The effects of neonicotinoids: imidacloprid and thiamethoxam on the development of *Xenopus laevis* tadpoles during tail regeneration

By

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Allegheny College

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Submitted in fulfillment of the senior thesis requirements of the Department of Biology at Allegheny College and approved by the senior thesis committee.

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the Allegheny College community as a whole.

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Pledge

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Abstract

Populations of several species of amphibians have been declining since the late 20th century. Pesticides such as neonicotinoids used for agriculture might contribute to amphibian decline not only because of direct effects but sub-lethal effects as well. Imidacloprid and thiamethoxam are neonicotinoids that leach into aquatic habitats and permeate the skin of amphibians living in these waters. Neonicotinoid insecticides were designed to be nicotinic acetylcholine receptor agonists of the insect nervous system. They disrupt development and metamorphosis in terrestrial invertebrates (Schneider et al., 2012); however, the consequences to the development of aquatic vertebrates have not yet been tested. *Xenopus laevis*, the African clawed frog, was used as a model system to investigate potential neonicotinoid effects on vertebrates. Imidacloprid (0.5, 1, and 5 µg/L) and thiamethoxam (0.5, 1, and 5 µg/L) addition to the water, after tail amputation, induced either deformities in regenerating tails, higher mortality, a decrease in total length, or change in body weight. Overall, it appears that 0.5 µg/L thiamethoxam elicited the most detrimental effects on *X. laevis* tadpoles.

Introduction

Worldwide populations of amphibians have been monitored since the 1970's, when signs of declines were detected, and the declines became more pronounced in the 1990's (Wake, 1991). While some amphibian populations are unaffected by the gross decline, currently more than 70% of the 6,600 amphibian populations are waning (Hayes et al., 2010). The population declines are more than simply just population fluctuations. Recently, the drastic fall of different species has led to extinctions of amphibians (Tavera-Mendoza et al., 2002). The most alarming extinctions are those of species from protected areas (Wake, 1991). In each habitat, amphibians fill niches, are frequently high on the food chain, and consume many invertebrates. Extinction of numerous species would have detrimental consequences to their ecosystems (Wake, 1991).

The cause of the decline in amphibian populations is speculated to be a result of a several factors. The two direct causes of population decline are death and reduced recruitment (Hayes et al., 2010). The five most plausible factors underlying death and reduced recruitment of amphibian populations are climate changes, habitat loss, invasive species, pathogens, and environmental pollutants; almost all are a result of human activities (Hayes et al., 2010). There is controversy surrounding which factors have bigger roles in the declining numbers of amphibians, but the examination of many pesticides has led to the notion that pesticides and other agricultural chemicals are the leading source of decline (Hayes et al., 2006).

The increase in the use of pesticides has paralleled the decrease in the population of amphibians (Tisler el al., 2009). Agricultural uses of pesticides are exceptionally dangerous because they can be carried far distances. For example, some chemicals have been detected

in habitats and precipitation 1,000 km from the source; as a result, only a small proportion of applied chemicals actually reach their intended targets (Hayes et al., 2010). Chemicals often spread throughout several environments causing harm to non-target organisms and impact human health (Tisler et al., 2009). Also, chemicals applied to terrestrial crops do not always remain in the soil; they can contaminate aquatic habitats by leaching into ground water, vaporizing into the atmosphere, and returning to surface water via rain (Hayes et al., 2010). Besides directly killing aquatic life, these chemicals can have sub-lethal effects. Some pollutants can affect development and reproduction and interfere with hormones such as corticosteroids. All of these factors disrupt normal physiological and behavioral functions which ultimately lead to decreased survival of populations (Hayes et al., 2010).

Various species, especially amphibians, serve as indicators of the health of an environment (Wake, 1991). Their porous skin is vulnerable to pollutants contaminating the water. Since they develop and reproduce in the water they are especially susceptible (Pancharatna et al., 2010).

The health of an ecosystem after pesticide application can be assessed by measuring corticosteroid levels in resident organisms like amphibians (Hayes et al., 2006). Corticosteroids, produced by the interrenal gland in amphibians are stress hormones that cycle through the body of *X. laevis* and other vertebrates (Krain & Denver, 2004). The most prominent corticosteroid found in the plasma of *X. laevis* is corticosterone (Krain & Denver, 2004). However, the major corticosteroid involved in metamorphosing *X. laevis* tadpoles is cortisol (Yadav, 2008).

Corticosteroids have several roles in the body: one is stimulating thyroid hormones that are the primary regulatory hormones for metamorphosis (Krain & Denver, 2004), and

another is regulating sodium and glucose balance (Wright et al., 2003). Both corticosteroids and thyroid hormones control gene transcription to regulate the timing of metamorphosis. In general, corticosteroids and thyroid hormone levels peak during the climax of metamorphosis, and then corticosteroid levels drop significantly (Hayes, 1997). In addition, corticosteroid levels vary in response to pressures from the environment. Therefore, when environmental factors cause stress to the animal, corticosteroid hormone levels rise, which modifies the timing of metamorphosis (Krain & Denver, 2004). When *X. laevis* tadpoles were exposed to a combination of nine chemicals, levels of corticosterone dramatically increased (Hayes et al. 2006). This is significant because abnormal levels of corticosteroids impede development, growth, and immunosuppression. These effects also can lead to declines in the population of *X. laevis* (Hayes et al., 2006).

Neonicotinoids are pesticides that have not been extensively studied in amphibians because they were thought to specifically affect insects such as hemipteran pests and not vertebrates (Matsuda et al., 2009). There are three subclasses of neonicotinoids, and each has a distinct chemical structure. The first subclass includes the insecticide imidacloprid (Fig. 1A) containing a heterocycle of chloropyridine. The second subclass includes thiamethoxam (Fig. 1B) containing a heterocycle of chlorinated thiazole, and the third subclass has a heterocycle of tetrahydrofluran (Shi et al., 2011). The cellular targets of these chemicals are nicotinic acetylcholine receptors (nAChRs). The multiple members of neonicotinoids have diverse agonistic actions on nAChRs that impair nerve functions (Matsuda et al., 2009). Currently, neonicotinoids are the fastest growing and leading insecticide in the world, but the sub-lethal effects to nontarget species have not been determined (Matsuda et al., 2009). Neonicotinoids are not intended to target aquatic species; however, whether they occur in

high concentrations from leaching, spray-drift, or run-off is a current topic of debate (Tisler et al., 2009).

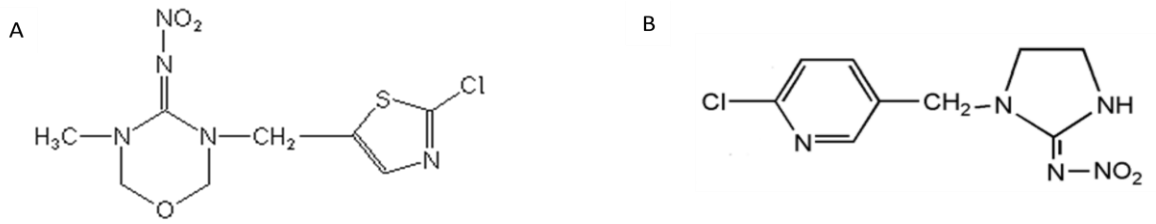

Figure 1. (A) Molecular structure for imidacloprid (Fossen, 2006) (B) and thiamethoxam (MSDS data sheet).

