Investigating Effects of the Piscicide Rotenone on Amphibians in Southwestern Montana Through Laboratory Experiments and Field Trials

By

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A thesis
submitted in partial fulfillment
of the requirements for the degree of
Master of Science in the Department of Biological Sciences
Idaho State University

October 2010

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Summary

Over the past two decades, amphibian populations have experienced significant decline world-wide as a result of habitat loss, habitat alteration, disease, and climate change. To better conserve amphibian communities, it is imperative to develop a knowledge base of how amphibians respond to habitat alterations and environmental stressors. Amphibian habitat changes in the western United States are occurring, in part, as a result of native fish restoration practices. To reverse the impacts of introduced, non-native fish species, fisheries managers are removing non-native species and restoring native species to historic habitats. A preferred and efficient method for removal of non-native fish species is through the use of approved piscicides, but these chemicals can have measurable, negative effects on amphibian populations. The focus of this research is to determine the effects of piscicide use on amphibians through controlled laboratory experiments and field investigations in southwestern Montana. Laboratory trials demonstrated that rotenone exposure at 1 mg/L (product) was lethal to tadpoles of two species – Columbia spotted frog *Rana luteiventris* and boreal toad *Anaxyrus boreas* – at all three age stages tested. In spotted frog tadpoles, the probability of mortality decreased as age increased, while age did not affect average mortality in boreal toad tadpoles. Tadpole species had a significant effect on mortality only at the oldest age stage, with spotted frog tadpoles experiencing lower mortality then boreal toad tadpoles ($p < 0.001$). Sub-lethal effects on morphology, although statistically different between control and exposed spotted frog survivors in two instances, were not consistent and were not considered biologically significant. My results further indicated that as the duration of rotenone exposure increased, spotted frog tadpole mortality increased, except at the oldest age stage. The results of the field investigations revealed that, in the 24 hours following application, rotenone was lethal to gill-breathing amphibian tadpoles and non-lethal to non-gill breathing metamorphs, juveniles, and adults. In the year(s) following, tadpole repopulation occurred at all water bodies treated with rotenone product. The information obtained from these two components will be used to better inform future fish restoration actions.

Chapter 1
Introduction

Many amphibian populations are currently in a state of significant decline and disruption world-wide (Knapp et. al 2007). These changes in abundance have been attributed to a variety of factors, such as disease, climate change, and habitat destruction. Because amphibians play a key role in structuring ecological communities, understanding these rapid declines is important (Alford & Richards 1999; Collins & Storfer 2003; Corn 2003; Blaustein & Bancroft 2007). Amphibians play a key role in structuring ecological communities. Adult amphibians feed largely on a variety of invertebrate species, while tadpoles maintain algal communities at levels conducive to invertebrates, an important food source for other aquatic organisms (Blaustein et al. 1994; Young et al. 2004). Additionally, amphibians are a key dietary component for a variety of organisms, including mammals, fish, reptiles, and birds (Blaustein et al. 1994; Young et al. 2004). Changes to the abiotic environment – climate change and habitat alteration – may be reflected in amphibian populations, thereby affecting other species and making some amphibian species useful indicators of ecosystem health (Young et al. 2004; Whiles et. al 2006; Patla et al. 2007). Useful information can be gleaned from understanding amphibian responses to alterations in their environments. This information can guide future conservation practices.

Amphibian conservation depends, in part, on amphibian resilience to human caused environmental stressors. Changes in water chemistry – water temperature, pH, input of chemicals like pesticides, herbicides, and fertilizers, and concentrations of dissolved oxygen, nitrogen, phosphorous, and ammonium – influence the life histories of

many amphibian species and affect the rate at which individuals move from the larval period to metamorphosis (Cummins 1989; Johansson 2000; Greulich 2003; Gerlanc 2005; Rose 2005; Griffis-Kyle & Ritchie 2007). Chemical input, in particular, can affect amphibian populations, and there are a wide variety of human-introduced chemicals that amphibians encounter.   From pesticides to herbicides to detergents, homeowners facilitate the introduction of a wide variety of household chemicals into the natural environment. These chemicals can be applied in spray, powder, or crystal form and many have been identified as having effects on non-target species of animals and plants.

Pesticide and herbicide use for large scale agricultural practices is highly prevalent in the United States. Between 1991 and 1998, over 1.5 billion pounds of pesticides were applied on California farms and agricultural fields (Kegley et al. 2000). That number continues to increase. At the national level, 200 million agricultural acres are treated with over 100 different kinds of chemicals each year (Hill 1995). Current studies on the effects of agricultural chemicals have focused almost exclusively on the impacts on eggs and tadpoles amphibian life stages (Cowman and Mazanti 2000). These studies indicate that the majority of chemicals comprising pesticides, insecticides, and herbicides cause mortality, deformity, or decreased performance in a wide variety of amphibian species (Cowman and Mazanti 2000).

Specific aspects of amphibian ecology and physiology make them particularly susceptible to the negative effects of introduced chemicals. Many species of amphibian inhabit small ponds that exist on the fringes of human development. These ponds can frequently receive toxins through aerial pesticide depositions and runoff (Henry 2000).

Additionally, because amphibians have multiple life stages, toxin exposure can occur multiple times at different stages during development (Henry 2000).

With regard to physiology, the skin of amphibians is particularly unique and is involved in various aspects of homeostasis (Pough et al. 2004). Amphibian skin is made up of a thin, outer epidermal layer and an inner dermal layer. Mucus glands produce secretions that protect individuals from dehydration and predators (Pough et al. 2004). Acting as a respiratory surface, amphibian skin absorbs substances, including chemicals, in the local environment (Ultsch et al. 1999). This incorporation of chemicals by both adult and larval amphibians can have direct and indirect effects on amphibian communities through mortality, limb deformity, and disruption of bodily processes (Henry 2000).

In addition to human input of chemicals, the presence or absence of predators influences amphibians. Predators can induce changes and adaptations in the life histories of their prey, including accelerating the rate at which amphibians move through vulnerable, non-reproducing stages (Abrams 2001). Multiple organisms prey on amphibian tadpoles, and tadpoles are faced with a trade-off between spending more time developing and growing larger, though increasing exposure to predators, and metamorphosing faster, but at a smaller size, to avoid predation (Rose 2005). In the red-eyed treefrog *Agalychnis callidryas*, adult females deposit eggs on vegetation situated above water bodies; tadpoles that hatch later and larger avoid larval predation better than tadpoles that hatch earlier (Warkentin 1999). The presence of egg eating snakes, detected by vibration, can push *A. callidryas* tadpoles to hatch/escape earlier than otherwise expected (Gomez-Mestre & Warkentin 2007). A trade-off exists because larval survival

increases with delayed hatching and an increase in size, but egg predation can accelerate hatching at the expense of development (Gomez-Mestre & Warkentin 2007). Tadpoles of multiple anuran species have exhibited predator-caused changes in behavior, morphology, and habitat choice (Relyea 2003; Relyea 2004; Relyea & Auld 2005; Richter-Boix et al. 2007).

A number of studies have further identified alterations in timing of hatching and metamorphosis in amphibians in the presence of aquatic arthropod and fish predators. Aquatic arthropods prey on both amphibian eggs and larvae, with the specific stage experiencing predation influencing the plasticity of the response. In a 2007 study, Ireland et al. found that green frogs *Rana clamitans* exposed to leeches, an egg predator, hatched earlier. Eggs exposed to dragonfly nymphs, a larval predator, hatched later, producing larger tadpoles (Ireland et al. 2007). Similar results have been seen in studies that compare the timing of hatching and other morphological characteristics between clutches exposed to either egg predators (beetles or crayfish) or larval predators (dragonfly larvae). Egg predators induce early hatching, through sensing of vibrations or chemical cues, while larval predators tend to induce morphological plasticity that selects for larger body size – either late hatching or tail developments – (Johnson et al. 2003; Saenz et al. 2003; LaFiandra & Babbitt 2004; Teplitsky et al. 2004; Yurewicz 2004; Kraft et al. 2005; Vonesh 2005; Wilson 2005).

Fish, non-native species in particular, have been documented to have negative effects on larval amphibians directly, through predation, and indirectly, by affecting behavior. The impacts of predation by fish, and their subsequent removal, on amphibians have been well documented (Bradford et al. 1993; Pilliod & Peterson 2000; Pilliod &

Peterson 2001; Vredenburg & Wake 2004; Mullin et al. 2004; Anholt et al. 2005; Knapp 2005; Ilsh et al. 2006; Walston & Mullin 2007; Boone et al. 2007). Amphibians detect the presence of fish by chemoreception, and fish can also therefore indirectly, negatively impact amphibian habitat use, time spent foraging, (Binckley & Resetarits 2003; Orizaola & Brana 2003; Bernard 2006; Barr & Babbitt 2007) and, most importantly, development. Embryonic palmate newts *Triturus helveticus*, for example, exposed to predator cues from brown trout *Salmo trutta* developed faster and metamorphosed earlier at a smaller size than control larvae (Orizaola & Brana 2005).

Non-native fish can also exert a negative influence on populations of native fish. Historically, fisheries management in this country focused on recreational fishing opportunities. In the western part of the United States, this focus led to stocking water bodies with non-native species. Populations of native fish, in particular cutthroat trout *Oncorhynchus clarkii*, have been completely extirpated, reduced in abundance, or compromised because of hybridization and/or competition with nonnative fish (Behnke 1992; Finlayson et al. 2005; Hamilton et al. 2009). While introduced fish populations are not the only factor contributing to native fish declines – population declines can also be attributed to habitat loss – their negative impact is both significant and reversible (Behnke 1992; Hamilton et al. 2009; Williams et al. 2009). Non-native fish removal is an effective method of native fish conservation, and, therefore, has become a central component of a growing number of fisheries management programs.

Fish removal can be accomplished by a variety of techniques, but an effective method for large scale, complete removal has been the use of the EPA approved piscicides, rotenone and antimycin (Finlayson et al. 2000; Finlayson et al. 2005; Moore et

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al. 2008). Chemical removal, especially in larger systems with complex habitat, is often more cost and time effective, with a higher probability of success than traditional methods, like gill-netting or electroshocking (Shepard et al. 2002). As a result, the use of piscicides in fisheries management is increasing (Mangum & Madrigal 1999; Finlayson et al. 2000; Ling 2002; McClay 2005; Hamilton et al. 2009). Rotenone products, in particular, have a proven record of consistent and efficacious removal of undesirable fish (Finlayson et al. 2000; McClay 2005).

Rotenone is an organic compound made from the roots of tropical legumes (Fontenot 1994). In water, it is readily absorbed across the gill epithelial layer of aquatic species and halts cellular respiration by preventing electron transfer in cell mitochondria. Specifically, rotenone prevents cells from converting NADH into useable energy (ATP) (Fontenot 1994) by occupying the binding sites ordinarily used by NADH in this conversion process (Oberg 1966).

The method and timing of rotenone applications is relatively standard across projects. Rotenone products are typically applied to streams and ponds at a dosage range of 0.5 to 1 mg/L (Grisak et al. 2007 (b)), but label recommendations can vary from as little as 0.1 mg/L for selective treatments to as much as 5 mg/L for preimpoundment treatments (EPA, CFT Legumine Label). In the Rocky Mountain west, rotenone applications typically occur during a small, 3 month-long window of time in mid to late summer through early fall (Grisak et al. 2007 (b)). This application period typically coincides with the larval life stage of local amphibian species.

Rotenone's effects on fish are well-documented (Meadows 1973, Amey 1984, Finlayson 2000, USFWS 2005, Britton 2006, Grisak et al. 2007 (a)), while its effects on

amphibians are less well known. Because rotenone enters through the gill membrane,

larval amphibians, have the potential to be negatively impacted by use of this piscicide

more than adult animals (Fontenot et al. 1994; McCoid & Bettoli 1996; Maxell 2000;

Patla 2005). Little research has been done with regards to the actual effects of rotenone

on amphibians, though rotenone has been used to remove fish to help conserve amphibian

populations (Mullin et al. 2004; Walston and Mullin 2007). In these instances, rotenone

was applied prior to amphibian breeding.

The most recent research on the effects of rotenone was conducted on three

species of Rocky Mountain stream dwelling amphibians. Grisak et al. 2007 (b) applied a

range of doses of a formulation of rotenone to Columbia spotted frog adults *Rana

luteiventris*, long-toed salamander tadpoles *Ambystoma macrodactylum*, and Rocky

Mountain tailed frogs tadpoles *Ascaphus truei* over a 96 hour period. The results

mirrored that which might be expected based on rotenone's mechanism of entry (i.e.

absorption across the gill membrane). The spotted frog adults survived exposure to

rotenone at 4.5 times the field dose (i.e. 1 mg/L), while long-toed salamander and tailed

frog larvae experienced mortality at doses significantly lower than the typical field dose

of 1 mg/L product (Grisak et al. 2007 (b)).

With the increase in rotenone applications to remove non-native fish, an improved

understanding of the chemical's effects on amphibians is needed. As mentioned

previously, rotenone will most likely have a negative effect on tadpoles while it appears

to not have lethal effects on adults. Information on the effects of specific factors, like age

and species and dosage level, on tadpole mortality could potentially enhance amphibian

conservation at fish restoration sites. Because these chemicals do not appear to have

lethal effects on adults, piscicide treated ponds will most likely be repopulated one

breeding season after application, but it is still unknown whether this repopulation will

occur at levels similar to those seen pre-treatment. Additionally, there is no data yet on

the long-term population level effects of removing an entire cohort of tadpole as a result

of piscicide application. In an effort to promote amphibian conservation in fish

restoration sites, this research seeks to determine the short and long-term effects of the

piscicide rotenone on amphibians in both a laboratory and field setting.

