Amphibian Exposure to Aquatic Herbicides:

Ecological Interactions with Invasive Plant Management

Amy Elizabeth Yahnke

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

Reading Committee:

Christian Grue, Chair

Marc Hayes

Aaron Wirsing

Program Authorized to Offer Degree:

School of Aquatic and Fishery Sciences

University of Washington

Abstract

Amphibian Exposure to Aquatic Herbicides:
Ecological Interactions with Invasive Plant Management

Amy Elizabeth Yahnke

Chair of the Supervisory Committee:
Christian E. Grue, Associate Professor
School of Aquatic and Fishery Sciences

Loss and degradation of wetland habitats are primary factors in amphibian declines. Wetland restoration may involve the use of aquatic herbicides to remove invasive plants, yet the impacts of aquatic herbicide tank-mixes on wetland fauna have rarely been considered. A paucity of data exists for native amphibian ecology, leading to data gaps for assessing risks of herbicide use to listed amphibians like Oregon spotted frogs (*Rana pretiosa*). To address those data gaps, a weekly field survey was conducted in Oregon spotted frog habitats during the aquatic weed management season, followed by ecologically-relevant toxicity tests. Detailed information is presented on life histories of Pacific Northwest amphibian species. Results suggest that metamorphic and post-metamorphic anurans and larval salamanders are most at risk of exposure to aquatic weed management. Laboratory toxicity tests were conducted using environmentally-relevant exposure scenarios with aquatic herbicide tank mixes. Exposure rates were estimated for

expected concentrations in 2 cm of water after direct over-spray. Anurans were exposed for 96-h, then reared in clean water for 2 mo to assess latent effects. Multiple endpoints were collected, including behavior, body condition, feeding rates, and liver condition. Juvenile Oregon spotted frogs were exposed to imazapyr + Agri-Dex tank mixes at high and low application rates for control of reed canarygrass (*Phalaris arundinacea*). No significant differences were observed for any endpoint. Metamorphosing northern red-legged frogs (*Rana aurora*) were exposed to a triclopyr + Competitor tank mix at a labeled rate for control of purple loosestrife (*Lythrum salicaria*). Metamorphs exposed to the triclopyr tank-mix were stressed during the 96-h exposure, and completion of metamorphosis was delayed by 1 d. Finally, because fear of predation is known to increase mortality of larval amphibians exposed to pesticides and aquatic predators, a proof-of-concept toxicity test incorporating terrestrial predator-prey ecology was developed. Northern red-legged frogs, housed in two different clean-water substrates, were exposed to the visual cue of garter snake (*Thamnophis sirtalis*) presence. Baseline behavior metrics and sample sizes that would be required for exposures with chemicals were determined. Frogs moved more and used more movements associated with evasion when exposed to snakes.

TABLE OF CONTENTS

Table of Contents iii
List of Figures vi
List of Tables viii
Acknowledgements x
Dedication xii
Executive Summary 1
Chapter 1: Amphibian Phenology Associated With Aquatic Weed Management 5
Introduction 6
Materials and Methods 7
Study sites 8
Vegetation surveys 9
Amphibian surveys 10
Statistical analyses 15
Results 16
Plot characteristics 16
Vegetation 16
Hydrology 17
Temperature 18
Amphibian survey summary 18
Amphibian life-stage phenology 20
Northwestern Salamander 20
Long-toed Salamander 21
Rough-skinned Newt 21
American Bullfrog 22
Pacific Treefrog 23
Northern Red-legged Frog 23
Oregon Spotted Frog 24
Habitat 25
Northwestern Salamander 26
Rough-skinned Newt 27
Pacific Treefrog 27
Northern Red-legged Frog 27

