Effects of a Neonicotinoid Insecticide on Larval Stages of the Green Frog, *Rana clamitans*

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Abstract

Agricultural use of insecticides to increase crop yield is potentially detrimental to the ecosystem due to potential harmful effects to non-target species living in the same area. Neonicotinoids are a new class of insecticide that appear less detrimental because of their increased rate of degradation and ability to bind more readily to the insect acetylcholine neurotransmitter, rather than that of vertebrates. Their effects on non-target species such as amphibians, however, are not well understood. In environmental studies, frogs are commonly used as an indicator taxon of ecosystem conditions because of their sensitivity to pollutants, ability to absorb chemicals through their skin, and ease of rearing in laboratory settings. We measured effects of a neonicotinioid insecticide, imidacloprid, on the green frog *Rana clamitans* by subjecting tadpoles of this species to different concentrations of imidacloprid. We examined effects on growth, development, survivorship and behavior of the tadpoles. The insecticide did not have measurable effects on tadpole growth or development. On the other hand, the insecticide had a measurable effect on both survivorship and behavior of green frog tadpoles at the two highest concentrations, 200 mg/L and 400 mg/L of insecticide. Observed effects could be a consequence of overstimulation of nerves, which can result in paralysis and/or death of an organism. Because this study was conducted in a laboratory setting following the acute toxicity testing protocol, it is unlikely tadpoles would be exposed to such concentrations in a natural setting. Therefore, although imidacloprid had detrimental effects on survivorship and behavior in our study, it is likely a safer alternative than other classes of insecticides if used as directed.

Introduction

There are many anthropogenic actions that, despite intended benefits, adversely influence ecosystems and have the potential to permanently or temporarily affect their inhabitants. In particular, modern agriculture uses substances like pesticides, which have been known to be detrimental to surrounding life (Peveling, 2001). Insecticides are routinely used to control insect pests that consume or otherwise blemish crops. Although insecticides can increase crop yield, they also negatively affect non-target species living in the area, including pollinators (Oldroyd, 2007), small mammals and amphibians (Jones et al., 2009, Relyea, 2009).

Many classes of insecticides are commonly used today. These classes are determined by the chemical makeup of the compound or the chemical process by which they work. The major classes of pesticides include in order of decreasing toxicity: organochlorine compounds, organophosphates, carbamates, pyrethroids, nicotinoids, neonicotinoids and biological agents such as bacteria and nematodes (Kraiss and Cullen, 2008). Organochlorine compounds, such as DDT, were introduced and widely used around the world, but due to harmful environmental effects, it has since been banned (Mannion et al. 2000). Organophosphates are also toxic and have an additive quality, meaning increased exposure to chemicals amplify the toxicity. Carbamates are similar to organophosphates, but are considered less toxic because of the shorter duration of the action of the insecticide on its target neurotransmitter. Pyrethroids are even less toxic to animals than carbamates and are used for household pests (Johnson et al., 2006).

Nicotinoids and neonicotinoids are very similar insecticides. This class of insecticides mimics the nicotine receptor of the nervous system (Tomizawa and Casida,

2005; Yamamoto et al., 1995) and is derived from natural sources such as the nicotine from tobacco. Neonicotinoids are the newest class of insecticides to be developed and represent the only major new class of insecticides to be developed in the past three decades (Massaro, 2002). Nicotinoids have many beneficial characteristics not seen in other insecticides because of their lower toxicity, meaning they are effective at much lower concentrations than other insecticides. Nicotinoids and neonicotinoids act on the nervous system of animals by mimicking the neurotransmitter acetylcholine (Ach) and binding to the acetylcholine receptor on the postsynaptic neuron (Matsuda et al., 2001). Thus, like Ach, these insecticides serve to transmit a nerve impulse across the synaptic cleft to the postsynaptic neuron. However, unlike acetylcholine, neonicotinoids are not broken down by the enzyme cholinesterase. Consequently, the neonicotinoid compound continually stimulates the postsynaptic neuron, resulting in overstimulation of the nervous system, which leads to paralysis and even death (Tomizawa and Casida, 2005; Yamamoto et al., 1995). When compared to nicotinoids, neonicotinoids are more beneficial because they are less toxic to vertebrate species, but are still effective in controlling insects. They bind more readily to the insect form of the Ach receptor than to the vertebrate form, and are thus referred to as neonicotinoids (Massaro, 2002; Yamamoto et al., 1995).

Neonicotinoids have also proven less toxic than other classes of insecticides, such as pyrethroids, to non-target invertebrates like crayfish (Barbee and Stout, 2009). Furthermore, relatively rapid degradation of neonicotinoids in both soil (half life = 39 days) and water (half life < 3 hours) is another key factor that makes this insecticide particularly promising (Kollman and Segawa, 1995; Moza, 1998). A compound known as

imidacloprid is one of the most widely used neonicotinoids (Massaro, 2002) and was the first compound in this class to be developed in 1991 (Massaro, 2002). Imidacloprid is a systemic insecticide, meaning that when a compound is sprayed on the leaves or soil surrounding of a plant, the plants absorb the chemical locally and transports it throughout the entire plant tissue. It is effective in controlling sucking insects (i.e. aphids, leafhoppers, whiteflies and thrips) and chewing insects, such as flea beetles. Imidacloprid is normally used on leafy vegetables, potatoes, grapes and citrus (Admire, 2010). Different manufacturers sell imidacloprid under various trade names including Gaucho™, Admire™, Advantage™, and Mallet™.

Despite the apparent advantages for pest control, neonicotinoids have the potential to be detrimental to non-target species. Furthermore regulations on insecticides do not provide sufficient information for all organisms that may be exposed to the insecticide. Neonicotinoids are suspected of contributing to colony collapse disorder in honeybees (Bonmatin, et al., Oldroyd, 2007), and concerns still remain over their safety for other non-target species, including vertebrates. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires that new pesticides used in the United States be registered by the Environmental Protection Agency (EPA). The application process usually filed by the chemical manufacturer requires data on product and residue chemistry, environmental fate and hazard information (Federal, 1972). Hazard information includes specific test data assessing toxicity, skin and eye irritation potential, and potential exposure by various routes (i.e. oral, dermal, inhalation) for humans, domestic animals and non-target organisms. This hazard information does not require data on effects of new pesticides on behavior, development and morphology of non-target

organisms (Tomizawa and Casida, 2005). Although the EPA sets upper limits for levels of pesticides used, it is uncertain if these levels are safe for non-target species and for all life stages of these species.

Amphibians are non-target organisms susceptible to effects of insecticides because of their sensitivity to pollution and other environmental contaminants (Relyea, 2010). Due to a highly permeable skin through which they breathe, amphibians readily absorb chemicals through the skin surface (Lannoo, 2005), leaving them particularly vulnerable to chemical exposure. Moreover, because amphibians live on both land and water, they may be exposed to pollutants originating from both terrestrial and aquatic sources. Frogs are often used as a representative indicator taxon within amphibians because they are abundant in many different ecosystems (Wilson and McCranie, 2003). Frogs serve as good indicator species with respect to effects of agricultural inputs to ecosystems because they breed in small ponds, like those found on many farms, where they could be exposed to chemicals through sprays or residual agricultural runoff (Lannoo, 2005; Yan et al., 2008). In addition, frogs prey on insects, fish and other small aquatic animals that may themselves have been exposed to chemicals in agricultural fields. Early stages of frog development, namely eggs and tadpoles, are particularly good for assessing how environmental contaminants may impact non-target species. Immature tadpole stages are relatively easy to rear and monitor in the laboratory and may be especially vulnerable to pollutants because of their small size and developmental process they undergo in these stages (Lannoo, 2005).

A standard method used to assess effects of chemicals on organisms is the acute toxicity test (ATT). This test determines appropriate concentrations of a chemical that

can potentially produce adverse effects on a specific percentage of the tested organisms during a short exposure (Standard, 2007). Measureable adverse effects include behavioral changes, immobility, and mortality. These tests have been performed on many different species of frogs, fish, and macroinvertebrates to predict effects on these organisms in field situations as a result of exposure under comparable conditions (Standard, 2007; Feng et al., 2004; Quan et al., 2006). ATT data are usually given in the form of an LC or LD50, which represents a concentration or dosage, respectively that kills half the tested population, and are measured in many different ways, including mg/L or parts per million (ppm). An LC50 value for one species cannot be compared to another species, thus there can be only one LC50 for a certain toxin tested on a distinct species. To the best of our knowledge, no acute toxicity tests have been done with neonicotinoids on the green frog, *Rana clamitans*, even though it is a common frog in the Eastern United States (Lannoo, 2005) and is known to inhabit small ponds near agricultural fields that could face exposure of certain harmful chemicals.

