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Effects of Roundup on Behavior, Growth, and Mortality of Larval Blue Dashers, Pachydiplax longipennis

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EFFECTS OF ROUNDUP ON BEHAVIOR, GROWTH, AND MORTALITY OF LARVAL BLUE DASHERS, *PACHYDIPLAX LONGIPENNIS*

A Thesis
Presented to
The Faculty of the Department of Biological Sciences
Murray State University
Murray, Kentucky

In Partial Fulfillment
of the Requirements for the Degree
Masters of Science

by
Kayleen K. Parker

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ABSTRACT

The objective of this study was to determine if Roundup® (active ingredient: glyphosate) causes negative effects on behavior, growth, and mortality of larval *Pachydiplax longipennis*, since other agrochemicals have been shown to cause drastic changes in aquatic environments and harm non-target organisms. In 2017, larvae were captured from rainwater-filled mesocosms at Hancock Biological Station in Murray, KY. Larvae were exposed to one of four concentrations of Roundup® (0mg/L, 2.5mg/L, 5mg/L, or 10mg/L). *Daphnia* consumption, seek refuge, and anti-predator trials were conducted at 7 and 14 days post-exposure. Growth and survival trials were carried out for eight weeks using different larvae. There were no significant differences among treatments for whether or not larvae ate offered *Daphnia* for Day 7 ($\chi^2 = 1.915$, df =3, P=0.5902) or Day 14 ($\chi^2 = 1.283$, df =3, P=0.7331). Latency for strike time and strike number were analyzed for the first *Daphnia* consumed. For strike time, the interaction between concentration and trial day (P=0.001) and body length ($P<0.001$) were significant. There was a significant difference between Day 7 and Day 14 for the control (P=0.011) and between the control and 5 mg/L for the Day 14 (P=0.005). For strike number, there were no significant differences. For the trials on Day 7, Roundup® concentration did not have a significant effect on the time the larvae took to consume 1 (P=0.130) or 4 (P=0.169) *Daphnia*. For the trials on Day 14, concentration did not have a significant effect on the time the larvae took to consume 1 (P=0.246) *Daphnia*; however, Roundup® significantly affected the time the larvae took to consume 4 *Daphnia* (P=0.029). In the seek refuge trials, there were no significant differences among treatments for the number of pokes required to elicit a behavioral response to hide during

Day 7 (χ^2 =9.458, df =6, P=0.1494) or Day 14 (χ^2=5.759, df =6, P=0.4507). In the anti-predator trials, there were no significant differences among treatments for the number of pokes required to elicit a fleeing response during Day 7 (χ^2 =1.336, df =3, P=0.7207) or Day 14 (χ^2 =1.976, df =3, P=0.5774). The behavioral response variables measured in the seek refuge and anti-predator trials were not significantly influenced by Roundup® concentration, trial day, or size of the larvae. Roundup® concentration had a significant effect on head width growth (P=0.020) and body length growth (P=0.049). There was a significant difference in head width growth between the 2.5 mg/L and 10 mg/L concentrations (P=0.014). Survival analysis showed that Roundup® concentration did not have a significant effect on number of days survived (P=0.394). Thus, Roundup® slowed prey consumption and significantly affected growth, suggesting that it could have a negative impact on larval dragonfly predation and growth rates. This study provides more detail into how a commonly used herbicide is harmful to a possible bio-indicator species, which in turn, shows that the environment overall is impaired by herbicide usage.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

INTRODUCTION

Agrochemicals can cause dramatic changes in aquatic environments and be harmful to non-target organisms. Agrochemicals include fungicides, insecticides, nematicides, and herbicides; the focus of this research was on herbicides. Annual use of herbicides worldwide is higher than the usage of insecticides or fungicides (Köhler and Triebskorn, 2013). However, the annual number of publications describing the effects of herbicides is much lower than publications addressing the effects of insecticides (Köhler and Triebskorn, 2013). Mammals have received more attention in lab observations for pesticide effect publications than any other organism (Köhler and Triebskorn, 2013). There are also publications on the effects of pesticides on insects such as beetles, flies, aphids, bees, and midges; odonates have received little publicized attention.

Herbicides can cause a shift in the phytoplankton community leading to a decrease of zooplankton and macro-invertebrate species due to changes in food quantity and quality (Hasenbein et al., 2017). Herbicides can also affect predator-prey relationships in an aquatic environment in that they can cause a decrease in populations of predator and/or prey of some organisms. If one prey option of a predator declines due to herbicide exposure, the predator must find another source of food or the predator population will decline as well. Aquatic habitats around the world are affected by herbicides and understanding the effects of herbicides on non-target organisms is important for determining the consequences of using them economically, ecologically, and for public health (Relyea, 2009; Bara et al., 2014).

Bioindicators are living organisms that reveal information on the health of an environment. When a population of a bioindicator declines, this suggests the environment is harmed by stressors such as herbicides. My research aims to provide more insight into the effects of an herbicide on a potential dragonfly bioindicator species.

Commonly Used Herbicides

Herbicides such as atrazine, 2,4-dichlorophenoxyacetic acid (2,4-D), metolachlor, glyphosate, and Roundup® are all currently applied to crops or aquatic environments in the United States (NPIC Product Research Online, 2017). Research on agricultural chemicals has shown many different negative effects on multiple organisms. Table 1 summarizes the herbicides listed above and the negative impacts they have on the specific organisms.

Atrazine. Atrazine is an herbicide used to treat corn crops (Campero et al., 2007); it has a wide ranged half-life that can surpass 100 days (Diana et al., 2000). Atrazine combined with predation risk in experimental treatments, decreased head width of the damselfly larvae of *Coenagrion puella* (Campero et al., 2007). Atrazine has also been shown to increase the time for a cannibalistic response in *Libellula luctuosa,* the widow skimmer dragonfly (St. Clair and Fuller, 2014). Atrazine enhances the body size and quantity of adult female mosquitoes, *Aedes aegypti* and *A. albopictus*, emerging from larval habitats and may increase the exposure risk of wildlife and humans to mosquito-borne pathogens (Bara et al., 2014). Atrazine is considered an endocrine-disrupting chemical due to induced morphologic gonadal abnormalities and altered gonadal function in fish and amphibians after exposure (Rohr and McCoy, 2010). Salamander embryos and larvae of *Ambystoma barbouri* exposed to ≥40 µg/L of atrazine showed accelerated water loss even four and eight months post-exposure, suggesting that the effects may be permanent (Rohr and Palmer, 2004). They also showed greater activity and fewer water-conserving behaviors when exposed to the same concentration. Green frog tadpoles, *Rana clamitans,* exposed to sublethal levels of atrazine had an increased susceptibility to infections by *Echinostoma trivolvis* cercariae (Rohr et al., 2008).

2,4-dichlorophenoxyacetic acid (2,4-D). Two, four-D has negative impacts on chemoreception of crayfish *Orconectes rusticus* (Browne and Moore, 2014). Sublethal levels of 2,4-D cause significant physiological and behavioral changes in these crayfish as well. Crayfish exposed to this herbicide walked more rapidly, took significantly longer to locate food, and showed a lower percentage of consumption of a food source compared to controls. Several species of fish exposed to 2,4-D displayed stress behaviors including anorexia, abnormal and restless swimming, vigorous jerks of the body, loss of balance, and respiratory difficulties (Farah et al., 2004; Sarikaya and Selvi, 2005).

Metolachlor. Metolachlor causes decreased walking speeds of the crayfish *O. rusticus* towards a food source (Wolf and Moore, 2002) and positive walking speeds of these crayfish toward an alarm signal (i.e. signal released from prey or predator during an act of predation) instead of fleeing from the source as did the controls (Cook and Moore, 2008). Sublethal concentrations may also interfere with the ability of crayfish to receive or respond to social signals. This in turn affects agonistic behaviors such as initiating fights with other crayfish.