Oregon Spotted Frog 28
Discussion 31
Implications for risk assessment 35
Conclusion 38
References 39
Appendix A. Amphibian observations during summer 2010 58
Appendix B. Timing of salamander metamorphosis in summer 2010 60
Chapter 2: Effects of the Herbicide Imazapyr on Juvenile Oregon Spotted Frogs 61
Introduction 62
Materials and Methods 65
Experimental design 65
Study animals 66
Animal husbandry 66
Tank mixes and analytical chemistry 69
Test endpoints 70
Statistical analysis 71
Results 73
Water quality 73
Behavior 74
Body condition 75
Liver condition 75
Gonads 76
Discussion 76
References 80
Chapter 3: Effects of the Herbicide Triclopyr on Metamorphic Northern Red-legged Frogs 93
Introduction 94
Materials and Methods 97
Experimental design 97
Study animals 97
Animal husbandry 98
96-h exposure 100
Behavior trial 102
Grow-out 103
Tank mix and analytical chemistry 106

Test endpoints 107
Statistical analyses 108
Results 110
Mortalities and deformities 110
Analytical chemistry 111
Water quality 111
Behavior 112
Metamorphosis 113
Cricket consumption 114
Body condition 114
Liver condition 116
Liver histology 116
Discussion 116
References 122
Chapter 4: Snakes on a Visual Plane: Adding Fear of Predation to Toxicity Tests With Anurans 145
Introduction 146
Materials and Methods 148
Experimental design 148
Study animals 148
Animal husbandry 149
Trials 152
Endpoints 153
Statistical analyses 153
Results 155
Water quality 155
Behavior 155
Sample sizes 156
Discussion 157
Conclusion 160
References 160

LIST OF FIGURES

Chapter 1: Amphibian Phenology Associated With Aquatic Weed Management

Figure 1.1 West Rocky Prairie plot, transect, and trap array locations..... 51

Figure 1.2 Dempsey Creek pond, channel transect, and trap array locations..... 52

Figure 1.3 Precipitation and mean air and water temperatures at West Rocky Prairie..... 53

Figure 1.4 Mean daily air and water temperatures at West Rocky Prairie..... 53

Figure 1.5 Distribution of northwestern salamander larvae and metamorph sizes..... 54

Figure 1.6 Distribution of rough-skinned newt larvae and metamorph sizes..... 54

Figure 1.7 Number of metamorphic frogs observed during weekly surveys..... 55

Figure 1.8 Number of young-of-year frogs observed during weekly surveys..... 55

Figure 1.9 Mean water temperatures in which Oregon spotted frogs were observed..... 56

Figure 1.10 Mean air temperatures in which Oregon spotted frogs were observed..... 57

Figure 1.11 Distribution of water depths and number of amphibians observed at each depth..... 58

Chapter 2: Effects of the Herbicide Imazapyr on Juvenile Oregon Spotted Frogs

Figure 2.1 Diagram of feeder float..... 88

Figure 2.2 Time frogs spent in the center ring of the float/feeding platform..... 89

Figure 2.3 Cumulative number of crickets consumed per day by the number of males..... 90

Figure 2.4 Mean crickets consumed per day by treatment..... 91

Figure 2.5 Body condition over time..... 92

Chapter 3: Effects of the Herbicide Triclopyr on Metamorphic Northern Red-legged Frogs

Figure 3.1 Diagram of experimental bucket design..... 134

Figure 3.2 Dissolved oxygen in control and tank mix buckets during the 96-h exposure..... 135

Figure 3.3 Mean time metamorphs spent moving in behavior arenas post-96 h exposure..... 136

Figure 3.4 Number of movements in behavior arenas immediately post-96 h exposure..... 137

Figure 3.5 Days to complete metamorphosis..... 138

Figure 3.6 Days post-metamorphosis to start of feeding..... 139

Figure 3.7 Interaction of limb deformity and treatment on number of crickets eaten..... 140

Figure 3.8 Body condition over time..... 141

Figure 3.9 Body condition over time of frogs with and without scoliosis..... 142

Figure 3.10 Boxplot of liver condition indices..... 143

Figure 3.11 Example of melanomacrophage cells in northern red-legged frog livers..... 144

Chapter 4: Snakes on a Visual Plane: Adding Fear of Predation to Toxicity Tests with Anurans