There are two opposing viewpoints about imidacloprid's concentration in surface water. Some research shows that imidacloprid does not leach into aquatic habitats and groundwater, but other research demonstrates it can seep into waterways. A study by the EPA found that imidacloprid leached into waterways and is a high risk to aquatic species (Miranda, el al., 2011) because imidacloprid has a high potency and affinity for nAChRs (Schneider et al., 2012). Thiamethoxam, another neonicotinoid, leaches into aquatic habitats as well and is considered a medium risk for aquatic organisms (Miranda et al., 2011).

Imidacloprid is infamous for being a major suspect in the declining honeybee population because it disrupts foraging behavior, decreases learning abilities, and decreases homing abilities (Schneider et al., 2012). It also disrupts fecundity and wing formation in brown planthoppers (Bao et al., 2009). Although thiamethoxam is not as frequently used as imidacloprid, thiamethoxam does have negative effects on development in species besides honeybees. Contrary to popular belief, neonicotinoids do affect mammals, as thiamethoxam affects the central nervous system in rats (Rodrigues et al., 2010). More specifically, thiamethoxam altered nicotinic acetylcholine receptors in the central nervous system of rats

that introduced negative behaviors related to symptoms of Alzheimer's disease (Rodrigues et al., 2010). Overall, imidacloprid and thiamethoxam interfere in processes of metamorphosis, feeding, and mobility of insects and other organisms (Sadeghi et al., 2009).

Neonicotinoids also affect the endocrine system in many species of amphibians including *X. laevis*, but no tests have been conducted to determine if they affect development (Hayes et al., 2010). *X. laevis* is an accepted model system to investigate development of the central nervous system, sensory-motor transformations, and motor commands during development (Straka & Simmers, 2011). Development is a complex process that is influenced by environmental factors and internal hormones (Hayes, 1997). Prior and during metamorphosis, external pressures affect hormones that regulate gene-expression and neural transformations, creating a collaborative system to investigate (Hayes, 1997). Metamorphosis is an intricate and multifaceted process that alters almost every organ system in *X. laevis* (Sillar et al., 2008). The physiological and behavioral transformation involved in becoming an adult frog requires remodeling of the visual, digestive, locomotive, nervous, and many other organ systems. Metamorphosis forces frogs to change the mode of respiration, from a monocular based visual system to binocular system, from an herbivorous to a carnivorous diet, from tail-based swimming to hindlimbs and forelimb propulsion movement, and the nervous system pathways within the spinal cord (Sillar et al., 2008).

X. laevis tadpoles have a relatively simple nervous system controlled by the spinal cord. Before metamorphosis the spinal cord consists of ten classes of neurons (Straka & Simmers, 2011). The important class of neuron is the descending excitatory interneuron (dIN). These neurons are multipolar, with axons in the hindbrain that have a long firing duration and are responsible for the excitation of other neurons (Roberts et al., 2012). They

excite and stimulate the firing of other neurons during swimming, have pacemaker properties, and co-release glutamate and acetylcholine. The dIN neurons are fairly active during swimming and locomotion for the period before the tadpole undergoes metamorphosis (Roberts et al., 2012). Neurons releasing acetylcholine exist, therefore there is most likely a type of acetylcholine receptor that can be affected by neonicotinoids. If neonicotinoids are presented in sub-lethal concentrations before metamorphosis, they could interfere with the transition of the nervous systems and many other physiological processes.

This study investigated whether thiamethoxam and imidacloprid affect the development of *X. laevis* tadpoles by measuring the total length, mass, death rates, cortisol levels, any time delays in the regeneration of tails, and behavioral changes. I predicted that there would be a delay in metamorphosis, higher mortality, and slower growth rates, and that there will be a more significant effect when *X. laevis* tadpoles are exposed to imidacloprid compared to thiamethoxam.

Materials and Methods

I. Rearing of eggs and tadpoles

X. laevis fertilized eggs and tadpoles (Xenopus 1, Michigan) were reared in one liter aquaria with dechlorinated water (Tavera-Mendoza et al., 2002). The photoperiod consisted of 12 hour light and 12 hour dark cycle. Air stones were placed in each aquarium, and the amount of air was limited to restrict the amount of vibrations in the tank, especially when the tadpoles were not mobile. About 50% of the water was changed each day, and the room temperature was 23°C (Bowes, 2009). Every five days the water was completely changed. In addition, for every 100 mL of dechlorinated water 100 µl of Amquel was added to protect the tadpoles from bacteria. Gentamicin sulfate, an antibacterial agent, was not necessary to add

to the water. After the tadpoles were mobile they were fed a “dime size” amount of "tadpole powder" once daily (Xenopus I, Michigan).

II. Water parameters

The pH of the water was checked once a week with a pH wand and was kept near a neutral pH. When the pH was not in the neutral range of 6.8 to 7.2, all of the water in the aquarium was replaced.

III. Treatments

There were nine treatment groups of 25 tadpoles each. The first group of tadpoles was kept in “pure” dechlorinated water. In 2008, the concentration of thiamethoxam in the surface waters of rivers in Denmark was on average 1µg/L (Van Dijk, 2010, Starner & Goh, 2012). Therefore, one group of tadpoles was exposed to 1 and another to 5 µg/L of thiamethoxam (Sigma, St. Louis). Imidacloprid has also been found in surface water around Denmark at five times above its maximum tolerable risk level. In addition, it has been found in various areas at 1µg/L (Anatra-Cordone & Durkin, 2005). Therefore, two treatments, of 1 and 5 µg/L of imidacloprid (Sigma, St. Louis), respectively, were used. Due to high mortality in the 5 µg/L imidacloprid treatment group and the 5µg/L thiamethoxam treatment, in my second group of amputations instead of 5µg/L treatments .5µg/L treatments were used. Each thiamethoxam and imidacloprid solution was added directly to the water in the aquaria. Thiamethoxam is more soluble in water (4.1 g liter−1) than is imidacloprid (0.51 g liter−1), but both pesticides were directly dissolved in water (Castle, et al., 2005). Furthermore, imidacloprid is stable in water at a neutral pH. Its half-life ranges from 42 to 129 days (Anhalt, et al., 2007). It does degrade faster in an alkaline solution; however, I kept the water at a neutral pH. Thiamethoxam, similar to imidacloprid in non-irradiated or hydrolytic

conditions, does not significantly degrade in water (Robinson, 2001). Both chemicals degrade faster when exposed to light, as a result, the stock solutions of both were kept in the dark until needed.

IV. Two amputation groups

The first 125 tadpoles that were amputated were at stage 34. After the amputation, body length and tail length were measured. Then, they were placed into five different treatment groups (1 and 5 µg/L) imidacloprid and thiamethoxam and a surgical control; each with the same amount of dechlorinated water, Amquel, and food. In addition, 25 tadpoles without tail amputations were also reared and kept under the same conditions. The second group of 125 tadpoles was amputated at stage 40. After the amputation, body length and tail length were measured, and animals were randomly placed into five groups: (.5 and 1 µg/L) imidacloprid and (.5 and 1 µg/L) thiamethoxam and a surgical control. Every five to eight days the tadpole's total length and mass were measured. The number of deaths was recorded every day.

