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Long-Term Assessment Of Predatory Fish Removal On A Pond-Breeding Amphibian Community In Central Illinois

Lee M. Gross
Eastern Illinois University

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Long-term Assessment of Predatory Fish Removal on a Pond-Breeding Amphibian

Community in Central Illinois

BY

Lee M. Gross

THESIS

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ABSTRACT

Habitat loss and fragmentation are important contributors to population declines in many species, so maintaining suitable habitat has become a priority for wildlife management. Removal of introduced fish can help restore suitable habitats for amphibian populations, although long-term assessments of such removals are lacking at the community level. In 2001, drift fences and pitfall traps were constructed around the majority of four ponds to monitor an amphibian community in a nature preserve in central Illinois. Rotenone™ was applied to ponds containing introduced fish in 2001, and again, in 2003. I collected data from 2005-2007 to determine the long-term impacts of fish removal on this amphibian community. There was no difference in species abundance across pond type and time period. Although no difference in species diversity was detected in either pond, before or after fish removal, species diversity tended to increase following fish removal in both pond types. The sizes of young-of-the-year *Ambystoma texanum*, *Lithobates sphenocephalus*, and *L. sylvaticus* young-of-the-year were smaller following fish removal. Small-mouthed Salamander (*A. texanum*) recruitment increased following fish removal, with treatment ponds responding better than control ponds. My results indicate that the removal of predatory fish has increased species diversity throughout WWNP, and also demonstrate the effectiveness of using Rotenone™ to remove predatory fish for improving habitat for pond-breeding amphibians without causing negative impacts on the latter group of species.

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DEDICATION

To Mom, Dad, Laura, and Matt, for their love and support while I was completing this thesis research.

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INTRODUCTION

Many amphibian species have experienced population declines throughout the world (Barinaga 1990, Blaustein and Wake 1990, Blaustein et al. 1994, Houlahan et al. 2000, Blaustein and Kiesecker 2002, Collins and Storfer 2003). Although these declines are well documented, there remains an on-going argument about their causes (Barinaga 1990). It is difficult to pinpoint the causes for the declines occurring in each population, because a particular cause might affect different amphibian species, or different populations within the same species, in various ways. Furthermore, multiple causes may act synergistically to affect the decline of an individual amphibian population.

Habitat loss and fragmentation are generally viewed as among the primary anthropogenic causes for the amphibian population declines (Gibbs 1998, Semlitsch 2000). Reductions in both species richness and individual abundance are often correlated with declines in forest cover and wetland habitat (Houlahan and Findlay 2003). Wetland and terrestrial habitats – both vital for amphibian survival – have been greatly altered for human use. Almost 53% of native wetland in the United States has been lost in the past 200 years due to human activities (Dahl 1990, Semlitsch 2000). The destruction of wetlands has been even more dramatic in Illinois, where almost 90% of native wetlands have been destroyed for agriculture and urban development (Suloway and Hubbell 1994).

In addition to habitat destruction, four other reasons for the declines in amphibian populations have dominated the scientific literature. Global climate change (*e.g.*, changes in temperature, rainfall, and UVB radiation), infection and diseases (*e.g.*, chytridomycosis, *Ambystoma tigrinum* virus, and trematode infections), environmental contaminants (*e.g.*, herbicides and insecticides), and the introduction of nonnative species

have been hypothesized by some as other reasons for the declines in amphibian populations throughout the world (Alford and Richards 1999, Houlahan et al. 2000, Blaustein and Kiesecker 2002, Collins and Storfer 2003, Kats and Ferrer 2003).

Introduced species are most commonly associated with having negative impacts on other members of their community. These taxa can increase inter- and intra-specific competition among the native species for resources such as food and habitat. Lawler et al. (1999) demonstrated that introduced American Bullfrog (*Lithobates catesbeianus*) larvae in the western United States can almost eliminate the recruitment of the native California Red-legged Frog (*Rana draytonii*), possibly through competition. Alien prey may also become an important food source for other introduced predators. It has been proposed that the introduction of the Puerto Rican Frog (*Eleutherodactylus coqui*) will become an important prey species to help sustain the introduced population of the Small Indian Mongoose (*Herpestes javanicus*) in Hawaii (Beard and Pitt 2006). Alien predators may also introduce exotic diseases, to which the native community lacks any immunity. Hanselmann et al. (2004) proposed that introduced *Lithobates catesbeianus* may be a reservoir for the fungal pathogen, *Batrachochytrium dendrobatidis*, which may be responsible for the outbreaks of chytridiomycosis and subsequent declines in amphibian populations in Venezuela. Introduced species may also include predators to which native prey species have no shared evolutionary history, which may limit defense mechanisms in those prey species.

Non-native species do not necessarily have negative impacts on the system in which they have been introduced. King et al. (2006) demonstrated that the introduced Round Goby (*Meogobius melanstomus*) has become the predominant prey source for the

threatened Lake Erie Watersnake (*Nerodia sipedon insularum*). An introduced predator that can suppress a native predator population (either through competition or direct predation) might indirectly help the populations of native prey species by lowering the density of the native predator. The introduction of Bluegill (*Lepomis macrochirus*) indirectly increased the survival of *Lithobates catesbeianus* larvae by removing native predators, such as Tiger Salamanders (*Ambystoma tigrinum*) and dragonfly larva (*e.g.*, *Anax junius*; Werner and McPeek 1994). It is relatively rare, however, for an introduced species to have a positive impact on the entirety of the community (Bially and MacIsaac 2000).

The introduction of predatory fish (typically for recreational purposes; Bahls 1992, Townsend 1996, Pilliod and Peterson 2001) has arguably become one of the greatest threats to pond-breeding amphibians (Kats and Ferrer 2003, Hartel et al. 2007). There are many ways in which introduced fish might negatively impact amphibian populations. Introduced fish directly impact amphibians through predation. Increases in fish predation on amphibian eggs and larvae have been associated with the declines in the Common Treefrog (*Hyla arborea;* Brönmark and Edenhamn 1994, Hartel et al. 2007), Gray Treefrog (*Hyla versicolor;* Smith et al. 1999), Columbia Spotted Frog (*Rana luteiventris*; Pilliod and Peterson 2001), Southern Mountain Yellow-legged Frog (*Rana mucosa*; Vredenburg 2004), Common Frog (*Rana temporaria*; Hartel et al. 2007), and some newt species (*Triturus* spp.; Orizaola and Braña 2006, Hartel et al. 2007). Amphibians may also experience declines because of competition with introduced fish for resources, such as food and/or the loss of structures used for breeding and refuge (Smith et al. 1999, Knapp and Matthews 2000, Pilliod and Peterson 2001, Knapp et al.

2005, Orizaola and Braña 2006). Introduced fish may also decrease amphibian populations by being a reservoir for exotic pathogens. Kiesecker et al. (2001) demonstrated that introduced fish can transmit pathogens to amphibian embryos, leading to increased mortality in the Western Toad (*Anaxyrus boreas*).

In addition to assessing the impacts of introduced fish on amphibian populations, recent research has attempted to eliminate these negative effects and reverse amphibian population declines. Most studies of this nature have either focused on a single amphibian species, or have only evaluated the short-term effects of fish removal on the amphibian community. Brönmark and Edenhamn (1994) documented successful reproduction in *Hyla aborea* a year after fish were removed from breeding habitat. Rapid improvements in *R. mucosa* population size were observed after the removal of introduced trout (Vredenburg 2004). There is still a need, however, for long-term evaluations of the impacts of fish removal on amphibian communities because of how each individual species may respond.

Herein, I present the results of a field experiment that evaluates the removal of introduced fish on an amphibian community in central Illinois. The purpose of this study is to assess the long-term effects of fish removal on an amphibian community by determining changes in: 1) within-species abundance; 2) community structure; 3) young-of-the-year (YOY) size for five species using the ponds; and, 4) Small-mouthed Salamander (*Ambystoma texanum)* larval recruitment. The results of my research can aid wildlife managers in improving habitat for pond-breeding amphibians, in that the managers can better predict population responses to predator removal.

METHODS

<u>Study Site and Focal Species</u>

All data was collected at Warbler Woods Nature Preserve (WWNP) in Coles County, Illinois (Fig. 1). WWNP totals 81.5 ha, and is under the management of the Illinois Nature Preserves Commission of the Illinois Department of Nature Resources (IDNR). There are four ponds located in the southeast section of WWNP. They are labeled from east to west: A, B, C, and D (Figure 1). Ponds A, B, and C are permanent ponds, while Pond D is an ephemeral pond that can dry as early as 25 June of each year. The four ponds vary in size, from 400 m^2 in size (Pond D) to 900 m^2 (Pond C). The ponds are surrounded by varying widths of secondary deciduous forest (primarily oak-hickory) beyond which lie, abandoned agricultural fields. The old field habitat has been planted with tree seedlings and saplings as part of an IDNR management plan to restore conditions at WWNP to those present in pre-settlement (pre-1840) times.

Ponds A and D have always been fishless ponds. Pond B contained a population of Black Bullhead (*Ameiurus melas*), and Pond C contained populations of two centrarchid species, Bluegill (*Lepomis machrochirus)* and Green Sunfish (*Lepomis cyanellus*). As described by Mullin et al. (2004), Rotenone™ was applied to Ponds B and C after the 2001 activity season, in a management effort to improve amphibian-breeding habitat. Rotenone™ was reapplied to Pond B in January 2003 to remove remaining Black Bullheads. Fish were never detected in either Ponds B or C after the second Rotenone™ application (Walston and Mullin 2007).

My drift fence-pitfall trap system was varyingly effective at capturing individuals representing 10 different amphibian species. Only changes in Small-mouthed

Salamander (*Ambystoma texanum*) recruitment were examined because this was the only species trapped at an adequate sample size for both adults and young-of-the-year (YOY) stages during my study. The Small-mouthed Salamander has a very small head and mouth compared to other *Ambystoma,* but is of medium body size (Petranka 1998). *Ambystoma texanum* are common in the state of Illinois and are usually found in poorly drained areas (such as woodlands, prairies, fields) under logs and leaf litter adjacent to breeding ponds (Phillips et al. 1999). Gender is easily determined in this species during the breeding season by the swollen papillose vents on males (Petranka 1998).

Sampling Procedure

In May 2000, a series of drift fences and pitfall traps was installed around the majority ($\geq$ 75 % coverage) of each pond at WWNP to monitor amphibian populations. The drift fence-pitfall trap system could not be placed completely around the ponds because of topography, dense vegetation, and in-flow/out-flow channels. The drift fence consisted of black construction silt fence (45 cm tall) supported by wooden stakes. The bottom 10 cm of the fence was buried below soil grade to prevent individuals from burrowing under the fence. Plastic containers (3-L buckets, 20 cm deep, and spaced every 7.5 m on both sides of the fence) were buried flush with the soil surface and used as pitfall traps. Each pitfall trap was uniquely labeled to differentiate it from other buckets, ponds, and position with respect to the fence (inside vs. outside).

Drift fences are a useful technique for long-term monitoring of amphibian movement to and from breeding sites (Dodd and Scott 1994). Individuals migrating to and from the pond move until they encounter the drift fence. The individual may choose to remain there or follow the fence, which will lead to being captured by falling into

pitfall traps on either side. Certain species of amphibians are more difficult to capture by this method than others. Large frog species (*e.g.*, *Lithobates* spp.) can jump over the drift fence (if not high enough) or out of pitfall traps (if not deep enough). Some treefrog species may also use their large toe pads to climb over drift fences and out of pitfall traps.

Traps were monitored every 48 hr during the activity season (usually late February to early December). During the non-active season, traps were covered to avoid accidentally trapping non-target organisms. Individuals found in the traps were first identified, then measured for snout-vent length (SVL; ±1 mm) and tail length (TL; ±1 mm), aged (either as adult or YOY), and sex was determined when possible. Before being released, each individual was toe-clipped to signify initial collection year and pond location. If a specimen was previously marked, the year and pond of original capture was recorded.

