THE IMPACTS OF ROUNDUP WEATHERMAX® AND IGNITE® 280 SL ON AMPHIBIANS IN THE SOUTHERN HIGH PLAINS

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ABSTRACT

The Southern High Plains (SHP) region of the United States is dominated by agricultural enterprise. In many areas, playa wetlands are the only remaining patches of native habitat. As such, they are vital for the persistence of flora and fauna in this region, including amphibians. Because most playas are embedded in cropland, SHP amphibians may encounter a variety of agricultural chemicals due to contaminated runoff or direct terrestrial exposure. However, no previous work has examined whether commonly applied herbicides pose a threat to larvae or juveniles of SHP species. Initially, I investigated the toxicity of widely used herbicides Roundup WeatherMAX® and Ignite® 280 SL to larval New Mexico and Plains spadefoot toads (*Spea multiplicata* and *S. bombifrons*, respectively) from three cropland and two grassland playas. To assess the effects of short-term herbicide exposure, I obtained Roundup WeatherMAX® and Ignite® 280 SL acute toxicity estimates (i.e., LC_{50} values) for both species. Because larvae may experience prolonged exposure under field conditions, I also investigated how survival of larval New Mexico and Plains spadefoot toads was affected by 30-day exposure to these herbicides at environmentally-relevant levels. I hypothesized that, due to historical differences in herbicide exposure, larvae from cropland playas would be less sensitive to Roundup WeatherMAX® and Ignite® 280 SL compared to those from grassland playas. The toxicity of formulated glyphosate herbicides (like Roundup WeatherMAX®) toward aquatic organisms is thought to result primarily from surfactants. To increase mechanistic understanding of surfactant toxicity toward larval amphibians, I also examined the histological impacts of a non-ionic surfactant (ADSEE 907®) on skin and gills of *Spea* spp. larvae. Because surfactants disrupt skin and gill structure of aquatic

organisms, I hypothesized that skin and gill lesions would be more extensive among *Spea* spp. larvae exposed to ADSEE 907®.

Following metamorphosis, juvenile SHP amphibians may disperse or inhabit moist areas near drying playas. This may result in direct exposure to agrochemicals. Therefore, I also investigated how the short-term survival of juvenile New Mexico spadefoot and Great Plains toads (*Bufo cognatus*) housed on soil or moist paper towels was affected by exposure to environmentally-relevant levels of a glufosinate-based herbicide (Ignite® 280 SL) and several glyphosate-based herbicides (Roundup WeatherMAX®, Roundup Weed and Grass Killer Super Concentrate®, and Roundup Weed and Grass Killer Ready-To-Use Plus®).

The hypothesis that Ignite® 280 SL or Roundup WeatherMAX® sensitivity would differ between spadefoot larvae from cropland and grassland playas was not supported. Acute toxicity tests indicated that New Mexico spadefoots were less sensitive to Ignite® 280 SL than Plains spadefoots. Ignite® 280 SL 48-hr LC_{50} values for both species (3.55-5.55 mg glufosinate/L) were well above environmentally relevant concentrations. These results agree with those from chronic toxicity tests; 30-day exposure to environmentally relevant levels of Ignite® 280 SL did not reduce survival among New Mexico or Plains spadefoot larvae. Acute toxicity tests with Roundup WeatherMAX® indicated no between-species variation in herbicide sensitivity. Roundup WeatherMAX® 48- and 216-hr LC_{50} values for New Mexico and Plains spadefoot larvae (1.65-2.30 mg glyphosate acid equivalents/L) were similar to environmental concentrations expected from accidental direct overspray. While chronic exposure to environmentally relevant levels of Roundup WeatherMAX® reduced survival of both species, the response of New Mexico spadefoots differed by landuse. Survival of New Mexico spadefoots from grassland playas was greater than those from cropland playas following the 30-day exposure.

Contrary to expectations, skin and gill lesions were not consistently more extensive among larvae exposed to ADSEE 907®, even though mortality was greater among these larvae compared to controls. The extent of gill lesions was similar among control larvae and those exposed to surfactant. While one type of skin lesion (apical hyperplasia) was more extensive among larvae exposed to surfactant, several other lesions (apical and skein necrosis) were more extensive among control larvae. Because larvae were collected from a SHP playa wetland, the expected histological response may have been obscured by prior contaminant induced lesions. It is also possible that other environmental stressors present in cropland playas contributed to observed skin and gill lesions. Additionally, the histological profile of larvae may have been influenced by normal tissue restructuring associated with metamorphosis. These results may indicate that larval skin and gills are not the primary target of non-ionic surfactants. For example, it is possible that general narcosis is the primary mode of toxicity.

Survival of New Mexico spadefoots and Great Plains toads juveniles was not affected by exposure to Roundup WeatherMAX® or Ignite® 280 SL on either paper towels or soil. However, New Mexico spadefoot and Great Plains toad survival was reduced by exposure to Roundup Weed and Grass Killer Ready-To-Use Plus® on moist paper towels or soil. The toxicity of this formulation may result from included "pelargonic and related fatty acids." However, since this product is intended for lawn and garden use, it is unlikely that large numbers of SHP amphibians will encounter this formulation under field conditions. Great Plains toads exposed to Roundup Weed and Grass Killer Super Concentrate® on paper towels also exhibited reduced survival. However, this result has limited ecological relevance to SHP amphibians because widespread exposure is unlikely and mortality occurred only on a highly artificial substrate (i.e., paper towels). Great Plains toad survival was not affected by exposure to this formulation under more realistic conditions (i.e., experimental tubs lined with soil).

Acute and chronic toxicity data suggest that Ignite® 280 SL does not pose a mortality risk to larval New Mexico and Plains spadefoots. However, Roundup WeatherMAX® may pose a mortality risk to larvae of these species. Further studies under increasingly realistic conditions are needed to determine if this is the case. Also, it is important to investigate whether Ignite® 280 SL and Roundup WeatherMAX® exert sublethal impacts on larvae of these species. When used properly, the agricultural herbicides tested (Roundup WeatherMAX® and Ignite® 280 SL) likely do not pose a threat to juvenile New Mexico spadefoots and Great Plains toads. Future studies should examine whether sub-lethal endpoints (e.g., growth, reproduction) are negatively affected by common agricultural herbicides, and if environmental factors modulate herbicide toxicity.

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CHAPTER I

INTRODUCTION

Amphibian population declines & pesticides

Over the past two decades, there has been increasing concern that amphibian populations are declining on a world-wide scale (Wyman 1990, Stuart et al. 2004). The most obvious threat to amphibians is the degradation or outright destruction of aquatic (wetland) and terrestrial habitats (Wyman 1990, Semlitsch 2000). Since the 1800s, 53% of the wetland area within the contiguous United States has been destroyed (Dahl 1990). Another factor that poses a serious threat to amphibian populations is chemical contamination of their habitats (Hall and Henry 1992, Bridges 1997, Howe et al. 2004). Many species breed in shallow wetlands (Mann and Bidwell 1999) embedded within areas commonly treated with agrochemicals (e.g., insecticides, herbicides, fertilizers) (Venne et al. 2008). These chemicals may enter adjacent aquatic habitat due to spray drift (Johansson et al. 2006), accidental overspray (Faber et al. 1998a) or run-off (Faber et al. 1998a, Johansson et al. 2006). This may result in exposure of developing amphibian eggs and larvae to pesticides at harmful levels (Howe et al. 2004). Also, because many amphibian species spend a significant portion of their lives in terrestrial habitat adjacent to breeding sites (Richter et al. 2001, Semlitsch and Bodie 2003), adults and juveniles may be threatened by terrestrial application of pesticides (Semlitsch 2003).

Recent evidence indicates that pesticide exposure can cause lethal and sublethal impacts on amphibians. Pesticides have been implicated in decreased larval (Chen et al. 2004,

Howe et al. 2004, Relyea et al. 2005, Relyea 2005a) and juvenile (Relyea 2005a) survivorship, body size (Howe et al. 2004), and activity levels (Bridges 1997, 1999, Bridges and Semlitsch 2000). Research has also demonstrated that pesticide exposure may cause increased time to metamorphosis (Boone and Semlitsch 2002, Howe et al. 2004, Relyea and Diecks 2008) and frequency of morphological deformations (Howe et al. 2004).

A closely related issue is whether intra- or inter-species differences in pesticide sensitivity exist. Recent evidence indicates that this is the case (i.e., among species - Mann and Bidwell 1999, Howe et al. 2004, Relyea 2004, Jones et al. 2009; among populations - Bridges and Semlitsch 2000) and that there are fitness costs correlated with increased pesticide tolerance (Semlitsch et al. 2000). This variation may have resulted from historical differences in contaminant exposure, which led to selection for tolerance (Bridges and Semlitsch 2000, Meyer and Di Giulio 2003). Another possibility is that differences in pesticide sensitivity resulted from physiological acclimation (Meyer and Di Giulio 2003). Determining whether pesticide susceptibility varies between or within species would help identify sensitive species/populations, and allow conservation efforts to focus on those species/populations most at risk (Bridges and Semlitsch 2000).

Objectives

The Southern High Plains (SHP) of Texas and New Mexico is dominated by shallow, circular-shaped wetlands called playas (Bolen et al. 1989). Because most playas are located within agricultural fields (Haukos and Smith 1994), it is important to determine whether SHP amphibians are negatively impacted by commonly applied agrochemicals. I investigated the toxicity of common glyphosate- and glufosinate-based herbicides toward several SHP amphibian species. Initially, I examined the acute and chronic toxicity of Roundup® WeatherMAX and Ignite 280 SL® to *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoots,

respectively) larvae from grassland and cropland playas (Chapter II). In this chapter, I integrate acute and chronic toxicity results and discuss whether between- and within-species (i.e., between landuse) differences in herbicide sensitivity exist. This work will offer insight about whether exposure to these herbicides at environmentally relevant levels threatens larvae of these two species. I also examined the histological impacts of acute exposure of *Spea* spp. larvae to a non-ionic surfactant to increase mechanistic understanding of surfactant toxicity toward amphibians (Chapter III). Because the effects of pesticides on post-metamorphic amphibians has received little study, I investigated the acute toxicity of a glufosinate- (Ignite 280 SL®) and several glyphosate-based herbicides (Roundup® WeatherMAX, Roundup Weed and Grass Killer Super Concentrate®, and Roundup Weed and Grass Killer Ready-To-Use-Plus®) to post-metamorphic *S. multiplicata* and *Bufo cognatus* (Great Plains toad) to determine whether terrestrial exposure at environmentally relevant levels poses an immediate threat to juveniles of these species (Chapter IV). My dissertation may ultimately help to quantify and manage any threat that these herbicides pose to SHP amphibians because laboratory toxicity tests play an important role in risk assessment (Thompson 2004).

Study area

The climate in the SHP region is subhumid continental (Haukos and Smith 1994). Mean annual precipitation ranges from 45 cm in the northeast to 33 cm in the southwest (Bolen et al. 1989). Mean high temperatures during summer near the center of the SHP (Lubbock, TX) were 30.4 °C (1971-2000; National Oceanic and Atmospheric Administration 2009). Playa wetlands display erratic hydroperiods, and naturally receive water only from precipitation and surface runoff associated with springtime thunderstorms (Smith 2003). These wetlands are the primary surface drainage feature on the SHP, and most exist within a distinct, closed watershed (Bolen et al. 1989). This region is one of the more heavily cultivated in the Western Hemisphere (Bolen

et al. 1989) and most playa watersheds are dominated by row crops, while a small proportion of playas are surrounded by native grassland (Haukos and Smith 1994). Since playas exist is an area dominated by agriculture, they provide required "islands of habitat" (Guthery and Bryant 1982) and are responsible for the persistence of nearly all flora and fauna that exist in this region (Haukos and Smith 1994). Playas also store flood waters, provide water for irrigation and livestock, and serve as recharge points for the Ogallala aquifer (Luo et al. 1997).

Playas are threatened by several factors. Many have been cultivated (Bolen et al. 1989), or modified to hold water for agriculture (Smith 2003). Playas surrounded by row crops receive excessive sediment inputs (Luo et al. 1997) that alter wetland hydroperiod (Tsai et al. 2007). While these processes do not immediately destroy playas, they degrade playa habitat by disrupting normal ecosystem function (Luo et al. 1997, Smith 2003).

SHP amphibians and herbicides

As is the case in other wetland ecosystems, amphibians are a vital component of playas (Smith 2003). They can be the dominant vertebrate during summertime (Anderson et al. 1999), serving both as predator and prey (Smith 2003). While 12 anuran amphibian species occur in the SHP (Smith 2003), the most common are *Pseudacris clarkii* (spotted chorus frog), *B. cognatus* (Great Plains toad), *S. bombifons* (Plains spadefoot), and *S. multiplicata* (New Mexico spadefoot) (Anderson et al. 1999). Only a single salamander species, *Ambystoma tigrinum* (tiger salamander), exists in the SHP (Smith 2003).

Because SHP amphibians are reliant upon playas for their continued persistence (Haukos and Smith 1994), destruction and degradation of this habitat poses a serious threat to these species. For example, sedimentation may decrease playa hydroperiod (Luo et al. 1997) to the point where amphibian community composition is affected (Ghioca and Smith 2008). Ghioca and Smith (2008) noted that *A. tigrinum* larvae were less abundant in playas embedded in

cropland compared to those surrounded by native grassland. Another factor that may threaten SHP amphibians is contamination of required habitat with agrochemicals (Venne et al. 2008). The amount of pesticides used in the state of Texas is one of the greatest (by volume) in the United States (Gianessi and Marcelli 2000). Because playas are at the lowest point in SHP watersheds (Luo et al. 1997), and most are imbedded within agricultural areas, these wetlands receive inputs of agrochemicals (Haukos and Smith 1994). One study found cotton or corn herbicides in 97% of 32 playas sampled within West Texas (Thurman et al. 2000). These chemicals enter playas via precipitation induced runoff (Haukos and Smith 1994, Thurman et al. 2000) and pesticide spray drift (de Snoo and de Wit 1998). Since many playas are surrounded by either cropland or native grassland and are structurally and functionally similar (Smith 2003), they represent an ideal opportunity to investigate how anthropogenic disturbance impacts amphibians in a replicated system (Gray et al. 2004).

Because pesticide sensitivity may vary among amphibian populations due to historical differences in contaminant exposure (Bridges and Semlitsch 2000), it is important to determine whether herbicide sensitivity differs among SHP amphibians from cropland and grassland playas. Previous work indicates that amphibians from these playa types differ in terms of individual level traits and overall community structure (Gray and Smith 2005, Ghioca and Smith 2008, Ghioca-Robrecht et al. 2009, McMurry et al. 2009). Gray and Smith (2005) found that amphibians inhabiting playas surrounded by grassland had larger post-metamorphic body size than those from cropland playas. McMurry et al. (2009) found that *S. bombifrons* and *S. multiplicata* larvae from grassland playas had greater body mass than those from cropland playas. This work also demonstrated that spleenic size and cellularity of *S. bombifrons* from grassland playas were greater than those from cropland playas. Ghioca and Smith (2008) found that *A. tigrinum* were present more often and in greater numbers in playas with grassland

watersheds. Ghioca-Robrecht et al. (2009) also determined that carnivore morph expression among *Spea* spp. larvae was related to landuse surrounding playas. This study found that playa water loss rate (a factor influenced by landuse, Tsai at al. 2007) was negatively associated with the proportion of carnivores present (Ghioca-Robrecht et al. 2009). Based on the above, it is possible that herbicides are influencing body size and immunology of playa amphibians (Gray and Smith 2005, McMurry et al. 2009).

Glyphosate- and glufosinate-based herbicides

Glufosinate- (e.g., Ignite® 280 SL) and glyphosate-based products (e.g., Roundup WeatherMAX®) represent two types of non-selective herbicides commonly used world-wide (Howe et al. 2004, Lee et al. 2005) to control weeds in agriculture and forestry, and for commercial applications to clear unwanted vegetation (Lee et al. 2005, Relyea 2005a). These herbicides are used extensively with herbicide-resistant crops (e.g. "Roundup-Ready" and "Liberty Link" cotton – Sankula and Blumenthal 2004) such as cotton, soybeans, and canola (Duke 2005). In Texas, application to cotton represents one of the most prevalent uses of glyphosate herbicides (National Pesticide Use Database 2004) and both types of herbicides are commonly applied with ground sprayers (Giesy et al. 2000). Glyphosate herbicides can be applied topically to herbicide-resistant cotton (Blair-Kerth et al. 2001) prior to the four leaf stage (Jones and Snipes 1999) and, therefore, glyphosate is often applied to cotton during mid to late June (Blair-Kerth et al. 2001). Glufosinate can be applied to herbicide-resistant cotton until the early bloom stage (Bayer CropScience LP 2005) which allows application until approximately late-July (Blair-Kerth et al. 2001). Therefore, SHP amphibians are likely exposed to these herbicides during the spring to summer breeding and larval development period (Strebbins 1954, Degenhardt et al. 1996).

The major components of glyphosate and glufosinate herbicides include the active ingredient and a surfactant (Koyama and Goto 1997, Giesy et al. 2000), which enhances penetration into plant leaves (Relyea 2005a). Glyphosate herbicides usually contain glyphosate and a polyethoxylated tallowamine (POEA) surfactant (Giesy et al. 2000). Some glyphosate products (e.g., Rodeo® - an herbicide intended for aquatic uses) are sold without a surfactant included, and require that one be added prior to application (Giesy et al. 2000). Glufosinate herbicides contain glufosinate-ammonium and a surfactant, often sodium polyoxyethylene alkylether sulfate (AES) (Koyama and Goto 1997). Both herbicides inhibit plant growth by interrupting important steps in the formation of required amino acids (Giesy et al. 2000, Lee et al. 2005).

Glyphosate and glufosinate are strongly adsorbed to soils and therefore, are usually present in low concentrations in surface runoff (Malone et al. 2004, Lee et al. 2005). Glufosinate, glyphosate and POEA degrade in soil or aquatic sediments via microbial activity (Rueppel et al. 1977, Smith 1988, Bartsch and Tebbe 1989, Giesy et al. 2000) and display limited environmental persistence (Banduhn and Frazier 1974 in Giesy et al. 2000, Marvel et al. 1974 in Giesy et al. 2000, Smith 1988, Smith and Belyk 1989, Oppenhuizen 1993 in Giesy et al. 2000). Time for dissipation of 50% (DT_{50}) of glyphosate applied to soils ranges from two and a half to six weeks (Oppenhuizen 1993 in Giesy et al. 2000), while that for glufosinate is three to 14 days (Smith 1988, Smith and Belyk 1989). In aquatic environments, the DT_{50} for glyphosate is one to two weeks (Goldsborough and Brown 1993, Giesy et al. 2000) and six to nine weeks for glufosinate (Faber et al. 1998a). The DT_{50} for the POEA surfactant used with glyphosate is approximately seven days in soil (Marvel et al. 1974 in Giesy et al. 2000) and three to four weeks in aquatic habitats (Banduhn and Frazier 1974 in Giesy et al. 2000). There is little information available that focuses on the AES surfactant used with glufosinate herbicides (Cox 1996).

Impacts of glyphosate and glufosinate herbicides on amphibians

Glyphosate herbicides. Until recently, it was widely believed that agrochemicals like those previously discussed pose only a minor risk to non-target organisms when applied properly (Relyea 2005a). However, there has been mounting evidence that exposure to glyphosate herbicides at environmentally relevant levels negatively impacts amphibians within terrestrial (Relyea 2005a) and aquatic habitats (Howe et al. 2004, Relyea 2004, 2005a). This evidence has been obtained from laboratory-based, single species and multi-species/community level experiments. In a laboratory single species study, Relyea (2004) determined that exposure to Roundup® at 1.5 mg glyphosate acid equivalents (ae)/L decreased growth and survival of *B. americanus, Rana clamitans,* and *R. catesbeiana*, but not *Hyla versicolor* or *R. pipiens*. Another laboratory experiment found that environmentally relevant levels (0.6 and 1.8 mg glyphosate ae/L) of two glyphosate formulations (Roundup Original® and Roundup Transorb®) caused reduced growth and survival among *R. pipiens* tadpoles (Howe et al. 2004). Relyea (2005a) also found that exposure of larval amphibians within aquatic mesocosms to Roundup® at levels simulating accidental direct overspray (2.9 mg glyphosate ae/L; maximum label rate - Relyea 2005a) caused reduced survival among all species tested (*R. pipiens*, *B. americanus*, and *H. versicolor*). This experiment also demonstrated that terrestrial exposure of juvenile amphibians to the same level of Roundup® resulted in significant mortality among all three species.

Results from several mesocosm studies support previously mentioned work: application of Roundup® at "direct overspray" levels caused significant larval mortality among some, but not all, amphibian species (Relyea et al. 2005, Relyea 2005b). However, an *in situ* enclosure study completed in natural wetlands found that Vision® (a glyphosate-based herbicide) caused no consistent negative impacts on larval survival, growth rate or size among *R. pipiens* and *R. clamitans* exposed to environmentally relevant concentrations (Wojtaszek et al. 2004). Relyea

(2005a) stated that these apparently contradictory results may be due to differences between the product applied (Roundup® vs. Vision®) or the experimental venue, though he did not speculate on the mechanistic nature or the specifics of such differences. These disparate results indicate that there are biotic and abiotic differences between mesocosms and *in situ* enclosures in natural wetlands that may cause amphibians to respond differently to herbicide exposure.

Previous work with glyphosate herbicides has indicated that toxicity is mainly due to the POEA surfactant (Mann and Bidwell 1999, Perkins et al. 2000, Edginton et al. 2004, Howe et al. 2004). Mechanistically, toxicity in amphibian larvae may result from negative impacts on respiratory surfaces. Surfactants have been shown to distort gill morphology in certain fishes (Brown et al. 1968, Abel and Skidmore 1975, Partearroyo et al. 1991, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008) and invertebrates (Lindgren et al. 1996). Edginton et al. (2004) investigated the impacts of Vision®, a glyphosate-based herbicide containing POEA surfactant, on four amphibian species. They found that mortality mainly occurred during developmental stages when gills were present. The above results suggest that the POEA surfactant present in many glyphosate formulations targets amphibian gills. However, no histological work has examined the pathological changes associated with amphibian exposure to POEA surfactant.

Glufosinate herbicides. While previous work has investigated the effects of glufosinate-based herbicides in other animals (Ebert et al. 1990, Kutlesa and Caveney 2001), little research has focused on how these herbicides impact amphibians. Kutlesa and Caveney (2001) found that *Calpodes ethylius* caterpillars fed leaves coated with glufosinate-ammonium displayed symptoms consistent with neurotoxicity: convulsions, tremors, and periods of paralysis prior to death. Ebert et al. (1990) determined that rats also developed similar symptoms following injection of glufosinate-ammonium. The authors posited that, because glufosinate-ammonium is structurally similar to glutamate, neurotoxic symptoms may result from glufosinate-ammonium

interfering with the role glutamate plays as a neurotransmitter.

Little work has examined whether glufosinate herbicides are toxic to amphibians. There is evidence that amphibians may experience indirect negative effects from these herbicides (Faber et al. 1998a,b). Faber et al. (1998a,b) found that important components of aquatic communities (phytoplankton – Faber et al. 1998a; zooplankton – Faber et al. 1998b) are negatively impacted by glufosinate-based herbicides. Amphibians may therefore be directly or indirectly at risk to effects resulting from exposure to glufosinate herbicide or alteration of the food web.

CHAPTER II

ACUTE AND CHRONIC TOXICITY OF ROUNDUP WEATHERMAX® AND IGNITE® 280 SL TO LARVAL *SPEA MULTIPLICATA* AND *S. BOMBIFRONS* FROM THE SOUTHERN HIGH PLAINS

Introduction

Amphibian population declines and pesticides

Amphibian populations are declining worldwide (Wyman 1990, Stuart et al. 2004). Many of these declines are caused by wetland and terrestrial habitat destruction (e.g., Wyman 1990). Within the contiguous U.S., approximately 53% of wetland area has been destroyed by human activity since the 1800s (Dahl 1990) and chemical contamination of remaining wetland habitat may also threaten amphibians (Howe et al. 2004). Many species reproduce in shallow wetlands (Mann and Bidwell 1999) located near areas commonly treated with agrochemicals (Boone and Semlitsch 2001). Because wetlands tend to concentrate chemicals applied in the surrounding landscape (Anderson and D'Apollonia 1978), amphibian larvae may be exposed to fertilizers, insecticides, and herbicides (Semlitsch 2003).

Herbicide background

Glyphosate- and glufosinate-based herbicides are commonly used worldwide (Howe et al. 2004, Lee et al. 2005) to control weeds in various settings (e.g., forests, farmland) (Lee et al. 2005, Relyea 2005a). Most formulations contain two main components: the active ingredient and a surfactant. Glyphosate-based herbicides (e.g., Roundup® formulations) commonly contain glyphosate and a polyethoxylated tallowamine (POEA) surfactant which improves active

ingredient absorption (Giesy et al. 2000). Glufosinate-based formulations are composed of glufosinate-ammonium and a surfactant, often sodium polyoxyethylene alkyether sulfate (AES) (Koyama and Goto 1997).

Glyphosate and glufosinate bind tightly to soil (Giesy et al. 2000, Lee et al. 2005) and therefore, surface runoff of these herbicides is usually limited (Malone et al. 2004, Lee et al. 2005). However, herbicides like these may enter aquatic habitats due to spray drift (Johansson et al. 2006), accidental overspray or run-off (Faber et al. 1998a, Johansson et al. 2006). Glyphosate herbicides are predicted to reach concentrations of 3.7 mg glyphosate/L [2.8 mg glyphosate acid equivalents (ae)/L] in aquatic habitats due to accidental overspray (Giesy et al. 2000), though the highest concentration yet reported is 2.6 mg glyphosate/L (2.0 mg glyphosate ae/L) (Feng et al. 1990, Horner 1990 in Giesy et al. 2000). Accidental overspray of aquatic habitats with glufosinate herbicides is predicted to result in concentrations of 1.0 mg glufosinate/L (Faber et al. 1998a).