Glyphosate and its General Effects

Glyphosate is a non-selective, post emergent herbicide widely used in agriculture around the world to control grasses and broad-leafed weeds (Dutra et al., 2010). One million eight hundred thousand tons of glyphosate has been used in the U.S. since 1974 and 9.4 million tons has been used worldwide. Glyphosate’s half-life in water ranges from 49 to 70 days (Mercurio et al., 2014; Bali et al., 2017). Pure glyphosate has been shown to have harmful effects on many organisms. Glyphosate based herbicides (GBHs) have a combination of adjuvants and surfactants that cause more harmful effects than pure glyphosate (Bonnet et al., 2006). The effects of pure glyphosate are discussed first, then the effects of GBHs.

Glyphosate affects both aquatic and terrestrial organisms. Glyphosate has been shown to affect the predatory interactions of two species of wolf spiders (Rittman et al., 2013). *Tigrosa helluo* detected and subdued prey more quickly when glyphosate was present. Although the timing of predation for *Pardosa milvina* was unaffected, glyphosate made prey capture more difficult for *P. milvina*, in that they performed more lunges to capture prey (Rittman et al., 2013).

Honeybees, *Apis mellifera,* had reduced sensitivity to sucrose when exposed to field-realistic concentrations of glyphosate; short term memory retention and learning also significantly decreased compared to controls (Herbert et al., 2014). The parasitoid wasp *Palmistichus elaeisis,* used as a biological control of *Anticarisa gemmatalis* in soybean crops (Pereira et al., 2013), had lower emergence rates when continuously exposed to glyphosate through a host fed on soybean leaves treated with glyphosate (Alcántara-de la Cruz et al., 2017).

In aquatic environments, prior research has also shown that glyphosate at 40mg/L causes a significant decrease in protein and lipid content in muscle and muscle pyruvate kinase activities for the freshwater red claw crayfish, *Cherax quadircarinatus* (Frontera et al., 2011; Avigliano et al., 2014). It also caused a reduction in weight gain for *C. quadircarinatus.* In human studies, glyphosate has been detected in brain and cerebrospinal fluid after exposure to commercial mixtures, indicating that the active component can pass through the blood brain barrier (Menkes et al., 1991; Sato et al., 2011; Bali et al., 2017). It can also cause increased necrosis and apoptosis in human cell lines (Gasnier et al., 2009; Mesnage et al., 2013; Bali et al., 2017).

Glyphosate binds with soil particles in the environment limiting its movement (Bonnet et al., 2006). This herbicide is mostly broken down by microbial metabolism producing a major metabolite, aminomethyl phosphonic acid (AMPA), which leads to the production of water, carbon dioxide, and phosphate (Rueppel et al., 1977; Forlani et al., 1999; Bonnet et al., 2006). AMPA has been found to be less toxic than glyphosate, based on values reported for ecotoxicity on fish, algae, and invertebrates, although its degradation process in the environment is generally slower (Agritox, 2006; Bonnet et al., 2006).

Glyphosate based herbicide (GBH) exposure may be neurotoxic to animals of various ages (Bali et al., 2017). This could impact brain development as well as behavior in adulthood. Bali et al. (2017) found that both subchronic (6 weeks) and chronic (12 weeks) exposure to GBH caused a decrease in weight gain and locomotor activity of mice. They also determined that it increased the level of anxiety and depression-like behavior. Their data also suggested that mice exposed to GBH from juvenile age through adulthood leads to neurobehavioral changes that arise from the damage to neuronal developmental processes. The toxicity of glyphosate related herbicides in decreasing order was Roundup > glyphosate acid > glyphosate-isopropylamine salt (Bonnet et al., 2006). Effects of GBHs could also be associated with the chemicals not specified on the label: surfactants, adjuvants, and others (Alcántara-de la Cruz et al., 2017).

Roundup®. Roundup® is one of glyphosate's main commercial forms (Avigliano et al., 2014). It is a non-selective, post emergent herbicide (Dutra et al., 2010) with a half-life of 7 to 70 days (Giesy et al., 2000). It enters aquatic environments in a number of ways: by runoff or aerial dispersion from fields or when applied directly to control aquatic weeds. Another cause of contamination is when the equipment used to apply herbicides, including Roundup®, is washed in or near local bodies of water (Vera et al., 2010; Geyer et al., 2016). When Roundup® is used in or near a wetland, it can be transported to parts of the wetland that are not generally exposed to these chemicals (Tsui and Chu 2008; Geyer et al., 2016). Careless handling, accidental spillage, or discharge of unprocessed wastes of Roundup® into waterways has harmful effects on aquatic life which may contribute to long-term biological effects (Jiraungkoorskul et al., 2001).

Roundup® Effects on Trophic Structure. Studies of aquatic organisms have shown a variety of effects. In a study completed by Geyer et al. (2016), Roundup® formulations had the most widespread effects on zooplankton community when compared to the effects of nutrient addition and the presence of non-native Western mosquitofish, *Gambusia affinis*; these effects varied between the formulations used and among the different taxa of zooplankton. The amphipod *Hyalella castroi* had a reduction in glycogen, proteins, lipids, and triglycerides reserves when exposed to Roundup® (Dutra et al., 2010). The cholesterol and Na+/K+ ATPase activity also decreased for these amphipods and survival rate was lower than the control animals. Amphipods are important links in the food chain of limnetic habitats and Roundup can cause significant changes in the trophic structure.

Roundup® Effects on Metabolic Chemicals.

When exposed to Roundup®, the fish *Leporinus obtusidens* had decreased levels of liver glycogen and acetylcholinesterase (AChE) in the brain (Salbego et al., 2009). Hepatic glucose levels were reduced in the fish exposed to the higher concentration of Roundup® (5 mg/L) and lactate levels in the liver and muscle increased at all exposure concentrations. Hepatic protein increased at the 5 mg/L exposure concentration but protein in the muscle decreased with increasing exposure. Overall, long-term exposure to Roundup® causes metabolic disruption in *L. obtusidens*.

Roundup® Effects on Reproduction and Survival. Roundup® has been shown to cause poorer sperm quality in *Poecilia vivipara*, adult male guppies (Harayashiki et al., 2013). It caused a reduction in plasmatic membrane integrity, DNA integrity, mitochondrial functionality, motility, motility period, and concentration of spermatic cells. Roundup® also has the potential to kill many species of anuran amphibians (*Rana sylvatica, R. pipiens, R. clamitans, R. catesbeiana, Bufo americanus, and Hyla versicolor*) under frequent stress of predators (Relyea, 2004) and 90%-100% of mortality occurred in the tadpole stage (Relyea, 2005). Stress itself can increase mortality; exposure to an herbicide can increase levels of stress, which in turn, increases the mortality level (McCauley et al., 2011).

Roundup® Effects on Habitat Availability. Female dragonflies lay their eggs in or near water, usually on plants. If vegetation is removed, either by another organism (e.g., cattle) or herbicides, there are fewer places for adult dragonflies to reproduce. This yields fewer larvae in the environment with fewer places to hide (Foote and Rice Hornung, 2005).

Table 1. Herbicides and their Behavioral and/or Physiological Effects on Specific Organisms