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Chapter 2
Toxicity of the piscicide Rotenone to Columbia spotted frog *Rana luteiventris* and boreal toad *Anaxyrus boreas* tadpoles

Abstract

The piscicide rotenone is commonly used to remove non-native fish from aquatic systems. While the effects of this chemical on fish are well documented, the impacts of rotenone on amphibians are less well known. I determined the toxicity of rotenone (CFT Legumine forumulation) to Columbia spotted frog *Rana luteiventris* and boreal toad *Anaxyrus boreas* tadpoles under laboratory conditions. Mortality after a 96 hour exposure period was examined by exposing tadpoles at three age stages to different doses of CFT Legumine (5% rotenone) (0 mg/L (control), 0.1 mg/L, 0.5 mg/L, 1 mg/L). Individuals surviving a 96 hour exposure period were part of a sub-lethal effects trial that measured delayed mortalities, time to metamorphosis, weight, and snout-urostyle length. An additional exposure duration trial was conducted with spotted frog tadpoles at three ages to determine survivability when exposed to CFT Legumine at 1 mg/L for 1, 2, 3, or 4 hours before being placed in rotenone-free water. My results demonstrated that rotenone exposure was lethal to tadpoles of both species at all three age stages. Individuals exposed to the common field application dose (1 mg/L product) experienced significantly greater mortality than control tadpoles ($p<0.001$). In spotted frog tadpoles, the probability of mortality decreased as age increased, while age did not affect average mortality in boreal toad tadpoles. Tadpole species had a significant effect on mortality only at the oldest age stage, with spotted frog tadpoles experiencing lower mortality than boreal toad tadpoles ($p<0.001$). Sub-lethal effects on morphology, although statistically different between control and exposed spotted frog survivors in two instances, were not consistent and were not considered biologically significant. My results further indicated that as the duration of rotenone exposure increased, spotted frog tadpole mortality increased, except at the oldest age stage. Fisheries managers can use these results to improve amphibian conservation in fish restoration areas.

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Introduction

      Native salmonid species are experiencing significant declines throughout much of the United States. In some cases, this decline has been attributed to the presence of non-native fish species (Behnke 1992; Finlayson et al. 2005; Hamilton et al. 2009).   In an effort to reverse this trend, a growing number of fisheries managers are utilizing non-native fish removals. This can be accomplished by a variety of techniques, but an effective method for large scale, complete removal has been the use of the EPA approved piscicides, rotenone and antimycin (Finlayson et al. 2000; Finlayson et al. 2005; Moore et al. 2008). Chemical removal, especially in larger systems with complex habitat, is often more cost and time effective, with a higher probability of success than traditional methods, like gill-netting or electroshocking (Shepard et al. 2002). As a result, the use of piscicides in fisheries management is increasing (Mangum & Madrigal 1999; Finlayson et al. 2000; Ling 2002; McClay 2005; Hamilton et al. 2009). Rotenone products, in particular, have a proven record of consistent and efficacious removal of undesirable fish (Finlayson et al. 2000; McClay 2005).

      Rotenone is an organic compound made from the roots of tropical legumes (Fontenot 1994). In water, it is readily absorbed across the gill epithelial layer of aquatic species and halts cellular respiration by preventing electron transfer in cell mitochondria. Specifically, rotenone prevents cells from converting NADH into useable energy (ATP) (Fontenot 1994) by occupying the binding sites ordinarily used by NADH in this conversion process (Oberg 1966). Oberg 1966 also observed rotenone molecules moving away from these binding sites, thereby reversing the lethal effects.

      Rotenone's effects on fish are well-documented (Meadows 1973, Amey 1984, Finlayson 2000, Britton 2006, Grisak et al. 2007 (a)), but effects on aquatic non-target

organisms, like amphibians, are not well understood. Because rotenone enters readily

across the gill membrane, larval amphibians have the potential to be negatively affected

by use of this piscicide (Fontenot et al. 1994; McCoid & Bettoli 1996; Patla 2005).

Because of this, some rotenone applications to aquatic systems have been timed to avoid

amphibian breeding (Mullin et al. 2004; Walston and Mullin 2007). However, in regions

with extended winters and late springs, like the Rocky Mountain west in the United

States, application of rotenone often coincides with the breeding season or larval period

of amphibians. This may have negative consequences for larval amphibian populations.

Amphibians are an important component of aquatic ecosystems. They are both a

prey and predator species and, as such, play a key role in structuring ecological

communities. In addition, changes to the environment – climate change, disease

outbreaks, and habitat alteration – may be reflected in amphibian populations, thereby

affecting other species and making amphibians useful indicators of ecosystem health

(Whiles et. al 2006; Patla et al. 2007). If a management strategy results in the reduction

or even loss of amphibian populations, it could have detrimental effects on the entire

ecosystem.

In the western United States, fish removal projects present an interesting

amphibian conservation dilemma. Introduced fishes can often reduce amphibian

populations through predation (Knapp et al. 2007). Further, non-native fish species can

exert greater predatory influence on all life stages of amphibians if they have a wider

gape than their native counterparts; removal of non-native fish from aquatic systems is

therefore predicted to have an overall positive effect on amphibian populations (Bradford

et al. 1993; Pilliod & Peterson 2000; Pilliod & Peterson 2001; Mullin et al. 2004;

Vredenburg & Wake 2004; Knapp 2005; Welsh et al. 2006; Walston & Mullin 2007). Ultimately, while the removal of non-native fish will most likely benefit local amphibian populations, the use of chemicals like rotenone to accomplish this removal has the potential to negatively impact larval populations.

Given the increased use of rotenone, it is important to develop an improved understanding of its effects on these organisms. Rotenone products are typically applied to streams and ponds at a dosage range of 0.5 to 1 mg/L (Grisak et al. 2007 (b)), but label recommendations can vary from as little as 0.1 mg/L for selective treatments to as much as 5 mg/L for preimpoundment treatments (EPA, CFT Legumine Label). Early research provides an initial understanding of the general effects of rotenone at a range of doses on both adults and larval frogs. Farringer (1972) reported LC_{50} values of greater than 3.2 mg/L (product) exposure (Noxfish formulation; produced by Prentiss Incorporated) for adult Northern leopard frogs *Rana pipiens*, indicating high tolerance of the piscicide in adults as non-gill breathers. 24-hour LC_{50} values for tadpoles of various species in another study, however, were below or within the range of doses used for fish removal, and demonstrated that rotenone applications could have negative effects on larval amphibians (Fontenot 1994). Interestingly, one of the earliest studies addressing the effects of rotenone (Noxfish formulation) on larval amphibians recorded LC_{50} values of 0.1 mg/L Noxfish product (Hamilton 1941). This perhaps indicates that, for this particular formulation, there is no concentration low enough to be tolerated by tadpoles that could still potentially negatively affect fish.

These studies begin to provide important information on the response of tadpoles to rotenone exposure, but the effects of dosage (treatment level), age, and species on

rotenone's toxicity to amphibian larvae need further investigation. Additionally, it is not known whether rotenone exposure results in sub-lethal effects or if all durations of exposure to rotenone are lethal to tadpoles. The objectives of this study were as follows: 1) determine the toxicity of rotenone to Columbia spotted frog *Rana luteiventris* and boreal toad *Anaxyrus boreas* tadpoles at four treatment levels; 2) determine the effects of tadpole age and species on mortality when treated at a typical field application dose (1 mg/L product); 3) determine whether rotenone exposure results in delayed mortality and sub-lethal effects on morphology; and 4) determine whether the duration of rotenone exposure affects the probability of tadpole mortality. By selecting two species that frequently coexist with fish in the intermountain west, the overarching goal of this study was to provide fisheries managers with information that would enable amphibian conservation in conjunction with native fish restoration.

Methods

The methodology for the laboratory trials was approved by the Idaho State University Animal Welfare Committee. Approximately 1,500 early stage (Gosner stage 21-25) (Gosner 1960) Columbia spotted frog and boreal toad tadpoles were collected from a single pond in southwestern Montana in early May 2008 or 2009 for experiments conducted in a given year. Tadpoles were collected by dip net from multiple clutches and transported 40 km back to a laboratory where they were evenly distributed among four 100 gallon outdoor holding tanks containing well water at ambient temperature. Well water, as opposed to city water, was used because it is not chlorinated. Animals were fed a mixed diet of algae wafers and Mazuri dry animal meal (National Aquatic Species Restoration Facility; Alamosa, CO) every one to two days. Ammonia and nitrate levels

in the holding tanks were monitored daily. Water was changed every other day or when

ammonia levels were greater than 0.25 mg/L.

Exposure Trials

   Exposure trials addressing treatment, age, and species effects were conducted as a

3 x 4 factorial experiment (four treatment levels tested at each of three age stages).

Tadpoles of both species at three different age stages – early (Gosner stage range 21-25),

middle (Gosner stage range 30-35), and late (Gosner stage range 40-45) – were exposed

to CFT Legumine (5% active rotenone; produced by Prentiss, Incorportated) at four

treatment levels. Treatment levels were as follows: 0 mg/L (control), 0.1 mg/L, 0.5

mg/L, and 1 mg/L, where 1 mg/L represents a commonly recommended field dose for

stream and pond treatments (Grisak et al. 2007 (b); EPA, CFT Legumine Label). Glass

fish tanks (7.6 L) with 6 L of un-chlorinated well water were used in the experiments

with eight replicate tanks per treatment level. One day prior to treatment, tadpoles were

removed from the outdoor holding tanks and held indoors in a 38 L tank for 24 hours.

   Because of laboratory constraints, spotted frog exposure trials were conducted in

2008, while boreal toad exposure trials were completed in 2009. However, in order to

test for species effects within the same year, a spotted frog exposure trial at the 1 mg/L

treatment level (all age groups) was repeated alongside the toad trials in 2009. For the

first two spotted frog age groups, ten tadpoles were randomly assigned to each of 32

experimental tanks (4 dosages x 8 replicates). For the late stage trial for this species, the

number per tank was decreased to five to protect against maintenance related mortalities.

For the youngest boreal toad tadpole trial, ten tadpoles were randomly assigned to each of

the 32 experimental tanks (4 dosages x 8 replicates). As toad tadpoles increased in age

and size, the number per tank decreased to five tadpoles (Gosner stage 30-35) and three

tadpoles (Gosner stage 40-45). Tanks were randomly assigned to treatment (dosage) levels. Tadpoles were given approximately one hour of acclimation time in the actual test tanks and were fed a portion of an algae wafer prior to treatment application. The temperature in the laboratory was approximately 20.2^0C.

A treatment solution was made by combining 0.25 mL of well-agitated CFT Legumine from a stock barrel and 625 mL of un-chlorinated well water. The amount of the solution delivered to each tank depended on the treatment level (0.1 mg/L, 0.5 mg/L, 1 mg/L). Control tanks received 15 mL of un-chlorinated well water. Different pipettes were used for each treatment group and tank water was swirled after receiving the treatment for even distribution. Because a large number of the oldest (Gosner 40-45) spotted frog tadpoles survived the highest treatment level (1 mg/L) in 2008, an additional trial at a treatment level of 2 mg/L was conducted. All methods for this trial were as described above.

Tadpoles were monitored for mortality immediately following treatment application. Mortality was determined visually (tail curling) and physically (lack of response to gentle prodding). Tadpole mortality was assessed every two hours for the first 10 hours following treatment and then twice a day for three subsequent days, as per ASTM standards (ASTM 2002). The exposure period lasted for 96 hours.

Data from the 2008 and 2009 exposure trials were used in separate analyses in the following ways. Dosage effects for spotted frog tadpoles were assessed by graphing 2008 mortality data by treatment level over time for each age group separately, while 2009 mortality data was used to determine dosage effects for boreal toad tadpoles. Age and species effects in both boreal toads and spotted frogs were determined separately at

the 1 mg/L treatment level using 2009 mortality data only. To assess age effects, mortality at the field application dose of 1 mg/L was compared across age stages using simple logistic regression, where tadpole age was a predictor of mortality, the binomial response. Tank effects were tested for by nesting tanks within treatment. The interaction between age stage and species at the 1 mg/L treatment level was tested using a cross-product test in the logistic regression analysis. All statistical analyses were run in MiniTab (MiniTab 15) unless stated otherwise.

Sub-Lethal Effects Trials

If, at the end of the 96 hour exposure period, tadpoles survived the 1 mg/L treatment, survivors were pooled together into a 10 gallon tank containing fresh, un-chlorinated water and monitored for sub-lethal effects until metamorphosis. Tadpoles were pooled at this time because of constraints on laboratory space. An equal number of control tadpoles were kept separately. The number of replicate tanks and tadpoles per tank varied, depending on the number of survivors and laboratory space, but tadpoles were allocated in an effort to minimize density related effects on growth and survival. The number of tadpoles per tank was the same between treatments, and food was dispensed as evenly as possible across tanks. Delayed mortalities were documented and visual assessments of development (changes in size, body shape, and tail absorption) were monitored. Metamorphosis was determined visually by the loss of the tail. Upon reaching this stage, metamorphs were weighed, measured (SUL), and then euthanized with an overdose of Tricaine Methane Sulfonate (MS-222). Time to metamorphosis was recorded.

Weight, SUL, and time to metamorphosis between exposed and unexposed tadpoles at each age stage for which there were data were compared statistically for

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spotted frogs using an ANOVA in SAS (v.9.1) and a Kruskal-Wallace test. Delayed mortality was defined as mortality that occurred after the initial 96 hr exposure trial was complete. Delayed mortalities of control and treated tadpoles were compared in Epi Info (v. 3.3) for spotted frogs using a Yates Corrected Chi Square test and for boreal toads using logistic regression.

