Sub-lethal effects of Roundup™ on tadpole anti-predator responses

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1. Introduction

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 toxicity. These areas are generally ideal for amphibians due to the presence of small, ephemeral water bodies. While Roundup™ has been shown to have lethal effects on many species of amphibians, effects on behaviour and sensory perception have yet to be considered. Here, we exposed wood frog tadpoles to a sub-lethal concentration of Roundup™ and showed that the ability of tadpoles to respond to injured conspecific cues, an important source of information regarding local predation risk, was impaired. Subsequent experiments revealed that impaired responses likely result from a chemical reaction between the Roundup™ and the cues and that tadpoles chronically exposed to Roundup™ had reduced basal movement rates compared with unexposed tadpoles. Our data demonstrate that environmentally-relevant concentrations of Roundup™ can drastically alter movement and anti-predator responses of tadpoles, with potential negative consequences for the population.

Keywords:
- Roundup™
- Sub-lethal effects
- Predation
- Risk assessment
- Injured conspecific cues
- Alarm cues
- Tadpole
- Ecotoxicology
- Amphibian decline

Roundup™ is a commonly used pesticide applied to agriculture and forest habitats. These areas are generally ideal for amphibians due to the presence of small, ephemeral water bodies. While Roundup™ has been shown to have lethal effects on many species of amphibians, effects on behaviour and sensory perception have yet to be considered. Here, we exposed wood frog tadpoles to a sub-lethal concentration of Roundup™ and showed that the ability of tadpoles to respond to injured conspecific cues, an important source of information regarding local predation risk, was impaired. Subsequent experiments revealed that impaired responses likely result from a chemical reaction between the Roundup™ and the cues and that tadpoles chronically exposed to Roundup™ had reduced basal movement rates compared with unexposed tadpoles. Our data demonstrate that environmentally-relevant concentrations of Roundup™ can drastically alter movement and anti-predator responses of tadpoles, with potential negative consequences for the population.

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mesocosm experiments, Relyea (2012) showed that Roundup™ became less lethal to tadpoles exposed to some predator odours, and that the Roundup™ changed the growth pattern of the tadpoles. For example, woodfrog tadpoles exposed to Roundup™ had relatively deeper tails of the same magnitude as the adaptive changes induced by predator cues. Relyea (2012) suggested that the herbicide might be activating the tadpoles’ developmental pathways used for anti-predator responses. Clearly, understanding how prey respond to risk in the presence of Roundup™ and other pesticides deserves further attention. In a study on Coho salmon (Oncorhyncus kisutch), Tierney et al. (2006) established that Roundup™ blocks olfaction in fish exposed to 1 mg a.e./L glyphosate acid. There was a significant reduction in electro-olfactogram (EOG) readings 10 min after the exposure started. This result raises the possibility that glyphosate, in sub-lethal concentrations, could inhibit anti-predator responses in larval amphibians due to alteration of olfactory function.

In the current study, we investigated the effects of Roundup™ exposure on the behaviour of woodfrog tadpoles, paying particular attention to how exposure influences responses to injured conspecific cues, a reliable indicator of risk. The ability of organisms to detect and appropriately respond to cues indicating risk is critical for survival. Many prey species, including woodfrog tadpoles, use injured conspecific cues in their assessment of risk (Ferrari et al., 2010a, 2007). These chemicals are released in the water column when an individual is injured or captured by a predator and serve as an early warning system to nearby individuals. These cues elicit an immediate overt anti-predator response in nearby conspecifics (Ferrari and Chivers, 2009) and also facilitate learning of unknown predators (Chivers and Ferrari, 2013; Ferrari et al., 2010b). Alterations in the structure of those cues or the perception of the cues could result in the loss of response to these critical information sources and consequently has potential to severely affect survival. Indeed, exposure to injured conspecific cues has been shown to dramatically increase survival of the prey during predator encounters (Mirza and Chivers, 2002).