Herbicide	Organism	Behavioral Effects	Physiological Effects	Literature Cited
Atrazine	*C. puella* (damselfly larvae)		Decrease in head width	Campero et al., 2007
	L. luctuosa (Widow Skimmer Dragonfly)	Increased time for a cannibalistic response		St. Clair and Fuller, 2014
	A. aegypti and *A. albopictus* (mosquitoes)		Higher emergence quanity and quality	Bara et al., 2014
	A. barbouri (Streamside Salamander)	Greater activity, fewer water conserving behaviors	Accelerated water loss 4 and 8 months post-exposure. Altered gonadal function	Rohr and Palmer, 2004 Rohr and McCoy, 2010
	R. clamitans (Green Frog tadpoles)		Increased susceptibility to infections by *E. trivolis* cercariae	Rohr et al., 2008
2,4-D	*O. rusticus* (crayfish)	Walked rapidly, took longer to locate food, and lower consumption of food		Browne and Moore, 2014
	Several species of fish	Abnormal/restless swimming and vigorous jerks of the body	Anorexia, loss of balance, and respiratory difficulties	Farah et al., 2004 Sarikaya and Selvi, 2005
Metolachlor	*O. rusticus* (crayfish)	Decreased walking speeds towards food, positive walking speeds towards an alarm signal, and interfere with the ability to receive or respond to social signals		Wolf and Moore, 2002 Cook and Moore, 2008
Glyphosate	*P.milvina* (wolf spider)	Made prey capture difficult (more lunges)		Rittman et al., 2013
	A. mellifera (honeybees)	Decreased short term memory and learning	Reduced sensitivity to sucrose	Herbert et al., 2014
	P. elaeisis (wasp parasitoid)		Low emergence when continually exposed	Alcantara-de la Cruz et al., 2017
	C. quadircarinatus (red claw crayfish)		Reduced weight gain, decrease in protein and lipid content and pyruvate kinase activities in muscle	Frontera et al., 2011 Avigliano et al., 2014
	H. sapiens (humans)		Found in brain and cerebrospinal fluid. Necrosis and apoptosis in cell lines	Menkes et al., 1991 Sato et al., 2011 Bali et al., 2017
GBH	Swiss Mice	Increased level of anxiety and depression-like behavior	Decrease in body weight gain and locomotor activity.	Bali et al., 2017
Roundup	Zooplankton		Significant effect on abundance	Geyer et al., 2016
	H. castroi (amphipod)		Reduced glycogen, proteins, lipids, and triglycerides reserves and reduced survival rate	Dutra et al., 2010
	L. obtusidens (fish)		Decreased AChE levels in the brain and caused metabolic disruption	Salbego et al., 2009
	P. vivpara (adult male guppies)		Caused poorer sperm quality	Harayashiki et al., 2013
	R.sylvatica, R. pipiens, R. clamitans, B. americanus, and H. versicolor (amphibian tadpoles)		Increased mortality with frequent stress of predators	Relyea, 2004

Dragonfly larvae, *Pachydiplax longipennis*

I used dragonfly larvae in my study because they are important indicators of water quality and environmental health (Watson et al., 1982; Clark and Samways, 1996;

Stewart and Samways, 1998). I examined the effects of field realistic concentrations of Roundup® on behavior, growth, and mortality of the dragonfly larvae, *Pachydiplax longipennis*. *Pachydiplax longipennis* is a summer species (late April-late September) and has a less synchronous emergence rate than any other common species. Dragonfly larvae are frequently the dominant predaceous insects in the littoral zones of aquatic ecosystems (Benke and Benke, 1975). Dragonfly larvae assist in controlling the population of pests such as mosquitoes (Fincke et al., 1997) and are possible important indicators of environmental health.

Dragonfly larvae use their respiratory system to escape possible predators (Hopper, 2001). Larvae move water in and out of the rectum lined with internal gills by contracting their abdominal muscles (Corbet, 1962). Water can be brought in through the anus and then squeezed out with enough pressure to thrust the larva forward at a high speed, fleeing quickly from the predator. I observed this type behavior in the anti-predator trials where I recorded the time it took for the larvae to flee and the total distance they traveled away from the "predator."

Hypotheses

I hypothesized that Roundup® affects predation and anti-predator behavior of *P. longipennis*. I predicted that exposure to Roundup® would increase the time it takes *P. longipennis* to consume *Daphnia*. I also predicted that Roundup® would increase the time it takes *P. longipennis* to seek refuge and to respond to a simulated predator attack. I also hypothesized that Roundup® negatively affects growth rates and increases mortality.

METHODS

Collection of Larvae

A dip net was used to sample the mesocosms at Hancock Biological Station in Murray, Kentucky. Roundup® and other herbicides are not used at or near the mesocosms. Over 100 *P. longipennis* larvae were collected in July 2017 and used in the behavioral trials. Over 70 *P. longipennis* were collected in August 2017 and used in the growth and mortality trials.

After transportation to the lab, the *P. longipennis* larvae were placed separately in 88.9 mm glass finger dishes containing aged tap water. Pictures were then taken of each of the larvae in the dishes; a camera was placed on a metal ring stand to maintain the same height for all pictures. A ruler was placed under the finger dishes before pictures were taken. I measured the larvae from tip of head to end of paraproct (body length) and head width (mm) using ImageJ (Java 1.6.0_24, Version 1.38).

Larval Maintenance

Larvae were maintained in the glass finger dishes throughout the experiment. Every three days, the larvae were fed four *Daphnia* and the water was changed. The Roundup® concentrations were kept constant throughout the experiment.

Two *Daphnia* cultures were started in May 2017 using *Daphnia* and water samples with algae collected from Dr. Howard Whiteman's cultures at Murray State University. *Daphnia* were housed in two 10-gallon, aerated tanks containing dechlorinated water. The *Daphnia* were fed TetraMin® Tropical Flakes fish food *ad libitum*.

Roundup Concentrations

Roundup Weed & Grass Killer Super Concentrate® was used to make the concentrations. This type of Roundup® has 3.6 pounds of glyphosate acid per US gallon and also contains isopropylamine salt. Stock solution 1 (SS1) of 10,000 ppm was made by diluting the concentrate with aged, dechlorinated tap water. SS1 was kept in a glass container and out of direct sunlight. Stock solution 2 (SS2) of 100 ppm was made by diluting SS1 with aged, dechlorinated tap water. SS2 was kept in a plastic 2L bottle and out of direct sunlight.

The final concentrations of 2.5, 5, and 10 mg/L were made by serial dilutions of SS2. All concentrations were kept in plastic 2L bottles out of direct sunlight. New batches of the concentrations were made every 5-7 days following the same procedure.

Exposure of Larvae

The larvae used in behavioral trials were housed in the lab for 5-7 days before they were exposed. After that time, 12 larvae were randomly assigned and exposed to Roundup® concentrations each day for a total of 60 larvae exposed. The larvae were maintained in one of four different concentrations of Roundup® (0, 2.5, 5, and 10 mg/L) from this time forward, with fifteen replicates per concentration of Roundup®. The larvae used in the growth and mortality trials were housed in the lab for 24 hours. After that time, all larvae were exposed to the randomly assigned concentrations of Roundup®, yielding at least 15 replicates for each concentration. All *P. longipennis* larvae were checked daily for mortality.

Behavioral Observations

Behavioral observations occurred on days 7 and 14 following initial Roundup®

exposure. The larvae were fed four *Daphnia* and the water was changed (with correct Roundup® concentration) every three days. After all behavioral trials were completed, the larvae remained in their Roundup® concentrations until day 21 to determine survival. Larvae that survived to day 21 were placed in a Ziploc bag and euthanized by freezing.

On each of the behavioral observation days, laptop computers and USB webcams (Microsoft® LifeCam HD-3000, Video Resolution: 1280 x 720, Frame Rate: 30 fps) were used to record each behavioral trial. The larvae were visually isolated from being disturbed by observer movement during trials by visual barriers. Plastic culture dishes (152.4mm in diameter) containing aged, dechlorinated water were used as arenas for all behavioral trials. Each culture dish was only used for larvae exposed to the same concentrations, to prevent cross-exposing the larvae, and the water was changed between trials with different animals. There were 12 larvae observed on each day of trials. Three types of behavioral trials were carried out: 1) *Daphnia* consumption, 2) seek refuge, and 3) anti-predator response.

Daphnia Consumption Trials:

A larva was placed in the center of a plastic culture dish. The larva was given five minutes to acclimate to the new environment. After the five minutes, four *Daphnia* were placed approximately 1mm in front of the larva. Once the larva consumed all four *Daphnia,* or after 3 hours elapsed, recording was stopped, and the larva was returned to its finger dish. Data collected included number of *Daphnia* consumed, latency of first strike time at first *Daphnia*, number of strikes to successfully capture first *Daphnia,* time to consume first *Daphnia,* time and total to consume all four *Daphnia* (summarized in Table 2).

Seek Refuge Trials:

One larva was placed in the center of a plastic culture dish. The dish contained a small portion of a leaf, approximately 15mm X 40mm in size, on the left side, for the larva to use as shelter. Recording started as soon as the larva was placed into the culture dish. The larva was given 30 minutes to hide on or under the leaf.

If the larva had not hidden by the time 30 minutes had elapsed, a small wooden dowel was used to poke the larva behind the second leg, to provide a stimulus to hide. The larva was poked at 1-minute intervals until they hid. Recording was stopped after the larva stayed hidden for 1 minute. The time it took for the larva to seek refuge, the total distance traveled, total average velocity, and the number of pokes needed were determined for these trials (summarized in Table 2).