V. Tail Amputation

The amputation occurred under a microscope without anesthesia or an antibiotic. The tadpoles were placed on a plastic petri dish submerged under a small drop of water; the tail was amputated in the middle using iridectomy scissors (Pancharatna et al., 2010). Following the amputations of the tails the tadpoles were randomly assigned to a container with water containing either imidacloprid (.5, 1, or 5 µg/L), thiamethoxam (.5, 1, or 5 µg/L), or the surgical control (dechlorinated water). The tadpoles continued to be fed tadpole powder (Xenopus 1) and the room was kept at 23°C.

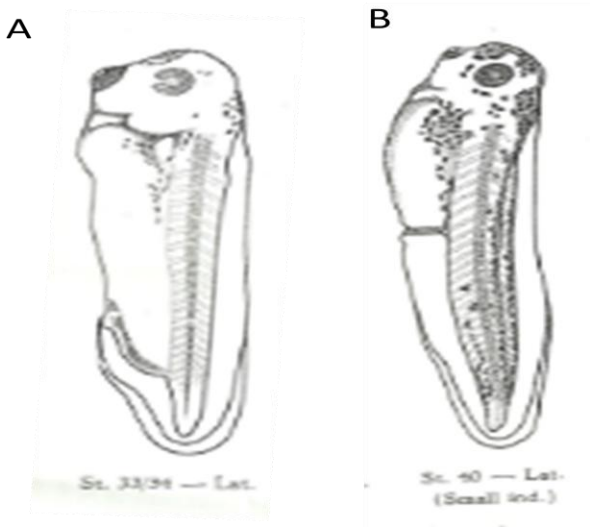

Figure 2. A) Stage 34 & B) Stage 40 from Gosner (1960)

VI. Appearance of tail regeneration

Every day I inspected the water and the tadpoles. Once every five days their overall appearance, and measured tail, limbs and total length of each tadpole was observed. Unfortunately, not a single tadpole developed hindlimbs or forelimbs.

VII. Tadpole preparation for cortisol determinations

I amputated a third group of tadpoles when the tadpoles were around stage 40. There were four treatments: untreated, surgical control, without any pesticide, 1µg/L imidacloprid, and 1µg/L thiamethoxam with 15 tadpoles in each. Each tadpole was placed in a single eppendorf tube with 150 µL of dionized water; samples were placed at -80ºC for one hour. Samples were then allowed to thaw, and the tadpoles were crushed with a Teflon homogenizer. Each tube was centrifuged for four minutes at 13,000 rpm and the supernatant was retained for cortisol analysis.

VIII. Cortisol levels

Cortisol levels were determined using an ELISA kit (ALPCO Diagnostics) and following the manufacturer's instructions.

IX. Statistical Analysis

Development, tail regeneration, total length, and weight at various intervals were examined using an ANOVA with StatView software. Mortality was analyzed with chi squared. In addition, levels of cortisol between each treatment group were analyzed as specified by ALPCO.

Results

I. Tail Regeneration Appearance

Overall, in each treatment group the tails of each tadpole regenerated. However, there were obvious differences in the way the tails regenerated. In the absence of pesticide the tadpole's tails regenerated straight and without any curl or bend. Any concentration of imidacloprid added to the water resulted in animals with curled tails (Fig 3 B, D & 2 B, D). The higher concentrations of imidacloprid induced more exaggerated curls. Thiamethoxam induced slight to extreme bends in tails initiating near the base of the body (Fig 3 E & 2 E). Maximal tail curvature was observed in tadpoles treated with .5 µg/L thiamethoxam (Fig 4 E).

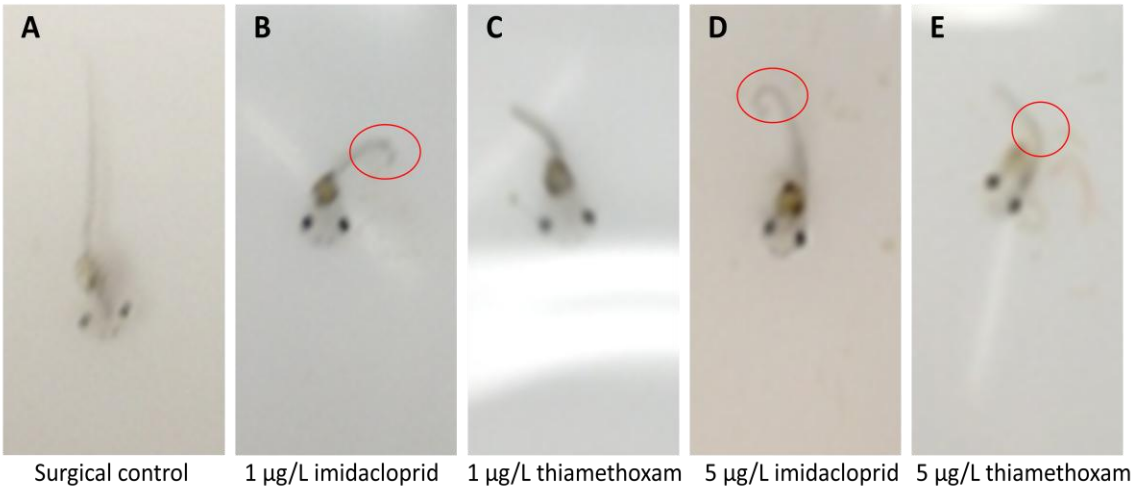

Figure 3. Regeneration of tails from the five different treatment groups 15 days after the amputations.

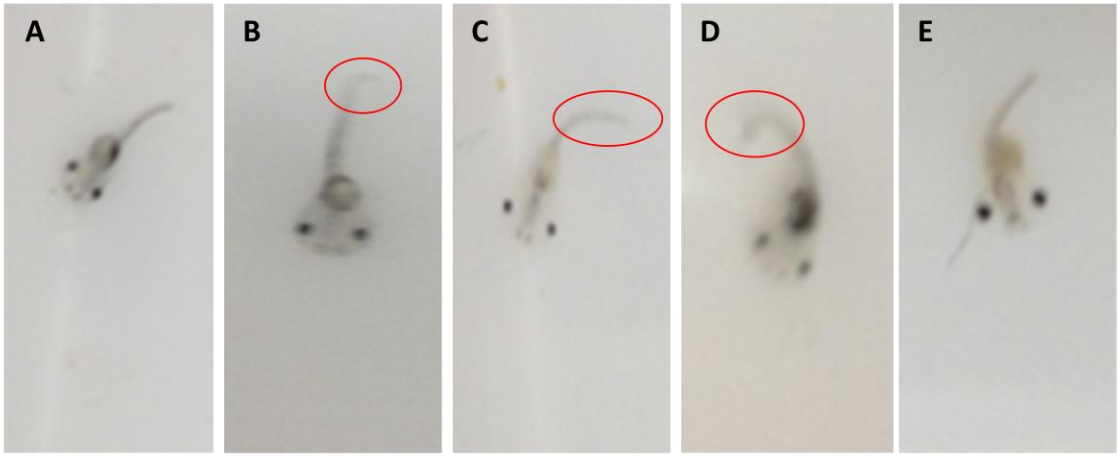

Figure 4. Regeneration of tails from the five treatments 15 days after amputations occurred.