Figure 4.1 Diagram of snake arena containing the frog box..... 169

Figure 4.2 Number of movements by frogs during exposure to snakes..... 170

Figure 4.3 Crickets consumed by frogs after exposure to snakes..... 171

LIST OF TABLES

Chapter 1: Amphibian Phenology Associated With Aquatic Weed Management

Table 1.1 Spearman Rank correlations for habitat variables..... 43

Table 1.2 Number of dips collected, depth, and proportion of dips that were dry..... 44

Table 1.3 Number and proportion of all amphibian observations..... 45

Table 1.4 Sizes of salamanders in all life stages..... 46

Table 1.5 Sizes of ranid frogs in all life stages..... 47

Table 1.6 Water depths where amphibians were found..... 48

Table 1.7 Spearman rank correlations for habitat and ranid relative abundance..... 49

Table 1.8 Summary of Oregon spotted frogs marked and recaptured..... 50

Chapter 2: Effects of the Herbicide Imazapyr on Juvenile Oregon Spotted Frogs

Table 2.1 ANOVA for frog size and body condition at the start of the experiment..... 83

Table 2.2 Water quality from treatment mixing buckets and frog tanks..... 84

Table 2.3 Estimates of linear mixed model fixed effects on feeding behavior..... 85

Table 2.4 Estimates of linear mixed model fixed effects on feeding rates..... 86

Table 2.5 Estimates of linear mixed model fixed effects on body condition over time..... 87

Chapter 3: Effects of the Herbicide Triclopyr on Metamorphic Northern Red-legged Frogs

Table 3.1 Number of frogs in each treatment by genetic group, sex, and status of deformities. 126

Table 3.2 Water quality pooled across days..... 127

Table 3.3 Observations of metamorphs in different locations during the 96-h exposure..... 128

Table 3.4 Movement behavior categories observed during behavior trials..... 129

Table 3.5 Estimates of linear mixed model effects on the interaction of treatment with limb deformities..... 130

Table 3.6 R^2 values from ordinary least squares regression of ln mass on ln snout-vent length. 131

Table 3.7 Body condition indices of frogs with and without scoliosis..... 132

Table 3.8 Distribution of lesion severity between the livers of control and tank-mix exposed frogs..... 133

Chapter 4: Snakes on a Visual Plane: Adding Fear of Predation to Toxicity Tests with Anurans

Table 4.1 Experimental design..... 162

Table 4.2 Water quality from frog boxes..... 163

Table 4.3 Frog behaviors observed during snake trials..... 164

Table 4.4 Number of frogs expressing behaviors before snakes were introduced..... 165

Table 4.5 Proportion of frog movements that occurred during specific snake behaviors..... 166

Table 4.6 Sample sizes required given behaviors from snake-exposed frogs..... 167

Table 4.7 Sample sizes required to detect differences in mean crickets consumed..... 168

ACKNOWLEDGEMENTS

My sincere gratitude goes to the members of my supervisory committee, each of whom contributed thoughtful guidance leading me to the completion of this work. I must especially acknowledge my Chair, Christian Grue, who provided me with a home base for the entirety of my graduate career, and taught me what it means to be a good scientist and a good teacher, without compromise. This project would not exist without the advice and support of Marc Hayes. Marc has also been a central figure in my graduate career and I look forward to continued discussions of all things related to amphibian ecology. Dave Beauchamp and Aaron Wirsing provided invaluable insight to predator-prey ecology and behavior. Each of them challenged me to see the broader implications of my research and I am grateful for their feedback and encouragement. Additionally Elaine Faustman (Graduate Student Representative) provided constructive comments that helped me to see the risk assessment context and the importance of reaching out to a broader, interdisciplinary audience. Several people provided technical assistance with field and laboratory work. My partner in the field surveys was Julie Tyson, who always had the right gear for the situation. Julie maintained the marked Oregon spotted frog database, spent long hours processing traps, and generally kept us all on track. Sasha Troiano and Schuyler Pearman-Gilman were my partners in the lab. They did a fantastic job of managing the process and keeping us moving through the tasks at hand. Sasha also assisted in the field work, as did J. Martin Grassley, Marc Hayes, Tiffany Hicks, Cole Roberts, and Michael Smith. Martin Grassley provided invaluable assistance with study design and in the lab. Robert Fisk also assisted in the lab. D. Heimer, Washington Department of Fish and Wildlife, provided the constituents of the imazapyr tank mix. SePro provided the triclopyr. Woodland Park Zoo provided the Oregon spotted frogs. Port Blakely Tree Farms provided access to the Dempsey