Larval Size and *Ambystoma texanum* Recruitment

The SVL measurement (±1 mm) of any juvenile emigrating from the ponds was used to quantify changes in the larval size at metamorphosis before and after fish removal. This was done for all species for which a large enough sample size of YOY individuals was captured and included: *Ambystoma texanum, Anaxyrus americanus*, *Lithobates catesbeianus*, *L. sphenocephalus*, and *L. sylvaticus*. The juvenile age class was restricted to larvae that had metamorphed the year of capture rather than individuals who were too young to breed. Recruitment in *Ambystoma texanum* was calculated by using the total number of captured YOY divided by the total number of breeding females captured during the spring migration (excluding any recaptures within the same season).

Statistical Analyses

Data collection for this thesis was started in June 2005 through the activity season of 2007. For analyses recorded below, my data were combined with those collected in 2001-2004. Data collected from 2000 were excluded from any analyses because that was an incomplete year of monitoring. A 2 x 2 analysis of variance (ANOVA) was performed to determine changes in overall abundance, as well as, changes in the populations of all species in both pond types (control and treatment) and time periods (pre- and post-fish removal). Abundances were standardized to pond size before all analyses. All abundances, except total abundance and *Lithobates catesbeianus*, were log-transformed to meet the normality requirements for the ANOVA. Species diversity was measured using the Shannon Diversity Index (H'), for each pond, before and after fish removal. I used a 2 x 2 ANOVA to detect any differences in species diversity between pond types (control and treatment) and time periods (pre-treatment and post-treatment). A 2 x 2 ANOVA was also use to detect any differences in larval size and *Ambystoma texanum* recruitment as a function of pond type and time period. Small-mouthed Salamander recruitment was standardized to pond size before any statistical analysis.

RESULTS

Since sampling began in 2001, a total of 11,447 individuals representing 10 amphibian species have been captured from WWNP (see Appendix I). The number of individuals captured in each year tended to be greatest in Pond B, especially before fish removal. Conversely, Pond D consistently had the smallest amount of individuals captured in each year, except in 2005, a drought year. There were no differences in overall abundance values (all species combined) between pond types, time periods, or their interaction ($F \leq 1.05$, $p \geq 0.36$). Similarly, abundances of each species did not differ as a function of pond type, time period, or their interaction (Table 1).

Before fish removal, American Toads (*Anaxyrus americanus*) were the most abundant amphibians, comprising 67.1% of the total individuals captured in control ponds and 90.7% in treatment ponds (Table 2, Fig. 2). American Toad abundance declined after fish removal, comprising only 3.0% of the total captures in control ponds and 9.9% in treatment ponds. A different trend can be seen in Small-mouthed Salamander abundance. Prior to fish removal, Small-mouthed Salamanders accounted for 16.2% of the total captures in control and 2.6% in treatment ponds, respectively. These values increased to 42.3% in control ponds and 45.7% in treatment ponds after fish removal (Table 2, Fig. 2). The abundance of other amphibian species increased in both control and treatment ponds following fish removal (Table 2), but not as dramatically as observed in *A. texanum*.

In control ponds, the Shannon Diversity Index score was 1.08 before fish removal and 1.35 after fish removal (Table 2). Treatment ponds experienced a similar trend in species diversity, with values increasing after fish removal. Although species diversity

increased in both pond types after fish removal, there were no differences between pre- and post-treatment H' values ($F = 0.05$, $p = 0.83$). There were also no differences in H' values as a function of pond type, or the interaction between pond type and time period ($F \leq 0.39$, $p \geq 0.57$).

The YOY body size at metamorphosis for some amphibian species at WWNP decreased after fish were removed in both treatment and control ponds (Table 3). The mean (±1 standard error) YOY size of Small-mouthed Salamanders in both pond types decreased from 37.3 ± 1.1 mm to 32.1 ± 0.1 mm following fish removal (Fig. 3). The mean YOY size for *L. sphenocephalus* was 35.9 ± 0.6 mm and 29.1 ± 0.1 mm before and after fish removal, respectively (Fig. 4). The mean YOY size for *L. sylvaticus* also decreased from 21.2 ± 0.6 mm to 19.1 ± 0.1 mm after fish removal (Fig. 5). There were no differences in YOY size as a function of pond type, or the interaction between pond type and time period, for these three species (Table 3).

Before fish were removed, the mean recruitment values for Small-mouthed Salamanders were similar in control (0.37 ± 0.24 metamorphs emerging per female entering a pond) and treatment ponds (0.36 ± 0.24; Fig. 6). After fish removal, however, the mean *Ambystoma texanum* recruitment increased in control ponds (2.00 ± 1.48 metamorphs emerging per female) and treatment ponds (6.51 ± 2.78). Small-mouthed Salamander recruitment was different before and after fish removal ($F_{1,2} = 162.57$, $p = 0.002$), with treatment ponds responding better than control ponds, as indicated by the significant interaction in this analysis ($F_{1,2} = 10.46$, $p = 0.03$).

DISCUSSION

Over the entire monitoring effect, the population-level responses of each pond-breeding amphibian species at WWNP did not follow a consistent pattern. My study has shown that non-native predatory fish can exert negative impacts on amphibian communities and populations, a result that is supported by previous research (Orizaola and Braña 2006, Welsh et al. 2006, Hartel et al. 2007, Werner et al. 2007). Predatory fish had a negative effect on species diversity within the amphibian community at WWNP, as evidenced by the lower diversity values in treatment ponds compared to control ponds. Treatment ponds also responded better than control ponds after fish removal, with diversity indices more than doubling in the absence of fish. The change in species diversity experienced at WWNP is likely due to the changes in relative abundance (discussed below) and not species richness, because I did not observe an increase in the number of amphibian species breeding at WWNP ponds.

My results indicate that the removal of predatory fish produced changes (sometimes dramatic) in the relative abundance of several amphibian species at WWNP. For example, *Lithobates sphenocephalus* relative abundance increased by 94% and 72% in treatment and control ponds, respectively, following fish removal. Spring Peepers (*Pseudacris crucifer*) experienced similar changes in relative abundance after fish were removed, with increases of 97% and 73% in treatment and control ponds, respectively. The increase in relative abundance values for both *L. sphenocephalus* and *P. crucifer* was greater in treatment ponds than in control ponds following fish removal. Both of these species are considered palatable to predatory fish (Kats et al. 1988, Gregoire and Gunzburger 2008), and it is likely that the fishes found in Ponds B and C suppressed

abundance through predation of eggs and larvae. Predatory fish have the potential to reduce or even eliminate many species of ambystomatid salamanders (Semlitsch 1987, 1988, Tyler et al. 1998). Larvae of *Ambystoma texanum* has been described as palatable to fish (Kats et al. 1988), which might explain the low relative abundance before fish removal. Small-mouthed Salamanders accounted for only 2.6% of amphibians captured in treatment ponds before fish removal and 16.2% in control ponds, respectively. Following the application of Rotenone™, *A. texanum* relative abundance increased in both treatment (48.3%) and control (58.5%) ponds.

Not all amphibian species at WWNP were negatively affected by the presence of predatory fish, as abundance for a few species declined following fish removal. American Toads appeared to coexist with fish, as they comprised 90% of the relative abundance in treatment ponds before fish removal. After Rotenone™ application, the relative abundance *Anaxyrus americanus* decreased to 9.9% and 3.0% in treatment and control ponds, respectively. American Toads are inferior competitors to many other larval amphibians, which might contribute to their decrease in abundance at WWNP following fish removal. Having a smaller body size than the larvae of many pond-breeding amphibians (Phillips et al. 1999), larval *A. americanus* might have an easier time avoiding detection by generalist predators, such as fish searching for amphibian larvae and other active prey types (Anholt et al. 1996). Skelly and Werner (1990) showed that *A. americanus* larvae reduce activity and change their spatial distribution in response to a predator's presence. Several *Anaxyrus* larvae are refused by predatory fish, presumably because of toxins produced by the larval integument (Kruse and Stone 1984, Kats et al. 1988, Welsh et al. 2006).

Anaxyrus americanus is not the only species that appeared to coexist with fish in the WWNP ponds. The relative abundance of American Bullfrogs (*L. catesbeianus*) was higher in Pond C before fish removal, compared to after fish removal. My results support previous research that addresses *L. catesbeianus* palatability and coexistence with predatory fish (*e.g.*, Kruse and Francis 1977). *Lithobates catesbeianus* larvae thrive in permanent ponds with centrarchid fish species, both directly (by having unpalatable larvae; Werner and McPeek 1994) and indirectly (by fish presence decreasing potential predators of bullfrog eggs and competitors with bullfrog larvae; Kats et al. 1988, Smith et al. 1999, Adams et al. 2003).

The use of chemical deterrents (*e.g.*, toxins produced by the integument) is not the only strategy employed by amphibians to reduce the risk of the predation by fish. Another strategy amphibians use to avoid aquatic predators is behavioral changes (*i.e.*, changes in activity). Lawler et al. (1999) found that California Red-legged Frogs (*Rana draytonii*) will decrease their activity in the presence of Mosquitofish (*Gambusia affinis*), even though Mosquitofish prefer other species of prey in laboratory feeding trials. *Ambystoma macrodactylum* and *A. gracile* have been documented to change their spatial distribution, by narrowing their range in substrate types used when fish are present (Tyler et al. 1998). Some of the change in activity level is caused by chemical cues detected from the presence of predators. Cope's Gray Treefrog, (*Hyla chrysoscelis*) detects Green Sunfish (*Lepomis cyanellus*) in the water by using chemical cues and reduces activity while seeking refuge to reduce predation risk (Petranka et al. 1987). Kats (1988) and Huang and Sih (1990) observed similar behaviors with *Ambystoma texanum* larvae that increased refuge use in the presence of olfactory cues from predatory fish.

Although not statistically distinct, the mean YOY size of *A. texanum* and *L. sylvaticus* tended to be larger in treatment ponds at WWNP compared to the control ponds both before and after fish removal (Figs. 3,5). Hecnar and M'Closkey (1997) reported a similar result wherein larval size was larger in ponds that contained fish compared to ponds without fish. Overall, there was a decrease in juvenile size at metamorphosis for three species (*A. texanum*, *L. sphenocephalus*, and *L. sylvaticus*) following fish removal in both pond types (Table 3, Figs. 3-5). The difference in *A. texanum* juvenile size can even be observed only two years after fish removal at WWNP (Walston and Mullin 2007). Many experiments have documented similar negative impacts of predatory fish on amphibian growth. A decrease in the larval body size for *A. maculatum* and *A. talpoideum* larvae occurred in the presence of *Lepomis macrochirus* (Semlitsch 1987). Larvae of *A. macrodactylum* and *A. gracile* also had reduced body size at metamorphosis when exposed to trout (*Oncorhynchus* sp.; Tyler et al. 1998).

Because differences in YOY body size of amphibians emerging from control and treatment ponds were not statistically distinct, my results indicate that predatory fish did not have an effect on size at metamorphosis for these three species at WWNP. This observation can be explained by a possible interaction between competition and predation. The increase in inter- and intra-specific for food and resources can be a limiting factor on growth and survival for many amphibians. A high larval density within a pond can cause a decrease in juvenile size at metamorphosis in *L. sylvaticus* (Berven 1990, Harper and Semlitsch 2007) and *Anaxyrus americanus* (Harper and Semlitsch 2007). Also, the risk of predation from other aquatic predators (*i.e.*, dragonfly larvae) has

been shown to decrease larval size of amphibians (Skelly and Werner 1990, Van Buskirk 2000).

Wilbur and Collins (1973) proposed a model to explain when an amphibian larva will begin metamorphosis. In this model, an individual must meet a certain minimum body size before beginning metamorphosis. If it does not reach this size, the individual must continue to grow. If at this time, the body size of an individual is smaller than maximum larval body size, the individual may still decide to begin metamorphosis if growth rate is slower than a species-specific intrinsic factor. The risk of staying in the pond is not worth the growth that may be obtained, therefore resulting in a smaller size at metamorphosis. When the growth rate is high (and the individual is smaller than the maximum size), the individual may not undergo metamorphosis and continue to grow until it can obtain the maximum size (Wilbur and Collins 1973). Larvae metamorphing at a larger body size increase their probability of survival (Semlitsch and Gibbons 1990).