Anti-Predator Trials:

A larva was placed directly in the center of the dish. The larva was given five minutes to acclimate to the new environment. After the acclimation period, pokes with a wooden dowel were administered behind the second leg, to simulate a predator attack, until the larva responded and moved from the original position. In research performed by Hopper (2001), a blunt metal probe was used to simulate a generic predatory attack. They tapped each larva on the thorax to simulate an unsuccessful attack from a fish or from a dragonfly larva that failed to hook the labium under the larva, but instead struck its prey on top of the thorax.

For these trials, the number of pokes needed, distance traveled after poke, time to stay still after poke, and the average velocity after poke were all recorded (summarized in Table 2).

Trial Order:

Before the trials were started, larvae were randomly assigned an order for the three types of behavioral observations. For the larvae that started with the *Daphnia* consumption trials, the next trial was the seek refuge trial, then the anti-predator trial. For the larvae that started with the seek refuge trials, the next trial was the anti-predator trial, then the *Daphnia* consumption trial. For the larvae that started with the anti-predator trials, the next trial was the *Daphnia* consumption trial, then the seek refuge trial. The larvae were returned to their finger dish for 1 hour after each trial before the next behavioral observation was conducted.

For the seek refuge and anti-predator trials, Veedub 64 and ImageJ computer programs were used to obtain data from videos. Veedub 64 provided still images every 10 seconds. The still images were then uploaded to ImageJ, where a global scale was set, using the diameter of the culture dish, to measure distance and velocity traveled for each larva.

Table 2. Measurements Taken for Behavioral Trials

Trial Type	Measurements
Daphnia Consumption	Number of *Daphnia* consumed
	Latency for first strike at first *Daphnia*
	Latency of strike number for first *Daphnia*
	Time to consume first *Daphnia*
	Total time to consume all *Daphnia*
Seek Refuge Trial	Time to seek refuge
	Total distance traveled
	Total average velocity
	Number of pokes needed
Anti-Predator Trial	Number of pokes needed
	Distance traveled after poke
	Time to stay still after poke
	Average velocity after poke

Growth and Mortality

Larvae collected in August 2017 were used in growth and mortality trials. The larvae were housed in glass finger dishes with aged, dechlorinated tap water for 24 hours. The larvae were then randomly assigned to a Roundup® concentration (0, 2.5, 5, or 10mg/L) and placed separately into the glass finger dishes. Mortality was checked daily. Water was changed, and larvae were fed Ostracods every three days. Photos were taken weekly of the larvae and ImageJ was used to measure the body length and head width. When a larva was found dead, a photo was taken to record the body length and head width at death. The growth and mortality experiment continued for 8 weeks, when only 4 out of the >70 larvae still survived. The remaining 4 larvae were euthanized by freezing.

Statistical Analysis

Data from the *Daphnia* consumption, seek refuge, and anti-predator trials were analyzed by base 10 log transforming the head width and body length measurements collected for each trial type and performing an ANOVA for each continuous or count response variable. Several models were generated and then body length or head width was chosen based on which model had the lowest AIC value. The analysis for the time to consume the fourth *Daphnia* excluded larvae that did not consume all four *Daphnia* during the 3 hours. Tukey's HSD Post Hoc Tests were performed if ANOVAs showed significant differences. Chi-square analysis was used to determine whether Roundup® concentration and trial day significantly impacted whether or not the larvae ate all offered *Daphnia.* Chi-square analysis was also used to determine whether Roundup® concentration and trial day significantly affected the number of pokes required in the seek refuge and anti-predator trials.

For the seek refuge trials, the number of pokes required to stimulate larvae to seek shelter after 30 minutes were separated into three groupings: none, low (1-5 pokes), and high (more than 5 pokes). For the anti-predator trials, the number of pokes required for the larvae to respond were separated into two groupings: 1 poke or more than 1 poke. Growth was analyzed by using ANOVAs on base 10 log transformed body length and head width data collected from the larvae. The mortality trials were analyzed using Cox regression for survival analysis.

RESULTS

***Daphnia* Consumption Trials**

There were no significant differences among treatments when comparing whether or not larvae ate all offered *Daphnia* on Day 7 (χ^2 =1.915, df =3, P=0.5902) or Day 14 (χ^2 =1.283, df =3, P=0.7331).

Variables and statistics for final models for latency of strike time and strike number for the first *Daphnia* are shown in Table 3. For the strike time, concentration (P=0.268) and trial day (P=0.988) were not significant; however, the interaction between concentration and trial day (P=0.001) and body length (P=0.000) were significant. There were significant differences between Day 7 and Day 14 within the control group (P=0.011; Figure 1A). There were also significant differences between the control and 5 mg/L on Day 14 (P=0.005; Figure 1A). For the strike number data, concentration (P=0.628), trial day (P=0.172), the interaction between concentration and trial day (P=0.954), and head width (P=0.474) were not significant (Figure 1B).

Variables and statistics for final models of each trial day and amount of time until consumption of 1 and 4 *Daphnia* are shown in Table 4. On Day 7, Roundup®

concentration did not have a significant effect on the time it took the larvae to consume the first (P=0.130; Figure 2A) or all 4 *Daphnia* (P=0.169; Figure 2B). For the trials on Day 14, concentration did not have a significant effect on the time it took to consume the first *Daphnia* (P=0.246; Figure 2A); however, Roundup® significantly affected the time it took to consume all 4 *Daphnia* (P=0.029; Figure 2B). For the consumption of 4 *Daphnia,* there were significant differences between 2.5 mg/L and 5 mg/L (P=0.019, Figure 2B).

Table 3. Statistics and Variables for Latency of Strike Time and Number Models

Model	**Variables in Final Model**	**F**	**P-value**
Log Strike Time	Concentration	1.33	0.268
	Trial Day	0	0.988
	Conc:Day	5.949	0.001
	Body Length	20.12	0
Log Strike Number	Concentration	1.4	0.247
	Trial Day	0.781	0.379
	Conc:Day	0.008	0.999
	Body Length	6.014	0.016

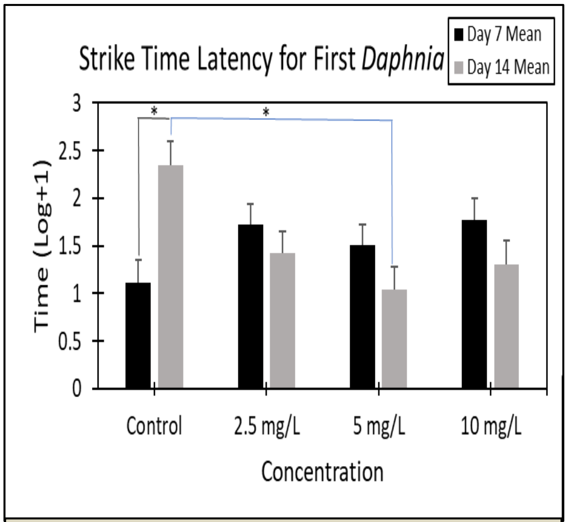

Figure 1A. Log-time of all concentrations for strike time latency of first *Daphnia* on Day 7 and Day 14. There were significant differences between Day 7 and Day 14 within the control group (P=0.011). There were also significant differences between the control and 5 mg/L on Day 14 (P=0.005).

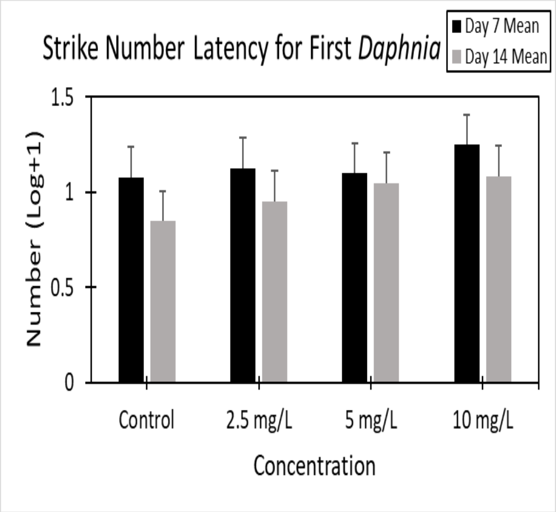

Figure 1B. Log-number of all concentrations for strike number latency of first *Daphnia* on Day 7 and Day 14. There were no significant differences.