Length of Exposure Trials

In 2009, spotted frog tadpoles, collected and housed as described above, at all three age stages were exposed to CFT Legumine for 6 treatment times: No rotenone exposure (control) and 1 mg/L for 1 hour, 2 hours, 3 hours, 4 hours, and 96 hours. Individuals in the 1, 2, 3, and 4 hour treatment groups were placed in rotenone-free water at the end of their assigned exposure period. For the youngest age trial, ten tadpoles were randomly assigned to each of the tanks. As tadpoles increased in size, the number per tank decreased to five tadpoles (Gosner stage 30-35) and three tadpoles (Gosner stage 40-45). Tadpole mortality was assessed for all treatment groups every two hours for the first 10 hours following treatment and then twice a day for three subsequent days, as per ASTM standards (ASTM 2002).

Mortality curves were used to visually assess the effects of different exposure durations on spotted frog tadpoles by age group. Analysis of the effects of exposure at 1 mg/L for 4 hours was compared to exposure to 1 mg/L for 96 hours using a Fisher's Exact test. This analysis was run for each age stage.

Results

There were no tank effects detected in any of the exposure or exposure duration trials (p-values ≤ 0.07). I could not determine tank effect in all of the sub-lethal trials because in some instances I only had one tank for each treatment group.

Exposure Trials

Rotenone exposure caused mortality in tadpoles in both species at all age stages tested, but mortality was not uniform across doses (Figures 2.1 and 2.2). In 2008, early age stage spotted frog tadpoles treated at 1 mg/L for 96 hours experienced average mortality of 100%. Average mortality at this treatment level declined to 73% and 57% at the middle and older age stages, respectively. Mortality of spotted frog tadpoles treated at 0.5 mg/L for 96 hours did not occur as quickly, and, with the exception of the youngest age stage, was, on average, less than that observed at the 1 mg/L treatment – 100%, 2%, and 25% for the early, middle, and late stages, respectively. Mortality at the 0.1 mg/L treatment was low for all age stages, but the 2 mg/L treatment caused 100% mortality after 96 hours in late stage spotted frog tadpoles (Figure 2.1).

In 2009, there was 100% mortality in early and middle age stage spotted frog tadpoles after 96 hours of exposure to CFT Legumine at 1 mg/L, while late stage tadpoles similarly exposed experienced only 6% average mortality (Figure 2.3). The difference between the mortality rates of late and early or middle age stage spotted frog tadpoles in 2009 was significant (Late vs. Early: $Z = -5.20$, $p<0.001$; Late vs. Middle: $Z = -4.51$, $p<0.001$). Exposed boreal toad tadpoles experienced average mortality of 99%, 83%, and 96% after 96 hours of exposure to 1 mg/L in the early, middle, and late stages, respectively (Figures 2.2 and 2.4). Age did not significantly affect mortality in boreal toad tadpoles at this treatment level (Figure 2.4; p-values ≥ 0.07). Similar to spotted frogs, mortality in boreal toad tadpoles treated at 0.5 mg/L for 96 hours occurred later and declined as tadpole age increased; 48%, 38%, and 17% for the early, middle, and late age stages, respectively (Figure 2.2).

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There was no effect of species on mortality in tadpoles exposed to CFT Legumine at 1 mg/L in 2009 at the early and middle age stage ($Z = -0.65$, $p = 0.51$). However, there was a significant effect of species in the late age stage. Spotted frog tadpoles at this stage (Gosner 40-45) appeared more resistant to the effects of rotenone than boreal toad tadpoles ($Z = -5.47$, $p < 0.001$).

Sub-Lethal Effects Trials

Spotted frog survivors used for these trials included exposed (1 mg/L) and unexposed tadpoles from the middle (Gosner 30-35) and late (Gosner 40-45) age stages treated in 2008. I had too few survivors in the early age stage to include in the sub-lethal effects analysis. There were no significant differences in measured traits between exposed and control tadpoles in the middle age stage. Among late age stage spotted frog tadpoles, individuals exposed at 1 mg/L were 0.38 grams heavier than their negative control counterparts ($F_{1,25} = 5.19$, $p = 0.031$). In 2009, there were an insufficient number of toad tadpole survivors at the early and late age stages, so the only individuals included in this portion of the research were exposed (1 mg/L) and unexposed middle age stage individuals. There were no significant differences in measured traits observed between exposed and control middle age stage boreal toad tadpoles (p-value always > 0.07).

There was no significant difference in delayed mortality between control and exposed middle age stage boreal toad tadpoles, though there was a substantial difference in delayed mortality between exposed and control spotted frog tadpoles in the middle age stage. In the middle age stage, treated (1 mg/L) spotted frog tadpoles had a total delayed mortality of 59% (N = 13 out of 22) compared to only 9% for the control tadpoles. When this total delayed mortality is added to the mortality observed during the actual exposure trial, cumulative mortality of the middle age stage spotted frog tadpoles treated at 1 mg/L

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in 2008 was 80% - closer to the 100% mortality seen in 2009. There were no observed

delayed mortalities in either control or treated surviving spotted frog tadpoles in the late

age stage trial.

Length of Exposure Trials

There was little mortality of spotted frog tadpoles exposed to 1 mg/L CFT

Legumine for less than 4 hours and then revived in fresh water (Figure 2.5), so only

results comparing 4 hours and 96 hours of exposure are reported here. There was

significantly less mortality between early and middle age stage spotted frog tadpoles

exposed at 1 mg/L for 4 hours and those exposed for 96 hours ($p<0.001$ at both age

stages) (Table 2.1), but not in the oldest age group. Survival of individuals exposed for 4

hours and then revived was, on average, 92%, 80%, and 83% for early, middle, and late

stage tadpoles, respectively. By comparison, there was no survival of early and middle

age stage tadpoles exposed at 1mg/L for 96 hours in 2009, while late age stage tadpoles

exposed for the full 96 hours experienced 94% survival (Table 2.1, Figure 2.5).

Discussion

This study suggests that rotenone is lethal to spotted frog and boreal toad tadpoles

at different dose levels, including one commonly used in fish removal projects (1 mg/L

product). These results are similar to those found in two recent studies that also showed

that rotenone is lethal to larvae of multiple amphibian species, often at doses well below

regularly used field application levels (Grisak et al. 2007; Little & Calfee 2008,

unpublished data). My results demonstrate to some extent, a dose response, indicating

that lower doses of CFT Legumine resulted in fewer mortalities. Treating at 0.5 mg/L in

a field setting would likely result in fewer tadpole mortalities than treating at 1 mg/L.

Age appeared to mitigate the effects of rotenone in spotted frog tadpoles, with mortality declining as tadpole age increased. Late age stage tadpoles were relatively resistant to the effects of rotenone, even after a 96 hour exposure period. There was no age effect seen in boreal toad tadpoles, as mortality was similar across age groups. Regardless of the species, if tadpoles were younger than Gosner stage 35, there was very high mortality when exposed to 1 mg/L CFT Legumine for 96 hours. At the youngest age stage, even treatment levels of 0.5 mg/L caused at least 50% mortality for both species. At the oldest age stage (Gosner stage range 40-45), spotted frog tadpoles were significantly less likely to die than boreal toad tadpoles when exposed at 1 mg/L product for 96 hours in this experiment.

The significant effect of age and species at the late age stage range seen in this portion of the research may have been a result of the physiological changes occurring in ranid (frog), but not bufonid (toad), tadpoles. Throughout the tadpole phase, members of the ranid family (i.e. spotted frogs) undergo lung development to supplement O_2 intake and, by the very late stages (Gosner 44-45), rely very little on gill-breathing. Bufonid tadpoles, by contrast, are fundamentally lungless, and remain gill-breathers throughout this life stage (McDiarmid & Altig 1999). Rotenone is absorbed across the gill membrane, and both the age and species effects seen in late stage spotted frog tadpoles may be attributed to the shift from gill to lung breathing.

The variation seen in late stage spotted frog tadpole mortality at 1 mg/L between 2008 and 2009 may also be a function of lung development in ranid tadpoles. Tadpoles used in the 2008 trial were within the assigned age stage range (40-45), but were, on average, on the younger side of the range (40-43). There was 57% mortality in this trial.

Tadpoles in the 2009 trial, however, were purposefully tested on the older side of the range (44-45), and experienced only 6% mortality. This difference in mortality could be a result of the quickening shift to lung breathing at the end of this life phase. The variation in mortality also gives some indication on how best to time rotenone treatments in order to cause the least amount of tadpole death.

Timing rotenone applications to avoid the larval stage altogether would be ideal, but, in areas like the Rocky Mountain west, accessibility can be difficult to impossible during the times in which larval amphibians are not present. Rotenone applications typically occur during a small, 3 month-long window of time in mid to late summer through early fall (Grisak et al. 2007 (b)). Though a suitable period of time for rotenone applications, the short growing season experienced in the Rocky Mountain region is also the breeding and larval period for local amphibian populations. Columbia spotted frogs and boreal toads, for example, begin breeding and egg-laying in May and June, often while ice and snow still cover portions of the lake or pond; tadpoles of both species typically take 2 to 3 months to metamorphose (Werner et al. 2004; Koch & Peterson 1995; Hovingh 1993). Given the overlap of the tadpole stage of many Rocky Mountain amphibian species and the optimal period for rotenone applications, fisheries managers could time applications to minimize tadpole mortality. My results demonstrate that, at least in spotted frogs, very late stage tadpoles (Gosner stage 40-45) experience lower rates of mortality, indicating that timing rotenone applications to coincide with this larval period could result in lower spotted frog tadpole death.

It should be noted that my laboratory results were obtained using precisely measured amounts of rotenone. During field treatments, it may be difficult to treat at

exactly 1 mg/L, for instance, because of irregularities in the shapes of the water body in question or overlap in drip station treatment in flowing water. In order to obtain 100% fish mortality, dosing at levels somewhat higher than 1 mg/L to compensate for irregularities in the calculations may be necessary or inadvertently occur. Limited evidence from my study suggests that treating at 2 mg/L can result in 100% tadpole mortality, even at the late age stage. If, however, field conditions allow for an application of rotenone at a treatment level lower than 1 mg/L, lower tadpole mortality could be expected. Additionally, while I was able to evenly agitate and distribute the CFT Legumine in the lab, uneven mixing and distribution of rotenone products may occur in the field which could potentially affect toxicity to tadpoles. The conclusions of this study should be interpreted with caution as they are derived from controlled laboratory experiments and may not reflect what happens under field conditions (i.e. the influences of environmental factors on rotenone's toxicity).

In the sub-lethal effects portion of this study, because the majority of tadpoles of either species at Gosner stage 35 or younger died when exposed to this dosage of CFT Legumine, I focused on the effects of rotenone on metamorphosis in late stage spotted frog tadpoles. Although this component was limited because of the lack of tank replication, rotenone did not appear to have negative effects on the size or timing of metamorphosis of late stage spotted frog tadpoles, and there was no delayed mortality. In fact, exposed late stage tadpoles were heavier than controls; this could have potentially resulted from slight differences in feeding and/or tadpole densities. The fact that I did not find consistent results for the variables tested in this portion suggests that feeding and tadpole density did not have an effect. The few surviving middle age stage spotted frog

tadpoles (n = 7) at this treatment level (1 mg/L product) did experience higher delayed mortality than their control counterparts. Despite the fact that my findings from the sub-lethal effects portion of the study appeared biologically insignificant, the importance of characteristics like weight, SUL, and time to metamorphosis cannot be understated given their influence on future survival as juveniles and adults (Bridges 2002; Buckley 2005; Capellan & Nicieza 2007). Indeed, other pesticides, like atrazine, have been demonstrated to cause significant sub-lethal effects in amphibians (Cowman & Mazanti 2000). Further research is warranted.

The length of exposure to rotenone also affected the level of mortality in spotted frog tadpoles, with shorter exposure periods resulting in fewer mortalities. This was particularly evident in the early (Gosner 21-25) and middle (Gosner 30-35) age stages. At these two age ranges, spotted frog tadpoles exposed to CFT Legumine at 1 mg/L for 4 hours experienced significantly lower mortality than those exposed for 96 hours (Early: $p < 0.001$; Late: $p < 0.001$; Figure 2.5). I did not run comparative analyses at each treatment level (i.e. exposure length), but the trends seen in Figure 2.5 give clear insight into the negative effects of exposure length on mortality and, more importantly, the point at which significant mortality begins to occur. At the youngest two age stages, significant mortality began at 6 hours. There was no discernable trend among late stage tadpoles; mortality in exposed tadpoles in this age group was never higher than 20%.

These findings provide options for tadpole conservation that should be considered while planning a rotenone treatment. If rotenone application must coincide with early or middle age stage spotted frog tadpoles or boreal toad tadpoles of any age, my results suggest that fisheries managers can reduce mortality in any of three ways. Given

available resources, larval amphibians in restoration areas could be conserved by removing them prior to rotenone application and reintroducing them when the rotenone is no longer active. According to my research, if tadpoles cannot be removed prior to application, they can be collected in the first 4 hours after treatment before significant mortality occurs and recovered in fresh, untreated water. Exposure to rotenone causes lethargy in tadpoles, making them easier to capture. Finally, if applications of less than 1 mg/L will still accomplish fish removal goals, managers could use lower dosages of rotenone to reduce impacts to larval amphibians. In these ways, fisheries managers can salvage the bulk of a tadpole population during a rotenone application instead of potentially losing the entire cohort.