To enhance our understanding of the potential effects of neonicotinoid exposure on an environmentally sensitive non-target species, we conducted acute toxicity tests (ATT) on tadpoles of the green frog, by exposing them to different concentrations of the insecticide imidacloprid. We assessed how this compound affected growth, survivorship, and behavior of the immature stages. We then continued to monitor the organisms following completion of the acute toxicity tests for an additional eight days in a long term effects test (LTFX) to investigate whether any long-term effects or recovery from chemical exposure occurred, as suggested by the Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates and Amphibian

(Standard, 2007). We hypothesized that growth would be stunted because we believed imidacloprid would interfere with growth of tadpoles by altering body chemistry and growth hormones. We also predicted, that survivorship and behavior would be affected by an increase in imidacloprid concentration. We believed the effect of the high toxicity of the insecticide would cause survivorship to decrease as imidacloprid concentration increased. Similarly, we hypothesized that tadpole activity and response to disturbance would decrease as concentration increases because imidacloprid may disrupt nerves that cause tadpoles to respond to disturbances. In addition, imidacloprid may disrupt nerves that prevent tadpoles from recognizing a disturbance has occurred. Thus, behavior of tadpoles in higher concentrations would be more sluggish and unresponsive.

Methods

Egg collection and tadpole rearing

Green frog *(Rana clamitans)* tadpoles used in this experiment hatched from egg masses collected from Hyla House Pond on the Pierce Cedar Creek Institute (PCCI) property located in Hastings, Barry County, Michigan. In past green frog studies at PCCI, Hyla House Pond has been used because of its large population of green frogs and its consistency as a location for green frog breeding and tadpole rearing. Hyla House Pond is located in a rural setting close to open fields, meadows and prairie meadows where insecticides are used. This is an ideal location to collect egg masses for this study because this habitat has the potential to be affected by insecticides. Daily searches, beginning June 9th and ending July 12th, 2010 took place in direct sunlight while wading in water with a depth between 0.3 m and 0.7 m. We collected two egg masses each containing over 800 eggs; one on June 30th and the second on July 6th. Egg masses were collected with pond water, brought to the laboratory and transferred to 700 mL plastic containers (GladWare®, Oakland, California) that were continuously aerated via aquarium bubblers. Excess organic matter (e.g. plant material) was removed from egg masses and eggs were subsequently allowed to acclimate overnight in the containers. The following day, we transferred eggs to tap water that had been kept in a bucket for several days to allow chlorine that might be present in the water to dissipate. The water used was from a drilled well and not chemically treated by PCCI.

Egg masses hatched approximately 4-5 days after collection. When eggs hatched, the date was noted so age of developing tadpoles could be recorded because actual date of oviposition was unknown. We transferred tadpoles to a 15 L plastic bucket that was

continuously aerated via aquarium bubblers. Tadpoles were fed algae pellets (HBH Frog & Tadpole Bites®, HBH Pet Products; Springville, UT) daily and water was changed every 1-2 days. We changed water by pouring off the majority of the water and then replacing it with fresh water.

Growth and development

Three body measurements were made to the nearest 0.5 mm using a plastic ruler while viewing the tadpoles through a dissecting microscope (Figure 1). Four individuals from each container were selected, placed into a Petri dish divided into four quadrants, measured and then returned to their respective container. Measurements of the tadpoles were taken before the acute toxicity tests began (Pre ATT), after the acute toxicity tests finished (Post ATT), and at the end of the long-term effects test (Post LTFX). Additionally, changes in morphology and development (e.g. disappearance of operculum, disappearance of gills, appearance of limb buds, disappearance of tail) were recorded at these same intervals.

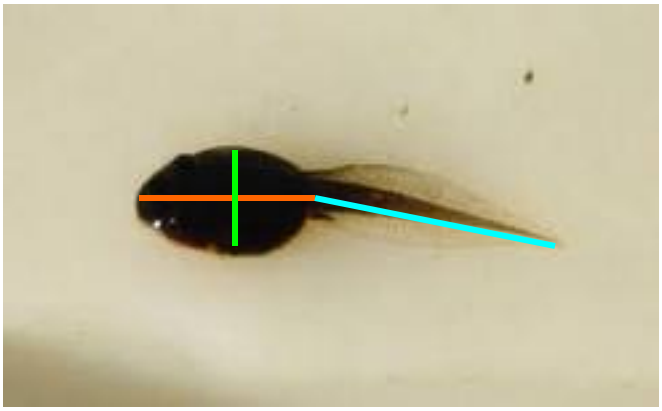

Figure 1. Schematic of the three body measurements taken before (Pre ATT) and after acute toxicity tests (Post ATT) and after the long term effects test (Post LTFX). The orange line represents snout to base of tail length (body length); blue represents base of tail to tip of tail length (tail length); green represents body width.

Acute toxicity tests (ATT)

We performed two sets of acute toxicity tests, one test with 10-day old tadpoles and another with 26-day-old tadpoles. For each experiment, we set up replicate plastic containers (1.89 L) (Meijer, Grand Rapids, Michigan), each filled them each with 1 L of treatment solution consisting of tap water and Admire Pro® (BayerCropScienceUS, Pittsburgh, Pennsylvania). The active ingredient in Admire Pro® is imidacloprid, which comprises 42.8% of Admire Pro®. Thus, treatment solutions were composed of tap water and the required amount of Admire Pro®, measured with a digital balance (Mettler PM400) to the nearest 1 mg, to yield imidacloprid concentrations of 0, 25, 50, 100, 200 and 400 mg/L. Choice of these concentrations of imidacloprid were based on previous acute toxicity tests with imidacloprid and other frog species (Feng et. al., 2004; Quan et. al., 2006). Each treatment was replicated six times using 10 tadpoles per replicate.

For 10-day-old tadpoles, three replicates of each treatment were run using the first batch of eggs, and three replicates were begun four days later using 10-day-old tadpoles from the second batch of eggs. For 26-day-old tadpoles, four replicates were run using the first batch of eggs, and two replicates were begun four days later using the second batch of eggs. We monitored survivorship of developing tadpoles over a four-day period. Organisms were observed, and number of surviving tadpoles recorded, at 3, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h after initial introduction of treatment solution as per ASTM guide for conducting acute toxicity tests (Standard, 2007). Dead tadpoles were removed from containers at this time. Containers were continuously aerated with aquarium bubblers, covered with lids to reduce evaporation, and exposed to a 16:8 h L:D cycle using fluorescent lights suspended 15 cm above the tops of the containers (Figure 2). Tadpoles

were not fed during the acute toxicity tests as per ATT standard protocol (Standard, 2007).

Figure 2. Experimental set-up of acute toxicity and long-term effects tests. The three columns on the left are the three replicates for the acute toxicity test (ATT). Once the acute toxicity test was finished after four days, tadpoles were transferred to the right side of the table for the additional eight day long-term effects test (LTFX). Rows in each column contain different concentrations of insecticide from 0 mg/L (foreground) to 400 mg/L (background).

We monitored water conditions regularly and completely exchanged treatment solutions in each container daily. We measured pH, dissolved oxygen content, and temperature of the water daily with pH strips (colorpHast, EM Science; Darmstadt, Germany) a dissolved oxygen meter (Hanna HI 9142) and thermometer (Fisher Scientific, Hampton, New Hampshire) respectively. Due to technical difficulties and lack of supplies on-site, these measurements were taken only for the latter half of the first experiment (10-day-old tadpoles) but for the entire length of the second experiment (26-day-old tadpoles).

Long-term effects tests (LTFX)

After an acute toxicity test was completed, surviving tadpoles were transferred to clean, reusable plastic containers (1.89 L) (Glad, Grand Rapids, Michigan) filled with 1 L

of tap water and monitored for an additional eight days for potential long term effects of ATT treatment exposure and recovery from treatment exposure. We monitored tadpole survivorship at 24 h intervals. Tadpoles were fed *ad libitum* throughout the LTFX test. Debris (e.g. uneaten food, organism waste) was removed daily and water was changed as needed by scooping out the majority of the water and replacing it with fresh water. We continued to record pH, dissolved oxygen content and temperature of the water daily.