Glyphosate degrades in aquatic habitats via microbial activity (Rueppel et al. 1977). While the mechanism of glufosinate degradation in aquatic habitats is unknown, it is likely the same as for glyphosate (Bartsch and Tebbe 1989). Time for 50% dissipation (DT_{50}) of glyphosate in aquatic environments is 1-2 weeks (Goldsborough and Brown 1993, Giesy et al. 2000), while that for glufosinate is 6-9 weeks (Faber et al. 1998a). The POEA surfactant in glyphosate herbicides persists longer in aquatic environments than the active ingredient, usually displaying a DT_{50} of 3-4 weeks (Banduhn and Frazier 1974 in Giesy et al. 2000). Little information is available concerning the environmental fate of the AES surfactant in glufosinate herbicides (Cox 1996).

Impacts of herbicides on amphibians

Recent laboratory and mesocosm experiments indicate that exposure to glyphosate herbicides at environmentally relevant levels may pose a risk to amphibian larvae (Howe et al. 2004, Relyea 2004, Relyea et al. 2005, Relyea 2005a,b). Several studies found that exposure at or below 2.9 mg glyphosate ae/L resulted in reduced growth (Howe et al. 2004, Relyea 2004) and survival (Howe et al. 2004, Relyea 2004, Relyea et al. 2005, Relyea 2005a,b) among certain species. Amphibians vary widely in their sensitivity to glyphosate herbicides (Mann and Bidwell 1999, Howe et al. 2004). Previous work found 24-hour LC_{50} values (i.e., the concentration causing 50% mortality among exposed individuals - Newman and Unger 2003) ranging from 2.0 - 16.6 mg glyphosate ae/L (Mann and Bidwell 1999, Howe et al. 2004), and 48-hour LC_{50} values ranging from 2.9 - 16.1 mg glyphosate ae/L (Mann and Bidwell 1999). To my knowledge, no previous studies have investigated the toxicity of glufosinate herbicides to amphibians. However, amphibians may be indirectly impacted because glufosinate herbicides can disrupt components of the aquatic food web (phytoplankton - Faber et al. 1998a, zooplankton - Faber et al. 1998b).

Southern High Plains amphibians and herbicides

Amphibians are a vital component of Southern High Plains (SHP) playa ecosystems because they can be the dominant vertebrate, serving as both prey and predator (Smith 2003). Because amphibians in this region rely on playa wetlands for continued persistence (Haukos and Smith 1994), any activities which degrade playas pose a serious threat to these species. Because playas are at the lowest point in SHP watersheds (Luo et al. 1997) and most are imbedded within agriculture, these wetlands receive inputs of agricultural chemicals via precipitation induced runoff (Haukos and Smith 1994). Thurman et al. (2000) found cotton or corn herbicides in 97% of 32 West Texas playa wetlands. One of the most widely applied herbicides in Texas is

glyphosate (National Pesticide Use Database 2004). Agricultural formulations (e.g., Roundup WeatherMAX®) are frequently used to control weeds in herbicide-resistant cotton (Blair-Kerth et al. 2001, National Pesticide Use Database 2004). Ignite® 280 SL, a glufosinate-ammonium herbicide used with FiberMax® LibertyLink® cotton, is an alternative to glyphosate formulations (Carter 2005). Both herbicides are applied during spring and summer (National Research Council 1975, Bayer CropScience LP 2005, Monsanto Company 2005).

SHP amphibians are likely exposed to agrochemicals (Venne et al. 2008) because herbicide application coincides with the breeding and larval development periods (Garrett and Barker 1987, Anderson et al. 1999). It is therefore important to determine whether larval amphibians in these wetlands are impacted by common pesticides. Dinehart et al. (2009) determined that the short-term survival of juveniles of several SHP species was not affected by exposure to environmentally relevant levels of common agricultural glyphosate and glufosinate formulations, Roundup WeatherMAX®, and Ignite® 280 SL.

A relevant question in the SHP is whether animals inhabiting cropland and grassland playas vary in terms of pesticide sensitivity. This phenomenon (i.e., adaptation or acclimation) has been demonstrated among invertebrates (Brausch and Smith 2009) and amphibians (Bridges and Semlitsch 2000). Brausch and Smith (2009) found that fairy shrimp (*Thamnocephalus platyurus*) from playa wetlands embedded in cropland were less sensitive to several pesticides compared to those from grassland playas. Bridges and Semlitsch (2000) demonstrated that contaminant susceptibility varied among amphibian populations, perhaps due to historical differences in contaminant exposure (Bridges and Semlitsch 2001). Amphibians from cropland and grassland playas are known to differ in terms of community structure (Gray et al. 2004, Ghioca and Smith 2008, Ghioca-Robrecht et al. 2009), body size (Gray and Smith 2005, McMurry et al. 2009), and immunological development (McMurry et al. 2009). However,

no previous studies have investigated whether landuse related differences in pesticide sensitivity exist among SHP amphibians.

Therefore, I examined the acute and chronic toxicity of a glyphosate- (Roundup WeatherMAX®) and glufosinate-based herbicide (Ignite® 280 SL) to New Mexico and Plains spadefoot toad larvae (*Spea multiplicata* and *S. bombifrons*, respectively) from SHP playa wetlands. To assess acute toxicity, I determined 48- and 216-hour (i.e., including post-exposure mortality) LC_{50} values for larvae of each species from playa wetlands embedded in cropland and native grassland landscapes. The latter LC_{50} values were calculated because traditional LC_{50} values may ignore post-exposure mortality and, therefore, significantly underestimate toxicity to field populations (Zhao and Newman 2004, Jones et al. 2009). While traditional acute toxicity tests provide insight about the immediate effects of short-term contaminant exposure (≤ 96-hours) (ASTM 2003), in nature, animals may experience prolonged exposure (Banduhn and Frazier 1974 in Giesy et al. 2000, Goldsborough and Brown 1993, Faber et al. 1998a, Giesy et al. 2000). I therefore also assessed how chronic exposure (30 day) to environmentally relevant levels of Ignite® 280 SL and Roundup WeatherMAX® affected survival of New Mexico and Plains spadefoot toads from cropland and grassland playas. In light of previous research, I hypothesized that sensitivity to Ignite® 280 SL and Roundup WeatherMAX® would vary between landuse, and that larvae from cropland playas would be less sensitive to herbicide exposure. I hypothesized that larvae from cropland playas would display greater LC_{50} values and increased survival following chronic herbicide exposure compared to those from grassland playas.

Methods

Study area

The SHP of Texas and New Mexico contain numerous shallow wetlands called playas (Bolen et al. 1989). The climate in this area is subhumid continental (Haukos and Smith 1994). Mean summer high temperatures near the center of the SHP (Lubbock, TX) were 30.4 °C (May-September, 1971-2000; National Oceanic and Atmospheric Administration 2009). Annual precipitation on the SHP averages from 45 cm in the northeast to 33 cm in the southwest (Bolen et al. 1989). Natural hydrologic inputs to playa wetlands come only from precipitation and surface runoff (Smith 2003). Because these wetlands occur in a region dominated by agriculture, they provide valuable wildlife habitat (Guthery and Bryant 1982) and support the persistence of most flora and fauna inhabiting the SHP (Haukos and Smith 1994).

Larval collection and housing

Playas were selected based on landuse within the surrounding watershed and the availability of *Spea* spp. (*S. bombifrons* and *S. multiplicata*; Plains and New Mexico spadefoots, respectively) larvae. These two species, in addition to *Pseudacris clarkii* (spotted chorus frog) and *Bufo cognatus* (Great Plains toad), are several of the most common anuran amphibian species found in the SHP (Anderson et al. 1999). Playas were considered "cropland" if at least 75% of the surrounding watershed was cultivated, and "grassland" if at least 75% was native grass (Anderson et al. 1999). Selected cropland playas were located in Floyd, Hockley, and Terry county, while grassland playas were located in Briscoe and Floyd county, TX. All sampled playas were separated by ≥ 1 km because most frogs and toads do not move greater than this distance away from their natal sites (Sinsch 1990). *Spea* spp. larvae were collected from three cropland playas on 19 May 2007 and from two grassland playas on 20-21 May 2007. Larvae were collected from only two grassland playas due to the limited availability of spadefoot larvae in

this landuse. New Mexico and Plains spadefoots were collected, held, and tested as a mixed species culture because protein electrophoresis is required to differentiate larvae of these species (Simovich and Sassaman 1986).

Following collection, larvae were transported to The Institute of Environmental and Human Heath at Texas Tech University (TIEHH) in Lubbock, TX in buckets containing playa water. All animal collection, housing, and research procedures were approved by the Texas Tech University Institutional Animal Care and Use Committee (protocol no. 06018-06). Larvae were held in a climate controlled room on a 14h-10h light-dark photoperiod for the duration of acclimation and toxicity testing. This photoperiod was used since it approximates that near the center of the SHP (Lubbock, TX) during May-September (U.S. Naval Observatory 2008). Larvae were housed in 39.7 L glass aquaria filled with aerated, aged tapwater from the same source used for dilution, and during toxicity tests (Mann and Bidwell 1999). Larvae were allowed to acclimate to laboratory conditions for nine days, during which time pulverized rabbit food was provided ad libitum (Relyea et al. 2005). Larvae were observed daily during acclimation. No obvious signs of stress were noted (i.e., <5% of larvae died during the acclimation period).

Temperature and water quality were monitored daily during acclimation. These variables were similar among all aquaria (mean ± 1 S.E.): temperature = 21.96 ± 0.04 °C, pH = 8.37 ± 0.02 , dissolved oxygen (DO) = 8.06 ± 0.08 mg/L, ammonia = 2.10 ± 0.10 mg/L. Temperature and pH were measured with a Hanna HI 9124 pH meter (Hanna Instruments, Woonsocket, RI), dissolved oxygen was measured with a Oakton DO 6 dissolved oxygen meter (Oakton Instruments, Vernon Hills, IL), and ammonia was monitored with Aquarium Pharmaceuticals test kits (Mars Fishcare North America, Inc., Chalfont, PA). An 80% water change (Meyer and Di Giuliuo 2003) occurred whenever ammonia exceeded 1 mg/L so that

ammonia levels during acclimation would not exceed those noted during similar studies (2.8-4.2 mg/L - Relyea 2004).

Acute toxicity tests

Methods. Tests procedures were based on American Society for Testing and Materials (ASTM) Guidelines for Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians (ASTM 2003). Roundup WeatherMAX® (Monsanto Company, St. Louis, MO) and Ignite® 280 SL (Bayer CropScience LP, Research Triangle Park, NC) were obtained from retail outlets (Table 2.1). To facilitate accurate pipeting during preparation of test concentrations, stock solutions [Roundup WeatherMAX®, 330 g glyphosate/L (269.9 g glyphosate ae/L); Ignite® 280 SL, 93.3 g glufosinate/L] were created by diluting formulated herbicides by half with aged tapwater.

Test chambers consisted of 18.95 L glass compartments created by dividing 37.9 L (10 gal.) aquaria in half with a glass divider secured with silicone Aquarium Sealant (Perfecto Manufacturing, Inc., Noblesville, IN). Fifteen liters of tapwater was added to each compartment and allowed to age for 48 hours. This water volume was selected to ensure that biomass loading limits (0.5 g/L) would not be exceeded (ASTM 2003). I measured water hardness ([$CaCO_3$] = 231 mg/L) with an Aquarium Pharmaceuticals test kit because differences in dilution water chemistry may affect acute toxicity estimates (Mann and Bidwell 1999).

Test concentrations were prepared immediately before the start of tests by pipeting the appropriate amount of stock solution into aquarium compartments. Solution in each compartment was gently mixed with a clean glass rod to ensure equal dispersion of stock solution. Larvae from cropland playas were exposed to eight herbicide concentrations [Roundup WeatherMAX® - 0.75, 1.5, 2.25, 3, 4.5, 6, 7.5, 10 mg glyphosate/L (0.61, 1.2, 1.8, 2.5, 3.7, 4.9, 6.1, 8.2 mg glyphosate ae/L); Ignite® 280 SL - 0.5, 2.5, 3.75, 5, 7.5, 10, 12.5, 15 mg glufosinate/L] and a control (aged tapwater only). The highest dose of each herbicide was excluded from tests

with grassland larvae due to their limited availability. The range of Roundup WeatherMAX® concentrations was based on previously determined amphibian LC_{50} values for glyphosate herbicides (Mann and Bidwell 1999, Howe et al. 2004), and was weighted toward lower concentrations more likely to include the actual LC_{50} value. Preliminary toxicity tests with *Spea* spp. larvae during summer 2006 indicated that the above range of Ignite® 280 SL test concentrations was appropriate.

Acute toxicity tests with cropland larvae commenced 29 May 2007. Testing with grassland larvae began 31 May 2007. Test solutions were prepared just prior to tests as described above. Larvae received food until transferred to test compartments because developing amphibian larvae are sensitive to starvation (Mann and Bidwell 1999). All larvae used for toxicity tests were Gosner-stage 29-30 (Gosner 1960). Twenty randomly selected *Spea* spp. larvae from each playa were blotted dry and weighed to quantify initial mass (Mann and Bidwell 1999).

Larvae from each playa were randomly allocated to test compartments. Cropland compartments contained three larvae from each of three cropland playas for a total of nine *Spea* spp. larvae. Grassland compartments contained four larvae from each of two grassland playas; the ninth *Spea* spp. larvae was randomly selected from the grassland holding tanks. Food was withheld during the first 48 hours of testing (Mann and Bidwell 1999) because uneaten food and fecal matter may influence the toxicity of chemicals (ASTM 2003). Four replicates (for each herbicide concentration and controls) were used, and test compartments were arranged in a randomized design.

Test compartments were checked every six hours. Any moribund larvae were euthanized by immersion in 1% MS-222 (Howe et al. 2004) and preserved at -80 °C for subsequent species differentiation. Larvae were considered moribund if they exhibited non-

responsiveness to prodding or loss of coordinated movement (ASTM 2003). After 48 hours, all remaining larvae were transferred to plastic containers filled with clean aged tapwater (Relyea 2004). Each container held 5 L of water and additional water was added daily to maintain a constant level. Larvae received pulverized rabbit food ad libitum. Larvae were observed every six hours for seven days to quantify any delayed (i.e., latent) mortality (ASTM 2003, Zhao and Newman 2004). Any moribund larvae were euthanized in MS-222 and preserved at -80 °C. An 80% water change (Meyer and Di Giuliuo 2003) occurred after four days or if ammonia levels surpassed 1 mg/L. After seven days, all remaining larvae were euthanized in MS-222 and preserved at -80 °C. Larvae were then identified as New Mexico or Plains spadefoots via protein electrophoresis following Simovich and Sassman (1986). Temperature, pH, dissolved oxygen, and ammonia levels were quantified initially and every 48 hours (ASTM 2003) in four randomly selected low, medium, and high herbicide concentration test compartments or associated plastic containers (ASTM 2003). The same water quality variables were measured in four control compartments and plastic containers. Soon after tests started, water samples (50 mL) were collected from these same test compartments and preserved in pre-cleaned environmental sample vials at -20 °C for subsequent analytical analysis to determine glyphosate and glufosinate concentrations.

Statistical analyses. Initial weight data for *Spea* spp. larvae from cropland and grassland playas were tested for homogeneity of variance using Levene's test and for normality with a Shapiro-Wilk test (SAS Version 9.2, SAS Institute, Cary, NC). These analyses indicated that variances were heterogeneous (Levene's test: $F_{1,98} = 24.01$, $P < 0.001$) and data were non-normally distributed; standard data transformations (i.e., log, natural log, square root) did not correct these problems. Therefore, weight data were compared with a Wilcoxon two-sample

test (PROC NPAR1WAY, SAS Version 9.2, SAS Institute, Cary, NC). This test is appropriate when the assumptions of a parametric t-test cannot be met (Sokal and Rohlf 1995).

Water quality data (temperature, dissolved oxygen, ammonia, and pH) from grassland and cropland test compartments were tested for homogeneity of variance using Levene's test (Table 2.2) and for normality with a Shapiro-Wilk test. Data for certain variables (48-hour exposure period: temperature, dissolved oxygen; 168-hour post-exposure monitoring period: temperature, ammonia) displayed heterogeneous variances (Table 2.2) and were non-normally distributed; data transformations (i.e., log, natural log, square root) did not correct these problems. Data for all remaining variables (48-hour exposure period: pH, ammonia; 168-hour post-exposure monitoring period: dissolved oxygen, pH) were non-normally distributed and standard data transformations did not correct this problem. Therefore, all water quality data were compared with Wilcoxon two-sample tests (PROC NPAR1WAY, SAS Version 9.2, SAS Institute, Cary, NC).

Forty-eight and 216-hour LC_{50} values and associated 84% confidence intervals were determined via probit analysis (PROC PROBIT, SAS Version 9.2, SAS Institute, Cary, NC). Probit analysis is a parametric technique commonly used for estimating LC_{50} values and associated confidence intervals (Perkins et al. 2000, Relyea 2005, Jones et al. 2009, Pereira et al. 2009). To calculate hazard quotients, I determined LC_1 values (48- and 216-hour) using the same technique (Table 2.3). LC_1 values are a preferable indicator of low effect concentrations because they are not strongly influenced by experimental design (Crane and Newman 2000). All LC values were calculated based on nominal concentrations because analytical chemistry indicated that dosing solutions were prepared accurately (see below). Because only a single LC_{50} value was determined for each species-landuse combination, between- and within-species (i.e., between landuse) LC_{50} values were compared by examining 84% confidence intervals for overlap (Jones et

al. 2009). This approach was adopted because simulations indicate that hypothesis tests using 84% confidence intervals have a type I error rate of 0.05 (Payton et al. 2003). Forty-eight hour and 216-hour LC_{50} values were compared using the same criteria as above. Effect size analysis (Cohen 1992) was used to estimate the magnitude of any between-landuse differences in LC_{50} values.

Chronic toxicity tests

Methods. Chronic toxicity tests with cropland and grassland larvae began 29 and 31 May 2007, respectively. Test chambers identical to those used during acute toxicity tests contained 15 L of tapwater aged for 48 hours. Test solutions were prepared just prior to tests as previously described. A control (aged tapwater) and two treatments were established for each herbicide. Roundup WeatherMAX® test solutions contained either 2.8 mg glyphosate ae/L (the highest concentration expected from accidental overspray of aquatic habitat - Giesy et al. 2000) or 2.0 mg glyphosate ae/L (the highest aquatic concentration yet reported - Feng et al. 1990, Horner 1990 in Giesy et al. 2000). Ignite® 280 SL test solutions contained either 1.0 mg glufosinate/L (the concentration expected from accidental aquatic overspray - Faber et al. 1998a) or 0.5 mg glufosinate/L. This set-up was replicated six times and compartments were arranged in a randomized design.

Larval care prior to chronic tests was the same as for acute tests. All larvae used for chronic toxicity tests were Gosner-stage 29-30 (Gosner 1960). Nine larvae were allocated to each test compartment in the same fashion as during acute toxicity tests. Each group of nine larvae was weighed as they were added to test compartments to quantify initial mean mass.

Test compartments were checked every six hours. Any moribund individuals (using the previously described criteria) were euthanized in 1% MS-222 and preserved at -80 °C for species differentiation. Larvae were provided pulverized rabbit food, with food levels constant among

all compartments. An 80% water change (Meyer and Di Giuliuo 2003) occurred every four days and herbicides were reapplied (i.e. static-renewal). Survival was monitored for 30 days, after which all remaining larvae were euthanized and preserved at -80 °C. Larvae were subsequently identified as New Mexico or Plains spadefoots via protein electrophoresis (Simovich and Sassaman 1986).

For each landuse-herbicide combination, basic water quality measurements including temperature, pH, dissolved oxygen, and ammonia levels were collected in four randomly selected control, low, and high herbicide concentration test compartments (ASTM 2002) initially and every 48 hours (ASTM 2002) during chronic toxicity tests. Just after tests started, water samples (50 mL) were collected from these same compartments and preserved at -20 °C for subsequent analytical analysis to determine glyphosate and glufosinate concentrations.

Statistical analyses. Weight data for *Spea* spp. larvae allocated to Roundup WeatherMAX® and Ignite® 280 SL cropland and grassland compartments were tested for homogeneity of variance using Levene's test and for normality with a Shapiro-Wilk test (SAS Version 9.2, SAS Institute, Cary, NC). These analyses indicated that variances were homogeneous (Levene's test: Roundup WeatherMAX® $F_{1,34} = 0.31$, $P = 0.58$; Ignite® 280 SL $F_{1,34} = 0.52$, $P = 0.47$) and data were normally distributed. Therefore, weight data were compared with t-tests (PROC TTEST, SAS Version 9.2, SAS Institute, Cary, NC).

Water quality data (temperature, dissolved oxygen, ammonia, and pH) from Roundup WeatherMAX® and Ignite® 280 SL grassland and cropland test compartments were tested for homogeneity of variance using Levene's test and for normality with a Shapiro-Wilk test (Table 2.2). Data for certain variables (Roundup WeatherMAX®: ammonia, pH; Ignite® 280 SL: ammonia, pH) displayed heterogeneous variances and were non-normally distributed (Table 2.2). Shapiro-Wilk tests indicated that data for all remaining variables (Roundup WeatherMAX®:

dissolved oxygen, temperature; Ignite® 280 SL: dissolved oxygen, temperature) were non-normally distributed. Standard data transformations (i.e., log, natural log, square root) did not correct these problems. Therefore, these data were compared with Wilcoxon two-sample tests (PROC NPAR1WAY, SAS Version 9.2, SAS Institute, Cary, NC).

Chronic survival data were analyzed using generalized linear models (PROC GENMOD, SAS Version 9.1, SAS Institute, Cary, NC) assuming a poisson distribution and a log link function (Littell et al. 2002). This analysis tested whether survival of New Mexico or Plains spadefoots were impacted by chronic herbicide exposure. Number of surviving larvae was the response variable, while treatment, landuse, and species were independent variables. Because data contained many zeros, 0.0001 was added to each datum so that the GENMOD model converged. Where necessary, CONTRAST statements were included in the GENMOD procedure to separate group means. When significant differences were present, the magnitude of all within group (i.e., between landuse) differences were estimated using effect size analysis (Cohen 1992).

Analytical chemistry

Acute controls and test solution samples with nominal concentrations above analytical detection limits were analyzed to compare nominal and measured glyphosate and glufosinate concentrations. Chronic controls and high herbicide concentration samples were also examined even though these nominal concentrations were below analytical detection limits. The amount of active ingredient (glyphosate or glufosinate) in test solutions was determined by gas chromatography of the TMOA-derivatized products following Tseng et al. (2004). To my knowledge, this procedure has not previously been used with formulated glyphosate or glufosinate herbicides. Commercially obtained glufosinate and glyphosate stocks (AccuStandard Inc., New Haven, CT) were used to construct calibration standards, calibration checks, and end calibration check standards.

Results

Analytical chemistry

Analysis of water samples from acute and chronic toxicity experiments indicated that an acceptable level of variability was present between measured and nominal concentrations (<30%, U.S. Environmental Protection Agency 1996). Acute test solution samples from the 6.0 mg glyphosate/L group actually contained (mean ± SD) 6.5 ± 1.8 mg glyphosate/L, and those from the 12.5 mg glufosinate/L group contained 15.4 ± 4.8 mg glufosinate/L. This level of variability (8% and 23% for glyphosate and glufosinate samples, respectively) is not surprising considering the uncertainty associated with using these analytical methods with formulated herbicides (Tseng et al. 2004). No glyphosate or glufosinate was detected in any control samples from acute tests. As expected, no glyphosate or glufosinate was detected in any herbicide test solution or control samples from the chronic experiment. The check standards used during the analysis revealed excellent recoveries for the analytical method (93% for glufosinate, 95% for glyphosate).

Acute toxicity

No mortality occurred in any control compartments, or in the lowest Roundup WeatherMAX® or Ignite® 280 SL test concentration (0.75 mg glyphosate/L and 0.5 mg glufosinate/L, respectively). Mean weight (± 1 S.E.) of *Spea* spp. larvae from cropland and grassland playas was 0.25 ± 0.01 g and 0.29 ± 0.02 g, respectively. Mean weight did not differ among larvae from cropland and grassland playas ($z = 1.30$, $P = 0.19$), but in general, grassland larvae weighed 16% more than cropland larvae.

Water quality. During the 48-hour exposure period, ammonia levels were similar among grassland and cropland test compartments (Table 2.4). Temperature and pH were higher in grassland compartments, while dissolved oxygen was higher in cropland compartments (Table

2.4). During the subsequent 168-hour post-exposure monitoring period, temperature and dissolved oxygen were similar among grassland and cropland test compartments, while ammonia levels and pH were greater in grassland test compartments (Table 2.4). All previously noted differences, except water temperature during the 48-hour exposure period, were small in magnitude and within the range of variation noted during acute toxicity studies with larval amphibians (Mann and Bidwell 1999, Howe et al. 2004, Relyea and Jones 2009). Even though test compartments were randomly arranged, mean water temperature was 3.28 °C higher in grassland compartments than cropland compartments during the first 48 hours of acute toxicity tests (Table 2.3). This difference likely resulted from air temperature variation in the room that housed experimental compartments. While this room is climate controlled, ambient air temperatures respond measurably to exterior (i.e., environmental) temperatures due to the room's design and location (i.e., one wall comprises the exterior of TIEHH and is subject to direct sunlight).

Roundup WeatherMAX®. LC_{50} values for Roundup WeatherMAX® (Table 2.5) are presented in units of glyphosate acid equivalents to facilitate comparison with other studies. No differences were present between 48- and 216-hour LC_{50} values for either species. Neither 48- or 216-hour LC_{50} values for Plains or New Mexico spadefoots differed between landuses or species. Effect size analysis indicated all between landuse differences were small (Table 2.5).