Another possible explanation for decreased juvenile size at metamorphosis for amphibians is chemical contamination. Atrazine, one of the most commonly used pesticides in the United States, was found in both the water and sediment in Ponds A and C (see Appendix II). Although Ponds B and D were not tested for atrazine contamination, it is likely that both ponds also contain atrazine because of the close proximity to Ponds A and C, and active agricultural fields that border WWNP. Within the United States, Illinois applies 5,000 t of atrazine to control broad-leafed weeds and grasses around corn crops (Solomon et al. 1996). Atrazine is highly water soluble, and the greatest risk of exposure from agricultural runoff in ponds generally occurs in early June through July (Solomon et al. 1996). During this time, many amphibian larvae are

exposed to high levels of atrazine while growing or undergoing metamorphosis. *Lithobates pipiens* were smaller at metamorphosis compared to control frogs when exposed to 0.1 μg/L of atrazine in a laboratory study (Hayes et al. 2006). Atrazine may also indirectly decrease amphibian larvae size by decreasing the prey abundance available in ponds (Rohr and Crumrine 2005). Other pesticides, such as metolachlor or alachlor, can work synergistically with atrazine, causing a decrease in juvenile size at metamorphosis (Hayes et al. 2006).

Although fish removal did not affect juvenile size at metamorphosis, fish presence did negatively impact *A. texanum* recruitment in my study. Recruitment is often a measure of reproductive success for amphibians. Predatory fish can completely eliminate ambystomatid salamander recruitment through predation of eggs and larvae (Semlitsch 1988). Most of the increase in *A. texanum* recruitment observed at WWNP can be attributed to greater numbers of Small-mouthed Salamanders emigrating from treatment ponds following fish removal (as seen by the interaction between pond type and time period). Only 14 juvenile Small-mouthed Salamanders were captured emigrating from treatment ponds before fish removal, compared to 988 juveniles captured the five years following fish removal. With consecutive years of high recruitment and capture success around all ponds, *A. texanum* has become the most abundant species in all four ponds in 2007.

Rotenone™ has been used as a management tool for over 50 years, but its use has declined in recent years due to safety concerns and public opinion (Finlayson et al. 2000, McClay 2000). My study demonstrates that Rotenone™ can be used safely and effectively to remove predatory fish from ponds, without any negative long-term effects

on the amphibians breeding in that habitat. Over-wintering larval *L. catesbeianus* were seen alive in the treatment ponds after Rotenone™ applications and successfully metamorphed the following years (Mullin et al. 2004). This outcome suggests that *L. catesbeianus* may have a higher tolerance to Rotenone™ than Black Bullhead (*Ameiurus melas*), which has a tolerance to Rotenone™ more than 10 times higher than most other fish species (Finlayson et al. 2000). Mullin et al. (2004) also applied Rotenone™ to the treatment ponds at WWNP at the end of the activity season, to limit amphibian exposure to the chemical. Because Rotenone™ degrades at a slower rate in colder water (Finlayson et al. 2000), an application of Rotenone™ late in the winter season could have possible negative effects on the amphibians breeding in the ensuing spring. Although species diversity values were not different across treatment periods, I am encouraged by the fact that diversity increased in both the control and treatment ponds following Rotenone™ application.

My study examined the responses of a pond-breeding amphibian community over a five-year span following the removal of predatory fish. The period encompassed by this data set is longer than many other studies, but still offers only a glimpse at the amphibian community. My results indicate that predatory fish had a negative impact on both species abundance within the community and the recruitment for Small-mouthed Salamanders. Size at metamorphosis of this species, as well as *L. sphenocephalus*, and *L. sylvatica*, did not appear to be influenced by fish presence. Abiotic factors can have pronounced effects on amphibian abundance, survival, and reproductive success. Changes in hydroperiod, temperature, and rainfall can cause fluctuations in amphibian abundance, survival, and recruitment (Pechmann et al. 1989, Semlitsch 2000, Ryan and

Winne 2001). More data is needed to discern if the fluctuations that amphibian populations experienced at WWNP are caused by the removal of predatory fish or if they are occurring stochastically, possibly in response to other factors. Overall, the removal of predatory fish has increased species diversity throughout WWNP. My study also reinforces the effectiveness of using Rotenone™ to improve habitat for pond-breeding amphibians without causing negative impacts on the community.

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Table 1: Results of analyses of variance that test the effects for time period (pre-treatment or post-treatment) and pond type (control or treatment) on the abundance of each amphibian species at Warbler Woods Nature Preserve in Coles County, Illinois from 2001-2007 (df = 1,2 in all cases).

Species	Source	F	*P*
Acris crepitans	Time Period	0.35	0.61
	Pond Type	5.95	0.13
	Time Period x Pond Type	2.38	0.26
Ambystoma texanum	Time Period	3.03	0.16
	Pond Type	0.59	0.49
	Time Period x Pond Type	0.56	0.50
Anaxyrus americanus	Time Period	1.65	0.27
	Pond Type	0.81	0.42
	Time Period x Pond Type	< 0.01	0.95
Pseudacris crucifer	Time Period	1.16	0.34
	Pond Type	0.75	0.44
	Time Period x Pond Type	0.15	0.72
Lithobates catesbeianus	Time Period	0.14	0.72
	Pond Type	4.26	0.11
	Time Period x Pond Type	1.12	0.35

Table 1, continued.

Species	Source	F	*P*
Lithobates sphenocephalus	Time Period	0.64	0.47
	Pond Type	< 0.01	0.96
	Time Period x Pond Type	0.02	0.89
Lithobates sylvaticus	Time Period	0.04	0.85
	Pond Type	0.41	0.56
	Time Period x Pond Type	0.10	0.77

Table 2: The relative abundance for each of the 10 amphibian species captured at Warbler Woods Nature Preserve in Coles County, Illinois, before and after Rotenone^{TM} application in control ponds (n = 2) and treatment ponds (n = 2) from 2001-2007. Shannon Diversity Index (H') values are displayed for both pond types and time period. Asterisks (*) indicate that there were no captures during that time period.

	Pre-Treatment		Post-Treatment	
Species	Control	Treatment	Control	Treatment
Acris crepitans	< 0.001	0.002	0.002	0.003
Ambystoma texanum	0.162	0.026	0.585	0.483
Anaxyrus americanus	0.671	0.907	0.030	0.099
Anaxyrus fowleri	< 0.001	< 0.001	< 0.001	*
Hyla versicolor	0.003	*	0.012	0.079
Pseudacris crucifer	0.012	0.003	0.045	0.111
Pseudacris triseriata	*	*	0.003	0.003
Lithobates catesbeianus	0.041	0.046	0.074	0.071
Lithobates sphenocephalus	0.029	0.007	0.103	0.127
Lithobates sylvaticus	0.079	0.009	0.145	0.024
H'	1.08	0.437	1.35	1.60

Table 3: Results of analyses of variance that test the effects for time period (pre-treatment or post-treatment) and pond type (control or treatment) on larval size at metamorphosis (±1 mm) for amphibian species at Warbler Woods Nature Preserve in Coles County, Illinois, between 2001-2007 (df = 1,2 in all cases).

Species	Source	F	*P*
Ambystoma texanum	Time Period	57.62	< 0.01
	Pond Type	6.47	0.08
	Time Period x Pond Type	0.11	0.76
Anaxyrus americanus	Time Period	0.42	0.56
	Pond Type	2.00	0.25
	Time Period x Pond Type	0.01	0.92
Lithobates catesbeianus	Time Period	1.45	0.31
	Pond Type	2.41	0.22
	Time Period x Pond Type	4.00	0.14
Lithobates sphenocephalus	Time Period	41.99	0.02
	Pond Type	0.50	0.55
	Time Period x Pond Type	8.24	0.10
Lithobates sylvaticus	Time Period	24.39	0.04
	Pond Type	0.29	0.64
	Time Period x Pond Type	2.37	0.26

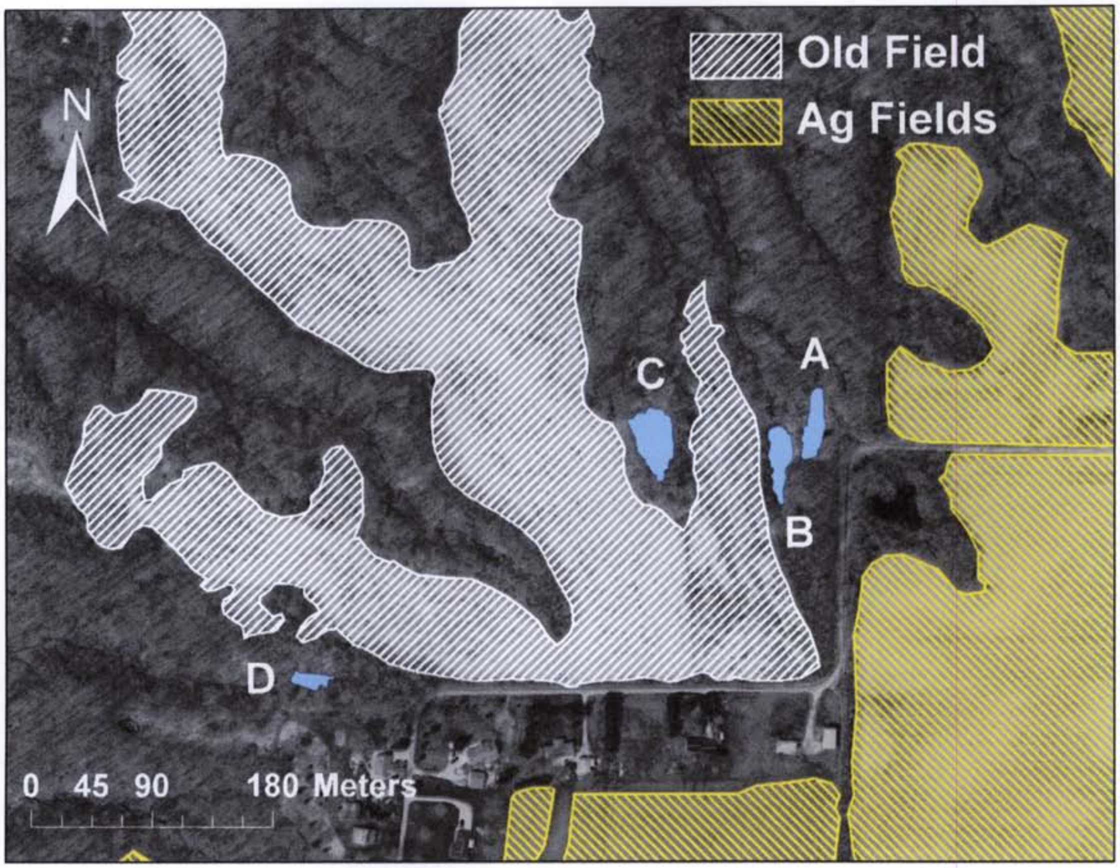

Figure 1: The southeastern portion of Warbler Woods Nature Preserve located in Coles County, Illinois. The four study ponds are highlighted in solid blue, with associated old fields in hatched white and forest cover unmarked. Neighboring agricultural fields have been highlighted in yellow-hatched areas. Ponds A and D (control ponds) are fishless ponds. Ponds B and C (treatment ponds) had fish introduced in the mid 1980's, but were completely removed in 2002 in Pond C and 2003 in Pond B.