Behavioral tests: response to disturbance

We monitored tadpole behavior daily before and after administering a disturbance to the holding container and ranked the behavior of tadpoles as a group in each container on the following five-point scale:

Rank	**Behavioral Characteristics**
1	No movement
2	Slight movement: tail flick or wiggle, no change of position.
3	Change of position; no greater than 5 cm from one previous location
4	Full swimming; less than 5 seconds in duration
5	Full swimming; greater than 5 seconds in duration

Behavioral rank was decided based on the number of individuals performing each behavioral characteristic. The most active individual was used to assign initial group rank. If less than 40% of individuals were active during the observation period, the final rank was reduced by 0.5. This was done to ensure that the group of ten individuals was not assigned a rank based on one or two active individuals that did not represent the activity of the entire group. If more than 40% of the individuals were active during the observation period, the rank of the most active individual was assigned to the group.

To obtain a pre-disturbance baseline of behavior, we watched each container for an observation period of one minute prior to disturbance. After pre-disturbance behavior

was ranked, we tapped the sides of the container four times in rapid succession to simulate a disturbance. Tadpoles were then observed for an additional period of 20 seconds to determine a post-disturbance behavioral rank. This method was adapted from Martin and Bateson (2007).

Disposal of insecticide and organisms

After experiments were concluded, we froze test organisms and disposed of them by burial according to Michigan's Department of Agriculture guidelines (Michigan, 1982). Treatment solutions were disposed of per suggestion of a BayerCropScienceUS representative. Because of the rapid breakdown of Admire Pro® and relatively low ratio of insecticide to water ratio, the BayerCropScienceUS representative suggested leaving treatment solution outside for two days in direct sunlight to break down before disposing of the solution in a field. The field had to be at least 8 m away from any bodies of water and not in any direct contact with honeybee populations (Admire, 2010).

Data analysis

Data were analyzed using Microsoft Excel and JMP software where appropriate. Statistical significance was set at $\alpha = 0.05$. Statistical significance was determined by one way analysis of variance (ANOVA) and Tukey HSD. In lieu of presenting F ratio and P value results from all ANOVA tests throughout the results section, presence of significant differences among means are shown in figures. All data are presented as means ± standard deviations, unless otherwise noted.

Results

Conditions of water to which tadpoles were exposed varied little over time or among treatments within days. Level of dissolved oxygen in the water (7.05 ± 0.6 mg/L) did not vary among concentrations within a day ($F_{5, 30} = 1.39$, $p = 0.23$) but did vary over time within concentrations ($F_{11, 60} = 16.2$, $p < 0.0001$; Figure 3). There was a general downward trend in dissolved oxygen over time, which contributed to the significant difference within concentrations present in all concentrations. Temperature of the water (22.2 ± 1.8 °C) showed long-term variation over a range of 2 °C, as temperature in the room varied, but daily temperature fluctuations were about 1 °C or less (Figure 4). The pH of the water (7.0 ± 0.5; data not shown) did not change over the course of the experiments. Patterns for both 10-day-old tadpoles and 26-day-old tadpoles for growth, survivorship and behavior were similar in data as well as in significance; therefore results from only 26-day-old tadpoles will be presented throughout the remainder of the report.

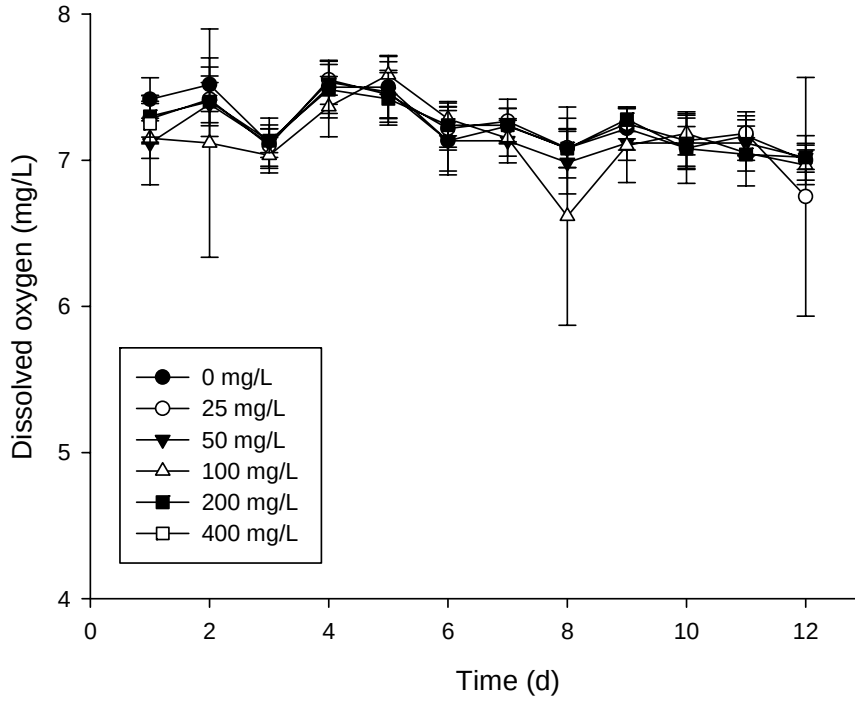

Figure 3. Dissolved oxygen of 26-day-old tadpoles varied little among treatments within days during the long term effect test. There is an overall downward trend in the data from the first day of the experiment to the last day. The trend is present in all concentrations of insecticide. Data presented as mean ± standard deviation.

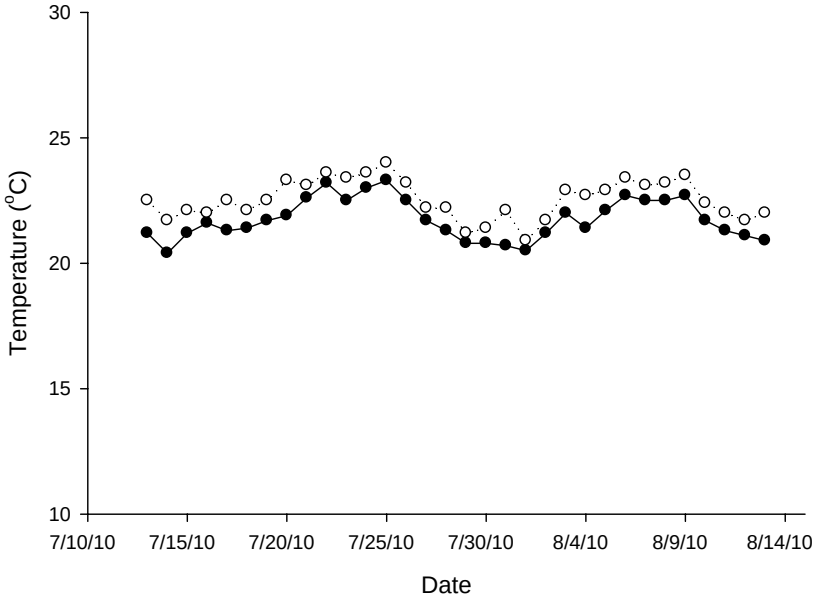

Figure 4. Water temperature during entire span of experiment on both ages of tadpoles varied within ~2 °C of the mean temperature of 22.2 °C. Data presented as maximum (white circles) and minimum (black circles) temperature per day of all containers measured.

Growth and development

Tail length, body length, and body width significantly increased over time during the experiment with 10-day-old tadpoles and 26-day-old tadpoles but did not differ significantly among treatments within a given time stage (pre ATT, post ATT, post LTFX) These trends are illustrated in Figures 5,6 and 7. No changes in morphology (i.e. regression of gills, appearance of limb buds) took place during the experiment in any treatment.