This study provides information on the effects of age, species, and exposure duration on the toxicity of rotenone to tadpoles, but much remains to be done. Continued field research and laboratory experiments addressing, among other things, the effects of the environment on rotenone's toxicity to larval amphibians are needed. As a compound, rotenone is not stable and degrades relatively quickly when applied to water. This decomposition is affected by environmental factors, such as sunlight, water temperature, organic debris, and water pH (Fontenot 1994), with rotenone products degrading rapidly when exposed to sunlight and warm water temperatures. Case in point, Fontenot (1994) cites a range of half-lives for the rotenone product Noxfish (Prentiss, Incorporated) of 10.3 days in water temperatures between 32 to 41° F and 0.94 days in water temperatures between 73 to 81° F. Rotenone's effects on amphibians in the field may ultimately be influenced by the chemical's interactions with water temperature, sunlight, substrate, and other environmental factors. Additionally, other environmental influences on amphibians

may affect their sensitivity to rotenone. Overall, it is clear that rotenone applications can

result in widespread larval mortality, but my results suggest that this mortality can be

avoided or partially mitigated. Native fish restoration and amphibian conservation are

not mutually exclusive, and the results of this research provide insight into ways in which

both can be accomplished.

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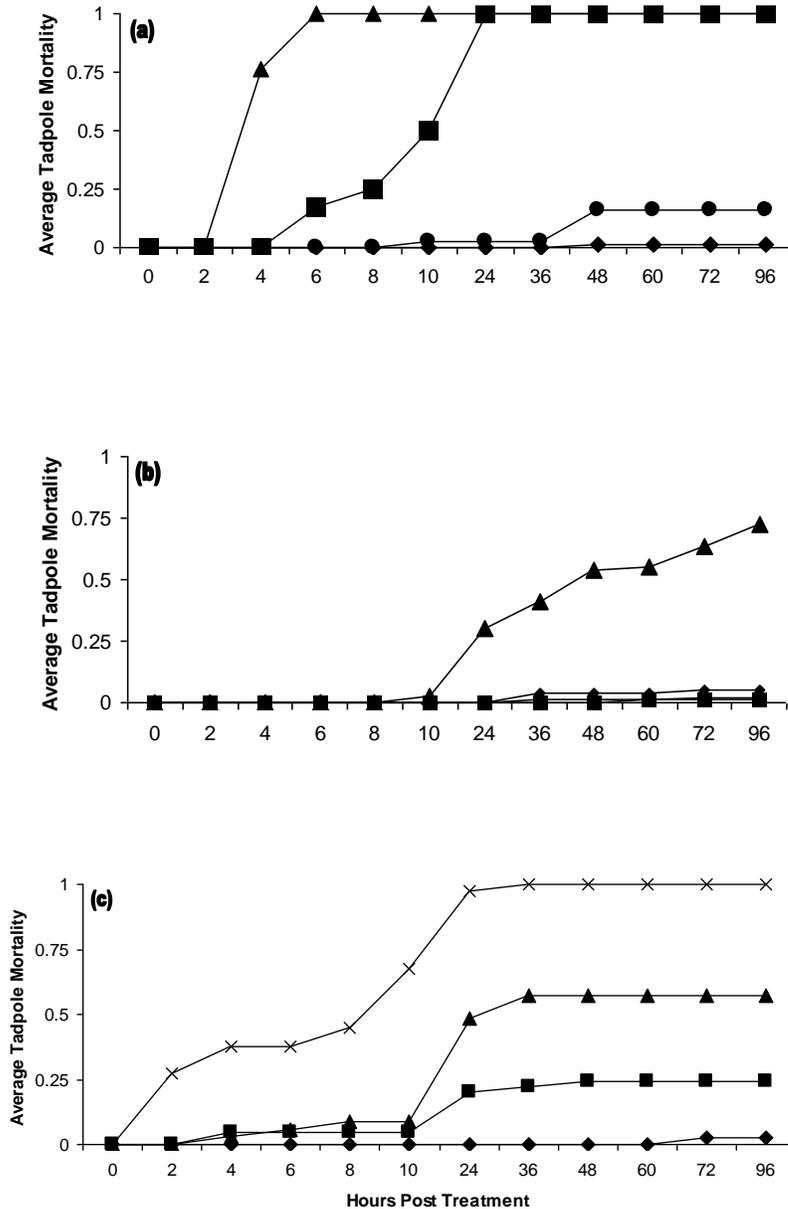
Figure 2.1–2008 cumulative mortality curves for spotted frog tadpoles at all three age stages. Age stages are graphed sequentially (i.e., 21-25 (a), 30-35 (b), 40-45 (c)). Triangles denote the 1 mg/L treatment, squares denote the 0.5 mg/L treatment, circles represent the 0.1 mg/L treatment, and diamonds denote the control group. In the trial of the Gosner stage 40-45 group (panel c), stars represent the 2 mg/L treatment. There were 10 tadpoles per tank in the first two age stage trials and 5 tadpoles per tank in the final age stage trial. Average mortality was standardized by dividing by the number of tadpoles per tank.
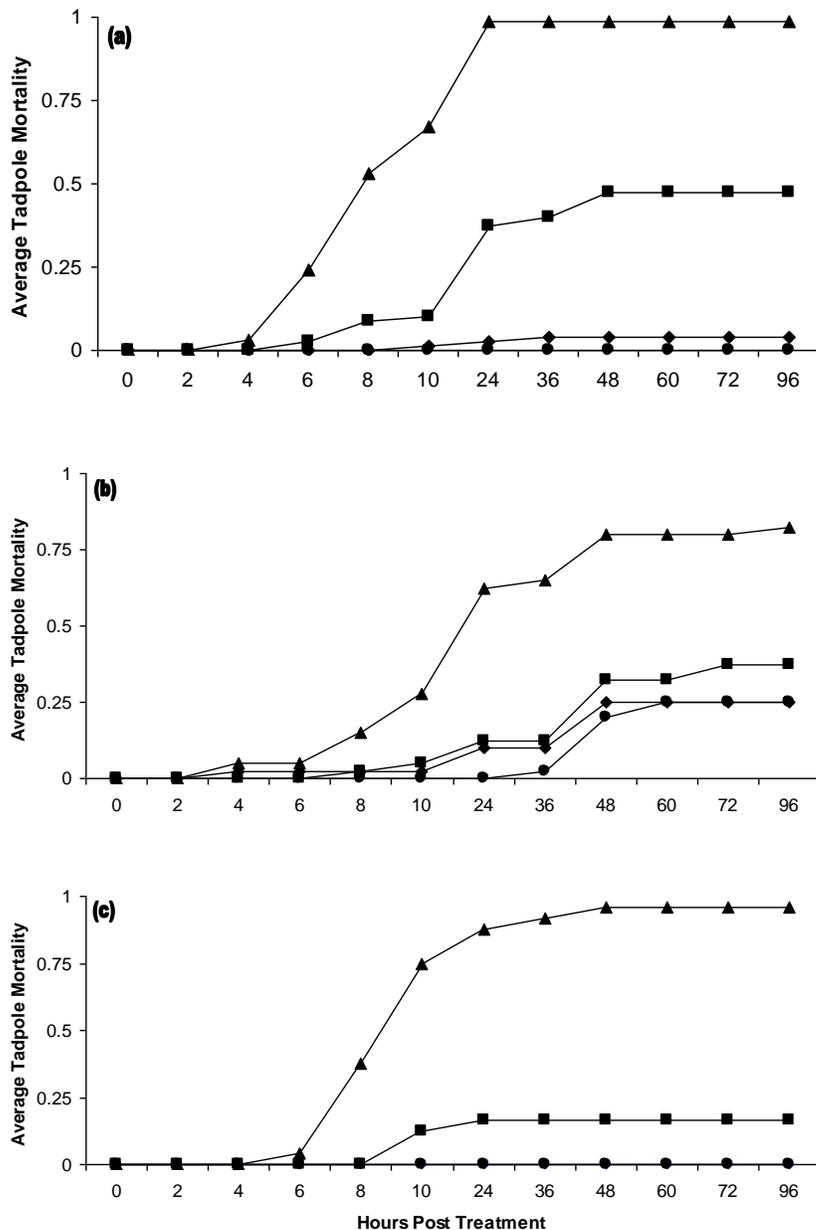
Figure 2.2–2009 cumulative mortality curves for boreal toad tadpoles at all three age stages. Age stages are graphed sequentially (i.e., 21-25 (a), 30-35 (b), 40-45 (c)). Triangles denote the 1 mg/L treatment, squares denote the 0.5 mg/L treatment, circles represent the 0.1 mg/L treatment, and diamonds denote the control group. There were 10 tadpoles per tank in the first age stage trial, 5 tadpoles per tank in the middle age stage trial, and 3 tadpoles per tank in the late age stage trial. Average mortality was standardized by dividing by the number of tadpoles per tank.
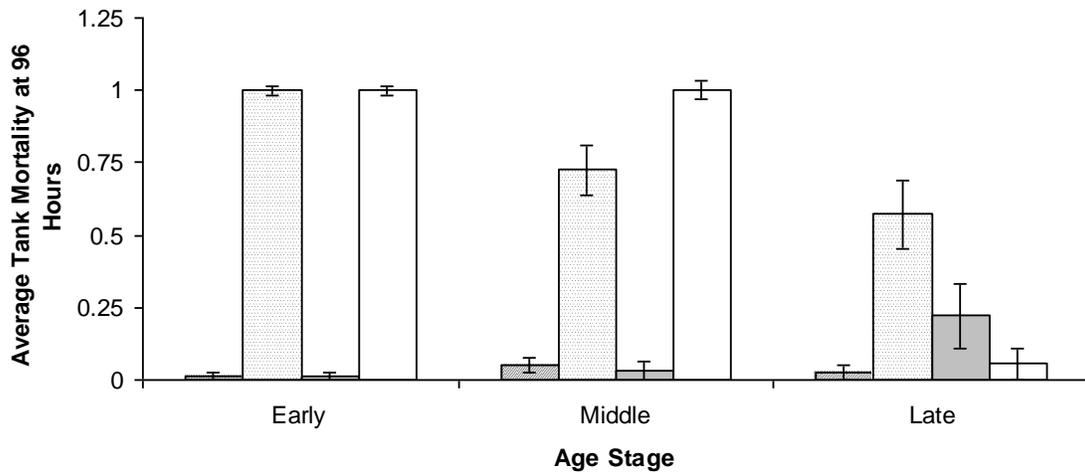
Figure 2.3–Average mortality by age stage of spotted frog tadpoles exposed to 1 mg/L CFT Legumine for 96 hours in 2008 and 2009. 2008 average mortality data is shown by dotted bars (exposed tadpoles) and striped bars (control tadpoles), while 2009 data is shown by open bars (exposed) and gray bars (control). Standard errors are represented by lines on top of each bar. There were 10 tadpoles per tank in the first age stage trial, 5 tadpoles per tank in the middle age stage trial, and 3 tadpoles per tank in the late age stage trial. Mortality in exposed tadpoles decreased as age increased. Average mortality was standardized by dividing by the number of tadpoles per tank.
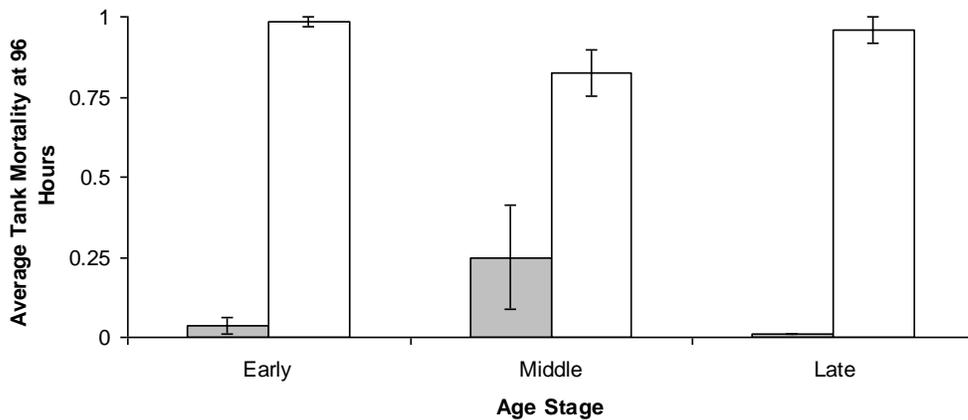
Figure 2.4–Average mortality by age stage of boreal toad tadpoles exposed to 1 mg/L CFT Legumine for 96 hours in 2009. Open bars represent mortality of exposed tadpoles while closed bars represent mortality of control tadpoles. Standard errors are represented by lines on top of each bar. There were 10 tadpoles per tank in the first age stage trial, 5 tadpoles per tank in the middle age stage trial, and 3 tadpoles per tank in the late age stage trial. Mortality in exposed tadpoles decreased as age increased. Average mortality was standardized by dividing by the number of tadpoles per tank.
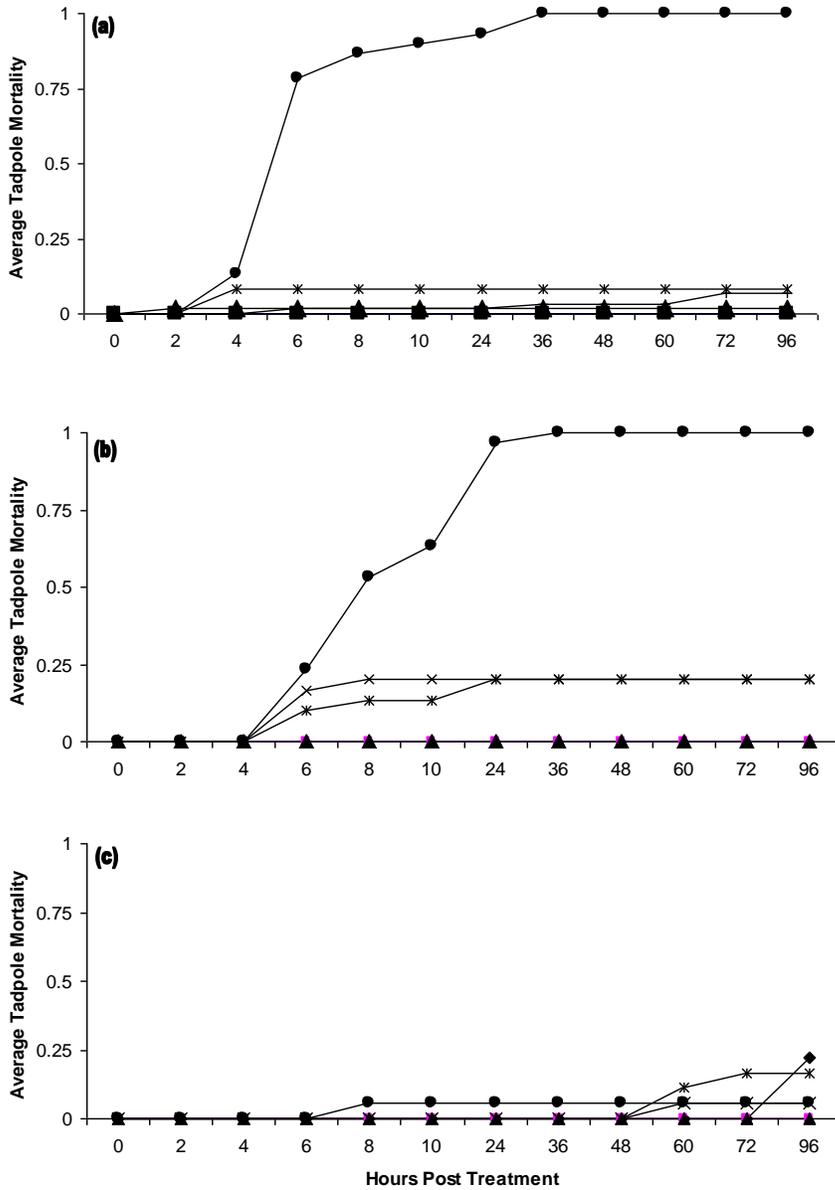
Figure 2.5–2009 average mortality by treatment group (i.e. exposure length) of spotted frog tadpoles over a 96 hour period at 1 mg/L product. Age groups graphed sequentially (i.e., 21-25 (a), 30-35 (b), 40-45 (c)). Circles denote the 96 hour exposure treatment, stars denote the 4 hour exposure treatment, crosses denote the 3 hour exposure treatment, triangles denote the 2 hour exposure treatment, squares denote the 1 hour exposure treatment, and diamonds denote the control group. There were 10 tadpoles per tank in the first age stage trial, 5 tadpoles per tank in the middle age stage trial, and 3 tadpoles per tank in the late age stage trial.

