rainbow trout causing similar effects to those observed herein. In a sub-chronic dosing period (three weeks), Roundup has the ability to alter the basal movement rates of tadpoles compared to unexposed and acutely exposed tadpoles. These results may be due to a few different mechanisms, including a change in salinity or acidity which could alter the chemical. These effects could have major implications for populations of wood frogs, and other anuran species, in the wild due to loss of anti-predator behaviour.

Chapter 4: General Discussion

4.1 The Effect on Roundup on Development

Although the experiment outlined in Chapter 2 did not produce many significant results, the possibility for differential effects of Roundup on different stages of tadpole development cannot be ruled out. There was a slight delay in development in the tadpoles exposed at stage 26. This may indicate that this stage is more susceptible than the older tadpoles (31+), but further studies are needed. This result was only observed in developmental stage and not observed in growth, therefore it may not be of high ecological relevance. There are many factors that could potentially alter the effects of Roundup on a tadpole's developmental process. My work used an environmentally relevant concentration of Roundup (0.5 mg a.e./L) for an acute time period (four days). Increasing both the concentration and exposure time could have led to a significant effect that would still be within environmentally relevant exposures. There are currently very few studies that show the effect of a chemical on later stage tadpoles (30+). It was hypothesized that embryonic tadpoles are more resistant to toxicants, due to reduced exposure to fully functional gills (Edginton et al. 2004). Surfactants act as gill irritants and decrease the ability of the gills to maintain osmotic balance (Partearroyo et al. 1991). Early stage tadpoles inevitably become more resistant to these effects because gill formation is not complete until stage 23, but this is not the case for stage 25-40 tadpoles, that have fully developed gills. More studies looking at the effects of chemicals on tadpoles stage 25 to metamorphosis need to be completed to fully understand the effects of chemicals over ontogeny. The proposed mechanism between early stage tadpoles (one to 21 vs. 21-25) does not apply to later stage tadpoles (25+) and future studies can help establish an appropriate mechanism for toxicity on these developmental stages.

The study presented in Chapter 2 controlled all factors other than developmental stage.This design can narrow the effect down to a single variable, but comes at the cost of environmental significance. Future studies should aim to build upon data conducted in this manner while adding other ecological factors (i.e. predator stress, multiple toxicants, tadpole density etc.). Both, simple studies, with one manipulated variable, and complex studies, with multiple manipulated variables, are needed to reach a definite conclusion as to how populations may be affected in nature. Mesocosms offer a good alternative to dosing natural water bodies and would be a good choice for studies similar to the one outlined in this thesis. The design of the study presented in

Chapter 2 should be able to detect differences in growth in tadpoles, if an effect were present. Additionally it could also be effectively performed using different endpoints or different methods for measuring complex stressors. A more realistic system with multiple, natural stressors (drying, predation and competition) could lead to increased susceptibility. For instance, a laboratory study by Relyea (2005c) used multiple species of amphibian species, combinations with multiple predator cue and Roundup, and showed that combining predator cues and Roundup increases mortality. Due to the difference in physiology and life strategies of tadpoles at different stages, effects on growth and toxicity may not remain consistent during the developmental period. Manipulating effects of predator odour, injured conspecific cues or testing anti-predator behaviour, using the methodology outlined in Chapter 2.2 would give us more insight to the complexity of tadpole development and the factors that alter it.

The effects of Roundup exposure during development on adult frogs have not been studied. This lack of research poses a challenging issue when looking at toxicology data. Raising tadpoles in a lab setting to metamorphosis is a difficult task, as it requires six to 15 weeks, frequent water changes (to avoid fouling) and large numbers of animals to allow for natural mortality. Froglets are also difficult to care for as they are challenging to feed in that they require a constant supply of very small, live food. Compared to stage 25 tadpoles, both raising and caring for metamorphic tadpoles is laborious and time consuming. However difficult to perform, the effects of many agricultural chemicals on post-metamorphic tadpole are still important for the survival of amphibians, and need to be studied.