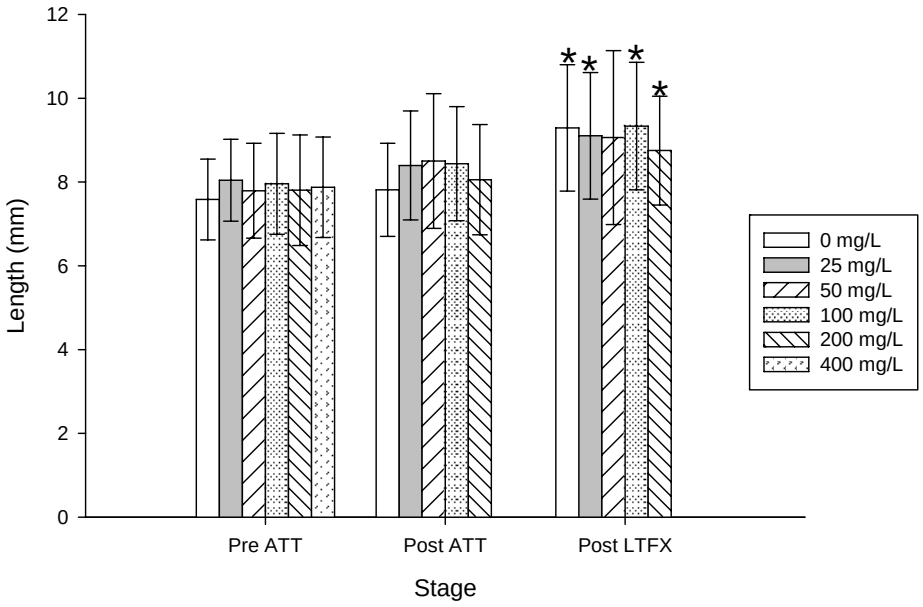

Figure 5. Tail length measurements (base of body to tip of tail) of 26-day-old tadpoles significantly increased over each time stage of the experiment, but did not vary among treatments during different time stages of the experiment. Pre ATT stage measurements were taken just before the ATT stage started; Post ATT was taken four days later between the ATT and LTFX stages; and Post LTFX was taken eight days later after the LTFX stage ended. Asterisks represent significant differences in growth over time in the overall ANOVA. With the exception of 50 mg/L treatment, tadpoles in all other treatments demonstrated significant growth in the Post LTFX stage. Data shown as mean ± standard deviation.

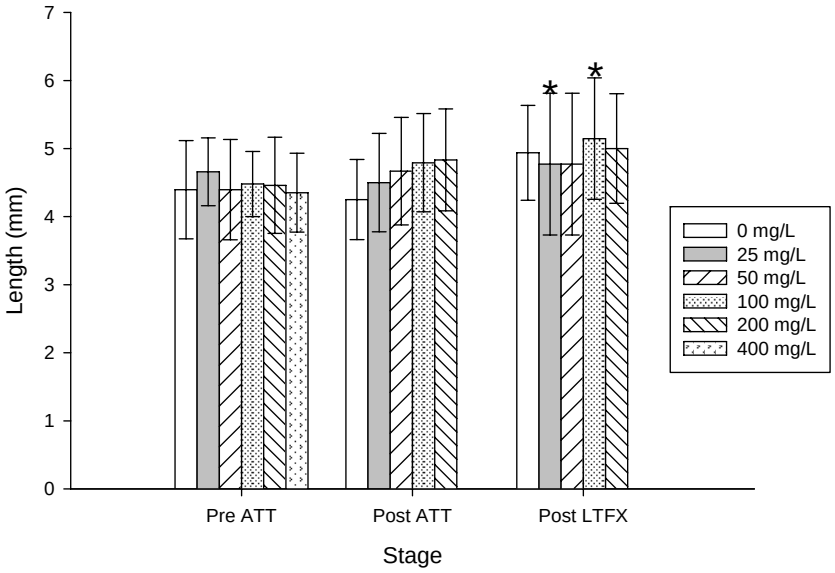

Figure 6. Body length measurements (base of body to snout) of 26-day-old tadpoles increased over each stage of the experiment, but did not vary between treatments during the experiment. Pre ATT stage measurements were taken before the ATT experiment started; Post ATT was taken four days later between the ATT and LTFX experiment; and Post LTFX was taken eight days later after the LTFX experiment ended. Asterisks represent significant differences in growth over time. Tadpoles in 25 mg/L and 100 mg/L demonstrated significant growth in the Post LTFX stage. Data shown as mean ± standard deviation.

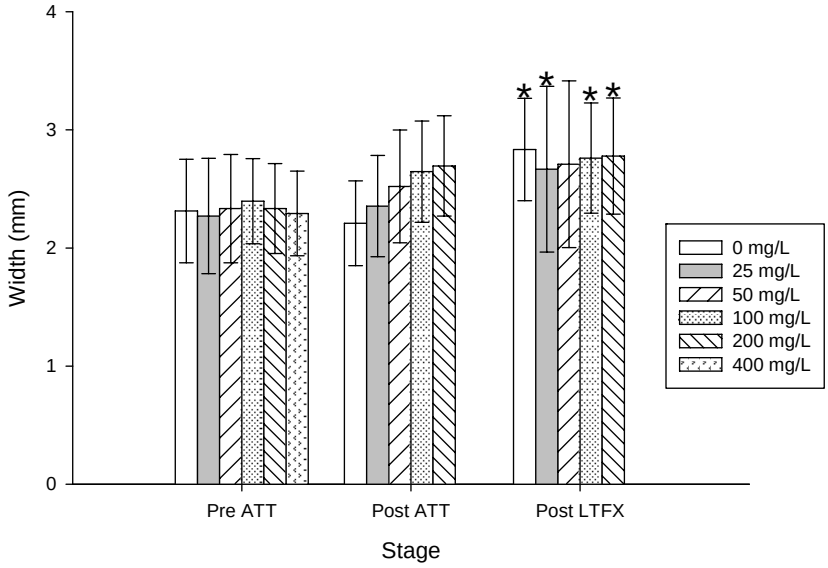

Figure 7. Body width measurements of 26-day-old tadpoles increased over each stage of the experiment, but did not vary between treatments during the experiment. Pre ATT stage measurements were taken before the ATT experiment started; Post ATT was taken four days later between the ATT and LTFX experiment; and Post LTFX was taken eight days later after the LTFX experiment ended. Asterisks represent significant differences in growth over time. With the exception of 50 mg/L treatment, tadpoles in all other treatments demonstrated significant growth in the Post LTFX stage compared to the other two stages. Data shown as mean ± standard deviation.

Acute toxicity tests (ATT)

Survivorship of both 10-day and 26-day-old tadpoles varied among treatments (Figure 8). All individuals exposed to the 400 mg/L treatment died after <12 h of exposure. Half of the individuals exposed to the 200 mg/L treatment also died, but did so gradually. Survivorship of tadpoles exposed to other treatments (i.e. 25, 50 and 100 mg/L) did not differ from that of tadpoles in tap water.

Long-term effects test (LTFX)

Overall survivorship of tadpoles did not change from day 1-8 of the LTFX stage (Figure 9). There was a significant difference in survivorship of both 10-day-old tadpoles and 26-day-old tadpoles when transferred from treatments of imidacloprid to tap water for an additional eight days for the long term effects test, but this occurred because of variation in number of individuals at the end of the ATT and onset of the LTFX test. This number persisted for the entire eight-day LTFX test. No additional significant deaths occurred after tadpoles were removed from treatment solutions and placed in tap water.

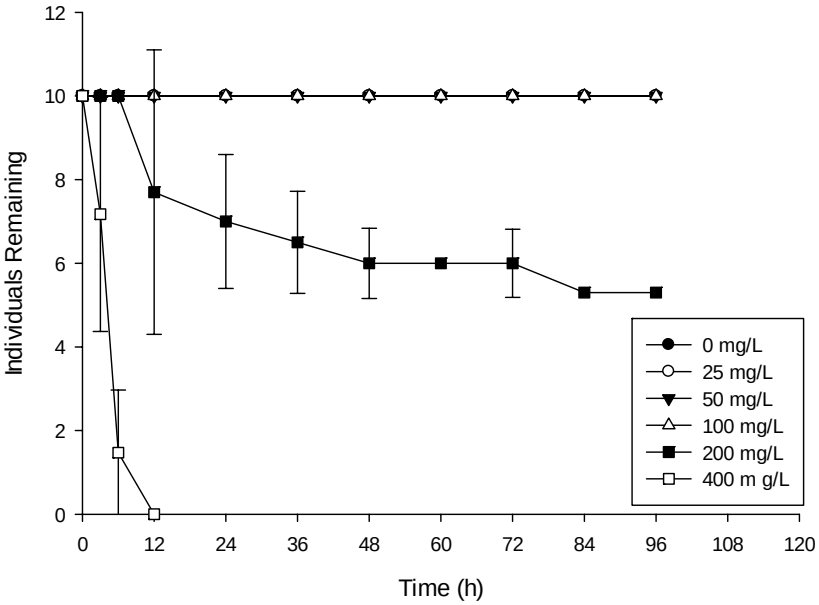

Figure 8. Survivorship of 26-day-old tadpoles during the acute toxicity test varied among treatments. Individuals in the 400 mg/L concentration died after < 12 hours, while almost half of the individuals in the 200 mg/L concentration died at the end of the 96 h test. Additional treatments had no significant deaths during the ATT. Data presented as mean ± standard deviation.

