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The Interactive Effects of Multiple Stressors on Lithobates catesbeianus and Anaxyrus americanus

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ABSTRACT

Amphibian populations worldwide have experienced dramatic declines, and many species have already become locally, regionally, or globally extirpated with thousands more being threatened with extinction. These declines have occurred more rapidly in amphibians than any other group of vertebrates, which is especially concerning to scientists because amphibians serve as indicator species of overall environmental health. Major causes for amphibian declines are discussed in Chapter 1 and include: habitat modification and destruction, commercial over-exploitation, introduced species, environmental contaminants, global climate change, and infectious diseases.

Chapter 2 discusses the major research aspects of the thesis by examining the interactive effects of multiple stressors on two species of larval amphibians. The study investigated the individual and combined effects of a major environmental contaminant (Glyphosate, commercial Roundup®), increased temperatures, and predatory cues on survival, growth, and development of tadpoles from two species (*Lithobates catesbeianus* and *Anaxyrus americanus*). Glyphosate reduced tadpole survival in both amphibian species and becomes more toxic to tadpoles as temperature increases. Increased temperature reduced survivorship over time in both species; however, survivorship decreased only when temperature interacted with glyphosate. Increased temperature also caused a decrease in growth in *L. catesbeianus* and an increase in growth and development in *A. americanus.* Accelerated growth and development caused by temperature may ameliorate the adverse effects of glyphosate by reducing larval period and increasing size at metamorphosis. Glyphosate caused significant anatomical shape variation in *L. catebeianus,* while increased temperature caused significant anatomical shape variation in *A. americanus*. The shape variations caused by the different stressors may lead to further developmental and behavioral abnormalities. Predatory cues had no effect on *A. americanus* survival, and only

decreased growth and development at intermediate glyphosate concentrations and temperatures; therefore, the effects of temperature and glyphosate concentration may have been enhanced in the presence of predatory cues. The study highlighted the importance of examining the interactions between multiple stressors on amphibian declines.

Chapter 3 focuses on potential solutions for global amphibian declines. Conservation efforts such as educational outreach, effective land management and water quality regulation guidelines, captive breeding programs, and several others are discussed.

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CHAPTER 1: INTRODUCTION

Recent Amphibian Declines

Over that past several decades, declines of amphibian populations have been occurring throughout the world (Alford and Richards 1999). Since the 1980s, amphibian species all over the world have experienced population declines, and species have disappeared at alarming rates, even in protected areas (Dodd 2010). Specifically, over one-third of amphibian species worldwide have experienced some form of population decline, and over 120 documented amphibian extinctions have occurred since 1980 (Whitfield et al. 2007). Data suggest that at least 2,468 amphibian species are experiencing some level of population decrease (Stuart et al. 2004). Species loss and decline is occurring more rapidly in amphibians than either birds or mammals, and declines seem to be non-random in terms of geographic range, taxonomic association, and ecological preference with Neotropical, montane, stream-inhabiting species being the most vulnerable (Stuart et al. 2004). Amphibian declines have become very concerning to many scientists, particularly because many species serve as indicator organisms of overall environmental health, and there does not seem to be a clear and simple cause for the recent declines (Collins and Storfer 2003). Knowledge of amphibian declines became widespread at the First World Congress of Herpetology in 1989, and since then the World Conservation Union Global Amphibian Assessment (GAA) has been working to determine threats to all amphibian species (Stuart et al. 2004). The International Union for Conservation of Nature (IUCN), which assesses the status of species on a global scale, lists 41% of amphibian species as threatened with extinction (Baillie et al. 2004). The major threats to amphibians include: habitat modification and destruction, commercial over-exploitation, introduced species, environmental contaminants, global climate change, and infectious diseases (Alford and Richards 1999, Baillie et al. 2004,

Stuart et al. 2004, Dodd 2010). Several of these causes (eg. global climate change, UV radiation, and environmental toxins) can interact to further increase amphibian susceptibility to disease and other pathogens (Alford and Richards 1999).

Anurans and other amphibians play a vital role in the ecosystem, particularly because they are capable of movement between and within aquatic and terrestrial environments (Duellman and Trueb 1994). By consuming large amounts of algae, tadpoles help to reduce the rate of natural eutrophication, over-enrichment of water with nutrients, and oxygen depletion in several aquatic ecosystems (Dodd 2010). Most anurans also convert about half of the energy they gain from food into new tissue which is then transferred to the next level of the food chain, allowing for sufficient energy flow from one level to the next (Dodd 2010). Without the presence of amphibians, a trophic cascade could be initiated which would affect many other species and lead to a loss in biodiversity. The absence of amphibians would lead to an over-abundance of their prey items (insects), while also reducing the population size of many of their predator species (birds, reptiles). Not only do amphibians serve vital ecosystem functions, but they are also important model organisms used in medical research and teaching.

Most anurans and other amphibians have aquatic larvae and lay their eggs in the water, however; some species may lay their eggs out of water, yet still have aquatic larvae. (Dodd 2010). Anuran larvae (tadpoles) differ highly in terms of morphology in comparison to adult anurans. Tadpoles require mouth parts and digestive systems that are very different from that of adult frogs (Duellman and Trueb 1994). Also unlike the adults, tadpoles possess a tail and well-developed caudal fins to help propel themselves through the water, while adult frogs have short, tailless bodies and long legs which are well suited for jumping (Duellman and Trueb 1994). The larval stage of anurans is one of the most sensitive life stages, as tadpoles are highly susceptible

to voracious aquatic predators such as: fish, turtles, predaceous diving beetles, dragonfly larvae, water scorpions, crawfish, and other amphibians (larval salamanders). The semi-permeable skin of tadpoles allows for easy absorption of compounds, which makes tadpoles very sensitive to environmental contaminants that can accumulate in aquatic ecosystems.

Tadpoles have also been seen to exhibit chemosensory predator recognition by reducing movement and increasing aggregation in the presence of predatory chemical cues (Marquis et al. 2004). There are debates regarding whether this predator recognition behavior is innate or learned, however; studies have shown that there is no difference in anti-predator behavior between wild-caught and laboratory-reared tadpoles in the presence of predatory chemical cues (Gallie et al. 2001). Lack of difference between wild-caught and laboratory-reared tadpoles in anti-predator behavior in the presence of predatory chemical cues suggests that this behavior is innate and present in certain tadpole species (Gallie et al. 2001). At the onset of metamorphosis, the tadpoles will be transformed, and they will develop the adult anuran morphology (Duellman and Trueb 1994).

Anthropogenic Effects

It is clear that there are many causes to recent amphibian declines, and many scientists agree that the combination and synergistic effects of multiple causes are responsible for the majority of amphibian declines (Alford and Richards 1999). Scientists also agree that anthropogenic activities are significantly related to the rapid degeneration of amphibian populations worldwide (Vitt and Calwell 2008). Humans can negatively affect amphibians in a number of direct ways: increasing habitat modification, commercial harvesting of amphibians, spread of disease, introduction of invasive species, and high use of pesticides and herbicides (Vitt and Caldwell 2008). Indirectly, humans negatively affect amphibians by increasing the rate

of climate change by inputs of carbon into the atmosphere and increasing the depletion of the ozone through use of chlorofluorocarbons and other chemicals (IPCC 2007, Blaustein et al. 2003). Unfortunately, with the global human population growing at a rate of over 211,000 people per day, the effects of human activities on amphibians in the future is only likely to increase unless changes occur in the way humans interact with and treat the environment (Vitt and Caldwell 2008).

Habitat Loss and Modification

Most scientists agree that the major threat to amphibian decline is habitat modification and destruction due to human activities such as landscape change from naturally forested areas to agricultural areas (Dodd 2010). Assessments show that habitat loss or modification has affected 183 amphibian species worldwide (Stuart et al. 2004). Creation of agricultural areas via habitat modification has become a major concern among many scientists not only because most amphibians are capable of moving among and within aquatic and terrestrial environments, but also because they have semi-permeable skins which may increase their susceptibility to environmental toxins such as chemicals present in agricultural runoff (Alford and Richards 1999).

Habitat modification can also create more proximate causes of amphibian decline. Local and regional populations as well as species can become extinct due to loss of suitable habitat and prevention of access to breeding ponds, which is caused by habitat modification (Collins and Storfer 2003). Deforestation can drastically alter microhabitats, increase soil compaction and desiccation, and reduce habitat complexity, which can lead to declines in terrestrial amphibian species such as salamanders (Alford and Richards 1999). Deforestation can also alter local climate patterns by reducing precipitation, evapotranspiration, and cloudiness, which can reduce

overall humidity at the local scale (Werth and Avissar 2002). Large scale logging of forests opens up the canopy and leads to significant increases in surface temperature (Shukla 1990). Increases in surface temperature can increase the flammability of the area by creating substantial amounts of combustible material in the form of dry litter (Malhi 2008). Local climate changes caused by deforestation such as warmer surface temperatures and decreases in precipitation and humidity will assuredly play a major role in amphibian declines.

Historical habitat loss can also lead to decreases in amphibian diversity (Hecnar 1997). Hecnar (1997) showed that historical drainage of wetlands and deforestation in Ontario, Canada destroyed most of the suitable habitat for local amphibian species (Hecnar 1997). Although construction of artificial ponds may have provided a beneficial solution to the problem of wetland drainage, only species that are highly adaptable to human-dominated landscapes were able to persist, leading to an overall loss in amphibian diversity in the area (Hecnar 1997). Allowing forests to regenerate and become mature can lead to recovery for many amphibian species, however; recovery to pre-disturbance levels can take a very long time and may never occur if forests are replanted as monocultures (Alford and Richards 1999).

Not only is habitat modification a major problem for amphibians in temperate areas, but it is also a major threat to tropical amphibian species (Gallant et al. 2007). Due to their ectothermic life histories and semi-permeable skin, the majority of amphibian species inhabit warm and moist areas, with tropical or subtropical environments containing the highest species richness (Gallant et al. 2007). Unfortunately, since the 20th century, the human population of the Earth has been growing exponentially, with the major growth occurring in tropical and subtropical regions (Gallant et al. 2007). With the huge increase in the human population also comes an increase in demands for resources such as food production. Increases in demand for

food production has led to conversion of more land in tropical and subtropical areas from forested areas to agricultural areas to support the growing demands of the human population (Gallant et al. 2007). By compiling maps of amphibian species richness, human population growth, and land cover changes, Gallant and others were able to determine that regions with the highest species richness and diversity of amphibians are also the regions with the highest amount landscape modification (Gallant et al. 2007). High amounts of landscape modification will undoubtedly lead to loss of amphibian species richness and diversity in these areas due to loss of suitable habitat and indirect effects of agriculture such as runoff of harmful pesticides and herbicides.

Commercial Over-Exploitation

Another major cause associated with global amphibian declines is commercial over-exploitation of amphibians for human use (Vitt and Caldwell 2008). The GAA estimates that commercial over-exploitation is the major cause of decline for fifty species of amphibians worldwide (Stuart et al. 2004). Decreases in amphibian diversity and richness due to over-exploitation also seem to be geographically non-random. The majority of declines caused by over-exploitation have occurred in East Asia or Southeast Asia, however; over-exploitation is causing declines in many other areas of the world as well (Stuart et al. 2004). Furthermore, the major amphibian families that are being threatened by over-exploitation are the Cryptobranchidae and Ranidae (Stuart et al. 2004).

One source of commercial use of amphibians is harvesting of frogs for human consumption as frog legs. Although harvesting of frogs has been occurring for decades, studies indicate that huge losses in species abundance can be caused by commercial harvesting (Collins and Storfer 2003). One study revealed that commercial harvesting of frog populations in one

Iowa county from 1920 to 1992 did, in part, lead to a decrease in frog abundance from 20 million individuals to around 50,000 individuals (Lannoo et al. 1994).

Disease

One of the most highly debatable causes for global amphibian declines is emerging infectious diseases caused by fungal and viral pathogens (Vitt and Caldwell 2008). The two most recognized pathogens involved with amphibian declines are the iridoviruses and chytrid fungus (Collins and Storfer 2003). Iridoviruses have been implicated as causes of mass amphibian extinctions worldwide, with the most common genera being ranaviruses (Daszak et al. 1999). Ranaviruses are highly virulent and can cause infections in adult and larval amphibians, however; larval amphibians (tadpoles) seem to be more vulnerable to infection (Daszak et al. 1999). Ranaviruses are still not completely understood and normally there is little sign of infection other than general weakness (Daszak et al 1999). However, mortality rates can reach 100% in infected larval amphibian populations, which would ultimately lead to decreases in adult amphibian populations (Daszak et al. 1999). Once infected by the virus, amphibians can develop an acute lethal disease in a very short incubation period (Daszak et al. 1999). The disease causes necrosis of hematopoitic and lymphoid tissues and leukocytes in most organs of the infected frog (Daszak et al. 1999). The disease can also lead to considerable hemorrhage in skeletal tissue, as well as increase the risk for secondary bacterial infections (Daszak et al. 1999). Ranaviruses can remain in the bottom of ponds for extended period of time and can be spread by fishing nets, boats, fishing rods, introduced fish during artificial stocking, or birds (Daszak et al. 1999). Although the link between ranaviruses and amphibian declines may not be as well understood as other diseases, ranaviruses do seem to have the potential to drastically reduce

amphibian population numbers, especially in isolated species and species with low fecundity rates (Daszak et al. 1999).

The most highly discussed disease related to recent amphibian declines would probably be chytridiomycosis. Chytridiomycosis is a disease that affects mainly mid to high elevation, stream-associated anuran species in tropical regions of Central and South America and Northern Australia (Skerratt et al. 2007). The disease is caused by the chytrid fungus *Batrachochytrium dendrobatidis*, which acts as a skin pathogen (Skerratt et al. 2007). Chytrid fungi are ubiquitous fungi and are found in aquatic habitats or moist soils where they degrade cellulose, chitin, and keratin (Daszak et al. 1999). Normally, parasitic chytrid fungi affect plants, protists, and invertebrates, however; *Batrachochytrium dendrobatidis* seems to be the only chytrid fungus to infect vertebrates (Daszak et al. 1999). Although signs of chytridiomycosis in anurans include: abnormal body posture, lethargy, loss of righting reflex, lesions, ulceration, and hemorrhages in the skin, muscles, or eyes, true diagnosis is only identified by the presence of intracellular flask-shaped sporangia within the epidermis (Daszak et al. 1999). The fungus uses keratin from the skin of adult anurans and the mouthparts of larval anurans as a nutrient and causes death in anurans by impairing cutaneous respiration and osmoregulation (Daszak et al. 1999).

Although chytridiomycosis has been reported as a potential cause of mass mortality and declines in many species of anurans in both Central America and Australia, the fungus was not present in histological surveys of museum specimens 10 years prior to population declines (Daszak et al. 1999). The absence of the fungus in museum specimens prior to declines suggest that chytridiomycosis has recently emerged as an anuran disease in both Central America and Australia (Daszak et al. 1999). Studies also suggest that the recent emergence of the disease may have been caused by anthropogenic introduction via pathogen pollution and global climate

change, which may have fostered increased transmission between individuals (Daszak et al. 2003). Although causes to the spread and introduction of chytridiomycosis are still being debated, many scientists agree that the impact that the disease has had on anurans is one of the largest losses to vertebrate biodiversity caused by any disease in history (Skerratt et al. 2007).