In Experiment 1, we raised tadpoles in pools free of any Roundup™ and then tested them for a response to injured conspecific cues or a water control in a container filled with either clean water or water spiked with an environmentally-relevant sub-lethal concentration of Roundup™ (0.5 mg a.e./L). The loss of an anti-predator response to injured conspecific cues under such conditions could result (1) from the cues being rendered inactivated or partly inactivated from a reaction with the Roundup™ or (2) from the lack of detection by the tadpole due to Roundup™-induced damage to the olfactory epithelium. These two alternatives need not be mutually exclusive. In order to start to address this issue, in Experiment 2, we raised tadpoles in clean water and then exposed them to a water control (negative control), injured conspecific cues in water (positive control), or injured conspecific cues mixed with 0.5 mg a.e./L of Roundup™. If mixing the predation cues with Roundup™ deactivates the injured conspecific cues, then tadpoles should fail to respond under these conditions. In order to address whether olfactory impairment could also explain impaired response to injured conspecific cues, we raised tadpoles for an extended period of time and then exposed them to injured conspecific cues as in Experiment 1. When we attempted to do this, we encountered a problem of not being able to satisfactorily characterize the anti-predator responses because of the inactivity of the tadpoles. In Experiment 3, we specifically address how chronic exposure to Roundup™ influences activity of tadpoles.

2. Materials and methods

2.1. Experimental animals

Several wood frog egg masses were collected from multiple ephemeral ponds north of Saskatoon in early May 2013. The egg masses were gently collected by hand and placed in 1-L containers and kept cool during transport to the University of Saskatchewan where they were assigned to 65-L outdoor stock tanks. Water changes were performed on the stock tanks every two days with half the water being renewed. We used filtered, dechlorinated city tap water [Ca²⁺ 43.5 mg/L, Mg²⁺ 18 mg/L, Na⁺ 26 mg/L, K⁺ 3 mg/L, Cl⁻ 11 mg/L, SO₄²⁻ 50 mg/L, hardness 157 mg/L, alkalinity 109 mg/L (both as CaCO₃), pH 7.6, dissolved organic carbon (DOC) 2.5 mg/L, Wang et al. (2014)]. Tadpoles in these tanks were fed ad libitum with commercial rabbit food after harvesting. Tadpoles were raised until stage 25 and were then used in Experiments 1 and 2 and kept for additional experiments. For Experiment 3, a single egg mass was placed into each of eight 10-L glass tanks. Half of the tanks were spiked with Roundup™ (0.5 mg a.e./L) during the egg stages and throughout development up to stage 25. These exposures were renewed every 4 days with a 100% water change.

2.2. Injured conspecific cues preparation

Injured conspecific cues were prepared following a standard protocol (Ferrari et al., 2009). A total 40 tadpoles were euthanized with a blow to the head, and homogenized in 800 ml of distilled water (average body area of tadpoles 9.66 mm², 0.177 g/tadpole), resulting in a concentration of stimulus of one tadpole per 20 ml of water. After homogenization, the tadpole solution was filtered through filter paper divided into small aliquots, and then frozen for later use in experiments.

2.3. Roundup™ preparation

Roundup™ Weathermax 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 airtight micro-centrifuge tubes at room temperature in a dark cabinet. These aliquots were later diluted to a concentration of 0.5 mg a.e./L for use in three experiments. The glyphosate concentration was within 3% of the nominal concentration (0.513 mg/L, ALS laboratory, Waterloo, Canada). This concentration was chosen to be a sub-lethal concentration based on multiple studies testing the toxicity of Roundup™ to wood frog tadpoles (Edginton et al., 2004; Relyea, 2003, 2005a, 2005b).