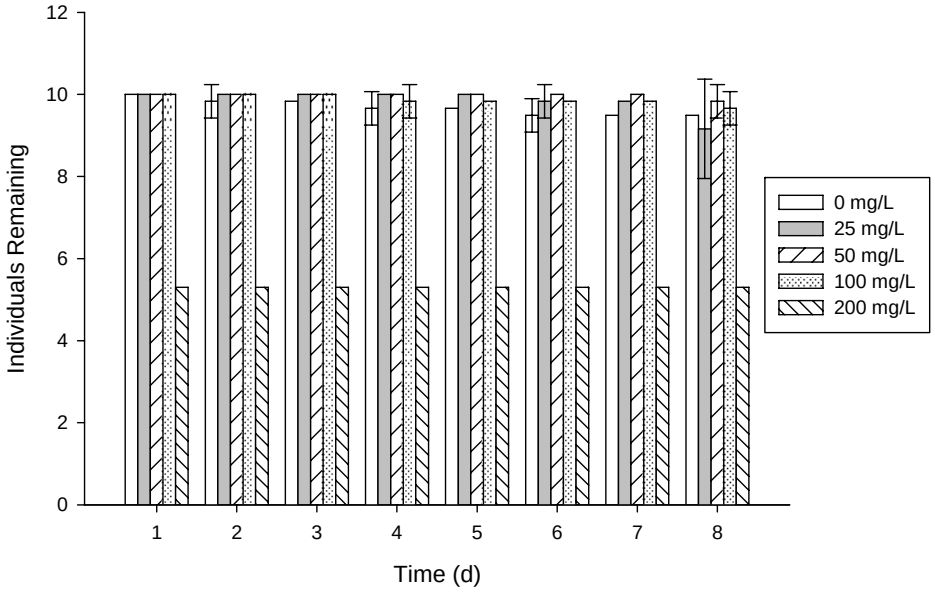

Figure 9. Survivorship of 26-day-old during the long term effects test tadpoles did not vary over time within treatments but did vary among treatments at each time. The 200 mg/L treatment started off with a different number of individuals due to deaths during the ATT; however no additional deaths during the LTFX occurred. The same pattern is present for all other concentrations. Data presented as mean ± standard deviation.

Behavioral tests: Response to disturbance

Acute toxicity test (ATT)

Tadpoles in different treatment concentrations differed in behavioral ranks in the pre-disturbance test periods, and pre-disturbance behavioral rank decreased over the four-day period in several groups (Figure 10). Behavior ranks of both the 10 and 26-day-old tadpoles decreased pre-disturbance over the four-day exposure during the acute toxicity test. There was a significant decrease in pre-disturbance behavior in both the 0 and 25 mg/L over time ($F_{3, 20} = 9.7$, $p < 0.004$; $F_{3, 20} = 17$, $p < 0.0001$, respectively) (Figure 10A). By day three, no change in rank occurred over time. Additionally, there was a significant difference in pre-disturbance behavior among all treatments on both day one ($F_{4, 25} = 3.40$, $p < 0.02$) and day two ($F_{4, 25} = 7.05$, $p < 0.0006$) (Figure 10B). On day one, individuals contained in the 200 mg/L concentration had a significantly lower behavioral rank. On day two, individuals contained in both 200 mg/L and 100 mg/L containers had a significantly lower behavioral rank. By day three, no change in rank occurred over time.

The post-disturbance behavioral rank of tadpoles was greater than that of the pre-disturbance behavioral rank, and this behavior varied significantly over time and among concentrations within a day (Figure 11). The post-disturbance behavioral rank of tadpoles in both the 100 and 200 mg/L containers significantly decreased over time (Figure 11A). Individuals contained in the 100 mg/L containers had significantly different behavior post-disturbance from day one to day four. Individuals contained in the 200 mg/L concentration had a significantly lower post-disturbance rank on days two, three and four when compared to the post-disturbance behavioral rank of day one. In

addition, the 200 mg/L behavioral rank was significantly different among all other concentrations within a day over all four days (Figure 11B).

Once disturbed, tadpoles demonstrated a significant increase in behavioral rank in all concentrations. There were significant differences in both the 25 mg/L concentration and the 200 mg/L concentration over time (Figure 12A). In the 25 mg/L concentration, behavioral rank difference on days one and two were significantly different than behavioral rank difference on days three and four. Furthermore, the change in behavioral ranking also showed significant differences among concentrations within a day (Figure 12B). The difference in behavioral rank was significantly lower in the 200 mg/L concentration at least one other concentration of insecticide, and this difference is seen on days two, three and four.

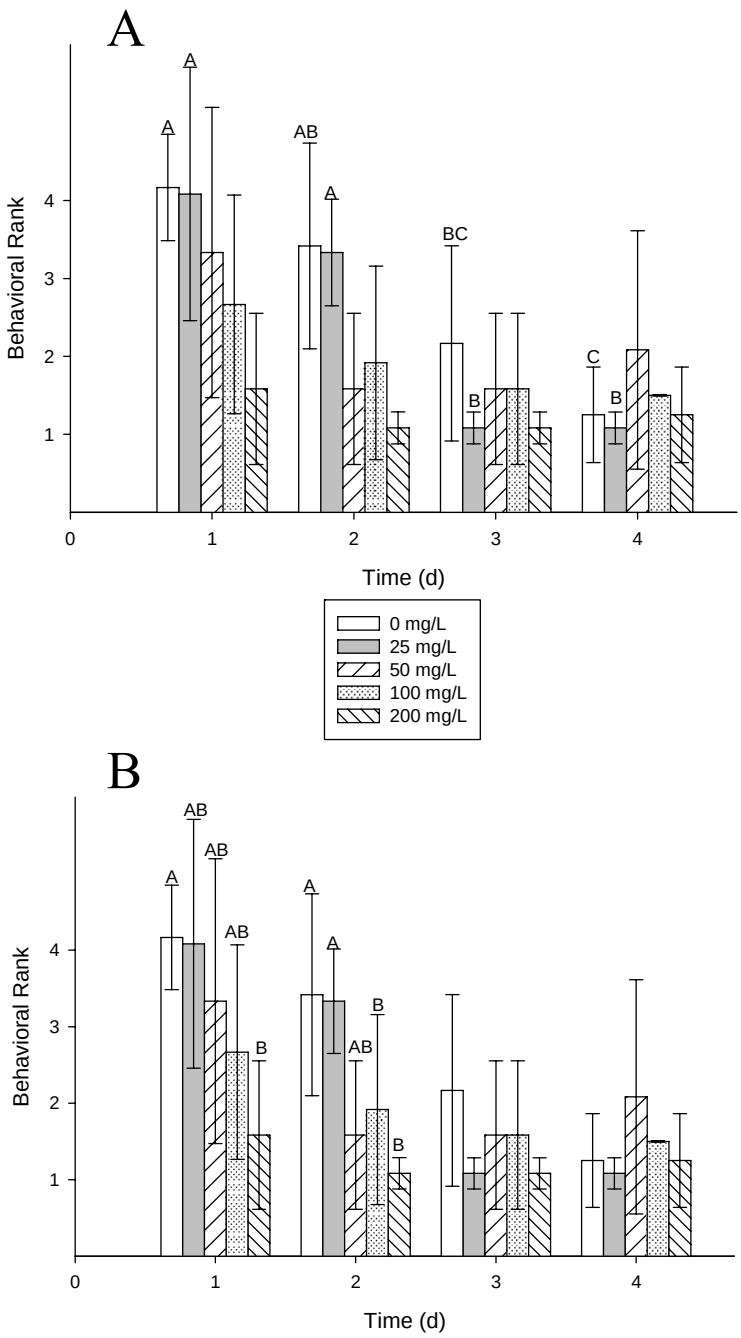

Figure 10. Pre-disturbance behavioral rankings of 26-day-old tadpoles during the acute toxicity test. Pre-disturbance behavioral ranking decreased over the four-day period in all treatments, and by day three no change in rank occurred over time (A). Behavior also decreased among treatments within a day for both day one and day two (B). Dissimilar letters denote significant differences within a concentration over time (A) and among concentrations in a day (B). Data shown as mean ± standard deviation.