Ignite® 280 SL. Only 48-hour LC_{50} values for Ignite® 280 SL are presented (Table 2.6) because they are identical to 216-hour values. LC_{50} values did not differ between landuses. Effect size analysis indicated that all between landuse differences were small. Toxicity estimates varied between species. LC_{50} values (48-hour) for New Mexico spadefoots from grassland and cropland playas were greater than those for Plains spadefoots from grassland and cropland playas (Table 2.6).

Chronic toxicity

Spea spp. larvae from grassland playas weighed more than those from cropland playas for Roundup WeatherMAX® (grassland = 0.29 ± 0.01 g, cropland = 0.24 ± 0.01 g) (t_{34} = -5.24, P < 0.001) and Ignite® 280 SL (grassland = 0.31 ± 0.01 g, cropland = 0.22 ± 0.01 g) (t_{34} = -8.31, P < 0.001) tests.

Water quality. Water quality variables were consistent among Roundup WeatherMAX® and Ignite® 280 SL test compartments throughout the 30-day exposure period (Table 2.7). For the Roundup WeatherMAX® test, pH, temperature, and ammonia levels were similar among grassland and cropland test compartments (Table 2.7). Even though test compartments were randomly arranged, dissolved oxygen was greater in grassland test compartments (Table 2.7). For the Ignite® 280 SL test, all water quality variables were similar among grassland and cropland test compartments (Table 2.7). All of the above differences were small in magnitude and within the range of variation noted during similar chronic toxicity tests (Relyea 2004, 2005).

Roundup WeatherMAX®. Survival differed among treatments (χ^2_2 = 181.99, P < 0.001), but not species (χ^2_1 = 0.44, P = 0.51) or landuse (χ^2_1 = 0.02, P = 0.88). There was a species-treatment interaction (χ^2_2 = 7.26 , P = 0.027), so I separated data by species and repeated the analysis. Survival of Plains spadefoots (n = 182) differed among treatments (χ^2_2 = 112.97, P < 0.001), but not by landuse (χ^2_1 = 0.25, P = 0.61). No treatment-landuse interaction was present (χ^2_2 = 0.00, P = 0.99). While control survival was high, no Plains spadefoots exposed to Roundup WeatherMAX® at 2.0 or 2.8 mg glyphosate ae/L survived the 30-day exposure period (Figure 2.1). All larvae exposed to 2.8 mg glyphosate ae/L died within 2 days, while some larvae exposed to 2.0 mg glyphosate ae/L survived as long as 11 days (Figure 2.2).

Survival of New Mexico spadefoots (n = 142) differed among treatments (χ^2_2 = 76.37, P < 0.001), but not by landuse (χ^2_1 = 0.01, P = 0.92). Survival of larvae exposed to Roundup

WeatherMAX® at 2.0 or 2.8 mg glyphosate ae/L was reduced compared to control larvae (Figure 2.3). No larvae exposed to 2.8 mg glyphosate ae/L survived the 30-day exposure period (Figure 2.3), and complete mortality occurred within five days (Figure 2.4). A treatment-landuse interaction was present ($\chi^2_2 = 6.18$, P = 0.046). To investigate this interaction, I compared survival between landuse for each treatment. Survival of control larvae and those exposed to Roundup WeatherMAX® at 2.8 mg glyphosate ae/L did not differ by landuse (control: $\chi^2_1 = 0.030$, P = 0.85; 2.8 mg glyphosate ae/L: $\chi^2_1 = 0.00$, P = 0.99). Larvae from grassland playas (n = 67) exhibited greater survival than those from cropland playas (n = 75) following chronic exposure to Roundup WeatherMAX® at 2.0 mg glyphosate ae/L ($\chi^2_1 = 6.41$, P = 0.011) (Figure 2.3). While all cropland larvae exposed to 2.0 mg glyphosate ae/L died within 18 days, mean survival of grassland larvae through 30 days was 20% (Figure 2.4). Effect size analysis indicated that this difference was a medium magnitude effect (Figure 2.3).

Ignite® 280 SL. Survival did not differ between landuses ($\chi^2_1 = 0.03$, P = 0.86), or among species ($x^2_1 = 0.00$, P = 0.97) and treatments ($\chi^2_2 = 0.14$, P = 0.93). Plains (Figure 2.5; n = 168) and New Mexico (Figure 2.6; n = 156) spadefoot survival was not affected by chronic exposure to Ignite® 280 SL at 0.5 or 1.0 mg glufosinate/L.

Discussion

Traditional toxicity estimates (48-hour LC_{50} values) for Roundup WeatherMAX® and Ignite® 280 SL did not differ from those that considered post-exposure mortality (216-hr LC_{50} values). Acute toxicity estimates indicate that Ignite® 280 SL is less toxic to New Mexico and Plains spadefoot larvae than Roundup WeatherMAX®. This likely resulted from differential toxicity of the active or "inert" (e.g., surfactants) ingredients in these formulations to Plains and New Mexico spadefoot larvae. Previous work with aquatic organisms (amphibian larvae and

fish) demonstrated that the toxicity of formulated herbicides varies based on differences in these ingredients (i.e., active or "inert" components) (Servizi et al. 1987, Mann and Bidwell 1999, Howe et al. 2004). Survival of New Mexico and Plains spadefoots was not reduced by chronic exposure to environmentally relevant levels of Ignite® 280 SL. In contrast, chronic exposure to Roundup WeatherMAX® at environmentally relevant levels resulted in extensive mortality among both species.

Toxicity of Ignite® 280 SL

To my knowledge, no studies have examined the toxicity of formulated glufosinate herbicides to amphibians. Work on sunfish (*Lepomis macrochirus*), a surrogate for amphibian larvae (U.S. Environmental Protection Agency 2009), indicated that technical grade glufosinate-ammonium is "slightly non-toxic," with LC_{50} values greater than 320 mg glufosinate/L (Kiernan and Orrick 2008). This estimate may be not accurately reflect toxicity of glufosinate herbicides because "inert" ingredients (e.g., surfactants) present in formulated products were lacking. Results from the current study indicate that Ignite® 280 SL would be considered "moderately toxic" to New Mexico and Plains spadefoot larvae ($1 < LC_{50} \leq 10$ mg active ingredient/L) according to U.S. Environmental Protection Agency (EPA) acute toxicity categories for pesticides (U.S. Environmental Protection Agency 2009).

Toxicity of Roundup WeatherMAX®

Mann and Bidwell (1999) investigated the acute toxicity of three glyphosate formulations to larvae of four Australian amphibian species (*Litoria moorei*, *Lymnodynastes dorsalis*, *Heleioporus eyrei* and *Crinia insignifera*). Roundup® and Touchdown® were much more toxic (48-hour LC_{50}: 2.9 - 16.1 mg glyphosate ae/L) than Roundup Biactive® (48-hour $LC_{50} \geq 328$ mg glyphosate ae/L). Roundup WeatherMAX® toxicity estimates (48-hour LC_{50}: 1.85 - 2.30 mg glyphosate ae/L) for Plains and New Mexico spadefoot larvae were lower than those reported

by Mann and Bidwell (1999). However, it is difficult to make comparisons between these studies because different herbicides were used, and toxicity of glyphosate formulations varies due to differences in "inert" ingredients (e.g., surfactants) (Mann and Bidwell 1999). Results from the current study indicate that Roundup WeatherMAX® would be considered "moderately toxic" to New Mexico and Plains spadefoot larvae according to U.S. EPA acute toxicity categories for pesticides (U.S. Environmental Protection Agency 2009).

While acute tests provide short-term toxicity estimates, glyphosate herbicides may persist for extended periods under field conditions (Goldsborough and Brown 1993, Giesy et al. 2000). Several laboratory, static-renewal studies have investigated this scenario (Howe et al. 2004, Relyea 2004). That work demonstrated that chronic exposure to glyphosate-based herbicides at or below 1.8 mg glyphosate ae/L reduced survival among larvae of several North American amphibian species. Chronic toxicity results from the present study agree with previous work: survival of Plains and New Mexico spadefoot larvae was greatly reduced by exposure to environmentally relevant levels of Roundup WeatherMAX® (2.8 and 2.0 mg glyphosate ae/L) under static-renewal conditions.

Risk posed by Ignite® 280 SL and Roundup WeatherMAX®

Laboratory toxicity tests like those completed during the current study play a key role in determining whether pesticides pose a risk to non-target organisms (Edginton et al. 2004). Under a tiered approach to ecological risk assessment, lower tier studies (i.e., highly controlled, acute and chronic toxicity tests) help evaluate potential for risk by assessing effects of contaminant concentrations above those commonly encountered under normal field conditions (Romeis et al. 2008). Should these tests indicate the potential for risk, higher tier studies that more closely reflect realistic exposure scenarios may be needed (Romeis et al. 2008). One way to evaluate the potential for risk is to calculate a hazard quotient (Edginton et al. 2004). This is

done by dividing environmentally relevant contaminant concentrations by effects values (e.g., LC_{50} values) from toxicity tests (Giesy et al. 2000). Hazard quotient values >1 indicate potential for adverse effects (Giesy et al. 2000), and that higher tier testing is required (Romeis et al. 2008).

Hazard quotients calculated with Ignite® 280 SL toxicity estimates (LC_{50} values) and the highest environmentally relevant glufosinate herbicide concentration (1.0 mg glufosinate/L) were all <0.71. This indicates that Ignite® 280 SL likely does not pose a mortality risk to New Mexico and Plains spadefoot larvae. Chronic toxicity data support this conclusion.

All hazard quotients calculated with Roundup WeatherMAX® toxicity estimates (LC_{50} values) and environmentally relevant aquatic glyphosate concentrations were >1. This indicates that Roundup WeatherMAX® may pose a mortality risk to New Mexico and Plains spadefoot larvae in SHP playa wetlands. Chronic toxicity results from the present study support this conclusion. Because various environmental factors (e.g., predator cues - Relyea 2003, pH - Chen et al. 2004) can increase herbicide toxicity, further tests under more realistic conditions are needed to determine whether Roundup WeatherMAX® poses a risk to New Mexico and Plains spadefoot larvae under field conditions.

Variation in sensitivity by landuse

Two previous studies have investigated whether sensitivity to herbicides or associated surfactants (e.g., POEA) varies among organisms from cropland and grassland playas (Brausch and Smith 2007, 2009). Brausch and Smith (2007) investigated the acute toxicity of three POEA surfactants to fairy shrimp (*Thamnocephalus platyurus*) from two cropland and grassland playas. While fairy shrimp from one grassland playa displayed the highest LC_{50} values, in general, there were no consistent differences in sensitivity by playa type. Subsequent work by Brausch and Smith (2009) indicated that fairy shrimp from playa wetlands surrounded by row crops were less

sensitive to the herbicide Karmex® DF (active ingredient = diuron) compared to those from grassland playas. Because these fairy shrimp experienced little or no prior agrochemical exposure, resistance among cropland individuals likely resulted from adaptation due to historical exposure to agrochemicals (Brausch and Smith 2009).

I hypothesized that New Mexico and Plains spadefoot larvae from cropland playas would be less sensitive to Roundup WeatherMAX® and Ignite® 280 SL. Acute toxicity tests indicated no difference in sensitivity among larvae from grassland and cropland playas for either herbicide. Chronic toxicity results are also contrary to the above hypothesis. New Mexico spadefoots from grassland playas exhibited greater survival than those from cropland playas following 30-day exposure to Roundup WeatherMAX® at 2.0 mg glyphosate ae/L. If this result was due to variation in herbicide sensitivity between grassland and cropland New Mexico spadefoot larvae, Roundup WeatherMAX® acute toxicity estimates (i.e., LC_{50} values) would likely also reflect this difference. Because New Mexico spadefoots from cropland and grassland playas displayed similar Roundup WeatherMAX® LC_{50} values, the above difference in chronic response must be viewed cautiously.

Selective pressure favoring the evolution of herbicide resistance among spadefoot larvae from cropland playas may have been lacking. This would be the case if Roundup WeatherMAX® and Ignite® 280 SL do not commonly attain environmental levels that affect larval fitness (e.g., negatively impact survival or growth). Few data describing aqueous herbicide levels in playa wetlands are available (Thurman et al. 2000), and none exist for glyphosate- or glufosinate-based herbicides. Even if environmental levels of Ignite® 280 SL negatively impact spadefoot larvae, it is unlikely that sufficient time has elapsed for resistance to develop. Brausch and Smith (2009) concluded that herbicide-resistant fairy shrimp were likely exposed for approximately 60 generations. Because glufosinate herbicides were first registered in the U.S. in

1993 (U.S. Environmental Protection Agency 1993 in Cox 1996), less than 14 generations of New Mexico and Plains spadefoots had been exposed to this herbicide at the time of this study.

It is possible that variation in herbicide sensitivity between cropland and grassland spadefoot larvae was confounded by the affects of dispersal. If inter-playa dispersal was frequent, this would tend to homogenize herbicide sensitivity within and among populations and slow the evolution of herbicide resistance among these species. Little is known about the propensity of post-metamorphic SHP amphibians to disperse, or the distances dispersing individuals may cover. Because most SHP wetlands are embedded in agriculture and receive herbicide inputs, dispersing individuals would likely encounter similar selective pressure, thereby favoring the development of population-level herbicide resistance. Future work should determine the degree of inter-playa movement displayed by SHP amphibian species. This knowledge would allow researchers examining variation in amphibian herbicide sensitivity between landuses to minimize any confounding affects of dispersal.

The only previous example of within-species variation in pesticide sensitivity among amphibians was observed for carbaryl (Bridges and Semlitsch 2000), an insecticide with a mode of toxicity that differs from that of Roundup WeatherMAX®. Carbaryl is a neurotoxic insecticide that disrupts acetylcholinesterase activity (Bridges 1997). Glyphosate-based herbicides are thought to be toxic toward amphibians and other aquatic organisms because surfactants (e.g., POEA) in these formulations disrupt gill structure and function (Swedmark et al. 1971, Abel 1974, Lindgren et al. 1996, Mann and Bidwell 1999). The mechanism of glufosinate herbicide toxicity toward amphibians is unknown.

It is possible that weight differences between cropland and grassland larvae influenced acute toxicity estimates and the chronic response of larvae to Roundup WeatherMAX® and Ignite® 280 SL. Mean weight of grassland *Spea* spp. larvae used during Roundup WeatherMAX®

and Ignite® 280 SL chronic toxicity tests was greater than cropland larvae. *Spea* spp. larvae used for acute toxicity tests exhibited the same trend. These weight differences may have affected my ability to detect variation in herbicide sensitivity between landuses. Heavier larvae may exhibit decreased sensitivity because higher herbicide concentrations are needed to attain a critical body burden, or because a smaller surface area-to-volume ratio decreases herbicide uptake (Wojtaszek et al. 2004). If greater larval mass conferred increased resistance to Roundup WeatherMAX® or Ignite® 280 SL, grassland larvae should consistently display greater LC_{50} values and increased survival following chronic herbicide exposure. Acute and chronic results do not consistently demonstrate this relationship. No significant differences were present among LC_{50} values for grassland and cropland larvae, although the above trend (i.e., heavier grassland larvae exhibited greater LC_{50} values) was evident for four out of six comparisons. Thirty-day survival was greater only among grassland New Mexico spadefoots exposed to Roundup WeatherMAX® at 2.0 mg glyphosate ae/L. The relationship between larval weight and glyphosate herbicide sensitivity is unclear (Mann and Bidwell 1999, Relyea and Jones 2009). Some evidence indicates that herbicide sensitivity and larval weight are inversely related (Mann and Bidwell 1999). Mann and Bidwell (1999) noted that *H. eyrei*, with mean weight approximately three times greater than the other species tested, was also the least sensitive to Roundup®. However, the sensitivity of *H. eyrei* to Touchdown® herbicide (a glyphosate-based formulation containing POEA and undisclosed surfactants) was similar to that of the species with the smallest mass (*L. dorsalis*). No clear relationship between larval weight and herbicide sensitivity was present among species displaying smaller weight differences (i.e., similar in magnitude to those I observed) (Mann and Bidwell 1999). Another study also found no consistent relationship between larval weight and herbicide (Roundup Original Max®) sensitivity among nine species of North American amphibians (Relyea and Jones 2009).

Another possibility is that water temperature differences between cropland and grassland compartments may have influenced acute toxicity estimates, thereby masking variation in herbicide sensitivity among grassland and cropland larvae. The relationship between water temperature and the toxicity of formulated glyphosate herbicides to aquatic organisms is unclear (Folmar et al. 1979, Tsui and Chu 2003). Tsui and Chu (2003) found no consistent relationship between the toxicity of formulated Roundup® to *Ceriodaphnia dubia* and water temperature. Folmar et al. (1979) compared the toxicity (24- and 96-hour LC_{50}) of formulated Roundup® to *Salmo gairdneri* and *Lepomis macrochirus* at three temperatures. For both species, a 10 °C increase in water temperature doubled the toxicity of Roundup® (i.e., reduced LC_{50} values by half). However, for three out of eight comparisons, a smaller increase in water temperature (+5 °C) resulted in a negligible increase or had no effect on Roundup® toxicity. During the present study, water temperatures were approximately 3 °C higher in grassland test compartments during the 48-hour acute exposure period. If Roundup WeatherMAX® or Ignite® 280 SL toxicity was affected by this temperature difference, grassland larvae would likely display lower LC_{50} values than cropland larvae. Results indicate that grassland and cropland LC_{50} values were not significantly different for either herbicide. Also, the above trend (i.e., grassland LC_{50} less than cropland LC_{50}) was not consistent for either herbicide, and was present for only two out of six comparisons. Therefore, it seems unlikely that acute toxicity estimates were affected by the above temperature difference.

Variation in sensitivity between species

No between species differences in Roundup WeatherMAX® sensitivity were present. However, acute toxicity estimates indicated that New Mexico spadefoots were less sensitive to Ignite® 280 SL than Plains spadefoots, irrespective of playa type. This is not surprising because pesticide sensitivity is known to vary among amphibian species (Mann and Bidwell 1999, Howe

et al. 2004, Relyea 2004, Jones et al. 2009) and by contaminant (Relyea 2004). Differences of this type have even been observed among species of the same genera (Jones et al. 2009). While several studies have noted difference in pesticide sensitivity among amphibian species with similar contaminant exposure histories (Howe et al. 2004, Relyea 2004, Jones et al. 2009), few explanations have been offered. New Mexico and Plains spadefoots may vary in their response to Ignite® 280 SL due to inherent physiological differences among species (Blanck 1984, Colavecchia et al. 2007, McKernan et al. 2009) that affect contaminant absorption, target site sensitivity, or metabolism (i.e., detoxification) (Croft 1990). While this has not been demonstrated among amphibians, McKernan et al. (2009) found that several avian species vary in their sensitivity to polybrominated diphenyl ether, at least partially due to among species variation in contaminant metabolizing enzymes.

Research needs

Studies estimating aqueous herbicide concentrations in SHP playa wetlands are needed. Also, my study only addressed impacts on survival. Previous work has demonstrated that pesticides cause sublethal impacts among larval amphibians by negatively impacting behavior (Bridges 1997) and growth (Howe et al. 2004, Relyea 2004). Sublethal effects like these can ultimately affect survival and reproduction (Relyea 2009). Future studies should investigate whether environmentally relevant levels of Ignite® 280 SL or Roundup WeatherMAX® exert sublethal impacts on New Mexico and Plains spadefoot larvae. Because various biotic (e.g., predators - Relyea 2003, Relyea et al. 2005) and abiotic (e.g., pH - Chen et al. 2004, Edginton et al. 2004; suspended sediment - Tsui and Chu 2003) factors are known to affect contaminant toxicity, further research is needed to determine whether environmental factors present in playa wetlands influence Ignite® 280 SL and Roundup WeatherMAX® toxicity. In addition, toxicity of these herbicides was only assessed among two playa amphibians. Because pesticide

sensitivity varies widely among amphibian species (Mann and Bidwell 1999, Howe et al. 2004, Jones et al. 2009) and by contaminant (Relyea 2004), the toxicity of other common pesticides toward other SHP amphibian species should be investigated.

Table 2.1. List of ingredients (by percent composition) in formulated herbicides used during larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) acute and chronic toxicity testing.

Herbicide formulation	Ingredients	Percent
Roundup WeatherMAX® [a]	Glyphosate (potassium salt form)	48.8
	Other Ingredients	51.2
Ignite® 280 SL [b]	Glufosinate-ammonium	24.5
	Other Ingredients	75.7

[a] Roundup WeatherMAX® contains 660 g glyphosate/L (equivalent to 540 g glyphosate acid equivalents/L).

[b] Ignite® 280 SL contains 280 g glufosinate-ammonium/L.

Table 2.2. Levene's test for homogeneity of variances for water quality data from cropland and grassland test compartments. Variables marked with an asterisk were non-normally distributed (Shapiro-Wilk test). Data were obtained from acute and chronic toxicity tests with *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoots, respectively) from playa wetlands embedded in cropland or grassland. Means for acute water quality were calculated during the first 48 hours and the subsequent 168 hour post-exposure monitoring period. Test chemicals were Roundup WeatherMAX® and Ignite® 280 SL.

Acute toxicity			
48-hr	*df*	F	P
Temperature *	1,126	64.97	<0.001
pH *	1,126	0.79	0.38
Dissolved oxygen *	1,126	22.23	<0.001
Ammonia *	1,126	1.21	0.27
168-hr			
Temperature *	1,189	8.92	0.003
pH *	1,189	1.14	0.29
Dissolved oxygen *	1,187	0.49	0.49
Ammonia *	1,189	6.89	0.009
Chronic toxicity			
Roundup WeatherMAX®	*df*	F	P
Temperature *	1,358	2.21	0.14
pH *	1,358	5.02	0.025
Dissolved oxygen *	1,358	1.55	0.21
Ammonia *	1,358	23.69	<0.001
Ignite® 280 SL			
Temperature *	1,358	0.14	0.71
pH *	1,358	5.81	0.017
Dissolved oxygen *	1,358	0.17	0.68
Ammonia *	1,358	11.29	0.001

Table 2.3. Acute toxicity of Roundup WeatherMAX® and Ignite® 280 SL to larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) from playa wetlands embedded in cropland or grassland. Both 48- and 216-hr (i.e., including post-exposure mortality) LC_1 values were calculated via probit analysis. Only 48-hour values are given for Ignite® 280 SL because they are identical to 216-hr values.

Roundup WeatherMAX® LC_1 (mg glyphosate acid equivalents/L)

S. bombifrons			
	n	48-hr	216-hr
Grass	208	1.09	1.00
Crop	175	0.97	0.72

S. multiplicata			
	n	48-hr	216-hr
Grass	80	1.32	0.82
Crop	113	1.01	1.01

Ignite® 280 SL 48-hour LC_1 (mg glufosinate/L)

	S. bombifrons		*S. multiplicata*	
	n		n	
Grass	181	1.41	107	2.78
Crop	142	1.75	146	4.20

Table 2.4. Water quality during Roundup WeatherMAX® and Ignite® 280 SL acute toxicity tests with larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) from playa wetlands embedded in cropland or grassland. Means were calculated for the first 48 hours and for the subsequent 168 hour post-exposure monitoring period. Water quality variables in grassland and cropland compartments were compared with Wilcoxon two-sample tests. Variables marked with an asterisk were non-normally distributed (Table 2.2). Those variables marked with a double asterisks also displayed heterogeneous variances.

	Mean ± 1 S.E.			
48 -hr	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Ammonia (mg/L)
Crop	19.00±0.04	8.67±0.01	8.35±0.03	0.26±0.05
Grass	22.28±0.09	8.75±0.01	7.96±0.09	0.19±0.04
168-hr				
Crop	22.09± 0.11	8.43±0.04	7.36±0.06	0.16±0.02
Grass	22.17±0.09	8.55±0.01	7.35±0.07	0.26±0.02

Wilcoxon two-sample tests

48-hr	z	P
Temperature **	-9.77	<0.001
pH*	-6.21	<0.001
Dissolved oxygen **	3.56	0.001
Ammonia*	1.34	0.18
216-hr		
Temperature**	1.05	0.29
pH*	5.35	<0.001
Dissolved oxygen*	-0.40	0.69
Ammonia **	3.26	0.001

Table 2.5. Acute toxicity of Roundup WeatherMAX® to larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) from playa wetlands embedded in cropland or grassland. Both 48- and 216-hr (i.e., including post-exposure mortality) LC_{50} values and associated 84% confidence intervals were calculated via probit analysis.

S. bombifrons		LC_{50} (84% confidence intervals), mg glyphosate acid equivalents/L	
	n	48-hr [a]	216-hr
Grass	208	2.03 (1.90-2.16) [A]	1.99 (1.85-2.13) [A]
Crop	175	1.85 (1.62-2.06) [A]	1.65 (1.42-1.87) [A]
		Effect Size [b]	
		48-hr	216-hr
Grass vs. Crop		0.10 (small)	0.19 (small)
S. multiplicata			
	n	48-hr	216-hr
Grass	80	2.30 (2.06-2.55) [A]	1.93 (1.68-2.20) [A]
Crop	113	2.11 (1.85-2.41) [A]	2.11 (1.85-2.41) [A]
		Effect Size	
		48-hr	216-hr
Grass vs. Crop		0.10 (small)	0.10 (small)

[a] For within-species (i.e., between landuse) comparisons, values that share a common letter are not significantly different due to overlapping 84% confidence intervals. Between-species comparisons were made by examining 84% confidence intervals for overlap.
[b] Effect size analysis was completed following Cohen (1992).

Table 2.6. Acute toxicity of Ignite® 280 SL to larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) from playa wetlands embedded in cropland or grassland. LC_{50} values and associated 84% confidence intervals were calculated via probit analysis.