II. Mortality

Initial rates of mortality varied significantly (Fig 5, day 3: $X^2 = 32.47$). Treatment with 5 µg/L imidacloprid induced the highest initial mortality (day 3: 16 deaths). Subsequently, the rate of mortality slowed, and animals treated with 1 µg/L imidacloprid had a faster mortality rate but slower than the animals treated with 5 µg/L imidacloprid (Fig 5). There were no significant differences between the mortality rate of tadpoles treated with 1 or 5 µg/L of thiamethoxam. By the termination of treatment at 35 days each treatment group had similar mortalities (day 24: $X^2 = 5.07$).

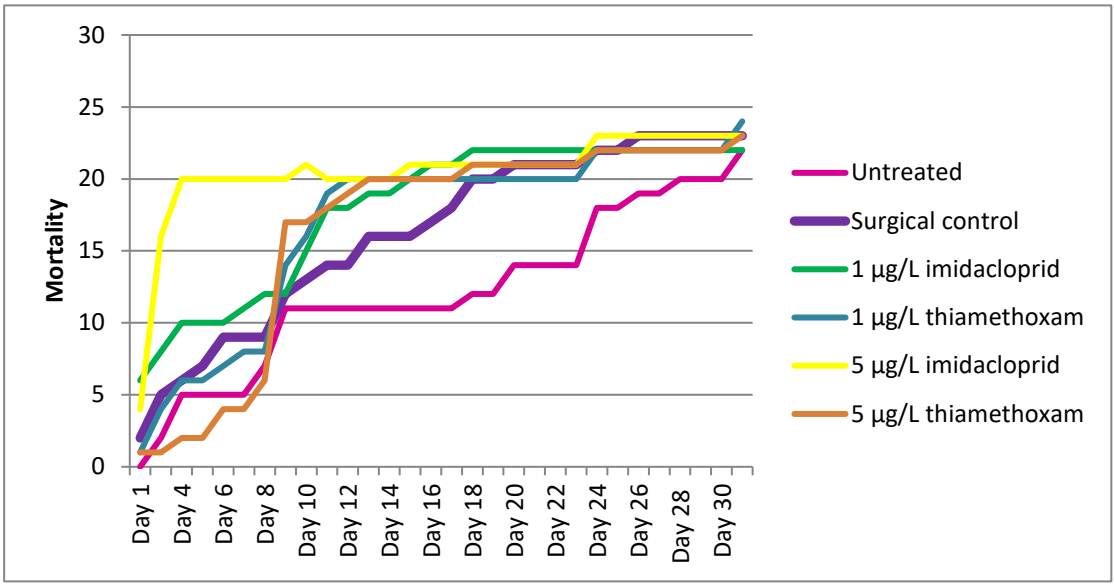

Figure 5. Daily mortality following tail amputations. Overall, mortality was significantly lower in untreated controls and significantly higher in those treated with 5 µg/L imidacloprid. Mortality is significant from day 3 to day 24 ($p < .01$).

In the second group of amputations all five groups had a low initial mortality (day 6: $X^2 = 3.12$) rate and the mortality rates leveled off around day 20 (Fig 6, day 15: $X^2 = 8.48$). The tadpoles treated with .5 µg/L thiamethoxam had the highest initial mortality rate. Surprisingly, there were very few differences between the surgical control and the other treatment groups.

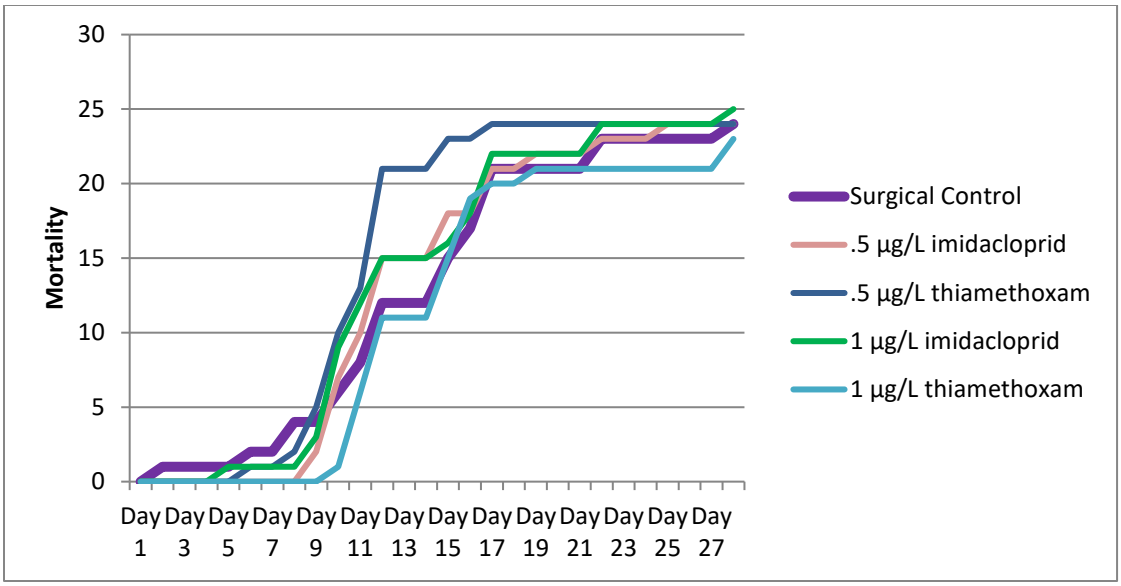

Figure 6. Daily mortality in the second group following tail amputations. Overall, the mortality is significantly higher in those treated with .5 µg/L thiamethoxam from day 10 to day 17 ($p < .01$).

III. Total length

Only day 4 and day 9 were statistically significant for the total length of the tadpoles (Fig 7b, $p<.0001$). The tails of the tadpoles grew and regenerated in each group; there were no consistent trends. Typically, the treatment with 1 µg/L thiamethoxam slowed tail regeneration except for the last two days. In addition, the water treated with 1 or 5 µg/L imidacloprid did not affect regeneration (Fig 7, $p >.0001$). For every day except one the animals treated with water with 5 µg/L thiamethoxam grew longer than those treated with 1 µg/L thiamethoxam (Fig 7b $p <.0001$). Overall the tadpoles developing in water treated with pesticide did not significantly regenerate or grow at a different rate than the tadpoles developing in "pure" dechlorinated water. For the second round of amputations the four groups of tadpoles treated with pesticide that did not develop (NOT significant) as much as the tadpoles treated with "pure" dechlorinated water. Also, the animals treated with .5 µg/L

imidacloprid had smaller body lengths than the tadpoles treated with water containing 1 µg/L imidacloprid, and after day 10, those treated with .5 µg/L thiamethoxam stopped developing and growing(Fig 8b, p <.0001). Lastly, the tadpoles treated with 1 µg/L imidacloprid and the tadpoles treated with 1 µg/L thiamethoxam averaged very similar total lengths (Fig 8).