Metamorphosis is not a new beginning for many species and latent effects that “carry-over” into adulthood are often observed. Latent effects on these species (i.e. weight at metamorphosis) have little effect on tadpole survival but a large effect on adult survival (Pechenik 2006). Also, adult frogs and toads are more resistant to Roundup than their aquatic juveniles (Relyea 2005b). Adult frogs show high resistance to Roundup in terms of lethality but an increased sensitivity to Roundup as larvae, therefore latent effects may be the only negative effects observed in adult anurans. Due to the novel nature of this field, most studies looking at effects of Roundup on tadpole development, lethality coupled with additional stressors, as well as adult toxicity, are important to determine whether this supposedly innocuous chemical is truly deleterious to frog populations.

4.2 The Effect of Roundup on Predator Recognition

The studies completed in Chapter 3 of this thesis provide some important insight as to the question “Does Roundup affect the anti-predator behaviour of Wood frog tadpoles?” The data suggests that Roundup is interfering with the chemical cue released from an injured tadpole, but provides no means to determine why this is occurring. At this point in time, the identities of the chemicals present in injured conspecific cues are unknown. When using a whole animal to create injured conspecific cues, many different chemicals may contribute to the anti-predator response it elicits. As the whole animal is homogenized to make injured conspecific cues, the alarm response could be due to different chemicals within the skin, gut, or any combination of organs making it extremely difficult and time consuming to isolate. Without knowing the chemical mechanism of injured conspecific cues, it is difficult to speculate why Roundup is causing this reduction in predatory response. EOG (electro olfactogram) studies begin to provide a more mechanistic answer to this problem but only if the Roundup is having some effect at the nasal epithelium, as opposed to altering the cue. Unfortunately, the experiment was not designed to derive a mechanism for the effect of Roundup on tadpoles. Rather, results demonstrated that Roundup causes a change in risk recognition, but have little capacity to answer how. Additional studies, using EOG technology, would be beneficial in deriving a mechanism of action.

They are few possible mechanisms for the lack of response elicited in Roundup exposed tadpoles to injured conspecific cues. The least probable mechanism is an interaction between Roundup and the olfaction system in the tadpole. The studies presented in Chapter 3 suggest this is not the case. It is possible that there is an instantaneous effect of Roundup reducing the olfactory abilities of tadpoles. However, this remains unlikely because of the extremely low concentration of Roundup present in the second study, where only 5 ml of a 0.5 mg a.e./L solution was added to the 0.5 L of water present in the container. Also, such a low concentration would need to have an immediate effect on the tadpole, to have the effect observed in Chapter 3. However improbable this effect is, it has been documented in Coho salmon at a concentration of 1 mg a.e./L but not at a concentration of 0.1 mg a.e./L (Tierney et al. 2006). Specifically, at a concentration of 1 mg a.e./L at 30 minutes of exposure, EOG measurements decreased to 66.1 ± 8.0%. The result observed by Tierney et al. (2006) required a higher exposure concentration and longer exposure period than the effect observed in this thesis, which happened within an hour of being exposed with 0.5 mg a.e./L. Due to the results observed in the study by Tierney et al. (2006), the possibility of inhibited tadpole olfaction due to Roundup cannot be ruled out.

To my knowledge, this is the first study to look at the effects of Roundup on anti-predator response, using injured conspecific cues, in amphibians. The field of behavioural ecotoxicology is an emerging field that could be an important addition to traditional toxicological testing. There are also multiple directions this research can be expanded upon. Looking at chronic exposures on tadpoles will allow us to discover if the effects seen in my study persist over time or if Roundup has the ability to eventually alter olfaction. The experiment presented herein show that a sub-chronic exposure to Roundup causes a reduction in basal movement, but was unable to test this sub-chronic exposure and the effect on anti-predator response due to lack of baseline activity. Studies conducted on adult amphibians can also be completed. These studies could determine the effects of Roundup on adult amphibian's ability to forage and avoid predators due to chemosensory impairment. Olfaction is important for adult amphibians to avoid predators, as these individuals can detect chemical cues left by predators and avoid them (Chivers et al. 1999). Roundup is applied directly to agricultural lands that adult anurans inhabit putting them at direct risk if there is an effect on chemosensory processing. Post metamorphic frogs disperse as metamorphs, migrate to hibernacula, breed and forage in territories potentially exposed to a variety of chemicals. The application of Roundup may affect important predator odours these species rely on to avoid being eaten while moving throughout their habitat.