	48-hour LC_{50} (84% confidence intervals), mg glufosinate/L [a]			
	S. bombifrons		*S. multiplicata*	
	n		n	
Grass	181	3.55 (3.20-3.87) [A]	107	5.55 (5.01-6.12) [A]
Crop	142	3.70 (3.26-4.14) [A]	146	4.85 (4.38-5.32) [A]

Effect Size [b]	*S. bombifrons*	*S. multiplicata*
Grass vs. Crop	0.04 (small)	0.17 (small)

[a] Only 48-hr LC_{50} values are given since they do not differ from 216-hr values (i.e., including post-exposure mortality). For within-species (i.e., between landuse) comparisons, values that share a common letter are not significantly different due to overlapping 84% confidence intervals. Between-species comparisons were made by examining 84% confidence intervals for overlap.

[b] Effect size analysis was completed following Cohen (1988).

Table 2.7. Water quality during chronic exposure (30 day) of larval *Spea multiplicata* and *S. bombifrons* (New Mexico and Plains spadefoot, respectively) to Roundup WeatherMAX® or Ignite® 280 SL. Larvae were obtained from playa wetlands embedded in cropland or grassland. Water quality variables in grassland and cropland compartments were compared with Wilcoxon two-sample tests. Variables marked with an asterisk were non-normally distributed (Table 2.2). Those variables marked with a double asterisks also displayed heterogeneous variances.

	Mean ± 1 S.E.			
Roundup WeatherMAX®	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Ammonia (mg/L)
Crop	22.39±0.10	8.51±0.01	7.33±0.05	0.06±0.01
Grass	22.65±0.11	8.50±0.01	7.55±0.07	0.03±0.01
Ignite® 280 SL				
Crop	22.51±0.08	8.44±0.01	7.18±0.06	0.16±0.01
Grass	22.52±0.08	8.48±0.01	7.20±0.06	0.14±0.01

Wilcoxon two-sample tests

Roundup WeatherMAX®	z	P
Temperature*	0.32	0.75
pH **	0.68	0.49
Dissolved oxygen *	-4.78	<0.001
Ammonia **	1.81	0.07
Ignite® 280 SL		
Temperature *	-1.05	0.29
pH **	1.82	0.07
Dissolved oxygen *	1.53	0.13
Ammonia **	-0.99	0.33

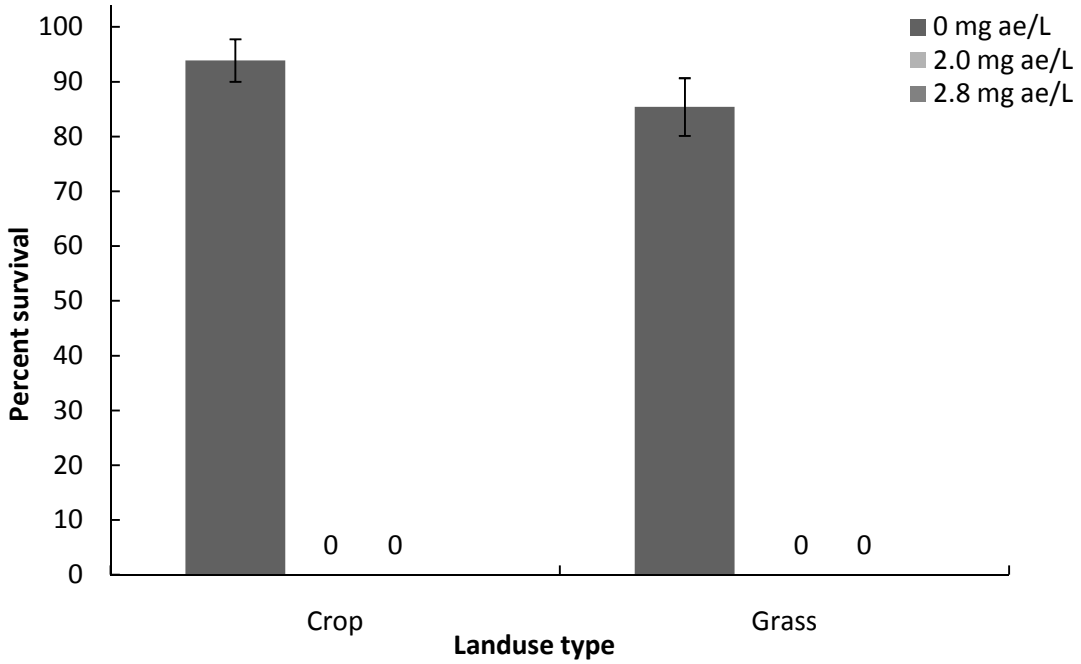
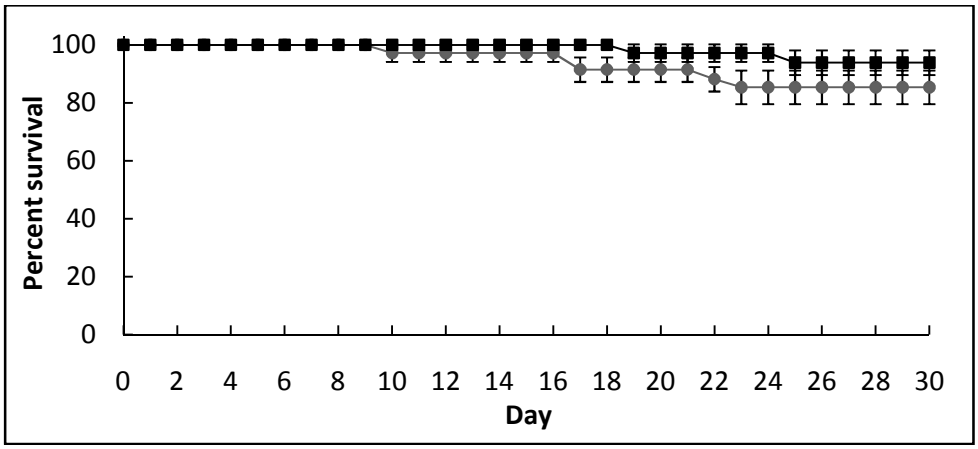


Figure 2.1. The survival (mean ± 1 S.E.) of *Spea bombifrons* (Plains spadefoot) larvae (n = 182) from cropland (n = 87) and grassland playas (n = 95) following chronic (30-day) exposure to Roundup WeatherMAX® [2.0 or 2.8 mg glyphosate acid equivalents (ae)/L] or aged tapwater in a static-renewal system.



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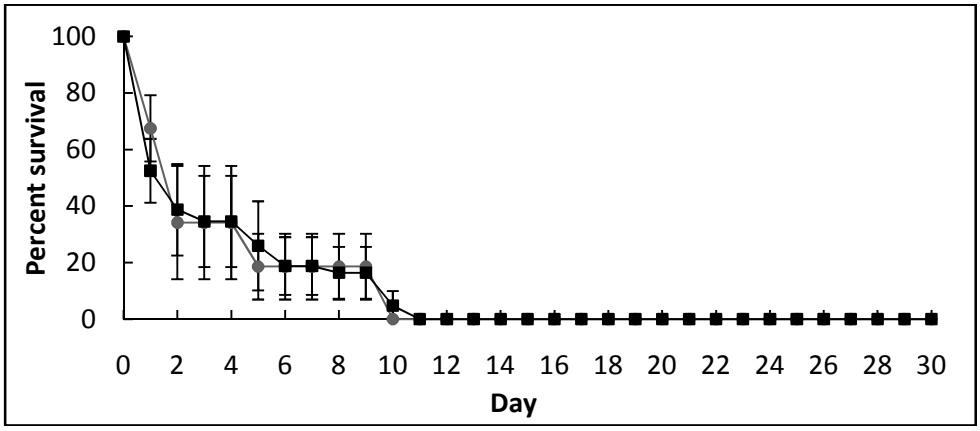
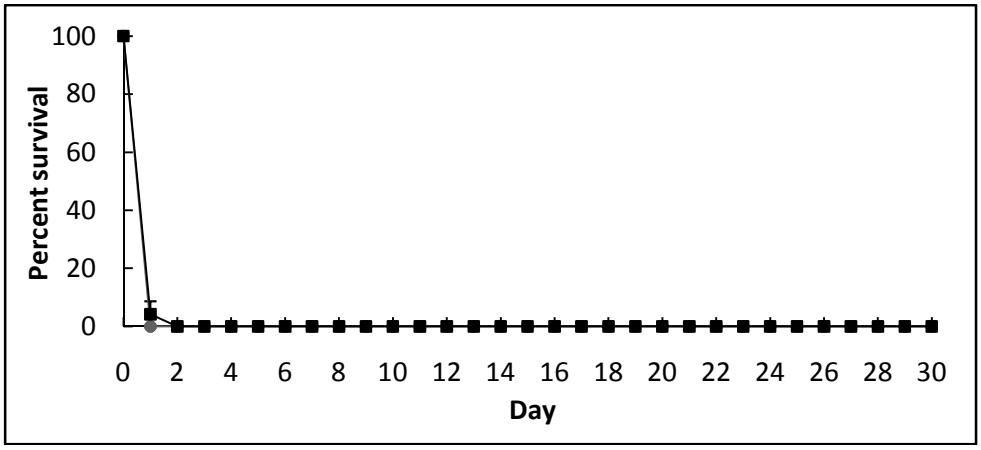


2.0 mg ae/L



2.8 mg ae/L

Figure 2.2. Survival (mean ± 1 S.E.) of *Spea bombifrons* (Plains spadefoot) larvae (n = 182) from cropland (squares; n = 87) and grassland playas (circles; n = 95) during chronic (30-day) exposure to aged tapwater, or Roundup WeatherMAX® at 2.0 or 2.8 mg glyphosate acid equivalents (ae)/L in a static-renewal system.

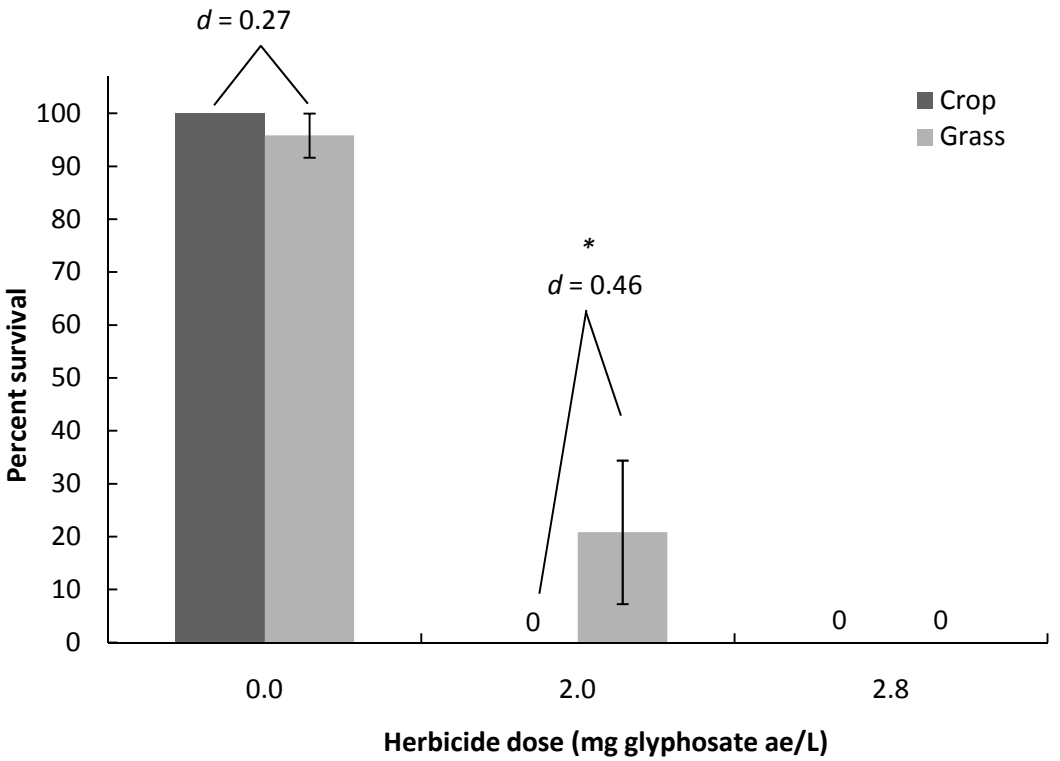
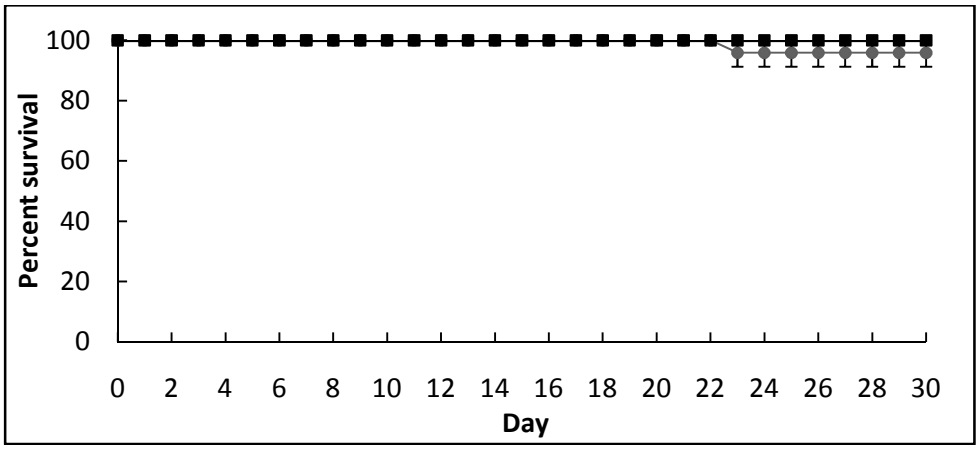


Figure 2.3. Survival (mean ± 1 S.E.) of *Spea multiplicata* (New Mexico spadefoot) larvae (n = 142) from cropland (n = 75) and grassland playas (n = 67) following chronic (30-day) exposure to Roundup WeatherMAX® [2.0 or 2.8 mg glyphosate acid equivalents(ae)/L] or aged tapwater in a static-renewal system. Between landuse type treatment means were compared with contrasts in GENMOD. Asterisks indicate means that are different at P<0.05. Effect size analysis values are indicated by *"d"*.



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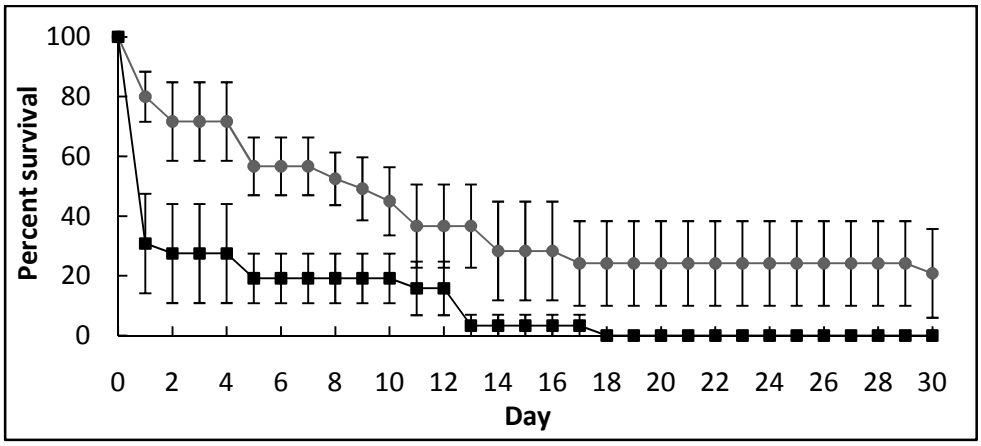
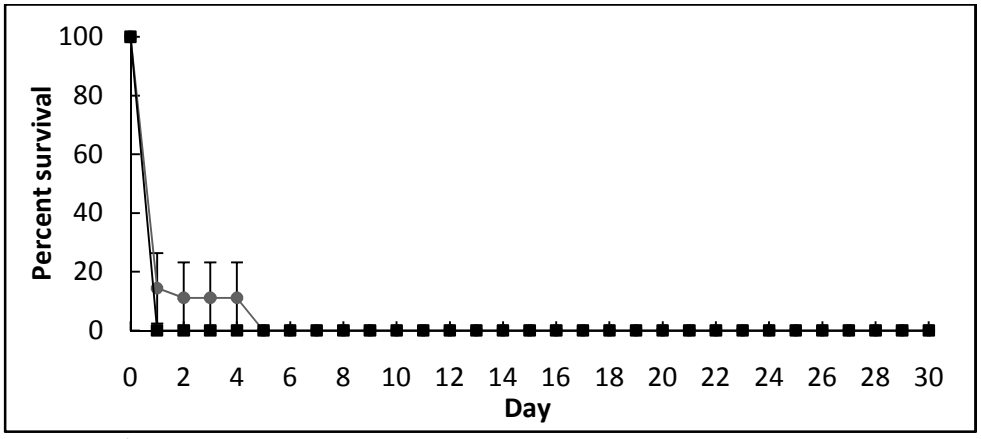


2.0 mg ae/L



2.8 mg ae/L

Figure 2.4. Survival (mean ± 1 S.E.) of *Spea multiplicata* (New Mexico spadefoot) larvae (n = 142) from cropland (squares; n = 75) and grassland playas (circles; n = 67) during chronic (30-day) exposure to aged tapwater, or Roundup WeatherMAX® at 2.0 or 2.8 mg glyphosate acid equivalents (ae)/L in a static-renewal system.

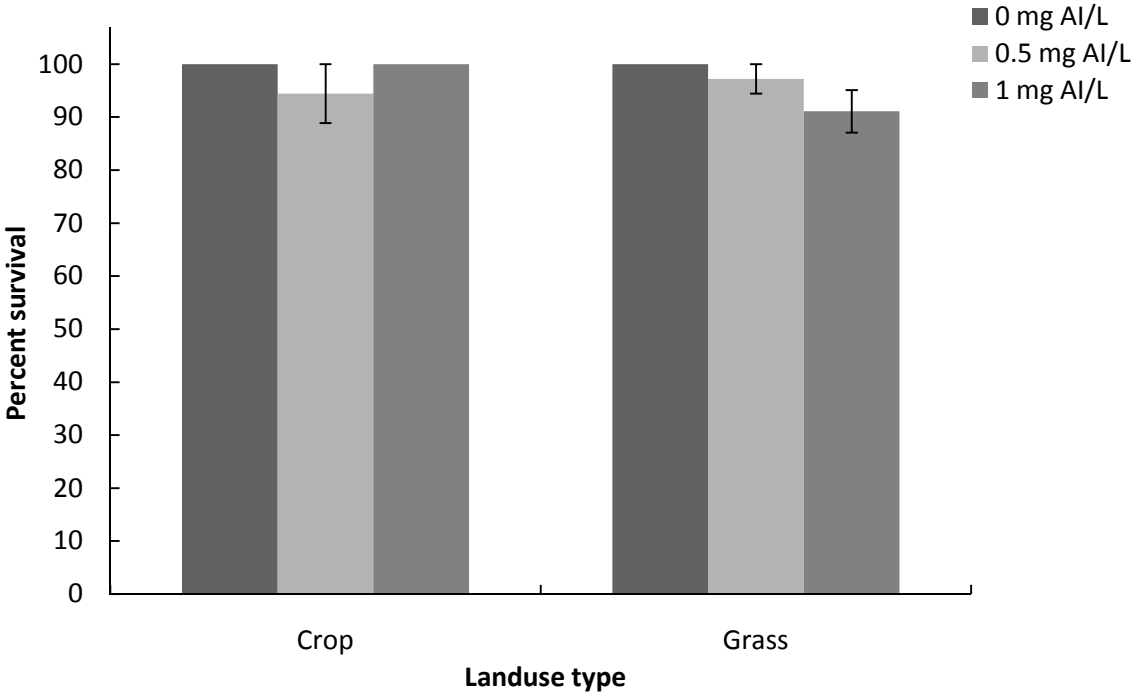


Figure 2.5. Survival (mean ± 1 S.E.) of *Spea bombifrons* (Plains spadefoot) larvae (n = 168) from cropland (n = 76) and grassland playas (n = 92) following chronic (30-day) exposure to Ignite® 280 SL [0.5 or 1.0 mg glufosinate (AI)/L] or aged tap water in a static-renewal system.

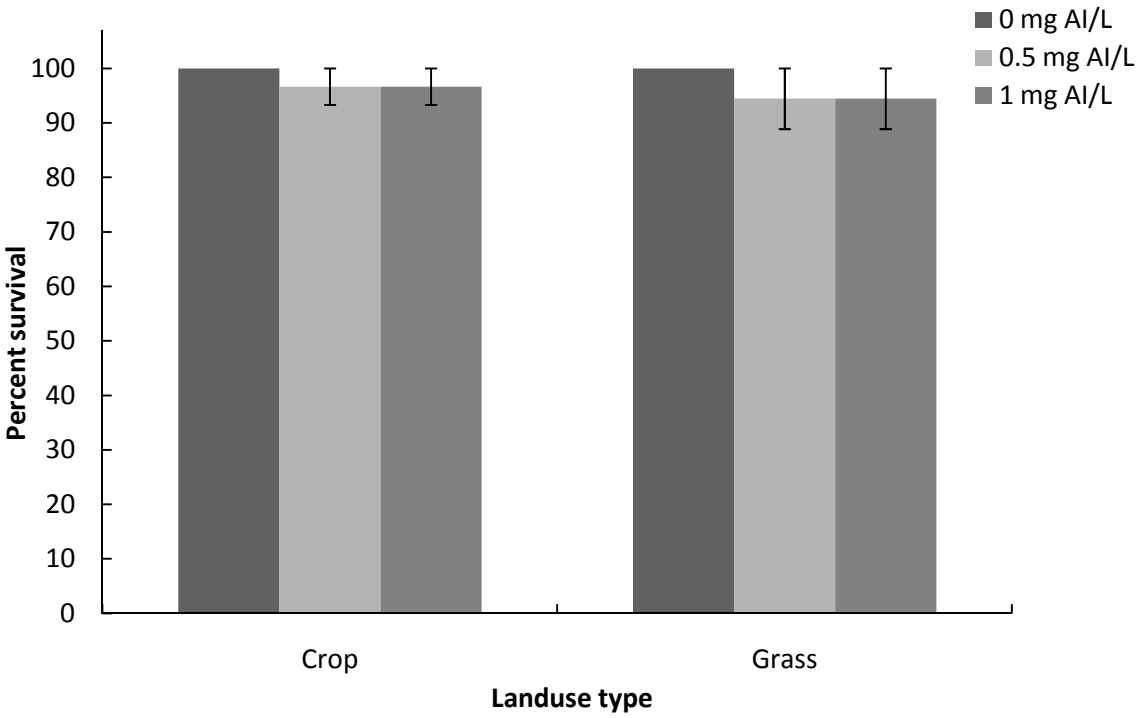


Figure 2.6. Survival (mean ± 1 S.E.) of *Spea multiplicata* (New Mexico spadefoot) larvae (n = 156) from cropland (n = 86) and grassland playas (n = 70) following chronic (30-day) exposure to Ignite® 280 SL [0.5 or 1.0 mg glufosinate (AI)/L] or aged tap water in a static-renewal system.

CHAPTER III

HISTOLOGICAL IMPACTS OF SURFACTANT EXPOSURE ON *SPEA* SPP. LARVAE FROM THE SOUTHERN HIGH PLAINS

Introduction

Amphibian population declines and chemical contaminants

There has been increasing concern that amphibian populations are declining world-wide (Wyman 1990, Stuart et al. 2004). Destruction of wetland and associated terrestrial habitat is undoubtedly a major driver of these declines (Wyman 1990, Stuart et al. 2004). Wetland loss within the contiguous U.S. has been extensive, with approximately 53% of wetland acreage destroyed since the 1800s (Dahl 1990). Environmental pollution is another factor that may threaten amphibian populations (Sparling et al. 2001, Howe et al. 2004). Many species reproduce and complete larval development in agricultural wetlands (Howe et al. 2004). Chemicals commonly applied in the surrounding landscape (e.g., insecticides - Boone and Semlitsch 2001) may contaminate adjacent wetlands due to accidental overspray (Faber et al. 1998a), run-off (Faber et al. 1998a, Johansson et al. 2006), or spray drift (Johansson et al. 2006). As a result, developing amphibian eggs and larvae may be exposed to pesticides (Howe et al. 2004).

While numerous studies have demonstrated that pesticides can negatively affect amphibians, limited research has investigated the mechanisms underlying toxicity (Honrubia et al. 1993, Lajmanovich et al. 1998, Lajmanovich et al. 2003, Bernabo et al. 2008). These studies indicated that exposure to a variety of formulated pesticides [Paraquat (herbicide), Endosulfan (organochlorine insecticide), ZZ-Aphox® (carbamate insecticide)] disrupts gill structure (Lajmanovich et al. 1998, Bernabo et al. 2008) and function (Bernabo et al. 2008) among larval amphibians. Gill damage was characterized by increased mucus production, enlarged intercellular spaces (Endosulfan - Bernabo et al. 2008), discontinuous epithelium (ZZ-Aphox® - Honrubia et al. 1993; Paraquat - Lajmanovich et al. 1998), and separation of epithelial cell layers (Endosulfan - Bernabo et al. 2008).

