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Negative Effects of Common Herbicides on Non-target Invertebrates

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NEGATIVE EFFECTS OF COMMON HERBICIDES ON NON-TARGET INVERTEBRATES

by

DEBRA ALBANESE

(Under the Direction of Lance Durden)

ABSTRACT

Three herbicide formulations, Roundup® Ready to Use Weed and Grass Killer, Bayer Advanced Southern Weed Killer for Lawns and Pure 20% Vinegar Solution were directly applied to eggs of four species of butterflies, *Danaus plexippus*, *Papilio cresphontes*, *Papilio polyxenes* and *Papilio troilus*. Roundup and Bayer Advanced containing 2,4-D, mecoprop-p and dicamba significantly reduced hatch success in all four species. Lethal and sublethal effects of these herbicides on the earthworm, Eisenia andrei, were also examined. Direct contact by *E. andrei* with a heavy dose of the herbicides applied to tops of soil 24 hours prior was lethal to earthworms. In a separate study, an amount equal to a light overspray was applied to soil on top of burrowing worms and no increase in mortality or reduction in reproduction was seen. However, we saw a trend of reduced reproduction coupled with increased adult weight in the light overspray with acetic acid that was significant and warrants further study.

INDEX WORDS: Glyphosate, Roundup 2,4-D, 2,4-Dichlorophenoxy acetic acid, *Eisenia andrei, Papilio cresphontes, Papilio polyxenes, Papilio troilus, Danaus plexippus*

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DEDICATION

This thesis is dedicated to my husband, Vince, for your support, encouragement and patience.

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TABLE OF CONTENTS

Page

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CHAPTER

2 THE EFFECTS OF HERBICIDES ON BUTTERFLIES

3 THE EFFECTS OF HERBICIDES ON EARTHWORMS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

OVERVIEW

Loss of insect biodiversity and decreased abundance of insects has become a worldwide problem (Conrad et al. 2006, Hallmann et al. 2017). The decline is due to a variety of factors, but habitat destruction, introduction of non-native plant species, climate change and increased use of pesticides (Fox 2013) are primary among those. Among all pesticides used in the U.S. in 2012, 61% were herbicides (U.S. EPA 2017). While herbicides are not intended to cause direct harm to insects they do indirectly affect phytophagous insects through loss of habitat and reduced nutritional value of host plants (Bohnenblust et al. 2013, Hahn et al. 2014, Watts et al. 2016). However, recent studies have shown that direct contact with herbicides either topically, or through ingesting treated food materials, did have lethal and sublethal effects on lady beetles (Freydier and Lundgren 2016), green lacewings (Schneider et al. 2009), parasitic wasps and honeybees (Gill et al. 2017). The impact on these beneficial insects is cause for particular concern as they provide biological control of insect and plant pest species. Without these beneficial insects the need for pesticides increases.

Pesticide approval by EPA is based on testing of active ingredients only. Yet, the active ingredient is rarely used in its pure form. Commercial formulations include adjuvants such as surfactants, crop oil concentrates, and ammonium fertilizers to improve the effectiveness of the herbicide (Hartzler 2019). Many of these additives are unregulated and thus are not required to be disclosed on labels. However, these adjuvants can change the effect on non-target invertebrates as well as change its persistence in the environment (Freydier and Lundgren 2016) and joint effects of mixtures can alter the toxic effect on organisms (Myers et al. 2016). The joint effects resulting from different combinations of adjuvants can have markedly different effects. For example, in a study of two glyphosate-based formulations, one was 4.5 times more lethal to earthworms after direct exposure than the second despite equal amounts of the active ingredient (Piola et al. 2013). Other studies have shown that Roundup® preparations are 3-42 times more toxic to some fish species than the active ingredient alone (HSDB

2015). Because inactive ingredients can drastically change the effects of the product on living organisms, ecotoxicological studies should test commercial formulations rather than active ingredients alone.

How herbicides are manufactured and used can also cause differential effects on the environment. First, the amount of herbicide that is delivered can vary widely depending on the spraying equipment, the technique of the applier, the accuracy of the dilution and the wind velocity (Qasem 2011)Heavy use of herbicides is common in all sectors. In many cases in agriculture, glyphosate-based herbicides are applied to fields before the main crop is planted, several times during the growing season and as a desiccant prior to harvesting (Danne et al. 2019, Givens et al. 2009). For control of lawn weeds, herbicide formulations are sprayed to large expanses of turfgrass to control a relatively small area of weeds. This heavy use of the herbicide increases opportunities for non-target invertebrates to come into contact with the chemicals.

There are a number of post-emergent herbicides in current use in the U.S. but nearly 50% of total pounds used is glyphosate-based. In the non-agricultural sector, 2,4-Dimethyphenoxyacetic acid, developed in the 1940’s is used as heavily as glyphosate (U.S. EPA 2017). The search for natural alternative herbicides is underway and acetic acid is one possible alternative that is effective against small weeds (Smith-Fiola and Gill 2007) and may provide a safer alternative to non-target invertebrates.

While herbicide use is intended to eliminate unwanted plants, organisms that rely on those plants for nutrition or habitat or that live in the soil below can be come in contact with the chemicals. Almost all Lepidoptera (butterflies and moths) have close relationships with specific plant species upon which they must lay their eggs for their larvae to survive (Hardy and Otto 2014). In terrestrial food webs, Lepidopteran larvae, rich in protein, calcium and carotenoids, are key components in the diets of birds, rodents, lizards and predatory insects (Narango et al. 2017, Strong et al. 2000). They also provide pollination services to nectar-producing trees, shrubs and herbs, including many species humans rely on for food and act as biological control agents by feeding on weed species (Fox 2013). A well-known butterfly, the Monarch butterfly, is threatened in North America due to loss of habitat to agriculture and coincident with increased use of herbicides in the Midwest (Pleasants and Oberhauser 2013). One recommended action to combat the population decline of the Monarch and other butterflies is to increase plantings of floral strips and gardens to provide habitat and food resources. However, this could inadvertently bring both eggs and larvae of butterflies into contact with herbicides in use in these areas (Majewska et al. 2018).

Earthworms are another invertebrate group that may inadvertently be exposed to herbicides that seep into soils after application to agricultural fields and lawns. Earthworms are important components of terrestrial food webs and provide ecosystem services by improving soil structure and breaking down nutrients in soil for use by plants. The earthworm, *Eisenia andrei*, is widely used in toxicity testing of chemicals and can be a biomarker for soil contamination. Moreover, *E. andrei* reproduction rates are reduced and their growth rates differentially affected when exposed to heavy metals and harmful organic compounds (Zaltuarskaite and Sodiene 2010). Because of their sensitivity to environmental toxins, use of *E. andrei* is required by international protocols for acute toxicity testing (Vernile et al. 2006).

There are several aims of this study, First, to determine if direct contact with formulations containing glyphosate, 2-4-D or acetic acid affect egg hatch success of four butterfly species, *Papilio polyxenes, Papilio cresphontes, Papilio troilus and Danaus plexippus*. Second, to determine if direct application of 10% and 20% acetic acid to larvae of these species increases their mortality compared to untreated controls. Third, to determine if direct contact with herbicide-contaminated soil increases adult mortality or affects the weight and reproduction of *E. andrei*.

CHAPTER 2

THE EFFECTS OF HERBICIDES ON BUTTERFLIES

INTRODUCTION

Butterflies exemplify the close relationship between plants and insects as larvae of many species are food plant specific. Larvae of over 50% of butterfly species will only feed on plants belonging to a single genus and another 25% feed only on plants belonging to a single one family. (Bernays and Chapman 1994). With many butterfly host plants being considered agricultural weeds (Marshall et al. 2013) butterfly eggs and larvae are at risk of direct exposure to herbicides that are sprayed to kill their host plants.

Butterflies lay their eggs on upper and lower surfaces of leaves, stems or flowers of host plants. The placement varies by species. Eggs are comprised of the oocyte or developing embryo, covered by a membranous vitelline envelope over which the chorion or outer shell is laid down. The chorion is porous with three types of openings present in different numbers depending on species. Aeropyles facilitate gaseous exchange, hydropyles allow water uptake and micropyles allow access of sperm for fertilization (Telfer 2009). Larvae chew through the shell and in many species consume it before beginning to feed on the host plant.

Despite their ecological importance, testing of herbicides has not been widely performed on Lepidoptera. Generally, the honeybee (*Apis mellifera*) has been used to satisfy ecotoxicology testing requirements for chemical companies seeking approval by EPA of their pesticide products (Whitford 2019). As such, there is a limited number of published studies on the effects of the three herbicides used in this study on insects and especially on lepidopteran species.

With respect to glyphosate, the active ingredient is rarely used alone as an herbicide but rather it appears in formulations of various concentrations containing adjuvants and other herbicides (Defarge et al. 2016). Various salt forms of the glyphosate acid have been developed to improve solubility and these compounds also create variability in the efficacy and toxicity of the product (Henderson et al. 2010). The best-known commercial formulation in the U.S. is Roundup®. The Roundup® formulation used in this study contains the isopropylamine salt form of glyphosate plus pelargonic acid along with other unnamed inert ingredients. Roundup® is sprayed on the leaf surface and stems and from there glyphosate is translocated throughout the plant via the phloem (Shuette 1998) where it inhibits the production of enzymes involved in amino acid production. (Heilig 2012). Glyphosate acts slowly within the plant so, nonanoic acid, also known as pelargonic acid, was added to this Roundup® formulation to destroy cell membranes and cause desiccation of stems and leaves within 24 hours (Wehtje et al. 2009, Kardasz et al. 2019). Pelargonic acid is a naturally occurring short chain fatty acid extracted from rapeseed oil (canola). However, despite being a natural product the European Food Safety Authority concluded that pelargonic acid had demonstrated high risk to non-target arthropods and earthworms when applied to open areas and could not conclude that pelargonic acid was a pesticide of low concern (EFSA 2013).

As the result of heavy use of glyphosate, increasing numbers of weed species have developed resistance to it. To combat the resistance, farmers are incorporating other herbicides in tank mixes with glyphosate or reaching back to older herbicides such as 2,4-D and dicamba in new formulations for a solution (Bohnenblust et al. 2013, Olszyk et al. 2015). Both 2,4-D and dicamba are synthetic plant hormones that cause overgrowth of stems and withering of leaves eventually killing the broadleaf (non-grass) weed. Acute contact with glyphosate was lethal to 50% of honeybees at a concentration of 100 µg per bee (Henderson et al. 2010).