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Table 2.1–2009 percent mortality of spotted frog tadpoles at all three age stages after 4 and 96 hours of exposure to CFT Legumine at 1 mg/L. Tadpoles exposed for 4 hours were significantly less likely to die than those exposed for 96 hours in both the early and middle age stage trials, while there was no difference in mortality at the late age stage. There were 10 tadpoles per tank in the early age stage (21-25) trial, 5 tadpoles per tank in the middle age stage (30-35) trial, and 3 tadpoles per tank in the late age stage (40-45) trial.

Age stage	Percent mortality (4 hours)	Percent mortality (96 hours)	Fisher's exact test
21-25	8.3	100	$p < 0.001$
30-35	20	100	$p < 0.001$
40-45	16.7	5.6	$p = 0.602$

Chapter 3
Temporal effects of rotenone application on amphibians in lentic habitats of southwestern Montana

Abstract

Throughout the western United States, fisheries managers are attempting to restore native cutthroat trout *Oncorhynchus clarkii* populations by removing non-native fish species. The Environmental Protection Agency approved piscicide rotenone is increasingly being used as a method to accomplish this removal. Fish restoration projects change the aquatic environment, and it is important to consider the impacts of fish restoration on non-target species, such as amphibians. I assessed the effects of fish removal on amphibians in two field situations by investigating the effects of rotenone to and the impacts of removing fish on local amphibian populations. To determine impacts, CFT Legumine (5% rotenone) was applied to a lake in Yellowstone National Park (YNP) in 2006 containing stocked Yellowstone cutthroat trout *O. c. bouvieri* and to two fishless ponds on the Flying D Ranch in southwestern Montana in 2008. Amphibian surveys were conducted at all water bodies immediately prior to and after the rotenone treatments to obtain tadpole population estimates and an estimate of mortality. A survey was conducted 1 year post-treatment at each treatment site to obtain tadpole abundance estimates in the year after application. In YNP, additional abundance and distribution surveys were conducted 2 and 3 years post-treatment to observe any long-term effects of fish removal and impacts of native fish stocking. Within 24 hours following application, rotenone caused nearly 100% mortality in gill-breathing, amphibian tadpoles, but did not affect non-gill breathing metamorphs, juveniles, and adults. In the year(s) following, tadpole repopulation occurred at all water bodies treated with CFT Legumine and population levels were similar to, or, in the case of YNP, higher than, pre-treatment levels. In YNP, tadpole abundance and distribution decreased after native westslope cutthroat trout *O. c. lewisi* were stocked in the treated lake.

Introduction

A growing number of fisheries management programs at the federal, state, and private levels are looking to non-native fish eradication in an attempt to restore populations of native salmonid species. Because of its proven efficacy (Shepard et al. 2002), chemical removal has become a common technique for accomplishing this task (Mangum & Madrigal 1999; Finlayson et al. 2000; Ling 2002; McClay 2005; Hamilton et al. 2009). In particular, application of rotenone products has increased because of its success and reliability in removing unwanted fish (Finlayson et al. 2000; McClay 2005). While much is known about the effects of rotenone on fish (Meadows 1973, Amey 1984, Finlayson 2000, Britton 2006, Grisak et al. 2007 (a)), very little is known about the impacts of rotenone applications on non-target species, like amphibians.

Understanding the impacts of chemical fish removal on amphibians is important because of the role amphibians play in aquatic and terrestrial ecosystems. Because amphibians are both prey and predator species, they help structure ecological communities. As a predator, adult amphibians contribute to the regulation of populations of a number of invertebrate species. In one recent study, populations of larval mosquitoes, a common disease vector, were denser in ponds that had experienced a reduction in amphibian predators (Chase & Shulman 2009). As a prey item, amphibians are a key dietary component for a variety of organisms, including mammals, fish, reptiles, and birds (Blaustein et al. 1994; Young et al. 2004). Thus, actions that impact amphibian communities could have cascading effects on the aquatic ecosystem.

The introduction of a piscicide can have immediate, negative impacts on non-target, gill-breathing, aquatic organisms, such as amphibians (Fontenot et al. 1994; McCoid & Bettoli 1996; Maxell 2000; Patla 2005). Laboratory work has demonstrated

that the rotenone product CFT Legumine causes significant mortality of boreal toad *Anaxyrus boreas* and Columbia spotted frog *Rana luteiventris* tadpoles (Billman et al. in review). Similarly, Grisak et al. (2007) documented mortality in a variety of native Montana amphibian larvae after exposure to rotenone products. The longer term consequences of this mortality if any (e.g., localized reduction or even loss of a larval year class), are not well understood.

On the other hand, removal of fish, particularly non-natives, can have positive impacts on amphibian populations over time by returning ecological communities to a more native state (Bradford et al. 1993; Pilliod & Peterson 2000; Pilliod & Peterson 2001; Vredenburg & Wake 2004; Mullin et al. 2004; Anholt et al. 2005; Knapp 2005; Welsh et al. 2006; Walston & Mullin 2007; Boone et al. 2007). Case in point, removal of introduced rainbow trout *O. mykiss* and brook trout *Salvelinus fontinalis* in the Sierra Nevada enabled significant recovery of declining yellow-legged frogs *Rana muscosa* (Vredenburg & Wake 2004). Additionally, amphibians have the ability to detect the presence of fish by picking up chemical cues, and introduced fish, especially in aquatic habitats that were historically fishless, can indirectly, negatively influence distribution, foraging time, (Binckley & Resetarits 2003; Orizaola & Brana 2003; Bernard 2006; Barr & Babbitt 2007) and, most importantly, development. The removal of introduced fish, whether permanent or temporary, could potentially have positive impacts on local amphibian populations.

In order to improve conservation of amphibian populations in fish restoration areas, it is necessary to expand on the knowledge from the above-mentioned laboratory experiments by measuring the effects of rotenone on amphibians under field conditions.

On-going fish removal projects provide an opportunity to document the impacts of rotenone application on amphibians under less controlled conditions. This research begins to address these effects by monitoring amphibian populations before and after rotenone treatments in lentic habitats in southwestern Montana, including High Lake in Yellowstone National Park (YNP).

In YNP, fisheries management was historically guided by the need to provide high quality fishing experiences for visitors. In Yellowstone, approximately 40% of the park's waters were originally fishless, but park managers stocked many of them, with both native and nonnative fish species, to turn the park into a fishing destination (Varley and Schullery 1998). In park waters, native cutthroat trout *Oncorhynchus clarkii* and Arctic grayling *Thymallus arcticus* populations have been completely extirpated, reduced in abundance, or compromised because of hybridization or competition with non-native fish. Under a new management paradigm, YNP fisheries managers currently seek to reverse this trend by removing non-native and hybridized fishes and restoring native cutthroat trout to historic habitat (Koel et al. 2006).

Westslope cutthroat trout (WCT) *O. c. lewisi* populations are a current focus of the park's native fish restoration goals (Koel et al. 2006). Yellowstone National Park is attempting to restore WCT by first removing all introduced non-native and hybridized fish from select areas, and, subsequently, restocking genetically pure WCT (Koel and York 2006). The East Fork Specimen Creek drainage, beginning with its headwater lake, High Lake, was chosen to be the initial focus of WCT restoration in YNP. Though historically fishless, High Lake was stocked in 1937 with Yellowstone cutthroat trout (YCT) *O. c. bouvieri*, which are not native to the lake's drainage. In order to restore

WCT to the drainage, chemical fish removal was scheduled for High Lake and its outflow, the East Fork Specimen Creek. With a resident breeding population of amphibians, High Lake provided an excellent opportunity to document the impacts of the rotenone application on this non-target organism.

While High Lake provided an excellent opportunity to study rotenone's effects on natural amphibian populations, I sought an additional study site to obtain more data. I similarly monitored the amphibian response to rotenone treatment at two small wetlands as part of a separate native trout restoration project on a private ranch in southwestern Montana in 2008. These wetlands were similar to each other in most aspects but different from High Lake, providing excellent contrast and allowing me to address research objectives in different aquatic environments.

The overall goal of this research was to document some of the short and longer-term impacts of rotenone exposure on amphibians in a natural setting. Specifically, the objectives of these field studies were to: 1) determine the effects of a commonly used rotenone product, CFT Legumine (5% rotenone), on amphibian populations under field conditions and 2) observe and describe the effects of fish removal and subsequent fish introduction on the amphibian population at High Lake. In doing so, I hope to provide information that will facilitate amphibian conservation in fish restoration areas.

Study Areas
Yellowstone National Park, WY

High Lake has a surface area of approximately 3.16 ha and is located in the northwestern corner of Yellowstone National Park at an elevation of 2638 m (8800 ft). The lake and an associated wetland complex form the headwaters of the East Fork Specimen Creek, a tributary to the Gallatin River in the upper Missouri River drainage

(Figure 3.1). Two rotenone treatments were scheduled at High Lake in early and mid-August 2006 to remove introduced Yellowstone cutthroat trout and establish westslope cutthroat trout – the native trout of the upper Missouri River drainage (Shepard et al. 2002). Amphibian monitoring was conducted at High Lake and two nearby, fishless wetlands (Figure 3.2) as part of the environmental effects assessment conducted by YNP during rotenone treatment. Two wetlands (North (0.17 ha) and South (0.13 ha)) were included as controls, or untreated sites, and were in close proximity to a known amphibian breeding site in the outlet channel of High Lake (Figure 3.2). The amphibian breeding site at the outlet channel was approximately 190 meters from the South wetland and 620 meters from the North wetland; the two wetlands were separated by approximately 760 meters. Water quality parameters for the three water bodies are documented in Table 3.1. The primary vegetation in all three water bodies was aquatic sedges/grasses, and the water bodies retain water throughout the summer season.

Flying D Ranch, MT

Monitoring of the effects of rotenone application on amphibians was conducted on the Flying D Ranch in southwestern Montana (Figure 3.1) at four small, fishless wetlands in the Cherry Creek drainage. This is a watershed in the Madison River drainage (upper Missouri River system), where a native cutthroat trout restoration project is currently underway. The four wetlands were located between 1463 and 1830 m in elevation and were known amphibian breeding sites. Wetlands #1, #2, and #3 were characterized as vegetated primarily by aquatic sedges/ grasses (genus: *Scirpus*) across 76-100% of the water body, while Wetland #4 was vegetated by bulrushes/cattails (genus: *Typha)* across 26-50%. All four wetlands varied in average depth and diameter.