2.4. Experiment 1: The effect of Roundup™ exposure on tadpole anti-predator responses

Tadpoles (stage 25) were taken from the outdoor stock tanks and randomly assigned to individual 0.5-L cups (10 cm diameter, 12 cm high) filled with either clean water or a solution of Roundup™ (final dilution of 0.5 mg a.e./L). The tadpoles were then left outdoors to acclimate for an hour, after which time behavioural trials were conducted using a well-established methodology (Ferrari et al., 2009). Tadpoles were observed for 4 min (pre-stimulus) during which time we recorded the number of times the tadpole crossed the median line of the cup (measure of activity). After the 4 min pre-stimulus period, a 30 s injection period occurred, in which either 5 ml of dechlorinated tap water (control solution) or 5 ml of injured conspecific cues were added to the
cup. Immediately afterwards, we continued recording line crosses for the 4 min post-stimulus period. Given that tadpoles reduce activity when they detect risk cues (Chivers and Mirza, 2001; Mathis et al., 2008), we calculated the change in activity from the pre-stimulus baseline, predicting no change in activity for tadpoles exposed to control cues and a reduction in movement for tadpoles exposed to injured conspecific cues in non-Roundup™ water. If Roundup™ impaired the response of tadpoles to injured conspecific cues, they should fail to exhibit an anti-predator response in the Roundup™ treatment. We tested 20 tadpoles in each treatment.

2.5. Experiment 2: Does Roundup™ render injured conspecific cues inactive?

The loss of an anti-predator response to injured conspecific cues for tadpoles tested in Roundup™-spiked water could result from the chemical alarm cue being rendered inactivated or partly inactivated from a reaction with the Roundup™ or from damage to the olfactory epithelium of the tadpole. The goal of this experiment was to address the first of these possibilities. We followed a similar protocol as in Experiment 1. Tadpoles (stage 25) were taken from the outdoor stock tanks and placed individually in 0.5-L cups filled with clean water and left to acclimate for one hour prior to conducting behavioural trials. In this experiment we exposed tadpoles to either (1) 10 ml of dechlorinated tap water, (2) 5 ml injured conspecific cues mixed with 5 ml of dechlorinated tap water, or (3) 5 ml injured conspecific cues mixed with 5 ml of 0.5 mg a.e./L Roundup™ solution. Solutions were mixed 15 min prior to being administered, to allow any possible interactions between the water, Roundup™ and injured conspecific cues to occur. Similar to Experiment 1, we predicted no response to the tap water control but a significant reduction in activity in the alarm cue treatment. If Roundup™ rendered the injured conspecific cues inactive, then tadpoles should fail to reduce activity in the mixed solution. We tested 20 tadpoles in each treatment.

2.6. Effect of Roundup™ on basal movement rates of tadpoles

The goal of this experiment was to test the effect of exposure to Roundup™ on activity of tadpoles. For this purpose, we quantified the activity of (1) tadpoles raised in clean water and transferred to clean water, (2) tadpoles raised in clean water and then transferred to water spiked with Roundup™ (final concentration of 0.5 mg a.e./L), and (3) tadpoles raised from eggs in water spiked with Roundup™ (3 weeks), and then transferred to containers with clean water. The first group served as a positive control, with tadpoles displaying full, unaltered behavioural profiles, while the other two served as treatment groups. Treatment group 2 provides information on the acute effects of Roundup™ exposure on baseline behaviour, while treatment 3 tested the effects of chronic Roundup™ exposure on the basal activity of tadpoles.

We placed 20 tadpoles from each of the three treatment groups (60 tadpoles in total) into individual 0.5 L cups and left them to acclimate overnight for 12 h. The cups were organised in groups of three (one of each treatment) with each group being watched for 15 s, every 4 min for 1 h the following morning. This observation schedule provided for 15 observations in the hour. If the tadpole moved during this time they received a score of 1. A score of 0 was given to inactive tadpoles. Tadpole activity was measured at 10:00 h and again at 19:00 h.

