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Bio-Accumulation of a Soil Fungicide in Western Barred Tiger Salamanders via Earthworm Prey

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BIO-ACCUMULATION OF A SOIL FUNGICIDE IN WESTERN BARRED TIGER SALAMANDERS VIA EARTHWORM PREY

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Western Tiger Salamander in eastern Nebraska have experienced population declines in recent years. In eastern Nebraska there is a high degree of agricultural production compared to other regions of the state. Pyraclostrobin fungicide, a substance that has been shown to cause numerous deleterious effects to amphibian species via direct contact, is applied to cornfields in large amounts. While a number of experiments have been conducted showing the effects of pyraclostrobin via dermal contact in amphibians, little is known about the effects of fungicide bioaccumulation. In this study, 24 salamanders were split into 4 groups with each group ingesting earthworms which had been exposed to differing concentrations of fungicide. Group 1 was fed uncontaminated worms as a control, groups 2, 3, and 4, were fed worms exposed to 2.3%, 4.6%, and 9.2% fungicide concentrations, respectively. Feeding occurred once per week over 4 weeks. Over the course of the study no mortalities occurred, no skin lesions were found, and the average length and mass gains over 4 weeks did not differ significantly between the control and experimental groups. At this time there is no evidence to suggest that fungicide bioaccumulation via earthworm prey causes any deleterious effects in Western Tiger Salamanders. The results of this experiment were largely inconclusive due to the lack of observable or measurable deleterious effects.

Preface

This study would not have been possible without the guidance of Dennis Ferraro, the use of his laboratory, and the help of the students who worked there each day and assisted me in conducting my experiment. Additional thanks to Dave Gosselin for his advice over the course of this project.

Introduction

Amphibian species have declined across the world at extraordinarily alarming rates. In the United States alone, amphibians populations decreased at a rate of 3.7% each year from 2002 to 2011 (Adams, et al, 2007). On a global scale, amphibians are facing declines dwarfing those seen in mammal and bird species, with almost one third of global amphibian species being threatened (Stuart, et al, 2004). Many theories have been suggested to explain this dreary phenomenon. As urban centers grow and agricultural zones follow suit, wetlands are filled or otherwise destroyed to make room for these new developments. Amphibians, which require bodies of water to survive or reproduce find themselves without a habitat and subsequently either experience high mortality rates or are unable to reproduce. Increasing populations of invasive species in native systems have outcompeted or brought new diseases to amphibian populations throughout the world. Climate change has altered the natural environments that amphibians are best suited for, drying up wetlands, killing plants or animals that amphibians need to survive, and making more common the species (such as chytrid fungus) that often threaten them (Bosch, et al, 2007). Pesticide usage in agricultural zones may have contaminated wetland environments, having deleterious effects on amphibians in the process. These theories are not mutually exclusive, and any or all of these causes may have led to the cataclysmic eradications amphibians have experienced in recent years.

Not unlike other amphibians across the globe, salamanders in Nebraska have also experienced declines. Western Tiger Salamander, *Ambystoma mavortium*, populations have dropped off in eastern Nebraska (Ferraro, 2015). Eastern Nebraska receives approximately twice as much precipitation (Nebraska Department of Agriculture, 2013) and the soil is of higher quality than in western Nebraska (Hayes, et al, 1926). Consequently, the east is farmed more intensively than the west. We believe that the high density of agricultural production in eastern Nebraska has played a large role in the reduction of salamander populations. Farmers seek to preserve their crops from pests and other biological threats by applying pesticides. While certain pesticides have been shown to harm amphibians, we want to focus specifically on the effects of fungicide (a type of herbicide) on salamander mortality. Nebraska farms are treated with a large amount of fungicide. In 2010 Nebraskan corn was treated with 100,000 pounds of fungicide (Hellman, 2015). Ten times as much fungicide was used in 2014 as was used in 2005 (Hartman, et al, 2014), and a number of studies have been conducted in recent years showing that certain fungicides can directly cause amphibian mortality.

Certain fungicides appear to disrupt nearly every stage and facet of an amphibian's livelihood. Strobilurin fungicides in particular appear to cause the most damage to amphibians. Strobilurin refers to a group of similar chemical compounds that act as the active ingredients in many commercial fungicide formulations. In a study conducted in 2014 Headline brand strobilurin fungicide was applied to *Bufo cognatus* tadpoles. The result was that the tadpoles developed five days quicker than they would have naturally, and 35% of individuals experienced mortality (Hartman, et al, 2014). In another study, scientists found that Headline brand fungicide killed 100% of Bufo tadpoles not only at ten times the recommended label rate but also at exactly the recommended label rate and even one tenth of the label rate (Belden, et al, 2010).