Water quality parameters for all four water bodies are documented in Table 3.2. The ponds retain water throughout the summer.

The High Lake and treated Flying D Ranch sites were relatively close in proximity and were similar in repopulation potential (i.e. were in close proximity to other wetlands containing amphibians). However, they differed in important ways. High Lake contained an established population of fish prior to rotenone application, while the Flying D Ranch sites did not. High Lake was a large, high elevation lake whereas the Flying D Ranch water bodies were small, mid to low elevation ponds.

Methods
Yellowstone National Park
An amphibian survey was conducted using USGS Amphibian Research & Monitoring Initiative protocols at each of High Lake and the two adjacent wetlands in early August 2006 (USGS ARMI Website

http://armi.usgs.gov/researchdevelopment.asp#DevelopmentofFieldProtocols). The initial pre-treatment survey at High Lake took place midday (1200-1530 hrs) on August 5, 2006, while the first surveys at the North and South wetlands occurred the following day between 1000 and 1430 hrs, with approximately equal time spent surveying at each wetland. These surveys consisted of walking the entire margin of the water body to both randomly and strategically (i.e. when tadpoles were observed) capture tadpoles by dip net to determine the presence and distribution of larvae. Captured tadpoles were identified to species and aged according to the Gosner staging system (Gosner 1960). Mark-recapture population estimates were obtained by marking each captured tadpole with a 2-3 mm tail clip during the initial survey, releasing them at or near the point of capture, and then collecting tadpoles during a second, recapture survey within 48 hours to count the number of

marked and unmarked individuals. High Lake was surveyed a second time on August 6 between 1445 and 1600 hours, while the recapture surveys at the North and South wetlands took place on August 8 between 1000 and 1230 hours.

On August 6, approximately 15 hours prior to the scheduled rotenone application in High Lake, three mesh sentinel cages holding captured individuals were placed at different locations around High Lake to ensure that any observed effects were a result of rotenone application and not other environmental factors. One cage, containing 18 tadpoles, was placed at the midway point along the east side of the outlet channel; a second cage with 18 tadpoles was placed along the margin at the north end of High Lake; and a third cage with three adult Columbia spotted frogs was placed at the midway point along the west side of the outlet channel. A control cage containing 19 tadpoles was placed in the untreated South wetland. All captured individuals, both adults and tadpoles, were Columbia spotted frogs, and all tadpoles placed in the sentinel cages were captured in the High Lake outlet channel. Sentinel cages were checked the following morning immediately prior to the rotenone treatment to assess any over-night mortality. During the treatment, a fifth sentinel cage containing 18 tadpoles was placed in the outflow of the piscicide treated lake to determine if moving water impacted tadpole survival differently than suspension in outlet or lake water.

Beginning at 0800 hours on August 7, CFT Legumine was applied to High Lake at an estimated concentration of 1 mg/L. Inflatable rafts with outboard motors were used to evenly distribute the piscicide within the lake, and backpack sprayers were used to apply rotenone to the lake margins and the outlet channel. A total of 17.5 gallons of piscicide were applied to the lake, outlet channel, inlet streams, and spring seeps, with

application ending by 1400 hours. In order to detoxify rotenone leaving High Lake, potassium permanganate was applied to the East Fork Specimen Creek at the end of the outflow channel. The North and South wetlands had no connectivity with High Lake and were not treated. Yellowstone cutthroat trout that died as a result of the rotenone application were collected during and after treatment. Total length (mm) and weight (gm) measurements were taken on the YCT removed from High Lake in the 24 hours post-treatment.

The outflow channel and all sentinel cages were visually inspected at 1100, 1400, and 1600 hours on the day of the rotenone application to assess treatment effects on amphibians. The following day, 24 hours after rotenone was first applied to the lake, a final tadpole survey of the outflow channel was conducted and sentinel cages were removed. Surviving individuals in sentinel cages were released.

In mid-July 2007, approximately one year post rotenone treatment, amphibian breeding and mark-recapture surveys were conducted at High Lake and the two adjacent wetlands, as previously described. I assessed tadpole distribution in the outlet channel and lake margin in the absence of fish. Immediately following these surveys, WCT eggs (via remote stream side incubators) and mixed-age individuals were stocked in High Lake. Weights and total lengths of the stocked fish were recorded. Similar mark-recapture surveys (late July) and fish stocking events were conducted in 2008 and 2009 at High Lake.

Data collected at High Lake and the two associated wetlands was analyzed as follows. Data from the mark-recapture surveys were used to obtain a tadpole population estimate at each of the three water bodies for each of the four years sampling took place.

Specifically, because High Lake and the two control wetlands met the basic assumptions of a closed system (i.e. no deaths, births, immigration, or emigration between the mark and recapture day), Chapman's modification of the Lincoln-Petersen equation (Thompson et al. 1998) with a 95% Confidence Interval (CI) was used to calculate the population estimates. These values were then compared graphically to assess whether pre-treatment tadpole abundance estimates differed from post-treatment estimates.

Flying D Ranch

In May 2008, several small, fishless wetlands in the Cherry Creek drainage on the Flying D Ranch were assessed for suitability as experimental rotenone treatment sites. Four of these wetlands were strategically chosen for inclusion in this study. Previous surveys of wetlands in this area documented breeding by only Columbia spotted frogs and boreal toads, but I attempted to choose wetlands that contained only spotted frogs to reduce the potentially negative impacts of rotenone application to boreal toads, a sensitive species. The sites were similar in size, configuration, habitat, and opportunity for spotted frog tadpole repopulation the following breeding season. The number of wetlands allowed to be treated for this experiment was limited to two by the Montana Department of Environmental Quality. Two wetlands were designated as controls, or untreated sites.

In mid-July 2008, pre-treatment amphibian surveys were conducted at each water body, as described previously for High Lake, to confirm the species present in the four wetlands and to obtain a pre-treatment population estimate. An age estimate of the tadpole population at each wetland was obtained by staging captured individuals according to the Gosner staging system (Gosner 1960). The initial surveys (i.e. marking tadpoles) were conducted between 1200 and 1400 hours on July 14 at wetlands #1 and #2

and on July 15 for wetlands #3 and #4. Recapture surveys were conducted at a similar time on July 16 for all four wetlands.

Following completion of the recapture surveys, wetlands #1 and #3 were treated at approximately 1 mg/L CFT Legumine (5% active rotenone). CFT Legumine was applied as evenly as possible by pumping pond water with a small gasoline pump and injecting rotenone via siphon into the pump discharge which was distributed evenly across the wetland surface and into the water column. The dosage of 1 mg/L CFT Legumine was selected as a commonly used field dose in pond and stream treatments and it matched the concentration used at High Lake. Wetlands #2 and #4 were control sites and were not treated.

I assessed the effects of treatment over two temporal windows. First, surveys were conducted 24 hours post-treatment to determine the short-term impacts of rotenone application on amphibians. Second, in July of 2009, one year after the initial rotenone application, amphibian surveys were again conducted at each of the four ponds to obtain a 1 year post-treatment tadpole population estimate. Pre- and post-population estimates were calculated using Chapman's modification of the Lincoln-Petersen equation (Thompson et al. 1998) with a 95% Confidence Interval (CI). The difference between the pre and 1 year post-treatment population estimates at each pond was calculated and, because of the small sample size, a Kruskal-Wallace test was used in MiniTab (MiniTab 15) to compare these calculated differences in treated and untreated ponds. The methodology of these field experiments was approved by the Idaho State University Animal Welfare Committee.

Results

Yellowstone National Park

        The only amphibian species documented breeding (e.g. presence of larvae) at High Lake was the Columbia spotted frog, while both spotted frogs and boreal chorus frogs *Pseudacris maculata* were sampled in the adjacent North and South wetlands. A single adult boreal toad was documented at High Lake from 2006-2008, but no eggs or larvae of this species were ever observed or sampled. The general age estimate for tadpoles at High Lake and the two control wetlands sampled during all 4 years was Gosner stage 40-43. Tadpoles were present in High Lake and both wetlands immediately prior to treatment (Table 3.3); however, tadpoles were only observed in the High Lake outlet channel during pre-treatment surveys and not in the lake or around the lake margins.

        Piscicide application had immediate, negative effects on tadpoles. Lake margin surveys conducted during and immediately after rotenone was applied indicated that tadpole mortality was 100% in High Lake, but non-gill breathing juveniles and adult stage frogs were unaffected. No live tadpoles were captured or observed in High Lake after treatment, and no dead juvenile or adult frogs were observed in or around the lake. There was 100% tadpole mortality in the two sentinel cages suspended in the lake, while the three adults held in a single sentinel cage survived the treatment. Comparatively, there was no tadpole mortality in the sentinel cage at the South wetland, and no tadpole or adult mortality was observed in either of the two untreated wetlands adjacent to the lake. There was 11% (2 out of 18) tadpole survival in the 5th sentinel cage which was placed in flowing water at the end of the High Lake outlet during treatment.

Pre-treatment tadpole population estimates (+/- 95% CI) obtained for the three water bodies were 115 (+/- 38), 96 (+/-36), and 84 (+/- 13) for High Lake, the South Wetland, and the North Wetland, respectively (Table 3.3). In 2007, one year post-rotenone treatment, the tadpole population estimate at High Lake was nearly 7 times greater than the 2006 pre-treatment estimate, while the estimates at the control wetlands were similar (Table 3.3). Following fish introductions in 2007, tadpole population estimates declined each year from the 2007 post-treatment high, but over the course of this study remained higher than the estimate obtained in 2006 before rotenone application and did not appear to be different from each other (Figure 3.3, Table 3.3).

Observational data detailing tadpole distribution and behavior at High Lake indicated changes in habitat use in the years following rotenone application. In 2006, tadpole distribution at High Lake was restricted to the margins of the outlet channel, while adults were documented throughout the lake. Tadpoles were skittish and cryptic, remaining strictly in the sedge-protected portions of the outlet margin. In contrast, tadpoles were observed in 2007 (prior to WCT introduction) throughout the outlet and in the margins around the main lake body. In 2008 and 2009, as WCT numbers and size increased, tadpole distribution became increasingly limited and, by the 2009 survey, was restricted once again to the outlet margins.

In 2006, 793 YCT were collected from High Lake as a result of the rotenone application, but only 301 individuals were weighed and measured. Lengths ranged from 72 mm to 400 mm (mean length of 246.8 mm (+/- 88.96)). The lengths of WCT transferred from Geode Creek to High Lake ranged from 42 mm to 289 mm, with mean

lengths varying little from 2007-2009. After the 2009 transfer, 3,003 WCT had been placed in High Lake over the course of the three years.

Flying D Ranch

July surveys at three of the four wetlands selected for my experiment documented breeding by only the Columbia spotted frog, while one wetland (#4), subsequently designated as a control, had both spotted frog and boreal toad tadpoles present. Spotted frog juveniles and adults were documented at all four wetlands. The general age estimate for tadpoles at all four wetlands in both surveys was Gosner stage 40-43.

Since spotted frog and boreal toad tadpoles were documented in both pre and post-treatment surveys at Wetland #4, the population estimates for this site are comprised of both species. Pre-treatment tadpole population estimates for the four wetlands were relatively similar (Figure 3.4 and Table 3.4). Rotenone application (1 mg/L CFT Legumine) caused immediate mortality in tadpoles at both treatment wetlands, but did not significantly affect tadpole population size in the following breeding season (Table 3.4). Tadpole surveys conducted 24 hours post-treatment revealed 100% mortality at each of the treatment ponds. There was no observed mortality among non-gill breathing juvenile and adult spotted frogs at either treated wetland. Tadpoles were plentiful at both control ponds, but no immediate post-treatment population estimates were collected. In 2009, 1 year post-treatment, tadpoles were again present at similar abundance levels at all 4 ponds (Figure 3.4). The Kruskal-Wallace test indicated that the calculated differences between pre and 1 year post-treatment population estimates did not differ significantly between treated and untreated ponds (H = 2.40, 1 df, p = 0.12).

Discussion

The results of this study demonstrate that rotenone applied at a common field dose caused significant and immediate mortality in spotted frog tadpoles but not in metamorphosed juveniles or adults. Application of rotenone at 1 mg/L product resulted in what appeared to be 100% tadpole mortality at both High Lake and the treatment ponds on the Flying D Ranch. No surviving tadpoles were seen 24 hours post-treatment. On the other hand, non-gill breathing life forms (i.e. adult, juvenile, metamorph) survived treatment at all sites. Consequently, tadpole population size in one to three breeding seasons post-treatment was not negatively impacted by rotenone treatment. Breeding at treated water bodies occurred in the season immediately following the late summer treatments, with either insignificant change (Flying D Ranch; Figure 3.4, Table 3.4) or an increase (High Lake; Figure 3.3, Table 3.3) in tadpole abundance.

Other research supports my findings that rotenone can have significant, immediate effects on larval stage amphibians, but not older, lung breathing stages under field and laboratory conditions. Application of rotenone at an unknown concentration to several ponds in the upper Santa Clara River drainage in California resulted in complete mortality of African clawed frog *Xenopus laevis* tadpoles, while adults were not affected and successfully bred after the treatment (McCoid & Bettoli 1996). In a controlled, laboratory setting, Grisak et al. (2007) found that rotenone exposure at a range of doses caused 100% mortality in tadpoles of two Rocky Mountain amphibian species, while adults of these same species showed no observable effects at concentrations well above those typically used in pond and stream treatments.