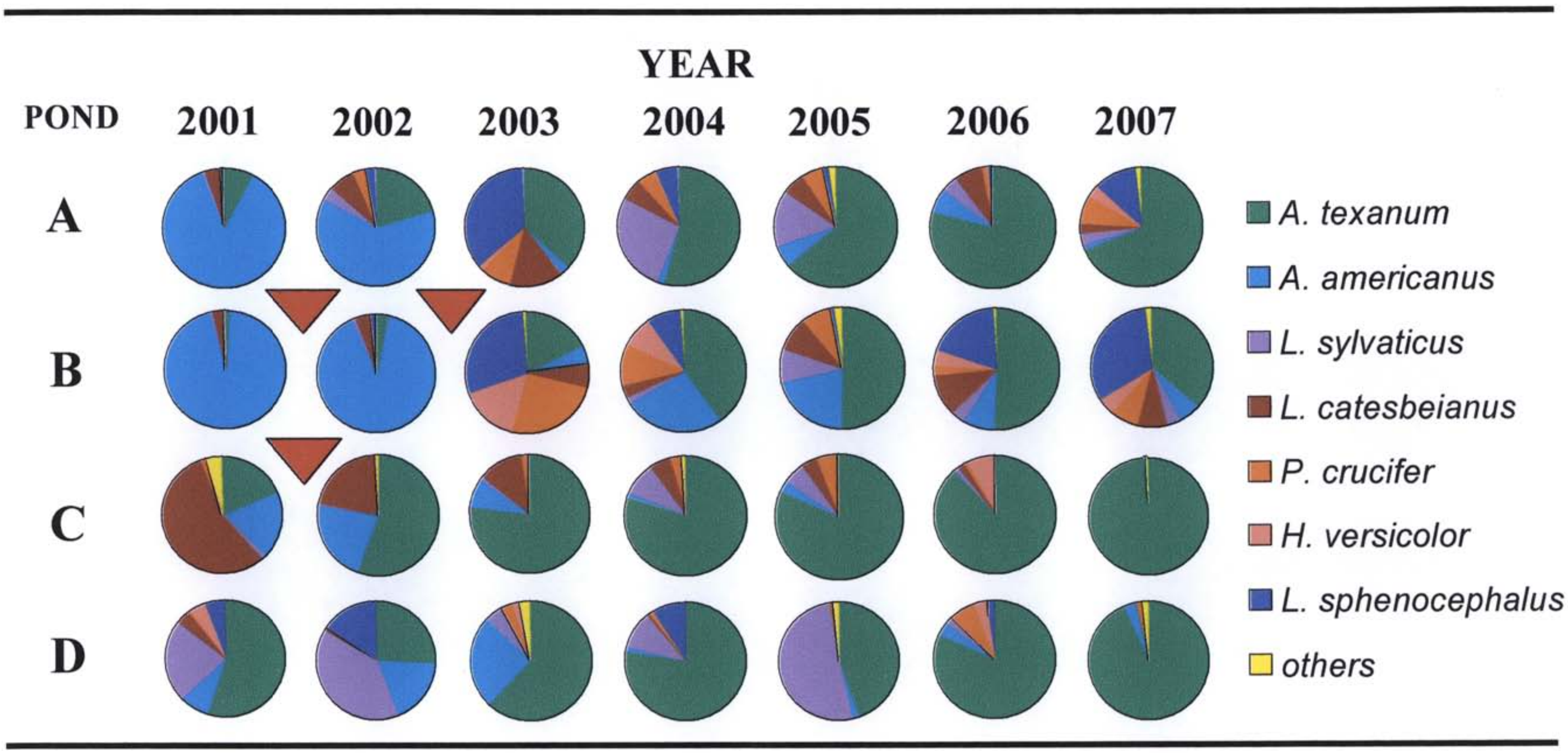

Figure 2: The proportional abundance of each species captured in each pond from 2001-2007 at Warbler Woods Nature Preserve in Coles County, Illinois. The "other species" is a combination of *Anaxyrus fowleri*, *Pseudacris triseriata*, and *Acris crepitans*. The red triangles represent when Rotenone™ was applied to Ponds B and C.

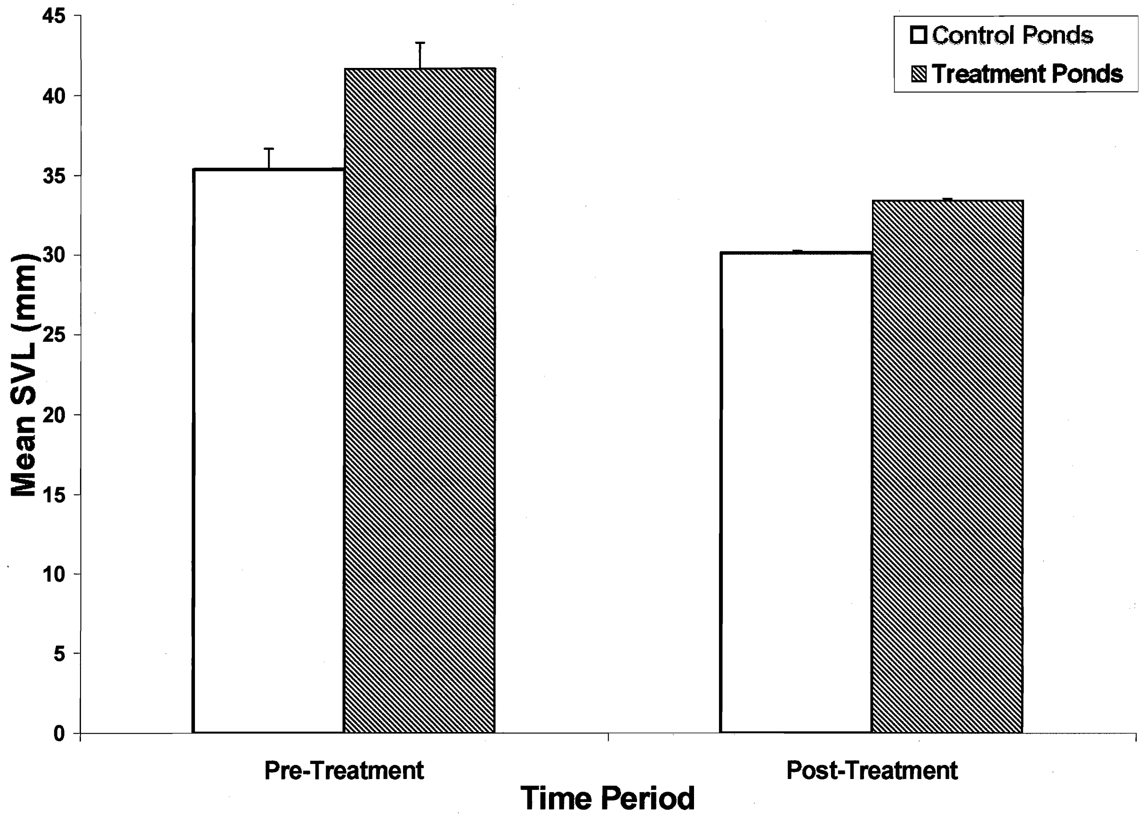

Figure 3: Mean snout-vent-lengths (+1 SE; mm) for young-of-the-year Small-mouthed Salamander (*Ambystoma texanum*) as a function of pond type (control and treatment) and time period (pre-treatment and post-treatment of Rotenone™) captured at Warbler Woods Nature Preserve in Coles County, Illinois.

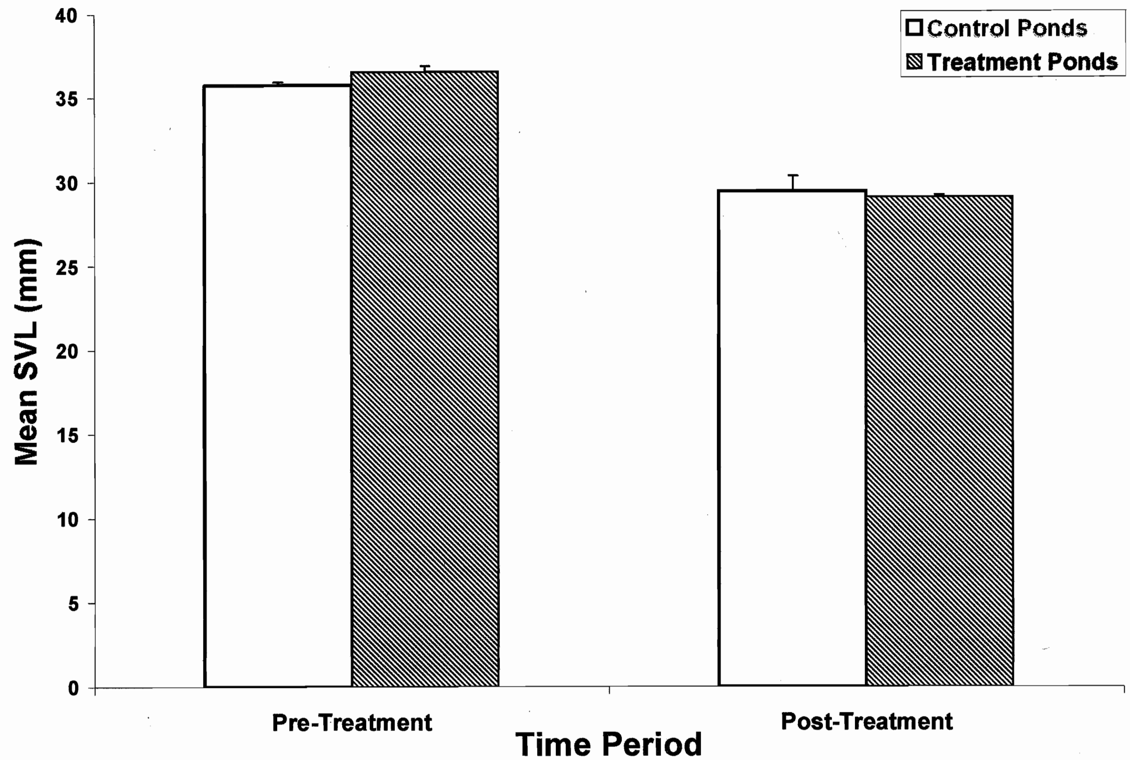

Figure 4: Mean snout-vent-lengths (+1 SE; mm) for young-of-the-year Southern Leopard Frogs (*Lithobates sphenocephalus*) as a function of pond type (control and treatment) and time period (pre-treatment and post-treatment of Rotenone^{TM}) captured at Warbler Woods Nature Preserve in Coles County, Illinois.

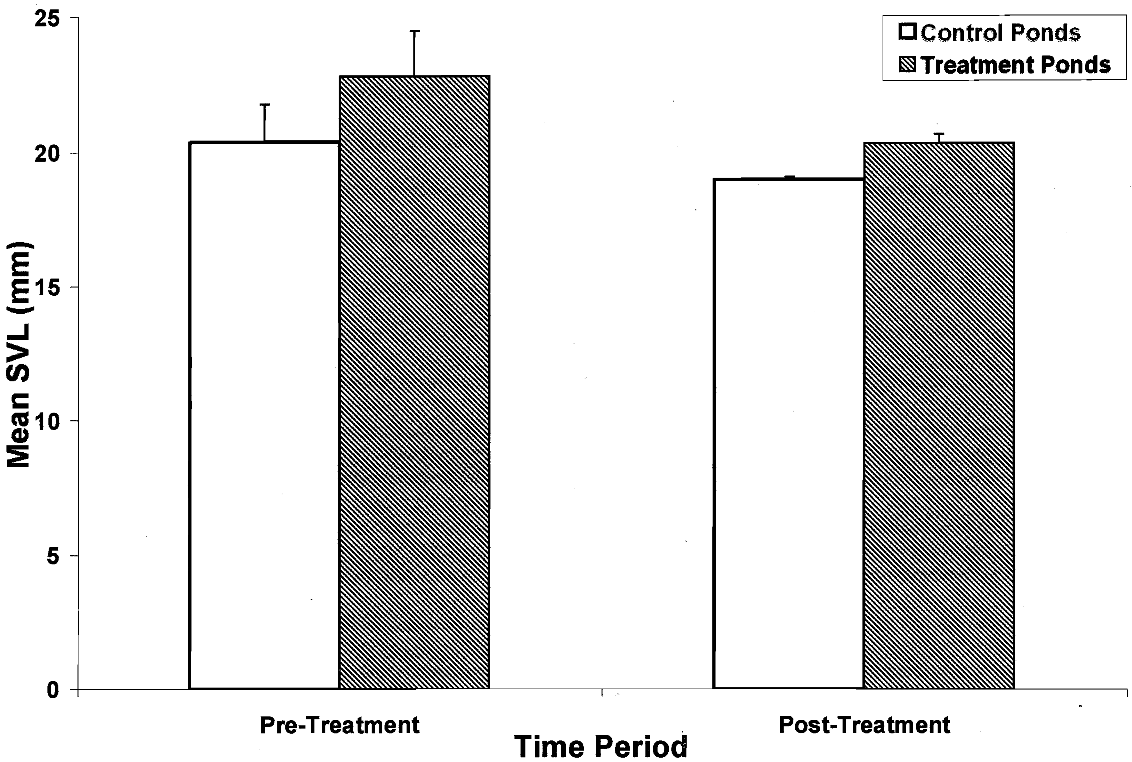

Figure 5: Mean snout-vent-lengths (+1 SE; mm) for young-of-the-year Wood Frogs (*Lithobates sylvaticus*) as a function of pond type (control and treatment) and time period (pre-treatment and post-treatment of Rotenone^{TM}) captured at Warbler Woods Nature Preserve in Coles County, Illinois.