The recommended label rate refers to the amount of the product the manufacturer recommends be applied to a crop. In a 2013 study by Bruhl, et al, scientists tested a number of fungicides on juvenile *Rana temporaria* frogs. In this case, the frogs were fully developed and terrestrial unlike the tadpoles of the two aforementioned studies. Fungicide was directly applied to soil in the enclosures. The study took place over a week to test the effects of the fungicides over time, however Headline fungicide killed 100% of frogs tested within one hour at the label rate (Bruhl, et al, 2013). Two brands of herbicide, one brand of insecticide, and two other brands of fungicide were also tested in this study. These chemicals only matched the mortality caused by Headline when applied at ten times the recommended rate. Given the apparent dangers of Headline fungicide on frogs and the popularity of its active ingredient on farms in Nebraska (83% of the 100,000 pounds of applied fungicide contained pyraclostrobin (Hellman, 2015), one of the members of the aforementioned strobilurin class of chemicals) it is an ideal chemical to study.

Fungicide is normally applied aerially when corn reaches its tasseling stage. While salamanders do not typically live within cornfields, they do live in wetlands in close proximity to farms. Wetland sediments act as a sink for hydrophobic contaminants—contaminants that are not attracted to water—such as pyraclostrobin fungicide (Smalling, et al, 2014). It is possible that salamanders are exposed to fungicide through their food sources, mainly earthworms. Earthworms spend their lives moving through, and ingesting, soils. If the soil is contaminated with fungicide, then salamanders may ingest fungicide when they consume worms. Given enough time, ingesting small amounts of fungicide through worms may cause significant mortality by bioaccumulation. Bioaccumulation is the accumulation of contaminants within an organism. Bioaccumulation occurs when an organism absorbs a contaminant faster than that contaminant can be expelled.

This study investigated the question of whether salamanders experience deleterious effects from the bioaccumulation of fungicide. To answer this proposition, we fed earthworms that lived in fungicide-contaminated soil to salamanders. Earthworms are an ideal prey for this experiment as they are ideal test organisms for biocides (Shnug, 2014) and are a main food source for salamanders in their natural environment. Our objectives were twofold. First we sought to discover whether the consumption of fungicide-exposed worms caused any adverse effects in salamanders. Additionally, we sought to determine whether salamanders exposed to higher concentrations of fungicide experienced worse heath than those exposed to lower fungicide concentrations. We hypothesized that salamanders will experience deleterious effects from fungicide bioaccumulation and exposure to higher fungicide concentrations will cause more harmful effects than lower concentration exposure.

The purpose of this experiment is to study pyraclostrobin bioaccumulation in salamanders. There are wider implications for this study because salamanders have not been exposed to fungicides in a lab setting, and bioaccumulation of fungicide through worms has not been studied in amphibians. Salamanders and other amphibians are an indicator species (Welsh and Oliver, 1998)—they are extremely sensitive to changes within their environment. Many wetland species rely on worms as a primary food source; if worms are indirectly affecting salamander populations through bioaccumulation this would suggest than many other species are threatened as well. If salamanders are riddled with fungicide this would threaten tertiary consumers—such as many fish species—that consume salamanders. This study is limited in several ways. Our low number of test subjects (24) may limit the assumptions that can be made from the results of our experiment. This investigation may not exactly mimic natural environmental conditions. Further studies will be needed regardless of the results of this experiment, but this study will supply primary data related to fungicide bioaccumulation in worm-feeding species.

Methods

In May 2015, dozens of salamander eggs were collected from wetlands in western Nebraska and transported to Lincoln, Nebraska to mature in a laboratory setting. This ensured that none of the subjects would have been previously been exposed to fungicides or other contaminants during earlier life stages. Any water the salamanders were exposed to in the laboratory was first tested for any contaminants. Salamanders were fed tubeworms during the larval stage and a mixture of earthworms and mealworms purchased from online vendors during subsequent stages of development.