Creek site and Washington Department of Fish and Wildlife provided access to West Rocky Prairie. The University of Washington Hatchery provided space for rearing the northern red-legged frogs. Funding was provided by Washington Department of Ecology through two Aquatic Weeds Management Fund Grants. Support for this work was also provided by the School of Aquatic and Fishery Sciences. The use of trade names or products does not constitute endorsement by the agencies affiliated with this research.

DEDICATION

To Ira Woyar.

EXECUTIVE SUMMARY

Restoration of wetlands often begins with the control of invasive plants, yet the impacts of invasive plant management on wetland fauna have rarely been considered. Presence of listed amphibians may require risk assessments for impacts of management to those species. Risk assessments are limited by available data, including a lack of ecological and toxicity data for amphibians. In lieu of data specific to the target species, risk assessments use data from fish and invertebrates as surrogates for impacts to aquatic (often larval) amphibian stages, and terrestrial birds and mammals as surrogates for terrestrial stages. Terrestrial risk assessment data use estimated consumption rates and feeding behavior, yet amphibian feeding ecology rarely corresponds to bird or mammal feeding ecology. Moreover, terrestrial-stage amphibians are likely to have multiple routes of exposure, including dermal absorption in addition to consumption of contaminated prey. To accurately assess the risks of invasive plant management on amphibians, one must consider the species and life stages at risk of exposure to management activities.

This dissertation was designed to address data gaps in risk assessment of Pacific Northwest amphibian exposure to invasive plant management in wetlands. It was funded by two Aquatic Weed Management Grants from Washington Department of Ecology. The first grant was designed to address the specific issue of reed canarygrass (*Phalaris arundinacea*) management in Oregon spotted frog (*Rana pretiosa*) habitats (Chapters 1 and 2). The second grant was designed to address management of broadleaf wetland weeds like purple loosestrife (*Lythrum salicaria*) and the interaction of herbicide application with physiological and ecological stressors (Chapters 3 and 4).

The project began with a high-intensity field survey of native amphibian species and life stages in locations with remnant Oregon spotted frog populations and reed canarygrass (Chapter 1). Results showed that post-metamorphic young-of-year Oregon spotted frogs remained in the wetland through the summer. In contrast, northern red-legged frog (*Rana aurora*) young-of-year left the surveyed areas of the wetlands soon after metamorphosis. However, northern red-legged frogs in the process of metamorphic climax were present in high numbers for short periods, and were observed later in the season than metamorphosing Oregon spotted frogs. These data suggest that the youngest life-stages at risk of exposure to late summer herbicide applications for control of reed canarygrass are young-of-year Oregon spotted frogs and metamorphosing northern red-legged frogs.

Data from the field study informed the subsequent laboratory toxicity trials described in Chapters 2 and 3. Oregon spotted frog young-of-year were exposed to imazapyr + Agri-Dex tank mixes (Chapter 2) and northern red-legged frog metamorphs were exposed to triclopyr + Competitor tank mixes (Chapter 3). Frogs were exposed for 96 h and then reared in clean water for 2 months to assess latent effects. Endpoints included behavior, cricket consumption over time, body condition over time, and liver condition. No statistically significant effects occurred with Oregon spotted frog exposure to imazapyr tank mixes. Northern red-legged frogs were stressed during exposure to the tank mix, which may have resulted in the statistically significant increase of 1 day in time required to complete metamorphosis. Moreover, an interaction with tank-mix exposure was observed in cricket consumption. Individuals with limb deformities that were inherent in the rearing population ate fewer crickets with exposure to the tank-mix than controls with and without deformities and tank-mix exposed frogs without limb deformities. The interaction may not be biologically significant given that frogs with limb deformities are unlikely

to survive, yet it warrants caution that prolonged exposure to a triclopyr + Competitor tank mix may interact with existing stressors to decrease consumption rates, potentially resulting in reduced overall fitness.

