ABSTRACT

Over the last few decades amphibian populations have been in decline. This decline is the result of numerous factors such as pathogens, introduction of non-native species, habitat destruction, and contaminants. Contaminants, particularly pesticides, are responsible for a large proportion of this decline. Some pesticides can have lethal effects while others cause sub-lethal effects, such as reduced performance with foraging, maternal behavior, escape from predators, hunting, and swimming ability, that are difficult to measure. Overall these exposures could result in the damage of an entire ecosystem. My experiment tested the effects of pesticide exposure on swimming ability of Northern Green Frog tadpoles (*Rana clamitans melanota*). Tadpoles were exposed to four different concentrations (0.0, 3.5, 5.0, and 7.5 mg/L) of the pesticide carbaryl. A different set of six tadpoles were placed in plastic containers for each concentration and exposed for 1, 24, and 48 hours. After each timed exposure all tadpoles were then placed in a track and prodded. Distance, time, and speed were calculated. Swimming speed data was then analyzed using a two-way ANOVA to determine if the pesticides had a significant effect. The analysis indicated that for each concentration, after 1 and 24 hours, there was a significant decrease in tadpole swimming speed. The 48 hour exposure time, for all concentrations, showed a decrease in swim speed; however, the times were not significantly different from the control tests. Overall this study shows that a small amount of contaminant can have a significant effect on swimming speed of Northern Green Frog tadpoles and lead to more studies about ecosystem-level consequences.

INTRODUCTION

Amphibian populations are experiencing a high rate of decline around the world (Blaustein et al., 2003). Currently more than 500 species of amphibians are in decline and many others are listed as special conservation concern. The sudden decline in populations is the result of numerous factors such as pathogens, introduced non-native species, contaminants, habitat destruction, and global environmental changes. More specifically a wide array of contaminants is contributing to this decline, including pesticides, herbicides, fungicides, fertilizers, and numerous other pollutants. Amphibians are especially susceptible to contaminants because of their moist and permeable skin, which allows them to breathe in water and intake toxic substances (Blaustein et al., 2003). One particular kind of contaminant is pesticides and they play a major role in the decline of the amphibian populations.

Pesticides are used to control undesirable organisms in terrestrial and aquatic habitats. These chemicals are designed to kill a select target group of organisms; however, the effect on other organisms that are not targeted is hard to predict (Relyea and Edwards, 2009). Although amphibians are usually not the target of pesticides, they can reach these animals by several means (Greulich and Pflugmacher, 2004). Habitats near agricultural areas that use pesticides are particularly vulnerable to pesticide exposure from runoff (Greulich and Pflugmacher, 2004). Some of these pesticides can have lethal and sub-lethal effects on amphibians. The lethal effects are easy to understand because they show that certain pesticides and concentrations can kill the amphibians. That provides data that can be automatically seen and provide proof that there is a problem. What are harder to test and determine are the sub-lethal effects of these pesticides. Sub-lethal effects are important because they can seriously affect an entire ecosystem and create problems that cannot be seen until it is too late. Sub-lethal effects can cause indirect mortality by

altering behaviors such as swimming, feeding, and/or predator avoidance (Bridges, 1999). These effects can also include reduced performance with motor tasks, foraging activities, maternal behavior, capacity to escape from predators, and diminished learning and memory processes (Punzo, 2005).

One important effect of sub-lethal doses of pesticides is the effect they have on swimming speed and distance. Both swimming performance and spontaneous swimming activity are important for carrying out vital life functions such as growth and development (Bridges, 1997). This lack of speed and endurance also causes a decline in both predator evasion and ability to catch prey. In turn, this could cause an unbalanced ecosystem. Amphibians, both predator and prey, use a variety of cues and methods to attack or escape. For example chemical signals can be picked up by the prey and warn them if a predator approaches. However the main way to catch prey and escape predators is visual cues. A predator needs to be seen in order for prey to start its escape and prey needs to be seen in order for the predator to attack. Rick Relyea and Kerry Edwards studied tadpoles and newts and found that after exposure to certain concentrations of pesticides (0.1 and 1.0 mg/L of carbaryl and malathion) tadpole and activity was lowered. When 0.1 mg/L of carbaryl and malathion were added to the newts' habitat they experienced little to no effect (Relyea and Edwards, 2009). This study concluded that low levels of pesticide exposure caused tadpoles to be unable to escape from the unaffected newts. Another study at the University of Missouri exposed both tadpoles (prey) and newts (predators) to different concentrations of the pesticide carbaryl and found that activity for both species was severely decreased (Bridges, 1999). Different concentrations can have different effects on predators and their prey. Because of a tadpoles' small size, tadpoles will be affected sooner by the pesticides, resulting in a decreased swimming ability, causing them to be more susceptible to

predators. Once the predators are exposed to a higher concentration of pesticides they too will have a decreased swimming ability and find it difficult to catch prey. If the predators and prey are exposed simultaneously to the pesticide their predator-prey relationship remains the same; however if one is exposed sooner than the other it can disrupt the predator prey dynamics (Bridges, 1999).