Once the salamanders had reached adulthood, 24 were chosen to act as test subjects. We wanted to use the largest sample size possible, and 24 was the largest number of individuals available. Each individual was placed into separate plastic enclosures containing uncontaminated soil and a dish of water. The salamanders were split into 4 groups of 6. Group 1 individuals were fed earthworms that had not been exposed to any fungicide. Groups 2, 3, and 4 were fed earthworms exposed to fungicide concentrations of 2.3%, 4.6%, and 9.2%, respectively. Because 4.6% is the standard application rate of pyraclostrobin fungicide, we chose to use the standard rate, 0.5x rate and 2x rate. In prior studies, the standard concentration proved harmful enough so as to cause mortality within an hour. As fungicide bioaccumulation has not thus far been studied, we experimented with a range of concentrations.

Hundreds of earthworms were placed into 4 separate containers containing soil and vegetables (for feed). In container 1, 200 milliliters of distilled water was applied on a weekly basis to the soil; worms in this container were prey to the control subjects. Two-hundred milliliters of 2.3%, 4.6%, or 9.2% pyraclostrobin fungicide solution was mixed into the soil of containers 2, 3, and 4, respectively.

From February 18, 2016 to March 17, 2016 fungicide was applied on a weekly basis to the worm enclosures and salamanders were fed 1 earthworm weekly. During each feeding period the salamanders were visually examined for mortality, skin lesions, or other signs of poor health. Individual salamander length and weights were measured and recorded prior to conducting the study and again on March 17, 2016 in order to determine whether any abnormal development had occurred.

Results

No mortalities occurred over the course of the experiment. Skin lesions or other deformities were not observed at any point in any single individual. There was no visual evidence of poor health in any of the salamanders. There were no observed difference between the control group salamanders or any of the individuals exposed to fungicide. Further, there were no observed differences between the salamanders exposed to differing concentrations of fungicide.

There were no abnormal variations in the length (fig. 1) or mass (fig. 2) growth patterns of the salamanders over the course of the experiment. Salamanders that consumed worms which had been exposed to 2.3% fungicide concentrations lost 0.3 cm in length and gained 0.6 g in mass on average. Those exposed to 4.6% fungicide concentrations gained 0.1 cm of length and

1.0 g of mass. The third group that had been exposed to 9.2% fungicide concentrations gained 0.2 cm in length and 1.0 g of mass. Control group salamanders saw no average change in length and an average gain of 1.1 g of mass.