4.3 Use of Roundup and Conservation Risk to Amphibians

Although innocuous to many species, Roundup is toxic to both fish and amphibians. These important groups cannot be forgotten when creating guidelines for environmental thresholds. Presently, guidelines for allowing agricultural chemicals to proceed to market evaluate chronic toxicity, acute toxicity and persistence with popular test species being Rainbow trout and Daphnia. Placing them at increased risk to agricultural chemicals, many amphibians actively choose ephemeral water bodies that other aquatic species avoid. These water bodies are not avoided when spraying agricultural chemicals and are inadvertently contaminated in the process of spraying, and to a much lesser extent by runoff (Solomon and Thompson 2003, Thompson et al. 2004). More studies and surveys need to be done to find realistic concentrations of Roundup after a direct overspray in an agricultural setting (rather than an experimentally designed scenario). Conducting routine surveys after Roundup sprays will give a reasonable range of what concentrations can be expected. Since spraying Roundup in water bodies is prohibited in agricultural settings (yet frequently impacts ephemeral bodies), there is little chance this practice will discontinue. This is especially true in very small water bodies, where many amphibians reproduce. Instead, a change in the lethal component of Roundup, the surfactant, may be beneficial.

One possibility to help avoid the lethality associated with Roundup is to design a non-lethal surfactant for use in areas where species may be at risk. Since the active ingredient (glyphosate) is relatively harmless, in terms of lethality, to amphibians or fish, a newly formulated surfactant may reduce mortality (Relyea 2005a). This solution may only solve the mortality issue for tadpoles. Unfortunately, changing the surfactant may only change the lethality and may not be a suitable solution to other effects caused by the herbicide. The effect Roundup has on tadpole injured conspecific cues, nor the mechanism for its ability to render injured conspecific cues ineffective at alarming tadpoles, are not fully understood. More research is required in the field of tadpole anti-predator behaviour, which may lead to an explanation as to why Roundup reduces anti-predator response in tadpoles. Knowing which chemical (or chemicals) in injured conspecific cues alarms the tadpole, will allow manufacturers to help reduce similarities in newly formulated chemicals. Testing different components of Roundup (glyphosate and surfactants) could also help determine why the lack of anti-predator response was observed. This can help reduce impacts on natural systems.

The population of tadpoles used in these studies were collected in close proximity to agricultural land that is sprayed with Roundup. The possibility for this population to be more resistant to Roundup than other populations is therefore theoretically high (Hua et al. 2013). The species used in this study, *L. sylvatica,* is a common species, which occupies many different habitat types, with a range that spans over much of Canada. For other Ranid species in Canada, the Wood frog may be a suitable surrogate to help protect other species including the locally, at risk, Northern Leopard frog (*L. pipiens*) (Animal Characterization Abstract for Saskatchewan-Leopard Frogs http://www.biodiversity.sk.ca/docs/factshts/ranpip.pdf). Using the wood frog as a surrogate to help protect this species allows for easy collection and will reduce disturbance to leopard frog populations. These species have similar life histories, similar sensitivity to

Roundup, occupy slightly different habitats and are known to experience the same stressors from agricultural chemicals.

4.4 Using Behavior as an Endpoint

One important aspect of this thesis was to differentiate the lethal and sub-lethal effects of Roundup on wood frog tadpoles. At a concentration of 0.5 mg a.e./L there was no lethality or change in growth or development but predator avoidance behaviour was completely lost. Many studies looking at contaminant impacts on amphibian health focus on growth, reproduction and mortality endpoints. The studies outlined in this thesis show that while there may be no impact of Roundup exposure (0.5 mg a.e./L) on these traditional endpoints, behaviour can be affected. The two approaches taken in this thesis show a more traditional approach to toxicology testing, paired with a new approach. While there is little effect of Roundup on tadpole development or growth at this environmentally relevant dose, there is a loss in predator avoidance. Recently, injured conspecific cues has been paired with agricultural chemicals which increases lethality (Relyea 2003, 2005c). My results compliment these studies demonstrating that in the short term, Roundup can alter the ability for these tadpoles to detect a predatory act and in the long term Roundup's lethality is increased with predator cues. In essence, exposure to Roundup can remove the ability for a tadpole to detect a predator-related event (shown in Chapter 3 of this thesis) and at higher exposure concentrations, the presence of a predator can increase the lethality of this chemical (as seen in the study by Relyea (2005)). This creates a deleterious situation with any combination of predator/conspecific cues and Roundup to tadpole populations.