Recovery time is also another significant factor in predator-prey dynamics. If the prey recovers first, predators will not be able to eat as much and if predators recover first, more prey will be eaten. A study done by Hopkins et al.(2004) showed the effects of swimming performance of two natricine snakes exposed to a cholinesterase-inhibiting pesticide, like carbaryl. They found that exposure to concentrations of 2.5 and 5.0 mg/L of carbaryl caused great reduction in swimming ability. They also noted that normal recovery time is about 96 hours, but the recovery time with their snakes was significantly slower, if full potential returned at all (Hopkins et al. 2004). A study done by Pamela Widder and Joseph Bidwell, from the Virginia Polytechnic Institute and State University, also showed that swim speed of frogs can be affected. They showed that another pesticide called chlorpyrifos can also inhibit swim speed and size and those pesticides can affect many species of frogs (Widder and Bidwell, 2008). They used environmentally realistic levels of the organophosphate pesticide (1, 10, 100, and 200 ug/L) and exposed it to four species of frogs for four days. Overall they found that the pesticide had an effect on swimming speed but that the effect differed between the species (Widder and Bidwell, 2008). Based on the previous studies stated above, it has been shown that pesticides can have a negative effect on aquatic ecosystems, particularly predator-prey interactions.

The goal of my study is to test the hypothesis that the pesticide carbaryl will have a negative effect on swimming speed of Northern Green Frog tadpoles (*Rana clamitans melanota),*

which in turn could result in a negative effect of the ecosystem as a whole.

Northern Green Frog tadpoles were chosen because they are native to North America, specifically Pennsylvania. They are found in permanent aquatic habitats such as springs, swamps, streams, ponds, and lakes. Tadpoles are dark brown or gray with some darker spots and it can take them anywhere from 3-22 months to begin metamorphosis, which is usually done in the winter. The tadpoles can eat the plant matter in water and have numerous predators, including aquatic insects, fish, birds, and salamanders (Rosamond Gifford Zoo, 2008).

Carbaryl (1-naphthyl-N methylcarbamate) is a broad-spectrum insecticide used to control insects and the United States currently uses about 2500-3500 metric tons per year (Punzo, 2005). Field concentrations of up to 4.8 mg/L have been reported immediately after application and may persist for over a year in certain ecosystems (Punzo, 2005). It has a widespread use and is applied to numerous habitats, including croplands, rangelands, forests, wetlands, oceans, and sewage treatment plants (Blaustein et al., 2003). Carbamates inhibit acetyl cholinesterase activity in the central nervous system causing paralysis and death at higher concentrations (Punzo, 2005). At sub-lethal concentrations it causes decreases in tadpole swimming activity and performance and slow growth and development, which in turn may have an adverse effect on adult fitness (Bridges, 1997). Another study also found that carbaryl had an interesting effect on metamorphosis. One and multiple exposures of carbaryl to the Green Frog, *Rana clamitans*, tadpoles were tested (Boone and Bridges, 2003). The results indicated that multiple exposures affected a population more than a single exposure but even a single exposure at different times in development can increase the rate of metamorphosis (Boone and Bridges, 2003). While they are unsure if increased rate of metamorphosis will be positive or negative, the effects should be viewed unfavorably because it is altering the natural state of the community (Boone and Bridges,

2003). Carbaryl has a short half-life in water, ranging from a few hours to only a few days, and is quickly metabolized by vertebrates (Bulen and Distel, 2011). Carbaryl was chosen because it is readily available and was the pesticide used for many other experiments pertaining to amphibian swimming ability.