Impacts of glyphosate herbicides on amphibians

Glyphosate herbicides are used world-wide to control weeds in agricultural, forestry, and domestic settings (Howe et al. 2004). Agricultural formulations are commonly applied to herbicide-resistant crops such as soybeans, canola, and cotton (Duke 2005). The major components of most glyphosate herbicides are the active ingredient (glyphosate) and a polyethoxylated tallowamine (POEA) surfactant (Giesy et al. 2000). POEA, a non-ionic alkylamine ethoxylate (ANEO) surfactant (Giesy et al. 2000, Brausch and Smith 2007), is added to improve absorption of the active ingredient across leaf cuticles and typically represents no more than 15% of glyphosate formulations (Giesy et al. 2000). Both glyphosate and the POEA surfactant degrade rapidly and display limited environmental persistence (Banduhn and Frazier 1974 in Giesy et al. 2000, Marvel et al. 1974 in Giesy et al. 2000, Oppenhuizen 1993 in Giesy et al. 2000). Previous work indicated that the toxicity of glyphosate herbicides toward amphibians is primarily due to the POEA surfactant (Mann and Bidwell 1999, Perkins et al. 2000, Edginton et al. 2004, Howe et al. 2004). These studies demonstrated that either glyphosate alone was less

toxic than the formulated product (containing POEA) (Mann and Bidwell 1999), or that the POEA surfactant exhibited greater toxicity compared to the formulated product (Perkins et al. 2000, Howe et al. 2004).

Impacts of surfactants on aquatic organisms

Histological studies examining surfactant exposure in aquatic organisms have commonly focused on gill alterations among fish (Brown et al. 1968, Abel and Skidmore 1975). Non-ionic surfactants, like POEA, are thought to be toxic to aquatic organisms primarily because they disrupt the respiratory function of gills (Swedmark et al. 1971, Abel 1974, Lindgren et al. 1996). Surfactant molecules become incorporated into cell membranes, resulting in the inactivation or removal of surface membrane proteins, as well as altered membrane permeability (Lindgren et al. 1996). Fish exposed to surfactants or formulated herbicides (containing surfactants) often display histological alterations (Abel 1974) including hypertrophy (Brown et al. 1968, Swedmark et al. 1971, Partearroyo et al. 1991, Ramirez-Duarte et al. 2008), hyperplasia (Brown et al. 1968, Partearroyo et al. 1991, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008) and necrosis (Brown et al. 1968, Partearroyo et al. 1991, Ramirez-Duarte et al. 2008) of gill epithelial cells, as well as detachment of epithelium from underlying tissue (Brown et al. 1968, Abel and Skidmore 1975, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008). It is important to note that these types of lesions are generally non-specific and most can result from exposure to a variety of contaminants (Mallatt 1985, Meyers and Hendricks 1985).

Gill lesions seem to follow a predictable sequence with increasing exposure time (Brown et al. 1968) or contaminant concentration (Ramirez-Duarte et al. 2008). Hypertrophy and hyperplasia develop initially (Brown et al. 1968), and have been observed within as little as 1.5 (Abel and Skidmore 1975) and 12 (Jauch 1979) hours. These structural alterations are thought to be protective because they increase the diffusion distance over which irritants must pass to

reach the bloodstream (Mallatt 1985). Hyperplasia also increases oxygen diffusion distance and may negatively impact respiration (Mann and Bidwell 2001). Should the irritant persist, epithelial cells may become detached from underlying tissue, and gill epithelium progressively disintegrates via cellular rupture and necrosis (Brown et al. 1968).

The mechanism underlying surfactant toxicity in amphibians is unclear (Mann and Bidwell 2001). No previous studies have investigated the histological changes associated with non-ionic surfactant (e.g., POEA) toxicity in amphibians. Toxicity in amphibians may result from surfactant-induced gill damage (Edginton et al. 2004). Edginton et al. (2004) determined that mortality among developing embryos and larvae of four amphibian species exposed to Vision® (a glyphosate herbicide containing POEA) was greatest when gills were present.

Southern High Plains amphibians and herbicides

Amphibians are a vital component of Southern High Plains (SHP) playa wetlands (Smith 2003) due to their great abundance during summertime (Anderson et al. 1999, Gray and Smith 2005) and because they occupy multiple trophic levels (Smith 2003). The most common species include *Spea bombifons* (Plains spadefoot), *S. multiplicata* (New Mexico spadefoot), *Pseudacris clarkii* (spotted chorus frog), and *Bufo cognatus* (Great Plains toad) (Anderson et al. 1999). Because SHP amphibians require playas for continued regional persistence (Haukos and Smith 1994), activities which destroy or degrade these wetlands are a serious threat to these species. Because playas are located at the base of SHP watersheds (Luo et al. 1997) and most are surrounded by agriculture (Haukos and Smith 1994), these wetlands receive agrochemical inputs via contaminated runoff (Haukos and Smith 1994). Thurman et al. (2000) found that 97% of 32 playas in West Texas contained cotton or corn herbicides. No analyses for glyphosate-based herbicides were completed.

In Texas, one of the most extensively applied herbicides is glyphosate (National Pesticide Use Database 2004). Agricultural glyphosate-based formulations are commonly used for weed control in cotton (National Pesticide Use Database 2004). These products can be applied to herbicide-resistant cotton (Blair-Kerth et al. 2001) prior to the four leaf stage (Jones and Snipes 1999) and, therefore, glyphosate herbicides are often applied in mid- to late-June (Blair-Kerth et al. 2001). SHP amphibians are likely exposed to agrochemicals (Venne et al. 2008) because the spring to summer breeding and larval development period of these species (Strebbins 1954, Degenhardt et al. 1996) coincides with application of common herbicides like glyphosate.

Objectives

I investigated how acute exposure to ADSEE 907® [a non-ionic ANEO surfactant (Krogh et al. 2003); formerly Berol 907®, hereafter ADSEE], at concentrations associated with mortality (Chapter II), impacted skin and gills of *Spea* spp. larvae. These tissues were examined because they are continuously exposed to environmental contaminants (Bernabo et al. 2008), and play a vital role in gas and ion exchange in amphibians (Boutilier et al. 1992). Because previous work indicated that acute surfactant exposure damaged the gills and skin of other aquatic organisms (e.g., fish) (Abel 1974, Partearroyo et al. 1991), I hypothesized that larvae exposed to ADSEE surfactant would exhibit more extensive skin and gill lesions compared to control animals.

Methods

Common irritant induced skin and gill lesions

Much of the work examining the histological impacts of irritants/contaminants on aquatic organisms has been completed with fish (Mallatt 1985). Common irritant induced gill lesions (i.e., structural abnormalities within organs, cells, or tissues - Meyers and Hendricks

1985) include epithelial hypertrophy, epithelial hyperplasia, epithelial cell lifting, and epithelial necrosis, loss of epithelial continuity, general necrosis (i.e., necrosis of epithelium and underlying tissue), mucous cell proliferation, excess mucous secretion, and altered chloride cells (Mallatt 1985). Fish also display a variety of irritant induced skin lesions including hypertrophy, hyperplasia, altered mucous secretion, increased vacuole prevalence, and necrosis (Meyers and Hendricks 1985). Exposure to formulated glyphosate herbicides resulted in numerous skin (epithelial hypertrophy, hyperplasia, and necrosis; altered mucous production - Ramirez-Duarte et al. 2008) and gill lesions (hypertrophy, hyperplasia, necrosis, and edema of lamellar epithelium; lamellar epithelial lifting - Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008) among fish.

Structure of amphibian gills

Larval amphibian gills are found in two branchial baskets adjacent to the heart (McIndoe and Smith 1984, Brunelli et al. 2004). The gills consist of supportive branchial arches from which gill tufts emanate ventrally (McIndoe and Smith 1984, Brunelli et al. 2004) and gill filters radiate dorsally (Brunelli et al. 2004). Gill tufts are composed of numerous, highly vascularized ramifications which normally display a distinct single or bi-layered epithelium (Lajmanovich et al. 1998, Bueno-Guimaraes et al. 2001, Brunelli et al. 2004, Bernabo et al. 2008). Gill filters consist of supportive tissue covered by an epithelium one to two cell layers thick (Brunelli et al. 2004). Several additional epithelial cell layers are present near the apex of gill filters (Brunelli et al. 2004).

Structure and development of amphibian skin

The development of amphibian skin has been studied extensively in *Rana catesbeiana* (Robinson and Heintzelman 1987, Tamakoshi et al. 1998, Utoh et al. 2000, Suzuki et al. 2001, Yoshizato 2007). Young larvae (Gosner stage 26-27) display a bi-layered epithelium consisting of

apical and skein cells (Tamakoshi et al. 1998, Utoh et al. 2000). A second skein layer begins to develop at approximately Gosner-stage 28 (Utoh et al. 2000, Suzuki et al. 2001). At this point in development, the skein layer consists of suprabasal skein cells interspersed with basal skein cells (Suzuki et al. 2001), and appears one cell layer thick (Utoh et al. 2000). Beginning at approximately Gosner-stage 29, basal skein cells are replaced by basal cells, indicating the transition to "pre-adult" skin (Utoh et al. 2000). The epidermis of Gosner stage 31-41 larvae is composed of a single apical layer and several (3-4) skein layers that overlie a developing basal cell layer (Robinson and Heintzelman 1987, Suzuki et al. 2001). Beginning at approximately Gosner-stage 41-42 (i.e., metamorphic climax), remaining skein and apical cells are eliminated by apoptosis and replaced by adult cells (Robinson and Heintzelman 1987, Yoshizato 1992). By Gosner stage 44-45, epidermal structure is completely transformed and resembles that of adults (Robinson and Heintzelman 1987). It is important to note that the onset of the above transformations varies by species (Yoshizato 2007, Fenoglio et al. 2009).

Larval collection and housing

Spea spp. (New Mexico and Plains spadefoot) larvae were collected from a cropland playa in Crosby County, Texas on 31 July 2007. Larvae were transported in buckets containing playa water to The Institute of Environmental and Human Heath at Texas Tech University in Lubbock, TX. They were held in glass aquaria (37.9 L) filled with aerated, aged tapwater for 48 hours to acclimate to laboratory conditions. Acclimation and subsequent experiments occurred in a climate controlled room on a 14h-10h light-dark cycle. This photoperiod was selected because it approximates that in Texas during summer (National Oceanic and Atmospheric Administration 2009). Water quality was monitored daily during acclimation. Temperature and pH were measured with a Hanna HI 9124 pH meter, dissolved oxygen was measured with a Oakton DO 6 dissolved oxygen meter, and ammonia was monitored with Aquarium

Pharmaceuticals test kits. Water quality was similar among holding aquaria (mean ± 1 S.E.): temperature = 22.16 ± 0.16 °C, pH = 8.41 ± 0.04 , dissolved oxygen (DO) = 8.16 ± 0.11 mg/L, ammonia = 0.83 ± 0.09 mg/L. Pulverized rabbit food was provided ad libitum (Relyea et al. 2005) during acclimation. Larvae were observed several times daily during acclimation and exhibited no obvious signs of stress (i.e., <5% of larvae died during acclimation). All collection and experimental procedures followed an approved Texas Tech University Institutional Animal Care and Use Committee protocol (no. 06018-06).

Surfactant exposure

Test compartments were created by dividing 37.9 L (10 gal.) glass aquaria in half with a glass divider secured with silicone Aquarium Sealant (Perfecto Manufacturing, Inc., Noblesville, IN). Compartments (18.95 L) were filled with 8.5 L of aerated tapwater on 1 August 2007 (30 hours prior to the start of tests). This water volume was selected to maintain larvae at a density similar to that recommended by American Society for Testing and Materials (ASTM) Guidelines for Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians (ASTM 2003).

A stock solution was created by diluting pure ADSEE surfactant (obtained from Akzo Nobel, Houston, TX) with two parts aged tapwater to allow for accurate pipeting. ADSEE is a mixture of tallowalkylamine ethoxylate and ethylene glycol (Krogh et al. 2003) that is added to herbicides to improve product effectiveness (Akzo Nobel 2008). This surfactant was used because it is a non-ionic ANEO surfactant (like POEA) and it was found in soils treated with a glyphosate herbicide (Krogh et al. 2003).

Test solutions (containing 1.44 mg ADSEE/L) were prepared just prior to tests by gently mixing 34.8 µL of the above stock solution into designated compartments with a glass rod. This surfactant dose was used to ensure sufficient mortality to address the experimental objective, since a 0.72 mg ADSEE/L dose (approximately equivalent to the 48-hour Roundup

WeatherMAX® LC_{50} for *Spea* spp. larvae from cropland playas) yielded no mortality during a pilot study. Roundup® formulations typically contain ≤15% surfactant (Giesy et al. 2000), with the actual value withheld as a trade secret. The above calculations assume that Roundup WeatherMAX® contains 15% POEA (Giesy et al. 2000, Howe et al. 2004).

The surfactant exposure experiment began 2 August 2007. Gosner-stage 34-36 larvae (Gosner 1960) were utilized due to their availability in the field, and for ease of subsequent dissection. Five randomly selected larvae were added to each of nine surfactant test compartments. An equal number of larvae were allocated to nine control compartments filled with aged tapwater only. Food was withheld throughout this experiment because the toxicity of chemicals can be influenced by uneaten food or fecal matter (ASTM 2003). Water quality was assessed in all experimental compartments initially and at the conclusion of the 48-hour exposure period. Test compartments were checked at least every 3 hours and any larvae exhibiting non-responsiveness to prodding or loss of coordinated movement (e.g., loss of righting reflex) (ASTM 2003) were considered moribund and euthanized in 1% MS-222.

Immediately following euthanasia, all larvae were rinsed in deionized water and weighed. Mean larval mass (± 1 S.E.) was 1.01 ± 0.03 g. Larvae were partially submerged (i.e., only the ventral body surface remained exposed) in 10% formalin to preserve tissue quality during dissection (Mary Hastert, Texas Tech University Imaging Center, *personal communication*). This process (i.e., initiating tissue fixation as rapidly as possible) should maintain tissue integrity (Meyers and Hendricks 1985). Two strips of ventral body epidermis, approximately 3 mm wide x 3 mm long x 1 mm thick, and both gills were removed from each larvae. Tissue samples intended for light microscopy (i.e., one skin sample and gill from each larvae) were placed in tissue cassettes and preserved in a buffered 10% formalin solution (Bueno-Guimaraes et al. 2001, Fivelstad et al. 2003). The other skin sample and gill were

preserved in a buffered 2.5% glutaraldehyde, 1.7% formaldehyde solution (McIndoe and Smith 1984) for potential analysis via electron microscopy. All tissue samples were refrigerated at 4° C until the experiment concluded. Preserved tissue samples have been stored in this fashion for up to four months without affecting tissue integrity (Robinson and Heintzelman 1987). After 48 hours, all remaining larvae were euthanized and processed as described above.

Mortality among larvae exposed to ADSEE surfactant was consistently high in all test compartments (Table 3.1). Six surfactant compartments (A, C, D, E, F, G) were randomly selected and these tissue samples were prepared for histological examination. Unexpectedly, mortality was also high in several control compartments (C, D, E, I; Table 3.1). Compartments D and E were excluded from further analysis since all larvae died within 48 hours. Also, compartment C was randomly selected from those remaining which displayed high mortality (3 of 5 larvae; C and I) and excluded. Tissue samples from larvae in the remaining six control compartments (A, B, F, G, H, I) were prepared for histological examination.

One set of tissue (i.e., gill and skin) from each larva in selected compartments was transported to the Texas Tech University Department of Anatomic Pathology and prepared for examination via light microscopy. These samples were embedded in paraffin, sectioned, mounted on slides and stained with hematoxylin and eosin (H&E) following standard techniques (Bueno-Guimaraes et al. 2001). The remaining tissue from each larva was transported to the Texas Tech University Imaging Center and embedded in paraffin. These samples are awaiting further processing should electron microscopic analysis be deemed useful in the future. While irritant induced tissue alterations can usually be observed with light microscopy (Brown et al. 1968, Abel and Skidmore 1975, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008), if none are apparent, electron microscopy can provide additional detail to detect pathological changes (Skidmore and Tovell 1972, Abel and Skidmore 1975, Meyers and Hendricks 1985).

Histological analysis

Previous histological studies have adopted a variety of approaches to evaluate pathological lesions (Austin et al. 1984, Honrubia et al. 1993, Haaparanta et al. 1997, Lajmanovich et al. 1998, Bueno-Guimaraes et al. 2001, Mondon et al. 2001, Bernabo et al. 2008, Jiang et al. 2009). Some studies were purely descriptive (e.g., Honrubia et al. 1993, Lajmanovich et al. 1998, Bueno-Guimaraes et al. 2001, Mondon et al. 2001, Bernabo et al. 2008), while others analyzed tissues based on the presence/absence of various lesions (e.g., Mondon et al. 2001), or obtained detailed quantitative information about a specific lesion type (e.g., Bueno-Guimaraes et al. 2001). I adopted a commonly utilized approach, and evaluated lesion intensity using a categorical index (e.g., Austin et al. 1984, Haaparanta et al. 1997, Jiang et al. 2009). Indices of this type score the prevalence of lesions based on percent coverage, though the number and range of categories vary by study (Austin et al. 1984, Haaparanta et al. 1997, Jiang et al. 2009). This approach can be used to assess a wide variety of lesions in a relatively concise, timely fashion (Jiang et al. 2009), and provides an intermediate level of detail.

A preliminary examination of the majority of slides indicated that a variety of skin and gill (i.e., gill tuft and gill filter) lesions were present. Gill lesions included epithelial hypertrophy (i.e., swelling of epithelial cells - Mallatt 1985, Meyers and Hendricks 1985), hyperplasia (i.e., abnormal proliferation of epithelial cells - Mallatt 1985, Meyers and Hendricks 1985) and necrosis (i.e., rupture and sloughing of epithelial cells - Mallatt 1985, Meyers and Hendricks 1985), epithelial cell lifting (i.e., separation of intact epithelium from underlying tissue - Mallatt 1985), loss of epithelial continuity (i.e., gaps in otherwise intact epithelium - Lajmanovich et al. 1998), and general necrosis (i.e., rupture of epithelium and underlying tissue - Mallatt 1985). Skin lesions included apical and skein hypertrophy (i.e., swelling), hyperplasia (i.e., abnormal proliferation), apical necrosis (i.e., cellular rupture and sloughing), and skein necrosis (i.e.,

rupture of skein tissue). Because these lesions were at least present on the majority of slides, I determined that a presence/absence-based scoring approach would yield little insight. Therefore, a categorical scoring index was designed to estimate the extent (i.e., percent coverage) of each lesion type (Haaparanta et al. 1997, Jiang et al. 2009). A preliminary examination of the tissue sections indicated that the following categories could be visually estimated (Haaparanta et al. 1997): 0 = absent, 1 = 1-19%, 2 = 20-49%, 3 = 50-74%, 4 = 75-89%, 5 = 90-100%. These categories resemble those used by Jiang et al. (2009), although the range of categories differs and an additional category was included to allow a more detailed analysis. The prevalence (percent coverage) of all lesions was evaluated in two skin and gill sections from each larva using the above categorical index (Haaparanta et al. 1997). All slides were examined blindly (Bueno-Guimaraes et al. 2001) using a Leitz Microlab light microscope. Low magnification (10x) was used whenever possible so that a large portion of each section could be viewed simultaneously (Haaparanta et al. 1997). Higher magnification (40x) was used as needed to resolve details not visible under low magnification (Haaparanta et al. 1997).

Statistical analysis

Water quality data (temperature, dissolved oxygen, ammonia, and pH) from control and surfactant test compartments were tested for homogeneity of variances using Levene's test and for normality with a Shapiro-Wilk test (SAS Version 9.2, SAS Institute, Cary, NC). Temperature data were homogeneous ($F_{1,34} = 1.83$, $P = 0.18$) and, while pH data were heterogeneous ($F_{1,34} = 4.78$, $P = 0.039$), a log transformation corrected this problem ($F_{1,34} = 4.04$, $P = 0.53$). Dissolved oxygen ($F_{1,34} = 7.45$, $P = 0.010$) and ammonia ($F_{1,34} = 69.06$, $P < 0.001$) data were heterogeneous and data transformations (i.e., log, natural log, square root) did not correct this problem. Shapiro-Wilk tests indicated that data for all variables were non-normally distributed. Standard data transformations (i.e., log, natural log, square root) did not correct this problem. Therefore,

water quality data were compared with a Wilcoxon two-sample test (PROC NPAR1WAY, SAS Version 9.2, SAS Institute, Cary, NC). This test is appropriate when the assumptions of a parametric t-test cannot be met (Sokal and Rohlf 1995).

I tested whether survival differed among control and surfactant exposed larvae using a chi-square test (PROC FREQ, SAS Version 9.2, SAS Institute, Cary, NC). For skin and gill samples, I tested whether lesion intensity differed among lesion type or treatment (control, exposed) using generalized linear models (PROC GENMOD) assuming a multinomial distribution and a cumulative logit link function (SAS Version 9.2, SAS Institute, Cary, NC). Lesion intensity was the dependent variable, and lesion type and treatment were the independent variables. If treatment or the lesion type-treatment interaction were significant, I compared within lesion type group means with an ESTIMATE statement [equivalent to a linear contrast comparing mean lesion intensity between treatments (control, exposed)] in GENMOD (Littell et al. 2002). To gain further insight into how surfactant exposure impacted skin and gill tissue, additional exploratory data analyses were completed with several reduced data sets.

Results

Survival was reduced among larvae exposed to surfactant compared to those housed in aged tapwater (control) ($\chi^2_1 = 15.32$, $P < 0.001$) (Table 3.1). Eighty-two percent of surfactant exposed larvae died during the 48-hour monitoring period compared to 42% of control larvae. Dissolved oxygen ($z = -0.73$, $P = 0.47$) and pH ($z = 1.89$, $P = 0.06$) were similar among control and surfactant test compartments during the 48-hour exposure period (Table 3.2). Temperature ($z = 2.03$, $P = 0.042$) and ammonia levels ($z = 2.35$, $P = 0.020$) differed among control and surfactant compartments. These differences were small in magnitude (Table 3.2), and within the range of

variability noted during previous studies investigating the toxicity of surfactants toward amphibians (Mann and Bidwell 2001).

Gill tissue

Gill (i.e., gill tuft and gill filter) lesion intensity differed among lesion types ($\chi^2_5 = 402.11$, $P < 0.001$) but not between treatments ($\chi^2_1 = 1.73$, $P = 0.19$), and there was no lesion type-treatment interaction ($\chi^2_5 = 2.92$, $P = 0.71$). For all lesion types, lesion intensity did not differ among control larvae and those exposed to surfactant (Figure 3.1). The most extensive lesions were epithelial hyperplasia and necrosis (Figure 3.1,3.2). Analyses of the reduced data sets (Figure 3.3 A-C) did not reveal any trends that differed substantially from the full data set.

Skin tissue

"Normal" skin epithelium (i.e., lacking dramatic lesions) displayed a distinct apical layer overlying one or several skein cell layers. No basal cell layer was evident. Lesion intensity differed among lesion type ($\chi^2_5 = 154.64$, $P < 0.001$) and between treatments ($\chi^2_1 = 3.47$, $P = 0.053$). A lesion type-treatment interaction was present ($\chi^2_5 = 80.94$, $P < 0.001$). ESTIMATE statements (equivalent to linear contrasts comparing treatment means) (Table 3.3) indicated that apical hyperplasia was more prevalent among surfactant exposed larvae, while apical necrosis and skein necrosis were more prevalent among control larvae (Figure 3.4, 3.5). Analyses of the reduced data sets (Figure 3.6 A-C) did not reveal any trends that differed substantially from the full data set.

Discussion

Based on previous work, I hypothesized that *Spea* spp. larvae exposed to surfactant would exhibit more extensive gill and skin lesions than control larvae. Unexpectedly, gill lesions were no more prevalent among larvae exposed to surfactant. Skin results were ambiguous;

while apical hyperplasia was more prevalent among larvae exposed to surfactant, several other lesions (apical and skein necrosis) were more extensive among control larvae. These results are surprising, especially in light of previous histological work investigating how non-ionic surfactants impact aquatic organisms.

While previous histological studies have investigated the mechanism of non-ionic surfactant toxicity in aquatic organisms, no work with amphibians has directly addressed this issue. Pathological lesions associated with non-ionic surfactant exposure have been noted frequently in the gills (Abel 1974, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008) and skin (Abel 1974, Ramirez-Duarte et al. 2008) of fish. Ramirez-Duarte (2008) found that acute (96 hour) exposure of *Piaractus brachypomus* to Roundup® herbicide (containing non-ionic surfactant) resulted in various skin (epithelial hypertrophy and hyperplasia, increased mucous production) and gill (hypertrophy, hyperplasia, and necrosis of lamellar epithelium; lamellar epithelial lifting) lesions. Jiraungkoorskul et al. (2003) determined that chronic exposure of *Oreochromis niloticus* to sub-lethal Roundup® doses resulted in gill lesions including hyperplasia and edema of lamellar epithelium, as well as lamellar epithelial lifting. In addition to the obvious negative effects of necrosis, the lesions noted above negatively impact gill function by increasing the distance across which gas exchange occurs (Meyers and Hendricks 1985, Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008). Several related studies with amphibians found that glyphosate herbicides containing non-ionic surfactants were more toxic to larvae compared to life stages that lacked gills (adult frogs - Bidwell and Gorrie 1995, developing embryos - Edginton et al. 2004), although no histological work was completed during these studies. Similar to Ramirez-Duarte (2008), skin epithelial hyperplasia (i.e., apical hyperplasia) was more prevalent among larvae exposed to ADSEE surfactant. This (i.e., hyperplasia) may be viewed as a protective response that limits chemical absorption (Mallatt 1985).