2.7. Statistical analysis

For Experiments 1 and 2, we computed a proportion change in activity from the pre-stimulus baseline ((post-pre)/pre) and used these values in our statistical analyses. In all cases, the data were normally distributed and the variance was homogeneous among the treatments. For Experiment 1, we used a 2-way ANOVA 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. For Experiment 2, we used a 1-way ANOVA to compare the effects of cue (water, injured conspecific cues and injured conspecific cues mixed with Roundup™) on tadpole anti-predator responses. In Experiment 3, we used a 2-way repeated-measure ANOVA to test the effect of Roundup™ exposure on basal activity of tadpoles. Treatment was a fixed factor and time was the repeated measure in this analysis. This tested the effect of time (morning or evening recording) and treatment group on the activity of tadpoles. Tukey post-hoc tests were used to determine where the significant differences occurred among the three groups.

3. Results

Experiment 1. The 2-way ANOVA revealed a significant interaction between Roundup™ exposure and test cue on tadpole activity (F1,76 = 4.1, P = 0.047, Fig. 1). In the absence of Roundup™, activity decreased significantly for tadpoles exposed to injured conspecific cues when compared to water control (F1,38 = 15.9, P < 0.001). However, in the presence of Roundup™ there was no difference in how tadpoles responded to water and injured conspecific cues (F1,38 = 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.

Experiment 2. The results of the 1-way ANOVA indicated a significant change in activity among tadpoles from the three treatment groups (F2,47 = 8.9, P < 0.001) (Fig. 2). Tukey post-hoc tests revealed that tadpoles exposed to water and the Roundup™+alarm cue 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...
Fig. 2. Mean (± SE) proportional change in line crosses of tadpoles exposed to control water, injured conspecific cues, or injured conspecific cues mixed with Roundup™ (0.5 mg a.e./L).

Fig. 3. Mean (± SE) proportion of time tadpoles spent moving. Tadpoles were raised in clean water and tested in clean water (control group), raised in Roundup™ water from eggs (~3 weeks) and tested in clean water (chronic Roundup™ exposure) or raised in clean water and exposed to Roundup™ water for 12 h prior to behavioural observations (acute Roundup™ exposure).

to both the control and the alarm cue + Roundup™ mixture (P < 0.001 and P = 0.003, respectively).

Experiment 3. The repeated-measures ANOVA indicated a significant difference in basal movement of tadpoles (F2,87 = 4.36, P = 0.016), but no significant effect of test time (morning vs. night, F1,87 = 0.20 P = 0.66) nor a treatment by time interaction term (F2,87 = 0.33, P = 0.72). Tukey post-hoc tests showed the group chronically exposed to Roundup™ was significantly different from both the control and acutely exposed groups (both P < 0.001, Fig. 3).

4. Discussion

The results of our experiments demonstrate that exposure to Roundup™ at a sub-lethal concentration has dramatic effects on behaviour and anti-predator responses of wood frog tadpoles. In our first experiment, we showed that tadpoles exposed to environmentally-relevant concentrations of Roundup™ fail to exhibit anti-predator behaviour whereas tadpoles tested in clean water show dramatic reductions in activity. Failure of the tadpoles to recognize this cue puts them at considerable risk, particularly given that injured conspecific cues are crucial for facilitating learned recognition of unknown predators (Ferrari and Chivers, 2011) – a pre-requisite for tadpoles to adaptively respond and avoid predation threats.