Global Climate Change

Global climate change can be interpreted in many ways and some of the most obvious observations include: increases in global average air and ocean temperatures, rising of the global average sea level, and increased melting of snow and ice caps (IPCC 2007). Temperatures are increasing around the globe with an average increase of 0.13°C per decade since 1956, which is almost twice the rate of temperature increase from 1906 to 1956 (IPCC 2007). Not only are temperatures increasing globally, but the rate of temperature increase is also rising with some areas experiencing a rise of 3.5°C since 1970 (IPCC 2007). Studies indicate that since 1996, almost every subsequent year is among the warmest years in global surface temperature in recorded history (IPCC 2007). Warming is not uniform across the globe, with increasing temperature rates being the highest over land and in higher northern latitudes (IPCC 2007). Increasing land temperatures are seen in a rise in average number of very hot days and nights and a decrease in cool to cold days and nights (IPCC 2007). The increased rate of warming in northern latitudes is causing polar ice caps to melt at high rates with the Artic sea ice extent decreasing by 2% per decade (IPCC 2007). Along with melting polar ice caps also comes increases in sea level by an average of 3.1 mm per year from 1993-2003 (IPCC 2007). With continuing increases in carbon emission global temperatures are expected to rise by up to 5.8°C by the year 2100, which can only lead to increased rates of melting ice and rises in sea level (IPCC 2007).

Not only is the temperature changing with global climate change, but changes in precipitation are also occurring. Studies show that there has been an increase in heavy precipitation events in most areas around the globe, however; there have been fewer precipitation events overall, which may lead to amphibian damaging droughts on a shorter scale (IPCC 2007). Some areas such as the Mediterranean, southern Africa, and parts of southern Asia are experiencing decreases in average annual precipitation (IPCC 2007). The decrease in precipitation in certain areas of the globe is leading to a growing likelihood of drought in many areas, which could be detrimental to amphibians (IPCC 2007).

Global climate change has been recognized by scientists worldwide, however; the effect of climate change on amphibian declines is only recently beginning to be understood. Assessments show that approximately 20-30% of plant and animal species worldwide, including amphibians, will experience an increased risk of extinction with a rise in temperatures of 1.5-2.5°C (IPCC 2007). Amphibians are at an especially high risk of decline and extinction caused by climate change because of their ectothermic lifestyles and their permeable skin which reduces the ability to retain moisture. Due to the unprecedented rate of change in climate, scientists predict that some species of amphibians may not be able to adapt to change quickly enough and may be limited to unsuitable habitats because of limited dispersal abilities (Collins and Storfer 2003).

Alterations in local climate caused by global climate change can affect the ecology of amphibians in many ways. In temperate environments, warmer spring temperatures can lead to snow cover melting faster. The increased rate of snow cover melting can in turn lead to earlier spawning and breeding times in amphibians (Alford and Richards 1999). With continued warming, many areas, especially high elevation areas, are projected to experience declines in

snowpack accumulation and snowmelt (Stewart 2009). Declines in snowpack accumulation and snowmelt can lead to alterations in river flow levels, which may affect stream breeding amphibian species (Arnell and Reynard 1996). As snowpack and glaciers become increasingly less abundant due to melting, there will also be less drainage into rivers which can decrease river flow and also impact amphibians.

In tropical environments, increased annual temperatures, extended dry seasons, and increasing variability in precipitation events can indirectly affect amphibians by decreasing the quality of leaf litter and prey availability (Alford and Richards 1999). A study conducted at La Selva Biological Station, Costa Rica suggests that climate driven reductions in leaf litter quality have caused a decline in terrestrial amphibians by 75% since 1970 (Whitfield et al. 2007). Another study conducted in the tropics reveals that many leaf litter anurans are already experiencing temperatures at or close to their critical thermal maximum (Holden and Whitfield 2011). Deforestation is only adding to the problem by reducing precipitation and humidity (Werth and Avissar 2002) and increasing surface temperatures (Shukla 1990). If temperatures in the tropics continue to rise at predicted rates and exceed species' critical thermal maximum, amphibians, especially leaf litter anurans, will be at a high risk of further decline and extinction if populations cannot adapt to climatic changes or shift to a more suitable habitat. Unfortunately, forest fragmentation has made the shift to a more suitable, higher elevation habitat very difficult, and scientists predict that there will be a net attrition of amphibians in lowland tropical areas (Colwell et al. 2008).

UV-B Radiation

Not only have humans helped to increase the rate of global climate change by carbon emission, but humans have also induced higher rates of ozone depletion and UV-B radiation

caused by the uses of chlorofluorocarbons (CFCs) and other chemicals (Blaustein et al. 2003). Studies also show that increased greenhouse gas emissions (caused by anthropogenic activities) induce stratospheric cooling, which results in further ozone depletion and more penetration of UV light through the atmosphere (Shindell et al. 1998). UV-B radiation is one of the most highly biologically damaging types of radiation to organisms and can cause decreases in growth rate and increases in immune dysfunction, mutation, and cell death (Blaustein et al. 2003). Normally, the majority of UV-B radiation is blocked from reaching the Earth's surface, however; depletion of the stratospheric ozone layer has led to an increase in the amount of UV-B radiation reaching the surface of the Earth in both temperate and tropical regions (Blaustein et al. 2003).

UV-B radiation can directly kill amphibians or it can work in concert with other causes such as environmental contaminants, pathogens, or climate change to adversely affect amphibians (Blaustein et al. 2003). Many studies have indicated that UV-B radiation can be extremely detrimental to amphibians during embryonic development by reducing the survival or hatching of amphibian embryos (Alford and Richards 1999). By comparing hatching success rates between groups of amphibian embryos exposed to ambient UV-B radiation and groups of amphibian embryos shielded from UV-B radiation, Blaustein and others found that overall hatching success rate was higher in the groups of embryos that were shielded from UV-B radiation (Blaustein et al. 2003). Although UV-B radiation did seem to decrease hatching success in most embryos, some embryos were not affected by UV-B radiation indicating that some species may be more vulnerable to UV-B radiation than other species (Blaustein et al. 2003). UV-B radiation can also interact with environmental contaminants such as pesticides and increase the toxicity of the contaminant (Blaustein et al. 2003). By working synergistically, UV

radiation and environmental contaminants can increase mortality on larval amphibian populations more than radiation or contaminant alone (Blaustein et al. 2003).

Although amphibian mortality (failure of embryos to hatch) can be caused by UV-B radiation, there are also several other sub-lethal effects on amphibians that can be caused by UV-B radiation. UV-B exposure has been seen to negatively alter behavior and induce developmental and physiological malformations such as skeletal abnormalities and eye damage in larval and adult individuals (Blaustein et al. 2003). Reductions in anti-predatory escape behavior have been seen in anuran tadpoles that were exposed to low levels of UV-B radiation (Kats et al. 2000). Deformations can be seen in up to 90% of individuals exposed to ambient levels of UV-B radiation compared to only 5% of individuals that were shielded from exposure (Blaustein et al. 2003). Physiological malformations in the eyes of anurans can damage photoreceptors and significantly reduce vision, which can indirectly lead to an increased risk of predation (Fite et al. 1998).

Studies show that embryos of certain amphibian species may have better defense against the harmful effects of UV-B radiation due to higher levels of photolyase, a photoreactivating DNA repair enzyme (Alford and Richards 1999). Photolyase can repair damaged segments of DNA by removing cyclobutane pyrimidine dimers (CPDs), which are caused by UV-B exposure (Blaustein et al. 2003). Selective pressures over evolutionary time have also led to mechanisms that reduce the species susceptibility to negative effects caused by UV-B radiation (Blaustein et al. 2003). Some of these mechanisms include: sunlight avoidance behavior, pigmentations in the skin that absorb UV light, UV-protective jelly that surrounds deposited eggs, or wrapping eggs in leaves and other plant material to prevent direct exposure to solar radiation (Blaustein et al. 2003).

Introduced/Exotic Species

Introduced and exotic species have also had a major effect on amphibian populations and helped to contribute to recent amphibian declines (Alford and Richards 1999). Introduced species can directly affect amphibian populations by increasing predation on native species, which ultimately leads to declines and even extinctions of native amphibian populations, even within protected areas (Collins and Storfer 2003). Some of the major alien predators to native amphibian populations include introduced fish, bullfrogs, cane toads, and crayfish (Kats and Ferrer 2003). A major problem species that has been introduced throughout the United States is the bullfrog, *Lithobates catesbeianus* (Alford and Richards 1999). Bullfrogs are established at very high densities outside of their natural range, and they consume smaller, native frogs and outcompete many native species leading to local declines (Alford and Richards 1999). Cane toads, *Bufo marinus*, have become one of the most invasive organisms in Australia and can suppress activity levels of native anuran species (Greenless et al. 2007). Native Australian anuran species also experience high levels of mortality when native tadpoles feed on toxic cane toad eggs (Crossland et al. 2008).

Although many areas are protected and managed to prevent habitat modification, the introduction of non-native fishes into protected areas is a common practice throughout the world and can drastically affect amphibian abundance and distribution (Knapp and Matthews 2000). Knapp and Matthews investigated the relationship between the decline of the mountain yellow legged frog, *Lithobates muscosa,* and introduced non-native fish species (trout) in the California's Sierra Nevada (Knapp and Matthews 2000). The study looked at over 1,700 sites in two adjacent historically fishless protected areas that differ mainly in the distribution of introduced non-native fish species (Knapp and Matthews 2000). The results reveal that

introduced fish species negatively affected the distribution of frogs at three separate scales: landscape, watershed, and individual water bodies (Knapp and Matthews 2000). At the landscape scale, data showed that the introduction of fish into the protected bodies of water negatively affected the distribution of the frogs (Knapp and Matthews 2000). At the watershed scale, results indicate that the total area of water occupied by fish was negatively correlated with the total area of water occupied by frogs (Knapp and Matthews 2000). Interpreting data at the individual water body scale revealed that frogs were three times more likely to be found and six times more abundant in sites without introduced fish species than water bodies with introduced fish species (Knapp and Matthews 2000). Another study investigating the relationship between the decline of the mountain yellow legged frog and introduced fish species proved that by removing the introduced fish species, rapid recovery of frog populations can occur (Vredenburg 2004). Even in protected areas, amphibian distribution and abundance can be affected by alien predatory species, however; recovery of amphibian populations is possible if introduced species are removed from the system (Knapp and Matthews 2000, Vredenburg 2004).

Not only can introduced species have a direct effect on the state of amphibian populations through predation, but non-native species can also have several negative indirect effects on amphibian populations such as: increasing competition between one or more life stages, introduction of pathogens by non-native species, and hybridization (Collins and Storfer 2003). Decreased growth and decreased size at metamorphosis when introduced predators are present has also been seen to occur in larval amphibian populations (Kats and Ferrer 2003). The decrease in growth and size at metamorphosis is due to reduced movement and reduced feeding by larval amphibians in the presence of introduced predators (Kats and Ferrer 2003). Dispersal of amphibians can also be affected by introduced species (Alford and Richards 1999). In areas were

fish are being introduced into only bodies of water, frogs may still persist in fish-free environments (Knapp and Matthews 2000). However, these frogs are unable to migrate and disperse to other sources of water because the surrounding bodies of water contain introduced predatory fish species (Alford and Richards 1999). By isolating amphibian populations from one another in this way, regional extinctions may occur because of problems in migration among local populations (Alford and Richards 1999).

Environmental Contaminants

The introduction of contaminants from anthropogenic activities is a major concern in recent amphibian declines (Dodd 2010). Among the most common environmental contaminants include pesticides, herbicides, and nitrogenous fertilizers, which are released into aquatic environments and can negatively affect larval amphibian populations (Vitt and Caldwell 2008). Other chemicals such as pharmaceuticals, estrogenic compounds, endocrine disrupting compounds, and other organic wastewater contaminants are also very common in aquatic systems and can interfere with tadpole development (Fraker and Smith 2004, Hogan et al. 2006). These chemicals can be released into the environment from farms, lawns, golf courses, and factories. Although the Clean Water Act regulates many point sources such as factories, there is little regulation of non-point sources such as farms and lawns (Clean Water Act of 1972).

Herbicides

Herbicides such as Roundup® (Monsanto Company), Atrazine® (Syngenta Group Company), 2,4-D® (Tenkoz Inc.), and Amitrole® (Nufarm Agriculture Inc.) are used widely used throughout the United States and many other parts of the world, and all can have devastating effects on amphibians (Relyea 2005a, Hayes et al. 2002, Mandrillon and Saglio 2007). The active ingredient in Roundup® is glyphosate, and glyphosate is one of the most

common commercially used herbicides in the world (Relyea et al. 2005). Mesocosm studies using 1,200 L cattle tanks as experimental habitats show that Roundup can reduce the overall species richness of all animal taxa present in the community by 22% (Relyea 2005a). Tadpole richness alone was reduced by 70% in the presence of Roundup® at the manufacturer's recommended application rate (Relyea 2005a). The presence of Roundup® completely eliminated two species (leopard frog and gray tree frog) and reduced the survival to only 2% in the wood frog (Relyea 2005a).

Relyea conducted another study to determine if the presence of soil in the aquatic system played a role in survival of larval amphibians (Relyea 2005b). Glyphosate is seen to be absorbed by soils, especially clay particles, and is subject to microbial breakdown, however; 96%-100% of larval amphibians died in the presence of Roundup® application at the recommended manufacturer's rate regardless of soil presence (Relyea 2005b). At the end of a three week period, only 2% of all individuals across all species survived (Relyea 2005b). A decrease in wood frog survival from 75%-2%, American toad survival from 97%-0%, and leopard frog survival from 98%-4% was seen when Roundup® was present (Relyea 2005b). In the same study, Relyea also investigated the effect of Roundup® on juvenile anuran species, and results show that 68%-86% of all juvenile individuals died in the presence of Roundup® (Relyea 2005b). Across all species, only 21% of the individuals survived after one day of exposure to Roundup® (Relyea 2005b).

Although these studies indicate that Roundup® can be extremely harmful to larval and juvenile amphibian populations, it does not include factors that may be experienced in nature such as predation. Reylea realized this problem and decided to test the effect of Roundup® on six larval anuran species in the presence and absence of predatory cues (Relyea 2005c). The

results indicate that the toxicity of Roundup® is increased in the presence of predatory cues, and the LC50 (concentration to kill 50% of a population) for Roundup® on the tadpole species decreased significantly in the presence of predatory cues (Relyea 2005b). Roundup® even became twice as lethal for wood frog tadpoles in the presence of predatory cues (Relyea 2005c). Roundup® can also cause indirect effects in tadpoles such as reducing biomass by 40% in certain species (Relyea et al. 2005). Clearly, the presence of Roundup® in aquatic systems can cause decreases in growth and survival of anuran tadpoles, yet many other herbicides can also produce negative effects in amphibian populations.