Studies of the effects of 2,4-D on insects are also limited. According to the EPA, when used at recommended levels, 2,4-D is not harmful to beneficial insects but carabid beetles exhibited 50% mortality after exposure to 1 gm^{-2} after 4 days (Jervais et al. 2008, Henderson et al. 2010). Again, several salt forms are sold commercially including an ester and amine form (de Castro et al. 2017). When 2,4-D was sprayed on host plants and ingested by the larvae of *Eupackardia calleta*, a southern Nearctic silk moth, larvae grew normally but emerged from pupal chambers 9 days to 6 weeks earlier than control larvae. Although small amounts of 2,4-D were found in the emerged adults, no increase in mortality occurred (Deml and Konrad 2001). In a study on the lady beetle, *Coleomegilla maculata*, a commercial formulation of 2-4-D containing 5,000 ppm of the ester form of the active ingredient reduced survival of second stage larvae by 80% while treatment with 2,4-D active ingredient was not different from the control. Neither the dicamba commercial formulations nor the active ingredient alone resulted in mortality different from control. However, both the active ingredients individually and commercial formulations of 2,4-D and dicamba reduced fecundity of the beetles (Freydier and Lundgren 2016). In another study, there was no evidence of direct toxicity of dicamba active ingredient to second and third instar larvae of two species of butterflies, the Painted Lady butterfly, *Vanessa cardui*, or the corn earworm moth, *Helicoverpa zea.* Only indirect effects from reduced nutrition provided by plants damaged by sublethal doses of dicamba were observed (Bohnenblust et al. 2013). Another concern with these two chemicals is that they volatilize and as such, are prone to drift during the application process potentially increasing the risk of damage to non-target plants and beneficial invertebrates (Bohnenblust et al. 2013). The use of 2,4-D active ingredient in agriculture had been steady since 1997 at 30 million pounds per year until 2012 when there was an increase to 40 million pounds used (U.S. EPA 2017) possibly in response to growing glyphosate resistance.

Due to environmental concerns over the impact of herbicides the search for alternatives for weed control is ongoing (Barker and Prostak 2009). Household vinegar which has been used historically as a natural herbicide (Dayan et al. 2009) may provide an alternative. Strong vinegar produces a quick burn-down effect when applied in sunlight but does not always kill the root and therefore regrowth of the plant can occur (Dayan et al. 2009). In some studies, concentrations of 20% vinegar approached the effectiveness of glyphosate (Chinery 2002) but in other studies, it was ineffective and costly (Snell 2014). It appears to be most effective on small weeds where leaf surfaces can be adequately covered (Evans et al. 2009). The U.S. EPA approved the pesticide registration of 20% acetic acid in 2015 (U.S. EPA 2015). To be used in certified organic crop production, a substance has to appear on the National List of Allowed and Prohibited Substances issued by the USDA. As of May 23, 2019, acetic acid had not been petitioned for addition to the list. (The National List of Allowed and Prohibited Substances 2010). Acetic acid had been approved by EPA for use as an unlabeled pesticide additive at concentrations up to 8% and is considered not harmful at this concentration. (Smith-Fiola and Gill 2007). Stronger concentrations, between 15 and 20%, are effective against redroot pigweed (*Amaranthus retroflexus)* and velvetleaf (*Abutilon theophrasti)*, two noxious weeds that impact row crops (Evans et al. 2009). Prior to the registration of 20% acetic acid as a pesticide, tracking of sales and usage by the EPA was not required, so the breath of use is uncertain. However, from an environmental perspective, acetic acid may be good alternative as it is a weak acid, has a short half-life of 14 days, does not adsorb to soil, and is not expected to volatilize from moist soil (Smith-Fiola and Gill 2007). Unfortunately, there has been no published direct testing of the effect of acetic acid on invertebrates, so additional testing on lepidopteran larvae was performed during this study.

The first study tested the effects of direct contact with Roundup®, Bayer 2,4-D and 20% acetic acid on the hatch success of butterfly eggs and of direct contact with first instar larvae with 10% and 20% acetic acid on survival. I predicted that mean hatch success of eggs and the time it took for of eggs to hatch after treatment with herbicides would differ from the control group but that the herbicide treatments would affect the number of days the larvae survived after treatment. I also predicted that mortality of neonate larvae treated with 10% and 20% concentrations of acetic acid and their amount of weight they gained in 8 days after treatment would not be different from the control group.

METHODS

Study organisms

Three species of Papilionidae were used. *Papilio polyxenes* is commonly called the Black Swallowtail butterfly. Larvae of this species feeds on plants belonging to the family Apiaceae and is found mainly in the Eastern United States (Hall 2014). Eggs are laid singly on the upper surface of leaves and flowers (pers. obs.). The Apiaceae family is comprised of 91 genera and includes carrots, dill and parsley among others. (USDA 2018) One member of this family, *Daucus carota*, commonly known as wild carrot or Queen Anne's lace, is considered to be a noxious weed in many southeastern states including Georgia (Georgia Invasive Species Task Force 2018) .The dill plant *Anethum graveolens* cv. "Mammoth" and water hemlock (*Cicuta douglasii*) from Apiaceae were selected as host plants for *P. polyxenes* for this experiment. Larvae of *Papilio cresphontes,* the Giant Swallowtail, feed on plants from Rutaceae, the citrus family which includes *Zanthoxylum clava-herculis*, the Toothache tree. It is widely distributed throughout the eastern United States and south into Mexico, the Caribbean and South America. Eggs are laid singly on the upper surface of the leaves of the host plant (McAuslane 2018). Larvae of *Papilio troilus,* the Spicebush Swallowtail, feed on plants in the family Lauraceae, including Sassafrass (*Sassafras albidum). P. troilus* is found throughout the eastern United States as far west as Texas and north to southern Canada. Eggs are laid singly on the undersides of leaves. Later stage larvae form shelters by spinning silk mats longitudinally along leaf surfaces causing the leaf to fold over. They remain in the shelter by day and feed at night (Hall 2014).

Danaus plexxipus, the Monarch butterfly (Family Nymphalidae, Subfamily Danainae), is among the best known butterflies in the United States. Its range is far reaching, extending from Mexico and Central America to both coasts of North and South America and the Caribbean. It is also found in Australia. It feeds on plants in the genus *Asclepias*, or milkweed. Eggs are laid singly on the undersides of leaves (Sourakov 2009). Eggs generally hatch within four days of being laid and up to 90% mortality of eggs and larvae is common (Oberhauser 2004).

Herbicides

Round-up® Ready-to-use Weed and Grass Killer – This product contains 2% glyphosate isopropyl amine salt, 2% pelargonic acid and related fatty acids and 96% unnamed ingredients. The directions for use on the Roundup® label call for thoroughly wetting the unwanted plant. Replanting any type of plant, ornamental or crop, is safe after three days.

Bayer Advanced Southern Weed Killer for Lawns – This herbicide contains 7.59% 2,4-D dimethylamine salt, 1.83% mecoprop-p, dimethylamine salt, 0.854% dicamba, dimethylamine salt and 89.74% unnamed ingredients. Directions for use per the label are to attach to a hose and spray. The recommended rate of application is 0.7 ml/m^2. The herbicide dilutes in-line when these directions are followed. For this study, however, the full-strength product was tested.

Acetic acid, 20% strength – A commercially available solution labelled "Pure 20% Vinegar Solution" was used for the egg hatch tests. It was manufactured by Factor Direct Chemicals in Copiague, New York, distributed by EcoClean Solutions, Inc. and purchased on Amazon.com. It was not labeled for use as an herbicide. For direct application to larvae, full strength acetic acid (JT Baker, CAS 64-19-7, 99.8%) was diluted with deionized water to form 10% and 20% solutions.

Experiment 1 – Direct Application of Herbicides to Eggs

Procedure

Eggs from each species were collected from host plants in a suburban garden or in the butterfly enclosure at the Coastal Discovery Museum on Hilton Head Island, SC. For *Papilio polyxenes*, the number of eggs obtained from the wild was supplemented with eggs from adults raised in the laboratory from wild-collected eggs. These adults originated from eggs obtained from the garden mentioned above. Sample sizes varied by species. The number of replicates by species were *Papilio polyxenes*, n= 10, *P. cresphontes* and *P. troilus*, n=18, and *Danaus plexippus*, n=20. Treatments were consistent among the four species tested and included Roundup®, Bayer 2,4-D and commercially prepared 20% vinegar. Deionized water was the control for the *Papilio* species, distilled water was the control for *D. plexippus*. Eggs were left on a small portion of leaf and transferred to prenumbered Petri dishes or clear plastic cups with lids with two filter papers moistened with deionized water. Treatments were assigned using a random number generator (Graphpad.com 2018). One drop from a dropper equal to 20 µl of each herbicide preparation and distilled water was applied directly to each egg of *P. polyxenes*. For all other species, a 10 µl drop was applied using a micropipette. The eggs were incubated at temperatures between 23°C and 25°C. One drop of deionized water was added each day to filter papers to maintain humidity levels within the containers. Eggs were checked daily for hatching. After hatching, *Papilio* larvae were raised in the insectary under a 12:12 light cycle. Leaves of the host plant were added as needed to the cups. At approximately four weeks post-hatch, the surviving larvae were placed in individual enclosures and fed until they formed pupae. *Danaus plexippus* larvae were not raised after hatching due to lack of host plants. Egg hatch success was calculated as the number of individuals hatched compared to the beginning total. For eggs that did hatch, the number of days from the treatment date to the hatch date was calculated. For larvae that did not survive, the number of days from the treatment date to the day the larvae died was calculated.

Experiment 2 – Direct application of acetic acid to larvae of Danaus plexippus

Procedure

Leaves of tropical milkweed containing eggs of *Danaus plexippus* were collected from a suburban garden on Hilton Head Island, SC during the week of October 19 – 26, 2018. Eggs collected at the beginning of the week were stored each day at 4°C until approximately 70 eggs had been gathered. All eggs were brought up to 23°C and allowed to hatch in plastic Petri dishes fitted with moistened filter paper. After hatching, fresh milkweed leaves were provided as needed until the day of treatment. Larvae were 1 to 5 days old at the time of treatment.