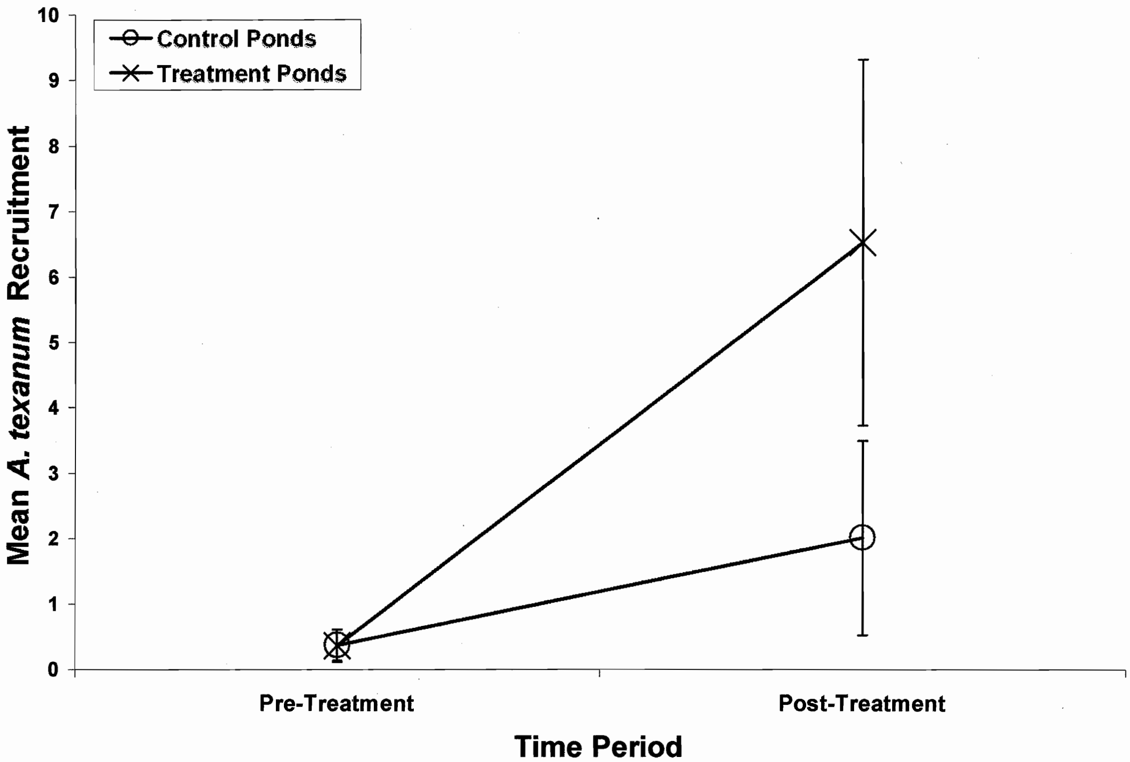

Figure 6: Changes in the recruitment values as a function of time period for Small-mouthed Salamanders (*Ambystoma texanum*) in control and treatment ponds at Warbler Woods Nature Preserve in Coles County, Illinois. Time periods represent pre- and post-application of Rotenone™ to remove fish from treatment ponds. Means are presented ±1 SE.

APPENDIX I

Appendix I: Number of amphibians collected entering or leaving each of four ponds at Warbler Woods Nature Preserve (Coles Co., Illinois). (A) Number of captures in each pond by year (pooled across all species); (B) Number of captures for each species by pond for all sampling years.

A

	Year							
Pond	2001	2002	2003	2004	2005	2006	2007	Total
A	871	286	550	1052	185	417	252	3613
B	1482	1097	1192	894	130	214	177	5186
C	70	221	91	516	102	382	201	1583
D	110	175	39	430	158	95	58	1065
Total	2533	1779	1872	2892	575	1108	688	11447

Appendix I (continued)

B

Sampling results 2001

	Pond				
Species	A	B	C	D	Total
Acris crepitans	0	2	3	0	5
Ambystoma texanum	69	17	13	60	159
Anaxyrus americanus	751	1405	13	9	2178
Anaxyrus fowleri	1	2	0	0	3
Hyla versicolor	0	0	0	5	5
Pseudacris crucifer	5	1	1	1	8
Pseudacris triseriata	0	0	0	0	0
Lithobates catesbeianus	33	46	39	4	122
Lithobates sphenocephalus	3	0	0	6	9
Lithobates sylvaticus	9	9	1	25	44
Total	871	1482	70	110	2533

Appendix I (continued)

Sampling results 2002

	Pond				
Species	A	B	C	D	Total
Acris crepitans	1	0	1	0	2
Ambystoma texanum	59	38	123	45	265
Anaxyrus americanus	177	984	48	32	1241
Anaxyrus fowleri	0	0	0	0	0
Hyla versicolor	0	0	0	0	0
Pseudacris crucifer	11	7	0	1	19
Pseudacris triseriata	0	0	0	0	0
Lithobates catesbeianus	21	36	47	1	105
Lithobates sphenocephalus	6	18	0	27	51
Lithobates sylvaticus	11	14	2	69	96
Total	286	1097	221	175	1779

Appendix I (continued)

Sampling results 2003

	Pond				
Species	A	B	C	D	Total
Acris crepitans	0	0	0	0	0
Ambystoma texanum	202	216	70	24	512
Anaxyrus americanus	14	55	7	10	86
Anaxyrus fowleri	1	0	0	1	2
Hyla versicolor	10	186	1	1	198
Pseudacris crucifer	45	298	1	1	345
Pseudacris triseriata	0	5	0	0	5
Lithobates catesbeianus	78	74	11	0	163
Lithobates sphenocephalus	197	358	0	0	555
Lithobates sylvaticus	3	0	1	2	6
Total	550	1192	91	39	1872

Appendix I (continued)

Sampling results 2004

	Pond				
Species	A	B	C	D	Total
Acris crepitans	2	6	2	0	10
Ambystoma texanum	567	354	412	333	1666
Anaxyrus americanus	18	237	6	5	266
Anaxyrus fowleri	1	0	0	0	1
Hyla versicolor	10	72	0	2	84
Pseudacris crucifer	46	99	19	4	168
Pseudacris triseriata	1	1	1	0	3
Lithobates catesbeianus	64	30	30	1	125
Lithobates sphenocephalus	59	80	0	39	178
Lithobates sylvaticus	284	15	46	46	391
Total	1052	894	516	430	2892

Appendix I (continued)

Sampling results 2005

	Pond				
Species	A	B	C	D	Total
Acris crepitans	0	0	0	0	0
Ambystoma texanum	118	65	83	70	336
Anaxyrus americanus	11	27	3	3	44
Anaxyrus fowleri	0	0	0	0	0
Hyla versicolor	0	0	0	0	0
Pseudacris crucifer	11	10	6	2	29
Pseudacris triseriata	3	3	0	2	8
Lithobates catesbeianus	11	12	4	0	27
Lithobates sphenocephalus	3	1	0	0	4
Lithobates sylvaticus	28	12	6	81	127
Total	185	130	102	158	575

Appendix I (continued)

Sampling results 2006

	Pond				
Species	A	B	C	D	Total
Acris crepitans	2	2	0	0	4
Ambystoma texanum	281	81	324	72	758
Anaxyrus americanus	25	12	2	6	44
Anaxyrus fowleri	0	0	0	0	0
Hyla versicolor	3	24	34	4	65
Pseudacris crucifer	8	9	3	10	30
Pseudacris triseriata	0	0	0	0	0
Lithobates catesbeianus	79	56	13	0	148
Lithobates sphenocephalus	5	25	1	2	33
Lithobates sylvaticus	14	5	5	2	26
Total	417	214	382	95	1108

Appendix I (continued)

Sampling results 2007

	Pond				
Species	A	B	C	D	Total
Acris crepitans	2	1	0	0	3
Ambystoma texanum	172	64	199	54	489
Anaxyrus americanus	4	11	1	2	18
Anaxyrus fowleri	0	0	0	0	0
Hyla versicolor	9	9	0	0	18
Pseudacris crucifer	19	13	0	1	33
Pseudacris triseriata	2	2	1	1	6
Lithobates catesbeianus	7	14	0	0	21
Lithobates sphenocephalus	29	57	0	0	86
Lithobates sylvaticus	8	6	0	0	14
Total	252	177	201	58	688

APPENDIX II

Do Limb Deformities Follow the Periodicity of Atrazine Contamination at a Nature Preserve in Central Illinois?

ABSTRACT

Large-scale agriculture is one of several factors known to contribute to declines in amphibian populations that have been reported during the last several decades. Row-crop farms are a predominant type of land use in Illinois and have impacted amphibian populations not only by increasing habitat fragmentation, but also by increasing agricultural runoff. At Warbler Woods Nature Preserve (WWNP; Coles County, Illinois) from the spring through summer 2006, two enclosures were placed in each of two ponds having differing proximities to drainage from agricultural fields (this experiment was repeated in 2007, but failed). Wood Frog (*Lithobates sylvaticus*) larvae were placed in each enclosure for the duration of their larval period (to completion of metamorphosis) and the incidence of mortality during that period was recorded. Concurrently (including 2007), water and sediment samples were analyzed from both ponds to quantify atrazine contamination; and drift fences were surveyed for amphibians with deformities. No external physical deformities were detected in any of the larvae from the enclosures used in the 2006 experiment; however, three species observed at WWNP had deformities observed, which directly corresponded to atrazine application. There were no differences in atrazine concentrations in water between the two ponds, but atrazine concentrations did vary by year. Majority of water samples collected from WWNP were above levels reported to have detrimental effects on amphibians, with some samples also being above the EPA's drinking water limit of 3.0 μg/L. Limb deformities in juvenile amphibians captured at WWNP were highest following peak atrazine concentrations in 2006 (n = 3)

and 2007 (n = 18). Pond C had more detectable atrazine in soil compared to Pond A, but year had no effect on atrazine detection in the soil. My results indicate that managed wildlife areas are not immune to pesticide contamination, and that the effects of agricultural chemicals should be mitigated to maintain stable amphibian populations.

INTRODUCTION

Habitat destruction and alteration are arguably among the greatest threats to amphibian populations (Semlitsch 2000, Blaustein and Kiesecker 2002). Pond-breeding amphibians depend on wetland and terrestrial habitats for individual and population survival (Semlitsch 2000). Unfortunately, throughout the Midwest of the United States, wetland and surrounding terrestrial areas have been lost to promote the increase demand for agriculture and urban development (Suloway and Hubbell 1994, Ribic et al. 1998). Declines in individual abundance and species richness in amphibian communities have been documented when suitable habitat is lost (Houlahan and Findlay 2003). Agricultural land use, predominant in Illinois, has negatively impacted amphibian populations not only by increasing habitat fragmentation, but also by increasing runoff from agricultural fields.

Agricultural runoff has two major implications for wetland habitat; it increases both sedimentation rates and chemical exposure. An increased build up of sediment can shorten the hydroperiod in wetland areas, thereby reducing reproductive success for aquatic breeding amphibians (Gray et al. 2004). Amphibians that inhabit and breed in wetland and forest habitats surrounded by agricultural landscapes run the risk of direct chemical exposure from agricultural runoff (Boone and Semlitsch 2001). This effluent often consists of pesticide chemicals that have been linked to physical and behavioral deformities in amphibians, and have been hypothesized to be a causative agent for amphibian population declines (Blaustein et al. 2003).