Atrazine® is the most commonly used herbicide in the United States and probably the world, and it also has the potential to devastate amphibian populations (Hayes et al. 2002). Atrazine® is found in almost all bodies of fresh water and can even reach 40 ppb (part per billion) in precipitation (Hayes et al. 2002). Atrazine®, which is a type of triazine herbicide, works as an endocrine disruptor in amphibians (Hayes et al. 2002).

Hayes and others conducted a study to determine the effects of environmentally relevant levels of Atrazine® on *Xenopus laevis* development (Hayes et al. 2002). By exposing tadpoles to Atrazine® levels ranging from 0.01-200 ppb, Hayes found that sexual development is disrupted and levels as low as 0.1 ppb can induce hermaphroditism in tadpole populations and demasculinization of male tadpoles (Hayes et al. 2002). Male tadpoles exposed to Atrazine® at these levels developed ovaries and had significantly smaller laryngeal size, which is important in male calling during breeding behavior (Hayes et al. 2002). Hayes also discovered that Atrazine® exposure at levels as low as 25 ppb can cause a 10-fold decrease in testosterone levels in sexually mature males (Hayes et al. 2002). The study further suggests that Atrazine® is disrupting steroidogenesis by inducing aromatase, which converts testosterone into estrogen

(Hayes et al. 2002). The results of this study are especially important because the levels of Atrazine® exposure used in the experiment are environmentally relevant and can be seen throughout nature, and even are found in precipitation (Hayes et al. 2002). Hayes suggests that to fully understand the effects of Atrazine® and other endocrine disrupting compounds on amphibians and other organisms in the environment, future studies must be integrative and invoke the disciplines of ecology, developmental biology, molecular biology, evolutionary biology, and cellular biology, as well as fields outside of biology such as chemistry, meteorology, and mathematics (Hayes 2005).

Another commonly used herbicide, Amitrole®, can also affect larval amphibians, however; the effect is indirect and does not directly reduce tadpole survival (Mandrillon and Saglio 2007). The presence of Amitrole® has been seen to negatively affect tadpole behavior in the presence of predators (Mandrillon and Saglio 2007). Tadpoles exposed to levels of Amitrole® ranging from 0.01-10.0 mg/L exhibited a lack of anti-predatory behavior when exposed to predatory salamanders (Mandrillon and Saglio 2007). Tadpoles became more active and decreased the amount of refuge use when exposed to Amitrole® in the presence of predators (Mandrillon and Saglio 2007). Although Amitrole® was not seen to directly affect tadpole survival, the lack of anti-predatory behavior in the presence of Amitrole® and predators may increase the likelihood for natural mortality via predation.

Monosodium Methanearsonate (MSMA) is a commonly used, arsenic-based herbicide that is used primarily on golf courses under the name Target 6.6® (Luxembourg-Pamol, Inc.) (Pichler 2008). Pichler investigated 28 golf course lakes in Florida, and found that arsenic concentration levels are up to 100 times higher in golf course lakes than non-golf course lakes (Pichler 2008). Furthermore, Pichler found that once the loading capacity of the sediment in golf-

course lakes is reached, arsenic from the golf course lakes can enter the local aquifer (Pichler 2008). Studies have shown that environmentally relevant levels of arsenic can negatively affect larval anuran behavior by decreasing swimming performance, which can increase larval anuran susceptibility to predation (Chen et al. 2009). Other studies have shown that tadpoles exposed to MSMA have a higher incidence of lordosis (inward curvature of the vertebrae) than tadpoles not exposed to MSMA (Britson and Threlkeld 1998). Britson and Threlkeld also found that tadpoles with lordosis have decreased feeding, which can also lead to decreases in growth or survival if the minimum tadpole size needed for metamorphosis is not reached prior to pond drying (Britson and Threlkeld 1998). Monosodium Methanearsonate has also been seen to significantly reduce survival in larval and juvenile Couch's spadefoot toad when applied at concentrations as low as one-eighth of the manufacturer's recommended application rate (Judd 1977).

Pesticides

Pesticides are another form of environmental contaminant that amphibian populations can be exposed to in nature (Vitt and Caldwell 2008). Some of the most commonly used pesticides in the United States that can negatively affect amphibian populations include: Malathion® (Hi-Yield Chemical Company), Sevin® (TechPac, LLC), and Endosulfan (Drexel Chemical Company) (Relyea 2004, Boone et al. 2004, Brunelli et al. 2009).

Malathion® is a widely used organophosphate pesticide that is sprayed over aquatic habitats to reduce mosquito densities that may carry malaria or West Nile virus (Relyea 2004). Studies have shown that Malathion® is moderately toxic to larval amphibians and can have a LC50 ranging from 1.25-5.9 mg/L (Relyea 2004). Relyea investigated the effect of exposure to Malathion® at levels ranging from 0-20 mg/L on the survival of six tadpole species in the presence and absence of predators (Relyea 2004). Malathion® can significantly reduce survival

in all six species of tadpoles at levels as low as 0.1 mg/L (Relyea 2004). Furthermore, 0% survival was seen in most species studied when exposed to 5 mg/L of Malathion® (Relyea 2004). In the presence of predators, Malathion® can become up to two times as lethal to gray tree frog tadpoles, however; the presence of predators did not affect the toxicity of Malathion® to the other five species of amphibians studied (Relyea 2004).

Malathion® exposure at the manufacturer's recommended application rate, decreases overall species richness decreased by 30% and significantly affected the survival of leopard frogs, wood frogs, and gray tree frogs (Reylea 2005a). Malathion® can also decrease survival in American toad tadpoles by up to 11% (Relyea et al. 2005). The decrease in toad tadpole survival, as well as the decrease in gray tree frog tadpoles became even more significant in the presence of predatory newts (Relyea et al. 2005).

Malathion® can not only directly affect amphibian populations by reducing tadpole survival, but it can also have an indirect affect amphibian populations (Relyea and Diecks 2008). Malathion® has been applied at rates ranging from 0.01-0.25 mg/L to aquatic mesocosm environments containing zooplankton, phytoplankton, periphyton, and leopard frog tadpoles (Relyea and Diecks 2008). Malathion® can indirectly affect leopard frog tadpoles by inducing a trophic cascade (Reylea and Diecks 2008). Concentrations of Malathion® as low as 0.01 mg/L can significantly reduce zooplankton abundance, which leads to an increase in phytoplankton (Relyea and Diecks 2008). The increase in phytoplankton results in greater competition with periphyton and ultimately reduces periphyton abundance (Relyea and Diecks 2008). The reduction in periphyton abundance causes significant decreases in tadpole growth and development, which leads to an increase in overall tadpole mortality (Relyea and Diecks 2008). These results are important because they imply that Malathion® can induce changes in the

community structure of aquatic environments, which can lead to an increased likelihood of tadpole mortality (Relyea and Diecks 2008).

Sevin®, with active ingredient carbaryl, is another commonly used pesticide throughout the United States (Relyea and Mills 2001). Sevin® is applied by direct overspray of croplands and can enter amphibian containing wetlands via direct overspray, aerial drift, terrestrial runoff, or erosion (Relyea and Mills 2001). Gray tree frog tadpoles exposed to concentrations of Sevin® ranging from 0.045-0.09 mg/L for only one week exhibited a significant increase in mortality (Relyea and Mills 2001). Results show that exposure to 0.05 mg/L of Sevin® can reduce tadpole survival to 40%, while exposure to 0.09 mg/L of Sevin® can reduce tadpole survival to 8% (Relyea and Mills 2001). By adding predatory cues to the environment, exposure to Sevin® at 0.05 mg/L became more lethal to gray tree frog tadpoles and reduced survival to only 3% (Relyea and Mills 2001). Among all treatments containing concentrations of Sevin®, the presence of predatory cues made Sevin® two to four times more lethal to tadpoles, killing 60-98% of individuals (Relyea and Mills 2001). Exposure to Sevin® can reduce tadpole growth by up to 50% (Relyea and Mills 2001). Relyea furthered his research and investigated the effect of Sevin® and predatory cues on several other species of tadpoles and (Relyea 2003). Exposure to Sevin® at concentrations as low as 3.2 mg/L can significantly decrease survival in tadpoles of green frogs, bullfrogs, leopard frogs, wood frogs, and American toads (Relyea 2003). Furthermore, in the presence of predatory cues, Sevin® became more toxic to green frogs, bullfrogs, leopard frogs, and American toads (Relyea 2003). Even very low concentrations of Sevin® can cause significant declines in larval amphibian populations, especially in the presence of predatory cues (Relyea and Mills 2001, Relyea 2003).

The mass at metamorphosis was significantly smaller in high density populations than in controls, with no effect in low density populations when Woodhouse's toad tadpoles were exposed to a concentration of 5 mg/L of Sevin® (Boone et al. 2004). Exposure to Sevin® can increase time to metamorphosis and decrease size at metamorphosis in American toads (Boone et al. 2007). In the presence of Sevin® and bullfrog tadpoles, the increase in time to metamorphosis and decrease in size at metamorphosis in American toad tadpoles is greater than exposure to Sevin® alone (Boone et al. 2007). With longer time to metamorphosis and decreased size at metamorphosis, tadpoles are experiencing a greater risk of predation at the vulnerably larval life stage (Boone et al. 2007).

Endosulfan® is a widely used, organochlorine pesticide that is directly sprayed in agricultural areas in to reduce insect pests (Brunelli et al. 2009). Endosulfan® has been seen to have neurotoxic effects on mammals and fish and can be found in aquatic systems at concentrations as high as 0.5 mg/L (Brunelli et al. 2009). Brunelli and others investigated the effect of Endosulfan® on the European toad at environmentally relevant levels ranging from 0.01-0.1 mg/L (Brunelli et al. 2009). At concentrations as low as 0.05, Endosulfan® can significantly increase tadpole mortality (Brunelli et al. 2009). Exposure to Endosulfan® at these levels was also seen to increase the time to metamorphosis, impair behavior, and increase the incidences of mouth and skeletal malformations (Brunelli et al. 2009). Impaired behavior was seen earlier in the trials than any other response as irregular swimming or immobility (Brunelli et al. 2009). Malformations were seen starting at day 8 of the trials and include: bloated heads, edema, depigmentation of the skin, ragged tissue around the snout and mouth, and asymmetric or bent tails (Brunelli et al. 2009). Furthermore, at even the lowest concentration of Endosulfan® (0.01 mg/L) significant decreases in body weight of individuals was recorded (Brunelli et al.

2009). Environmentally relevant levels of Endosulfan® that occur in nature can cause severe damage to larval amphibian populations (Brunelli et al. 2009).

Nitrogenous Fertilizers

Nitrogen pollution is another major source of environmental contaminants, and it can enter aquatic systems through runoff of nitrogenous fertilizers from agricultural areas and areas containing livestock (Rouse et al. 1999). In the environmental nitrate levels in aquatic environments can range from <1.0-100 mg/L, and sublethal effects on amphibians can be seen in nitrate levels as low as 2.5 mg/L (Rouse et al. 1999). A survey of 8,545 bodies of water found that 19.8% of sources surveyed contained nitrate levels that exceed levels that are sublethal to amphibians (Rouse et al. 1999). Furthermore, these levels of nitrate can have negative effects on amphibian prey, which can in turn lead to declines in amphibian populations (Rouse et al. 1999).

Ammonium nitrate can also negatively affect tadpoles at environmentally relevant levels (Ortiz et al. 2004). After only eight days of exposure to ammonium nitrate at low levels (50 mg/L), there was a significant increase in mortality in the common tree frog (Ortiz et al. 2004). The Iberian painted frog and European toad also experienced significant increases in mortality after fifteen days of exposure to ammonium nitrate at the same level (Ortiz et al. 2004). At the highest concentration (200 mg/L), western spadefoot and natterjack toad tadpoles experienced decreased growth and high levels of abnormalities that include edemas and bent tails (Ortiz et al. 2004). Levels of ammonium nitrate that occur in nature are sufficient to have negative impacts on larval amphibian populations (Ortiz et al. 2004).

Ammonium nitrate can also indirectly affect amphibian populations (Ortiz-Santaliestra et al. 2010). Antipredatory behavior in the presence of red crayfish was significantly reduced with exposure to environmentally relevant levels of ammonium nitrate in Iberian painted frog and

spadefoot toad tadpoles (Ortiz-Santaliestra et al. 2010). Tadpoles exposed to ammonium nitrate were consumed significantly faster by red crayfish than control tadpoles (Ortiz-Santaliestra et al. 2010). Control tadpoles also exhibited specific defensive and antipredator behaviors while those tadpoles exposed to ammonium nitrate did not exhibit these behaviors (Ortiz-Santaliestra et al. 2010). The results of this study indicated that ammonium nitrate can negatively affect larval amphibians by indirectly increasing their risk to predation (Ortiz-Santaliestra et al. 2010).

Pharmaceuticals/Organic Wastewater Compounds

Organic wastewater compounds and pharmaceuticals such as caffeine, acetaminophen, and triclosan have been documented in several aquatic ecosystems and can have negative effects on amphibian populations (Fraker and Smith 2004). Studies show that exposure to ecologically relevant levels of triclosan can affect tadpole behavior by lowering startle response and reducing overall activity, which can indirectly increase susceptibility to aquatic predators (Fraker and Smith 2004). Fraker and Smith also found that high concentrations of caffeine can reduce overall growth and body size of northern leopard frog (Fraker and Smith 2004). Another study conducted by Smith and Burgett revealed that high concentrations of acetaminophen and intermediate concentrations of triclosan can significantly increase mortality in American toad tadpoles (Smith and Burgett 2005).

Endocrine disrupting compounds (EDCs) such as estradiol, ethinylestradiol, and 4-*tert*-octylphenol are other pharmaceuticals that commonly occur in aquatic environments as waste from power plants (Hogan et al. 2006). Hogan and others exposed wood frog and northern leopard frog tadpoles to several concentrations of estradiol, ethinylestradiol, and 4-*tert*-octylphenol, and found that all three EDCs are toxic to both species of anuran (Hogan et al. 2006). Furthermore, EDCs also decreased tadpole body weight, which may have long-term

effects on the rate of metamorphosis and cause anurans to remain in the larval stage for longer periods of time (Hogan et al. 2006).

Conclusion

Global amphibian declines are still not completely understood, and the major cause of these declines has yet to be discovered. Amphibian decline is not a simple problem and the causes of decline have been seen to interact to further increase amphibian susceptibility to pathogens (Alford and Richards 1999). To fully understand amphibian declines, multiple stressors must be examined together. Amphibians experience multiple stressors simultaneously in nature, and this interaction of stressors may be the proximal cause of amphibian population declines.