Table 4. Statistics and Variables for each Daphnia Consumption Model

Model	Variables in Final Model	F	P-value
Day 7, Daphnia 1	Body Length	11.909	0.001
	Concentration	1.97	0.13
Day 7, Daphnia 4	Head Width	8.805	0.005
	Concentration	1.759	0.169
Day 14, Daphnia 1	Head Width	0.288	0.594
	Concentration	1.43	0.246
Day 14, Daphnia 4	Body Length	11.477	0.002
	Concentration	3.434	0.029

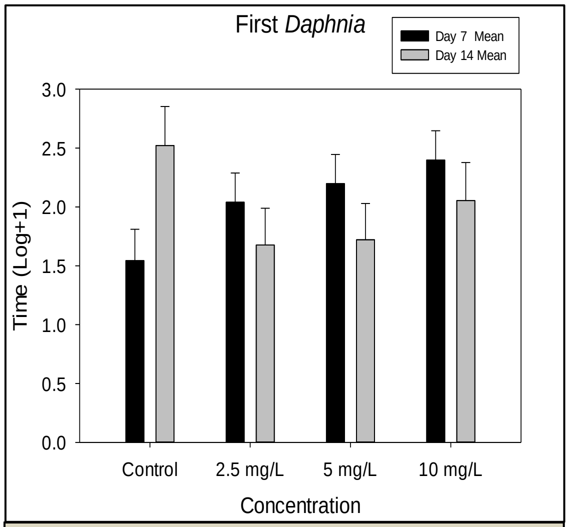

Figure 2A. Log-time of all concentrations for consumption of first *Daphnia* on Day 7 and Day 14. There were no significant differences.

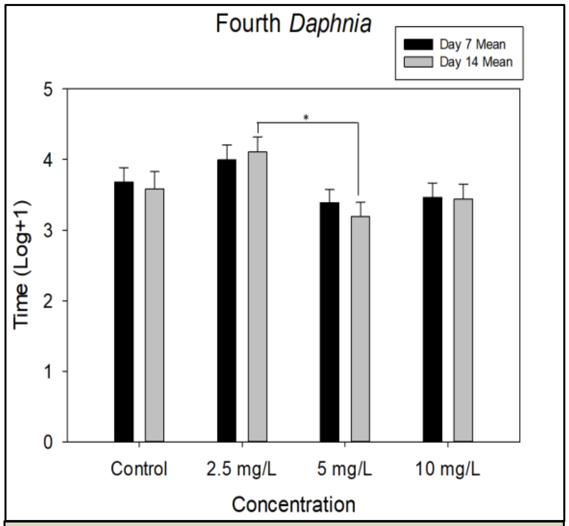

Figure 2B. Log-time of all concentrations for consumption of fourth *Daphnia* on Day 7 and Day 14. There were significant differences between the 2.5 mg/L and 5 mg/L concentrations at Day 14 (P=0.019).

Seek Refuge Trials

There were no significant differences among treatments in the number of pokes required on Day 7 (χ^2 =9.4584, df =6, P=0.1494) or Day 14 (χ^2 =5.7589, df =6, P=0.4507).

Variables and statistics for the final models for the seek refuge trials are shown in Table 5. Log distance traveled was not significantly influenced by Roundup® concentration (P=0.782; Figure 3A), trial day (P=0.077; Figure 3B), or the interaction between concentration and trial (P=0.845). Time to seek refuge was not significantly influenced by Roundup® concentration (P=0.835), trial day (P=0.282), or the interaction between concentration and trial (P=0.075). Log mean velocity was not significantly influenced by Roundup® concentration (P=0.272), trial day (P=0.334), or the interaction between concentration and trial (P=0.091). Data are only shown for the log distance traveled to provide an example of non-significant results.

Table 5. Statistics and Variables for each Seek Refuge Model			
Model	**Variables in Final Model**	**F**	**P-value**
Log Distance Traveled	Concentration	0.36	0.782
	Trial Day	3.19	0.077
	Conc:Trial	0.273	0.845
	Body Length	3.819	0.053
Time to Seek Refuge	Concentration	0.287	0.835
	Trial Day	1.167	0.282
	Conc:Trial	2.362	0.075
	Body Length	3.875	0.051
Log Mean Velocity	Concentration	1.318	0.272
	Trial Day	0.941	0.334
	Conc: Trial	2.208	0.091
	Body Length	0.002	0.964

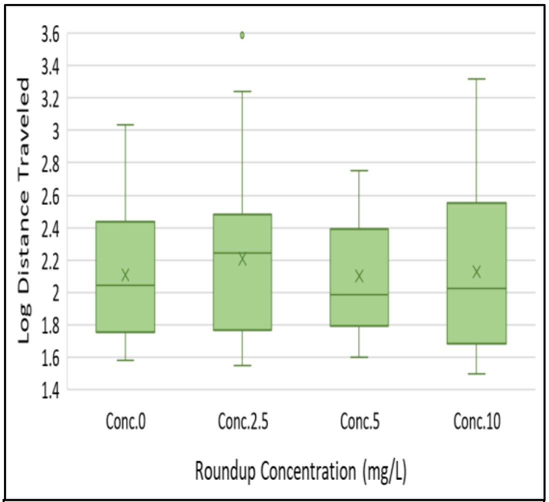

Figure 3A. Log distance traveled based on Roundup concentration for both trial days. There were no significant differences among the treatments.

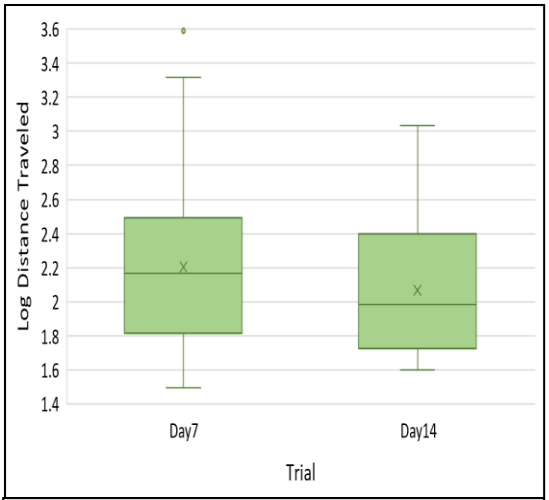

Figure 3B. Log distance traveled. There were no significant differences between the trial days.

Anti-Predator Trials

There were no significant differences among treatments in the number of pokes required on Day 7 (χ^2 =1.3355, df =3, P=0.7207) or Day 14 (χ^2 =1.9758, df =3, P=0.5774).

Variables and statistics for final models of the response variables from the anti-predator trials are shown in Table 6. Log distance traveled was not significantly influenced by Roundup® concentration (P=0.539; Figure 4A), trial day (P=0.949; Figure 4B), or the interaction between concentration and trial (P=0.412). Log mean velocity was not significantly influenced by Roundup® concentration (P=0.471), trial day (P=0.690), or the interaction between concentration and trial (P=0.570). Log time to stay still was not significantly influenced by Roundup® concentration (P=0.856), trial day (P=0.581), or the interaction between concentration and trial (P=0.585). Data are only shown for the log distance traveled to provide an example of non-significant results.