In contrast to these findings, Billman et al. (in review) found low mortality (6%) in late age stage spotted frog tadpoles exposed to CFT Legumine at 1 mg/L in a recent

laboratory challenge. There could be two key explanations for this discrepancy. First, the age of spotted frog tadpoles appears to play a significant role in susceptibility to the lethal effects of rotenone – very late age stage tadpoles were more resistant than younger individuals. Tadpoles exposed to CFT Legumine as part of the Billman et al. (in review) laboratory trials were aged at Gosner stage 44-45, while tadpoles in the field experiments described here were younger. This could explain the different mortality rates (i.e. 6% (laboratory trials) vs. 100% (field trials)). Secondly, tadpoles in the laboratory challenge received exactly 1 mg/L CFT Legumine. While the sites in this research were treated at 1 mg/L CFT Legumine, the effective concentration in the margins of the treated water bodies – where tadpoles are found – was most likely greater than 1 mg/L. This potentially occurred either because of differences in the way the chemical distributed once applied or because these areas were treated multiple times and received a dosage closer to 2 mg/L product. Indeed, findings from the laboratory challenge revealed 100% tadpole mortality, regardless of age, at 2 mg/L.

It is important to recognize the potentially negative impacts of piscicide-induced larval mortality on the aquatic system. In the short-term (i.e. immediately post-treatment) loss of an entire tadpole cohort removes an important link in the food webs of aquatic and terrestrial systems. Tadpoles maintain algal communities at levels conducive to some invertebrates, an important food source for aquatic organisms, including fish (Blaustein et al. 1994; Young et al. 2004). In the long-term, the effects of removing an entire tadpole generation from a water body on the amphibian population itself in later years when the removed individuals would have been sexually mature are unknown. Some amphibian species, such as Columbia spotted frogs, may have highly variable recruitment.

Substantial recruitment events (i.e. > 100 metamorphs), which are important to the maintenance of local amphibian populations, may occur infrequently (Trenham et al. 2003; Greenberg & Tanner 2005). If rotenone were applied during the year of a recruitment event and an entire tadpole cohort was lost, there could be significant, negative consequences for the overall population in future years. Because of these potentially significant, negative consequences, efforts to mitigate mortality should be made.

Piscicide applicators might consider a number of strategies for minimizing tadpole mortality in the field. Timing treatment treat until tadpoles are no longer present is the best option, but other research suggests that waiting until tadpoles are at a very late age stage, or treating at lower dosages are other ways in which fisheries managers can decrease tadpole loss as a result of fish removal. Tadpole mortality can also be mitigated collecting tadpoles, either before or during treatment, and holding them until the rotenone is no longer active in the treated water body. This technique may prove to be most applicable for fisheries managers in the Rocky Mountains, in particular, where rotenone applications coincide with the larval period (Grisak et al. 2007; Billman et al. in review). If fish restoration project guidelines call for multiple rotenone applications, I suggest conducting treatments within the same year instead of across consecutive years to avoid loss of multiple tadpole cohorts.

When addressing the long-term impacts of tadpole mortality as a result of rotenone treatment, managers should consider general factors, including 1) the conservation status of the amphibian species in the restoration area; 2) proximity of other amphibian populations to the restoration site; and 3) life history of amphibians at the

restoration site. Species prevalence can be a useful tool in determining how much effort can or should be allotted for tadpole preservation at any given site. If the species of amphibian breeding in the restoration area is common over a wide range, efforts to capture and hold tadpoles or otherwise minimize impacts may not be critical for that species. If, however, the species in question is threatened or in decline, it may be important to salvage as many tadpoles as possible.

The proximity of wetland habitat that can provide colonizers to a restoration site is an important consideration. Knapp et al. (2001) suggests that amphibian recovery in high alpine lakes where fish had been removed was augmented by breeding adults from neighboring source ponds. High Lake and the two treated sites on the Flying D ranch were characterized as having at least one wetland containing breeding adult amphibians within 500 meters. I do not, however, know whether any adults from neighboring ponds bred at either High Lake or the treatment ponds on the Flying D Ranch in the years after treatment. The repopulation seen in the breeding season after treatment could have been accomplished by established, resident adults. In isolated habitats, it may be important to preserve tadpoles to protect against the unknown, extended effects of losing an entire age class that cannot be replaced by emigrating individuals. Determining the degree of isolation that would necessitate action to conserve tadpoles should be done on a site-by-site basis, especially since adults of some species of Rocky Mountain amphibians have documented movements to breeding sites of up to 3 kilometers (Pilliod & Peterson 2000; Pilliod & Peterson 2001).

The life histories of amphibians at high and low elevation differ in important ways that can affect the impacts of tadpole removal on a population. Generally speaking,

at low elevation, amphibians have a shorter life-span, longer activity period, and can reach sexual maturity earlier than individuals at high elevation where conditions and resource availability push adults toward a shorter activity period with an increase in longevity and time to sexual maturity (Cvetkovic et al. 2009). Populations at low elevation tend to experience higher rates of turn-over because adults breed at an earlier age, while, at high elevation, because individuals breed later in life, population turn-over does not occur as quickly. Loss of an entire tadpole cohort in fish restoration sites at high elevation poses a greater risk because it may take longer to replace their breeding contribution. Efforts should be made to minimize tadpole mortality at these sites.

In addition to direct effects from chemical application, the manipulation (e.g. removal or change in species) of the fish population in a water body being restored can also impact amphibians. The absence of fish after treatment appeared to impact tadpole distribution and abundance at High Lake. At High Lake in 2007, tadpole abundance increased significantly in the absence of fish following the 2006 rotenone application. As WCT were stocked into the lake from 2007-2009, and presumably increased in size and abundance, the tadpole population at High Lake decreased, as did the extent of tadpole distribution throughout the lake itself. Tadpoles were documented throughout the lake in 2007 in the absence of fish, but were restricted to the sedge-protected margins of the outlet channel by 2009 – similar to pre-treatment conditions.

Changes in tadpole distribution at High Lake could have been caused by a number of factors. Predation can affect tadpole distribution by reducing tadpole numbers or eliminating them from a particular area. Predators have been shown to influence the behavior, morphology and habitat choices of tadpoles of multiple amphibian species

(Relyea 2003, 2004, 2005; Richter-Boix et al. 2007). Specifically, tadpoles can chemically detect the presence of fish, thereby negatively affecting distribution and foraging time (Binckley & Resetarits 2003; Orizaola & Brana 2003; Bernard 2006; Barr & Babbitt 2007). The diminished tadpole distribution at High Lake in the presence of fish in 2006, 2008, and 2009 was consistent with this concept of fish avoidance.

This research provides important information on the effects of rotenone and fish removal, but much remains to be investigated. Environmental factors, such as substrate and water depth, or application patterns could have significant impacts on the toxicity of rotenone to tadpoles and should be more thoroughly investigated. This research does not address the impacts of removal of an entire tadpole cohort on future population size. At High Lake I have obtained population estimates for 3 years after tadpole removal, but I have yet to determine whether the loss of an entire cohort of breeding individuals will significantly impact the High Lake spotted frog population. Spotted frogs in the Rocky Mountains typically reach sexual maturity 4 to 5 years after their first summer (Koch & Peterson 1995), making monitoring this population in the next 1 to 2 years highly important in determining whether tadpole removal has negative extended effects.

This research and results obtained from recent laboratory studies provide fisheries managers with information that can be used to conserve amphibian populations at fish restoration sites. Rotenone will negatively impact tadpoles when applied at typical field application doses, but these negative effects appear to be short-term. Repopulation of treated water bodies will occur in the breeding season following application. Taking these results and other factors discussed above into account will enable managers to

develop effective methods for mitigating the overall impacts of fish removal on

amphibians.

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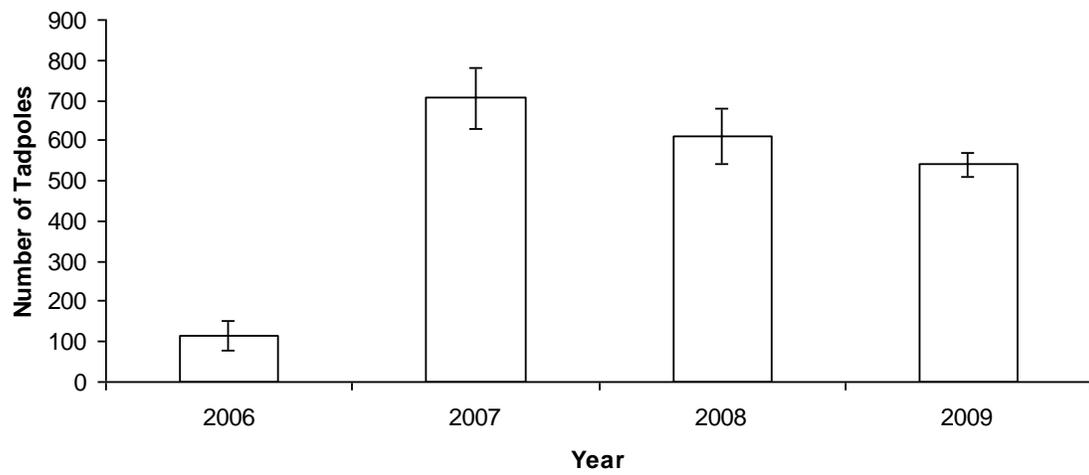
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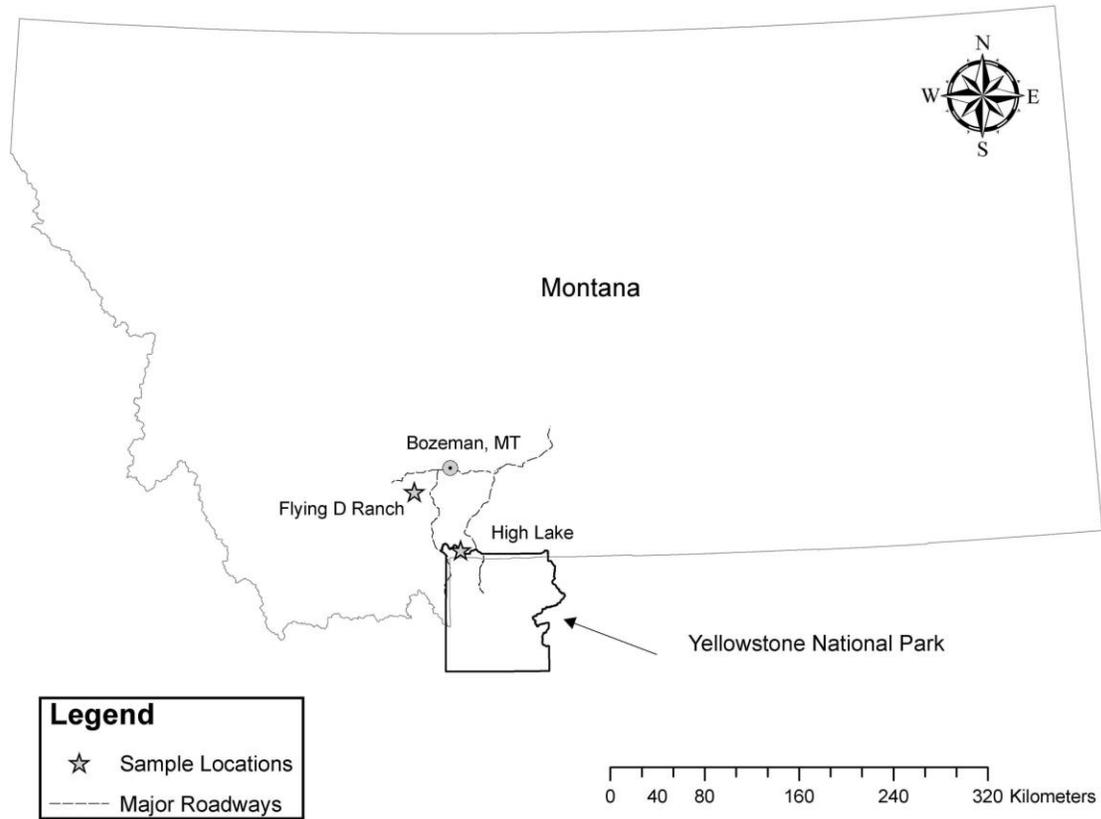
Figure 3.1.- Locations of the High Lake and Flying D Ranch study areas.
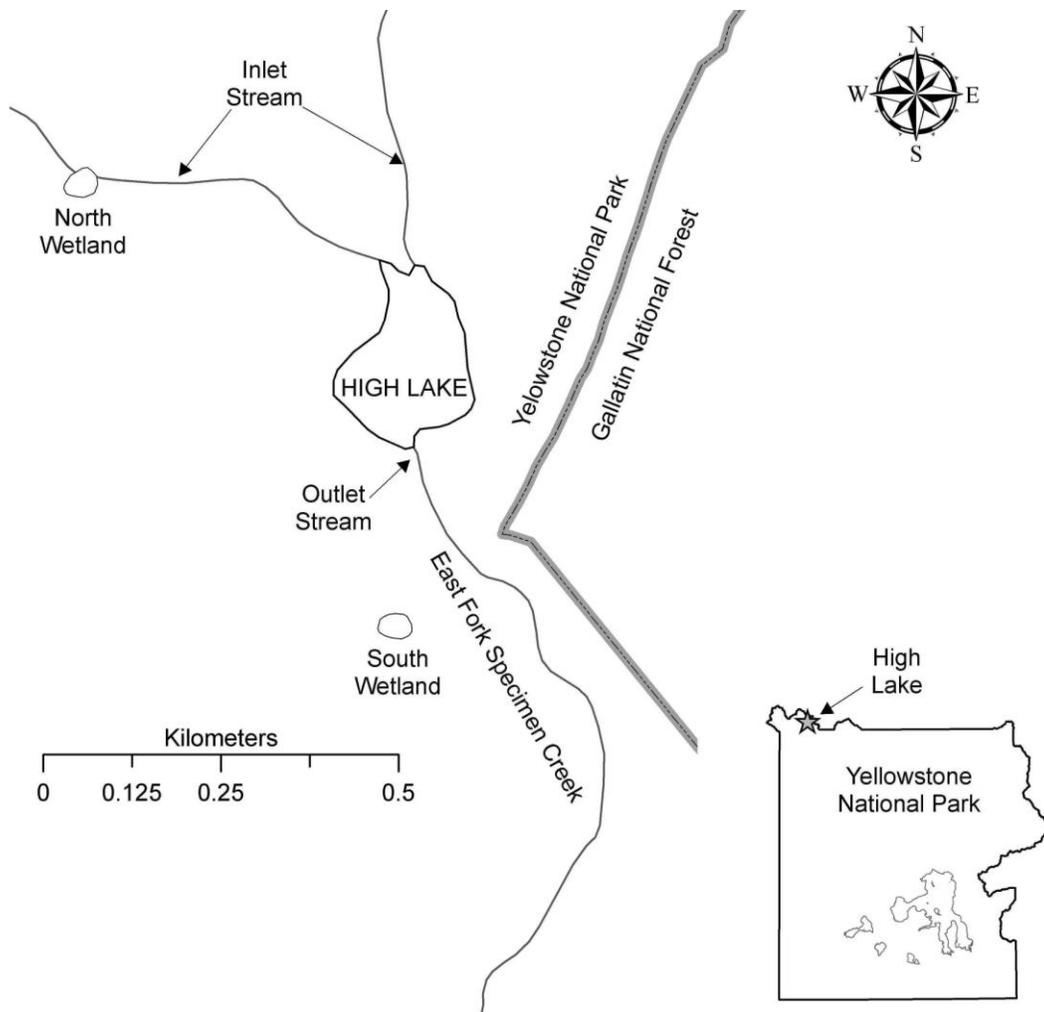
Figure 3.2.- High Lake area, including the outlet channel (amphibian breeding site), North wetland, and South wetland.