Larvae exposed to surfactant exhibited greater mortality than control larvae. This difference is not easily reconciled with the histological results. While extensive gill damage undoubtedly contributes to surfactant induced mortality (via decreased respiratory capacity or inability to maintain ionic or osmotic stability - Swedmark et al. 1971, Abel 1974), it is unclear whether gill damage is the primary cause of death (Abel 1974). Several previous studies with fish noted that exposure to herbicides containing surfactants negatively impacted a variety of organs (Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008). Ramirez-Duarte et al. (2008) found that *Piaractus brachypomus* exposed to Roundup® herbicide (containing POEA surfactant) for 96 hours exhibited gill, skin, kidney, and brain lesions. Chronic exposure to Roundup® (1-6 months) resulted in pathological lesions within gill, liver, and kidney tissue of Nile tilapia (*Oreochromis niloticus*) (Jiraungkoorskul et al. 2003). Mann and Bidwell (2001) assessed the toxicity of two non-ionic agricultural surfactants (nonylphenol ethoxylate and alcohol alkoxylate) to larvae of six amphibian species. They concluded that several factors may have contributed to surfactant toxicity including gill damage or membrane narcosis (Schuurmann 1990, van Wezel and Opperhuizen 1995). Membrane narcosis is a nonspecific disruption of membrane function which may result in decreased activity levels, slowed response to external stimuli, depressed cardiovascular function, and loss of balance (van Wezel and Opperhuizen 1995) with mortality ultimately due to widespread disruption of cellular function (Mann and Bidwell 2001). In general, the toxicity of nonionic surfactants to aquatic organisms is thought to result primarily from membrane narcosis (Roberts 1991, Joshi et al. 2007). Based on previous research, it is possible that membrane narcosis (Mann and Bidwell 2001) contributed to the toxicity of the nonionic surfactant ADSEE toward *Spea* spp. larvae, or that rapid narcosis induced mortality precluded the development of severe lesions among some larvae exposed to surfactant.

As developing amphibian larvae approach metamorphosis, gills (Atkinson and Just 1975) and certain skin structures (Fox 1981, Robinson and Heintzelman 1987, Yoshizato 1992) degenerate. Research by Atkinson and Just (1975) indicated that measurable gill degeneration (reflected by gill weight) becomes apparent in Gosner-stage 42 *R. catesbeiana* larvae. They concluded that degenerating cells were continually removed or sloughed (Atkinson and Just 1975). Histological data indicated that degenerating gills become black in appearance, and atrophy via apoptosis (i.e., degenerating cells are contained within well defined membranes) (Atkinson and Just 1975). Beginning around Gosner-stage 41-42, the epithelial skin of *R. catesbeiana* larvae undergoes dramatic changes as nearly all apical and skein cells degenerate (via apoptosis) and are replaced by adult skin cells (Robinson and Heintzelman 1987, Yoshizato 1992). According to Robinson and Heintzelman (1987), degenerating apical cells are enucleated, clear, and primarily contained within intact cell membranes. The chronology of the above processes have been well studied in only a few species (Atkinson and Just 1975, Robinson and Heintzelman 1987, Yoshizato 2007), and are known to vary among species (Yoshizato 2007, Fenoglio et al. 2009). It is possible that a portion of the skin and gill lesions observed in the present study resulted from normal metamorphic tissue restructuring. However, in light of previous research, this seems unlikely. Skin and gills from control individuals displayed lesions that appeared necrotic (i.e., rupture of cell membranes - Wyllie 1981), rather than the apoptotic changes associated with amphibian metamorphosis (Atkinson and Just 1975, Robinson and Heintzelman 1987).

The expected histological response may have been obscured by lesions resulting from prior contaminant exposure. Due to their limited availability in the field during late summer, larvae were collected from an aquatic habitat prone to chemical contamination (i.e., a SHP playa wetland; Thurman et al. 2000, Venne et al. 2008). Lesions like those observed can be caused by

a variety of contaminants (Brown et al. 1968, Abel 1974, Meyers and Hendricks 1985) including pesticides (Honrubia et al. 1993, Lajmanovich et al. 1998, Jiraungkoorskul et al. 2003, Bernabo et al. 2008, Ramirez-Duarte et al. 2008), surfactants (Abel and Skidmore 1975, Partearroyo et al. 1991), and metals (Baker 1969, Skidmore and Tovell 1972). While previous work detected pesticides in playa water (Thurman et al. 2000) and sediment (Venne et al. 2008), no pesticide residue data relevant to the present study exist.

Environmental stressors other than contaminants may have also contributed to observed skin and gill lesions (Meyers and Hendricks 1985). Amphibian larvae inhabiting playa wetlands surrounded by agriculture likely encounter non-contaminant stressors (e.g., decreased wetland hydroperiod - Tsai et al. 2007). While no experimental evidence indicates that altered wetland hydroperiod causes lesions among larval amphibians, previous work suggests that other environmental stressors (e.g., dietary deficiencies) were responsible for gill lesions among fish (Rucker et al. 1952, Wood and Yasutake 1957). Previous research demonstrated that environmental stressors can affect morphological (Newman 1994), developmental (Newman 1994, Denver 1998) and immunological (McMurry et al. 2009) traits among larval anurans. For example, Newman (1994) found that decreased food availability caused *Scaphiopus couchii* larvae to metamorphose earlier and at a smaller size. Denver (1998) indicated that decreasing water levels stimulated rapid metamorphosis among larval *Scaphiopus hammondii*. McMurry et al. (2009) found that *Spea* spp. from agriculturally impacted playa wetlands displayed altered immunological development. While the laboratory surfactant exposure likely exacerbated gill and skin lesions to some degree, this response may have been masked by previous environmentally induced lesions (Eller 1975).

It is possible that results were influenced by experimental methodology. Experimental compartments were identical to those used during acute and chronic toxicity tests (Chapter II)

and had been previously filled only with clean, aged tapwater. It seems unlikely that these factors contributed to observed mortality or lesions among control or surfactant individuals because no control larvae housed in identical compartments filled with similar dilution water died during acute toxicity tests (Chapter II). All larvae were euthanized in MS-222 prior to dissection and tissue preservation. This method of euthanasia was selected because it is commonly used during histological studies with aquatic organisms and does not affect tissue structure (Robinson and Heintzelman 1987, Jiraungkoorskul et al. 2003, Bernabo et al. 2008, Fenoglio et al. 2009). Immediately following euthanasia, larvae were also partially submerged in 10% formalin (prepared by Mary Hastert, Texas Tech University Imaging Center), and desired tissues were removed and preserved in the same formalin solution. It is unlikely that this affected tissue quality because previous histological studies demonstrated that 10% formalin was an effective tissue preservative (Honrubia et al. 1993, Bueno-Guimaraes et al. 2001, Suzuki et al. 2001). All subsequent tissue processing and slide preparation was completed by experienced personnel (Mary Hastert or personnel at the Texas Tech University Department of Anatomic Pathology). Based on the above, it is doubtful that tissue collection or processing contributed to observed lesions.

Histological studies are prone to several general types of error (Mallatt 1985). Investigators may either mistake artifacts for pathological lesions, or overlook lesions that are subtle and difficult to detect (Mallatt 1985). The former can result from errors in tissue fixation and processing, or the use of unhealthy animals that exhibit abnormal responses (Mallatt 1985). As previously mentioned, it seems unlikely that tissue fixation or processing contributed to observed lesions. All *Spea* spp. larvae appeared outwardly healthy at the beginning of the current study, and no signs of disease were evident upon histological examination. It is possible that I misidentified normal tissue structure as pathological lesions, or that I failed to detect

subtle lesions. However, if these types of error were present, they were likely independent of treatment group because slides were scored blindly (Mallatt 1985). Therefore, while errors of this type may have affected the intensity of certain lesions, it is unlikely that the magnitude or direction of differences among treatment groups (i.e., control versus surfactant) was affected.

Research needs

The current study offers limited insight about the mechanisms underlying surfactant toxicity in larval amphibians. However, several topics deserve further study. Future research should examine *Spea* spp. skin and gill structure throughout larval development and metamorphosis to determine when larval tissue degradation begins. This would clarify whether any lesions observed in the present study resulted from normal tissue restructuring. Previous histological work demonstrated that exposure to formulated pesticides can affect various tissues/organs among aquatic organisms (Jiraungkoorskul et al. 2003, Ramirez-Duarte et al. 2008). To clarify the mode of surfactant toxicity to larval amphibians, future work should examine whether surfactant exposure produces lesions in other tissues/organs (e.g., kidney, liver). Histological work completed with sublethal surfactant levels would support the development of sensitive biomarkers of exposure (Sepici-Dincel et al. 2009), and perhaps allow for the detection of surfactant contamination prior to extensive larval mortality. Additional work could be completed to analyze skin and gill tissue via electron microscopy. However, in light of the current light microscopy results, it seems unlikely that electron microscopic analysis would dramatically alter the direction/magnitude of observed effects or offer additional insight. Laboratory studies on these topics should utilize larvae from fresh, field-collected egg masses because this would minimize the possibility of confounding effects due to prior contaminant exposure.

Table 3.1. Mortality (by test compartment) among *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant at 1.44 mg/L or aged tapwater (control) for up to 48 hours. Each test compartment initially held five larvae.

	Compartment								
Group	A	B	C	D	E	F	G	H	I
Surfactant	3	5	4	4	5	5	4	4	3
Control	0	0	3	5	5	1	1	1	3

Table 3.2. Water quality variables in control and surfactant compartments (mean ± 1 S.E.) during 48-hour exposure of *Spea* spp. (New Mexico and Plains spadefoot) larvae to aged tapwater or ADSEE 907® surfactant at 1.44 mg/L.

	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Ammonia (mg/L)
Control	21.76 ± 0.06	8.59 ± 0.03	8.84 ± 0.08	0.07 ± 0.03
Surfactant	21.57 ± 0.05	8.55 ± 0.03	8.81 ± 0.05	0.00 ± 0.00

Table 3.3. Comparison of mean skin epidermal lesion intensity among *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant (1.44 mg/L) for up to 48 hours versus those housed in aged tapwater.

Comparison [a]	*df*	χ^2	P
Apical hypertrophy [b]	1	0.41	0.070
Apical hyperplasia	1	24.60	<0.001
Apical necrosis	1	13.14	<0.001
Skein hypertrophy	1	10.33	0.20
Skein hyperplasia	1	7.96	0.32
Skein necrosis	1	22.58	<0.001

[a] Means were compared with an ESTIMATE statement (equivalent to a linear contrast comparing treatment means) in GENMOD.

[b] See methods for definition of terms.

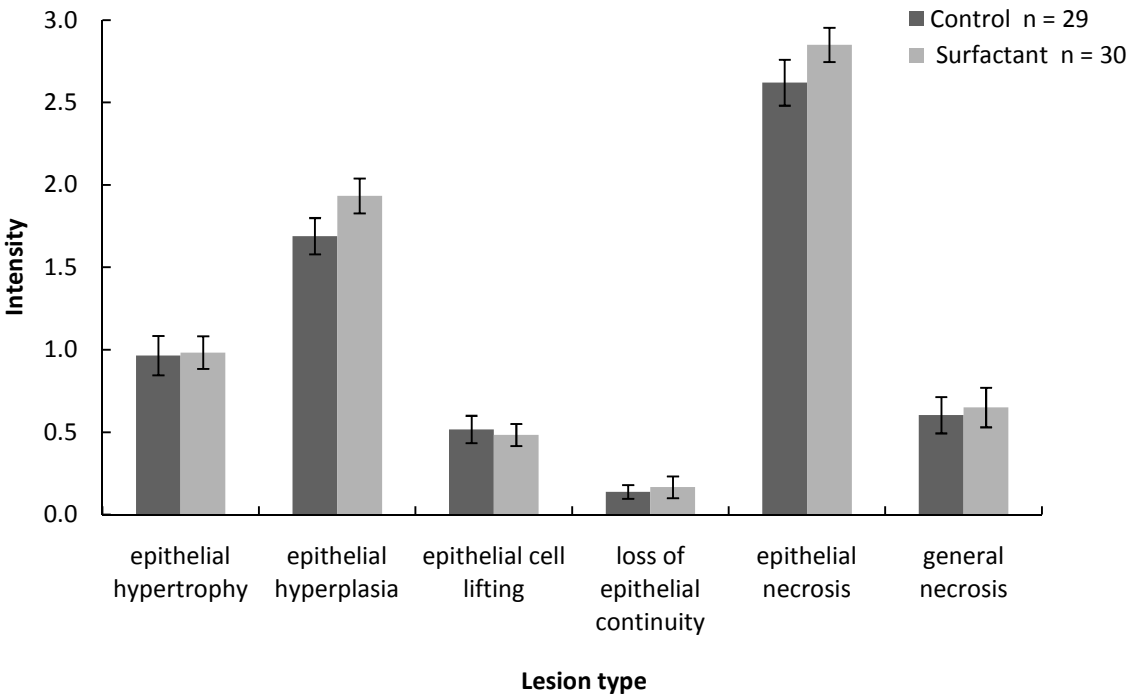


Figure 3.1. Histological response of *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. Gill tissue (gill tuft and gill filter) was evaluated via light microscopy and scored on a categorical scale. Intensity reflects percent coverage by a given lesion type: 0 = 0% (absent), 1 = 1-19%, 2 = 20-49%, 3 = 50-74%, 4 = 75-89%, 5 = 90-100%.

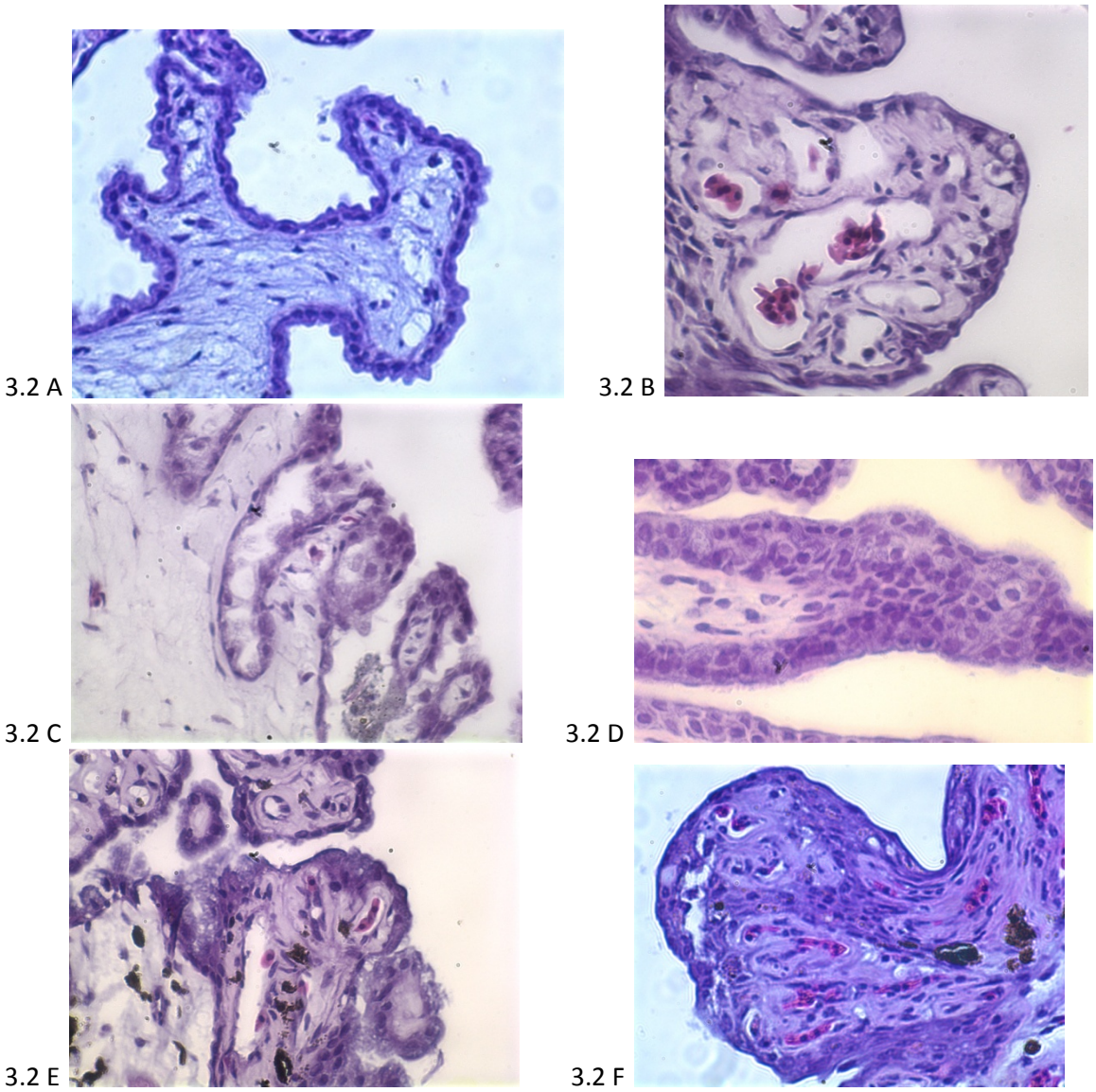
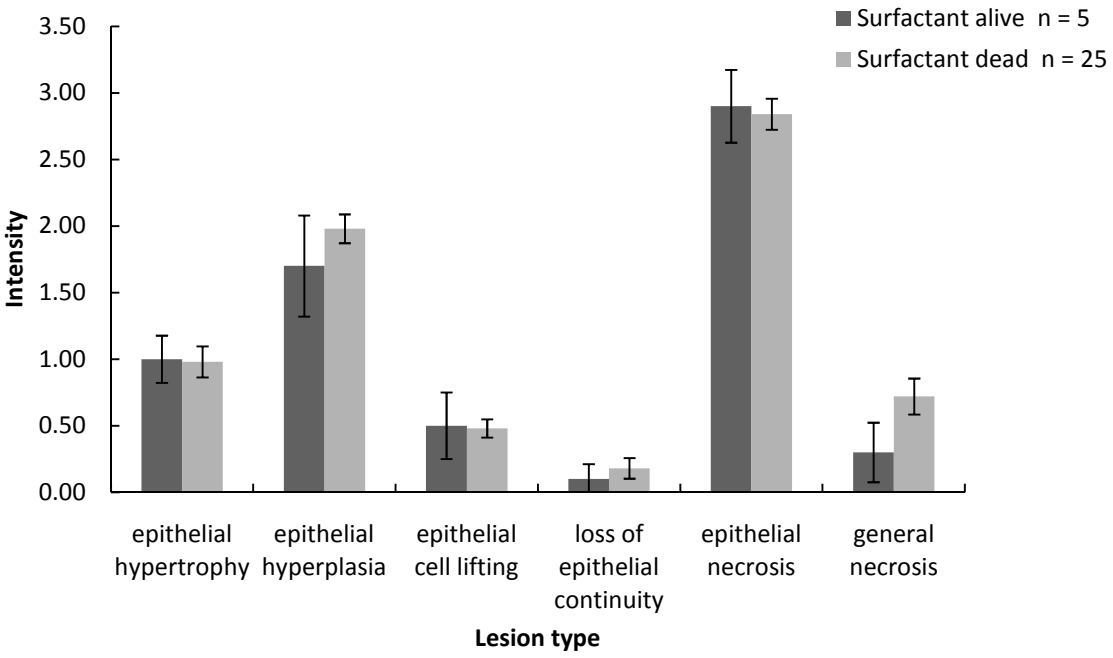
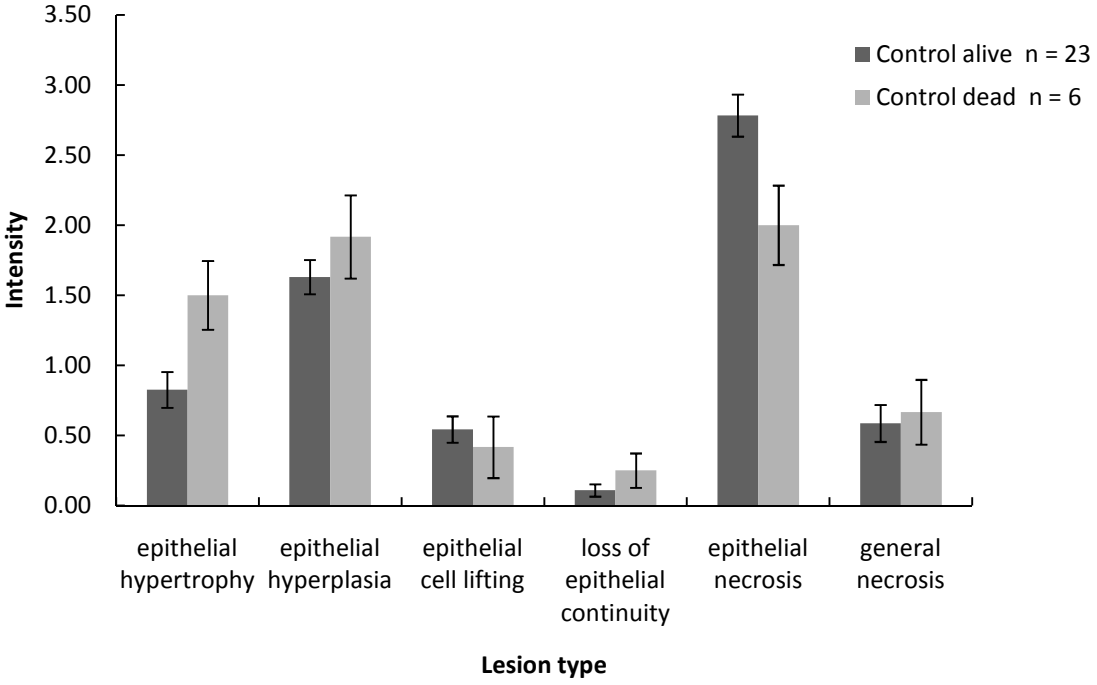


Figure 3.2 A-F. Gill tissue from *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. A: Structure of normal gill filter. B: Structure of normal gill tuft. C-F: Frequently observed gill lesions. C: Gill filter epithelial necrosis. D: Gill filter epithelial hyperplasia. E: Gill tuft epithelial necrosis in a control animal. F: Gill tuft epithelial hyperplasia. All images were taken at 60x using a Spot Insight color camera (Diagnostic Instruments, Inc.) attached to a Nikon BX 41 microscope.


Surfactant alive n = 5
Surfactant dead n = 25
Intensity
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
epithelial hypertrophy
epithelial hyperplasia
epithelial cell lifting
loss of epithelial continuity
epithelial necrosis
general necrosis
Lesion type

3.3 A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Control dead n = 6
Intensity
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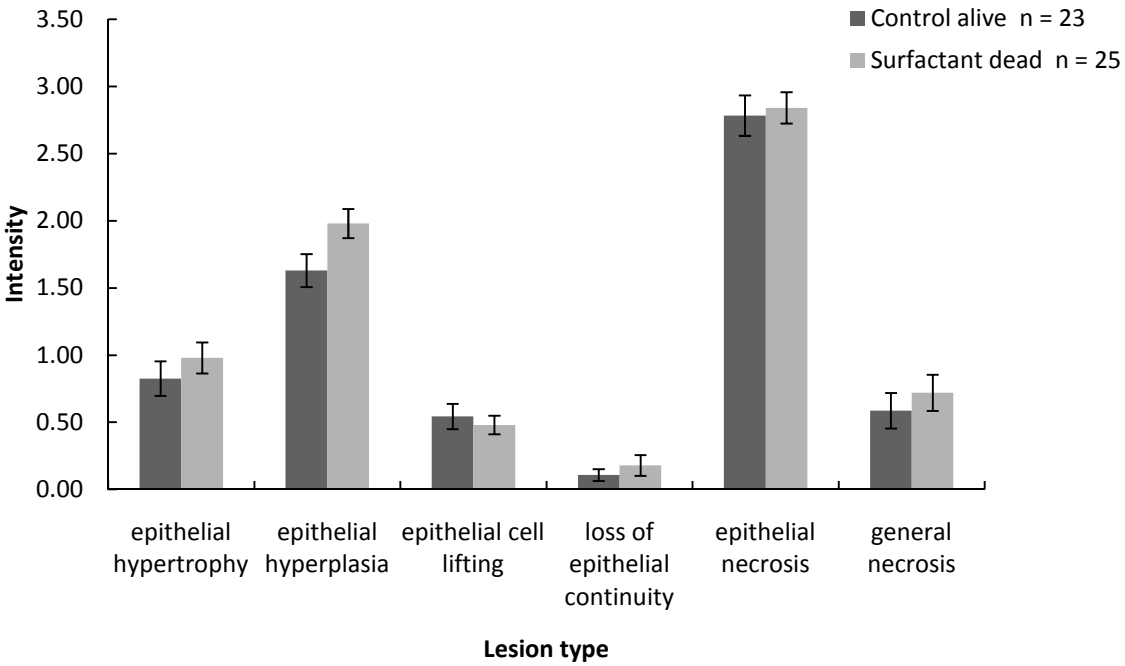
3.00
2.50
2.00
1.50
1.00
0.50
0.00
epithelial hypertrophy
epithelial hyperplasia
epithelial cell lifting
loss of epithelial continuity
epithelial necrosis
general necrosis
Lesion type

3.3 B



3.3 C

Figure 3.3 A-C. Histological response of *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. Gill tissue (gill tuft and gill filter) was evaluated via light microscopy and scored on a categorical scale. Intensity reflects percent coverage by a given lesion type: 0 = 0% (absent), 1 = 1-19%, 2 = 20-49%, 3 = 50-74%, 4 = 75-89%, 5 = 90-100%. A: Larvae exposed to surfactant that survived versus those that died during the exposure period. B: Control larvae that survived versus those that died during the exposure period. C: Control larvae that survived versus larvae exposed to surfactant that died.