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CHAPTER 2: SYNERGISTIC EFFECTS OF HERBICIDE, PROJECTED INCREASED TEMPERATURE, AND PREDATION ON LARVAL ANURAN SURVIVAL, GROWTH, AND DEVELOPMENT

Abstract

Several studies have examined the effects of individual stressors on amphibians; however, few studies have determined the interactive effects of multiple stressors on amphibians. I investigated the individual and combined effects of a major environmental contaminant (Glyphosate, commercial Roundup®), increased temperatures, and predatory cues on survival, growth and development of tadpoles of two species, *Lithobates catesbeianus* and *Anaxyrus americanus*. Glyphosate reduced tadpole survival in *L. catesbeianus* and *A. americanus* by as much as 100 percent. An interaction between glyphosate and temperature indicated that elevated temperatures increased glyphosate toxicity to both amphibian species, as seen in lowered glyphosate LC50 values as temperature increases. Increased temperature reduced growth in *L. catesbeianus* tadpoles and accelerated growth and development in *A. americanus* tadpoles, even in the presence of glyphosate. Accelerated growth and development may ameliorate the adverse effects of glyphosate by reducing the larval period and exposure time to toxins, increasing size at metamorphosis, and providing survivors with a competitive advantage. Increased temperatures also caused significant anatomical shape variation in *A. americanus*, and glyphosate caused significant anatomical shape variation in *L. catesbeianus*. Variation in anatomical shape may lead to further developmental and behavioral abnormalities. Predatory cues had no effect on *A. americanus* survival, and only decreased development at intermediate glyphosate concentrations and temperatures. The observed interaction suggested that the effects of temperature and glyphosate concentration may have been enhanced by the presence of predatory cues. I highlight the importance of examining the interactions between multiple

stressors on amphibian populations, and further studies will be needed to better comprehend the synergistic effects of multiple stressors on global amphibian declines.

Introduction

Worldwide, amphibian populations have experienced dramatic declines over the past several decades, even in protected areas (Alford and Richards 1999). At least 2,468 species of amphibians are experiencing some form of population decline (Stuart et al. 2004) and over 120 amphibian species have been extirpated since 1980 (Whitfield et al. 2007). Declines in amphibian populations are also occurring more rapidly than in any other taxa such as birds or mammals, with Neotropical, montane, stream-inhabiting species being the most vulnerable to further decline and extirpation (Stuart et al. 2004). The highly permeable skin of amphibians and the presence of both aquatic and terrestrial life stages can increase amphibian susceptibility to environmental toxins and fluctuations in temperature or rainfall patterns, which may help to explain why amphibian populations are declining at a faster rate than other vertebrate groups (Alford and Richards 1999).

The possibility of a global pattern in amphibian declines and loss first became apparent at the First World Congress of Herpetology in 1989, where the World Conservation Union Global Amphibian Assessment (GAA) was formed to determine the major threats to all amphibian species (Alford and Richards 1999, Stuart et al. 2004). Amphibian declines have become a major concern to scientists worldwide, particularly because many amphibian species serve as indicator organisms of overall environmental health (Collins and Storfer 2003), and because amphibians play a vital role in nutrient cycling and energy flow in both aquatic and terrestrial ecosystems (Duellman and Trueb 1994, Dodd 2010). Although scientists do agree that amphibian populations are declining at alarming rates worldwide, there is still debate on the immediate

cause of these declines and there does not seem to be a clear and simple answer (Collins and Storfer 2003). The International Union for Conservation of Nature (IUCN) Red List of Threatened Species currently lists 41% of amphibian species as threatened with extinction (Baillie et al. 2004), with the major causes being habitat modification and destruction, commercial over-exploitation, introduced species, environmental contaminants, global climate change, and infectious diseases (Alford and Richards 1999, Baillie et al. 2004, Stuart et al. 2004, Dodd 2010). Studies also suggest that many of these causes can interact with each other to further increase amphibian susceptibility to pathogens and disease (Alford and Richards 1999).

Global climate change has become a major concern in amphibian declines (Alford and Richards 1999). Temperatures are increasing on average 0.13°C per decade on a global scale ,and with continuing increases in global carbon emissions, temperatures are expected to rise by up to 5.8°C by the year 2100 (IPCC 2007). Assessments show that approximately 20-30% of plant and animal species worldwide, including amphibians, will experience an increased risk of extinction with a rise in temperatures of 1.5-2.5°C (IPCC 2007). Amphibians are at an especially high risk of decline caused by increasing temperatures because of their ectothermic lifestyles and permeable skin, and some scientists predict that many species of amphibians will not be able to adapt to temperature changes quickly enough and may not be able to disperse to more suitable habitats (Collins and Storfer 2003). Many amphibians, especially in the Neotropics, are already experiencing temperatures at or near their thermal maximum (Holden and Whitfield 2011). Global climate change also causes variation in precipitation levels, leading to decreases in annual precipitation and increases in drought in many areas around the world (IPCC 2007). Decreased precipitation, along with declines in snowpack accumulation and snowmelt, may reduce potential

breeding areas for amphibians and further accelerate amphibian declines (IPCC 2007, Stewart 2009, Arnell and Reynard 1996).

Another major threat to amphibians is habitat modification and destruction (Dodd 2010), with over 183 species of amphibians being affected worldwide (Stuart et al. 2004). Not only does habitat modification directly affect amphibian populations by destroying suitable habitats, especially for breeding (Collins and Storfer 2003), conversion of habitat to agricultural areas also increases the amount of environmental toxins, from agricultural runoff, in aquatic environments that amphibians inhabit (Alford and Richards 1999). Studies also suggest that areas of highest amphibian species richness are also the areas of highest habitat modification and conversion of natural landscape to agricultural areas (Gallant et al. 2007).

The introduction of environmental contaminants from anthropogenic activities such as agriculture has become a major concern in amphibian declines (Dodd 2010) and among the most common include herbicides, pesticides, and nitrogenous fertilizers that are introduced via agricultural runoff into aquatic ecosystems (Vitt and Caldwell 2008). These chemicals can be introduced into the environment from direct overspray of agricultural fields, lawns, factories, and golf courses; however, there is little regulation of most of these sources (agricultural fields, lawns, golf courses) under the Clean Water Act (Clean Water Act of 1972).

Many studies have been conducted to determine the indirect and direct effects of chemical contaminants found in agricultural runoff on amphibians populations (Boone et al. 2004, Boone et al. 2007, Britson and Threlkeld 1998, Brunelli et al. 2009, Chen et al. 2009, Judd 1977, Hayes et al. 2002, Hayes 2005, Mandrillon and Saglio 2007, Ortiz et al. 2004, Ortiz-Santaliestra et al. 2010, Pichler 2008, Relyea and Mills 2001, Relyea 2003, Relyea 2004, Relyea 2005a, Relyea 2005b, Relyea 2005c , Relyea et al. 2005, Relyea and Diecks 2008, Rouse et al.

1999). Herbicides such as Roundup® (Monsanto Company), with the active ingredient glyphosate, are used widely throughout the United States and other parts of the world and have been shown to reduce overall amphibian species richness and survival when applied at the manufacturer's recommended rate (Relyea 2005a, Relyea et al. 2005). Other commonly used herbicides such as Amitrole® (Nufarm Agriculture Inc.) and Atrazine® (Syngenta Group Company) can also have indirect effects on amphibian populations (Mandrillon and Saglio 2007,Hayes et al. 2002). Applied at or below the manufacturer's recommended rate, Amitrole® can negatively affect amphibians by reducing anti-predatory behavior in tadpoles, which increases susceptibility to predation (Mandrillon and Saglio 2007). Atrazine® can also indirectly effect amphibian populations by inducing hermaphroditism and decmasculinization in tadpole populations at concentrations so low that they can be found in precipitation (Hayes et al. 2002).

Several herbicides and pesticides that are found in agricultural runoff and make their way into aquatic ecosystems can interact with other stressors such as predation and temperature to further increase negative effects on amphibian populations (Boone and Bridges 1999, Boone et al. 2007, Broomhall 2002, Relyea and Mills 2001, Relyea 2003, Relyea 2004, Relyea 2005b, Relyea 2005c, Relyea et al. 2005, Rohr et al. 2011). The toxicity of Roundup® increases and can become up to twice as lethal to certain tadpole species in the presence of predatory cues (Relyea 2005b, 2005c). Malathion® (Hi-Yield Chemical Company) and Sevin® (TechPac, LLC), two of the most commonly used pesticides in the world, can also become more lethal to tadpoles in the presence of predatory cues (Relyea 2004, Relyea et al. 2005, Relyea 2003, Relyea and Mills 2001). Other studies have shown that in the presence of predators, Sevin® can indirectly effect tadpole populations by reducing size at metamorphosis and increasing larval period, which can further increase exposure to environmental toxins and aquatic predators

(Boone et al. 2007). Increased temperature can increase the toxicity of Sevin® to tadpoles, resulting in further decreases in survival (Boone and Bridges 1999). Other studies suggest that increased temperature may increase tadpole growth and ameliorate the adverse effects of environmental toxins by reducing exposure to toxins and reducing exposure to highly voracious and abundant aquatic predators (Rohr et al. 2011). Increased temperatures cause increased metabolic rates in amphibians which can lead to increased excretory processes and possible increase in detoxification (Duellman and Traub 1994).

Herbicides are used widely throughout the United States with almost one million farms and 41 million households using some form of herbicide (Grube et al. 2011). Glyphosate (commercial names: Roundup®, Rodeo®) is the most commonly used herbicide in the agricultural sector, with 180-185 million pounds used in the United States alone in 2007 (Grube et al. 2011). In 2007, glyphosate was also the second most commonly used herbicide in everyday home and gardening, with 5-8 million pounds used in the United States (Grube et al. 2011). Glyphosate inhibits the synthesis of essential amino acids within plants (Tomlin 2006) and is widely used in agriculture, forestry, industrial weed control, lawns, gardens, and aquatic environments (Tomlin 2006). Glyphosate can be found in terrestrial environments where it is applied via direct overspray; however, spray drift and agricultural runoff can lead to glyphosate entering aquatic ecosystems where it is highly soluble in water (Schuette 1998). In soil, glyphosate has a half-life ranging from 3-130 days and a soil dissipation half-life averaging 44-60 days (Schuette 1998). In water, the hydrolysis half-life of glyphosate is > 35 days, and ranges from 35-63 days in water obtained from natural sources (Schuette 1998). Glyphosate loss is primarily caused by sedimentation absorption (especially in aquatic ecosystems) and microbial degradation, with rates of decomposition depending on microbial population types and

abundance (Schuette 1998). Water tends to have fewer microorganisms than most soils, therefore glyphosate is able to persist longer in aquatic environments, where larval amphibians inhabit, than in terrestrial environments (Schuette 1998). The maximum glyphosate concentration expected for aquatic habitats in nature after a single application via direct spraying for terrestrial or aquatic weeds at the manufacturer's recommended rate is 3.7 mg/L (Giesy et al. 2000). Other estimates predict that glyphosate may reach concentrations up to 10.1 mg/L in aquatic environments when applied at the manufacturers recommended rate (Mann and Bidwell 1999); however, the highest concentration of glyphosate to be observed in natural wetlands is 6.9 mg/L (Edwards et al. 1980). By simulating direct overspray, past studies have shown LC50 estimates (concentration to kill 50% of the population) for glyphosate on tadpoles to range from 0.55 mg/L (Relyea 2005c) to 15.5 mg/L (Mann and Bidwell 1999) depending on species. After being applied to a target site, glyphosate will be absorbed by the plants or soil and has very little pre-emergent activity; therefore application of glyphosate can occur many times throughout the year to ensure weed control (Schuette 1998).

Herein, I test for an interaction between multiple stressors (glyphosate, increased temperatures, and predatory cues) on larval anuran survival, growth, and development. The focal amphibian species are common, non-threatened species (*Lithobates catesbeianus*, *Anaxyrus americanus*) that can be found co-occurring in aquatic ecosystems throughout Arkansas (Trauth et al. 2004). The predator species (Green darner dragonfly nymph) is a natural predator of the focal amphibian species (Trauth et al. 2004). Both amphibian and predator species can be found in aquatic environments that may be subject to environmental runoff (Trauth et al. 2004). The temperature range used is within the range of projected increased temperatures resulting from global climate change and is expected to occur in natural aquatic environments where tadpoles

live (IPCC 2007). All temperatures used are within the natural voluntary thermal tolerances of the focal amphibian species; however, the highest temperature used is representative of a temperature above the optimal thermal range for both amphibian species (Lucas and Reynolds 1967, Brattstrom 1963).

The main objectives of the study are: (1) To determine the effects of varying concentrations of glyphosate on survival, growth, and development of two tadpole species, *L. catesbeianus* and *A. americanus*, (2) To determine the effects of varying temperatures on survival, growth, and development of *L. catesbeianus* and *A. americanus*, (3) To determine the effects of predatory cues on survival, growth, and development of *L. catesbeianus* and *A. americanus*, and (4) To determine if any interaction exists between glyphosate, temperature, and predatory cues in reference survival, growth, and development of *L. catesbeianus* and *A. americanus*.

The main hypotheses of the study are: (1) As glyphosate concentration increases, survival, growth, and development will decrease in *L. catesbeianus* and *A. americanus*, (2) As temperature increases, survival will decrease; however, growth and development will increase in *L. catesbeianus* and *A. americanus*, (3) The presence of predatory cues will decrease survival, growth, and development in *L. catesbeianus* and *A. americanus*, and (4) Increased temperatures and the presence of predatory cues will increase the toxicity of glyphosate and further decrease survival, growth, and development in *L. catesbeianus* and *A. americanus*.

Methods and Materials

Animal Collection and Maintenance

Bullfrog tadpoles (*Rana catesbeianus*; renamed *Lithobates catesbeianus*) (Collins and Taggart 2009) were obtained from Carolina Biological Supply Company in March 2012.

Carolina Biological Supply Company breeds *L. catesbeianus* tadpoles throughout the entirety of the year and has multiple developmental stage classes available. *Lithobates catesbeianus* tadpoles obtained from Carolina Biological Supply Company were 1-2 inches in total length and identified as being at Gosner stage 25 (Gosner 1960). To ensure a hap-hazard sample of the available genetic variation, *L. catesbeianus* tadpoles obtained from Carolina Biological Supply Company were randomly selected from multiple broods of developing *L. catesbeianus* tadpoles, all of which were at the same developmental life stage.

American toad tadpoles (*Bufo americanus*; renamed *Anaxyrus americanus*) (Collins and Taggart 2009) were collected from an unidentified stream located near Pettigrew, AR in the Ozark National Forest, Newton County, AR, USA (35°49'22.41"N, 93°27'45.42"W) on May 12, 2012. *Anaxyrus americanus* tadpoles were collected from small pools along a 100 m segment of the creek. Tadpoles collected were obtained from multiple broods of tadpoles along the creek to ensure hap-hazard sample of the available genetic variation within that stream.