Table 6. Statistics and Variables for each Anti-Predator Model

Model	Variables in Final Model	F	P-value
Log Distance Traveled	Concentration	0.725	0.539
	Trial Day	0.004	0.949
	Conc:Trial	0.965	0.412
	Head Width	0.272	0.603
Log Mean Velocity	Concentration	0.846	0.471
	Trial Day	0.16	0.69
	Conc:Trial	0.673	0.57
	Head Width	0.195	0.659
Log Time to Stay Still	Concentration	0.257	0.856
	Trial Day	0.306	0.581
	Conc:Trial	0.649	0.585
	Head Width	1.985	0.162

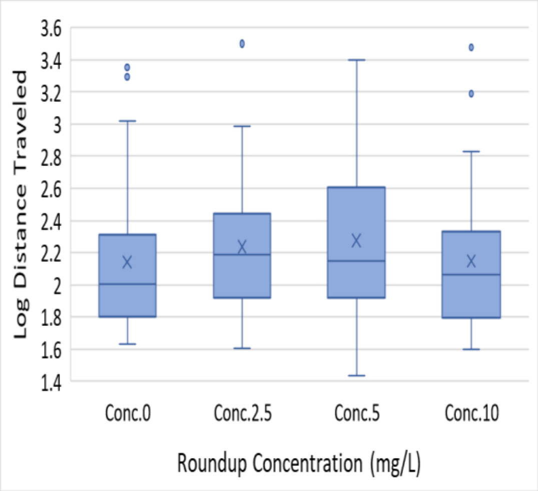

Figure 4A. Log distance traveled based on Roundup concentration for both trial days. There were no significant differences among the treatments.

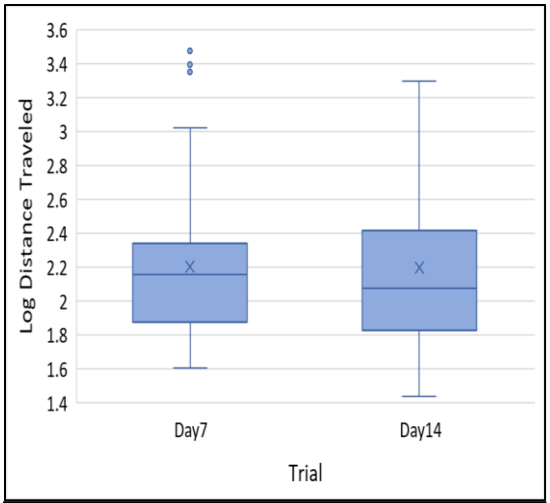

Figure 4B. Log distance traveled. There were no significant differences between the trial days.

Effects on Growth Rates

Variables and statistics for the ANOVAs of the growth models are shown in Table 7. Roundup® concentration had a significant effect on log head width and log body length growth (P=0.049). Log initial body length also had a significant effect on log body length growth (P=<0.001). Figure 5 and Figure 6 show the growth trend of log head width and log body length during the 8-week trial period. Both figures show that as exposure time increases, growth rate decreases. For mean head width growth, there were significant differences between 2.5 mg/L and 10 mg/L (P=0.014, Figure 7A). For mean body length growth, there were no significant differences between the Roundup® concentrations (Figure 7B).

Table 7. Variables and Statistics for each Growth Model

Model	Variables in Final Model	F	P-value
Log Head Width Growth	Concentration	3.496	0.02
Log Body Length Growth	Concentration	2.753	0.049
	Log Initial Body Length	18.731	0

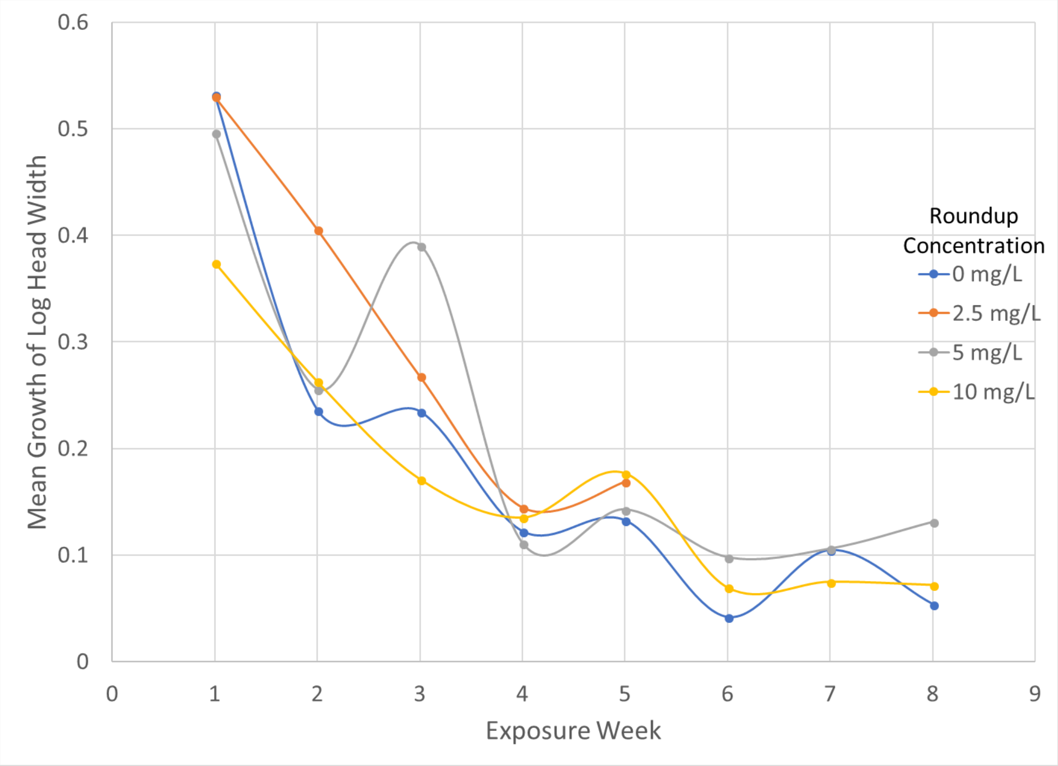

Figure 5. Mean growth (head width) over the 8-week trial period. There were no significant differences between the concentrations. Growth rate decreased with increased exposure time.

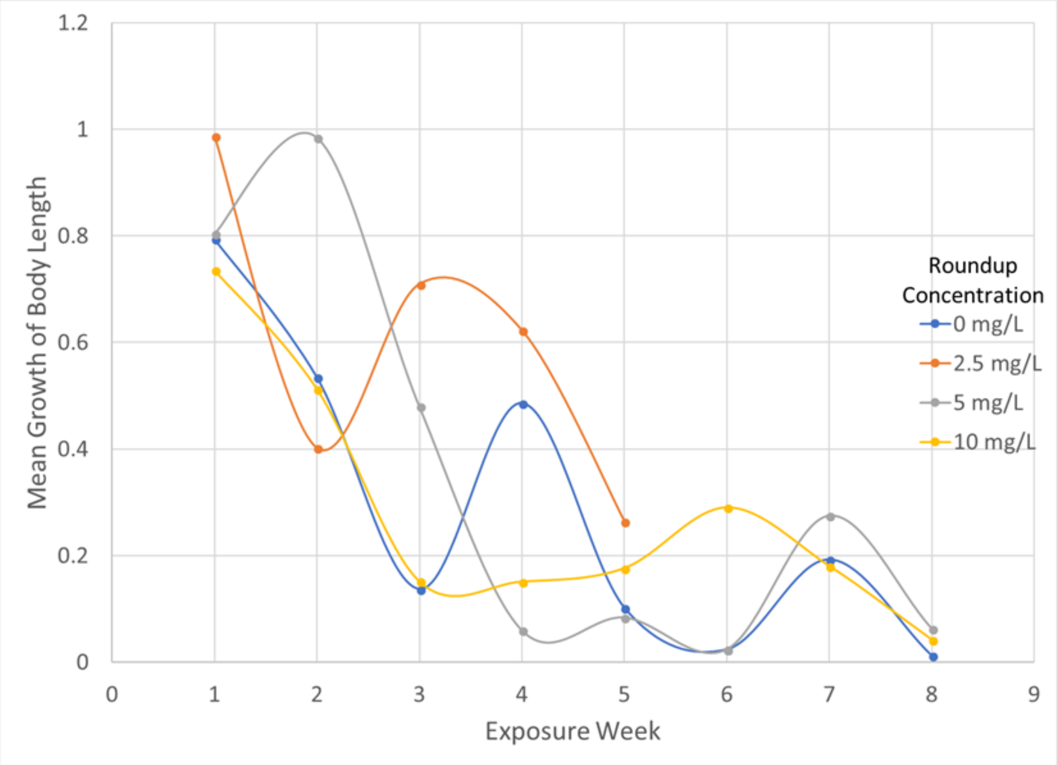

Figure 6. Mean growth (body length) over the 8-week trial period.
There were no significant differences between the concentrations. Growth rate decreased with increased exposure time.