Atrazine is one of the most commonly used herbicides in the United States, with up to 50 million kg applied annually (Eisler 1989, Sass and Colangelo 2006). In fact,

more tonnage of atrazine is applied in Illinois than any other of the United States, particularly on corn crops in the central part of the State (Solomon et al. 1996). The primary use of atrazine is to control weeds in areas planted with corn, sugarcane, and other crops (Solomon et al. 1996). Atrazine is very water-soluble and can lead to ground water contamination (Eisler 1989, Solomon et al. 1996). Because there is such a high risk of ground water contamination, the European Union has banned atrazine use since October 2003 (Sass and Colangelo 2006). Depending on turbidity (atrazine is photodegradable) and pH of a water body, atrazine concentration can be detected in water up to 742 days following application (Solomon et al. 1996). Similarly, residency of atrazine in sediment varies depending on composition. Most studies show that the residence time in sediment can be up to 365 days, but the pesticide is usually detectable only during the first 35 days following application, and within the first 30 cm (Eisler 1989, Solomon et al. 1996, Müller et al. 2003).

It is well known that atrazine can produce adverse physiological effects in amphibians, including endocrine disruption that produces feminization of adult males, hermaphrodites, and gonadal deformities (Hayes et al. 2002, 2003, Hayes 2004). Atrazine was shown to produce gonadal abnormalities in the majority (92%) of *Lithobates pipiens* used in a laboratory study (Hayes et al. 2002, 2003, Hayes 2004). Reeder et al. (1998) found hermaphroditic Northern Cricket Frogs (*Acris crepitans*) more frequently in areas contaminated with atrazine. Endocrine disruptors in general can result in lower reproductive rates and individual fitness and, over time, lead to declining amphibian populations. Therefore, monitoring areas that are designated as nature preserves or sanctuaries for amphibians for both pesticides such as atrazine as well as

amphibian health is critical to determine the risk that such "islands" in a sea of agriculture may impose to these species.

In addition to gonadal deformities, atrazine exposure has been linked to decreases in both body mass and length in metamorphosing amphibians exposed to concentrations greater than 200 μg/L (Diana et al. 2000, Rohr et al. 2004). Forson and Storfer (2006) demonstrated that Tiger Salamander (*Ambystoma tigrinum*) larvae exposed to moderate levels atrazine had increased susceptibility to *Ambystoma tigrinum* virus because of lowered leukocyte levels. Atrazine can decrease the functioning of the immune system in *L. pipiens* and *L. sylvaticus,* which may lead to increased limb deformities (Houck and Sessions 2006). Lastly, ecologically relevant levels of atrazine mixed with other commonly-used pesticides increased the mortality, and decreased growth rate and body size, in *L. pipiens* at rates greater than the individual pesticides alone (Hayes et al. 2006). Likewise, glyphosate, the active ingredient in the pesticide "Round-up" and the second most common pesticide used in the Midwest (after atrazine; Aspelin 1997) may also induce anuran feminization; however, the induction is most likely due to interactions with other stressors. Therefore, it has been suggested that any monitoring of the effects of atrazine in the environment should not ignore the presence of glyphosate (Howe et al. 2004, Battaglin et al. 2005).

Limb deformities are a common outcome in amphibians exposed to various chemicals (Quellet et al. 1997, Loeffler et al. 2001, Blaustein and Johnson 2003) and their occurrence has been documented in 44 of the United States (Loeffler et al. 2001, Kiesecker 2002). Such abnormalities are generally characterized as missing limbs, truncated limbs, extra limbs, or skin webbings (Gardiner and Hoppe 1999). Abnormal

limb developments were shown to be more common in *L. sylvaticus* that inhabit areas exposed to agricultural runoff (Kiesecker 2002). Agricultural chemicals containing carbaryl produced limb deformities in *L. pipiens* (Bridges and Semlitsch 2000). Gardiner and Hoppe (1999) reported multiple limb deformities associated with retinoid exposure, and suggested that chemicals that behave like retinoids may have similar results. Pesticide exposure may decrease function in amphibian immune systems, resulting in higher frequencies of trematode infections that can lead to limb deformities (Kiesecker 2002, Johnson and Sutherland 2003, Johnson et al. 2006).

The majority of toxicological studies involving amphibians and agricultural chemicals have been laboratory-based experiments. Relatively few toxicological studies exist for agricultural chemical runoff *in situ* and its impacts on natural amphibian populations, due to the difficulty of making a direct link between agricultural chemical exposure and adverse ecological effects (Bridges and Semlitsch 2000, Boone and Semlitsch 2001). The number of physical deformities observed among amphibians in the field is relatively low and, therefore, it is difficult to link their occurrence to chemical exposure from surrounding agricultural areas (Newman and Unger 2003).

The purpose of this study was to monitor amphibian populations at the WWNP through drift fence collections as well as an enclosure to quantify limb deformities and explore if there is a relationship with atrazine exposure in both time and space. Therefore the objectives of this study were to determine: 1) the occurrence of limb deformities in wild amphibian populations at Warbler Woods Nature Preserve (WWNP; Coles County, Illinois); 2) the amount and periodicity of atrazine in water and sediment samples from two WWNP ponds that are hydrolically linked; 3) determine the concentration and

periodicity of another common pesticide, glyphosate, to document its potential contribution and/or interaction with atrazine in this drainage system; and, 4) if limb deformities can be induced in native Wood Frog (*L. sylvaticus*) larvae that one allowed to develop *in situ* in WWNP ponds.

METHODS

Study Site

All data were collected from Warbler Woods Nature Preserve (WWNP) in Coles County, Illinois (Fig. 1). The Illinois Nature Preserves Commission of the Illinois Department of Nature Resources (IDNR) manages the 81.5 ha area. There are 3 permanent ponds and 1 ephemeral pond located in the southeast section of WWNP. They are labeled from east to west in alphabetic order. Pond D is the ephemeral pond that dries by 25 June of each year and has the smallest surface area (400 m^2). Pond C has the largest surface area (900 m^2) of all ponds at WWNP. The four ponds have varying coverages of duckweed (*Lemna* sp.) on the surface of the water in each activity season, with the occurrence of this aquaphyte being most prevalent at Ponds B and C. A secondary deciduous forest (primarily oak-hickory) surrounds all four ponds and provides a closed canopy. There is also an old agricultural field between Ponds B and C that has been planted with tree seedlings and saplings as part of an IDNR management plan to restore WWNP to resemble pre-settlement conditions. Residential and agricultural areas lie adjacent to WWNP, with a county road separating the property from these other land uses. Pesticide contamination of wetland habitat at WWNP is possible via agricultural runoff from adjacent fields. Pond C has the greatest physical separation from agricultural fields, while Ponds A and B are closest to these possible sources of contamination (Fig. 1).

Monitoring of Amphibian Limb Deformities at WWNP

Drift fences (constructed out of 45-cm tall silt fencing) and pitfall traps (3-L plastic containers) were installed around the majority ($\geq$ 75 % coverage) of each pond at

WWNP to monitor amphibian populations starting in May 2000. To prevent amphibians from burrowing under the fence, 10 cm of the fence was buried below soil grade. Uniquely labeled traps were buried flush with the soil surface and spaced every 7.5 m on each side of the fence. Traps were monitored every 48 hours during the activity season (usually late February to early December); otherwise, they were sealed to prevent the capture non-target organisms.

Specimens found in the traps were identified, measured for snout-vent length (SVL; ±1 mm) and tail length (TL; ±1 mm), aged as either adult or young-of-the-year (YOY), and sex was determined (when possible). Any limb deformities observed in specimens caught between June 2005 and December 2007 were recorded. Before being released, each individual was toe-clipped to indicate initial collection year and pond location. If a specimen was previously marked, the year and pond of original capture were recorded.

Water and Sediment Samples

To determine if atrazine was present at the site, a pilot water sampling routine was established in 2006. At this time, one 125 ml water sample from Pond A and Pond C were taken weekly from early March through April. Once the field enclosure experiment began (described below), the frequency of water sampling was increased to three 125 ml samples per week per pond. After the enclosures were removed, the water sampling frequency was decreased to once per month until the end of November. Three 125 ml water samples were taken on a weekly basis, beginning in early March through mid-December in 2007, from Ponds A and C. All water samples were frozen until atrazine analysis could be completed. Atrazine concentration analysis was completed using the

protocol from the competitive magnetic particle atrazine enzyme linked immunosorbent assay (ELISA) kit produced by Abraxis LLC (Warminster, PA). Samples were processed in duplicate to determine the variability in the measured atrazine concentration. Concurrently, these same water samples were analyzed for glyphosate, although not at the same intensity. Specifically, 14 water samples (collected between 20 March 2006 to 31 August 2007) from Pond A and 9 samples from Pond C were analyzed for glyphosate, with 100% duplication. Glyphosate concentration was determined by using a competitive magnetic particle ELISA test kit produced by Abraxis LLC (Waminster, PA).

Single sediment samples from each pond were taken once a week using a soil core sampler (diameter = 2.54 cm, coring depth = 0.51 m) from March through June, then monthly from July until end of November in 2006. In 2007, single sediment samples from each pond were taken during March and April, and then increased to three sediment samples per pond per week from May through first week of December. After collection, sediment samples were placed in a freezer until further analysis. To measure atrazine levels present in sediment samples, they were first allowed to thaw overnight. The sediment samples were then dried for a week in an oven at 30 °C. A methanol based extraction protocol produced by Abraxis LLC (Warminster, PA) was followed to prepare sediment samples for analysis. A centrifuge was used during the soil extraction, as described in the alternative protocol produced by Abraxis LLC; otherwise, all other steps of the standard protocol were followed. Atrazine concentrations were determined using an atrazine high sensitivity ELISA test kit produced by Abraxis LLC. Sediment samples were processed in duplicate to determine variability in atrazine concentration. A repeated

measures analysis of variance (ANOVAR) was conducted to determine differences in atrazine concentration between pond type and year for water samples. A logistic regression analysis was conducted to test for differences in atrazine detection between pond type and year in sediment samples. To evaluate the significance of variable coefficients, a Chi-square test that employed a Wald statistic was used.

Lithobates sylvaticus **Field Experiment**

In Spring 2006, adult Wood Frogs (*Lithobates sylvaticus*) were collected from sites around Coles County, Illinois (excepting WWNP), and maintained in an outdoor enclosure (360.7 x 175.3 x 86.4 cm) constructed out of chicken wire fencing on the campus of Eastern Illinois University. This species was chosen because it is present at WWNP, has a relatively short larval period, and has been observed with limb deformities within natural populations (Table 1). During the spring of 2006 and 2007, the collected adults reproduced in a shallow (20.3 cm depth, 101.6 cm diameter) pool of water constructed within the enclosure. The resulting egg masses were transferred into 19-L aquaria filled with aged tap water, and maintained at 21 °C and a 12:12 L:D photoperiod (K. Yager, pers. comm.). All larvae hatching from eggs were fed leaves of iceberg lettuce (*Lactuca sativa* L.) *ad libitum* (K. Yager, pers. comm.) and remained in the lab until released within the WWNP enclosures.

Four enclosures, measuring 76 x 76 x 76 cm, using polyvinyl chloride tubing as the framing were constructed. Two sizes of Nitex® bolting cloth were affixed to the framing to form the walls of each of the two enclosures. These sizes were chosen because of their relevance to parasitic infections of the developing larvae – a 500 μm pore size could admit trematode metacercariae within the enclosure, whereas the 80 μm pore

size prohibited the access by metacercariae (Kiesecker 2002). One enclosure of each pore size was placed in Ponds A and C, respectively. On 13 April 2006, 125 *L. sylvaticus* larvae (at Gosner stages 24-26; Gosner 1960) were placed within each of the four enclosures. In April 2007, this procedure was repeated with 150 larvae per enclosure (at Gosner stages 22-25). The number of Wood Frog larvae was increased from 2006 to potentially increase the number of individuals completing the experiment (however, all larvae died or were depredated in 2007). The remaining larvae in each year were preserved in 70% ethanol so that the age could be determined (Gosner 1960) and tissue samples obtained. Sections of a fishing seine (pore size = 2 mm) were secured over the top of the enclosures to deter predation on the frog larvae. The larvae were allowed to complete their development through metamorphosis before being euthanized. Newly-metamorphed frogs were collected every four days until no specimens remained in any enclosure. Collected metamorphs were euthanized by freezing to preserve possible contaminant uptake and physical deformities.