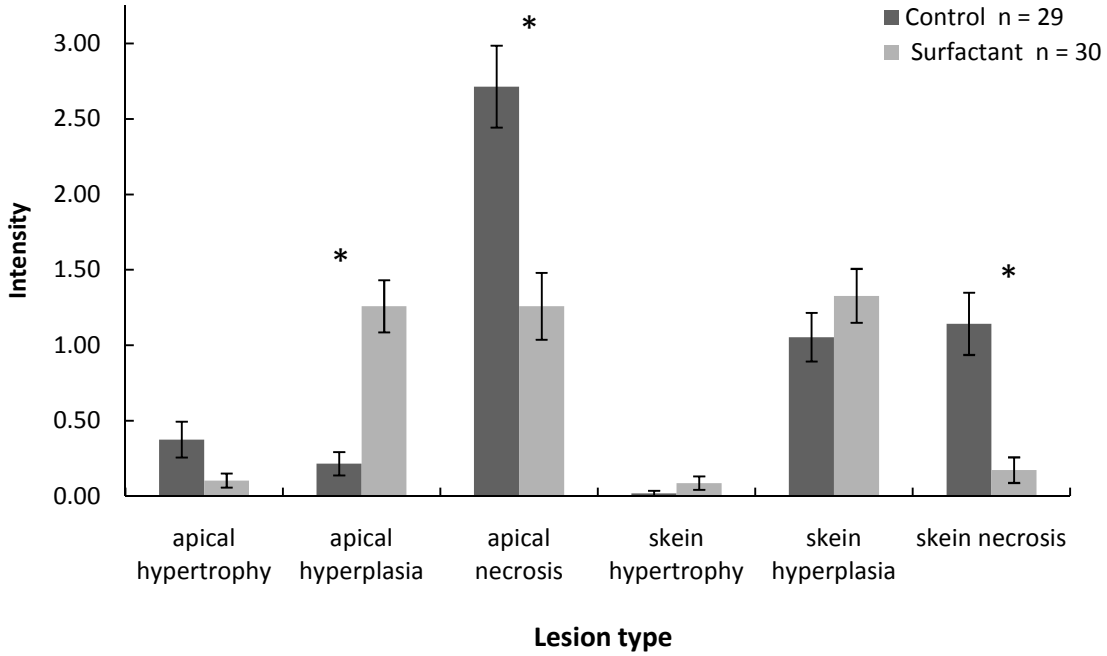
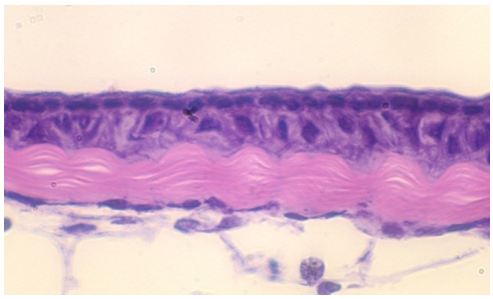
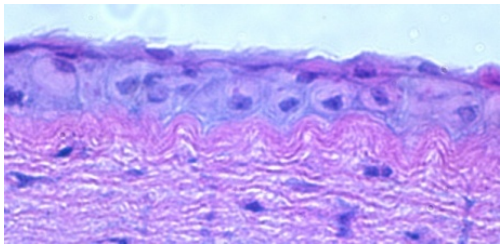
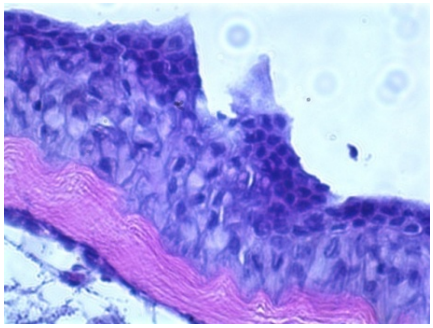
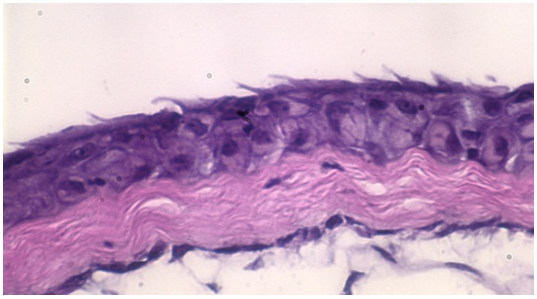


Figure 3.4. Histological response of *Spea* spp. larvae (New Mexico and Plains spadefoot) exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. Skin samples (ventral body epidermis) were evaluated via light microscopy and scored on a categorical scale. Lesions in the outermost apical cell layer and underlying skein layer were evaluated. Intensity reflects percent coverage by a given lesion type: 0 = 0% (absent), 1 = 1-19%, 2 = 20-49%, 3 = 50-74%, 4 = 75-89%, 5 = 90-100%. Within lesion type group means were compared with an ESTIMATE statement in GENMOD; asterisks indicate significant differences at $p<0.05$.

3.5 A 

3.5 B 

3.5 C 

3.5 D 

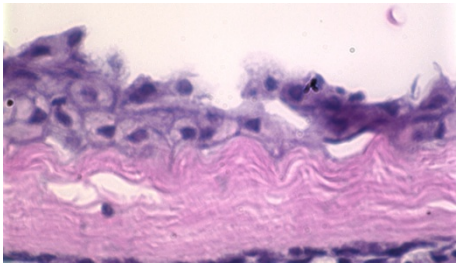
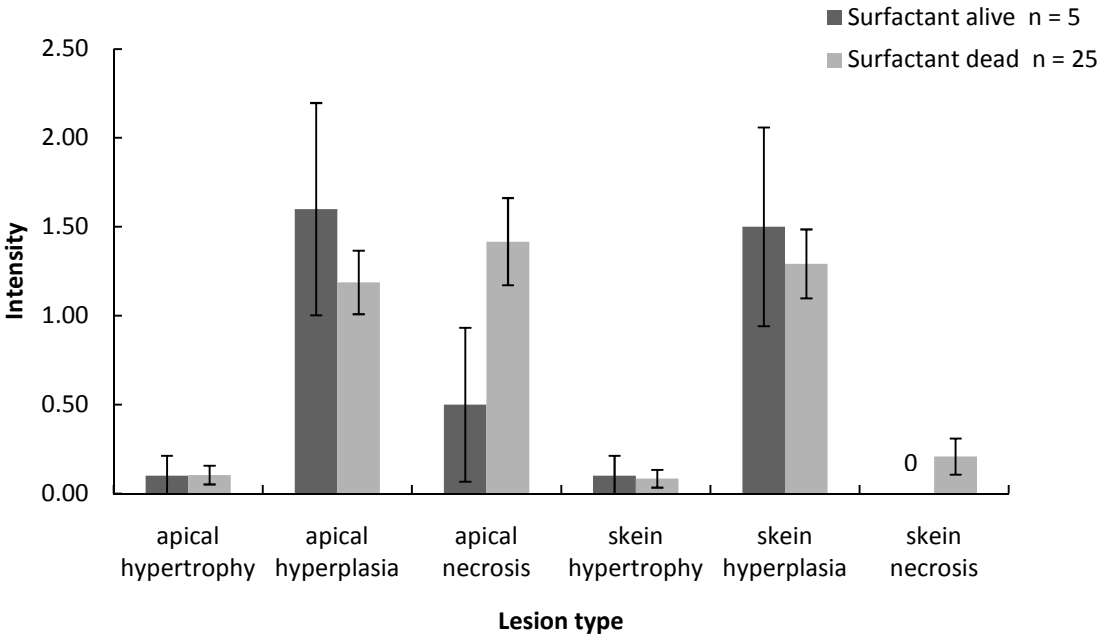
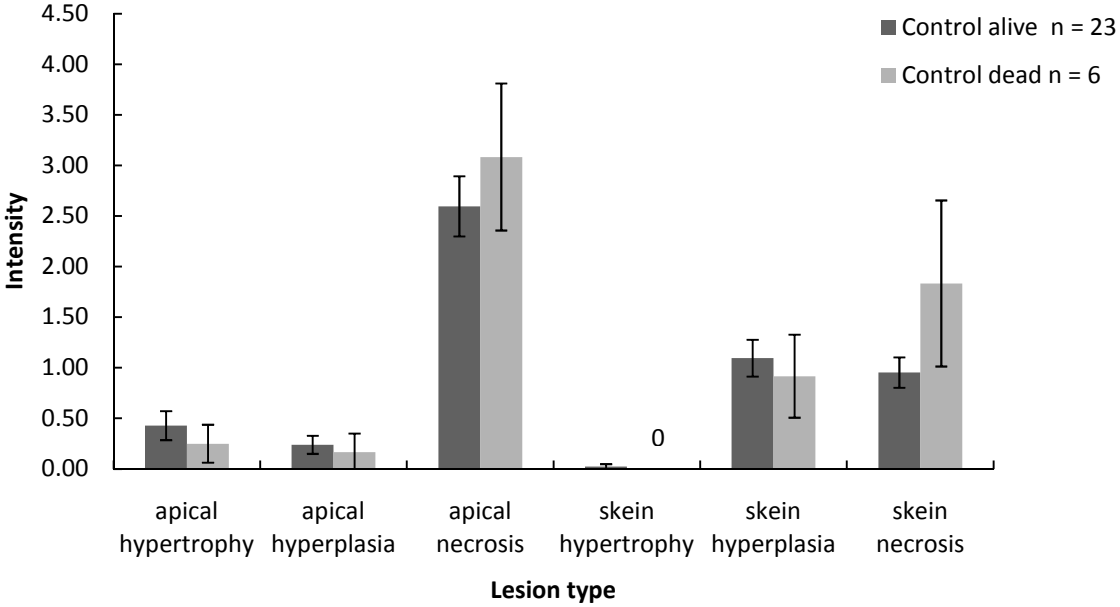
3.5 E 

Figure 3.5 A-E. Skin from *Spea* spp. (New Mexico and Plains spadefoot) larvae exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. A: Normal epithelial structure. B-E: Frequently observed epithelial lesions. B: Apical necrosis. C: Apical and skein hyperplasia. D: Apical necrosis in a control animal. E: Apical and skein necrosis in a control animal. All images were taken at 60x using a Spot Insight color camera (Diagnostic Instruments, Inc.) attached to a Nikon BX 41 microscope.


Surfactant alive n = 5
Surfactant dead n = 25
2.50
2.00
1.50
1.00
0.50
0.00
Intensity
0
apical hypertrophy
apical hyperplasia
apical necrosis
skein hypertrophy
skein hyperplasia
skein necrosis
Lesion type

3.6 A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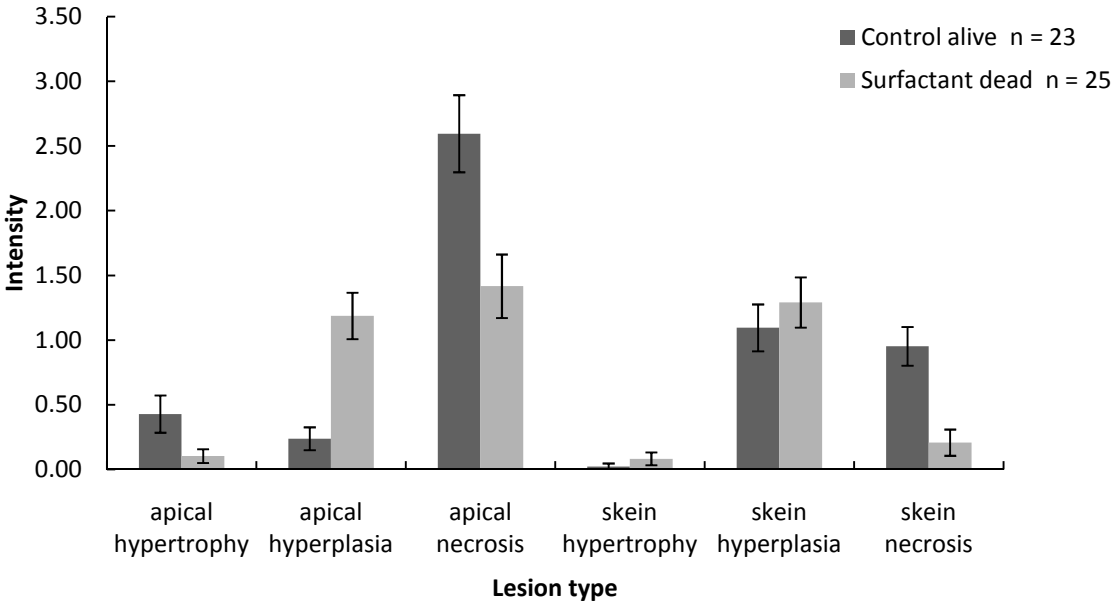
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Intensity
0
apical hypertrophy
apical hyperplasia
apical necrosis
skein hypertrophy
skein hyperplasia
skein necrosis
Lesion type

3.6 B



3.6 C

Figure 3.6 A-C. Histological response of *Spea* spp. larvae (New Mexico and Plains spadefoot) exposed to ADSEE 907® surfactant (1.44 mg/L) or aged tapwater (control) for up to 48 hours. Skin samples (ventral body epidermis) were evaluated via light microscopy and scored on a categorical scale. Lesions in the outermost apical cell layer and underlying skein layer were evaluated. Intensity reflects percent coverage by a given lesion type: 0 = 0% (absent), 1 = 1-19%, 2 = 20-49%, 3 = 50-74%, 4 = 75-89%, 5 = 90-100%. A: Larvae exposed to surfactant that survived versus those that died during the exposure period. B: Control larvae that survived versus those that died during the exposure period. C: Control larvae that survived versus larvae exposed to surfactant that died.

CHAPTER IV

TOXICITY OF A GLUFOSINATE- AND SEVERAL GLYPHOSATE-BASED HERBICIDES TO JUVENILE AMPHIBIANS FROM THE SOUTHERN HIGH PLAINS, USA

Introduction

Amphibian populations are declining worldwide, due in large part to the degradation of wetland and terrestrial habitats (e.g., Wyman 1990). Chemicals, such as insecticides, herbicides, and fertilizers used in agricultural activities may also contaminate aquatic and terrestrial habitats required by amphibians and pose a threat via direct toxicity (Semlitsch 2003). Glyphosate (e.g., Roundup®) and glufosinate-ammonia (e.g., Ignite®) based herbicides are used worldwide (Howe et al. 2004, Lee et al. 2005) to control weeds in farmland and forests (Lee et al. 2005, Relyea 2005a). Glyphosate-based herbicides are also frequently applied in residential settings (Relyea 2005a).

Most glyphosate-based herbicides contain two basic components: the isopropylamine (IPA) salt of glyphosate and a surfactant (the most common being a polyethoxylated tallowamine, POEA, surfactant) (Giesy et al. 2000). Glufosinate herbicides contain glufosinate-ammonium and a sodium polyoxyethylene alkylether sulfate (AES) surfactant (Koyama and Goto 1997). Both glyphosate and glufosinate-ammonium adsorb strongly to soil (Malone et al. 2004, Lee et al. 2005), degrade rapidly via microbial activity and have limited environmental persistence (Faber et al. 1997, Giesy et al. 2000). In terrestrial situations, the POEA surfactant displays environmental fate similar to glyphosate (Giesy et al. 2000). Little information on the

fate of the surfactant used in glufosinate herbicides is available. Since the major components of glyphosate herbicides bind tightly to soil and rapidly degrade, it is often assumed that they pose little risk to non-target organisms (Relyea 2005a). However, recent work indicates that exposure to these chemicals can negatively affect amphibians within terrestrial (Relyea, 2005a) and aquatic habitats (Howe et al. 2004, Relyea 2004, 2005a).

Numerous studies have investigated effects of glyphosate formulations on larval amphibians and results indicate that the surfactants, rather than the active ingredient, may be responsible for observed mortalities (Mann and Bidwell 1999, Howe et al. 2004, Relyea 2004, Relyea et al. 2005, Relyea 2005a,b). Non-ionic surfactants, such as POEA, exhibit their negative effects primarily by disrupting the respiratory surfaces of aquatic organisms (Lindgren et al. 1996). Following metamorphosis, many amphibian species occupy terrestrial habitats. Yet few studies (Bidwell and Gorrie 1995, Mann and Bidwell 1999, Relyea 2005a) have examined how post-metamorphic amphibians are affected by exposure to commonly applied herbicides. No work has examined whether natural environmental factors (e.g., soil) modulate the toxicity of herbicides toward post-metamorphic amphibians. Further research conducted under increasingly realistic conditions is necessary to fully understand how common agrochemicals affect amphibians (Relyea 2005a).

My purpose was to estimate juvenile survival of two of the most abundant amphibian species (*Spea multiplicata*, New Mexico spadefoot; *Bufo cognatus*, Great Plains toad) from playa wetlands of the Southern High Plains (SHP) following exposure to common herbicides at environmentally relevant levels. The SHP of Texas and New Mexico is one of the most heavily cultivated regions in the world (Bolen et al. 1989). It is therefore not surprising that the total volume of pesticides applied in Texas is among the greatest in the United States (Gianessi and

Marcelli 2000). Application to cotton represents one of the most prevalent uses of glyphosate-based herbicides (National Pesticide Use Database 2004).

Because the nearly 25,000 SHP playas are principally embedded throughout an intensively farmed region, terrestrial margins of many playas likely receive overspray during applications of agrochemicals. Following metamorphosis, juvenile amphibians inhabit areas near playas while the soil remains moist (Voss 1961, Graves and Kruppa 2005, Morey 2005). New Mexico spadefoots and Great Plains toads often occupy shallow burrows (Degenhardt et al. 1996) and emerge primarily for nocturnal foraging (Bragg 1944, Garrett and Barker 1987). However, recently metamorphosed individuals may also disperse away from drying playas (Degenhardt et al. 1996). Due to this behavior and the fact that herbicides are applied to cotton at various times throughout the spring and summer (National Research Council 1975; Bayer CropScience LP 2005, Ignite® 280 SL herbicide product label, Research Triangle Park, NC; Monsanto Company 2005, Roundup WeatherMAX®: complete directions for use, St. Louis, MO), juvenile SHP amphibians may be exposed to common herbicides. During this study, juvenile amphibians were exposed to environmentally relevant concentrations of a glufosinate-ammonium based herbicide [Ignite® 280 SL (IG)] and several glyphosate-based herbicide formulations [Roundup WeatherMAX® (WM), Roundup Weed and Grass Killer Super Concentrate® (WGKC), and Roundup Weed and Grass Killer Ready-To-Use Plus® (WGKP)] while housed on moist paper towels or natural soil and survival was monitored for 48 hours following application.

Materials and methods

Recently metamorphosed Plains and New Mexico spadefoot toads were collected on 27 June 2007 adjacent to a cropland playa wetland in Hale County, TX, USA. A mixture of the two

species was collected because at a young age the two are difficult to distinguish (Degenhardt et al. 1996). Great Plains toad juveniles were collected near a cropland playa in Hale County, TX on 8 July 2007. Similar sized individuals were collected to ensure that animals used for subsequent toxicity testing were of similar developmental stage. The specific exposure history of the populations from which animals used in this study were drawn is unknown. However, these amphibian populations likely experienced previous pesticide exposure because they inhabit wetlands surrounded by agriculture. All subsequent animal care and experimental procedures (with exceptions noted) were the same for both spadefoot and Great Plains toads. This research was completed under a Texas Tech University Institutional Animal Care and Use Committee approved protocol (No. 06018-06). After collection, animals were transported to The Institute of Environmental and Human Health at Texas Tech University in Lubbock, TX. They were held in 37.9 L glass aquaria containing 6 cm of moistened natural soil obtained from Terry County, TX. The physiochemical characteristics of this sandy loam soil were previously determined by A&L Midwest Laboratories (Omaha, NE). The soil displayed the following properties: 74% sand, 10% silt, and 16% clay, 1.3% organic matter, and pH of 8.3 (Zhang et al. 2006). Though this soil was not tested for glyphosate- or glufosinate-based herbicide residues, significant chemical contamination is unlikely because the soil was obtained from an area where no pesticides have been applied for at least five years. Small crickets were provided *ad libitum* to juveniles throughout the following experiments. Fluker's Orange Cube Complete Diet (Fluker's Cricket Farm, Inc., Port Allen, LA) was provided to all crickets for at least 6 hours.

Spadefoot and Great Plains toads were allowed to acclimate to laboratory conditions for three and four days, respectively. The spadefoot toad experiment commenced on 30 June 2007, while that with Great Plains toads began 13 July 2007. Experimental compartments were 11.4 L (31.5 cm long by 20.1 cm wide) plastic tubs lined with either paper towel or the previously

described natural soil (260 g - dry weight). The soil covered the bottom of each tub evenly without allowing metamorphs to bury themselves. A 946.4 mL (32 oz.) garden spray bottle was used to spray both substrates with aged well water until they were visibly moist. Paper towel lined containers received 14 g of evenly dispersed water, while soil lined containers received 28 g of water. Ten randomly selected juveniles were then added to each tub and allowed to acclimate for six hours prior to herbicide application. Due to a counting error, a single tub received only nine spadefoot juveniles.

All herbicides were applied at the maximum rate allowed for a single application. This was done to simulate direct exposure by terrestrial overspray (Relyea 2005a; Table 4.1); my study therefore represented a "worst-case" exposure scenario. WM was applied at a rate of 0.16 mL glyphosate/m^2 (44 fl oz WM/ac) (Monsanto Company 2005, Roundup WeatherMAX®: complete directions for use, St. Louis, MO), WGKC at a rate of 1.33 mL glyphosate/m^2 (2.5 fl oz WGKC/ft^2) (Monsanto Company 2006, Roundup Weed and Grass Killer Super Concentrate® product label, St. Louis, MO), and IG at 0.21 mL glufosinate/m^2 (29 fl oz IG/ac) (Bayer CropScience LP 2005, Ignite® 280 SL herbicide product label, Research Triangle Park, NC). Because no application rate was provided for WGKP, it was applied at a rate (based on amount of glyphosate) equivalent to that recommended for the other residential-use formulation (WGKC).

Herbicide solutions were applied using 946.4 mL garden spray bottles. Initially, each bottle was calibrated so that 10 sprays delivered a consistent amount of water into an empty 11.4 L tub. Over the course of six such 10-spray trials, the amount of water delivered by each bottle was consistent among treatments (mean ± 1 S.E.: WM, 8.64 ± 0.03 g; WGKC, 8.73 ± 0.02 g; WGKP, 8.32 ± 0.02 g; IG, 8.96 ± 0.04 g; aged well water, 8.74 ± 0.01g). Herbicides were diluted with aged well water so that they could be applied at the previously stated rate. The proper

dilution for each herbicide was determined as follows. Based on the desired application rate for each product, the amount of herbicide required for an area the size of the experimental tubs (633.15 cm^2) was calculated. Pure herbicide was then diluted so that 10 sprays from the appropriate bottle would deliver the desired amount of herbicide to each tub. Spray bottles were filled with diluted herbicide solution, the calibration of each was checked, and adjustments were made if necessary. Herbicides were then applied to experimental tubs via 10 evenly spaced sprays from the appropriate bottle. Control tubs received 10 sprays of well water. All calibration and herbicide applications were performed by the same person. There were five treatments (four herbicide formulations plus a control) that were replicated four times for each of two substrates (soil or paper towel) for each species.

Following herbicide application, survival was monitored for 48 hours to assess the acute response to herbicide exposure. Tubs were checked every six hours and moribund individuals euthanized by immersion in a 1% MS-222 solution (Howe et al. 2004). Animals were considered moribund if they exhibited lethargy or non-responsiveness to prodding. At end of the experiment, all remaining spadefoot toads were euthanized. Protein electrophoresis, following the techniques of Simovich and Sassman (1986), was used to identify juveniles as New Mexico or Plains spadefoots. All spadefoot juveniles were weighed at the time of death. The mean mass of Plains spadefoots (±1 S.E.) was 1.81 ± 0.04 g, and that of New Mexico spadefoots was 1.85 ± 0.02 g. Great Plains toads were weighed as they were distributed to experimental tubs; mean mass (±1 S.E.) was 0.74 ± 0.01 g.

Electrophoresis identified 337 of the spadefoot juveniles as New Mexico spadefoots, 59 as Plains spadefoots, and 2 as hybrids. Because New Mexico spadefoots dominated all experimental tubs, statistical analysis was only possible for this species and Great Plains toads. Generalized linear model (PROC GENMOD, SAS Version 9.1, SAS Institute, Cary, NC), assuming a

poisson distribution with a log link function (Littell et al. 2002), were used to test whether New Mexico spadefoot and Great Plains toad survival was influenced by pesticide exposure. Number of surviving juveniles was the response variable, and herbicide formulation and substrate (soil or paper towel) were the treatment effects. Since the data contained many zeros, 0.001 was added to each data value so that the GENMOD model converged. Treatment means (number surviving) were separated by including CONTRAST statements in the GENMOD procedure.

Glyphosate and glufosinate concentrations in treatment solutions used in the terrestrial exposure experiment were determined by gas chromatography analysis of the TMOA-derivatized products using a published procedure (Tseng et al. 2004). To my knowledge, the method had not been previously tested on formulated glyphosate or glufosinate products. This analysis was conducted in order to compare these measured concentrations to nominal concentrations (determined gravimetrically based on product label information). Calibration standards, calibration checks, and end calibration check standards were all constructed using certified glyphosate and glufosinate stocks obtained commercially (AccuStandard Inc.).

Results

New Mexico spadefoot survival was affected by herbicide formulation ($\chi^2_4 = 106.21$, $P < 0.0001$) and substrate ($\chi^2_1 = 4.95$, $P = 0.03$), but there was no herbicide formulation-substrate interaction present ($\chi^2_4 = 1.79$, $P = 0.77$). After 48-hours, New Mexico spadefoot survival was greater on soil than on paper towel (Figure 4.1). Post-hoc contrasts were used to compare treatment means (survival) between control animals and those exposed to each of four herbicide formulations. These analyses indicated that New Mexico spadefoots exposed to WGKP exhibited lower survival (Figure 4.1, Table 4.2) than control animals. All New Mexico spadefoots exposed to WGKP on paper towel and soil died within 48-hours of exposure.