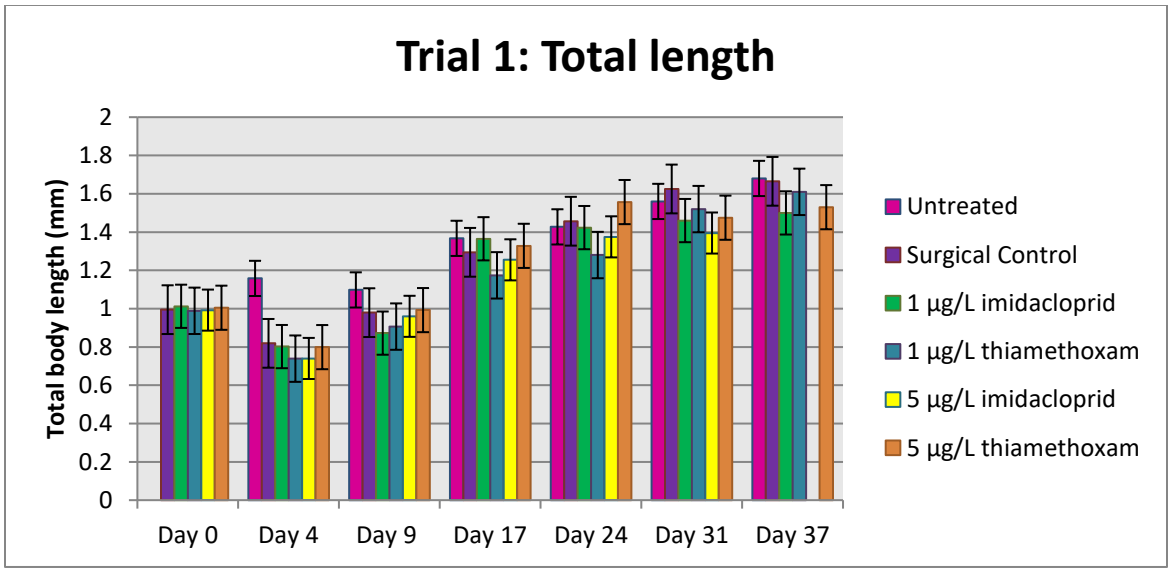

Figure 7. Average total lengths of tadpoles of six treatment groups. Measured each tadpole and calculated the average total length. The only significant differences were on days 4 and 9 ($p < .001$). Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

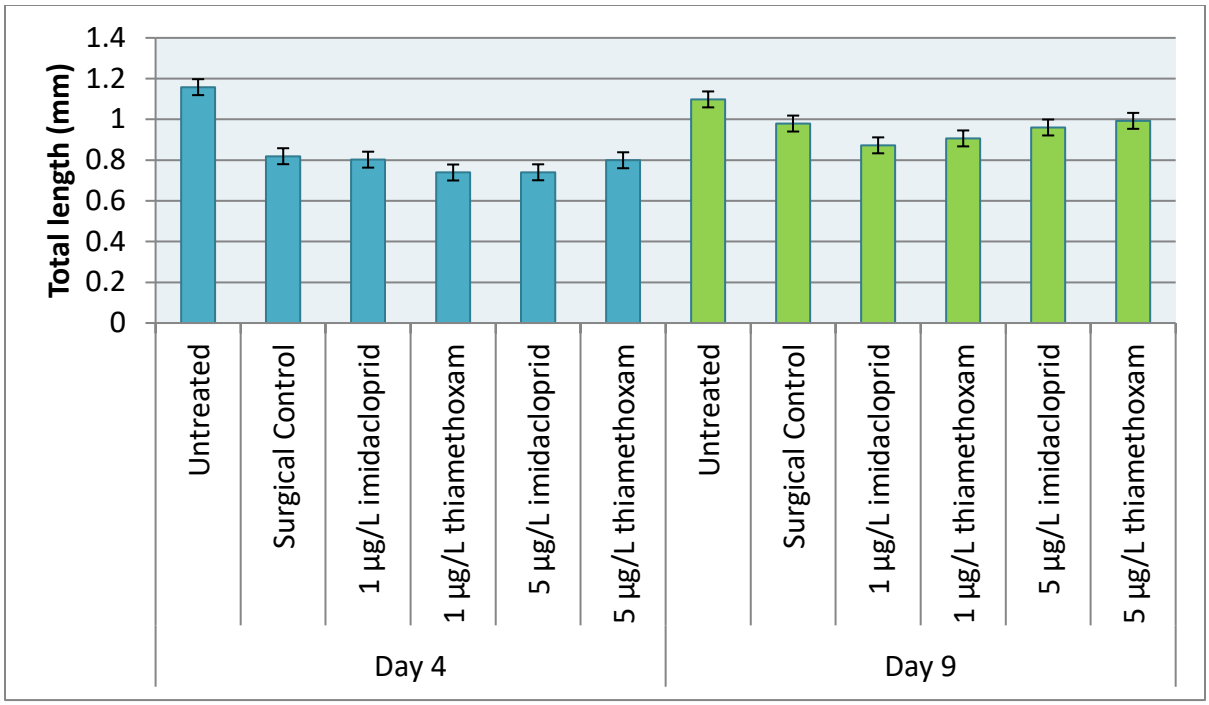

Figure 7b. Average total lengths of tadpoles on day 4 and day 9. Different letters represent significant differences. Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

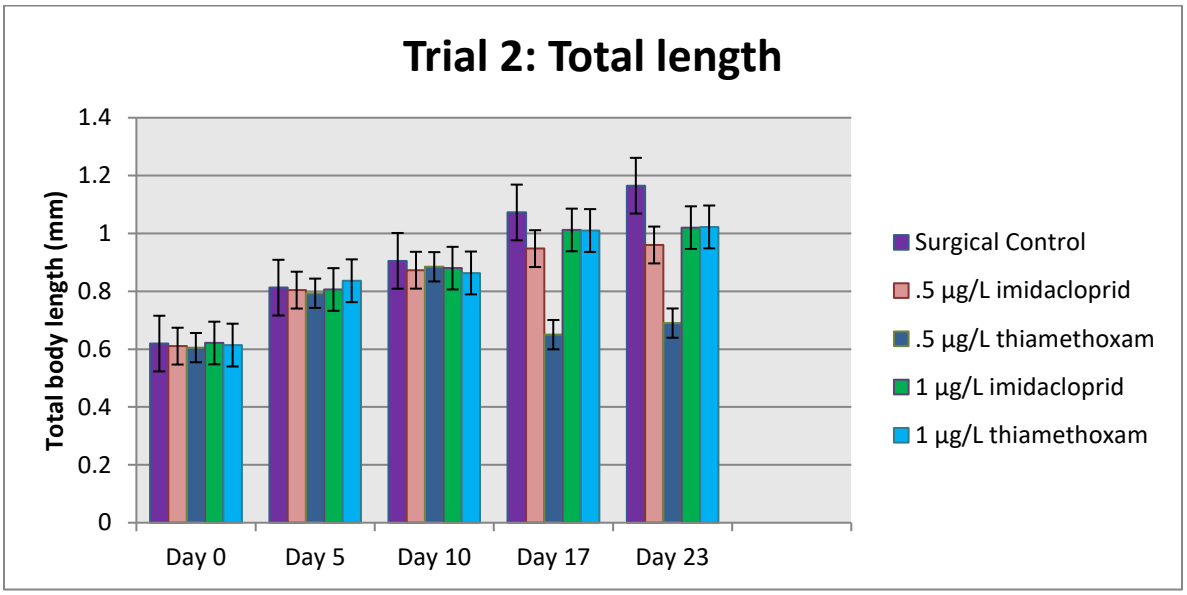

Figure 8. Average total lengths of tadpoles of six treatment groups. Measured each tadpole and calculated the average total length. The only significant difference was on days 17 ($p < .0001$). Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