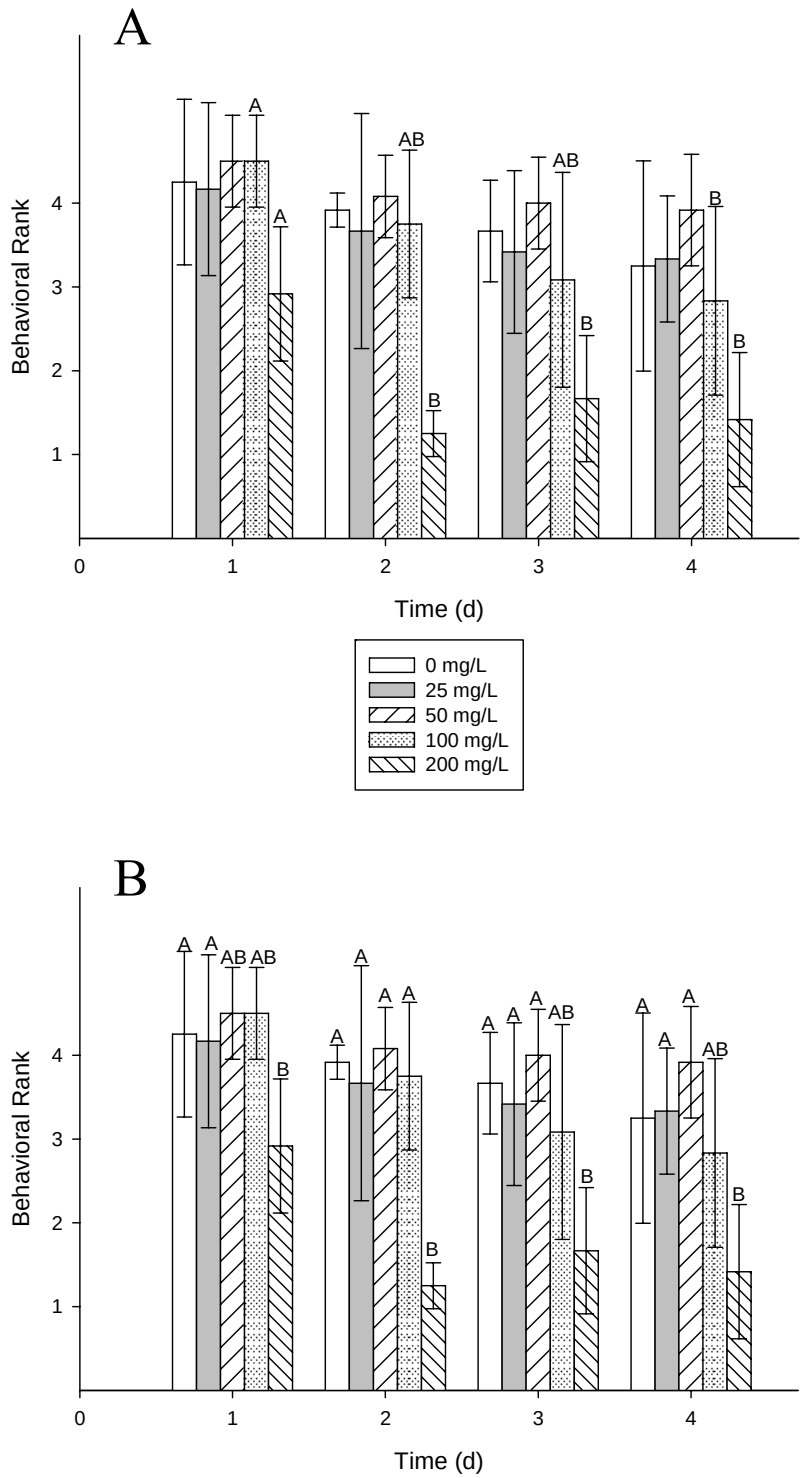

Figure 11. Post-disturbance behavioral rankings of 26-day-old tadpoles during the acute toxicity test. Post-disturbance behavioral ranking was greater than that of pre-disturbance behavioral ranking, but varied among treatments. There were significant differences in both the 100 mg/L and 200 mg/L concentrations over time (A). There were also significant differences with individuals contained in the 200 mg/L among all other concentrations within a day over all four days (B). Dissimilar letters denote significant differences over time (A) and within concentrations in a day (B).

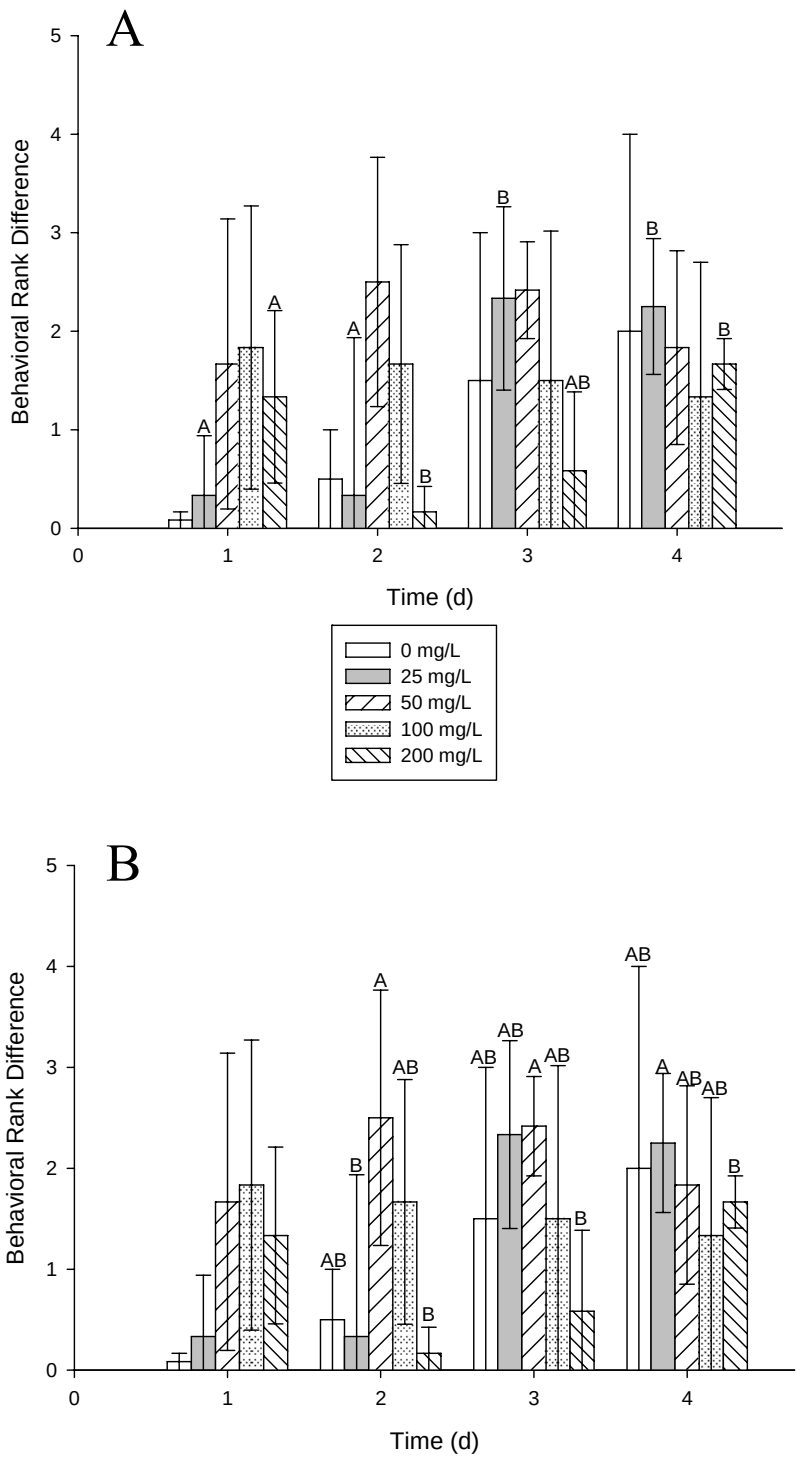

Figure 12. Behavioral rank difference between post-disturbance and pre-disturbance rankings of the 26-day-old tadpoles during the acute toxicity test. Significant differences appear in the 25 mg/L concentration as well as the 200 mg/L concentration over time (A). Significant differences also occur within concentrations in a day over all four days (B). Dissimilar letters denote significant differences over time (A) and within concentrations in a day (B).

Long Term Effects Test (LTFX)

Behavioral rank of tadpoles changed little after tadpoles were transferred to clean tap water for the long-term effects study (data not shown). Pre-disturbance behavioral ranks of 26-day-old tadpoles did not vary over time or among treatments within days. Although the behavioral rank increased post-disturbance for all groups, revealing that tadpoles in all groups responded to disturbance, no clear trends in responsiveness were found among treatments on any given day, nor of one treatment throughout the entire eight-day test. The difference between pre- and post-disturbance behavioral ranking did not vary among treatments over time, nor among treatments on any given day.