The studies presented in this thesis are good examples showing why behaviour can be a sensitive endpoint, where other endpoints may not detect a significant change. The same phenomena has also been documented in some species of freshwater fish, where a change in a behavioural endpoint is detected at a much lower concentration than the lowest observed effect concentration (LOEC) of a growth endpoint (Saglio and Trijasse 1998, Kane et al. 2005). These behaviour trials may be able to determine new no-observed effects concentration (NOEC) simply due to the endpoint tested. Amphibians and many fish species are excellent study models for behavioural-based toxicological testing approach because of their repeatable predator avoidance behaviour (Ferrari et al. 2009, Ferrari et al. 2010, Hoover et al. 2012). When exposed to alarm cue (or injured conspecific cues), these species respond in a consistent manner that can be altered by environmental stressors, i.e. salinity, acidity and addition of a toxicant (Saglio and Trijasse 1998, Hoover et al. 2012). The loss of anti-predator behaviour also poses a serious threat to the survival of these species, thus helping validate the use of behaviour as an endpoint.

Literature Cited

Agriculture, S. M. o. 2011. Guide to Crop Protection: Weeds, Plant Diseases, Insects. Saskatchewan Ministry of Agriculture Regina, SK.

Belden, L., I. Moore, R. Mason, J. Wingfield, and A. Blaustein. 2003. Survival, the hormonal stress response and UV-B avoidance in Cascades Frog tadpoles (Rana cascadae) exposed to UV-B radiation. Functional Ecology **17**:409-416.

Belden, L., E. Wildy, A. Hatch, and A. Blaustein. 2000. Juvenile western toads, *Bufo boreas*, avoid chemical cues of snakes fed juvenile, but not larval, conspecifics. Animal Behaviour **59**:871-875.

Blaustein, A. R., and D. B. Wake. 1995. The puzzle of declining amphibian populations. Scientific American **272**:52-57.

Boone, M. D., and C. M. Bridges. 2003. Effects of carbaryl on green frog (*Rana clamitans*) tadpoles: timing of exposure versus multiple exposures. Environmental Toxicology and Chemistry **22**:2695-2702.

Bridges, C. 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Archives of Environmental Contamination and Toxicology **39**:91-96.

Bridges, C. M. 1999. Effects of a pesticide on tadpole activity and predator avoidance behavior. Journal of Herpetology **33**:303-306.

Bridges, C. M. 2002. Tadpoles balance foraging and predator avoidance: effects of predation, pond drying, and hunger. Journal of Herpetology **36**:627-634.

Bridges, C. M., and R. D. Semlitsch. 2000. Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. Conservation Biology **14**:1490-1499.

Carey, C., and C. J. Bryant. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives **103**:13.

Cauble, K., and R. Wagner. 2005. Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. Bulletin of environmental contamination and toxicology **75**:429-435.

Chivers, D. P., J. M. Kiesecker, E. L. Wildy, L. K. Belden, L. B. Kats, and A. R. Blaustein. 1999. Avoidance response of post-metamorphic anurans to cues of injured conspecifics and predators. Journal of Herpetology **33**:472-476.

Cothran, R. D., J. M. Brown, and R. A. Relyea. 2013. Proximity to agriculture is correlated with pesticide tolerance: evidence for the evolution of amphibian resistance to modern pesticides. Evolutionary Applications **6**:932-841.

Crump, M. L. 1989. Effect of habitat drying on developmental time and size at metamorphosis in *Hyla pseudopuma*. Copeia **1989**:794-797.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging infectious diseases **5**:735.

Davis, A. K., L. L. Connell, A. Gross, and J. C. Maerz. 2008. A fast, non-invasive method of measuring growth in tadpoles using image analysis. Herpetological Review **39**:56-57.

Dill, G. M., C. A. CaJacob, and S. R. Padgette. 2008. Glyphosate-resistant crops: adoption, use and future considerations. Pest Management Science **64**:326-331.

Duke, S. O., and S. B. Powles. 2009. Glyphosate-resistant crops and weeds: now and in the future. AgBioForum **12**:346-357.