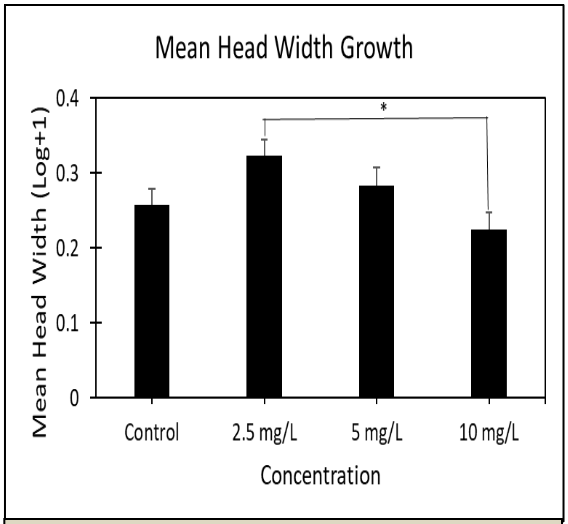

Figure 7A. Mean log head width growth over the 8-week trial period. There was a significant difference between the 2.5 mg/L and 10 mg/L Roundup® concentrations.

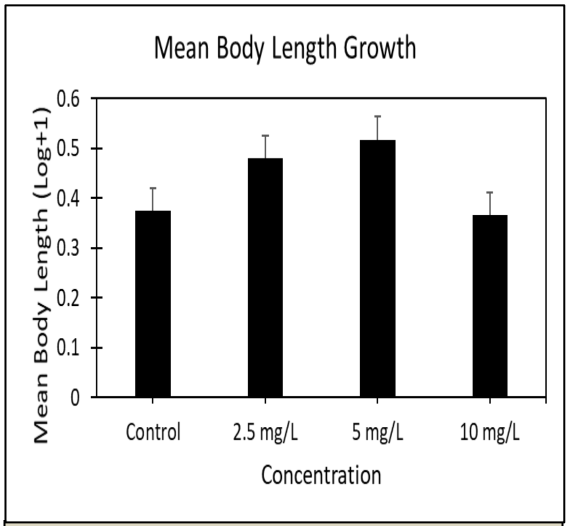

Figure 7B. Mean log body length growth over the 8-week trial period. There were no significant differences among the Roundup® concentrations.

Mortality Trials

The Cox regression of survival analysis for the mortality trials showed that Roundup® concentration did not have a significant effect on the number of days that the larvae survived (P=0.394). Figure 8 shows the number of days that the larvae survived based on Roundup® concentrations. The points where the data are shown as crosses means that those larvae survived the entire trial time (4 larvae).

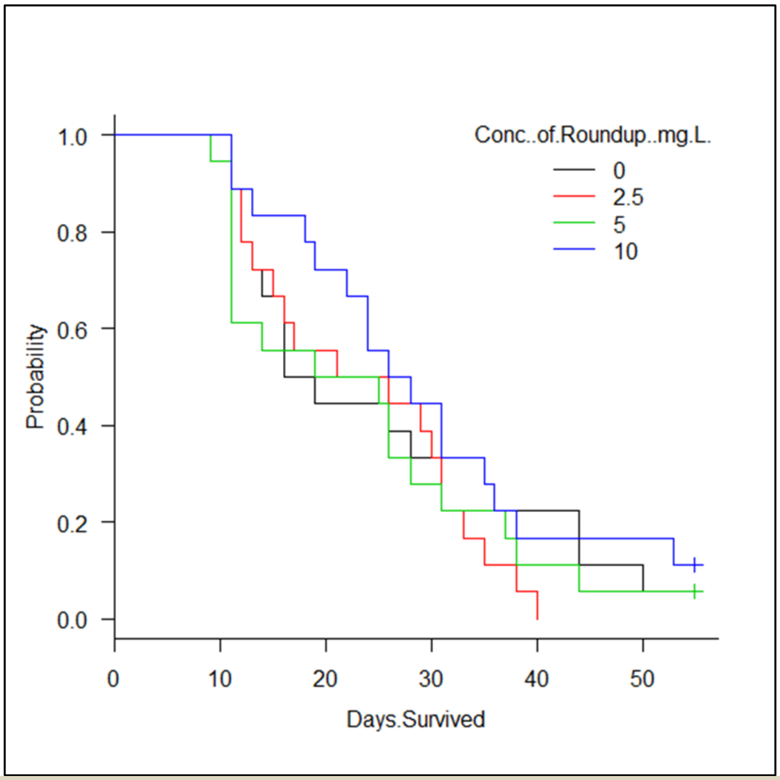

Figure 8. The probability of days survived for larvae based on Roundup® concentration. There was no significance on days survived based on Roundup concentration. Four larvae survived the entire trial period, shown by crosses on the graph: 2 from 5 mg/L and 2 from 10 mg/L.

DISCUSSION

I conducted this study to gain an understanding of the effects of different Roundup® concentrations on larval *P. longipennis*, a potential bioindicator of ecosystem health. I hypothesized that Roundup® affects predation and anti-predator behavior of *P. longipennis*. I also hypothesized that Roundup® negatively affects growth rates and mortality.

I predicted that as the Roundup® concentrations and exposure period increased, there would be a decrease in the number of *Daphnia* larvae consumed and an increase in the time it took them to feed. The hypothesis that Roundup® affects predation was not supported by the number of *Daphnia* consumed but was supported by the strike latency and rates of *Daphnia* consumption. It took longer for the larvae exposed to Roundup®

for 7 and 14 days to eat 4 (2.5 mg/L concentration on day 14) *Daphnia.* In nature, the larvae exposed to Roundup® may be more exposed to predation by larger dragonfly larvae or by fish if the larvae take longer to capture and consume prey. Interestingly, the lowest concentration of Roundup® (2.5 mg/L) caused an increase in the time it took *P. longipennis* larvae to consume 4 *Daphnia* when compared to the other two concentrations.

I predicted that increased Roundup® concentration would cause an increase in the time it took larvae to seek refuge, to flee from a "predator," and that they would move at slower rates. The hypothesis for these trials was not supported as the results showed that Roundup® concentration and trial day did not have significant effects on the time it took larvae to seek refuge, to flee from a "predator," or their rate of movement.

I predicted that higher concentrations of Roundup® would cause a significantly lower growth rate than the control. I also predicted that as the exposure time increased, there would be a decrease in the rate of growth. The hypothesis that Roundup® negatively affects growth rates was not supported because there was no significant difference between the control and the three Roundup® concentrations; the only significance among concentrations was between larvae exposed to 2.5 mg/L and 10 mg/L. In addition, all larvae showed lower growth rates with time.

I predicted increased mortality rates with higher Roundup® concentration. This hypothesis was not supported as Roundup® concentration did not have significant effects on the number of days larvae survived. In fact, two larvae exposed to the 5 mg/L concentration and two larvae exposed to the 10 mg/L concentration survived the entire trial period.

The results for the *Daphnia* consumption trials showed that Roundup® concentration and trial day did not affect whether or not *P. longipennis* larvae consumed all offered *Daphnia* but did show that exposed larvae captured prey more slowly. These results differ from another herbicide's (2,4-D) effects on crayfish, where exposed crayfish consumed a lower percentage of food than controls (Browne and Moore, 2014). The 2,4-D herbicide did cause crayfish to take longer to locate and consume food, similar to my study. These results also differ from glyphosate's effects on *P. milvina* wolf spiders prey capture in that *P. milvina* required more lunges but took the same amount of time compared to the control (Rittman et al., 2013). The latency data for the time of first strike at a *Daphnia* showed that the 5 mg/L concentration took significantly less time to strike than the control on Day 14. Roundup concentration decreased the time to strike at the first *Daphnia*, but did not significantly affect the number of strikes the larvae performed before a successful capture of the *Daphnia.*

The results for the seek refuge and anti-predator trials showed that Roundup® concentration did not have significant effects on the time it took *P. longipennis* to seek refuge, the distance traveled, or the velocity traveled. These results differ from prior research observing metolachlor's effects on *O. rusticus* crayfish walking speeds (Wolf and Moore, 2002). These researchers found that metolachlor caused a decrease in walking speeds of the crayfish. These results also differ from the effects of atrazine on *A. barbouri* salamanders, which causes greater activity (Rohr and Palmer, 2004).