Discussion

Ambient conditions (i.e. dissolved oxygen, temperature and pH) did not vary in the experiment. Although dissolved oxygen decreased over the course of the experiment, the overall downward trend was present in all concentrations (Figure 3). Temperature varied little (22.2 ± 2 °C) over the course of the experiment (Figure 4). In addition, pH did not change. Because ambient conditions did not vary during the experiment, we can conclude that imidacloprid concentration is the only variable that affected tadpoles.

Growth of tadpoles was unaffected by amount of imidacloprid present in treatment solution. Although tadpoles did grow significantly from the beginning of the experiment to the end, growth was not significantly different among concentrations within a stage. This evidence leads us to reject our hypothesis that imidacloprid stunts growth. Significant differences in both pre- and post-disturbance ranking over time and among concentrations of insecticide within a day occurred during the acute toxicity test.

Contrary to growth, survivorship of green frogs was significantly reduced with exposure to both the 400 mg/L and 200 mg/L concentrations of insecticide. These data are contrary to our assumptions that as imidacloprid concentration increased, survivorship would decrease. Instead, imidacloprid gave a threshold effect, only affecting two of the concentrations while the remaining three concentrations had the same survivorship as tap water. We also discovered that once tadpoles were removed from imidacloprid, no additional tadpoles died, meaning that tadpoles were indeed dying due to imidacloprid exposure and its effects were reversible.

On the other hand, significant differences were not found in any pre- or post-disturbance behavioral rankings during the long-term effects study. This suggests that

tadpole behavior was indeed affected by imidacloprid, but these affects were reversible after being transferred to tap water. Our hypothesis, which stated that tadpoles would become more sluggish and unresponsive, was correct.

Growth and development

Growth of tail and body did not vary among treatments, indicating growth was not affected by imidacloprid. Based on these data, we can conclude that imidacloprid did not have a significant measurable effect on growth during the period of exposure. Since this experiment focused on acute exposure and followed the standard 96 h length of experiment, it is possible that tadpoles exposed long-term would have significant differences in growth. Also, due to the fact that no changes in morphology took place over the course of both the ATT and LTFX test, a time period of 12 days, it is possible that exposing tadpoles long-term to imidacloprid would cause developmental and morphological differences among treatments. In other longer studies, growth of organisms was stunted as a result of pesticide use (Webb and Crain, 2006, Brodman et al., 2010).

Acute toxicity test (ATT)

Tadpole survivorship was affected at the two highest concentrations of imidacloprid, but was not affected at lower levels of exposure, indicating imidacloprid caused the death of tadpoles in the highest concentrations. Survivorship patterns were similar across both ages of tadpoles, indicating that age was not a factor of survivorship effects of imidacloprid on tadpoles. All tadpoles exposed to the 400 mg/L concentration died within twelve hours of exposure to the insecticide, whereas approximately half of tadpoles contained in the 200 mg/L concentration died by the end of the four-day ATT

study (Figure 8). Because of its status as a neonicotinoid, imidacloprid targets the nervous system to kill insects. It is possible that tadpole death was caused by paralysis of the nerves controlling breathing. If imidacloprid bound to the acetylcholine receptor but was not broken down by cholinesterase, the nerves would be continuously stimulated resulting in paralysis. If paralysis occurred in the gills of the tadpole, they would then die due to asphyxiation. These results are contrary to what we expected, because we thought survivorship would me much more gradual, with the most tadpoles dying at the 400 mg/L concentration, but also a few dying at the 25 mg/L concentration. Based on this change in assumption, water solutions may have to contain a specific amount of imidacloprid for any deaths to occur. If a specific amount of imidacloprid were not needed, we would have seen more the number of deaths gradually increasing from the 25 mg/L concentration to the 100 mg/L concentration. This suggests that the lethal dose for tadpoles of the green frog is between 100 mg/L and 200 mg/L. This acute toxicity concentration is close to other literature values that have presented an LC50-48h value of 218.8 mg/L for *R. nigromaculata* (Quan et al., 2006), 219 mg/L for *R. nigromaculata* (Feng et al., 2004) and 165 mg/L for *R. limnocharis* (Feng et al., 2004).

Long-term effects test (LTFX)

After being transferred into tap water, no additional tadpoles died (Figure 9). Although survivorship (or starting number) of tadpoles was statistically significant both at the beginning and the end of the LTFX test, these differences persisted throughout the experiment. The difference in number of individuals at the beginning of the LTFX test occurred because of deaths during the acute toxicity test. From these observations, we can conclude the cause of death was indeed exposure to imidacloprid, and there was no

additional measurable effect on the tadpoles in the eight-day long-term effects tests. It is possible that effects of imidacloprid are reversible, and will stop after exposure to imidacloprid ceases.

Behavioral tests: Response to disturbance

Acute toxicity test (ATT)

Tadpoles in different treatment concentrations differed in both pre-disturbance (Figure 10) and post-disturbance (Figure 11) behavioral rankings. Tadpoles in the two lowest concentrations varied over time (Figure 10A). This difference results from the acclimation of the tadpoles to a new environment. Therefore, after the tadpoles fully acclimated on day three, we see no significant difference. Additionally, there was a significant difference in pre-disturbance behavior among all treatments on both day one and day two (Figure 10B). This difference, causing individuals in the 200 mg/L concentration (day one and day two) and 100 mg/L concentration (day two) to be significantly different from the other concentrations results from the tadpoles becoming more sluggish as a result of the imidacloprid. The sluggish nature of tadpoles reflects the lower pre-disturbance rank not present in lower concentrations of imidacloprid. In a study by Nauen (1995), he indicated that behavioral effects affected *Mysus persicae* with low concentrations of imidacloprid. Releya et al. (2010) found similar results.

In comparison to pre-disturbance ranking, post-disturbance behavioral ranking was greater. Post-disturbance behavioral rank of tadpoles in both the 100 and 200 mg/L containers were significantly different over time (Figure 11A). This explains that the insecticide's effects on tadpoles increase over time, causing their responsiveness to decrease. Furthermore, tadpoles in the 100 mg/L concentration were affected more

slowly (day four) than tadpoles in the 200 mg/L concentration (day two), implying that higher levels of insecticide take less time to show significant behavioral differences.

The sluggish and unresponsiveness of tadpoles in the 200 mg/L concentration is also supported when comparing treatments within a day (Figure 11B). During all four days of the ATT, individuals contained in the 200 mg/L concentration had a significantly lower response to the disturbance than the other treatments. This again supports the theory that imidacloprid behaviorally affected tadpoles because tadpoles contained in higher concentrations are more sluggish before a disturbance, and also less responsive after a disturbance. In previous studies with other insecticides, tadpoles displayed bent tales and unusual swimming behavior (Webb and Crain, 2006).

However, based on the data, even tadpoles in low levels of insecticide were less excitable at the end of the acute toxicity test than tadpoles in containers without insecticide, as demonstrated in the difference between pre-disturbance and post-disturbance behavioral ranks (Figure 12). This suggests that, while not lethal, imidacloprid was having an effect on tadpoles even at lower concentrations. We suspect that imidacloprid can have effects on prolonged exposure on survivorship. All tadpoles responded positively to a disturbance, the difference in behavioral rank from the tadpoles in the 200 mg/L concentration was much lower than the other concentrations. The calming effect during the span of the four-day ATT explains why no significant data was recorded on day three and day four.

Behavioral observations are most likely caused by the lack of breakdown of neonicotinoids in tadpoles during continuous exposure. Neonicotinoids are not broken down by cholinesterase, and as a result continuously stimulate the nerve, causing

paralysis and even death. Looking at the data, we can conclude that partial or full paralysis of the tadpoles occurred at concentrations of 200 mg/L of imidacloprid and can account for their lack of an increased response to a disturbance. We can also conclude that death occurred in tadpoles contained in both the 200 mg/L and 400 mg/L containers because of the same continuous stimulation of the nerve.

Moreover, once tadpoles were transferred to tap water for the LTFX test, behavioral rank of tadpoles in all concentrations changed little. Pre- and post-disturbance behavioral ranks did not vary over time or among treatments. Also, there was no significant difference in behavioral rank among treatments or over time. Much like the survivorship data, tadpoles seem to recover after being transferred to tap water. We know this because the pre- and post-disturbance behavioral rank of all surviving tadpoles was the same, regardless of what treatment tadpoles were in for the ATT.