Edginton, A. N., P. M. Sheridan, G. R. Stephenson, D. G. Thompson, and H. J. Boermans. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environmental Toxicology and Chemistry **23**:815-822.

Edwards, W., G. Triplett, and R. Kramer. 1980. A watershed study of glyphosate transport in runoff. Journal of Environmental Quality **9**:661-665.

Ferrari, M. C., G. E. Brown, F. Messier, and D. P. Chivers. 2009. Threat-sensitive generalization of predator recognition by larval amphibians. Behavioral Ecology and Sociobiology **63**:1369-1375.

Ferrari, M. C., B. D. Wisenden, and D. P. Chivers. 2010. Chemical ecology of predator-prey interactions in aquatic ecosystems: a review and prospectus The present review is one in the special series of reviews on animal-plant interactions. Canadian Journal of Zoology **88**:698-724.

Flowers, M. A., and B. M. Graves. 1997. Juvenile toads avoid chemical cues from snake predators. Animal Behaviour **53**:641-646.

Folmar, L., H. Sanders, and A. Julin. 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Archives of Environmental Contamination and Toxicology **8**:269-278.

García-Muñoz, E., F. Guerrero, and G. Parra. 2009. Effects of copper sulfate on growth, development, and escape behavior in *Epidalea calamita* embryos and larvae. Archives of Environmental Contamination and Toxicology **56**:557-565.

Gasnier, C., C. Dumont, N. Benachour, E. Clair, M.-C. Chagnon, and G.-E. Séralini. 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology **262**:184-191.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**:183-190.

Grube, A., D. Donaldson, T. Kiely, and L. Wu. 2011. Pesticides industry sales and usage. Environmental Protection Agency, US.

Health Canada. 2011. Pesticides and Pesticide Management. In Consumer Product Safety. Retrieved January 3rd 2014 from http://www.hc-sc.gc.ca/cps-spc/pest/faq-eng.php

Hoover, Z., M. C. Ferrari, and D. P. Chivers. 2012. The effects of sub-lethal salinity concentrations on the anti-predator responses of fathead minnows. Chemosphere **90**:1047-1052.

Hopey, M. E., and J. W. Petranka. 1994. Restriction of wood frogs to fish-free habitats: how important is adult choice? Copeia **1994**:1023-1025.

Howe, C. M., M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry, and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry **23**:1928-1938.

Howe, G. E., R. Gillis, and R. C. Mowbray. 1998. Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. Environmental Toxicology and Chemistry **17**:519-525.

Hua, J., N. I. Morehouse, and R. Relyea. 2013. Pesticide tolerance in amphibians: induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. Evolutionary Applications **6**:1028-1040.

Jefferson, D. M., K. A. Hobson, and D. P. Chivers. 2013. Understanding the information value of repeated exposure to chemical alarm cues: what can growth patterns tell us? Pages 237-246 *in* Annales Zoologici Fennici. BioOne.

Jones, D. K., J. I. Hammond, and R. A. Relyea. 2010. Roundup® and amphibians: the importance of concentration, application time, and stratification. Environmental Toxicology and Chemistry **29**:2016-2025.

Kane, A., J. Salierno, and S. Brewer. 2005. Fish models in behavioral toxicology: automated techniques, updates and perspectives. Methods in aquatic toxicology **2**:559-590.

Leduc, A. O. H. C., E. Roh, M. C. Harvey, and G. E. Brown. 2006. Impaired detection of chemical alarm cues by juvenile wild Atlantic salmon (*Salmo salar*) in a weakly acidic environment. Canadian Journal of Fisheries and Aquatic Sciences **63**:2356-2363.

Levine, B. 2003. Principles of forensic toxicology. Amer. Assoc. for Clinical Chemistry.

Luz, R., R. Martínez-Álvarez, N. De Pedro, and M. Delgado. 2008. Growth, food intake regulation and metabolic adaptations in goldfish (*Carassius auratu*s) exposed to different salinities. Aquaculture **276**:171-178.

McCallum, M. L. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology **41**:483-491.

Partearroyo, M. A., S. J. Pilling, and M. N. Jones. 1991. The lysis of isolated fish (*Oncorhynchus mykiss*) gill epithelial cells by surfactants. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology **100**:381-388.