Various agrochemicals, heavy metals, and surfactants have been shown to be info-disruptors for numerous taxa, even at low concentrations (Lurling and Scheffer, 2007). I predicted that Roundup® would have harmful effects on *P. longipennis* response time to consume food, to seek refuge, and to flee from a simulated predator. Various studies have shown that pollution might increase the risk of disease and predation by affecting species' perception of fear (Lurling and Scheffer, 2007; Rohr et al., 2009). Even though Roundup® did not have significant effects on the seek refuge and anti-predator trials, it did disrupt the time it took for the larvae to consume *Daphnia*. This suggests that Roundup® is an info-disruptor for *P. longipennis*.

There are two main predators of dragonfly larvae: insectivorous fish in communities with fish and large larval dragonfly species in communities without fish (Hopper, 2001). A study performed by Hopper (2001) showed that the escape behavior of *P. longipennis* differs between communities based on different predator types, as well as waterborne cues from those different predator types. Large larval *Anax* dragonfly species were found at the mesocosms where I collected *P. longipennis* larvae. There were no fish found in those mesocosms, therefore I predicted that the larvae would respond to the "predator" in the anti-predator response trials as if it were a larger larval dragonfly species. The larger larval dragonfly species replace fish as the main predator in those systems (Hopper, 2001).

Fleeing from an invertebrate predator can be an effective escape behavior (McPeek et al., 1996), but fleeing from a fish may increase the level of detection, attack, and capture by that fish (Henrikson, 1988). Species that coexist with fish swim slowly and less frequently, and usually do not flee from an attack (Hopper, 2001). These species even remain motionless when they encounter an invertebrate predator in a staged setting, which results in death (McPeek, 1990). The species that inhabit fish-free waters move more often and quickly than the species that coexist with fish. They readily swim away from approaching predators. In a laboratory setting, *Enallagma* species of dragonfly from fish-free lakes are more active, suggesting that they are more susceptible to predation by fish (Blois-Heulin et al., 1990; McPeek, 1990). The *Enallagma* species from lakes containing fish are less active, which causes them to be more susceptible to dragonfly predation. Henrikson (1988) found that dragonfly species residing in lakes with fish swam away from a simulated attack only 10% of the time and froze the other 90%, whereas species residing in fishless lakes swam away 70% of the time. In the anti-predator trials in my research, the larvae did flee from the simulated predator as if it were a larger larval dragonfly.

The results from the growth trials show that the Roundup® concentrations did not have a significant effect on log head width and log body length when compared to the controls. These results differed from atrazine’s effects on *C. puella* larvae, where head width decreased (Campero et al., 2007). Another study found that early juvenile crayfish exposed to chronic levels of glyphosate had reduced growth rates (Avigliano et al., 2014). Juvenile fish (*Leporinus obtusidens*) exposed to 1 mg/L and 5 mg/L Roundup® presented a 10%-15% lower length over a 90-day trial period (Salbego et al., 2009). While growth rate did decrease with prolonged exposure in my research, it was not significant compared to the controls.

The results from the mortality trials showed that Roundup® concentration and increased exposure time were not significant. These results differed from prior studies on the effect of Roundup® on the amphipod *H. castroi* (Dutra et al., 2010) and multiple species of amphibian tadpoles (Relyea, 2004), where exposure caused increased mortality.

I found dose response relationships where there were differences between the low doses and high doses of Roundup® for the time it took the larvae to consume 4 *Daphnia* (Figure 2B) and the growth of the larvae (Figure 7A & 7B). Larvae exposed to low levels of Roundup® captured prey more slowly and grew faster than the higher Roundup® treatments. This type of response curve I saw for growth is a common phenomenon called hormesis (Jager et al., 2013). There are three options for explaining hormesis: acquisition, allocation, and medication (Jager et al., 2013).

Acquisition is when an organism obtains more energy from food sources (Jager et al., 2013). A possible cause for this need for an increase in energy acquisition is that the higher levels of energy assist with the organism's energy loss due to exposure to a toxin, such as an herbicide. Some organisms may obtain higher amounts of energy than are needed. This could lead to physiological changes such as increased growth or higher fat reserves. The ramification of these changes is that the organisms may be more exposed to predation if they grow larger or increase activity to obtain food. Allocation is when an organism distributes energy to other traits where that energy is needed more. An example for this explanation of hormesis would be if an organism distributes energy that it normally uses in reproduction to increase its growth instead. The organism distributes the energy to the most important process in order to survive longer. Medication by a toxin may cure an organism with an infection. The toxin may assist in fighting infections that the organism may have, which in turn, helps the organism survive or grow better than others would that are still infected.

From my data, the 2.5 mg/L concentration had a negative effect on the time it took to 4 *Daphnia* compared to the higher exposure level. This did not follow the general terms of hormesis in that the lowest concentration did not stimulate responses; instead, it inhibited the response time. The acquisition explanation of hormesis is still relevant to my research because the larvae exposed to 5 mg/L concentration consumed *Daphnia* faster than the larvae exposed to the 2.5 mg/L, supporting that the higher concentration may influence the amount of energy obtained from faster feeding. For the allocation explanation, the larvae had spurts of growth over the exposure period, but all concentrations had a decrease in growth rate. The larvae exposed to the 2.5 mg/L concentration consumed *Daphnia* more slowly but had a larger size throughout the experiment showing that the larvae distributed energy in a different manner. For medication, I did not determine if any type of infection existed in the larvae. If there were infections in the larvae, that would assist in explaining why the larvae exposed to the 5 mg/L and the 10 mg/L concentrations did not consume prey at a slower rate, have significant effects on growth, and die at a significant rate.

Although my results only showed weak effects of Roundup®, in combination with other studies, I recommend the use of alternative methods, such as incorporating alfalfa in annual crop succession or sowing mixed crops, instead of herbicides (Meiss et al., 2010; Gaba et al., 2015). In a study performed by Gaba et al. (2016), crop yields and herbicide use did not have a significant relationship. Herbicides were found to be better at controlling less abundant plant species than the abundant weed species that farmers were trying to control. Herbicides reduced the survival of more abundant weed species only when high doses of herbicides were applied in a small number of cases. Wheat yield loss due to weeds was found to be less than 8% in fields exposed to herbicides but weeds in organic farms have an adverse effect on crop yield. Abundant weed species do not decrease crop yields and herbicides are not suspected to help control those abundant weeds. This information supports that the use of herbicides should be reduced or terminated to protect the environment from any more degradation (Gaba et al., 2016). More research should be completed in the use of herbicides on crops to provide more information if herbicides are truly needed to increase crop production.

Glyphosate is the only herbicide that is certified by the US Environmental Protection Agency (USEPA) for use in aquatic environments (USEPA 1993; Rzymski et al., 2013). Glyphosate has nearly no mobility in water and is removed quickly to the sediments and suspended particulate matter after ionization (Solomon and Thompson, 2003). This does not inhibit its potential toxicity to living organisms, especially those inhabiting the bottom layers of water bodies, such as *P. longipennis,* and those feeding on the particulate matter. In other studies, pesticides have strong selection on invertebrates in aquatic systems (Köhler and Triebskorn, 2013). A study performed by Rzymski et al. (2013) indicated that GBHs may cause harmful effects on aquatic organisms including macroinvertebrate communities. All levels of organisms can be affected in some way by herbicide exposure.

Herbicide use in natural surface waters and terrestrial ecosystems near aquatic environments should have stricter limitations and monitoring procedures in place. My research, and many prior studies show that herbicides cause negative effects on many organisms as well as continuing degradation of the environment. With so much information of these harmful effects, it is surprising that many toxic herbicides are still in use. Not only should there be limits on the levels of usage, but usage of some herbicides should be terminated based on chronic effects on organisms exposed to them. My study provides more detail into how a commonly used herbicide is harmful to a possible bio-indicator species, which in turn, shows that the environment overall is impaired by herbicide usage. This research should be replicated in the future and also determine if higher concentrations of Roundup® would have a more significant effect on dragonfly larvae.

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