Based on our results, it is possible that the effects of imidacloprid are temporary. In a previous study on aphids, insects seemed to improve behavior and overall function after stopping exposure to plants treated with imidacloprid (Nauen, 1995). It is also possible that the tadpoles were able to metabolize the imidacloprid absorbed in their systems and break down the majority of the compound in a small amount of time. Flies, for example can metabolize imidacloprid in as little as 24 hours (Nishiwaki, 2004). In another study, Nishiwaki et al. (2004) showed that houseflies could eliminate 20% of the compound in 24 hours. These studies are consistent with the results of the LTFX survivorship, which indicated that few tadpoles died after being transferred to tap water.

Because of the standard protocol followed, this experiment took place in a laboratory with controlled conditions to determine a concentration range of insecticide

that is safe for green frog tadpoles and other amphibians. Our goal was to measure the acute effects of imidacloprid. In our experiment, treatment solutions containing imidacloprid were changed daily, but it is unlikely that such conditions will ever be observed in a natural wetland ecosystem, such as Hyla House Pond. There are very few scenarios where an acute exposure, such as the exposure in this study, would ever occur in a natural environment. Moreover, because the insecticide is designed to break down quickly by photolysis under natural light conditions, insecticide levels would decline to safe levels for tadpoles in a matter of hours. Since imidacloprid has a rapid half-life of 45 minutes, even if an acute concentration of 400 mg/L of insecticide were accidentally introduced into a wetland ecosystem, levels would be safe in less than two hours. Tadpoles would only be exposed to unsafe levels for a short amount of time, and effects of the insecticide would probably not significantly affect growth, survivorship or behavior.

A more likely case of exposure of imidacloprid in a natural environment comes from an insecticide like AdmirePro® itself, which is sprayed on leaves. Because AdmirePro® is a systemic insecticide, it is sprayed directly onto the leaves immediate soil surrounding a plant to protect the plant from insects. Warning labels for AdmirePro® prohibit its use 24 h before a rainstorm, and also prohibit its use closer than 25 yards from a stream, pond, lake or any other body of water. The most likely way for imidacloprid to be introduced in such a habitat is through runoff after a heavy rainfall. Even if a heavy rainfall did occur, the possibility of a lethal concentration (or of a concentration that would cause growth or behavioral effects) of imidacloprid reaching a pond is minimal (Anatra-Cordone, 2005; Kollman and Segawa, 1995).

Based on these data, imidacloprid, appears to be a safe insecticide in so far as it does not harm non-target species if used at appropriate dosages as directed by the manufacturer. Imidacloprid, as a representative of the class of neonicotinoids, illustrates that this class is indeed a safer alternative to other classes of insecticides such as pyrethroids. Future studies could measure the effects of imidacloprid on frog eggs and adult reproduction. Although much is already known about imidacloprid, other neonicotinoids and other classes of insecticides, there is still always more to discover and investigate, with the goal of minimizing anthropogenic effects on not only ecosystems, but also future generations.

Literature Cited

"Admire Pro Systemic Protectant: Product Information: BayerCropScienceUS" *BayerCropScience: Crop Protection, Pest Control Products.* 15 Jan 2010.

Anatra-Cordone, M. and P. Durkin. 2005. Immidacloprid—Human Health and Ecological Risk Assessment—Final Report. Forest Health Protection. USDA, Forest Service.

Barbee, G. C. and M. J. Stout. 2009. Comparative acute toxicity of neonicotinoid and pyrethroid insecticides to non-target crayfish (*Procambarus clarkii)* associated with rice-crayfish crop rotations. *Pest Management Science*. 65:1250-256.

Bonmatin, J. M., I. Moineau, R. Charvet, M. E. Colin, C. Fleche, E. R. Bengsch. Behaviour of Imidacloprid in Fields. *Toxicity for Honey Bees.* 44.

Brodman, W., W.D. Newman, K. Laurie, S. Osterfeld and N. Lenzo. 2010. Interaction of an Aquatic Herbicide and Predatory Salamander Density on Wetland Communities. *Journal of Herpetology.* 44(1):69-82.

"Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)" 1972. United States Environmental Protection Agency.

Feng, S., Z. Kong, X. Wang, L. Zhao, P. Peng. 2004. Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell.* 56(5): 457-63.

Johnson, R.M. Z. Wen, M.A. Schuler, and M. R. Berenbaum. 2006. Mediation of Pyrethroid Insectidide Toxicity to Honey Bees (Hymenoptera: Apidae) by Cytochrome P450 Monooxygenases. *Journal of Economic Entomology.* 99(4):1046-50.

Jones, D.K., J.I. Hammond, and R.A. Relyea. 2009. Very highly toxic effects of endosulfan across nine species of tadpoles: Lag effects and family-level sensitivity. *Environ. Toxicol. Chem.* 28:1939-1945

Kollman and Segawa. 1995. DPR Pest Chem Database. Department of Pesticide Regulation.

Lannoo, M. 2005. *Amphibian Declines: The Conservation Status of United State Species*. New York: University of California.

Mannion, C.M., H. E. Winkler, D.I. Shapiro, and T. Gibb. 2000. Interaction Between Halofenozide and the Entomopathogenic Nematode *Heterohabditis marelatus for* Control of Japanese Beetle (Coleoptera: Scarabaeidae) Larvae. *Journal of Economic Entomology.* 93(1):48-53.

Martin, P. and P. Bateson. 2007. *Measuring Behavior—An Introductory Guide.* Cambridge: Cambridge University Press.

Massaro, E. J. 2002. Handbook of Neourtoxicology. New Jersey: Humana Press Inc.

Matsuda, K., S. D. Buckingham, D. Kleier, J. J. Rauh, M. Grauso and D. B. Sattelle. 2001. Neonicotinoids: insecticides acting Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci.* Nov; 22(11):573–580.

"Michigan Legislature-Act 239 of 1982." *Michigan Legislature – Home.* 30 Sept. 2010.

Moza, P.N., K. Hustert, E. Feicht, and A. Kettrup. 1998. Photolysis of imidacloprid in aqueous solution. *Chemosphere,* 36(3), 497-502.

Nauen, R. 1995. Behaviour modifying effects of low systemic concentrations of imidacloprid on *Mysus persicae* with special reference to an antifeeding response. 44(2): 145-53.

Nishiwaki, N., K. Sato, Y. Nakagawa, M. Miyashita, and H. Miyagawa. 2004. Metabolism of Imidacloprid in Houseflies. *J. Pestic. Sci.* 29:110-6.

Nishiwaki, N. 2004. Studies on the Structure-Activity Relationship and Mode of Actions of Neonicotinoid Insecticides. *J Pestic. Sci.* 29:222-3.

Oldroyd, B. P. 2007. What's Killing American Honey Bees? *PLoS Biol* 5(6):1195-9.

Peveling, R. 2001. Environmental conservation and locust control—possible conflicts and solutions. *Journal of Orthoptera Research.* 10(2):171-187.

Quan, L., S. Feng, Y. Yun, C. Shulan, L. Longfeng and X. Haijuan. 2006. Toxicity of a novel pesticide, imidacloprid, on tadpoles and frogs of Rana nirgmoaculata Hallowell. *Nanhua daxue Xuebau, Yixueban.* 34(2): 181-4.

Relyea, R.A., and K. Edwards. 2010. What doesn't kill you makes you sluggish: How sublethal pesticides alter predator-prey interactions. *Copeia*: 558-567

Relyea, R.A. 2009. A cocktail of contaminants: How pesticide mixtures at low concentrations affect aquatic communities. *Oecologia* 159:363-376

Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. E 729-96. 2007. Pennsylvania: ASTM International.

Tomizawa, M.J. and J. E. Casida. 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* 45: 247-68.

Webb, C.M. and D.A. Crain. 2006. Effects of ecologically relevant doses of malathion on developing *Xenopus laevis* tadpoles. ***BIOS.*** 77(1):1-6

Wilson, L. D. and J. McCranie. 2003. Herpetofaunal indicatior species as measures of environmental stability in Honduras. ***Caribb. J. Sci.*** 39.1:50-67.

Yamamoto, I., G. Yabuta, M. Tomizawa, T. Saito, T. Miyamoto, S. Kagabu. 1995. ***J Pesticide Sci.*** 20:33-40.

Yan, D., X. Jiang, S. Xu, L. Wang, Y. Bian, and G. Yu. 2008. Quantitative structure-toxicity relationship study of lethal concentration to tadpole (***Bufo vulgaris formosus)*** for organophosphorous pesticides. ***Chemosphere*** 71:1809-15.