RESULTS

Incidence of Amphibian Limb Deformities

Three amphibian species exhibited limb deformities at WWNP in 2006, with Small-mouthed Salamanders (*Ambystoma texanum*) accounting for the majority of limb deformities observed (n = 9). Limb deformities in this species were present in Ponds A and B in 2006, and were the only species to be observed with a limb deformity in Pond B (Table 1). Of all species captured in 2006, Wood Frogs had the highest prevalence of limb deformities at 20% (number of individuals with deformities / total number of individuals captured). No captures from Pond D exhibited limb deformities in 2006 (Table 1).

In 2007, a total of 31 individuals captured at WWNP displayed some type of limb deformity, a 182% increase from 2006. The Small-mouthed Salamander again accounted for the largest number of individuals with limb deformities (n = 26), and was the only species observed with limb deformities in Ponds C and D in this year (Table 1). American Bullfrogs (*Lithobates catesbeianus*) had the highest prevalence of limb deformities in 2007 (29% in Pond A), which was also the highest incidence rate during the study period (Table 1). Limb deformities in American Toads (*Anaxyrus americanus*) were observed in individuals from Pond B whereas, in 2006, deformities were observed in captures in this species from Pond A only. No limb deformities were detected in *L. sylvaticus* captured in 2007.

During both 2006 and 2007, limb deformities were most common among individuals trapped in the month of March (n = 21), all of which were part of the adult age class (Table 2). In 2006, limb deformities in the young-of-the-year (YOY) age class

were observed in the months of August, September, and October (Table 2). The incidence of limb deformities in YOY individuals increased six-fold in 2007, and occurred in June, July, August, and October (Table 2). The incidence of limb deformities observed in juvenile amphibians at WWNP was highest following peak atrazine concentrations in both years (Table 2, Fig. 2).

Water and Sediment Samples

Atrazine was found in measurable levels in water samples from both ponds at WWNP throughout 2006 and 2007 (Fig. 2). Water samples from Pond A had a mean (±1 standard error) atrazine concentration of 0.80 ± 0.12 μg/L, and Pond C had a mean atrazine concentration of 0.72 ± 0.10 μg/L in 2006, when corn was planted in surrounding agricultural fields. The mean atrazine concentrations were lower in water samples of both ponds in 2007, when soybeans were planted in the surrounding fields, with Pond A having a mean concentration of 0.25 ± 0.02 μg/L and Pond C with a mean concentration of 0.21 ± 0.01 μg/L. There was no difference in atrazine concentration detected in water between Pond A and Pond C at WWNP ($F_{1,120} = 0.27$, $p = 0.60$; Fig. 3). There was a difference in atrazine concentration in water samples between years of the study ($F_{1,120} = 18.25$, $p = < 0.001$; Fig.3), with samples averaging 0.76 ± 0.08 μg/L and 0.23 ± 0.01 μg/L in 2006 and 2007, respectively. Glyphosate was also detected from water samples collected from Ponds A and C in 2006 and 2007 (Fig. 2). The mean glyphosate concentration in Ponds A and C was highest before atrazine concentrations peaked in 2006, but both atrazine and glyphosate concentrations peaked around the same time in 2007 (Fig. 2).

Of the 245 sediment samples collected, only 18 samples contained detectable atrazine concentrations. There was a difference in detectable atrazine concentration in sediment samples, with Pond C accounting for 78% of the total number of detectable samples (Wald $\chi^2 = 5.47$, $p \leq 0.02$; Table 3). The detectable atrazine in sediment samples ranged from 5.98 to 9.83 μg/L in Pond C, and from 5.97 to 7.11 μg/L in Pond A. There were no differences in atrazine concentration as a function of the year of the study, nor any interaction between pond and year (Wald $\chi^2 \leq 1.26$, $p \geq 0.26$).

***Lithobates sylvaticus* Field Experiment**

In 2006, Wood Frog larvae began completing metamorphosis on 1 June, and all larvae had either completed metamorphosis or perished by 29 June. Also in this year, 92 tadpoles (73.6%) completed metamorphosis in Pond A in the enclosure having cloth walls with a large pore size (500 μm). Within the enclosure having walls of smaller pore size (80 μm) in Pond A, a total of 47 tadpoles (37.6%) completed metamorphosis. No tadpoles completed metamorphosis in either enclosure type in Pond C in 2006. In 2007, none of the larvae completed metamorphosis, regardless of enclosure type or pond. Due to low sample size of surviving *L. sylvaticus* larvae, the incidence of limb deformities was not statistically compared.

DISCUSSION

Enzyme-linked immunosorbent assays (ELISA) have become increasingly useful in toxicological studies and in the monitoring of environmental contaminants. Competitive-based magnetic particle ELISA is useful because of its ability to accurately detect trace amounts of atrazine in water and soil samples (ng/L), relatively low costs, and short time it takes to analyze samples (Lopez-Avila and Charan 1994, Hottenstein et al. 1996). Graziano et al. (2006) obtained accurate and precise results using the ELISA test kits from Abraxis to detect atrazine concentration in drinking water. This study also demonstrates the use of ELISA kits can be a quick and reliable method for effectively detecting atrazine in water and sediment at WWNP.

Based on the hydrological features of wetland complexes, they contain a history of the land-use within their respective watershed. Atrazine and glyphosate are the most prevalent pesticides used in the Midwest and both are heavily used in agriculture. The ELISA results showed that the magnitude and periodicity of these two pesticides mimicked each other in WWNP and therefore are likely to both have impacts on amphibian health. Although no studies to date have directly linked glyphosate to limb-deformities, the potential interaction of these two pesticides should not ignored, but rather documented when exploring issues regarding pesticide exposure in amphibians. In 2006 and 2007, the highest detection of atrazine in water samples occurred in the first week of June, which is when many larval amphibians are exposed during growth and development. The results of this study are consistent with those of Solomon et al. (1996) and Graziano et al. (2006), who found atrazine detection was generally higher in the months of June and July in the Midwest. In 2006, one water sample from each of Ponds

A (6.8 μg/L) and C (5.2 μg/L) had atrazine concentrations above the EPA's 3.0 μg/L standard for drinking water (EPA 2003). Atrazine detection in sediment samples taken at WWNP was very patchy, but limited to concentrations higher than 5.94 μg/L because of the extraction protocol used. This is not an uncommon result because of the extremely variable nature of atrazine in soil and sediment samples (Eisler 1989). Atrazine was detected in sediment samples taken in Pond C more frequently than in samples taken from Pond A. This result might be attributed to the thick layer of duckweed (*Lemna* sp.) that grows on Pond C during the activity season, which limits atrazine decomposition by ultraviolet radiation. Pond A has little to no amounts of duckweed coverage on the water surface.

These data indicate that a cyclic pattern of both atrazine and glyphosate contamination may be occurring temporally at WWNP. Both pesticides were in higher concentrations in water samples of both ponds in 2006, when corn was planted in surrounding agricultural fields, compared to 2007, when soybeans were planted. Atrazine can be applied to soybean fields to control broad-leafed grasses, but its use generally limited to corn, sorghum, and wheat crops in the United States (Eisler 1989). Therefore, during years when soybeans are planted in agricultural fields surrounding WWNP, atrazine contamination is likely originating from agricultural fields that are more distant to the study site. Glyphosate is used on both corn and soybean fields, however, and was likely coming from the surrounding landscape at constant rate. Interestingly, glyphosate peaked at the same time as atrazine in 2007, which may be due to tillage practices. People using no-till farming practices sometimes apply more glyphosate before the planting season to eradicate weeds. This is one likely explanation for the

mimicry in periodicity between the two chemicals, especially in 2006 when there was a spike in glyphosate just before the growing season. Even with the difference in atrazine concentration that occurred between years, a peak in atrazine concentration in water samples occurred in June of both years (irrespective of crop planted on adjacent land).

These results indicate that the pesticide concentrations detected in water and sediment samples taken at WWNP may potentially to have detrimental impacts on the inhabiting amphibian populations. Larval *Lithobates sylvaticus*, *Pseudacris crucifer*, and *Anaxyrus americanus* exposed to 3 μg/L of atrazine have lower survival than larvae exposed to higher concentrations (Storrs and Kiesecker 2004). *Lithobates pipiens* exposed to low levels of atrazine (< 0.2 μg/L) have been reported to experience adverse effects in survival, growth, and gonadal development (Hayes et al. 2003, 2006). Rohr et al. (2006) found that Streamside Salamanders (*Ambystoma barbouri*) exposed to atrazine levels greater than 4 μg/L had higher mortality a year after exposure compared to a control group. Low levels of atrazine exposure can suppress the immune systems in amphibians (Houck and Sessions 2006), which can increase susceptibility to helminth infection and disease. Moreover, glyphosate has been found to completely eradicate two species of tadpoles and nearly wipe out a third species, resulting in a 70% decline in the tadpole species richness (Relyea 2005).

Frogs account for 77.8% of the documented cases of limb malformations in North America, with the remaining salamander species accounting for only 22% of limb deformity cases (Loeffler et al. 2001). Based on current literature searches, this study is the first to document a relatively high incidence of limb deformities in a wild population of Small-mouthed Salamanders (*Ambystoma texanum*). This species accounted for the

majority of limb deformities detected in 2006 (82%) and 2007 (84%) at WWNP. Overall, the prevalence of *A. texanum* individuals that exhibited limb deformities was 1.2% in 2006 and 5.3% in 2007. In both years, adult *A. texanum* with limb deformities were captured while immigrating to the ponds for breeding, although it is unknown if successful reproduction occurred.

In addition to A. *texanum* showing a higher incidence of limb deformities in 2007, *Anaxyrus americanus*, *Lithobates catesbeianus*, and *L. sphenocephalus*, also showed an increased frequency of limb deformities in the second year of my study. Wood Frogs were the only species that had a higher rate of limb deformities in 2006 than 2007. Eaton et al. (2004) found that less than 2% of Wood Frogs in western Canada displayed some type of limb deformity. The absence of limb deformities among trapped *L. sylvaticus* in the 2007 data is more consistent with Eaton et al. (2004) than is the 2006 data, when a 3.8% prevalence of limb deformities was observed (individuals all using Pond C).

Lithobates sylvaticus larvae raised in the field enclosures within Ponds A and C experienced a high mortality in both years of this study, a pattern that could be attributed to several factors. Although predators could not enter the enclosures, their presence in the surrounding areas may have induced stress on the *L. sylvaticus* larvae, as they had no refuge within the enclosures. Multiple stressors, including the combination of pesticides and predators, has been shown to have detrimental effects on some larval amphibians (Boone and Semlitsch 2001, Rohr et al. 2004, Rohr and Crumrine 2005). The pesticide levels detected at WWNP may have directly and/or indirectly lead to the mortality experienced in the enclosures. Another possibility is that the enclosures restricted natural movements of the larvae, which in turn, limit their ability to obtain food and other

resources (Boone and James 2005). Enclosure studies allow specimens to be surrounded with all the variability found in the environment, but one's ability to single out the exact cause for any one pattern in the data is limited (Boone and James 2005).

Recurring toxicant exposure might render the populations of some amphibian species less resilient to other anthropogenic disturbances (Semlitsch et al. 2000). Although only Ponds A and C were tested for pesticides, it is likely that Ponds B and D have a similar contamination because of the short distance between the ponds. Future water and sediment samples are needed to validate this possibility. The close proximity of agricultural fields to WWNP might allow other agricultural chemicals to runoff into the water and sediment at WWNP, and these should also be subject to screening and research. Although *L. sylvaticus* larvae within the enclosure experiment exhibited low survival rates, it would be worth repeating this study with *Ambystoma texanum* larvae because this species had the highest prevalence of limb deformities at WWNP. These results indicate that managed wildlife areas are not immune to pesticide contamination, and that the effects of these agricultural chemicals should be mitigated to maintain stable amphibian populations.

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Table 1: The prevalence of limb deformities (number of captures with limb deformities divided by the total number of individuals captured) observed in amphibian species at each of four ponds at Warbler Woods Nature Preserve, Coles County, Illinois, in 2006 and 2007.