Each caterpillar was weighed and its length measured. Eighteen larvae were separated by size as follows; under 0.001 g, 0.001 – 0.002 g, and > 0.002 g. Individuals were sorted by size into small and large. From each size group, the larvae were haphazardly assigned to one of three treatment groups; 10% acetic acid, 20% acetic acid and deionized water for the control group. There were 6 replicates. Using a micropipette, 5 µl of each treatment was applied directly to the abdomen on the dorsal surface. Larvae were checked daily and mortality noted. Larvae were weighed and measured after 1 hour, 24 hours and 8 days and the change from the initial size calculated. One larva from the 10% treatment group escaped its enclosure and was removed from the results.

Statistical Analysis

Egg hatch success was assessed using Chi-square (χ^2) tests. The number of days to hatch and the number of days to death were analyzed using ANOVA. Data were first tested for normality (Kolmogorov – Smirnov's test) and homogeneity of variances (Levene's test). For the days to hatch results for *P. troilus,* the non-parametric Kruskal-Wallis test was used due to heterogeneous variances. [All statistical analyses were performed in JMP 13.1.0. (SAS Institute, Inc. Cary, N.C.)]

RESULTS

Experiment 1 - direct application of herbicides to butterfly eggs

For all four butterfly species tested, the percentage of eggs that hatched after direct exposure to herbicides was significantly different among the treatment groups. Eggs in the control group hatched at rates that ranged from 50% to 100%. The lowest hatch rates were seen in eggs treated with Roundup®, which ranged from 0 -15% and Bayer, 2,4-D, which 5.6% to 10%. Eggs treated with 20% acetic acid fared better than the other herbicide treatments with hatch rates ranging from 35% to 72.2%.

For *Papilio polyxenes,* treated with 20µl drops, no eggs treated with Round-up® or 20% acetic acid hatched and only 10% of eggs treated with Bayer, 2-4-D hatched compared to 50% hatch success in the control group ($\chi^2_3 = 13.5$, p = 0.004) (Figure 1). Unhatched eggs treated with Round-up® and Bayer, 2-4-D appeared dry and were easily crushed. Conversely, unhatched eggs treated with acetic acid appeared to have yolk sacs but did not develop. For *P. cresphontes*, 94% of eggs hatched from the control group and 72% from the acetic acid treatment group, yet only 6% of Roundup® treated eggs, 22% of Bayer 2,4-D treated eggs hatched. The difference in hatch success among the treatment groups was significant ($\chi^2_3 = 44.0$, $p < 0.0001$). For *P. troilus* 100% of eggs hatched in the control group and 72% of eggs that had been treated with acetic acid hatched, while 6% of Roundup® treated eggs and 6% of Bayer 2,4-D treated eggs hatched. The difference in hatch success among the treatment groups was again significant ($\chi^2_3 = 63.6$, $p < 0.0001$). Finally, for *Danaus plexippus*, 65% of the control group eggs hatched, 6% of each of the Roundup® and Bayer, 2, 4-D groups eggs hatched and 35% of the acetic acid group eggs hatched ($\chi^2_3 = 17.7$, $p = 0.005$) (Table 1).

The number of days it took for larvae to hatch after treatment ranged from 2.0 to 4.3 for *Papilio cresphontes* and *P. troilus*, and from 9.0 to 9.3 days for *Danaus plexippus* (Table 1). For *P. cresphontes*, the mean number of days to hatch ranged from 2.0 ± 0.0 days for the Roundup® treatment to 4.3 ± 0.5 days for the Bayer 2,4-D treatment. Only one egg out of 18 hatched from the Roundup® treated batch and it did so after two days. However, the difference in the mean number of days to hatch among the treatment groups was not significant ($F_{3,31} = 1.65$, $p = 0.20$). For *P. troilus* the mean number of days to hatch ranged from 3.0 ± 0.0 days for the Roundup treated group to 4.3 ± 0.6 days for the acetic acid treated group. Again, the difference in the mean number of days to hatch by treatment group was not significant (Kruskal-Wallis, p=0.43). Only 1 egg of 18 hatched from each of the Roundup® and Bayer, 2,4-D treatments for *P. troilus* after 4 and 3 days, respectively. Finally, for *Danaus plexippus*, the mean number of days to hatch also did not differ among the treatment groups ($F_{(3,21)} = 0.25$, $p=0.86$). The number of days to hatch for this species was longer than for the swallowtails and ranged from 9.0 ± 0 days for the Bayer, 2,4-D treatment group to 9.3 ± 0.5 for the Roundup treated group.

The fate of Papilio larvae that successfully emerged from eggs varied among species and among treatment groups. Larvae of *Danaus plexippus* were not monitored after they hatched. Length of survival, measured as the number of days between the treatment and the day of death, was not different among the treatment groups for any of the three Papilio species tested.. For *Papilio cresphontes*, the control group survived on average 19.1 ± 9.1 days before dying and the Roundup® group survived an average of 21 days. Survival for the Bayer 2,4-D and acetic acid groups was lower at 10.3 ± 4.0 and 13.3 ± 3.0 days, respectively. However, days to death was not statistically different among treatment groups ($F_{3,24} = 2.53$, p =0.08). *For Papilio troilus*, the control group survived on average 12.1 ± 5.5 days and the acetic acid group survived an average of 14 ± 5.0 days. Only one larva survived in each of the Roundup® and Bayer 2,4-D treatment groups and they lived 4 and 14 days, respectively. Days to death was not statistically different among treatment groups ($F_{3,19} = 1.13$, p =0.36). No *P. troilus* larvae treated with Roundup® or Bayer, 2,4-D survived to pupation while 22% of acetic acid-treated larvae and 22% of the control group pupated. All of the eggs of *P. polyxenes* that hatched including one treated with Bayer, 2,4-D and 5 from the control group, survived to form pupae (Table 2).

For the three Papilio species combined, no eggs treated with Roundup® lived long enough to form pupae (Table 2). In the Bayer, 2,4-D treatment groups for all three Papilio species, only one larva survived to form a pupa. Three *P. cresphontes* larvae treated with acetic acid formed pupae and four *P. troilus* larvae formed pupae. No eggs of *P. polyxenes* treated with acetic acid hatched.

In the second experiment, the odds of dying were 3.2 times higher after treatment with 10% acetic acid compared to the control group and the odds of dying were 2.5 times higher after treatment with 20% acetic acid. However, the proportion of individuals that died by the end of the 8-day period was not statistically different among the treatment groups ($\chi^2_2 = 0.82$, p = 0.66). All the larvae that perished in the treatment groups were less than 0.002 g at the time of treatment, while no larvae less than 0.002 g in the control group died. Only one larva in the control group died and it was larger than 0.002 g. In addition, mean weight increase for those larvae surviving to the 8th day after treatments was not different among the treatment groups ($F_{(2,9)} = 1.72$, p = 0.23) (Figure 2).

DISCUSSION

For butterflies treated with herbicides, the hypothesis that treatment with herbicides would reduce egg hatch success was supported. However, hatch success rates in the control groups were lower than expected for *Papilio polyxenes* and *Danaus plexippus*. Based on personal observation, I expected egg hatch rates in the laboratory for the control group to be near 90%. In a study of the species, *Papilio polytes*, monthly hatch rates of eggs raised in laboratory conditions over the course of one year ranged from 40% to 100%, with a mean annual hatch rate of 93.5% (Atluri et al. 2001). In this study, however, the hatch rate for *Papilio polyxenes*, in the control group was only 50% and for *Danaus plexippu*s the rate was 65%. For the other two species, *P. cresphontes* and *P. troilus,* control hatch rates were as expected at 94% and 100%, respectively. For butterflies, a number of factors can affect the viability of eggs, such as health and nutrition of the parents, genetics of the offspring and abiotic factors like temperature and humidity. The rearing conditions of eggs were similar for all four species, so it is unlikely that abiotic factors influenced hatch rates differentially. However, some of the eggs of *P. polyxenes* were obtained from the mating of two individuals raised from eggs gathered in the same small garden. As females were observed laying multiple eggs on one plant, there is a chance that the individuals that mated were a brother-sister pair. As such, the lower than expected hatch rate in the *P. polyxenes* control group may have been the result of reduced egg viability due to inbreeding depression. In two studies of the Glanville fritillary (Nymphalidae: *Melitaea cinxia*) in Europe, hatch rates were 37% for brother-sister pairs compared to 69% for non-related pairs and 71% for brother-sister pairs compared to 90% in non-related pairs (Saccheri et al. 1998; Nieminen et al. 2001). The hatch rate for the control group of *D. plexippus,* was also lower than expected. Inbreeding likely would not have been a factor as all eggs were obtained from the wild. It is possible that 65% is a normal hatch rate for this species. One study reported hatch rates for *D. plexippus* in a laboratory setting 66.8% (Mongue et al, 2016).

The amount of herbicide used may have influenced the results in the treatment groups for *Papilio polyxenes*. Eggs of *P. polyxenes* was treated with 20 µl droplets for all treatments while the other three species were treated with 10 µl droplets. The larger droplet size allowed, in some cases, small pools of herbicide to collect around the base of the eggs which remained attached to host plant leaves. The pooling of herbicide around the base of the egg would have increased the time the egg was in contact with the herbicide and increased damage to the egg resulting in lower hatch success for each treatment. For the remaining three species, the droplet size was reduced by one-half to avoid pooling of herbicide around the egg.