MATERIALS AND METHODS

Northern Green Frog *(Rana clamitans melanota)* tadpoles were wild-caught and collected from ponds and streams in the Meadville, PA area on November 4, 2011. The pesticide carbaryl was purchased from the local Home Depot in the form of Sevin® Concentrate Bug Killer with carbaryl being the active ingredient at 22.5 % by weight. Experimental groups included 149 Green frog tadpoles that ranged in size from .57 to 5.46 grams with an average size of about 1.55 grams. The tadpoles were placed in a 36L aquarium filled with de-chlorinated water. The aquarium was filled with rocks and sediment provided by Allegheny College Biology department. To simulate more natural habitat, water from the ponds the tadpoles were located in was also added to the aquarium. The water was kept around 18-21 degrees Celsius with no special lighting. The tank was cleaned every seven days. The tadpoles were feed a diet of a mixture of TetraMin® large tropical fish flakes and Buckeye Rabbit pellets every other day. Upon arrival the tadpoles were placed into the aquarium and allowed to acclimate for a period of 24 hours. After 24 hours six tadpoles were moved to a plastic container containing 750mL of de-chlorinated water, which was aerated for one hour. This container contained 0.0 mg/L of carbaryl. After one hour the tadpoles were removed and individually tested for their swim speed five times with an average recorded. This test was repeated with a different set of six tadpoles for periods of 24 and 48 hours. After the control groups were tested, carbaryl was added to the plastic containers in the following amounts: 3.5mg/L, 5.0mg/L, and 7.5mg/L. Again each

container received six tadpoles and was left for one hour. The tadpoles were taken out and their swimming ability was recorded. This test again was repeated after 24 and 48 hours for each concentration. The entire series of testing was also repeated for a second time. During testing each individual tadpole was placed in a plastic track filled with de-chlorinated water. The track was one cm wide, 2.54 cm high and 71 cm long, with the bottom being marked in one centimeter increments. Each tadpole was then prodded with the back end of a pen until a calculable distance was swum. A calculable distance was considered to be one steady swimming period. Distance swam was recorded and time it took to swim that distance was found using a stopwatch and subsequently recorded. Speed was then finally calculated by cm/sec. After each tadpole was done their weight was taken and they were placed in a second aquarium containing the same materials as the first.

For disposal the anesthetic MS222 was used. 0.5 grams of MS222 was placed in a plastic container containing one liter of water. All of the tadpoles were then placed in to the container. Once the tadpoles ceased movement they were scooped up and placed in a plastic bag, which was then placed in a freezer. Once the tadpoles were frozen they were placed in a hazardous waste container. The MS222 that was used was also placed in an appropriate hazardous waste container and disposed of. All remaining containers were cleaned and their contents properly disposed of as well.

Swimming speed data were analyzed with a two-way ANOVA, in which concentration (four levels) and exposure time (three levels) were used as factors and Tukey's HSD post hoc test, $p>0.05$. Analysis was performed using JMP 8.0 for Mac OSX.

RESULTS

Tadpoles showed decreased swimming ability in a concentration-dependent and time-dependent when exposed to pesticides. As shown in table 1, the results of the ANOVA test indicate that the interaction between concentration and time was statistically significant.

Table 1. The results of the two-way ANOVA test.

Effect Tests				
Source	DF	Sum of Squares	F Ratio	Prob >F
Concentration (mg/L)	3	1992.6271	27.7516	<0.0001
Time (hours)	2	139.8870	2.9223	0.0575
Concentration (mg/L) X Time (hours)	6	627.5840	4.3702	0.0005

Control tadpoles with 0.0 mg/L of carbaryl in their water had the highest swimming speeds throughout 1, 24, and 48 hours (Fig. 1). They also had a very quick reaction time. Many times during the experiment even of a shadow would cause them to sprint down the track. When carbaryl was added the tadpole's speeds significantly decreased. However, there was not a significant difference in speed when pooling the three concentrations and comparing them to the control values,(Tukey's HSD post hoc test, $p>0.05$). Also over time the effects of carbaryl diminished and after 48 hours the tadpoles speed almost returned to the speed observed in the control test, Tukey's HSD post hoc test, $p>0.0\ 5$.