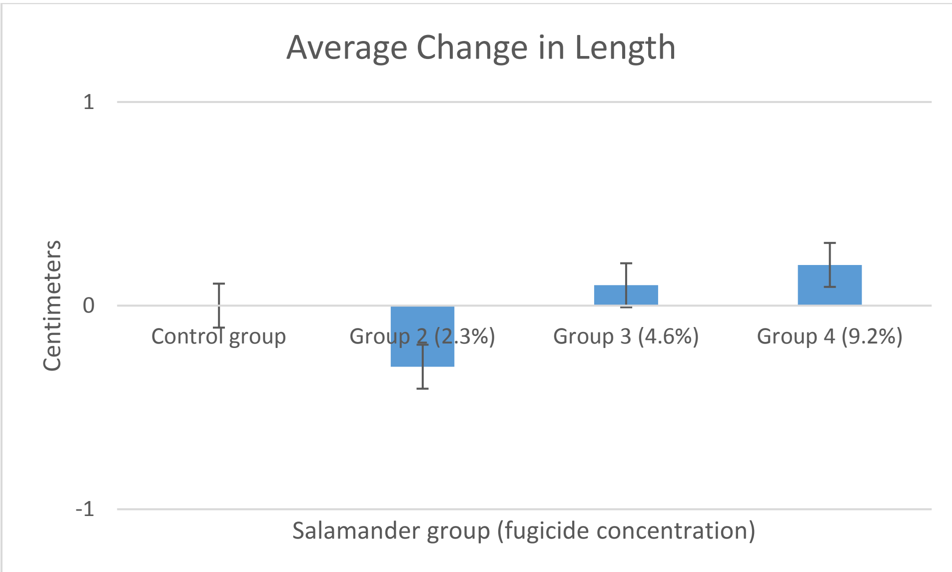

Figure 1

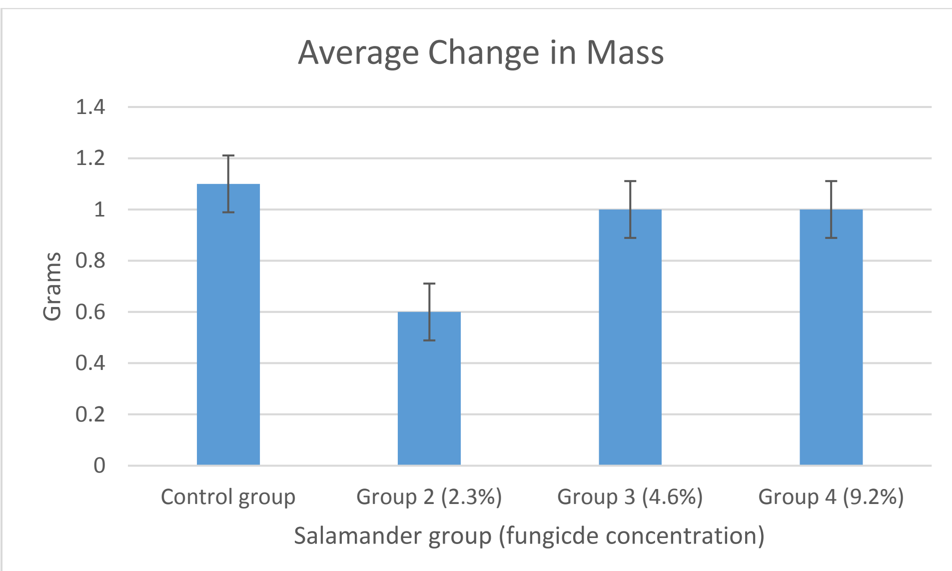

Figure 2

Discussion

This study did not answer definitively the question of whether fungicide bioaccumulation causes deleterious effects in salamanders. The lack of mortalities and observable deformities means that there is no evidence to support the hypothesis that fungicide bioaccumulation is harmful to salamanders. Additionally, the rates of the growth in both length and mass of the experimental groups were not significantly different than the growth rates of the control group. There were also no significant differences between the growth rates of the 3 experimental groups. Group 2 (2.3% exposure) appears to have lost length on average, however this is almost certainly due to human error in measurement. There is nothing to suggest that salamanders exposed to higher concentrations of fungicide via earthworm prey experience worse health than those exposed to lower concentrations.

There have not been any studies thus far examining the effects of bioaccumulation of pyraclostrobin fungicide in amphibians so the result of this experiment cannot be said to be either typical or unusual. However, a number of studies have been conducted wherein pyraclostrobin was directly applied to amphibian species. Typically, direct contact with the fungicide led to mortality, abnormal development, or disfigurement. Fungicide ingestion via earthworm prey appears to cause fewer deleterious effects than direct application of fungicide.

Conclusion

This study was conducted in an effort to better understand population declines of Western Tiger Salamanders observed in eastern Nebraska. The pyraclostrobin fungicide that is applied in large quantities to Nebraskan corn fields has been shown to cause deleterious effects in a variety of amphibian species. Little is known about the effects of fungicide bioaccumulation in amphibians. We sought to answer whether fungicide bioaccumulation causes deleterious effects in salamanders, and whether higher concentration exposure resulted in more deleterious effects than lower concentrations.

There was no evidence to suggest fungicide ingestion caused any effects, or worsening effects with higher concentrations of pyraclostrobin. The lack of mortalities, disfigurements, or abnormal changes in length or mass suggest that at this time there is no definitive answer as to whether fungicide bioaccumulation is harmful to salamanders. The results of this study do not suggest fungicide ingestion cannot cause deleterious effects—rather, the data is inconclusive. Due to the body of evidence showing pyraclostrobin often causes mortality when applied directly to amphibians, it is still possible—if not likely—that fungicide ingestion can cause mortality.

Additional studies will be necessary to better understand the possible effects of fungicide bioaccumulation. This study should be repeated and conducted over a larger period of time—this will allow for more feeding opportunities and larger amounts of bioaccumulation. Raising the concentration rates drastically to levels that are not necessarily reflective of the natural environment would help to definitively answer whether pyraclostrobin ingestion is harmful. If earthworm prey that has been exposed to concentrations of 20% or more is ingested over a period of multiple months and no deleterious effects are observed it is unlikely that lower concentrations are harmful. In future experiments the test subjects should be euthanized after completion of trials in order to test their tissues for pyraclostrobin. This would determine whether fungicide is accumulating within the salamanders. Furthermore, the reproductive health of the salamanders should be tested in future studies. It is possible that reproductive deformities have decreased fitness and led to population declines in Nebraska. More studies must be conducted in order to determine whether pyraclostrobin ingestion is harmful to amphibians.

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