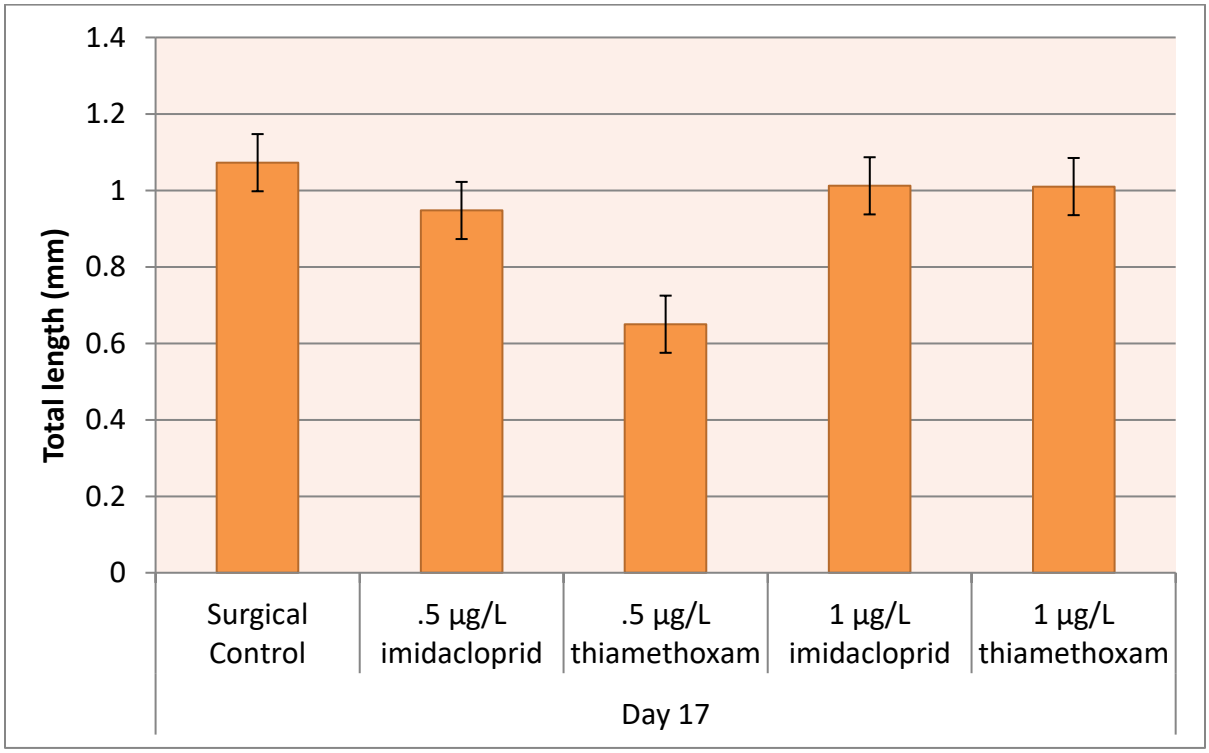

Figure 8b. Average total lengths of tadpoles on day 17. Different letters represent significant differences. Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

IV. Mass

Overall, the mass of untreated animals was the lowest, and that of the surgical controls the highest (Fig 9). In addition, the mass of animals treated with 1 µg/L imidacloprid was similar to that of the surgical controls. However, the animals treated with thiamethoxam consistently weighed the least compared with tadpoles of other pesticide treatments except for day 9 (Fig 9).

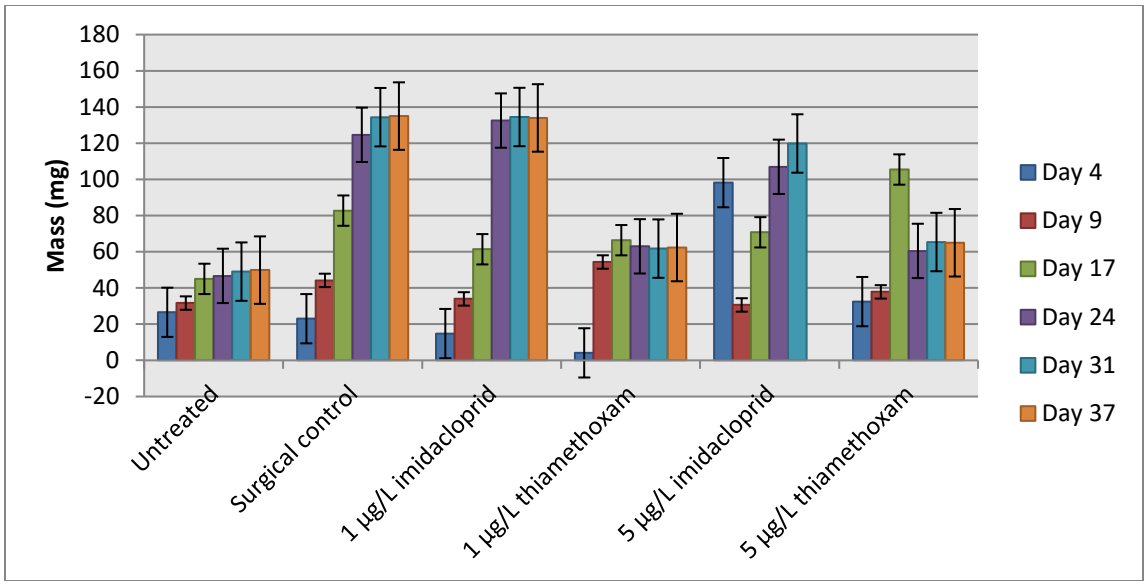

Figure 9. Average mass of one tadpole in the six treatment groups in the first trial. Weighed all animals and took the average. Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

For the second trial, the tadpoles treated with .5 µg/L thiamethoxam weighed the most and always weighed more than those exposed to 1 µg/L thiamethoxam except for day 10 (Fig 10). The surgical controls weighed consistently more than every other group except those treated with .5 µg/L thiamethoxam. Finally, the respective thiamethoxam treated tadpoles weighed more than the imidacloprid treated tadpoles (Fig 10).