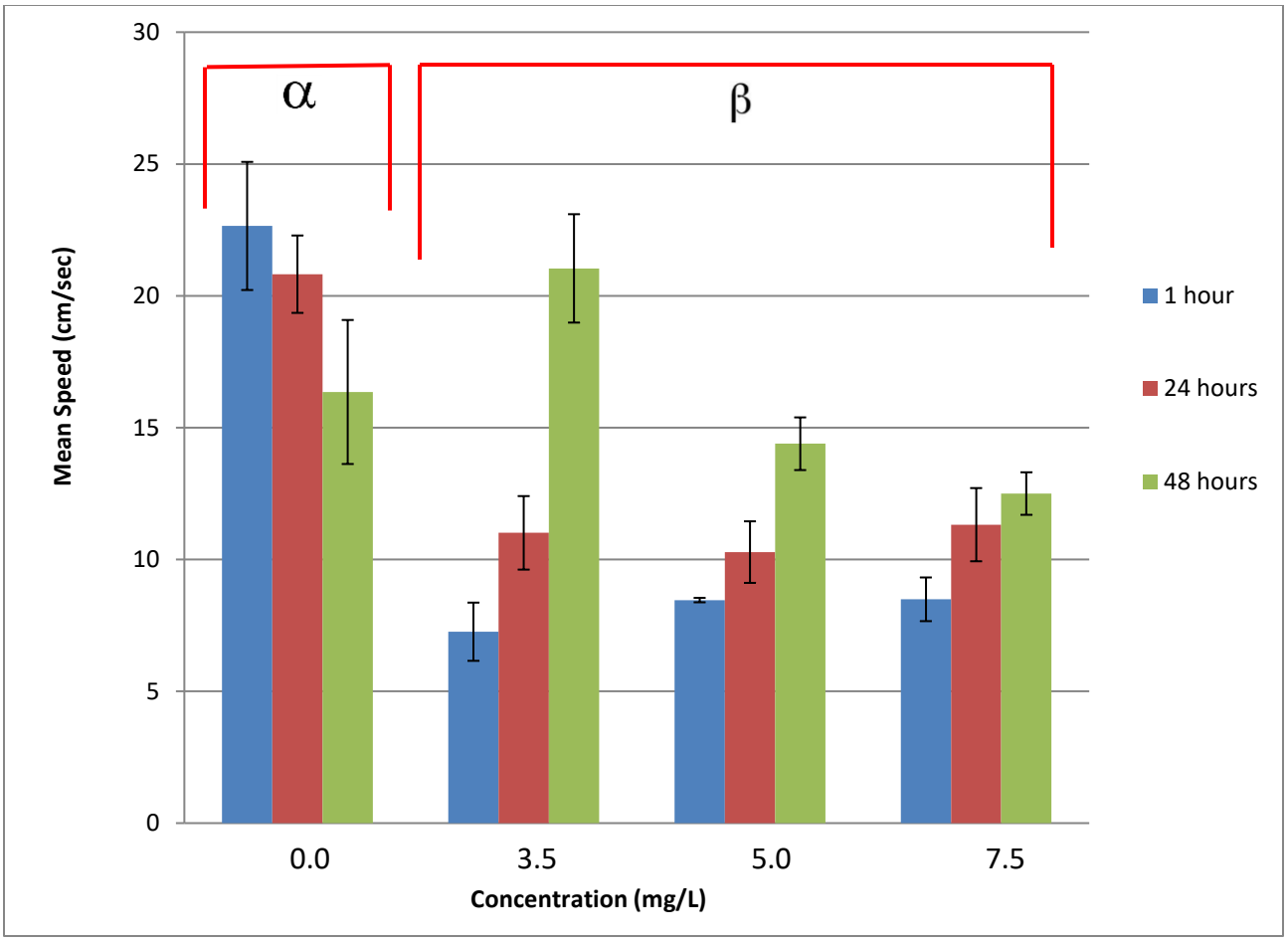


Figure 1. Mean Speed vs. Concentration. Speed vs. concentration shown in groups of 1, 24, and 48 hours. In standard error.

Control tadpoles were also significantly faster than the tadpoles that were exposed to all three concentrations for one and 24 hours (Tukey's HSD) but not significantly faster than tadpoles exposed to concentrations for 48 hours (Tukey's HSD). There was also no significant difference of swimming speed among the three concentrations (β) at any time when comparing them together with the control values (α).

DISCUSSION

Determining the effects of pesticides on amphibians may have important implications in terms of conservation. Recent estimates suggest that more that 40% of amphibian species are in decline or close to extinction (Buck et al. 2012). Sub-lethal concentrations can cause changes in immune response, reproduction, physiology, morphology and behavior, and can also affect amphibians indirectly, often through effects on the food web (Buck et al. 2012). Every behavior in an amphibian's environment has a benefit or a consequence. On a regular basis amphibians need to perform activities such as finding food, avoiding predators, swimming, avoidance, feeding, mating, growing, etc. It is a delicate ecosystem and if it is at all tampered with the effects can be detrimental. Pesticides can have those effects and at different concentrations different species of amphibians are affected. For example decreased tadpole activity was observed in eight ranid species after exposure to 2.5 mg/l of carbaryl in the lab, which can impede development through reduced food consumption and an inability to escape from predators (Bulen and Distel, 2011).