Roundup® and Bayer, 2,4-D, significantly reduced the hatch success of eggs regardless of species. Eggs of *P. cresphontes* and *P. troilus* that did not hatch were discolored and dehydrated while unhatched eggs of *D. plexippus* were discolored and deflated, but not dried out. The fact that the herbicide harms eggs of different species differently could be related to the fine structure of the eggs in each species. Eggs of Papilio species are spherical and smooth while eggs of *D. plexippus* are torpedo-shaped and grooved (pers. obs.). One can imagine that the grooves in the *D. plexippus* eggs may create spaces for herbicides to linger, lengthening the time of contact with the compound. In addition, damage to eggs might not be the result of the active ingredient in the herbicides but rather of the adjuvants added to the herbicide formulations. Manufacturers are not required to reveal the full list of ingredients in their formulas and there is mounting evidence that formulations can be more toxic than the active ingredients alone (Myers, et al. 2016). The main additives to herbicides used to improve herbicide effectiveness fall into three categories, surfactants, crop oil concentrates and ammonium fertilizers (Hartzler 2019). Of these, surfactants, which reduce surface tension of spray droplets, and crop oil concentrates, that provide better coating of surfaces, could be particularly damaging to eggs by clogging the aeropyles in the chorion and thereby preventing gaseous exchange by the developing embryo. Testing of the effect of adjuvants on butterfly eggs should be performed as these may be damaging on their own despite being classified by the EPA as non-harmful (U.S. EPA 2016).

The effect of acetic acid on egg hatch success was less clear. Eggs of *P. cresphontes* and *P. troilus* hatched at a rate of 72% while no eggs of *P. polyxenes* hatched and only 35% of *D. plexippus* eggs hatched. While the 20 µl droplet size flowed over the egg surface, the 10 µl droplet size of acetic acid remained in a bubble in many cases. Because there are no surfactants in the acetic acid formulation, the surface tension of small droplets was high and droplets remained as bubbles on the surface of eggs after application. Although the droplet remained in contact with the egg surface, high surface tension of the droplet could have protected the egg from full exposure to acetic acid.

None of the herbicide treatments affected the time it took for eggs to hatch for *P. cresphontes, P. troilus* or *D. plexippus*. If eggs hatched, they did so in a time frame consistent with the control group. Even in favorable laboratory conditions that removed the risk of predation and parasitism, none of the larvae from hatched eggs treated with Roundup® or Bayer, 2,4-D survived to the last instar. While mortality of first instar larvae is high and variable among butterfly species in general, ranging between 9 to 96% in field and laboratory conditions (Zalucki 2002), treatment with Roundup® and Bayer, 2,4-D resulted in rates far below these norms and could quickly decimate local populations. For eggs of *P. cresphontes* and *P. troilus* treated with acetic acid, some larvae, 17% and 22%, respectively, survived long enough to pupate. Therefore, the effect of acetic acid on butterfly populations may be less damaging than that of Roundup® or Bayer 2,4-D. However, the long-term impact of acetic acid on butterfly populations needs to be studied further.

The second study on the direct application of two strengths of acetic acid, 10% and 20%, to early instar larvae of *Danaus plexippus* was inconclusive. While the odds ratios indicate that larvae treated with acetic acid were at least 2.5 times more likely to die than the control group, the proportions that died were not statistically different among the treatment groups. This was due primarily to a small number of larvae that hatched resulting in lower statistical power. Of the 70 *D. plexippus* eggs collected only 25% hatched compared to a hatch rate of 65% in the first experiment. Eggs stored in the refrigerator for 1 to 3 days may have perished. Zalucki (1982) calculated that below 12.2°C, egg development ceases. For future studies, cold storage is not advised and the application of the treatment of the larvae could be staggered as eggs are collected. In addition, many of the *D. plexippus* eggs gathered were covered with black spots which I believe were the spores of the protozoan, *Ophryocystis electroscirrha* (OE). OE is known to reduce larval survival in infected 1st and 3rd instar Monarch larvae. Larvae inoculated with 10 or 100 spores reduced survival of larvae by 80% for 3rd instar larvae and 65% for 1st instar larvae in a laboratory experiment (Altizer 1999). Another possibility affecting larval mortality is that treatment immediately after ecdysis may be more harmful than treatment applied after the cuticle is allowed to harden. Therefore, it would be important to apply the treatment at a fixed time after the caterpillar sheds it's exoskeleton.

A number of studies have concluded that the effect of herbicides on butterfly population is mainly the result of reduced nutrition of the host plants or a change in the dynamics of the habitat due to loss of the host plants (Bohnenblust 2013, Hahn 2014, Watts 2016, Sharma et al. 2018). However, this study shows that direct contact with Roundup® and Bayer 2,4-D reduces the percentage of eggs that hatch and reduces the number of larvae that survive to form pupa.

Table 1: Egg hatch success and development time for *Papilio cresphontes*, *Papilio troilus* and *Danaus plexippus* after direct exposure to 10 µl of herbicides. (*) indicates data are mean ± SD.

	P. cresphontes (n=18)				*P. troilus (n=18)*				*D. plexippus (n=20)*		
	# hatched	Percent hatched	Days to hatch (*)	Days to death (*)	# hatched	Percent hatched	Days to hatch (*)	Days to death (*)	# hatched	Percent hatched	Days to hatch (*)
Control	17	94.4	3.7 ± 1.1	19.1 ± 9.1	18	100	3.8 ± 1.2	12.1 ± 5.5	13	65	9.2 ± 0.9
Roundup	1	5.6	2.0 ± 0.0	21.0 ± 0.0	1	5.6	4.0 ± 0.0	4.0 ± 0.0	3	15	9.3 ± 0.5
Bayer 2,4-D	1	22.2	4.3 ± 0.5	10.3 ± 4.0	1	5.6	3.0 ± 0.0	14.0 ± 0.0	2	10	9.0 ± 0.0
Acetic acid	13	72.2	4.2 ± 1.2	13.3 ± 3.0	13	72.2	4.3 ± 0.6	14.0 ± 5.0	7	35	9.0 ± 0.8

Table 2: Number of butterfly larvae of *Papilio polyxenes, Papilio cresphontes and Papilio troilus* that survived to form pupae after direct exposure to herbicide treatments. *P. polyxenes* was treated with 20 µl of each herbicide while *P. cresphontes* and *P. troilus* were treated with 10 µl of each herbicide. Control groups were treated with deionized water.

	P. polyxenes (n =10)		*P. cresphontes* (n=18)		*P. troilus* (n =18)	
Treatment	# survived	% survived	# survived	% survived	# survived	% survived
Control	5	50%	5	28%	4	22%
Roundup	0	0%	0	0%	0	0%
Bayer 2,4-D	1	10%	0	0%	0	0%
20% acetic acid	0	0%	3	17%	4	22%

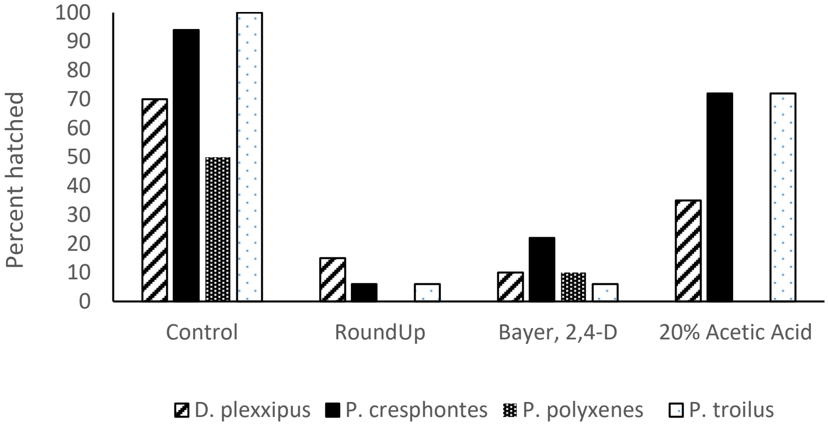

Figure 1: Percentage of *Danaus plexippus* (n=18), *Papilio cresphontes* (n =20), *Papilio polyxenes* (n =10) *and Papilio troilus* (n=20) eggs that hatched after exposure to direct application of herbicide to each egg. Control was either distilled or deionized water. Amount of treatment applied to eggs for *P. polyxenes* was 20 µl. All other species received 10 µl treatments. Within each species, differences among the hatch rates by treatment were significant.

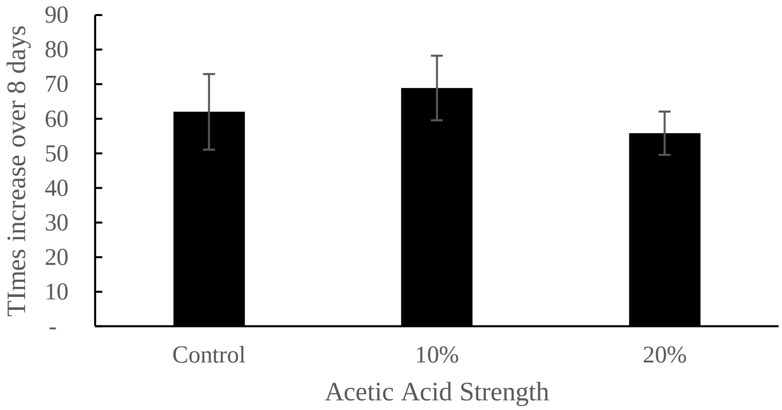

Figure 2: Increase in mean weight of *Danaus plexippus* caterpillars that survived to 8 days post-treatment with 10% and 20% acetic acid and control group. Error bars represent standard error. (n=5 for control, n=4 for 20% acetic acid, n=5 for 10% acetic acid).

CHAPTER 3 – HERBICIDE EFFECTS ON THE EARTHWORM, *EISENIA ANDREI*

INTRODUCTION

The application of herbicides typically involves some degree of overspray onto soil. Yet the amount of overspray will vary depending on the equipment used and the zeal of the applier. This overspray on soil could be a route of unintended exposure to these chemicals by soil-dwelling organisms. Earthworms are one group that could be exposed to soil-borne chemicals. Earthworms are soil-dwelling invertebrates that provide important terrestrial ecosystem services by improving soil function. Their burrowing and tunneling in the soil relieve compaction and increases the flow of water. They feed on surface leaf litter and seeds after dragging them into soil where degradation is slowed and carbon is sequestered, having a positive effect on climate. They improve the growth of plants and other soil-dwelling microorganisms and insects by making nutrients bioavailable (Lavelle et al. 2006). The earthworm *Eisenia andrei* and its close relative, *Eisenia fetida*, are important species in ecotoxicology studies. They are native to Europe but have been introduced worldwide as composting worms.