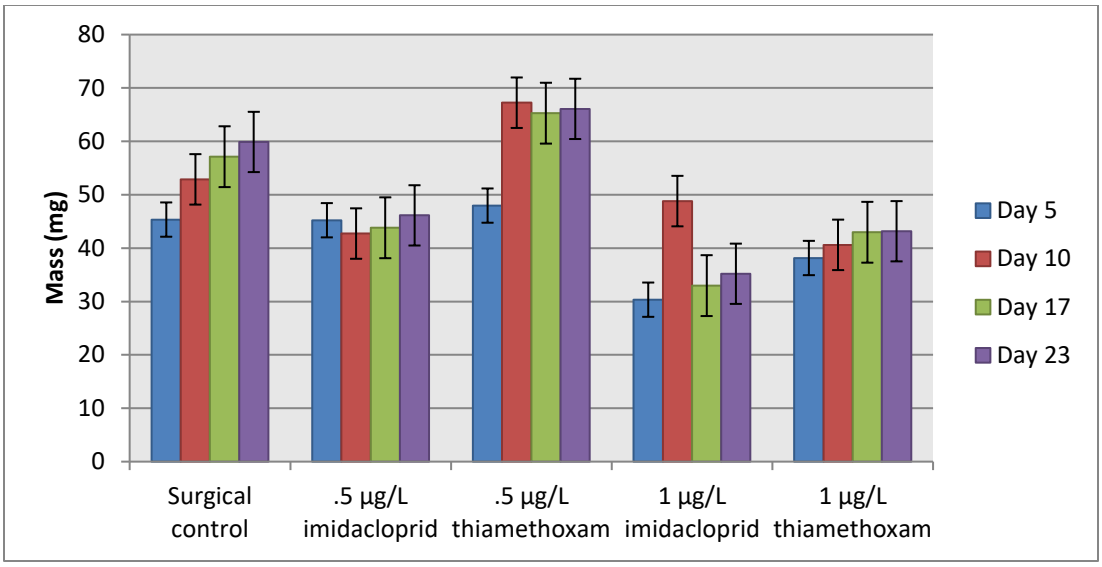

Figure 10. Mass of one tadpole in the six treatment groups in the first trial. Weighed all animals and took the average. Each histogram represents the mean +- standard error mean of 20-30 individual measurements.

V. Cortisol Levels

The cortisol concentrations did not differ significantly across treatments; however, there is an apparent trend. The tadpoles in untreated water had the highest levels of cortisol and 1 µg/L thiamethoxam had the lowest levels of cortisol (Fig 11).

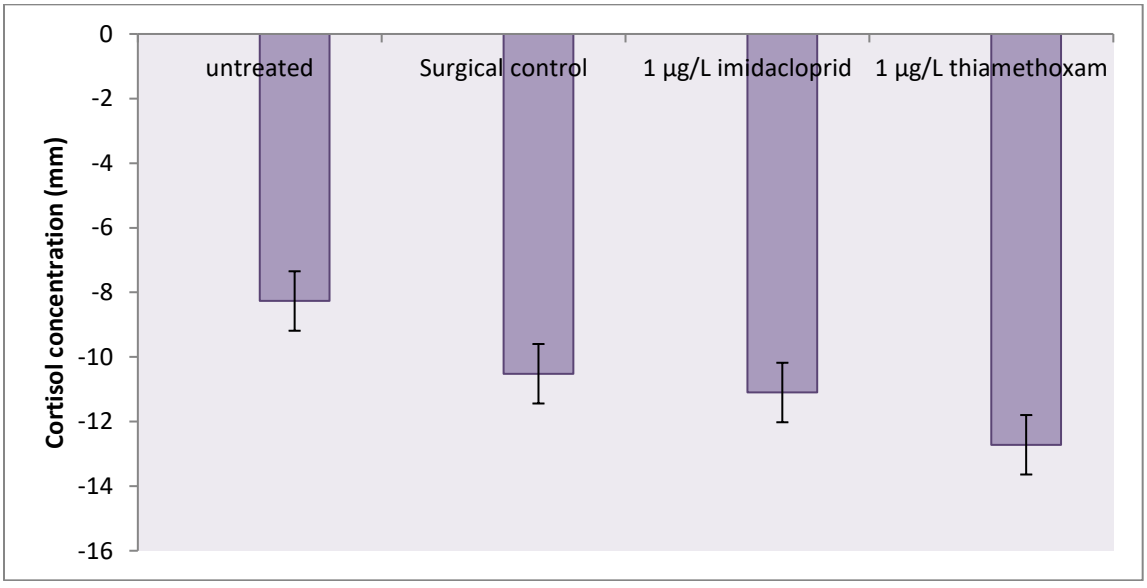

Figure 11. Average concentrations of cortisol in each treatment group, analyzed using a cortisol ELISA kit (ALPCO). The level of cortisol in neither group is significant.

Discussion

I. Overview

Pesticides applied for agriculture purposes affect nontarget organisms, especially amphibians. When developing or using pesticides it is important to consider both lethal and sub lethal affects on nontarget organisms that may result from long-term exposure to low concentrations because most organisms in the environment are exposed to low concentrations over a long period of time (Bridges, 2000). Many pesticides occur in concentrations that do not cause direct mortality but instead promote deformities, behavioral changes, decrease in growth, decrease in weight, and changes in hormone levels. These effects can decrease in fitness, reproduction, immune defenses, and ultimately lead to a decrease in survival and population decline (Bridges, 2000). Whole ecosystems including amphibians such as *X. laevis* can be affected by low concentrations of pesticide (Relyea, 2009).

II. Appearance and behavior

Exposure to pesticides promoted deformities in tadpole's tails. Exposure to any concentration of imidacloprid resulted in abnormal curvature of regenerating tails; similarly, thiamethoxam exposure resulted in abnormal bends in regenerating tails. Increases in amphibian deformities are occurring throughout the United States (Kiesecker, 2002). Southern Leopard frog tadpoles (*Rana sphenocehpala)* introduced to low concentrations of carbaryl, also developed deformities (Bridges, 2000). Such findings suggest that deformities can and have been caused by chemical run-off. Indeed, limb deformities in amphibians are more common in areas near agricultural run-off (Kiesecker, 2002). Moreover, an increase in limb deformities occurs in larval wood frogs exposed to low concentrations of atrazine and malathion (Kiesecker, 2002). It is likely that contaminants induce stress, which suppresses the immune response.

Immune suppression might then lead to an increase in trematode infection, and cause subsequent limb deformities (Kiesecker, 2002). Whether the deformity is severe or minor, these abnormalities in amphibians can decrease fitness and survival. If a tadpole's tail is bent or curled, swimming ability is decreased, and susceptibility to predators is thereby increased (Bridges, 2000). Furthermore, malformations might diminish juvenile recruitment and adult reproduction and overall survival resulting in a smaller population (Bridges, 2000).

After the amputations the tadpoles exposed to 5 µg/L imidacloprid exhibited dramatic behavioral changes. The addition of water to aquaria usually elicits movement away from the site of water entry. In contrast, tadpoles treated with 5 µg/L imidacloprid remained stationary as water entered the immediate area. This is consistent with behavioral changes caused by neonicotinoids in bees and other invertebrates (Sadeghi et al., 2009). Neonicotinoids disrupt foraging behavior, decrease learning abilities, and decrease homing abilities in honeybees (Schneider et al., 2012). It is also consistent with other studies that have documented behavioral alterations in tadpoles after exposure to sublethal concentrations of pesticides (Bridges, 2000).