Metamorphosis timing can also be affected by pesticides. Metamorphosis timing is important; it determines how long a tadpole is in each stage and how long it takes for completion. Pesticides can have a negative effect depending on what stage of development each tadpole is in (Bulen and Distel, 2011). For example if too little tadpoles live through metamorphosis than predators will not have enough food and have a harder time surviving. Or if tadpoles stay in a particular stage too long they may be more susceptible to predation. Predator cues may also be affected. Amphibians mostly use sight and movement sensations to sense a potential predator. However some predators emit certain chemicals that can be picked up by the prey and warn them that danger is approaching. Exposure to pesticides can cause the cues to be

misinterpreted or not sensed causing prey to be more unlucky (Qin et al. 2011).

The pesticide carbaryl is widely used and has been shown to directly impact the survival of larval amphibians (Davis et al. 2011). Carbaryl, a neuro-toxic insecticide, is one of the most commonly used pesticides in the United States. It tends to be short-lived in aerobic aquatic environments, but degradation and toxicity vary widely and may depend on pH, oxygen levels, temperature, and presence of ultraviolet light and application may occur at several times during the agricultural seasons. Carbaryl, like many other pesiticides, may also indirectly affect survival through complex food web and competitive interactions (Davis et al. 2011). In a study done by Groner and Relyea (2011) pond communities containing zooplankton, phytoplankton, periphyton, and leopard frog tadpoles were exposed to medium to high concentrations of acetyl cholinesterase inhibiting insecticides, malathion and carbaryl. They found that the two insecticides had a comparable tropic cascades with diverging effects on amphibians and decreases with everything else (Groner and Relyea 2011). Carbaryl is also not the only pesticide to cause problems. Axolotl *Ambystoma mexicanum* was exposed to various concentrations of chlorpyrifos. It was found that the salamanders experienced a reduction in walking, frequency and speed, and swimming and frequency of prey attack by about 90% (Robles-Mendoza et al. 2011). In this study it is important to note that there was a reduced frequency of prey attack. This feature has a direct impact on the ecosystem, as fewer attacks leads to an abundance of prey, which has yet to be determined if this is a positive or negative change. Future studies will be needed to determine ecosystem-level consequences.

It is important to understand the basic mechanics of an ecosystem. The studies mentioned above support the theory that an ecosystem exists in a delicate balance and that any tampering with that balance can have negative effects, such as the huge decline in amphibian species. My

study was designed to test a single consequence of pesticide exposure to further support the theory that pesticides can severely affect an environment in very small amounts. The study focused on the straight swimming speed of Northern Green Frog tadpoles. Tadpoles were exposed to certain concentrations of the pesticide carbaryl because it is the most common pesticide used in the United States and is easily accessible to the public. Concentrations of 3.5, 5.0, 7.5 mg/L were chosen based on the concentrations used in previous experiments (Punzo 2005). After completion of the experiment it can be concluded that swimming speed is significantly reduced after exposure to pesticide. Slower swimming speeds of tadpoles could set off a chain reaction and cause many components of the ecosystem to change, such as losing other species because their food supply is too easy to catch and the population declines. This study supports the findings of Punzo, 2005 and Bridges, 1997 who tested the effects of pesticides on the Rio Grande Leopard Frog *(Rana berlandieri*) and the Plains Leopard Frog(*Rana blairi*) respectively. It was found that exposure for 24 hours caused the highest decrease in swimming ability. The study done by Punzo (2005) examined the effects of pesticides on not only swimming speed but tadpole activity as well. The tadpoles were exposed to concentrations of pesticide at 3.5, 5.0, and 7.5 mg/L. Time spent in activity was significantly decreased after 24 hours, for all concentrations. Full recovery was also observed when tadpoles were exposed to the 3.5 mg/L after 48 hours (Punzo, 2005). This differs slightly to my results, which seen in figure 1 indicates that for each concentration the slowest speeds were observed after only one hour. Also for each concentration full recovery was not observed for any time of exposure. Bridges (2007) also tested activity and swimming performance of tadpoles using 3.5, 5.0, and 7.5 mg/L of carbaryl. This study more closely matched my results showing that sprint speed and distance decreased significantly following exposure, which supports my findings that swim speed is

significantly decreased only after one hour of exposure. Also slight recovery was observed after 24 and 48 hours but no significant full recovery was observed (Bridges, 1997). The differences in my results compared to others could be because of the half-life of carbaryl which can be hours-days and also carbaryl's effectiveness can be dependent on certain qualities such as pH and temperature of the water.