Lithobates catesbeianus and *A. americanus* tadpoles separated by species, and all individuals from each species were placed in single 38 L aquaria containing 20 L of dechlorinated water and mixed thoroughly. Tadpoles were fed goldfish pellets every two days and maintained in aquaria at a 12-h light photoperiod until experiments began. Water was replaced in each aquarium once a week. *Lithobates catesbeianus* experiments were conducted first, and *A. americanus* experiments began once tadpoles reached Gosner stage 25 to ensure that both *L. catesbeianus* and *A. americanus* tadpoles were at the same developmental life stage (Gosner 1960). Once experiments began, individual tadpoles were randomly selected and transferred into 0.47 L polyethylene experimental cups containing 0.36 L of dechlorinated water. Before transferring, each tadpole was blotted with paper towels and weighed to determine initial

mass. Experimental cups were then placed in growth chambers set at varying temperatures and maintained at a 12-h light photoperiod throughout the duration of the experiment. Tadpoles were continually fed goldfish pellets every two days throughout the experiment.

Green darner dragonfly nymphs (*A. junius*) were collected from a pond located in the Wedington Wildlife Management Area, Washington County, AR, USA (36° 4'33.71"N, 94°22'23.19"W) throughout May and June 2012. Individual *A. junius* nymphs were placed in 2 L aquaria containing 0.5 L of dechlorinated water. *A. junius* nymphs were fed two conspecific toad tadpoles once a week and maintained at a 12-h light photoperiod until experiments began.

Herbicide Dosing

Lithobates catesbeianus and *A. americanus* tadpoles were exposed to varying concentrations of commercial-grade glyphosate (Round-up Ready®, Montanto Company) ranging from 0.0-10.0 mg/L. *Lithobates catesbeianus* tadpoles were exposed to five different concentrations of glyphosate (0.0, 0.5, 1.0, 5.0, 10.0 mg/L), while *A. americanus* tadpoles were exposed to eight different concentrations glyphosate (0.0, 2.5, 4.0, 5.0, 5.5, 6.0, 7.5, 10.0 mg/L). Although *A. americanus* tadpoles were exposed to eight different concentrations of glyphosate, only seven different concentrations, selected from the total eight concentrations, were used for each temperature treatment to allow for better estimation of the LC50 value (concentration to kill 50% of the population). A stock solution of glyphosate was created every four days immediately prior to dosing. The stock solution was created by mixing 20 mL of Round-up Ready® commercial herbicide to 180 mL of dechlorinated water. Experimental cups were treated with either 1.8 ml of dechlorinated water, or 0.09, 0.18, 0.45, 0.72, 0.9, 0.99, 1.08, 1.35, or 1.8 ml of stock solution to produce the concentrations of 0.0, 0.5, 1.0, 2.5, 4.0, 5.0, 5.5, 6.0, 7.5, 10.0 mg/L

of glyphosate, respectively. To prevent water from fouling, full water and glyphosate solution changes were conducted every four days throughout the experiment.

Predator Dosing

A. americanus tadpoles were exposed to predator treatments by creating a predator stock solution. A predator stock solution was created every four days immediately prior to dosing. The stock solution was created by placing two *A. junius* nymphs in 0.5 L of dechlorinated water and adding four conspecific *A. americanus* tadpoles. *A. junius* nymphs were allowed to consume conspecific tadpoles, and then nymphs were removed and placed back into the housing aquaria. Conspecific tadpoles used in the predator stock solution can release alarm chemicals from their skin, which can be recognized by other tadpoles via chemoreception and cause other tadpoles to exhibit anti-predatory responses (Petranka 1989, Petranka and Hayes 1998). Experimental cups were treated with 2 ml of predator stock solution to create the predator cue. Predator stock solution was replaced and added to experimental cups every four days during the full water and glyphosate solution changes.

Exposure

For the *L. catesbeianus* experiment, one environmental chamber was set at 22°C and one environmental chamber was set at 28°C. A 6°C spread was selected to examine the potential effects of increasing temperatures, which are predicted to increase by as much as 5.8°C by 2100 (IPCC 2007). Each chamber contained 30 replicate experimental cups of each of the five glyphosate concentrations (0.0, 0.5, 1.0, 5.0, 10.0 mg/L). Individual tadpoles were randomly assigned to each temperature and glyphosate treatment, and temperature was verified in each chamber using a standard glass thermometer submerged in 0.4 L of water. Total sample size of *L. catesbeianus* tadpoles for each treatment is recorded in Table 1.

For the *A. americanus* experiment, three environmental chambers were used and set at 22°C, 25°C, and 28°C, respectively. Each chamber contained 12 replicate experimental cups with predator cue present and 12 replicate experimental cups with predator cue absent at each of the seven glyphosate concentrations. The chamber set at 22°C contained glyphosate concentrations of 0.0, 2.5, 5.0, 5.5, 6.0, 7.5, and 10.0 mg/L. The chambers set at 25°C and 28°C contained glyphosate concentrations of 0.0, 2.5, 4.0, 5.0, 6.0, 7.5, and 10.0 mg/L. The experiments were run sequentially and the concentrations for each chamber were modified from the first experiment and selected to allow a more accurate estimation of the LC50 of glyphosate for each temperature. Individual tadpoles were randomly assigned to each temperature, predator, and glyphosate treatment, and temperature was verified in each chamber using a standard glass thermometer submerged in 0.4 L of water. Total sample size of *A. americanus* tadpoles for each treatment is recorded in Table 2.

Both *L. catesbeianus* and *A. americanus* experiments lasted for a total of 16 days, which has been used in previous studies to simulate continued agricultural runoff (Relyea 2005c). The sixteen day trials represent a large fraction of the larval period for *A. americanus*, and a small fraction of the larval period for *L.* catesbeianus (Trauth et al. 2004). Everyday each cup was checked to determine if tadpoles were dead or alive, and dead individuals were removed on a daily basis. At the end of the 16 days, any surviving tadpoles were blotted dry with paper towels and weighed to determine final mass. Final Gosner stage was also determined for any surviving toad tadpoles. After weighing, tadpoles were euthanized using MS-222, and then each tadpole was photographed using Leica Application Suite (LAS) imaging software. Any tadpoles seen to undergo complete metamorphosis prior to the end of the experiment were removed from the cups, weighted, euthanized, photographed, and included in the total number of surviving tadpoles

for their respective treatments. Any tadpoles that jumped out of the cups throughout the experiment were removed from the data set.

Table 1. Sample size for each treatment for *L. catesbeianus* tadpoles.

Glyphosate Concentration (mg/L)	0.0	0.5	1.0	5.0	10.0
22°C	27	30	29	29	28
28°C	29	26	28	30	30
N Total = 286					

Table 2. Sample size for each treatment for *A. americanus* tadpoles.

Glyphosate Concentration (mg/L)		0.0	2.5	4.0	5.0	5.5	6.0	7.5	10.0
22°C	**Predation**	11	12	0	12	12	12	12	12
	No Predation	12	12	0	12	12	12	12	12
25°C	**Predation**	12	12	12	12	0	12	12	12
	No Predation	12	12	12	11	0	12	12	12
28°C	**Predation**	12	11	12	12	0	12	12	12
	No Predation	12	12	12	12	0	12	12	12
N Total = 501									

Statistical Analysis

All statistical analyses for *A. americanus* did not include samples from 4.0 mg/L and 5.5 mg/L because these treatments were not used in all temperatures (not included in full factorial design). All analyses for both amphibian species used an alpha value of 0.05, and data is presented as mean data for each treatment with ±2 standard error.

Survival and Mortality

Survivorship for *L. catesbeianus* and *A. americanus* tadpoles was analyzed as a function of the overall mortality at the end of the experiments (day 16) and the survival probability over time (16 days). These variables were used to determine differences in overall survival for each species and each treatment used in the experiments. The assumption of normal distribution was checked using boxplots and histograms, and the assumption of homogeneity of variance was checked using a Levene's test. All samples were also independent of each other due to the random assigning of tadpoles to treatments. After checking and ensuring that assumption, logistic regression using the binary response variable of dead/alive and the nominal predictor variables of temperature and glyphosate concentration were conducted to determine any significant differences in survival among the treatments for *L. catesbeianus* and *A. americanus* tadpoles. Repeated measures survival analyses were also conducted using survival probability over time as the response variable, dead or alive (zero or one) as the censor variable, and temperature and glyphosate concentration as the nominal predictor variables. These variables allow for comparison of survival probability among treatments over time. The nominal predictor variable of predation was also included in the logistic regression and survival analyses for *A. americanus* tadpoles. All survival and mortality analyses were conducted in JMP 9.0 or SYSTAT 13.0 software.

Growth

Growth was analyzed for *L. catesbeianus* and *A. americanus* tadpoles as a function of the difference between final and initial dry mass for each of the treatments. Mass difference for *L. catesbeianus* tadpoles was compared for each temperature treatment and glyphosate concentration. Mass difference for *A. americanus* tadpoles was compared for each temperature

treatment, predation treatment, and glyphosate concentration. The assumption of normal distribution was checked using boxplots and histograms, and the assumption of homogeneity of variance was checked using a Levene's test. All samples were also independent of each other due to the random assigning of tadpoles to treatments. After checking and ensuring that all assumptions were met, analysis of variance tests (ANOVA) using the continuous response variable of mass difference (final mass minus initial mass) and nominal predictor variables of temperature and glyphosate concentration were conducted to determine any significant differences in growth among the treatments for *L. catesbeianus* and *A. americanus* tadpoles. The nominal predictor variable of predation was also included in the ANOVA tests for *A. americanus* tadpoles. All growth analyses were conducted using JMP 9.0 or SYSTAT 13.0 software.

Development

Development was analyzed for *A. americanus* tadpoles as a function of final Gosner stage for each of the treatments. To better analyze the data, Gosner stages were assigned in a ranking categorical order for all analyses on development. The categorical level that each of the Gosner stages was assigned is seen in Table 3.

Table 3. Gosner categorical levels for development analyses of *A. americanus* tadpoles.

Gosner Developmental Stage	Gosner Categorical Level
25	1
26-30	2
31-36	3
37-39	4
40	5
41	6
42	7
43	8
44	9
45	10
46	11

Final Gosner categorical level was compared for each temperature treatment, predation treatment, and glyphosate concentration. The assumption of normal distribution was checked using boxplots and histograms, and the assumption of homogeneity of variance was checked using a Levene's test. All samples were also independent of each other due to the random assigning of tadpoles to treatments. After checking and ensuring that all assumptions were met, ANOVA tests using the continuous response variable of final Gosner categorical level and the nominal predictor variables of temperature, predation, and glyphosate concentration were conducted to determine any significant differences in development among the treatments. All analyses were conducted using JMP 9.0 or SYSTAT 13.0 software.

Development was also analyzed using geometric morphometry to analyze differences in shape among *L. catesbeianus* and *A. americanus* tadpoles based on anatomic landmarks. Tadpole images for each species were compiled into single treatment files using Thin-plate spline (tps) utility software (tpsUtil). Using Thin-plate spline digitize software (tpsDIG), four landmarks were placed on predetermined structures of each tadpole image. Landmarks were the same within a species treatments, but different between species. The program assumes symmetry; therefore landmarks were only placed on half of the specimen. A reference distance of 2 mm was also included in each tps image. Example images of *L. catesbeianus* and *A. americanus* are seen in Image 1 and Image 2, respectively.

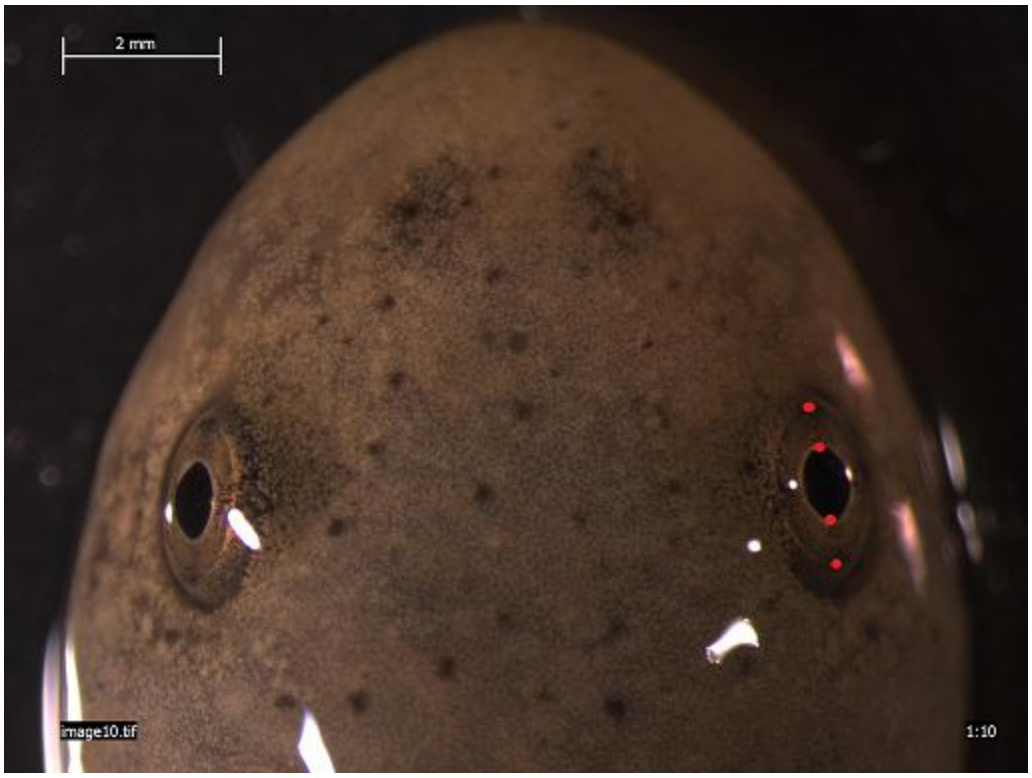

Image 1. Image of *L. catesbeianus* tadpole at end of trial. Location of digitized landmarks indicated by red dot.

Image 2. Image of *A. americanus* at the end of trial. Location of digitized landmarks indicated by red dot.

After placing landmarks and scaling each image, landmark relative warp scores were obtained using Thin-plate spline relative warps analysis (tpsRelw) from distances calculated by comparing each individual's configuration to a consensus configuration. The assumption of normal distribution was checked using boxplots and histograms, and the assumption of homogeneity of variance was checked using a Levene's test. All samples were also independent of each other due to the random assigning of tadpoles to treatments. After checking and ensuring that all assumptions were met, relative warps scores were used simultaneously as response variables in a multivariate analysis of variance test (MANOVA) to determine if differences in shape occur among treatments for *L. catesbeianus*. Differences in shape for *L. catesbeianus* tadpoles were compared for each temperature treatment and glyphosate concentration. A

multivariate analysis of covariance test (MANCOVA) was conducted using *A. americanus* data because final Gosner stage was recorded and used as a covariate for *A. americanus*. Relative warps scores were also used as response variables in MANCOVA for *A. americanus*. Differences in shape for *A. americanus* tadpoles were compared for each temperature treatment, predation treatment, and glyphosate concentration. MANOVA and MANCOVA analyses were conducted using JMP 9.0 and SPSS 20.0 software.