	2006				2007			
	Pond				Pond			
Species	A	B	C	D	A	B	C	D
Ambystoma texanum	0.02	0.01	0.00	0.00	0.07	0.09	0.03	0.04
Anaxyrus americanus	0.04	0.00	0.00	0.00	0.00	0.09	0.00	0.00
Lithobates catesbeianus	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.00
Lithobates sphenocephalus	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
Lithobates sylvaticus	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00

Table 2: The number of amphibians captured (using pitfall traps) in each month that exhibited limb deformities at Warbler Woods Nature Preserve, Coles County, Illinois. The values are shown for young-of-the-year (YOY) and adult age classes in each month of the 2006 and 2007 activity seasons.

	2006		2007	
Month	YOY	Adult	YOY	Adult
March	0	8	0	13
April	0	0	0	0
May	0	0	0	0
June	0	0	4	0
July	0	0	11	0
August	1	0	1	0
September	1	0	0	0
October	1	0	2	0

Table 3: Detectable concentrations of atrazine in sediment samples collected at Warbler Woods Nature Preserve, Coles County, Illinois, during 2006 and 2007 in Pond A and Pond C. Only 7% of 245 sediment samples had detectable atrazine concentrations.

Date	Pond	Sample Number	Atrazine Concentration (µg/L)
06/29/2006	C	1	6.00
08/30/2006	C	1	5.98
09/30/2006	C	1	8.99
10/31/2006	A	1	5.97
10/31/2006	C	1	6.00
04/12/2007	C	1	8.02
05/11/2007	C	1	9.83
06/16/2007	C	3	8.60
06/22/2007	C	3	6.68
07/12/2007	C	2	8.02
07/12/2007	C	3	6.55
08/10/2007	C	2	6.64
08/17/2007	C	2	6.01
09/21/2007	C	2	6.54
09/28/2007	A	3	7.11
11/10/2007	C	1	6.85

Table 3: continued.

Date	Pond	Sample Number	Atrazine Concentration (μg/L)
11/16/2007	A	1	6.26
11/23/2007	A	3	6.24

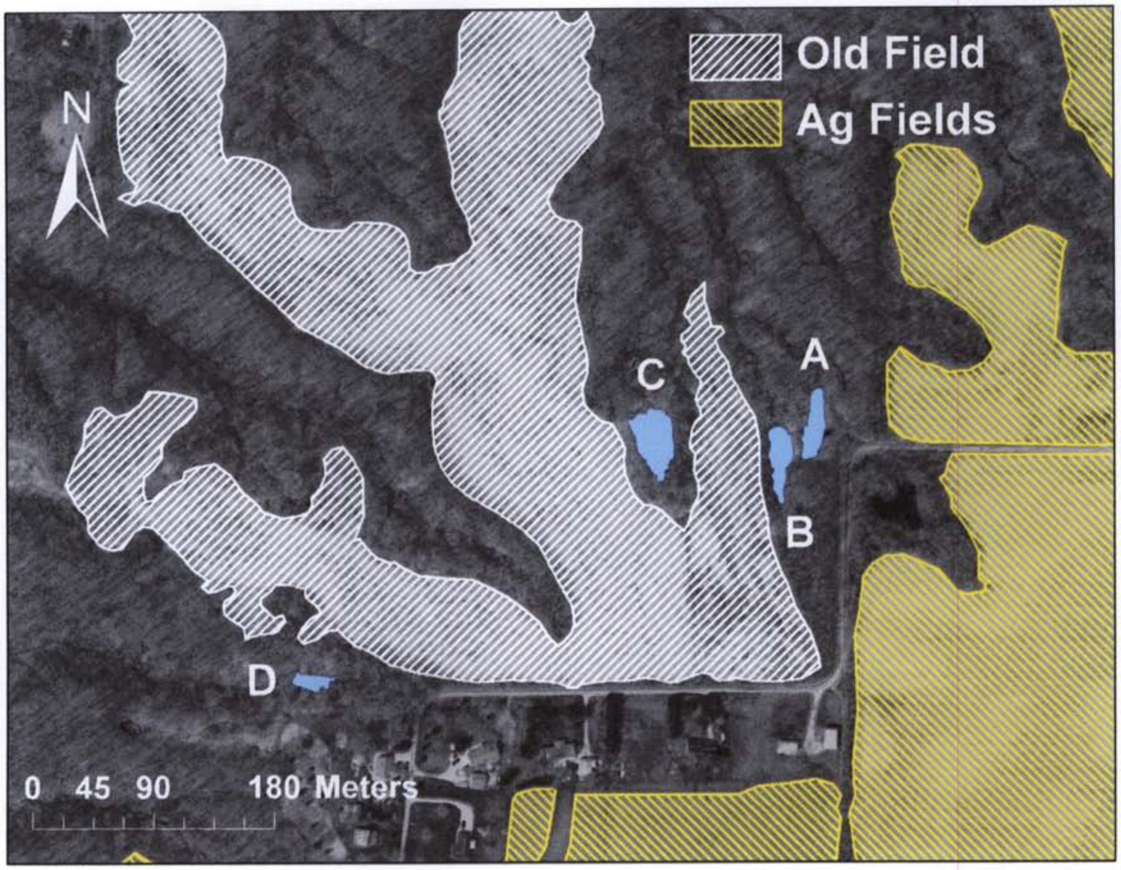

Figure 1: The southeastern portion of Warbler Woods Nature Preserve (WWNP), Coles County, Illinois. The four study ponds are highlighted in solid blue, with associated old fields in hatched white and forest cover unmarked. Neighboring agricultural fields have been highlighted in yellow-hatched areas. Pond A is closest to currently active agricultural fields, while Pond C has the greatest habitat buffer within WWNP.

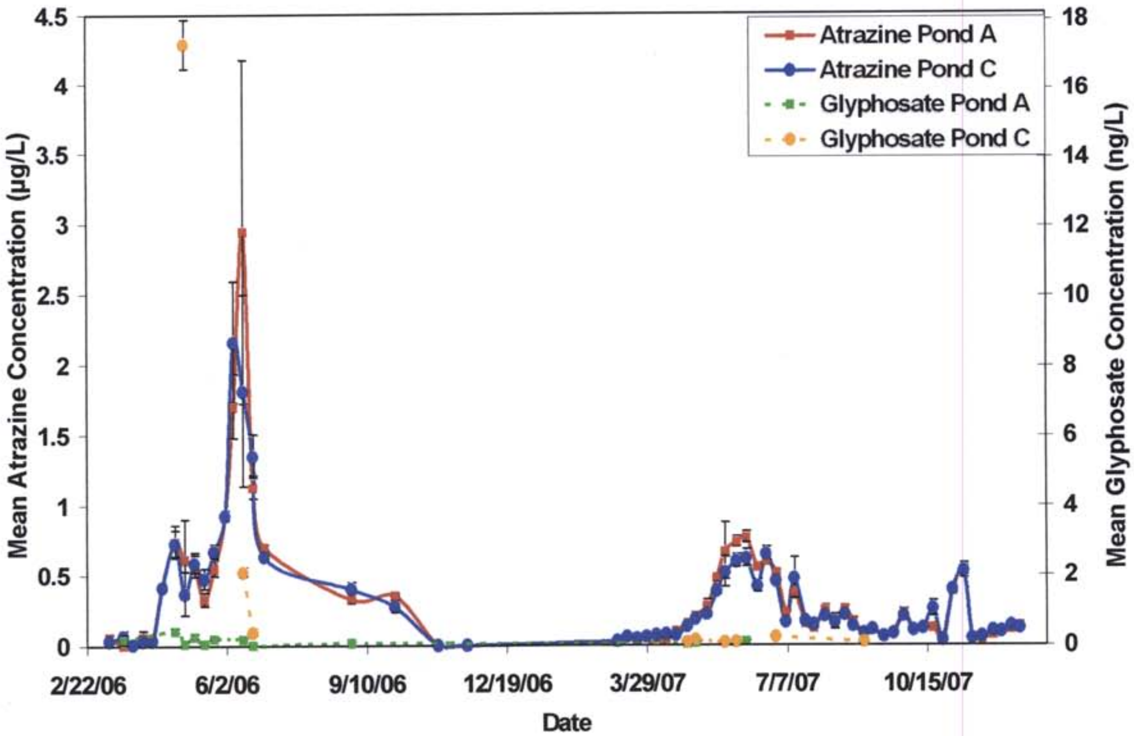

Figure 2: Mean (±1 SE) atrazine concentration (µg/L) of water samples collected from Ponds A (red) and C (blue) at Warbler Woods Nature Preserve, Coles County, Illinois, from March 2006 to December 2007. Mean (±1 SE) glyphosate concentration (ng/L) of a subset of water samples collected from Ponds A (green) and C (orange).

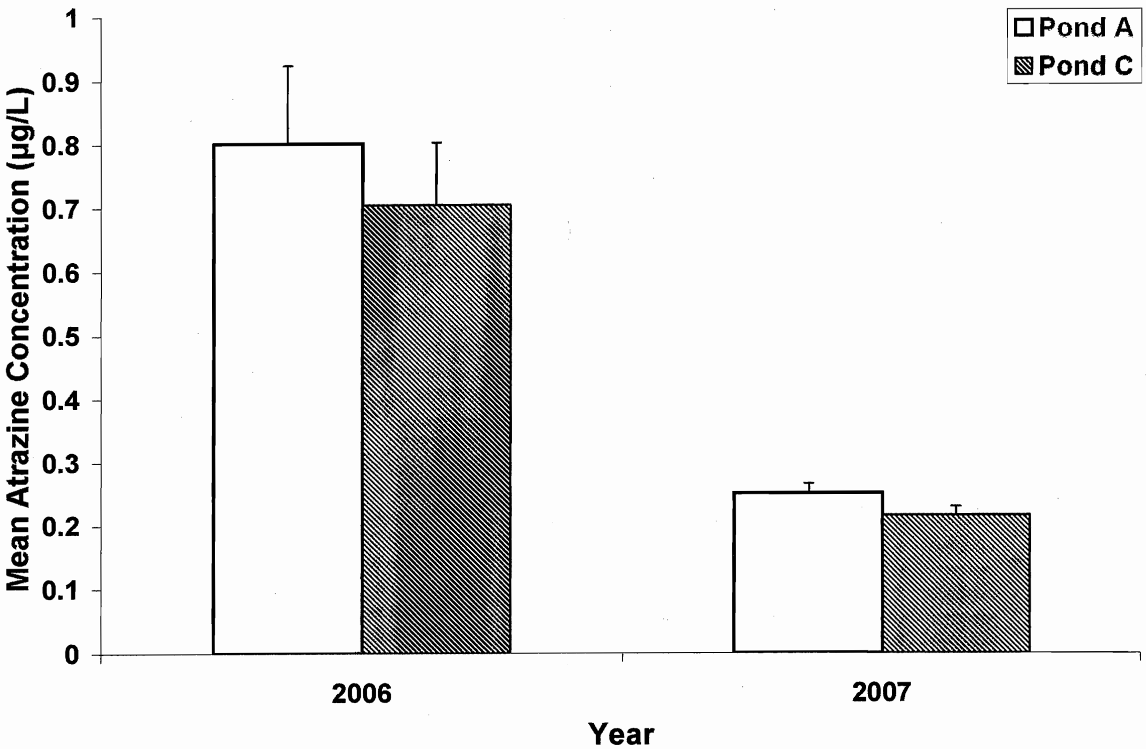

Figure 3: Mean (+1 SE) atrazine concentration (µg/L) of water samples taken from Ponds A and C at Warbler Woods Nature Preserve, Coles County, Illinois during 2006 and 2007.

APPENDIX III

Appendix III: The prevalence and mean intensity of helminthes found in 23 Bullfrogs (*Lithobates catesbeianus*) collected from Warbler Woods Nature Preserve in Coles County, Illinois in 2006 and 2007. Mean abundance of helminthes was 16.7 ± 1.8 worms per frog. Codes for position in frog: L = lung, S = stomach, SI = small intestine, LI = large intestine, and UB = urinary bladder.

	Position in frog	Prevalence (% infected)	# worms per infected frog (intensity; mean ± SD)
Digenea			
Gorgodera sp.	SI, LI, UB	56%	8.8 ± 6.2
Haematoloechus sp.	L	78%	7.5 ± 5.0
Megalodiscus sp.	LI	39%	7.4 ± 4.9
Nematoda			
Aplectana sp.	S, SI, LI	56%	6.5 ± 5.8
Spinitectus sp.	S	26%	3.3 ± 1.7