III. Mortality

In general pesticide treatment resulted in high initial mortality rates; subsequently rates of mortality became similar among all treatment groups. Tadpoles treated with 5 µg/L imidacloprid had the highest initial mortality rate (first trial), although those exposed to .5 µg/L thiamethoxam also had low initial survivorship (second trial). Pesticides of varying concentrations can increase mortality rates in *X. laevis* (Christin et al., 2003), including imidacloprid at different concentrations (Pestana et al., 2009). In the laboratory and in situ locations, prolonged exposure to low concentrations of Dithane DG affects growth,

deformities, and mortality (Harris et al., 1998). Mancozeb, the active ingredient in Dithane DG is a type of carbamate. Neonicotinoids are substitutes for carbamates and organophosphates and have similar mechanisms because they both affect the nervous system. Overall, tadpoles in the laboratory introduced to 2.8- 5 µg/L Dithane DG, have low survivorship, and pesticide influxes in pond waters are environmental stressors that decrease the population size of tadpoles (Harris et al., 1998).

Carbaryl, a carbamate, causes high mortality to *R. sphenocephala* tadpoles (Bridges, 2000) after day 11 to day 14 of exposure. Later, the rate of mortality slowed, suggesting a sensitive period when tadpoles are most affected by this pesticide and mortality increases (Bridges, 2000). This high mortality can be attributed to (a) the strength of the pesticide used and (b) the pesticide effects on the tadpole's immune system. Comparison of effects of classes of pesticides revealed that cholinesterase pesticides or acetylcholine pesticides were most strongly associated with declines in amphibian populations in California (Davidson, 2004). These pesticides affect acetylcholine receptors, similar to neonicotinoids, which are an integral part of the nervous system, and can have detrimental consequences to amphibians when affected. The immune system of *X. laevis* can be affected by other pesticides as well. A mix of over six agricultural pesticides decreased the number of splenocytes in *X. laevis* tadpoles, which potentially results in an inefficient immune system (Christin et al., 2004). In *X. laevis* tadpoles the ability to recognize and kill foreign tissue does not begin until day 12, and is not complete until the end of metamorphosis (Carey & Bryant, 1995). Therefore, *X. laevis* larvae are vulnerable to toxins and diseases, which is increased by the addition of pesticides that worsens the immune system.

IV. Total length

After amputation, the tails began to regenerate. Although differences were not always statistically significant, tails of the treated groups generally grew more slowly than those of the controls. Tadpoles treated with 5 µg/L imidacloprid appeared to have a two day delay in tail regeneration (first trial). Treatment with .5 µg/L thiamethoxam also significantly slowed tail regeneration by the end of the treatment period (second trial).

Reduced tadpole growth has been observed in many field studies and duplicated in the laboratory following exposure to carbamates and organophosphates (Harris et al., 1998). For example Diathane DG inhibits growth early in development at concentrations seen in the environment (Harris et al., 1998). The reduction in growth might be attributed to the drive to eat and energy availability. Usually the primary environmental determinant of growth is food availability; however, all groups received the same amount of food and each tadpole had the same opportunity to eat. Therefore, imidacloprid and thiamethoxam potentially disrupted the tadpole's foraging and eating behavior. Imidacloprid decreases foraging behavior in honeybees and might have a similar effect on the *X. laevis* tadpoles (Schneider et al., 2012). In support of this hypothesis the aquaria with the tadpoles treated with .5 µg/L thiamethoxam had considerably less excretions than did other aquaria. Secondly, tadpoles developing in water with these chemicals may expend energy in response to the toxins (Bridges, 2000). This allocation of energy would reduce growth and result in smaller tadpoles. The decrease in size can negatively affect the population because a decrease in size at metamorphosis or a delay in metamorphosis could lead to decline or even extinction of a population (Bridges, 2000).

The animals treated with the lower concentrations of thiamethoxam were affected more than those at higher concentrations. This is likely an example of hormesis. A hormesis response is a dose-response curve that is not linear but u-shaped (Calabrese & Baldwin, 2003). Essentially, the effects of many hormones and chemicals can be worse with lower concentrations than higher concentrations (Calabrese & Baldwin, 2003). Higher concentrations can be detected and can produce a defensive response that the lower concentrations might not evoke.

V. Mass

Surprisingly, the more the tadpoles grew in length the less they increased in mass. Thus, the tadpoles treated with thiamethoxam lengthened more slowly, however they weighed more than the tadpoles treated with imidacloprid. The untreated tadpoles weighed less than every other treatment group and the animals treated with .5 µg/L thiamethoxam consistently weighed the least. This is somewhat similar to the effects of carbaryl on *Rana sphenocephala* because the treatments of different concentrations did not significantly affect the mass of tadpoles (Bridges, 2000). Instead of total length being an indication of weight, the tadpoles' width may be an indication of weight; as a result width and mass could have a direct relationship, and should be measured.

VI. Cortisol levels

There were no significant differences in cortisol concentrations because there was very little cortisol in any of the tadpoles. The untreated group did have the highest levels of cortisol and the tadpoles treated with 1 µg/L thiamethoxam had the least. The *X. laevis* tadpoles at day 10 were small; therefore the samples could have been diluted excessively, which caused an insignificant amount of cortisol to be detected. Corticosteroids have a daily and seasonal

rhythmic cycle in tadpoles (Norris, 2007). The amount of cortisol in the day and in the afternoon for *Rana catesbeiana* varied substantially and could not be tracked (Wright et al., 2003). Therefore, depending on the cycle and stage the tadpoles were in, the animals could have had almost no cortisol circulating. Also, in amphibians cortisol is primarily found in the plasma and not in the saliva (Norris, 2007).

On the other hand, the neonicotinoid may not have been enough stress to induce an increase or change in cortisol levels. Hypoxia nor capture methods did not cause a significant change in plasma or glucose levels in *Lithobates catesbeianus* tadpoles (Rocha et al., 2010).

VII. Conclusion

Neonicotinoids induced changes in mortality rates, total length, and weight. Additionally imidacloprid and thiamethoxam induced deformities in regenerating tails. Despite this, my hypothesis was not completely supported because it appears that .5 µg/L thiamethoxam elicited the most detrimental effects on *X. laevis* tadpoles. Both pesticides are harmful to other species, including honeybees and bumblebees. These effects were so great that they are both considered class 4, “highly toxic contaminants” (Mommaerts et al., 2010) which helps support my hypothesis that neonicotinoids can cause harmful effects to *X. laevis* tadpoles.

VIII. Future Directions

In the future there are many directions to pursue. The duration the tadpoles are exposed can be increased to metamorphosis or even past metamorphosis. Deformities in the growth of hindlimbs and forelimbs can be observed and any growth or behavioral changes after metamorphosis can be examined because the majority of *X. laevis* growth occurs after metamorphosis (Carey & Bryant, 1995). Lastly, instead of measuring the concentrations of cortisol, the levels of corticosterone could be measured. Corticosterone is another prominent

corticosteroid found in all amphibians (Yadav, 2008). This could be a better indicator of stress in tadpoles because cortisol sometimes is not an adequate indicator of stress levels in tadpoles like *Rana catesbeiana* (Rocha et al., 2010). In addition, when stressors such as density and hypoxia were induced in *Lithobates catesbeianus* tadpole aquaria cortisol, again was not sufficient to increase the level of cortisol in the animals' plasma (Teixeira et al., 2012). However, when *Rana temporaria* tadpoles were introduced to predators at different latitudes and corticosterone was measured, it was an adequate indicator because the amount of corticosterone significantly increased (Dahl et al., 2012).

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