The life cycle of *Eisenia andrei* makes this species a suitable study organism for toxicology studies. They are hermaphroditic, so sex ratios need not be considered. They sexually mature within 4 weeks at 25°C. The closely related species, *Eisenia fetida* emerges from cocoons 3 weeks after fertilization followed by a slow increase in weight over the next 3 weeks. Each cocoon produces an average of 2.5 juveniles (Van Gestel et al. 1992). Changes in their body weight follow a predictable pattern. Between week three and week five after emerging from the cocoon, there is a rapid increase in weight, peaking at week five and followed by a slight decline in weight thereafter (Neuhauser et al. 1979). Despite the similar growth rate to *E. andrei*, *E. fetida* had nearly 30% lower reproduction than *E. andrei* under control conditions (Dominguez et al. 2004).

Sublethal effects of toxins on weight and reproduction have been observed in this species. Earthworms eat organic materials in soil and along with food material, any toxins that have been bound to the soil are ingested. In studies of the effects of earthworms exposed to contaminated soils, abnormal reductions in weight have been documented and it is hypothesized that this is a result of energy being diverted from growth to metabolizing toxins (Nahmani et al. 2006). However, weight reductions were found to be a less sensitive measure of the sublethal effects of toxins than cocoon production in earthworms (Van Gestel et al. 1992). Effects on reproduction in the form of reduced cocoon production and cocoon hatching, to varying degrees, were seen in all samples of metal contaminated soils (Nahamini et al. 2006).

Aside from ingesting toxins, earthworms can also take up chemicals through their skin (Jager et al. 2003). Jager (2003) showed that compounds with n-octonol-water partition coefficients (log K_{ow}) less than 5.0 were likely to diffuse across the skin of earthworms. All three herbicide formulations and disclosed adjuvants tested in this study have log K_{ow} below 3.42 (Appendix) making uptake through the epidermal membrane possible. Respiration also occurs through the skin as oxygen and carbon dioxide molecules diffuse across the cuticle, aided by moisture in the mucus-coating, into blood vessels in the body wall (Laverack 1963). While they have a simple closed circulatory system, oligochaete blood contains hemoglobin molecules in suspension in plasma rather than contained in red blood cells as seen in vertebrates (Laverack 1963). Without the protection of the red blood cell, earthworm hemoglobin is more directly exposed to the effects of toxins that can cross the epidermal membrane of the worm (Smith et al. 1997). A breakdown product of hemoglobin is porphyrin, a pigment that is deposited in the integument and gives *Eisenia andrei* its dark red color (Laverack 1963).

Soil is a complex substrate that makes the analysis of the effects of toxins in the environment challenging. Soil composition, the abundance of soil microorganisms, the type and amount of minerals and salts present, the type and amount of metals present and the amount of available water, can all affect the bioavailability of toxins in the soil. The fate of glyphosate in soil illustrates this complexity. Glyphosate initially adsorbs to soil particles mainly through interactions with minerals in the soil, especially iron oxides (Shushkova et al 2010). However, in soils rich in phosphorous-containing compounds, sorption sites may have already been bound by phosphorous so glyphosate cannot adsorb to soil particles. Conversely, sandy soils with a low volume of organics have low cation-exchange capacity and therefore cannot bind glyphosate. If bound to soil particles, glyphosate is immobilized. If unbound, glyphosate can accumulate and be flushed into ground waters (Hebert et al. 2019, Shushkova et al. 2010). Glyphosate that remains in soil is degraded over time by microorganisms (Van Bruggen et al. 2018). The half-life in soil ranges from 2-197 days (NIH U.S. National Library of Medicine 2018). This wide range in values is the result of a number of factors that influence degradation time: the abundance and type of microorganisms in the soil (Shuette 1998), the relative amount of clay versus sand in the soil, and the pH of the soil (Van Bruggen et al. 2018). A number of studies have concluded that glyphosate in soil had negative sublethal effects on *Eisenia* that are manifested in lower production of cocoons and survival of juveniles plus reduced weight in adults (Correia and Moreira 2010, Gill et al. 2017, Pochron et al. 2019).

The fate of 2,4-D in soil is similar to that of glyphosate. It strongly adsorbs to soil particles high in organic matter and free iron (Hermosin and Cornejo 1991). In fact, the adsorption is so strong it protects 2,4-D from biodegradation by microorganisms (Ogram et al. 1985). However, the 2,4-D dimethylamine salt form in many commercial solutions quickly degrades to the acid form which instead of adsorbing to soil becomes soluble in water and can leach into ground waters (Howard 1991). The half-life of 2,4-D in soil is 10 days; therefore, persistence in the environment is not likely (Jervais et al. 2008). In laboratory studies, negative effects of 2,4-D on earthworms have been consistently reported. The calculated 48-hour lethal concentration at which 50% of individuals died (LC_{50}) for the earthworm *Lumbricus rubellus* is 61.6 $\mu g/cm^2$ (NIH U.S. National Library of Medicine 2019). *Eisenia fetida,* had a 14-day LC_{50} of 350 mg/kg soil and showed sublethal effects on development and reproduction at concentrations as low as 10 mg/kg (Correia and Moreira 2010, de Castro et al. 2017). In a study on *Eisenia andrei*, exposure to sublethal doses of 2,4-D of 3.5, 7.0 and 14 mg/kg in soil resulted in slowed weight gain compared to control groups and increased lysosomal membrane stability, a stress biomarker (Hattrab et al 2015).

No studies on the effect of acetic acid used as an herbicide on earthworms have been published. Earthworms are also known to be sensitive to changes in pH and so are rarely found in soils with pH levels lower than 4.5 (Laverack 1963). The earthworm, *Eisenia fetida*, died within one week at pH less than 5.0 and greater than 9.0 (Kaplan et al. 1980). The sensitivity to pH may be due to low presence of calcium in low pH soils or interference with osmosis (Laverack 1963). To further the testing of herbicides on non-target invertebrates, two studies using *Eisenia andrei* were carried out; direct contact by worms with soil that had been treated with herbicides 24 hours previously and indirect contact by burrowing worms with herbicides lightly sprayed on the top of soil. In this study, the commercial formulations of the various herbicides were poured or sprayed onto the tops of soil to mimic a real-world exposure scenarios.

Based on the results from the test of herbicides on butterflies reported above, and because *Eisenia andrei* is known to be sensitive to chemicals and low pH, I tested the hypothesis that contact with Roundup®, Bayer, 2,4-D and 20% acetic acid is harmful to *Eisenia andrei*. I predicted that mortality would increase compared to the control group after exposure on top of the soil to the full-strength concentration of Roundup®, Bayer, 2,4-D and 20% acetic acid. The second hypothesis tested was that a light overspray onto soil tops under which *E. andrei* has burrowed would not lethal but would negatively affect reproduction and growth. Based on the low concentrations of herbicides applied, I predicted that there would be no difference in the number of survivors among treatments. I further predicted, there would be reduced production of cocoons and juveniles and a decrease in adult weight compared to the control group.

METHODS

Study organisms

Eisenia andrei, were obtained from Uncle Jim’s Worm Farm, Spring Grove, Pennsylvania, a commercial provider of vermiculture worms.

Procedure – General

One-liter cylindrical plastic containers (15 cm X 9 cm) were each filled with 400 g dry-weight of locally sourced soil composed of 88% sand, 8% silt and 4% clay. The depth of the soil was approximately 7 cm. To each container, 150 ml of deionized water was added to moisten soil but leave no standing water in the bottom of the container resulting in a moisture level of 27% (w/w). Adult worms were selected for each container and visually examined to ensure lack of lesions and presence of developed clitella. The group of worms was cleaned with tap water and allowed to crawl on moistened paper towels to remove soil prior to being weighed collectively. Worms used in the experiment were from two separate batches. The first batch of 500 worms was acclimated in local soil for 3 weeks prior to the beginning of the test and liberally fed a combination of vegetable scraps and commercial worm food during that period. The second batch of 1,000 worms was acclimated in soil within each container for one week prior to the treatment date. After treatment, plastic covers were placed lightly on top of containers.

Experiment 1 – Direct contact with herbicides by *Eisenia andrei*

Five herbicide treatments were tested, Roundup®, Bayer 2,4-D, 20% acetic acid, 10% acetic acid and 5% acetic acid. The control was deionized water. For each treatment, 30 ml of herbicide was applied directly to the top of the soil and allowed to seep into the soil for 24 hours. The amount of treatment was the lowest amount that would completely saturate the top layer of the soil in the container leaving no untreated soil. Ten adult worms were selected from the acclimation chamber, weighed and then placed on top of the treated soil in each container. There were five replicates of each treatment. Mortality was noted at 4, 24 and 48 hours and 10, 20 and 41 days after treatment. Deceased worms were removed. Containers were maintained at 21°C - 23°C with a 10:14 hour light cycle. Commercial dried worm food (3 g) was added to the top of soil weekly. Some earthworms migrated out of the lightly covered containers.

The weight and number of adults were measured after 10, 21, 30, and 41 days post-treatment. The number of cocoons and juveniles was counted at 10, 21, 30, and 41 days post-treatment. Total reproduction was calculated as the number of cocoons times 2.5 (Van Gestel 1992) plus the number of juveniles.

Experiment 2 – Exposure of burrowing *Eisenia andrei* to herbicides

Three herbicide treatments were tested, Roundup®, Bayer 2,4-D and 20% vinegar. Ten worms were selected, weighed and deposited onto untreated soil in each of 32 containers. Commercial worm food (5 g) was added to the top of each container. After one week of acclimation, 1 ml of each herbicide was diluted with 2 ml of distilled water and poured into a miniature spray bottle. The solution was sprayed uniformly over the top of the soil. The amount of 1 ml of was selected to simulate a light overspray on soil. The control treatment was 3 ml of distilled water. There were 8 replicates in each treatment. Containers with worms were maintained in temperatures ranging from 22°C to 24°C under a 13:11 light cycle. Temperatures reached 27°C briefly on two days in week five. Commercial worm food (5g) was added to the containers weekly. Containers were periodically opened to allow air flow and left for six weeks. The number of adults, number of cocoons, number of juveniles and collective weight of adults were measured over a four-day period.