Figure 3.3.- Tadpole population estimates and 95% Confidence Intervals at High Lake for each of 2006-2009. The tadpole population expanded in 2007 in the absence of fish, but declined as the number of fish in High Lake increased.
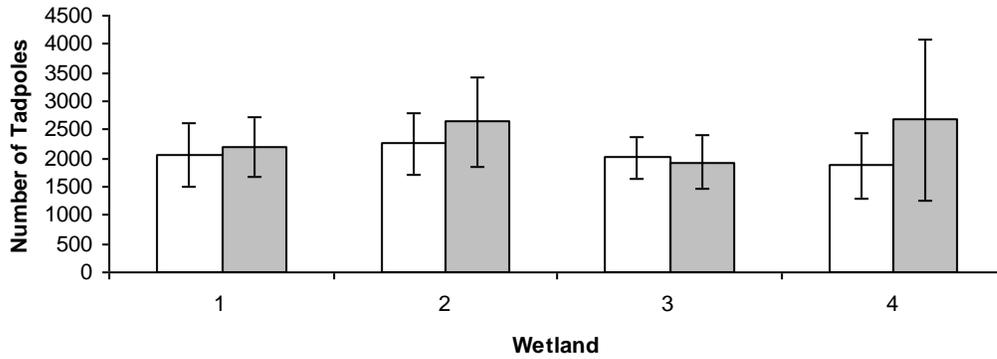
Figure 3.4. - Tadpole population estimates and a 95% Confidence Interval at control ponds (n = 2) and treated ponds (1 mg/L; n = 2). Open bars represent estimates taken in 2008 (pre-treatment) and shaded bars represent estimates taken in 2009 (1 year post-treatment). Ponds #1 and #3 were treated with CFT Legumine while Ponds #2 and #4 were untreated. There was no significant difference between pre and post-treatment tadpole estimates in either treated or control ponds.

Table 3.1.- Water quality parameters measured at High Lake and the two associated wetlands (2006 & 2009).

	pH	Temperature (C)	Conductivity
Lake - 2006	9.5	15.2	27
South Wetland - 2006	6.6	15.9	22
North Wetland - 2006	7.4	18.9	30
Lake - 2009	7.3	24.3	20
South Wetland - 2009	7.2	25.4	30
North Wetland - 2009	7.7	26.3	20

Table 3.2.- Water quality parameters measured at treatment and control sites on the Flying D Ranch (2008 & 2009).

	pH 2008	pH 2009	Temperature (C) 2008	Temperature (C) 2009	Total Dissolved Solids 2008	Total Dissolved Solids 2009
Wetland #1 (Treatment)	7.61	6.92	26.3	24.6	70	50
Wetland #2 (Control)	7.13	6.7	27.4	26	50	N/A
Wetland #3 (Treatment)	7.53	8.28	23.3	20.5	270	240
Wetland #4 (Control)	7.8	8.24	29.3	27.3	370	330

Table 3.3.- Lincoln-Petersen tadpole population estimates (+/- 95% Confidence Interval) obtained at High Lake and the 2 control wetlands in each of 2006, 2007, 2008, and 2009. The 2007 estimate was roughly 7 times the 2006 estimate, though the population estimates declined in 2008 and 2009.

	2006	2007	2008	2009
Lake	115 (+/- 38)	705 (+/- 75)	612 (+/- 69)	541 (+/- 29)
South Wetland	54 (+/- 36)	57 (+/- 12)	98 (+/- 2)	95 (+/- 5)
North Wetland	69 (+/- 13)	78 (+/- 81)	71 (+/- 34)	95 (+/- 41)

Table 3.4.- Lincoln-Petersen tadpole population estimates obtained at control and treated ponds on the Flying D Ranch before (2008) and after (2009) the rotenone treatment (1 mg/L product).

	2008 Population Estimate (+/- 95% CI)	2009 Population Estimate (+/- 95% CI)
Wetland		
1	2068 (+/- 561)	2199 (+/- 538)
2	2266 (+/- 542)	2638 (+/- 786)
3	2008 (+/- 380)	1927 (+/-476)
4	1869 (+/- 562)	2677 (+/- 1408)

Chapter 4
Conclusions

Fish restoration projects can change aquatic ecosystems by removing some or all of the fish population and potentially impacting non-target species. Amphibians and macroinvertebrates are important non-target components of aquatic and terrestrial ecosystems, and understanding the impacts of fish removal practices on these organisms is critical to the ultimate success and viability of native fish restoration as a whole. Understanding the effects of the actual method of removal is of particular importance. This research sought to address the effects of rotenone, a chemical commonly used in fish restoration, on larval and adult amphibians in both laboratory and natural settings. The overall goal of this research was to develop recommendations to mitigate any negative effects of rotenone application on amphibians.

In the laboratory, I tested whether factors such as dosage, tadpole age, tadpole species, or duration of exposure affected mortality. Based on the fact that rotenone is absorbed across the gill membrane, it was entirely plausible that widespread, undifferentiated mortality would occur when treatments were applied to different species and different tadpole life stages; however, my results demonstrated that mortality varied with both treatment dose and the duration of exposure. Specifically, I found that mortality increased with an increase in either dose or exposure length. Tadpole age played a significant role in observed spotted frog tadpole mortality, while it did not affect mortality of boreal toad tadpoles. There was a significant species effect, but it was only observed at the latest age stage tested. Finally, I found that, in spotted frog tadpoles

(boreal toads were not evaluated), the effects of rotenone were reversible and that shorter exposure periods followed by revival in untreated water resulted in lower mortality.

The laboratory experiments provided needed information of rotenone's effects under controlled conditions, but the field experiments provided an opportunity to observe the impacts of rotenone in a natural setting. Knowledge of rotenone's mechanisms informed the hypotheses developed for both field treatments. Because rotenone enters across the gill membrane, adult amphibians are thought not to be affected by the chemical. I hypothesized that, because breeding adults would not die as a result of rotenone application, tadpole repopulation in the breeding season following treatment would occur similarly to pre-treatment levels. My hypothesis was supported by trials on the Flying D Ranch, where treated and control ponds were repopulated by tadpoles at a level statistically similar to that seen pre-treatment. In Yellowstone National Park, while repopulation of High Lake 1 year post-treatment occurred, tadpole abundance was actually greater than pre-treatment levels. This may have been because there were no longer fish in the lake. Removal of predatory fish, such as rainbow, brook, and brown trout, has been demonstrated elsewhere to result in larger tadpole populations (Knapp 2005; Knapp et al. 2007). The general trend demonstrated that rotenone applied at a typical field level dose (1 mg/L product) caused immediate tadpole mortality but, because breeding adults were not harmed, short-term repopulation of the water body occurred similarly to pre-treatment levels.

Because I documented tadpole repopulation in the short-term following a rotenone treatment, it might suggest significant effort to protect amphibians in restoration areas are not necessary; however, there is no data yet collected on how a local amphibian

population is affected in the extended-term by removal of an entire tadpole cohort. In other words, I have yet to determine whether the loss of a tadpole generation significantly effects tadpole production in the year(s) during which the removed tadpoles would have begun to breed. In Yellowstone National Park, the opportunity to collect these data is upcoming, with years 4 and 5 post-treatment in 2010 and 2011. In the absence of these data, it is important for fisheries managers to attempt to minimize tadpole mortality at fish restoration sites. This research was ultimately able to provide a number of suggestions for mitigating the impact of rotenone applications on local amphibian populations. These strategies are based on both biological and logistical factors, and are intended to be used on a project-by-project basis.

Because it was demonstrated, both in the lab and the field, that rotenone can cause high, if not complete, tadpole mortality, the first, and optimal, strategy is to apply rotenone products when tadpoles are not present. Presumably, this time frame could be either before eggs are laid or after tadpoles have metamorphosed. In geographical locations with temperate weather patterns this is a viable option for tadpole conservation. In regions, like the Rocky Mountain west, that have extended winters and short summers, however, accessibility of lakes and streams, especially at high elevation, becomes restricted to the same period that encompasses the larval stage of local amphibians.

In these instances, creative solutions taking into account factors like tadpole ecology and physiology are necessary. Spotted frog tadpoles, as a member of the Ranidae family, appear to be resistant to the effects of rotenone at the very late end of the tadpole phase. I speculate that this resistance is a function of an almost complete shift to lung breathing by the end of the tadpole life stage. Bullfrog *Rana catesbeiana* tadpoles

have also shown this respiratory change, indicating that this may be a characteristic shared by other ranid tadpoles. If it is impossible to refrain from applying rotenone until after metamorphosis, the second strategy is to wait until the late tadpole stage (Gosner 44), thereby reducing tadpole mortality.

A third suggestion for minimizing tadpole mortality in fish restoration areas is based on the results of the exposure duration laboratory trials. Spotted frog tadpoles appear to be able to survive limited rotenone exposure. Tadpole collection before or during a treatment followed by holding them in untreated water is another method by which widespread tadpole mortality can be avoided. This strategy may work across species, though it was only evaluated in spotted frogs in this study.

Treating at lower dosages resulted in lower tadpole mortality, both in spotted frogs and boreal toads, making this another strategy for minimizing tadpole loss. The laboratory trials revealed that rotenone applied at a dose of 0.5 mg/L product resulted in lower tadpole mortality, both in spotted frogs and boreal toads. While the tendency is to treat at 1 mg/L for most stream and pond/lake projects, 0.5 mg/L is included in the CFT Legumine dosage recommendations for these types of restoration projects. Admittedly, aspects of the aquatic environment can affect the efficacy of rotenone, making treating at higher doses, like 1 mg/L or 2 mg/L, a more promising option for ensuring complete fish kill. However, because treating at lower levels, like 0.5 mg/L, does appear to result in lower amphibian mortality while still killing fish (EPA, CFT Legumine label), this is an important option for reducing effects to tadpoles in fish restoration projects.

The field work in Yellowstone National Park provided some initial, observational information on the benefits of fishless habitat and the subsequent effects of fish

introduction on tadpoles. The significant increase in the tadpole population at High Lake

when there were no fish in the lake and the decrease in the population that occurred as

fish were returned are consistent with other researchers that report the positive impacts of

fishless habitat on amphibians (Knapp 2001; Knapp 2005; Knapp 2007). Returning

historically fishless habitat to its original state can, therefore, create suitable breeding and

summer habitat, especially for pond breeding amphibian species. Returning ponds and

lakes in Yellowstone National Park, in particular, to their historically fishless states can

provide more habitat for tiger salamanders *Ambystoma tigrinum* and, potentially, boreal

chorus frogs since these species cannot coexist with or breed in the presence of fish

(Koch & Peterson 1995). It is important to keep this in mind when determining the

ultimate fate of water bodies where fish have been removed. While creating genetic

sanctuaries for native trout is important, it may come at the cost to some native

amphibians that cannot coexist with fish. Returning historically fishless waters to their

original status, a result of fish removal practices, can therefore be an important tool in

amphibian conservation.

This study provides much needed information on the effects of age, species, and

exposure duration on the toxicity of rotenone to tadpoles, but additional research remains.

While it appears as though application of rotenone products at dosage levels currently

used to kill fish will result in significant tadpole mortality, it is important for fisheries

managers to continue to investigate techniques that will lessen this impact. In

conjunction with this, continued field research and laboratory experiments addressing,

among other things, the effects of the environment on rotenone's toxicity to larval

amphibians are needed. Determining ways to mitigate the effects of fish removal and

restoration on amphibians is both important and achievable.

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