In our experiments we attempted to consider the mechanism(s) responsible for the impaired alarm responses. A lack of response could be a result of complete or partial deactivation of the alarm cue by the Roundup™. Indeed, other human-induced environmental change has led to dramatic shifts in predator-prey interaction due to the deactivation of conspecific cues. For instance, Leduc et al. (2004, 2006) documented that this was the mechanism responsible for impaired alarm responses in Atlantic salmon (Salmo salar) exposed to episodic bouts of acid rain. When the pH of stream drops to ~6.4, salmon fail to respond to injured conspecific cues and this impairs their ability to learn to recognize predators (Brown et al., 2012). However, there is evidence of local adaptation to those new environmental conditions, for instance, by increasing the reliance of prey to visual cues (Elvidge et al., 2013). In our case we added enough Roundup™ to the test containers to reach a concentration of 0.5 mg/L, but this tiny amount of acid was not enough to significantly alter the pH of our water from 7.6 (water vs. Roundup™-spiked water, N=10/treatment, P > 0.7). If deactivation is the only mechanism responsible for impairing anti-predator responses, then impairment of alarm responses should be predictable based on the presence of a given concentration of Roundup™ in the environment, just as is the case with episodic bouts of acid rain. Clearly, investigations designed to understand factors influencing the half-life of Roundup™ would be particularly welcome, given that deactivation should be dependent on Roundup™ concentration. If Roundup™ also impairs chemosensation, then the temporal effects of Roundup™ exposure could be greater if it takes considerable time for the olfactory system to recover following exposure. Experiments that consider temporal aspects of exposure are more pressing given the advent of Roundup™ ready crops, which allow for multiple applications per year. There is the potential for waterbodies where tadpoles are developing to receive multiple exposures of Roundup™ in a single season.

We have considerable evidence that injured conspecific cues are deactivated by Roundup™. In Experiment 2, tadpoles failed to reduce activity when we mixed the alarm cue solution with Roundup™. In this experiment, there was a small amount of Roundup™ added to the test container with the tadpoles. Can we be sure that the lack of a response is not due to the Roundup™ impairing the olfactory epithelium? Tierney et al. (2006) showed that it takes approximately 10 min of exposure for 1.0 mg/L of Roundup™ to impair the olfactory system, and an increase in concentration increases the speed of the impairment. In our experiment, we mixed the 5 ml of 0.5 mg/L Roundup™ solution with 5 ml of alarm cue and introduced it to a container with 0.5 L of water giving a final concentration of 0.05 ml a.e./L. Given that our concentration is 2.5 orders of magnitude lower and our experiment last only 4 min, we are confident that the lack of a response in this experiment was due to inactivation of the cue and not impairment of the olfactory system.

An experiment designed to test whether long-term exposure to low concentrations of Roundup™ impairs olfaction would be welcome. We originally set out to do this, but we found it difficult to quantify the activity of the tadpoles under chronic Roundup™ exposure. In Experiment 3, tadpoles chronically exposed to Roundup™, and then transferred to clean water showed significant reductions in their basal movement. This is in contrast to tadpoles that were raised in clean water and tested in clean water. We know that tadpoles exposed to an acute concentration (0.5 mg a.e./L for 12 h) of Roundup™ do not significantly alter their activity when compared to the control tadpoles. If chronic exposure to Roundup™ influences basal movement rates, we should expect
that this could influence feeding and growth and hence provides a mechanism to explain why Relyea (2003) found that predator odours can influence the lethal effects of Roundup™. Studies testing whether chronic exposure to Roundup™ causes permanent damage to the olfactory system in tadpoles could tell us whether there is likely loss of recognition of other important stimuli including feeding and kin recognition cues.

Here, we showed that an environmentally-relevant concentration of Roundup™ can significantly alter the ability of tadpoles to respond to injured conspecific cues. This cue is important in alerting tadpoles of a nearby danger and is the key trigger for learned predator recognition. Woodfrogs find themselves in wetlands with high variation in the types and number of predators, hence any chemical that impairs their ability to learn to recognize novel predators is potentially devastating (Jefferson et al., 2013). Although we realize that the mechanism of impairment is of little consequence when we consider the survival consequences of Roundup™ exposure, we found that chemical inactivation of the alarm cue by Roundup™ was in part responsible for the effects we saw in one of our experiments. The field of chemical ecology lacks critical information on the identity of those injured conspecific cues (Ferrari et al., 2010a; Wisenden, 2000). If we knew the identity of woodfrog cues, we could create a predictive framework to identity potential effects of other agricultural pesticides on tadpole alarm responses. Understanding whether other Ranid frogs use the same chemicals would be particularly valuable because some species, such as northern leopard frogs (Rana pipiens), are in serious decline in areas where Roundup™ exposure is increasing.

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