Statistical analysis

Assumptions of normality and homogeneity of variance for data used were tested using Shapiro-Wik *W* test and Levene’s test, respectively. Adult mortality was analyzed using ANOVA followed by Tukey’s post-hoc test to assess pairwise significance. The results are expressed as mean ± SD (standard deviation). In experiment two, an ordinary least squares regression model was used to see how ending pH affected reproduction by adult. ANOVA was used to determine if there were differences in survival and reproduction by adult among the treatment groups. Differences among treatment groups for mean ending weight of adults was analyzed using the Kruskal-Wallis test as ending weight was not normally distributed. A model was fit using least squares regression to determine how ending weight and treatment affected reproduction by adult in the second experiment. Statistical significance was set at $p = 0.05$. [All statistical analyses were performed in JMP 13.1.0. (SAS Institute, Inc. Cary, N.C.)]

RESULTS

Experiment 1 – Direct contact with herbicides

Mortality after 4 hours was statistically different among the treatment groups ($F_{(5,24)} = 31.8$, $p < 0.0001$) (Table 3). Worms exposed to 20%, 10% and 5% acetic acid had mortality rates of 94%, 90% and 82%, respectively, after 4 hours. By 24 hours after treatment, all worms in all acetic acid concentrations had perished. Examination of the expired worms showed abnormal swelling and constriction of body segments plus loss of color typically at the posterior end (Figure 3). After 4 hours in the Bayer, 2,4-D treated containers, only 2 earthworms had died. Live worms remaining on the surface of the soil exhibited abnormal movement and twitching. After 24 hours, 72% of all Bayer 2,4-D treated worms had died. Four percent of these survived to 10 days after treatment but none survived to 20 days after treatment. For the Roundup® treated worms, mortality after 4 hours was 88% and remained so through 41 days. The only survivors were in one container where six worms found a gap between the soil and the container side and were able to burrow into the soil. The other 4 worms in this container perished on top of the soil. Reproduction in the Roundup® group after 41 days was 50% lower than the control group with 13.8 juveniles per adult compared to a mean of 27.8 juveniles per adult in the control group.

In the control group, of the 50 worms tested, 4 worms died and 6 were not found and may have migrated out of the containers during the 41 days period. Migrations were treated as mortalities in the analyses and doing so did not affect the outcome. There was no correlation between mean weight of adults after 41 days and the reproduction rate ($r^2 = .061$) (Figure 4). Only 6% of the total variation in reproduction was explained by the linear relationship between adult weight at 41 days and reproductive output. Estimated reproduction was calculated as ((# cocoons X 2.5) + # juveniles) / # of surviving adults). Reproduction per adult in 41 days was 25.1 juveniles or 4.2 juveniles per week per adult. Adult weight varied with time and peaked at around 20 days followed by a decrease in weight thereafter. Cocoon production was at its highest at 30 and 40 days (Figure 5).

Experiment 2 – Indirect contact with herbicides

There were no dead earthworms observed in any of the containers at the end of the test period. However, after 10 days, earthworms began escaping from containers and there were losses from containers in each treatment group. Additionally, in some containers, the number of adults increased as juveniles grew to full adult size. By the end of the trial, the mean number of adults that remained in each container was not statistically different among the treatment groups ($F_{(3,28)} = 1.444$, $p = 0.25$). The number remaining as a percentage of the original 80 worms per treatment was 70% for the control, 69% for Roundup°, 74% for Bayer 2,4-D, and 98% for the acetic acid treatment (Table 4).

There was no significant difference in mean reproductive output per adult earthworm among the treatment groups ($F_{(3,28)} = 2.14$, $p = 0.118$) (Figure 6). Additionally, the mean ending weight of adults did not differ significantly among the treatment groups (Kruskal-Wallis $\chi^2_3 = 6.25$, $p = 0.10$) nor did the mean change in weight from the beginning of treatment ((Kruskal-Wallis $\chi^2_3 = 7.40$, $p = 0.06$) (Table 4). However, the mean weight increase in adults for the acetic acid treatment was 3.6 times higher than for the control and Roundup® treatments and 2.2 times higher than the Bayer, 2,4-D treatment while the mean reproduction in the acetic acid treatment was just over half the mean reproduction of the control group.

Although ending adult weight did not differ significantly among the treatment group, the interaction between ending adult weight and treatment was significant and the full model is significantly different from the null model ($F_{(7,24)} = 3.39$, $p = 0.01$) (Figure 7). The combination of ending adult weight and treatment explained 49.7% of the variation in reproductive output of adults ($r^2 = 0.497$) (Table 5). However, in one replicate in the Bayer, 2,4-D treatment group, the mean reproduction was much higher than for any other replicate as only two adult worms were present at the end of the test period. It appears that the worms reproduced prior to migrating out of the container. Excluding this outlier, ending weight and treatment combined explain only 33% of the variation in reproduction and the full model is no longer significantly different from the null model ($F_{(7,23)} = 1.62$, $p = 0.18$). The power analysis for this experiment based on reproduction per adult was 77%.

DISCUSSION

The hypothesis that direct contact with herbicides applied to the soil surface would be lethal to *Eisenia andrei* was supported by the data. To analyze the results, one has to consider the complexity of the soil system. The degree to which the chemicals applied to the top of soil affected the worms would have been influenced by the toxicity of the active ingredient, the toxicity of adjuvants plus the combined effects of all these compounds. In addition, mobility of the compounds in the soil can change the concentration of chemicals present on the soil top that is in contact with the worms. Mobility in the soil is a function of the composition of the soil, the degree to which the compound adsorbs to soil particles and the solubility of the compound in water.

Direct contact with Roundup® was lethal to 88% of individuals within 4 hours in contrast to the results of the Correira and Moreira (2010) study. The concentration of the Roundup® formulation I applied (30 ml) had the equivalent amount of active ingredient (1,034 mg/kg) equal to the highest amount applied by Correia and Moreira (2010). However, they reported no mortality at that level while in this study 88% mortality occurred quickly. There were four key differences with the Correia and Moreira (2010) study that may explain the difference in results. First, they used the closely related *E. fetida* for testing which was shown to respond differently to toxins. Moreover, when *E. fetida and E andrei* were exposed to sewer sludge, *E. andrei* accumulated greater amounts of cadmium and zinc than *E. fetida* (Rorat et al. 2016). While these two species are closely related, their response to glyphosate may have been different. Second, Correia and Moreira (2010) tested the pure active ingredient whereas the commercial formulation was tested here. In a study of two glyphosate formulations, the first containing 72% glyphosate acid equivalent and the second containing 85.4% glyphosate acid equivalent by weight, the first formulation was found to be 4.5 times more lethal than the second (Piola et al. 2013). Toxicity values based solely on active ingredients may not be represent the true toxicity of formulations in use. Third, in the Correia and Moreira (2010) study, the solution was distributed evenly throughout the soil in each container. In my study, the glyphosate formulation was applied to the soil surface and allowed to percolate through the soil overnight to mimic real world applications. However, it is likely that by applying the glyphosate formula to the top of the soil and allowing it to percolate resulted in a gradient of herbicide concentration within the container with a higher concentration near the top and a lower concentration near the bottom of the container. This would mean that worms in my study contacted a higher effective dose than in Correira and Moreira (2010). Finally, soil composition has a significant effect on the bioavailability of compounds in soil. Correira and Moreira (2010) used local soil comprised of 60% sand, 28% clay, 11% silt and 1% organic carbon with a pH of 5.5. There may have been more opportunity for glyphosate molecules to bind to soil particles in the Correira and Moreira (2010) study than in my study which had only 12% clay and organic content. This reduced amount of unbound glyphosate available in the Correira and Moreira (2010) may have lessened the effect on the earthworms. Additionally, Rampazzo (2012) showed that the presence of iron oxides in soil are more important than organic content of soil in determining the amount of glyphosate adsorption to soil particles. Neither Correira and Moreira (2010) nor I quantified the amount of iron oxides in our soil samples and this may be an important consideration for future studies.

In the first experiment, six earthworms survived the test of direct exposure to Roundup® in one container by burrowing along a gap in the side of the container. Even though these individuals survived to the end of the 41-day trial, reproduction was compromised with the number of juveniles per adult reduced by half over the control group. Correia and Moreira (2010) had no reproduction in any container treated with the glyphosate active ingredient distributed evenly throughout the container. However, Correira and Moreira (2010) reported a reproduction rate in their control group of only 0.04 cocoons per adult per week. This rate is far below previously reported rates ranging from 2.0 per adult per week over 15 weeks at 20°C (Dominguez et al. 2004) and 3.4 per week over 20 days as 25°C (Reinecke and Kriel 1980). I observed a mean rate of 4.2 cocoons per adult at 22-24°C. This raises the possibility that an experimental artifact affected results in the Correira and Moreira (2010) study. Additionally, it is not clear whether the worms in my study made full contact with the initial dose of the Roundup® or were able to avoid contact perhaps by moving on top of each other. As each of the periodic measurements were made, the herbicide would have become mixed into the soil. This would then put the worms in contact with the chemical in a manner consistent with the Correira and Moreira (2010) study. However, whether the worms suffered the sublethal effect of reduced reproduction from that initial contact on the soil top or from ongoing contact with chemicals distributed through the soil is not known.

Direct contact by earthworms with soil treated with Bayer, 2,4-D was also lethal by the end of 24 hours. The equivalent amount of active ingredient in 30 ml of Bayer, 2,4-D used in this study was 4,398 mg/kg which is well above the 14-day LC_{50} of 350 mg/kg (World Health Organization (WHO) 1997). This dose also exceeded the lowest lethal dose in the study by Correia and Moreira (2010) by more than 8 times. In that study, worms treated with 500 and 1000 mg/kg died within hours and mortality was 30-40% for all concentrations between 1 and 100 mg/kg. While the formulation I used was intended to be attached to a hose and diluted in-line, the dilution rate was not disclosed. I wanted to see the effects of formulations taken directly form the bottle testing a misuse of this product.