Survival of Great Plains toad was also affected by herbicide formulation ($\chi^2_4 = 77.56$, $P < 0.001$) and substrate ($\chi^2_1 = 6.99$, $P = 0.008$). Since a significant herbicide formulation-substrate interaction was present ($\chi^2_4 = 14.84$, $P = 0.005$), data were separated by substrate and the analysis repeated. These analyses indicated that survival of Great Plains toads was affected by herbicide formulation on each substrate (soil: $\chi^2_4 = 62.65$, $P < 0.001$; paper towel: $\chi^2_4 = 29.75$, $P < 0.001$). Post-hoc contrasts indicated that, compared to control animals, Great Plains toads exposed to WGKP exhibited greatly reduced survival on both soil (Figure 4.2, Table 4.3) and paper towel (Figure 4.3, Table 4.3). Of the Great Plains toad metamorphs exposed to WGKP on soil, only 22.5% survived for the entire monitoring period, while all of those exposed on paper towels died within 48 hours. Contrasts also indicated that Great Plains toads exposed to WGKC on paper towel exhibited lower survival compared to control animals (Figure 4.3, Table 4.3). Only 47.5% of the Great Plains toads metamorphs exposed to WGKC on paper towel survived for 48 hours. Additional contrasts were used to compare within-treatment survival means between substrates. These analyses indicated that survival of Great Plains toads was greater among those exposed to WGKC and WGKP on soil compared to paper towels (Table 4.4).

Analysis of the herbicide solutions used in this study revealed that, overall, measured concentrations were consistent with nominal values (Table 4.5) especially considering the uncertainty associated with the use of the derivatization method (Tseng et al., 2004) on formulated products. However, some difficulty was encountered during these tests as the treatment solutions contained both the active ingredient and the “inerts.” In some instances, it appeared that these inert ingredients interfered with the derivatization reaction, particularly for WGKC and IG. Multiple attempts to alter the ratio of derivatization reagent to sample improved some of the analyses; however, the IG sample was particularly difficult. In contrast, no

difficulties were encountered with the derivatization and subsequent analysis of the individual active ingredients.

Discussion

Ultimately, we want to understand whether pesticides negatively impact amphibian communities. To achieve this goal, there must be a transition from highly artificial laboratory experiments toward research completed under more realistic conditions (Relyea et al. 2005, Relyea 2005a). Since I included natural soil as an exposure substrate, this study represents an important step in this direction. I exposed individuals of two recently metamorphosed SHP amphibian species to environmentally relevant concentrations of a variety of herbicide formulations while housed on moist paper towel and natural soil. I used formulated herbicides because these are the chemicals that juvenile amphibians encounter in their natural habitats. The survival of both species tested was reduced only by exposure to those formulations not intended for agricultural application.

Effects of glyphosate-based herbicide exposure on post-metamorphic amphibians have been examined in just a few studies, and no data exists for the effects of glufosinate-based herbicides. Adult and newly metamorphosed *Crinia insignifera*, a southwestern Australian frog species, exposed to Roundup 360® exhibited 48-hour LC_{50} values ranging between 65.9 and 69.1 mg glyphosate/L (Bidwell and Gorrie 1995, Mann and Bidwell 1999). Frogs in this study were exposed by partial submersion to a solution of aged tap water and Roundup 360®. Relyea (2005a) sprayed juveniles of three North American amphibian species, while housed on moist paper towels, with Roundup Weed and Grass Killer® (1.9% glyphosate) at a rate of 1.6 mL glyphosate/m^2 to assess the effects of unintended overspray during agricultural applications. Survival of all three North American species (*Rana sylvatica*, *Bufo woodhousii fowleri*, and *Hyla*

versicolor) was greatly reduced within 24 hours, as only 32%, 14%, and 18%, respectively, of exposed animals survived. I exposed SHP playa amphibians to several glyphosate-based herbicide formulations at a similar or lower rate (WGKC and WGKP, 1.33 mL glyphosate/m^2; WM, 0.16 mL glyphosate/m^2) on paper towel and soil. It is unknown how the composition of WGKP compares to the formulation used by Relyea (2005a). My results show that WGKP reduced survival of both species tested on both substrates, while WGKC reduced survival of only Great Plains toads exposed on paper towel. WM had no effect on 48-hour survival of either species tested on either substrate.

Unexpectedly, the response of juveniles amphibians exposed to WGKC versus WGKP differed. While both formulations were applied at the same rate (1.33 mL glyphosate/m^2), mean survival of both species was dramatically reduced on both substrates only among animals exposed to WGKP. The only known difference between the two formulations is that WGKP contains pelargonic and related fatty acids, suggesting these compounds are the ingredients responsible for mortality in my study, not glyphosate or the surfactants included in the inert ingredients. Pelargonic acid is a natural fatty acid that acts as an herbicide by quickly desiccating plant tissues (Pline et al. 2000). Although toxicity testing with pelargonic acid revealed little or no toxicity toward non-target organisms (e.g., fish, birds, honeybees) (U.S. Environmental Protection Agency 2000), my results indicate that further evaluations of pelargonic acid toxicity in amphibians may be warranted. Finally, because WGKP and WGKC contain other proprietary ingredients, I cannot discount the possibility that the mortality arising from exposure to the formulations could be explained by the presence of unidentified “inert” ingredient(s) (e.g., the surfactant).

Because glyphosate, glufosinate, and POEA surfactant bind rapidly to soil (Giesy et al. 2000, Malone et al. 2004, Lee et al. 2005) and therefore become less biologically available for

uptake, one would expect that juvenile amphibian survival would be greater in soil lined compared to paper towel lined containers. To my knowledge, no previous work has addressed this question. Relyea (2005a) demonstrated that the presence of soil in aquatic mesocosms did not mitigate the toxicity of Roundup Weed and Grass Killer® toward amphibian larvae, stating that any protective effects of soil were probably superseded by the rapid onset of tadpole death. My results indicate that, in general, New Mexico spadefoots exhibited greater survival on soil compared to on paper towel. Survival of Great Plains toads exposed to WGKP or WGKC was also greater on soil. These results illustrate the importance of including natural environmental factors when investigating the effects of pesticides on amphibians (Relyea 2005a,b). Failure to do so can lead to inaccurate conclusions about the risk that these chemicals pose to non-target organisms in field situations.

The herbicide formulations evaluated in this study vary widely in their intended use. WGKC and WGKP are commonly applied to residential lawn and gardens, whereas WM and IG are commercial agricultural products. WGKP was the only product that significantly reduced survival among both species tested for both substrates. Users of this product need to be aware of the importance of avoiding direct application to terrestrial amphibians. WGKC also reduced the survival of Great Plains toads exposed on paper towel. Since WGKP and WGKC are not intended for agricultural use, results related to these formulations reveal little about how post-metamorphic playa amphibians are affected by the application of common agricultural herbicides. I included these formulations since previous work (Relyea 2005a) examining the affects of glyphosate-based herbicide on terrestrial amphibians used products intended for lawn and garden use. It seems more relevant to evaluate the toxicity of agricultural formulations that amphibians are likely exposed to in field situations (e.g., WM and IG). These formulations did not reduce survival at 48-hours following exposure for either playa amphibian species tested.

My results indicate that when the agricultural formulations examined in this study are used as intended they do not pose an immediate risk to Great Plains toads or New Mexico spadefoots.

Although the current study increases our understanding of how common herbicides impact post-metamorphic amphibians, it also highlights areas that merit further research. Impacts of herbicide exposure on survival of post-metamorphic amphibians were examined in only two common playa species. Previous research with larval amphibians has demonstrated that variation in herbicide sensitivity exists between species (Mann and Bidwell 1999). It is therefore prudent to determine how commonly applied agrochemicals impact juveniles of other amphibian species. Also, my study only examined the effects of a "worst-case" exposure level. I chose this single dose because I framed my study within a tiered approach to ecological risk assessment (Romeis et al. 2008). My work represents a lower tier study used to determine whether the potential for risk exists. If a lower tier study such as this indicates the potential for risk, higher tier studies that more accurately reflect real-world exposure scenarios should be undertaken (Romeis et al. 2008). While my results indicate that the agricultural formulations tested did not pose a threat to juvenile New Mexico spadefoot and Great Plains toads, many abiotic and biotic factors present in amphibian habitats were absent during my study. Previous work has demonstrated that the toxicity of pesticides toward amphibians changes when additional natural stressors (e.g., predators) are present (Relyea 2003, Relyea et al. 2005). Therefore, further research completed under increasingly natural conditions is necessary to understand whether common herbicides pose any risk toward amphibian populations. Additionally, the current study monitored only a single endpoint (survival) for a short period of time. Previous studies have shown that pesticides can have sublethal impacts on amphibians by negatively affecting growth (Howe et al., 2004), behavior (Bridges 1997) and reproduction (Hayes et al. 2002). The vast majority of studies examining such sublethal effects have focused

on larval amphibians. More work is needed to determine whether pesticide exposure causes sublethal impacts on post-metamorphic amphibians, and what implication such effects have in terms of the persistence of amphibian populations (Relyea 2005a).

Conclusion

Many amphibian species occur in areas where pesticide use is common. While extensive research has examined how these chemicals impact amphibian larvae, few studies have investigated how pesticide exposure affects post-metamorphic amphibians. I exposed juveniles of two Southern High Plains amphibian species to environmentally relevant concentrations of several widely used herbicides. Natural soil was included as a substrate to increase environmental realism. Roundup Weed and Grass Killer Ready-to-Use Plus®, an herbicide intended for lawn and garden use, caused significant mortality among both species. The agricultural formulations (Roundup WeatherMAX® and Ignite® 280 SL) that juvenile amphibians likely encounter in real-world scenarios did not affect the short-term survival of either species tested. While these agricultural herbicides likely do not pose an immediate threat to the species tested, further research is needed to determine whether exposure to these herbicides causes more subtle, sublethal affects.

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Table 4.1. List of ingredients present (by percent composition) in each herbicide formulation sprayed onto juvenile *Spea multiplicata* (New Mexico spadefoot) and *Bufo cognatus* (Great Plains toad).

Herbicide Formulation [a]	Ingredient	Percent
WM	Glyphosate	48.8
	Other Ingredients	52.2
WGKP	Glyphosate	2
	Pelargonic and related fatty acids	2
	Water and minor formulating ingredients	96
WGKC	Glyphosate	50.2
	Other Ingredients	49.8
IG	Glufosinate-ammonium	24.5
	Other Ingredients	75.5

[a] WM = Roundup WeatherMAX®, WGKP = Roundup Weed and Grass Killer Ready-To-Use Plus®, WGKC = Roundup Weed and Grass Killer Super Concentrate®, IG = Ignite® 280 SL.

Table 4.2. Pair-wise comparisons of mean survival of juvenile *Spea multiplicata* (New Mexico spadefoot) 48-hours after direct exposure to aged well water (control) or an herbicide [a] in plastic tubs.

Contrast [b]	*df*	χ^2	P
Control vs. IG	1	0.01	0.91
Control vs WM	1	0.19	0.66
Control vs WGKC	1	0.81	0.37
Control vs WGKP	1	79.49	<0.001

The degrees of freedom (*df*), test statistic (χ^2) and associated probability (P) are given.

[a] Ignite® 280 SL (IG), Roundup WeatherMAX® (WM), and Roundup Weed and Grass Killer Super Concentrate® (WGKC) were applied at the maximum rate allowed for a single application: IG = 0.21 mL glufosinate/m^2, WM = 0.16 mL glyphosate/m^2, WGKC = 1.33 mL glyphosate/m^2. Roundup Weed and Grass Killer Ready-To-Use Plus® (WGKP) was applied at a rate equivalent to that recommended for WGKC.

[b] Means were compared with a CONTRAST statement in GENMOD.

Table 4.3. Pair-wise comparisons of mean survival of juvenile *Bufo cognatus* (Great Plains toad) 48-hours after direct exposure to aged well water (control) or an herbicide [a] in plastic tubs lined with paper towel or soil.

		Paper towel		Soil	
Contrast [b]	*df*	χ^2	P	χ^2	P
Control vs. IG [c]	1	1.18	0.23	0.01	0.91
Control vs WM	1	0.22	0.64	0.01	0.91
Control vs WGKC	1	7.04	0.01	0.01	0.91
Control vs WGKP	1	54.00	<0.001	20.21	<0.001

The degrees of freedom (*df*), test statistic (χ^2) and associated probability (P) are given.
[a] For herbicide application rates see Table 4.2.
[b] Means were compared with a CONTRAST statement in GENMOD.
[c] For herbicide formulation codes see Table 4.1.

Table 4.4. Pair-wise comparisons of mean survival of juvenile *Bufo cognatus* (Great Plains toad) on soil versus paper towel 48-hours after direct exposure to aged well water (control) or an herbicide [a].

Contrast [b]	*df*	χ^2	P
Control	1	0.00	1.00
IG [c]	1	1.43	0.23
WM	1	0.33	0.56
WGKC	1	7.64	0.006
WGKP	1	12.42	<0.001

The degrees of freedom (*df*), test statistic (χ^2) and associated probability (P) are given.
[a] For herbicide application rates see Table 4.2.
[b] Means were compared with a CONTRAST statement in GENMOD.
[c] For herbicide formulation codes see Table 4.1.

Table 4.5. Accuracy (relative error) of analyses of glyphosate and glufosinate treatment solutions.

Herbicide Formulation	Nominal Concentration (mg/L)	Measured Concentration (mg/L) [a]	Relative Error (%)
WM [b]	30	29.8	-0.67
WGKP	1014	1092	7.7
WGKC	954	628	-34
IG	41	138	236

[a] Mean of 3 determinations.

[b] For herbicide formulation codes see Table 4.1.

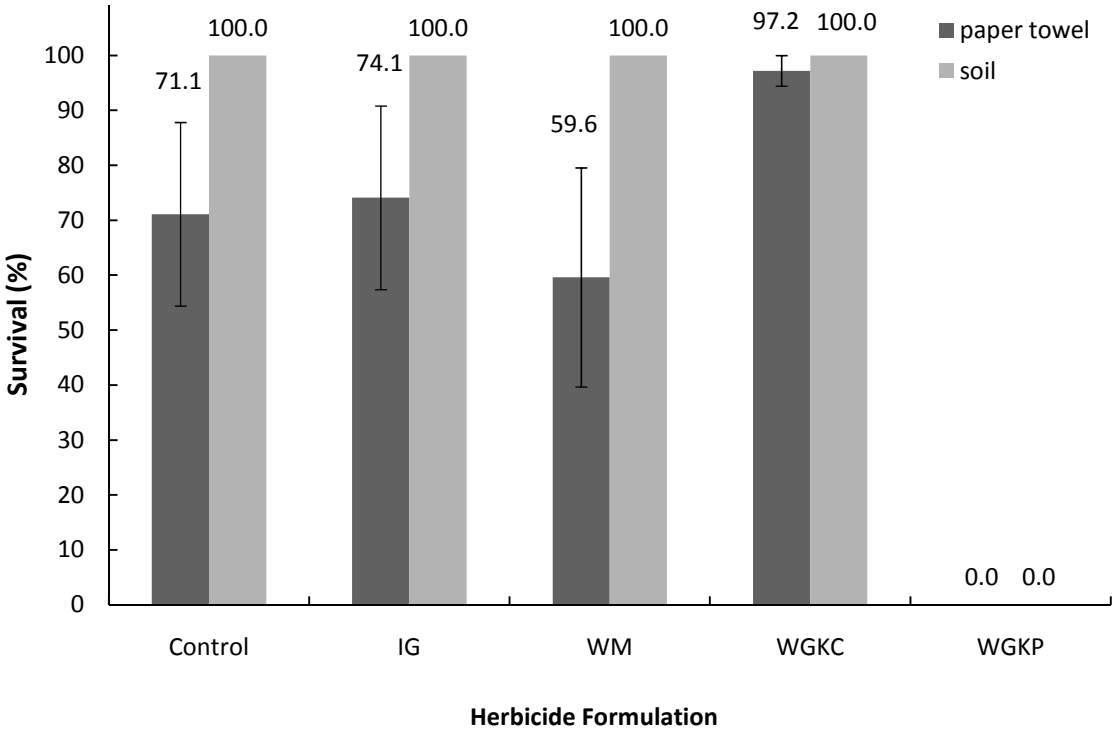

Figure 4.1. The survival (mean ±1 S.E.) of juvenile *Spea multiplicata* (New Mexico spadefoot) 48-hours after direct exposure to aged well water (control) or an herbicide at the given rate: Roundup Weed and Grass Killer Ready-To-Use Plus® (WGKP), 1.33 mL glyphosate/m^2; Roundup Weed and Grass Killer Super Concentrate® (WGKC), 1.33 mL glyphosate/m^2; Roundup WeatherMAX® (WM), 0.16 mL glyphosate/m^2; Ignite® 280 SL (IG), 0.21 mL glufosinate/m^2. Animals were exposed in plastic tubs lined with soil or paper towel.

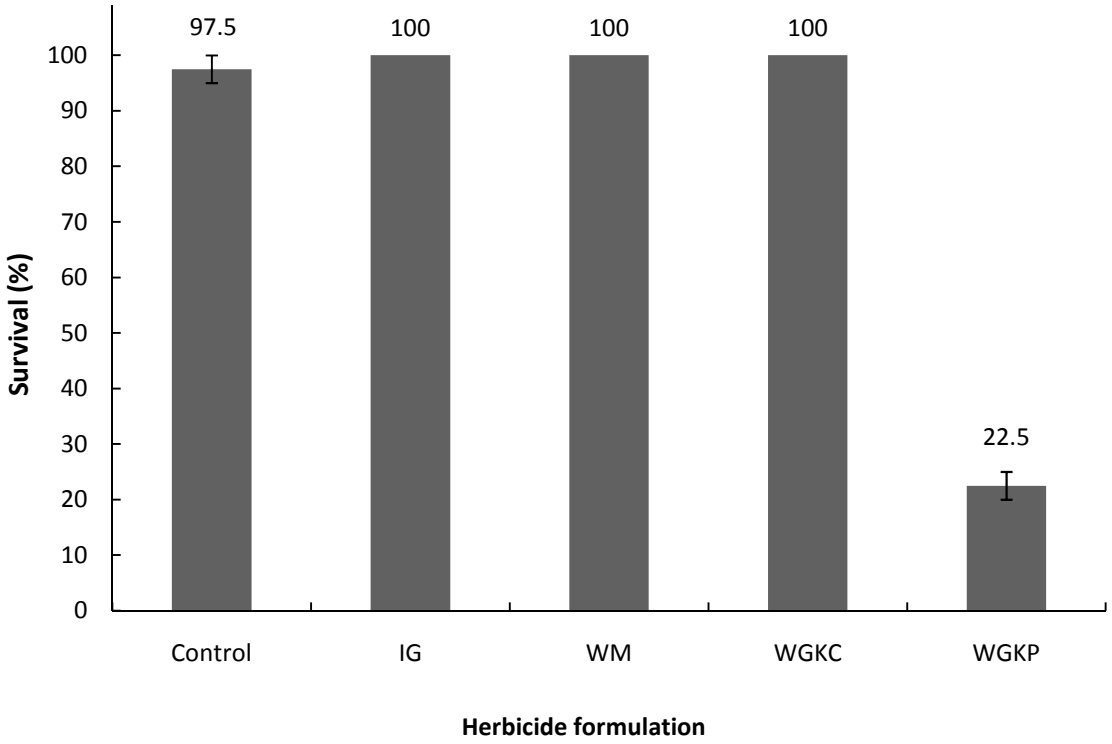

Figure 4.2. The survival (mean ±1 S.E.) of juvenile *Bufo cognatus* (Great Plains toad) 48-hours after direct exposure to aged well water (control) or an herbicide at the given rate: Roundup Weed and Grass Killer Ready-To-Use Plus® (WGKP), 1.33 mL glyphosate/m^2; Roundup Weed and Grass Killer Super Concentrate® (WGKC), 1.33 mL glyphosate/m^2; Roundup WeatherMAX® (WM), 0.16 mL glyphosate/m^2; Ignite® 280 SL (IG), 0.21 mL glufosinate/m^2. Animals were exposed in plastic tubs lined with soil.

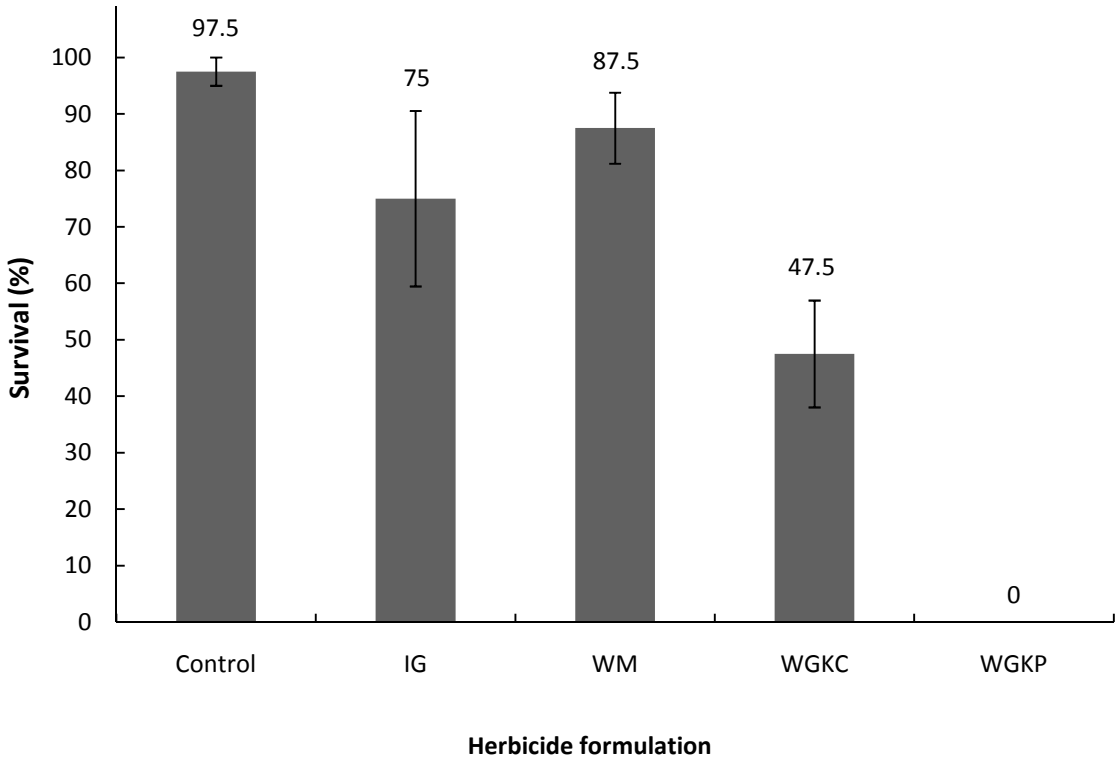

Figure 4.3. The survival (mean ±1 S.E.) of juvenile *Bufo cognatus* (Great Plains toad) 48-hours after direct exposure to aged well water (control) or an herbicide at the given rate: Roundup Weed and Grass Killer Ready-To-Use Plus® (WGKP), 1.33 mL glyphosate/m^2; Roundup Weed and Grass Killer Super Concentrate® (WGKC), 1.33 mL glyphosate/m^2; Roundup WeatherMAX® (WM), 0.16 mL glyphosate/m^2; Ignite® 280 SL (IG), 0.21 mL glufosinate/m^2. Animals were exposed in plastic tubs lined with paper towel.

CHAPTER V

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Dissertation: THE IMPACTS OF ROUNDUP WEATHERMAX® AND IGNITE® 280 SL ON AMPHIBIANS IN THE SOUTHERN HIGH PLAINS

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Title of Study: THE IMPACTS OF ROUNDUP WEATHERMAX® AND IGNITE® 280 SL ON AMPHIBIANS IN THE SOUTHERN HIGH PLAINS

Pages in Study: 111 Candidate for the Degree of Doctor of Philosophy

Major Field: Zoology

Scope and Method of Study: Most playa wetlands in the Southern High Plains (SHP) region are embedded in cropland. SHP amphibians may encounter agricultural chemicals due to contaminated runoff or direct terrestrial exposure. I compared the acute and chronic toxicity of widely used herbicides Roundup WeatherMAX® and Ignite® 280 SL to larval New Mexico and Plains spadefoots (*Spea multiplicata* and *S. bombifrons*, respectively) from playas embedded in cropland or grassland. I also examined how the survival of juvenile New Mexico spadefoots and Great Plains toads (*Bufo cognatus*) housed on soil or moist paper towels was affected by exposure to environmentally relevant levels of a glufosinate-based (Ignite® 280 SL) herbicide and several glyphosate-based (Roundup WeatherMAX®, Roundup Weed and Grass Killer Ready-To-Use Plus®, Roundup Weed and Grass Killer Super Concentrate®) herbicides. The toxicity of glyphosate herbicides toward amphibians is thought to result primarily from surfactants. To increase knowledge on this subject, I investigated the histological impacts of a non-ionic surfactant (ADSEE 907®) on skin and gills of *Spea* spp. larvae.

Findings and Conclusions: No consistent difference in herbicide sensitivity was present among larvae from cropland and grassland playas. Toxicity data suggest that Ignite® 280 SL does not pose an immediate threat to larval New Mexico and Plains spadefoots. However, Roundup WeatherMAX® may pose a risk to larvae of these species. Roundup Weed and Grass Killer Ready-To-Use Plus® was highly toxic to New Mexico spadefoot and Great Plains toad juveniles. However, it is unlikely that amphibians will encounter this formulation under field conditions because this product is intended for lawn and garden use. When used properly, the agricultural herbicides tested (Roundup WeatherMAX® and Ignite® 280 SL) likely do not pose an immediate threat to juvenile New Mexico spadefoots and Great Plains toads under field conditions. Skin and gill lesions were not consistently more extensive among larvae exposed to ADSEE 907®. This unexpected histological response may have resulted from prior contaminant induced lesions, or tissue restructuring associated with metamorphosis. It is also possible that membrane narcosis (i.e., widespread disruption of cell membranes that negatively impacts cellular function) contributes to the toxicity of non-ionic surfactants toward amphibians.

ADVISER'S APPROVAL: Loren M. Smith