Results

Survival

Whole model (2-factor) logistic regression showed that glyphosate had a significant effect on *L. catesbeianus* survival ($p < 0.0001$) (Figure 1 and Figure 2). Temperature did not have a significant effect on tadpole survival ($p = 0.9991$); however, tests revealed a significant interaction between temperature and glyphosate concentration ($p = 0.0026$). Single-factor regression analyses were conducted to determine the specific levels of each factor that influence the interaction. As glyphosate concentration increased, *L. catesbeianus* tadpoles exhibited a reduced survival at both 22°C ($p < 0.0001$) and 28°C ($p < 0.0001$). Although increases in glyphosate concentration significantly reduced tadpole survival at both temperatures, increased temperatures only significantly reduced tadpole survival at the highest glyphosate concentration of 10.0 mg/L ($p < 0.0001$). An inversion of the logistic regression, which allows prediction of x-values from y-values, was conducted to estimate LC50 values (Figure 1 and Figure 2). The LC50 value for *L. catesbeianus* tadpoles at 22°C was 11.46 (Figure 1), while the LC50 value for *L. catesbeianus* tadpoles at 28°C was 6.98 (Figure 2).

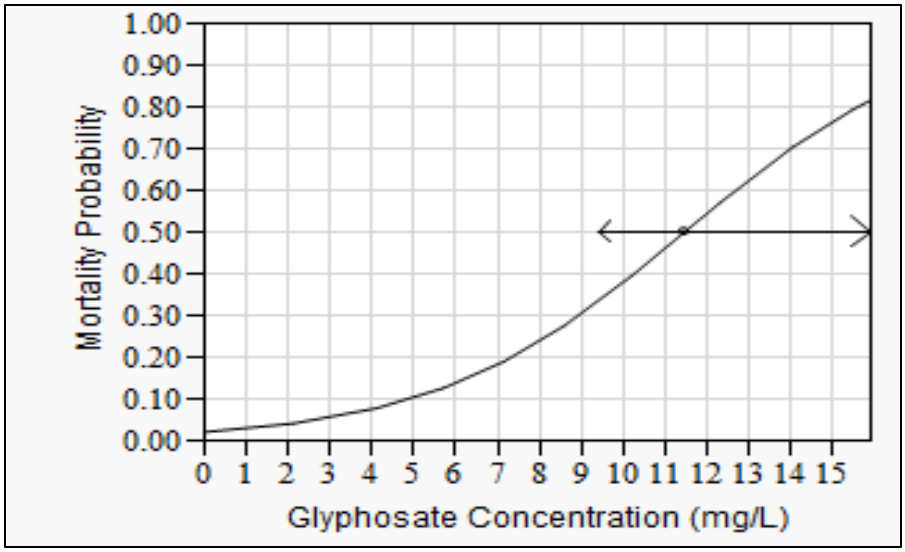

Figure 1. *L. catesbeianus* tadpole mortality probability at 22°C for each glyphosate concentration, 0.5 mortality probability shown with 95% confidence intervals.

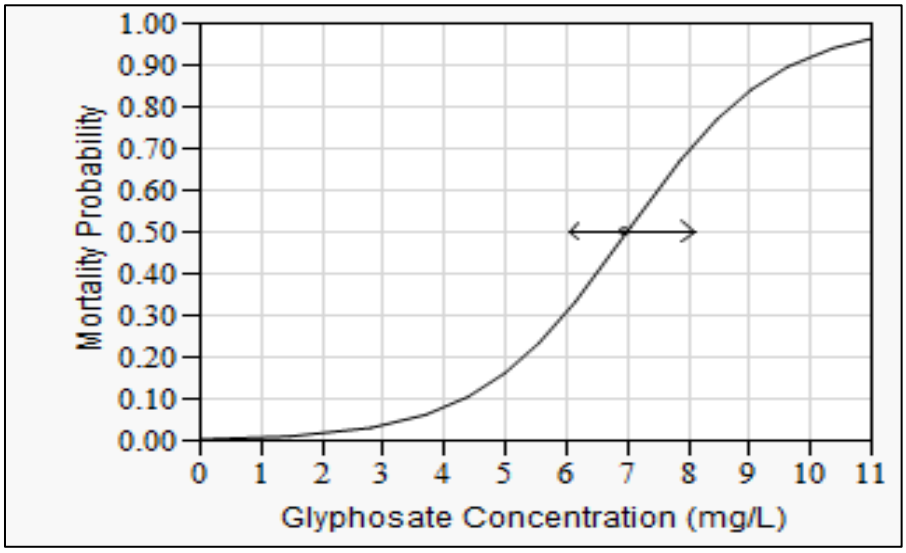

Figure 2. *L. catesbeianus* tadpole mortality probability at 28°C for each glyphosate concentration, 0.5 mortality probability shown with 95% confidence intervals.

Repeated measures survival analyses revealed a significant individual effect of temperature and glyphosate concentration on *L. catesbeianus* tadpole survivorship ($p < 0.0001$) (Figure 3).

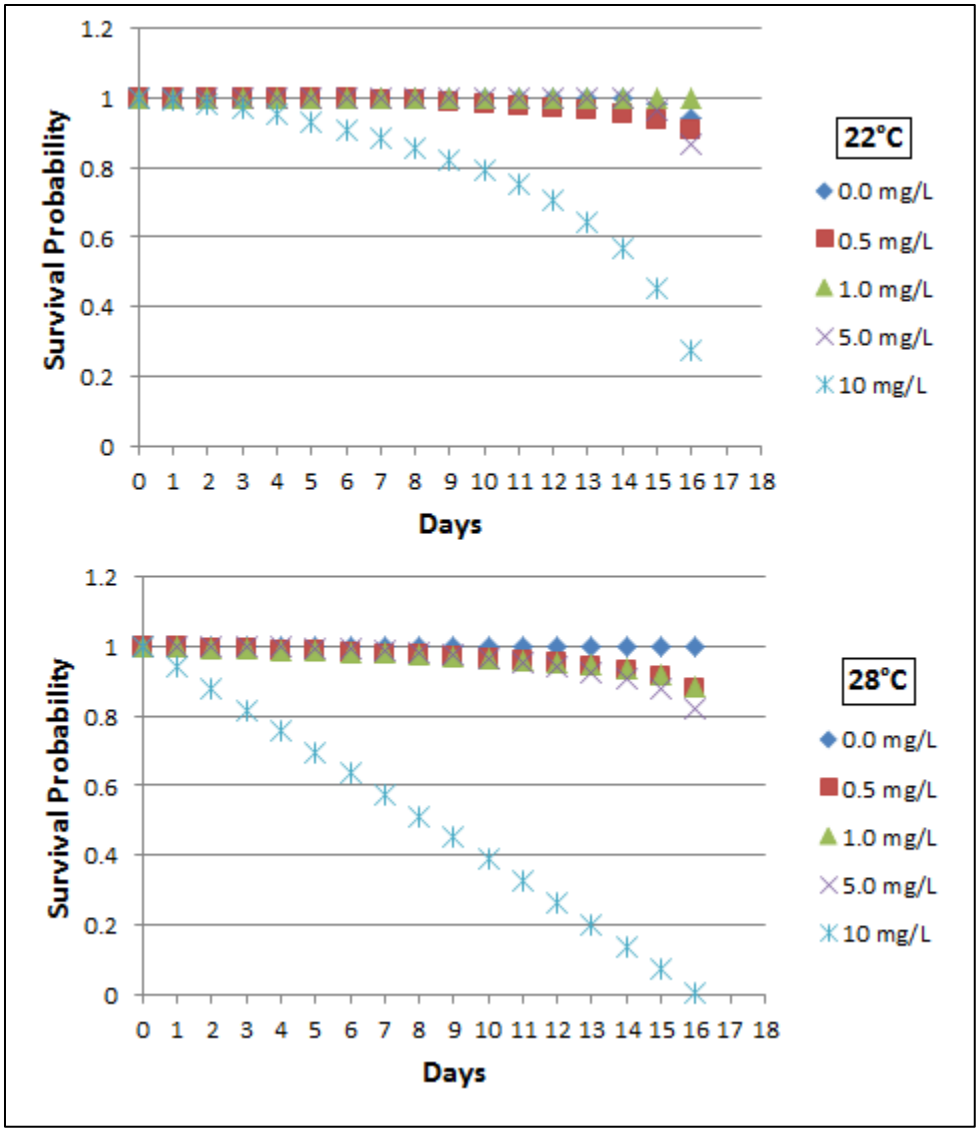

Figure 3. *L. catesbeianus* tadpole mean survivorship over a 16 day trial period in varying temperatures and glyphosate concentrations.

Whole model (3-factor) logistic regression indicated that glyphosate concentration had a significant effect on *A. americanus* survival ($p < 0.0001$); however, temperature and predatory cues had no significant effect on survival ($p > 0.05$) (Figure 4, Figure 5, and Figure 6). An inversion of the logistic regression, which allows prediction of x-values from y-values, was conducted to estimate LC50 values (Figure 4, Figure 5, and Figure 6). The LC50 value for *A.*

americanus tadpoles at 22°C was 5.49 (Figure 4), at 25°C was 4.94 (Figure 5), and at 28°C was 3.54 (Figure 6).

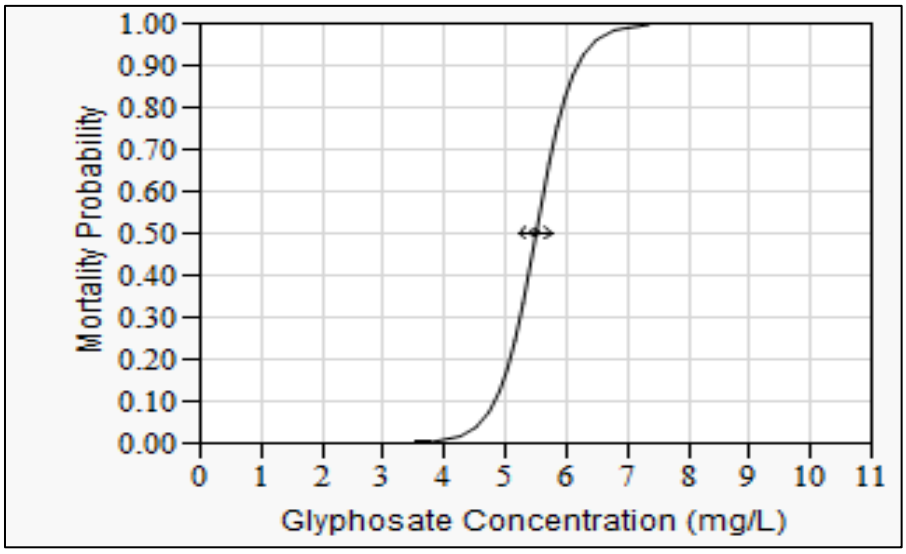

Figure 4. *A. americanus* tadpole mortality probability at 22°C for each glyphosate concentration, 0.5 mortality probability shown with 95% confidence intervals.

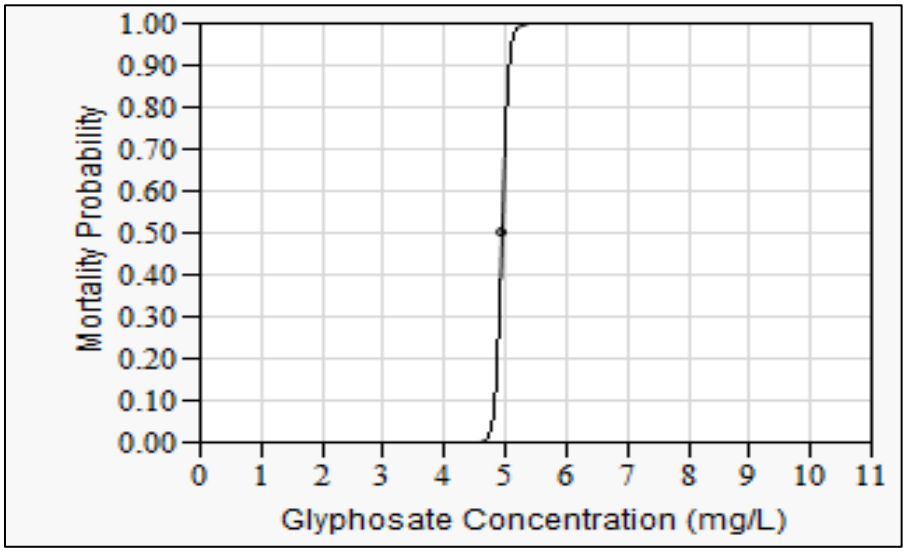

Figure 5. *A. americanus* tadpole mortality probability at 25°C for each glyphosate concentration, 0.5 mortality probability shown with 95% confidence intervals.

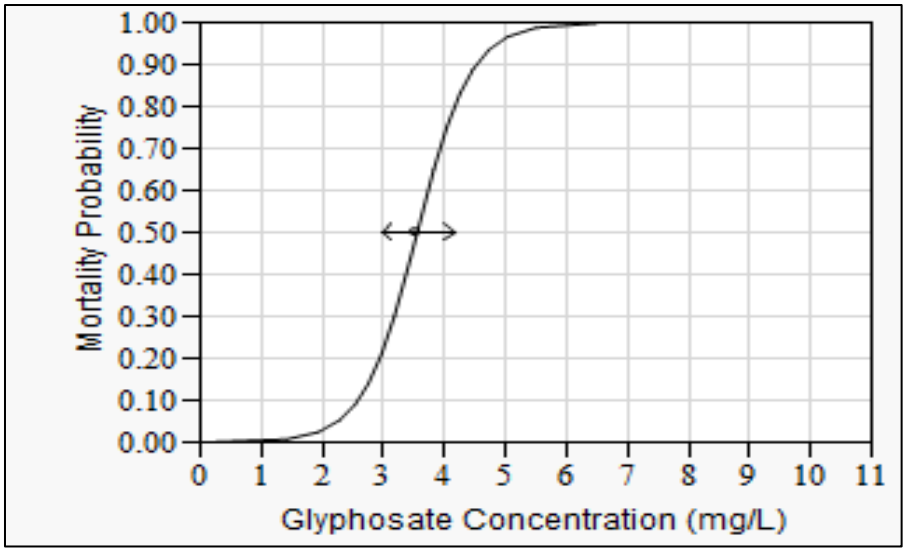

Figure 6. *A. americanus* tadpole mortality probability at 28°C for each glyphosate concentration, 0.5 mortality probability shown with 95% confidence intervals.

Repeated measures survival analyses revealed a significant effect of temperature and glyphosate concentration on *A. americanus* tadpole survivorship ($p < 0.0001$); however, the presence of predatory cues had no significant effect on tadpole survivorship ($p > 0.05$) (Figure 7).