Direct application of acetic acid at 5%, 10% and 20% was also lethal to the earthworms and mortality occurred quickly. Most individuals died within 4 hours and any remaining died within 24 hours. Although acetic acid is a weak acid, the pH of the 20% formulation was 2.4. It is well known that most earthworm species are not found in acidic soils but the reason is not clear (Laverack 1963). The leaching of color from their bodies in response to the acetic acid treatments may indicate interference with the porphyrin pigment in their integument and so perhaps the hemoglobin molecule in the blood was also affected. In *Eisenia fetida,* hemoglobin exposed to a solution of pH 4.6 dissociates into fragments that retain their affinity for oxygen, but the total oxygen that can be bound decreases (Mainwaring et al. 1986). More study of the effect of acetic acid on earthworms is necessary to understand these results.

In the second study of a light overspray of 1 ml of herbicides applied onto soil tops over burrowing worms, the hypothesis was partially correct. The herbicide treatments did not affect the number of adults that survived among the different treatment groups. For the Roundup® treatment group, these results were consistent with other studies that tested low concentrations of glyphosate in soil. There was no mortality at 1ppm of active ingredient (Correria and Moreira 2010) and none in field-treated soil with < 0.05 ppm glyphosate (Casabe et al. 2007). However, for the 2,4-D treatment group, Correira and Moreira (2010) observed 30 – 40% mortality in soil treated to 1 ppm while no worms in my study died despite treatment equivalent to 157 ppm active ingredient.

The second hypothesis was not supported by the data. I expected to see sublethal effects on reproduction and ending weight of all three herbicide treatments. However, the overspray of 1 ml of herbicides, equal approximately to one release of a hand spray, on soil did not have any adverse effects. With regard to glyphosate, Correira and Moreira (2010) reported a complete absence of reproduction in all concentrations between 1 – 1000 ppm. On the other hand, a study using soil obtained from Roundup Ready® treated soybean fields testing at 0.05 ppm glyphosate reported no difference in the number of cocoons compared to controls after 10 days in soil (Casabe et al. 2007). The equivalent amount of active ingredient in my study was 37 ppm and this concentration did not appear to affect reproduction. As for 2,4-D, Correira and Moreira (2010) reported an absence of reproduction in 2,4-D treated-soil at concentrations as low as 1 ppm yet, in my study, 157 ppm of 2,4-D active equivalent was applied to soil tops and had no significant effect on reproductive output. The difference in the results among these three studies may be due to the mobility of the chemicals in the different soil types used and how the chemical was distributed throughout the soil. In both the Correira and Moreira (2010) and Casabe et al. (2007) studies, the herbicide was distributed throughout the container placing the earthworms in constant contact with the chemical. In my study, herbicide was applied to the top of the soil as would occur in real world applications. Therefore, contact by the worms with the herbicide would have depended on whether the compound leached into the soil to the depth of the earthworms' burrows or remained adsorbed onto soil tops. If herbicide adsorbed to tops of soil, it would be contacted by the worms casually when they rose to the surface to feed or to defecate. Because both glyphosate and 2,4-D have low log K_{ow} values, absorption through the skin of the earthworm is possible (Jaeger 2003). The continuous exposure to contaminated soil in the Correira and Moreira (2010) study compared to periodic exposure in my study may be the cause of the different outcomes on reproduction. Again, the low reproduction in the Correira and Moreira (2010) control group may indicate a problem with the experimental design rather than an effect of the glyphosate.

The use of weight gain or loss as a measure of the sublethal effects of compounds on earthworms needs to be carefully considered. In addition to the effect of toxins, weight fluctuations are normal in *Eisenia* species and are related to the worm's reproductive cycle (Neuhauser et al. 1979) or can be a function of moisture content in soil (Dominguez and Edwards 1997). Neuhauser (1979) observed that weight is lost just after cocoons are produced and slowly regained in the weeks after (Neuhauser et al. 1979). In the control group from experiment 1, weight gain peaked at day twenty just prior to the increase in cocoon production at day 30 and there was no correlation between the ending weight of the adults and the number of offspring ($r^2 = 0.06$). By the 40th day, mean weight rebounded to near pre-reproductive levels. Worm raised in soil with 65-75% moisture by weight grow at a daily rate 60% lower than worms raised in soil with 80 -90 % moisture by weight over 44 days. The increase weight at higher moisture levels also accelerated the development of sexual maturity in juvenile worms (Dominguez and Edwards 1997) potentially affecting the amount of reproduction. In studies of the effects of herbicides on the weight of earthworms, results were not consistent. Correia and Moreira (2010) found that worms exposed to glyphosate from 10 – 1000 mg/kg concentrations showed a gradual reduction in body weight of 50% over 56 days and worms exposed to 2,4-D lost 30% of their initial weight at 10 and 100 ppm. Piola et al. (2012) showed concentration dependent weight reductions of 15% to 40% for two formulations of glyphosate in a 72-hour filter paper test. However, Pochron et al. (2019) found that worms treated with Roundup Ready-to-Use-III® at a rate of 26.3 mg/kg dry soil did not suffer reductions in body weight after 29 days and in my study, there was no significant different in ending mean weight among the treatment groups. These differing results plus the number of variables shown to affect weight of earthworms possibly makes weight an unreliable measure of sublethal effects.

The sublethal effects on earthworms in the acetic acid treatment group were somewhat surprising. Although the results were not statistically different from the other treatment groups, there was a trend of higher mean weight per adult worm and lower juvenile production. There are two possible explanations for this. First, acetic acid may have interfered with or delayed the ability of adults to produce cocoons. Alternatively, cocoons or juveniles may have been harmed by the acetic acid resulting in fewer living juveniles at the end of the test period. As juveniles and cocoons of *E. andrei* were clustered on food particles near the top of soil, cocoons and juveniles may be exposed to the direct effects of the acetic acid while burrowing adults are protected from its effects. In both scenarios, the lower numbers of offspring would result in less food competition allowing for adults to achieve higher weight. A separate study whereby the containers are opened periodically during the test period to remove cocoons and allow them to develop separately may help resolve this question.

There were three issues in my experimental design that I would change if this study were to be repeated. First, there were a number of migrations from containers from all treatment groups. In the first study of direct contact, only 10% of individuals migrated out during the 41-day test period. In this study, migrations were 26%. Because migrations occurred from all four treatment groups, they do not appear to have affected the results. However, the migrations affect calculations of average reproduction per adult and make comparisons across different studies difficult. Migrations out of the containers are normal and could be in response to food competition or excretion of urine and feces that foul the containers (Neuhauser et al. 1979). Pochron et al. (2019) reported migrations up to 25% as well. For future studies, a modification to the top of the container to prevent migrations could be helpful. Second, the moisture content of soil in this experiment was 33.3% at the beginning of the experiment which is low compared to that used in other experiments. Casabe et al. (2007) and Correira and Moreira (2010) used a 60% moisture content. However, because worms will die in overly wet soil and because soil in my study had a high sand content, I used an amount of water which would maximize moisture levels but minimize standing water at the bottom of each container. I did not monitor the moisture levels in the containers during the study period, but as containers were kept covered, evaporation would have been minimal. *Eisenia fetida* prefers to deposit cocoons at 60-70% moisture and juvenile growth rate can be negatively affected at low moisture levels (Reinecke and Venter 1987). Differences in moisture levels among the containers could have contributed to the high variability in cocoon production within and among treatment groups. For future studies, a method for maintaining a standard moisture level across all replicates should be considered. Third, containers remained unopened until the end of the study period. This was chosen to mimic real-world conditions and prevent mixing the herbicides into the soil which would inevitably occur in the process of removing cocoons. However, in the Casabe et al. (2007) study, cocoons were removed periodically and moved to separate containers for hatching. This allowed the authors to measure cocoon viability as well as production and they noted reduced cocoon viability in Roundup®-treated soil.

Table 3: Percent mortality of *Eisenia andrei* after direct exposure to 30 ml of herbicide applied to top of soil. Data are percent mortality ± SD. Superscripted letters indicate statistically similar groups based on Tukey post-hoc tests for pairwise comparisons. (n=5).

	4 hours post-treatment	24 hours post-treatment	41 days post-treatment
Treatment	Mortality %	Mortality %	Mortality %
Control	0 ± 0^a	0 ± 0^a	20 ± 23^a
Roundup®	88 ± 27^b	88 ± 27^b	88 ± 27^b
Bayer, 2,4-D	4 ± 5^a	72 ± 5^b	100 ± 0^b
20% acetic acid	94 ± 13^b	100 ± 13^b	100 ± 0^b
10% acetic acid	90 ± 22^b	100 ± 22^b	100 ± 0^b
5% acetic acid	82 ± 22^b	100 ± 22^b	100 ± 0^b

Table 4: Survival percent, reproduction, weight increase, ending weight and range of ending weights of burrowing *Eisenia andrei* 44-47 days after treatment with 1 ml of herbicide applied to top of soil. (+) indicates data are mean ± SE. Superscripted letters indicate statistically similar groups based on Tukey post-hoc tests for pairwise comparisons. (n=8)

	Survival Percent	Reproduction per adult (+)	Weight increase (g) (+)	Ending Weight adults (g) (+)	Ending Weight range (g)
Control	70	41.8 ± 6.8	0.07 ± 0.02^{b}	0.42 ± 0.02^{ab}	0.34 – 0.52
Roundup	69	35.9 ± 4.8	0.07 ± 0.02^{ab}	0.42 ± 0.02^{b}	0.34 – 0.51
Bayer, 2,4-D	74	47.3 ± 11.1	0.11 ± 0.02^{ab}	0.45 ± 0.02^{ab}	0.35 – 0.50
Acetic acid - 20%	98	21.8 ± 5.6	0.24 ± 0.08^{a}	0.58 ± 0.08^{a}	0.30 – 0.98

Table 5: Results of least squares regression on reproduction by adults using ending average weight and treatment with 1 ml herbicide treatments applied to top of soil of burrowing *Eisenia andrei* as compared to the control group (n=8). Relative to the null model, the full model was significant ($F_{(7,24)} = 3.39$, p =0.01).

	Coefficient	SE	p
Constant	96.0	22.8	0.00*
Roundup® treatment	0.49	7.2	0.95
Bayer, 2,4-D treatment	3.04	6.2	0.63
Acetic acid treatment	-7.8	6.4	0.23
Ending avg weight	-127.7	51.8	0.02*
(End avg wght – 0.46)* Roundup®	145.0	95.3	0.14
(End avg wght – 0.46)* Bayer, 2,4-D	-317.6	106.9	0.01*
(End avg wght – 0.46)* Acetic acid	69.8	56.4	0.23

Figure 3: *Eisenia andrei* 4 hours after treatment with 30 ml of 5% acetic acid applied to top of soil (left) compared to healthy *Eisenia andrei* before treatment (right).