Pechenik, J. A. 2006. Larval experience and latent effects—metamorphosis is not a new beginning. Integrative and Comparative Biology **46**:323-333.

Pimentel, D., H. Acquay, M. Biltonen, P. Rice, M. Silva, J. Nelson, V. Lipner, S. Giordano, A. Horowitz, and M. D'amore. 1992. Environmental and economic costs of pesticide use. BioScience **42**:750-760.

Pollet, I., and L. I. Bendell-Young. 2000. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. Environmental Toxicology and Chemistry **19**:2589-2597.

Relyea, R. A. 2002. Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. Ecological Monographs **72**:523-540.

Relyea, R. A. 2003. Predator cues and pesticides: a double dose of danger for amphibians. Ecological Applications **13**:1515-1521.

Relyea, R. A. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environmental Toxicology and Chemistry **23**:1737-1742.

Relyea, R. A. 2005a. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications **15**:618-627.

Relyea, R. A. 2005b. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications **15**:1118-1124.

Relyea, R. A. 2005c. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology **48**:351-357.

Relyea, R. A. 2012. New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. Ecological Applications **22**:634-647.

Relyea, R. A., and D. K. Jones. 2009. The toxicity of Roundup Original MAX® to 13 species of larval amphibians. Environmental Toxicology and Chemistry **28**:2004-2008.

Saglio, P., and S. Trijasse. 1998. Behavioral responses to atrazine and diuron in goldfish. Archives of Environmental Contamination and Toxicology **35**:484-491.

Saskatchewan Agriculture, Food and Rural Revitalization. 2002. Fertilizer, Pesticide and Irrigation use, 2001 Census. (http://www.agriculture.gov.sk.ca/Fertilizer_Pesticide_Irrigation_Use) Last accessed January 3rd 2014

Saskatchewan Conservation Data Center. 2006. Animal Characterization Abstract for Saskatchewan. Retrieved January 3rd 2014 from http://www.biodiversity.sk.ca/docs/factshts/ranpip.pdf

Schuette, J. 1998. Environmental fate of glyphosate. Published by Environmental Monitoring & Pest Management Department of Pesticide Regulation Sacramento, California ISSN:95824-95624.

Schulz, A., D. Sost, and N. Amrhein. 1984. Insensitivity of 5-enolpyruvylshikimic acid-3-phosphate synthase to glyphosate confers resistance to this herbicide in a strain of *Aerobacter aerogenes*. Archives of microbiology **137**:121-123.

Skelly, D. K. 1996. Pond drying, predators, and the distribution of Pseudacris tadpoles. Copeia **1996**:599-605.

Solomon, K., and D. Thompson. 2003. Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. Journal of Toxicology and Environmental Health Part B: Critical Reviews **6**:289-324.

Stella, J., and M. Ryan. 2004. Glyphosate herbicide formulation: a potentially lethal ingestion. Emergency Medicine **16**:235-239.

Thompson, D. G., B. F. Wojtaszek, B. Staznik, D. T. Chartrand, and G. R. Stephenson. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian larvae in forest wetlands. Environmental Toxicology and Chemistry **23**:843-849.

Tierney, K. B., P. S. Ross, H. E. Jarrard, K. Delaney, and C. J. Kennedy. 2006. Changes in juvenile coho salmon electro-olfactogram during and after short-term exposure to current-use pesticides. Environmental Toxicology and Chemistry **25**:2809-2817.

Tome, M. W., C. E. Grue, and L. R. DeWeese. 1991. Ethyl parathion in wetlands following aerial application to sunflowers in North Dakota. Wildlife Society Bulletin **19**:450-457.

Tsui, M. T., and L. Chu. 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere **52**:1189-1197.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences **105**:11466-11473.

Welsh, H. H., and S. Droege. 2001. A case for using plethodontid salamanders for monitoring biodiversity and ecosystem integrity of North American forests. Conservation Biology **15**:558-569.

Wilbur, H. M. 1980. Complex life cycles. Annual review of Ecology and Systematics **11**:67-93.

Wilbur, H. M. 1987. Regulation of structure in complex systems: experimental temporary pond communities. Ecology **68**:1437-1452.

Williams, G. M., R. Kroes, and I. C. Munro. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regulatory Toxicology and Pharmacology **31**:117-165.