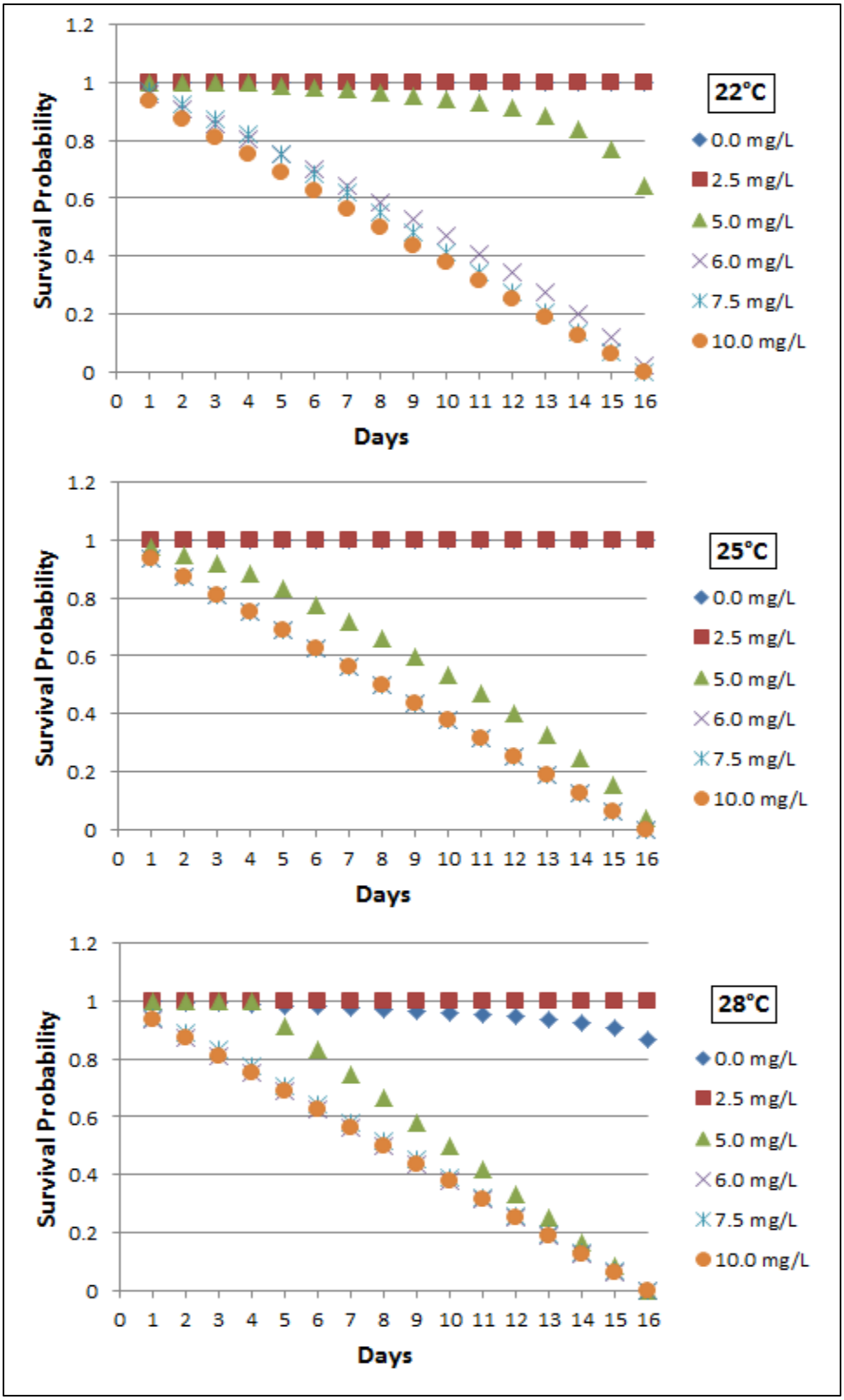

Figure 7. *A. americanus* tadpole mean survivorship over a 16 day trial period in varying temperatures and glyphosate concentrations.

Growth

Whole model (2-factor) ANOVA showed that glyphosate concentration had no significant effect on *L. catesbeianus* tadpole growth (as measured by weight difference) ($p = 0.872$) (Figure 8). Whole model ANOVA also indicated that temperature had no effect on tadpole growth ($p = 0.125$) and there was no significant interaction between glyphosate concentration and temperature ($p = 0.993$) (Figure 8).

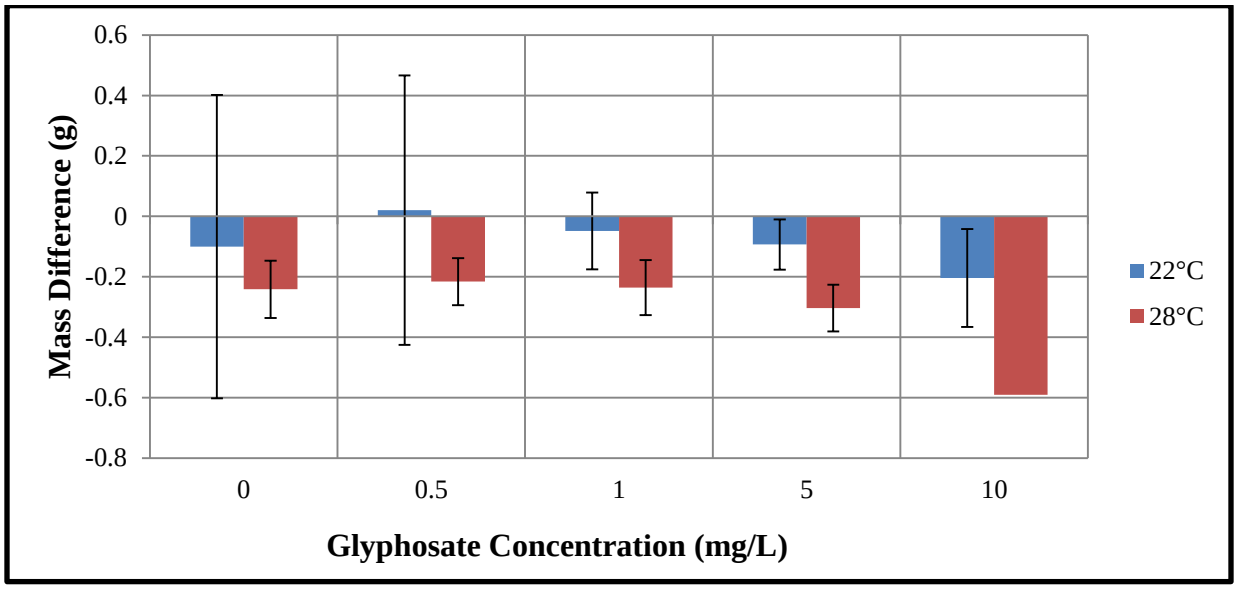

Figure 8. *L. catesbeianus* tadpole mean growth at 22°C and 28°C for each glyphosate concentration (± 2 SE).

The whole model (3-factor) ANOVA indicated that temperature caused a significant difference of *A. americanus* tadpole growth ($p < 0.0001$); however, glyphosate concentration had no significant effect on tadpole growth ($p = 0.893$) (Figure 9). Predatory cues also had no significant effect on *A. americanus* tadpole growth ($p = 0.413$) (Figure 9). Tests also indicated a significant interaction between temperature and glyphosate concentration ($p = 0.005$) and a significant interaction among temperature, glyphosate concentration, and predation ($p = 0.001$). Tukey's tests were conducted to compare means among all treatments, and levels not connected by the same letter are significantly different ($p < 0.05$) (Figure 9). Tukey's tests showed that the

predatory cues caused a significant difference in *A. americanus* tadpole growth at 22°C in the 6.0 mg/L glyphosate concentration ($p < 0.05$) (Figure 9). Samples in the 7.5 mg/L and 10.0 mg/L glyphosate died off before the end of the experiment and were not included in the analyses of growth.

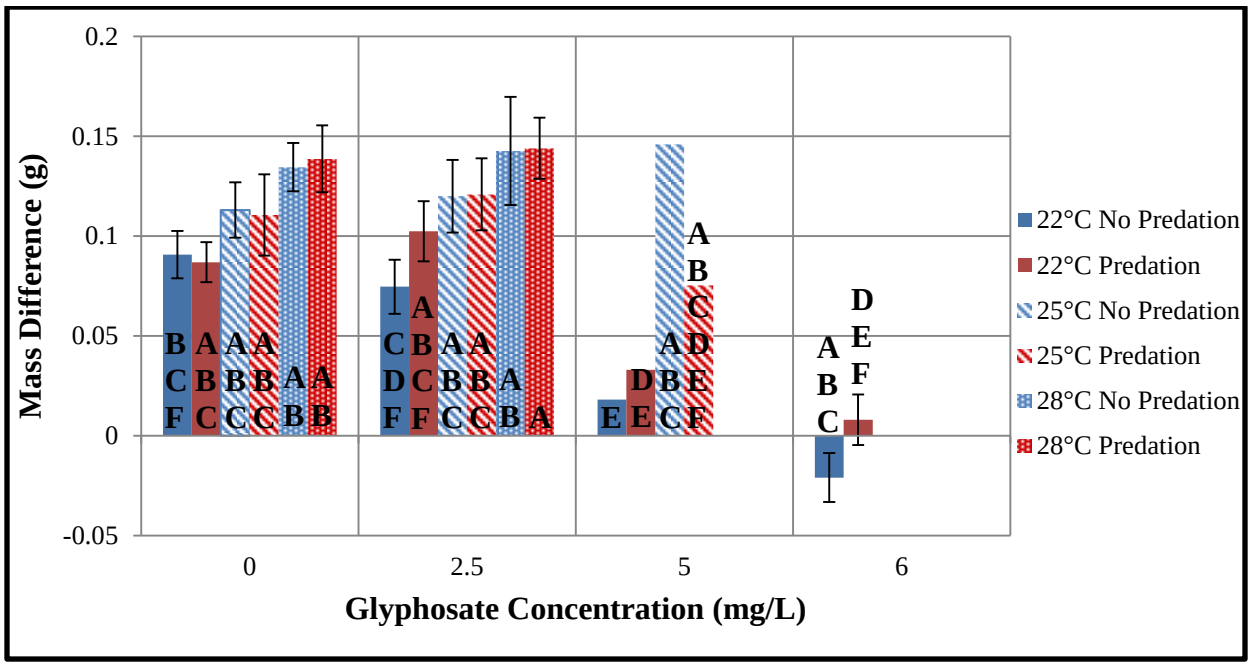

Figure 9. *A. americanus* tadpole mean growth in the presence and absence of predatory cues at 22°C, 25°C, and 28°C for each glyphosate concentration (± 2 SE).

Development

The whole model (3-factor) ANOVA test revealed that temperature had a significant effect on *A. americanus* tadpole development (as measure by end Gosner level) ($p < 0.0001$); however, glyphosate had no significant effect on tadpole development ($p = 0.064$) (Figure 11). Predatory cues also had no significant effect on tadpole development ($p = 0.874$). Tests also indicated a significant interaction between temperature and glyphosate concentration ($p = 0.003$) and a significant interaction between temperature, glyphosate concentration, and predatory cues ($p < 0.0001$). Tukey's tests were conducted to compare means among all treatments, and levels

not connected by the same letter are significantly different ($p < 0.05$) (Figure 10). Tukey's tests indicated that predatory cues caused a significant difference in *A. americanus* development at 25°C in the 5.0 mg/L glyphosate concentration ($p < 0.05$) (Figure 10). Samples in the 7.5 mg/L and 10.0 mg/L glyphosate died off before the end of the experiment and were not included in the analyses of development.

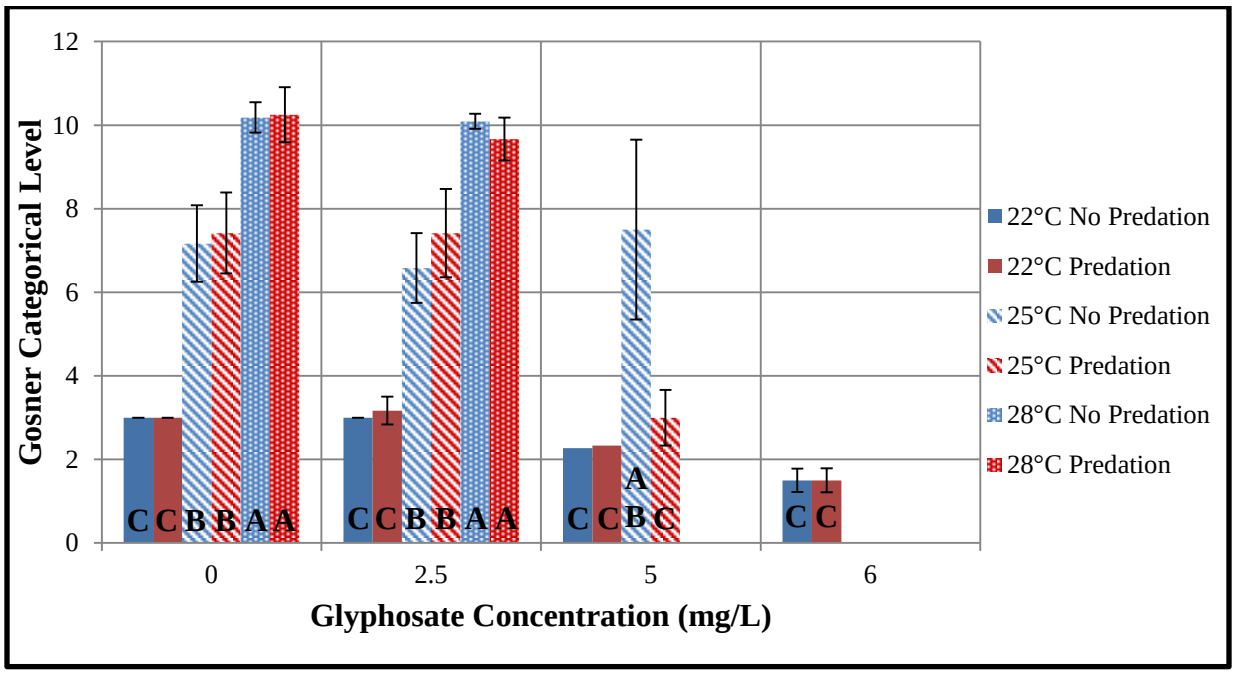

Figure 10. *A. americanus* tadpole mean development in the presence and absence of predatory cues at 22°C, 25°C, and 28°C for each glyphosate concentration (± 2 SE).