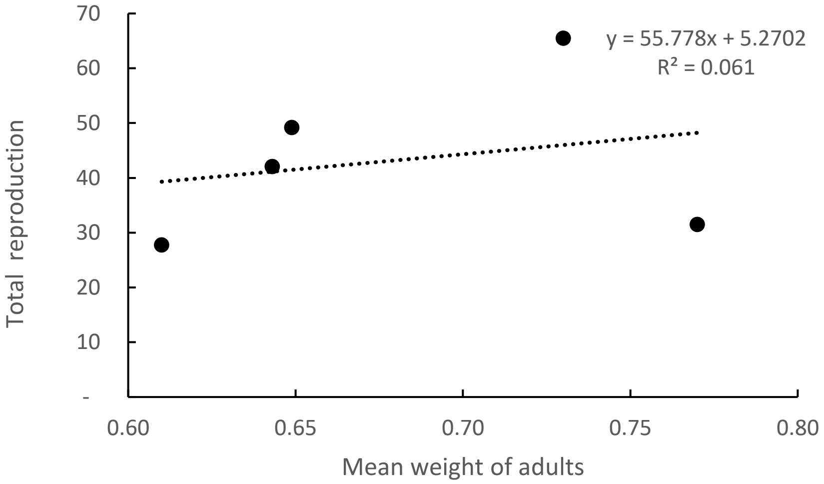

Figure 4: Comparison of mean weight of *Eisenia andrei* adults to total estimated reproduction (# cocoons X 2.5 + juveniles) in the control group after 41 days (R^2 = 0.061).

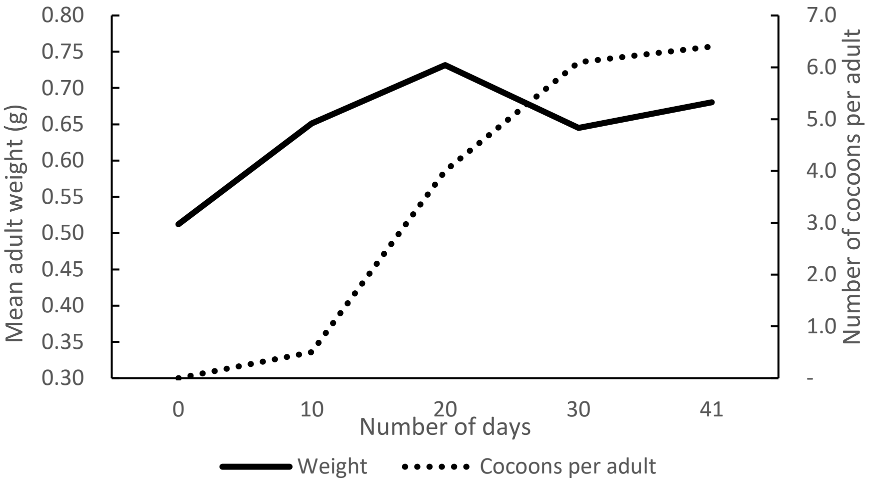

Figure 5: Change in mean weight of *Eisenia andrei* adults in the control group and mean number of cocoons present per adult from 0 to 41 days.

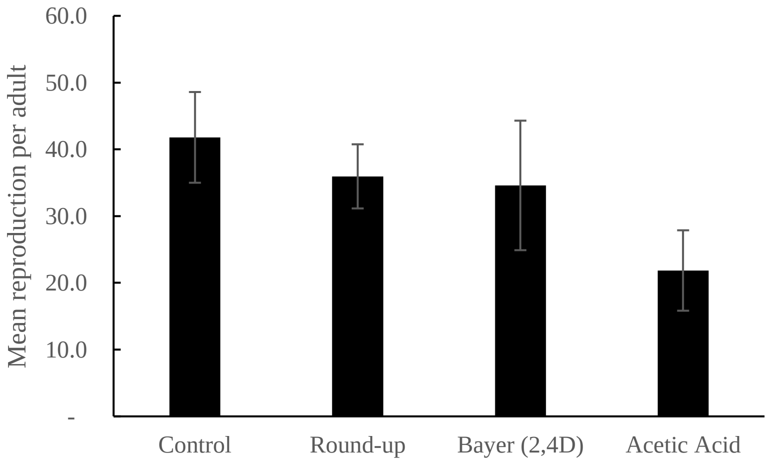

Figure 6: Mean reproduction of *Eisenia andrei* per adult after treatment with 1 ml herbicide applied to top of soil after 44-47 days in soil. Differences in mean reproduction among treatment groups were not significant. Error bars represent SE (n=8).

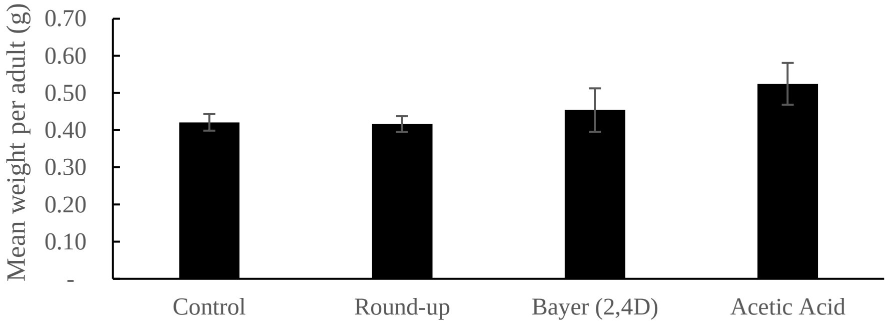

Figure 7: Mean weight per adult(g) of *Eisenia andrei* after treatment with 1 ml herbicide applied to top of soil after 44-47 days in soil by treatment group. Error bars represent SE (n=8).

CHAPTER 4 CONCLUSIONS

Awareness of the potential harmful effects of herbicides to humans is increasing, but the level of use of these chemicals in agriculture remains high. The role these chemicals have had in the decline of terrestrial invertebrates and beneficial insect species is not completely understood. This study was undertaken to provide important information about the effects on non-target invertebrates by two commonly used herbicides, Roundup® with glyphosate and Bayer Weed and Grass Killer with 2,4-dichlorophenoxyaceticacid and dicamba, and one natural alternative, acetic acid. These three herbicide formulations were tested on butterflies and earthworms and the effects on each were different. The hatching of butterfly eggs was significantly reduced after contact with all three herbicides. Earthworms of the species *Eisenia andrei* that made direct contact with heavy overspray of herbicides suffered high mortality rates. However, earthworms burrowing in soil that was treated with a light overspray did not suffer increased mortality, abnormal weight changes, or reduced reproduction that was statistically different from the control group. The effect of direct contact on butterfly larvae with 10% and 20% acetic acid was not conclusive.

The testing on terrestrial invertebrates that was part of the EPA approval of glyphosate and 2,4-D, was based on acute toxicity testing on honeybees and earthworms using the active ingredient only. However, many studies discussed above show significant reductions in fecundity after exposure to both active ingredients and commercial formulations of glyphosate and 2, 4-D which could decimate populations of beneficial invertebrates. Moreover, studies of commercial herbicide formulations had significantly different results from those using the active ingredient only. In my study using Roundup®, the commercial formulation was lethal to *Eisenia andrei* at an equivalent dose that was found to be safe for the isolated glyphosate active. After completing this study, I believe that testing of the chronic effects of herbicides on non-target organisms should be mandatory prior to pesticide approval. Legal requirements to perform additional environmental testing of commercial formulations may be appropriate when product usage in society reaches certain high levels.

Unfortunately, the use of acetic acid as an herbicide was not shown to be a good natural alternative as direct contact was lethal to *E. andrei* and it showed trends of sublethal effects on weight of adults and their reproduction that require more testing. The ongoing search for environmentally friendly herbicides is necessary as agricultural practices will continue to demand herbicides due to the high cost savings they provide (Gianessi 2005).

As the result of this work, several opportunities to further our understanding of the effects of these three herbicide formulations are apparent. First, a dose response study of overspray amounts on soil covering burrowing earthworms to determine the highest safe amount of herbicide that will not cause sublethal effects on growth and reproduction of the burrowing earthworms should be performed. Second, the Correira and Moreira (2010) study appears to be flawed in that the level of reproduction observed in the control group was far below normal. As this is the only published study on the effects of the active ingredient glyphosate on earthworms in soil, it would be helpful to repeat this study in artificial soil according to the OECD protocol. Third, additional studies to determine the impact of sublethal effects of herbicides on butterfly populations should be performed. The results here did not show an effect on days to death or days to eclosure on Lepidopteran species as sample sizes were limited due to the negative effect on hatch success.

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APPENDIX A

Table 1: Chemical properties of herbicides used in this study.

Chemical	Molecular formula	Molecular Weight (g/mol)	Log K_{ow}	K_{oc}	Solubility in Water	LC_{50} to earthworms	Half-life in soil
Glyphosate, isopropylamine salt	$C_6H_{17}N_2O_5P$	228.19	-5.4	na	High	5000 ppm	2-197 days, 47 days typical
Glyphosate acid	$C_3H_8NO_5P$	160.07	-3.24	na		na	na
2,4-dichlorophenoxy acetic acid, dimethylamine salt	$C_{10}H_{13}Cl_2NO_3$	266.13	2.81	Low[b]	High[b]	61.6 µg/cm	10 - 59[b] days
2,4-dichlorophenoxy acetic acid	$C_8H_6Cl_2O_3$	221.03	2.81	Low[b]	High[b]	61.6 µg/cm	10 - 59[b] days
Pelargonic acid	$C_9H_{18}O_2$	158.24	3.42	Low[c]	Moderate[d]	na	na
Dicamba	$C_8H_6Cl_2O_3$	221.03	2.21	na	Low	na	na
Acetic acid	$C_2H_4O_2$[c]	60.05[c]	-0.17[c]	None[c]	High[c]	na	< 14 days[c]

Data from NIH U.S. National Library of Medicine, 2018 except where separately noted.

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