For tadpoles, swimming speed is important for carrying out certain life functions such as growth and development (Punzo, 2005). A decrease in swimming speed may result in higher predation rates and increased mortality leading to a distressed environment (Punzo, 2005). The main conclusion indicated on figure 1 is that in comparison with the control group, pesticide exposure significantly decreases swimming speed. This finding also could potentially support the findings of Relyea and Edwards, (2010). They introduced a 0.1 and 1.0 mg/L concentration of two pesticides, carbaryl and malathion, to Gray Treefrogs, *Hyla veriscolor*; Green Frogs, *Rana clamitans*; and American Bullgrogs, *R. catesbeiana* as well as three species of their predators. The results indicated that predator-prey interactions were affected because the tadpoles affected had reduced swimming ability after exposure to pesticides.

It is also important to note that difference in swimming speeds, for this experiment, for the three concentrations were not significant when comparing them together with the control. This finding may indicate that the concentrations were too close together in value and that further study may be needed to determine the concentration range between no effect and death of certain amphibian species.

Carbaryl has a relatively short half life, hours-days, and swimming speed after 48 hours almost returned to the same values as the controls making that difference not significant. Hopkins and DuRant (2004) researched the effects of carbaryl on two species of snakes, the

neonate black swamp snakes (*Seminatrix pygaea*) and diamond water snakes (*Nerodia rhombifer*). Maximum swim velocity was determined for each snake after exposure to two pesticide concentrations, 2.5 mg/L and 5.0 mg/L. Each group showed a reduction in speed and a full recovery only after 96 hours (Hopkins and DuRant. 2004). This demonstrated that carbaryl has a relatively short half life and full recoveries are possible after a few hours, with the number varying slightly between species.

The effects of carbaryl on swimming speed were noticeably significant, but there were also other observations that were not measured. Carbaryl works by disrupting the nervous system of its intended prey and some unfortunate bystanders. After exposure some of the tadpoles exhibited interesting behavior when trying to swim the track. Some were twisting their bodies into a C-curve, periodically twitching, having difficulty swimming in a straight line, as well as completely rolling over with their underside exposed. Also some of the affected tadpoles had the ability to reach high speeds by performing a single sprint after being prodded. It is interesting to note however that for the control group that just the sight of a shadow, not even a touch, would cause them to sprint down the track. The affected tadpoles, however, had to be poked and prodded several times before any movement was observed. In the study done by Robles-Mendoza et al. (2011) axolotl *Ambystoma mexicanum* were exposed to chlorpyrifos in concentrations of 0.05 and 0.1 mg CPF/L for 48 hours. Their motor activity was observed and it was reported that salamanders that were exposed had a swimming pattern that was almost absent and when it was observed, it was very slow and only one swimming event per treatment was observed. Also swimming speed was significantly reduced (Robles-Mendoza et al. 2011). Garcia-Munoz et al. (2009) studied the effects of copper sulfate on growth, development, and escape behavior in *Epidalea calamita* embryos and larvae. The specimens were exposed to

different copper sulfate, a toxic substance used to control fungi growth, concentrations, ranging from 0.05 to 0.40 mg Cu L (-1), after 96 hours. Embryonic and larval mortality, development, growth and larval escape behavior were evaluated. All larvae from the control treatments needed just one stimulus to react, while the larvae from the other treatments needed more stimuli (Garcia-Munoz et al. 2009).

A predator should not have the ability to come up to prey and touch it several times before the prey realizes anything is going on and its brain signals it to swim away. The predator will have an easy time catching prey that could result in a significant decrease in population.

For future research it would be interesting to change certain aspects of the original study. For example the amount of pesticides used can be changed to gain a better idea of the range between unaffected and death. Different types of pesticides can be used to determine the most effective for pest control and the safest for the environment. The species of amphibian can be different to get an idea of how pesticides affect different species. Other effects of pesticides, besides swimming, can also be tested such as foraging behavior, maternal instincts, mating, etc.

Overall it is shown that a very small concentration of pesticide can have a large effect on tadpole swimming speed. Further studies will be needed to see the effects pesticides have on other species of amphibians. But based on previous research pesticides have a negative effect not only on individual species but also on their interactions between each other resulting in an overall amphibian decline and an alerted ecosystem. Hopefully this study and others will contribute support for protection of amphibians and the environment they live in.

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