Temperature had no significant effect on *L. catesbeianus* tadpole shape (as measured by 2-factor MANOVA on tps relative warps scores) ($p = 0.9120$); however, increases in glyphosate concentration caused significant variation in shape ($p = 0.0135$), with 10 mg/L glyphosate causing the largest amount of variation. Although temperature had no significant effect on *L. catesbeianus*, temperature and predatory cues did have a significant effect on anatomical shape variation (as measured by 3-factor MANCOVA) in *A. americanus* tadpoles ($p < 0.001$). The highest amount of shape variation was seen at 25°C followed by 22°C, with 28°C having the

lowest amount of shape variation. More anatomical shape variation was seen in the presence of predatory cues than in the absence of predatory cues. Glyphosate concentration had no significant effect of shape variation in *A. americanus* ($p = 0.197$). MANCOVA tests also showed a significant interaction between temperature and predatory cues ($p = 0.02$). Temperature had a significant effect on *A. americanus* tadpole shape variation in both the presence and absence of predatory cues ($p < 0.0001$). However, the presence of predatory cues only significantly increased tadpole shape variation at 25°C ($p = 0.005$) and 28°C ($p = 0.019$).

Discussion

Glyphosate has the ability to reduce tadpole survival in both *L. catesbeianus* and *A. americanus*, and perhaps other amphibian species. Many other studies indicate similar results (Relyea 2005a, Relyea 2005b, Relyea 2005c, Relyea et al. 2005). The highest mortality was seen in the highest glyphosate concentration in both species of amphibians tested. The lowest concentrations of glyphosate had little overall effect on *L. catesbeianus* and *A. americanus* tadpole survival over the sixteen day trial period; however, longer exposure times are possible and may result in decreased survival. The results also show that in the lowest temperature, tadpoles of both species did not begin to experience mortality until very late in the trial period, except with treatments that had a glyphosate concentration above 5.0 mg/L (Figure 3 and Figure 7). Tadpoles in treatments with glyphosate concentrations over 5.0 mg/L began to die-off much earlier in the trial period than other treatments (Figure 2 and Figure 7). Glyphosate concentration also seemed to become more lethal to tadpoles (especially *L. catesbeianus*) as temperature was increased, which matches other studies using different environmental toxins (Boone and Bridges 1999). The interaction between glyphosate concentration and temperature indicates that if

temperatures continue to rise as expected (IPCC 2007), glyphosate may become more toxic to amphibian larvae and lead to further decreases in survival.

Increased temperature alone did not cause a reduction in tadpole overall survival in both *L. catesbeianus* and *A. americanus*; however, increased temperature did decrease survivorship over time (Figure 3 and Figure 7). More individual tadpoles died off earlier in the trial period at high temperatures than at the lower temperatures. Although temperature did not significantly decrease overall survival at the end of the trial period, increased temperature did result in an increased toxicity of glyphosate for both amphibian species. The increased toxicity of glyphosate caused by increased temperature is shown by a decrease in LC50 values from 28°C (LC50 = 6.98) to 22°C (LC50 = 11.46) in *L. catesbeianus* and a decrease in LC50 values from 28°C (LC50 = 3.54) to 25°C (LC50 = 4.94), to 22°C (LC50 = 5.49). All decreases in survival, except for the *L. catesbieanus* 22°C treatment, were seen at concentrations predicted to occur in nature after one direct overspray application (3.7 mg/L-10.1 mg/L) (Giesy et al. 2000, Mann and Bidwell 1999). Furthermore, if glyphosate is applied to the same habitat multiple times in the growing season, which studies suggest may be occurring due to the low pre-emergent activity of glyphosate (Schuette 1998), glyphosate may accumulate in wetlands and reach concentrations that could significantly reduce tadpole survival, especially in higher temperatures.

Although glyphosate was seen to reduce tadpole survival, glyphosate does not significantly reduce *L. catesbeianus* or *A. americanus* tadpole growth and development. The lack of effect of glyphosate on tadpole growth and development differs from other studies which have shown that glyphosate reduces tadpole biomass by 40% in certain species (Relyea et al. 2005). A decrease in growth was also seen in *L. catesbeianus* tadpoles as temperatures increased (Figure 8). However, past studies have indicated that as temperature increases, so does growth

and development in amphibians (Duellman and Trueb 1994, Dodd 2010). The decreases in growth and development seen in *L. catesbeianus* and *A. americanus* tadpoles may also indirectly increase further susceptibility to environmental contaminants or aquatic predators by increasing larval period and time to metamorphosis, which will increase exposure time to these stressors.

On the other hand, *A. americanus* tadpoles showed a significant increase in growth and development as temperature increased, which agrees with other studies on amphibian species (Dodd 2010, Duellman and Trueb 1994, Rohr et al. 2011). Temperature and glyphosate concentration, as well as temperature, glyphosate concentration, and presence of predatory cues also showed significant interactions in reference to *A. americanus* tadpole growth. The interaction may indicate that although increased temperature does increase growth in *A. americanus* tadpoles, temperature has a lesser effect on growth in higher concentrations of glyphosate and in the presence of predatory cues. The increase in growth seen by *A. americanus* in higher temperatures may also ameliorate the adverse effects of glyphosate by decreasing larval period and time to metamorphosis. By decreasing the larval period, *A. americanus* tadpoles will not be exposed to environmental toxins such as glyphosate or to highly voracious and abundant aquatic predators for as long of time. The idea of decreased exposure to environmental toxins caused by increased temperature is also suggested by Rohr and others (2011) in the case of atrazine exposure on *Ambystoma barbouri*. The difference between *A. americanus* and *L. catesbeianus* in the case of growth may be attributed to the robust tolerance and high resilience seen in *L. catesbeianus* tadpoles, which have the ability to overwinter as tadpoles and may be more accustom to dramatic shifts in the environment throughout the seasons (Trauth et al. 2004). *L. catesbeianus* tadpoles also grow at slower rates and can retain their larval stage over a much

longer period of time than *A. americanus* tadpoles, and significant effects on *L. catesbeianus* growth may not have been able to be detected in the short trial period.

The results of the geometric morphometry analyses reveal that increased glyphosate concentrations caused significantly more variation in overall body shape in *L. catesbeianus*, with the highest glyphosate concentrations causing the largest amounts of shape variation. Although glyphosate had an effect on *L. catesbeianus* tadpole body shape, glyphosate had no significant effect on *A. americanus* tadpole shape. However, temperature did cause significantly more shape variation in *A. americanus* tadpoles, with the highest shape variation being at the intermediate temperature (25°C). The sample size in the highest temperature treatment for *A. americanus* was very small and more samples may have indicated that the highest amounts of shape variation were in the highest temperature treatment. Temperature had no effect on shape variation in *L. catesbeianus*. The presence of predatory cues significantly increased anatomical shape variation in *A. americanus* tapoles; however, the interaction between predatory cues and temperature indicates that predatory cues may only significantly increase shape variation in higher temperatures. The differences seen in shape variation between the two amphibian species tested may be accredited to the location of the anatomical landmarks that were placed on the tadpole images, as well as the different life-histories of the two amphibian species (Trauth et al. 2004). Analyses only reveal information about variation in shape at the location of the landmarks, and therefore may not be sufficient to describe shape changes in a functional sense. Different location of landmarks may have revealed different results; however, landmarks were specifically chosen to include all individuals across all treatments. Landmarks must be placed in exactly the same location on all individuals, which can become very challenging when individuals are at different stages of development. The differences of developmental stage may have also

accounted for some of the differences in shape variation. The increase in shape variation caused by glyphosate in *L. catesbeianus* and temperature and predatory cues in *A. americanus* may lead to further developmental problems such as asymmetry, skeletal formation abnormalities, problems in sexual development, or impaired behavior which has been documented in the presence of other environmental toxins (Britson and Threlkeld 1998, Brunelli et al. 2009, Hayes et al. 2002).

Although predatory cues did have an effect on anatomical shape variation in *A. americanus* tadpoles, the presence of predatory cues did not seem to have any effect on survival in *A. americanus*. However, *A. americanus* tadpoles experienced significantly reduced growth in the absence of predatory cues at 22°C in the 6.0 mg/L glyphosate concentration (Figure 9). *Anaxyrus americanus* tadpoles also experienced significantly reduced development in the presence of predatory cues at 25°C in the 5.0 mg/L glyphosate concentration (Figure 10). A plausible explanation may be the reduction in *A. americanus* tadpole development caused by predatory cues is attributed to the significant interaction between temperature, glyphosate concentration, and predatory cues. Therefore, the reduction in development may actually be caused by increased glyphosate concentrations and increased temperature, rather than the presence of predatory cues alone. The fact that predatory cues did not cause any significant effect on survival is contradictory to past studies, which suggest that predatory cues can increase the toxicity of different environmental contaminants and lead to further decreases in survival than contaminants alone (Relyea and Mills 2001, Relyea 2003, Relyea 2004, Relyea 2005b, Relyea 2005c, Relyea et al. 2005). Other studies also indicate that the presence of predatory cues should decrease growth in certain amphibian species (Boone et al. 2007), which was not seen in the current study's results. The results of this study may have also been influenced by the relatively

small sample size, caused by die-off in the 5.0 mg/L glyphosate and 25°C and the 6.0 mg/L glyphosate and 22°C treatments or a possible flaw in experimental methodology. Future studies will integrate a different approach of placing caged predators inside the experimental aquaria with the tadpole species to allow for better chemosensory recognition of predatory cues.

This study suggests that glyphosate can decrease survival in larval amphibian species at ecologically relevant concentrations. Furthermore, glyphosate seems to become more toxic and lethal to tadpoles in the presence of elevated temperatures indicating that amphibian larvae may become more susceptible to desiccation from environmental toxins in the near future where temperatures are projected to rise by up to 5.8°C by the year 2100 (IPCC 2007). Decreases in tadpole survival will undoubtedly result in further declines in amphibian populations, which could alter nutrient and energy cycling and ultimately lead to a trophic cascade and losses in diversity and abundance of many other taxa. Although glyphosate and increased temperatures do cause decreases in tadpole survival, the individuals that do survive to adulthood may have an indirect competitive advantage. The survivors will have less competition for food resources, habitat, and mating which may increase their overall fitness. Increased temperature can also lead to decreases in growth in certain larval amphibian species, which can indirectly increase susceptibility to environmental toxins and aquatic predators by increasing larval period. By increasing larval period and susceptibility to different stressors, more larval amphibians may die and even greater decreases in survival may be expected. Increased temperature may also lead to increases in growth and development in certain tadpole species. Increased growth and development will lead to a shorter larval period, larger size at metamorphosis, and decreased susceptibility to stressors found in aquatic habitats. The larger biomass caused by increased

temperatures may also give those individuals a competitive advantage by increasing mobility, distribution, and predator avoidance.

This study indicates that climate change could increase the toxicity of environmental contaminants to larval amphibians, while also decreasing exposure time to environmental contaminants and voracious predators found in aquatic habitats. More studies must be conducted in larger settings and in more ecologically relevant conditions to adequately understand the interactive effects of climate change, pollution, and other stressors on amphibian populations. However, one thing is certain, amphibian populations are declining at alarming rates worldwide and if critical actions and initiatives are not taken to understand and cease these declines, many more amphibian populations will begin to dwindle and more species will be extirpated within the near future.

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CHAPTER 3: CONCLUSION

Potential Solutions and Conservation Efforts

Although there are no clear and easy solutions to the problem of recent amphibian declines, there are steps that humans can take to help preserve the remaining global amphibian populations. One of the major ways to do this is to implement and enforce strict water quality guidelines and rules regarding the use of herbicides, pesticides, and fertilizers. The use of these chemicals will undoubtedly always be beneficial to mass agriculture and food production, however; if these chemicals are to be used, they must be prevented from entering surrounding bodies of water where many amphibians breed and deposit eggs. One way to prevent chemicals from agricultural runoff entering water sources is to create physical barriers, natural (vegetative) or unnatural (physical structures), between the areas where the chemicals are being used and the bodies of water (Blanco-Canqui et al. 2006, Spaan et al. 2005, Fiener and Auerswald 2003, Moss et al. 2005). Unfortunately, direct aerial overspray will continue to dump large amounts of chemicals used for agriculture into bodies of water, but the chemicals that enter the water from runoff can be reduced by barriers (Blanco-Canqui et al. 2006, Spaan et al. 2005, Fiener and Auerswald 2003, Moss et al. 2005). Switching to shade-growing farming practices, especially in the tropics, can also be beneficial to amphibians by increasing leaf litter and creating more area of suitable habitat (Siebert 2002). If policies are enacted to help create barriers and the use of non-sustainable and harmful agricultural practices is policed, localized amphibian populations may be able to recover and further declines due to environmental contaminants may be reduced.

Another important solution to the problem of amphibian declines is effective land management practices. Although many wetlands and riparian zones are maintained by USDA conservation programs, more focus on critical areas is needed. Critical areas where amphibians

are declining must be protected by creating reserves and refuges in these areas. These reserves must also take into account amphibian population range shifts that may occur due to global climate change (Colwell et al. 2008). This includes creating corridors between reserves and areas of suitable habitat so that amphibian populations will be able to shift their range if needed. By modeling the effects of climate change on the environment, reserves and corridors can be created in areas that will help to ensure the survival of dwindling populations (Colwell et al. 2008). Also, if these reserves are created, limited human use must occur within the reserves. If the reserves are completely open to the public, human disturbance could be detrimental to amphibian conservation.

More effective policing policies on the transportation of amphibians around the globe must also be implemented. One of the major reasons for amphibian declines is the spread of disease and introduced species, both of which were influenced by transportation of amphibians across the landscape (Vitt and Caldwell 2008, Collins and Storfer 2003, Alford and Richards 1999). If policies are created to more effectively police the transportation of amphibians, the future spread of disease and introduction of non-native species into different environments may be better controlled. Also, in areas where non-native species have already been introduced and seen to have negative effects on native amphibian population, those introduced species must be removed from the system to allow the native species to rebuild (Vredenburg 2004).

Captive management and translocation programs may also help the diminishing state of amphibian populations (Gascon et al. 2007, Dodd and Seigel 1991, Snyder et al. 1996, Kraaijeveld-Smit et al. 2006., Griffiths and Pavajeau 2008). Threatened species that lack other effective conservation alternatives, can be farmed and placed in breeding programs such as those that currently occur in zoos around the globe for other threatened animals (Snyder et al. 1996).

Breeding programs will ensure the survival of the species even if the natural populations go extinct; however, breeding program will not work for all species and should be considered a last resort (Snyder et al. 1996). High success rates can be established through long term commitment in breeding programs and repatriation or augmentation of species, which may allow populations to rebuild in their natural environments (Griffiths and Pavajeau 2008).

All of these solutions have advantages and disadvantages. For any of these to have a chance at helping to preserve populations of amphibians worldwide, humans must be educated on the current state of declining amphibians. By starting educational programs to inform the general public about amphibian declines and the importance of amphibians to the environment, more people may start to take initiatives and change their ways to help amphibian populations. These programs can be implemented in elementary schools to ensure that the next generation of humans will see the importance of amphibians. Also, by using the media such as radio, newspaper, and television, older generations will learn about the state of amphibians and may change their ways and act in more sustainable manners. Although these changes may only be small and may not directly help amphibians in the very near future, if amphibian populations are to survive and cease declining every small effort can be beneficial. With more and more people becoming educated and developing a more sustainable lifestyle, hopefully remaining amphibian populations will be able to be preserved and cease from further declines.

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