The need for more ecological realism in toxicity tests is addressed in Chapter 4 by the design and baseline testing of a method to incorporate fear of predation into toxicity tests for terrestrial-stage amphibians. Amphibians are important prey for snakes, and the field results in Chapter 1 indicated overlap of garter snake (*Thamnophis* spp.) presence with that of metamorphosing and juvenile frogs. Stress associated with fear of predation can increase mortality of larval amphibians when they are exposed to a combination of pesticides and the chemical scent of predators. Visual cues from predators may be more important for terrestrial life stages of amphibians, yet no method exists by which visual cues are incorporated into toxicity tests. Young-of-year northern red-legged frogs were housed in one of two (clean water or damp terrestrial) substrates in a clear plastic box inside a larger arena where a snake was visible, but could not access the frog in the box. Frogs were exposed to the snakes three times over the course of 4 days, and behavior was assessed for differences among snake and control (non-snake) treatments and the substrates. Data were used to determine baseline behavior metrics and sample sizes that would be required for exposures with chemicals. No differences in frog behaviors were observed between the substrates, but frogs exposed to snakes were more likely than controls to move, and they used movements generally indicative of evasive behavior, whereas controls did not. Evasive movements by snake-exposed frogs included jumping and swimming. Sample size estimates for toxicity tests with predator stress were large for some metrics, but within the range of standard toxicity test requirements for metrics including cricket consumption and one of the behaviors associated with flight (jump).

Data showing minimal effects often go unpublished, yet they are important for managers and policy-makers trying to address competing ecological needs and a concerned public. Results from the field work indicate that existing toxicity data for larval amphibians may not be relevant to the species and life stages at risk of exposure to aquatic weed management in the region. Toxicity tests with aquatic herbicides show minimal effects of tank mixes used in wetland restoration, yet suggest caution when transitional life stages are concerned. Finally, a new method for ecologically-relevant toxicity tests was developed. This dissertation addresses data gaps that will improve our ability to assess risks of wetland restoration to amphibians in the Pacific Northwest.

CHAPTER 1: AMPHIBIAN PHENOLOGY ASSOCIATED WITH AQUATIC WEED MANAGEMENT

Abstract – Invasion by non-native plants leads to wetland loss and degradation, a primary factor in amphibian declines. Herbicides are often used to manage invasive wetland plants, but data gaps that exist in native amphibian ecology limit the effectiveness and scope of risk assessments for use of herbicides in amphibian habitats. To address those data gaps, a high-intensity field survey of native amphibian species and life stages was conducted in locations with remnant Oregon spotted frog (*Rana pretiosa*) populations and invading reed canarygrass (*Phalaris arundinacea*). Surveys were timed to correspond with the weed management season to identify species and life stages at risk of exposure to herbicide applications to manage reed canarygrass. Results showed that young-of-year Oregon spotted frogs remained in the wetland habitats through the summer. In contrast, northern red-legged frog (*Rana aurora*) young-of-year left the surveyed areas of the wetlands soon after metamorphosis. However, northern red-legged frogs in the process of metamorphic climax were present in high numbers for short periods, and were observed later in the season than metamorphosing Oregon spotted frogs. These data suggest that the youngest life-stages at risk of exposure to late summer herbicide applications for control of reed canarygrass are young-of-year Oregon spotted frogs and metamorphosing northern red-legged frogs. Data from field surveys of amphibian ecology that reflect relevant time-frames and identify the actual species and life stages at risk of exposure are important to inform accurate risk assessments and minimize impacts of herbicide use in wetland habitats.

Keywords – Amphibians, Phenology, Habitat, *Rana pretiosa*, Risk Assessment

INTRODUCTION

Wetland loss and degradation are considered primary factors in amphibian declines [1]. Invasion by non-native plant species is a common mechanism by which habitat degradation progresses [2]. Work to restore wetland habitats often involves management of invasive plant species through removal or control by mechanical, cultural, or chemical means. Managers working to restore wetland habitats for species of concern may be limited in weed control options without sufficient data to assess risk of herbicide exposure. Risk of exposure includes the hazard (toxicity) of the herbicide and the exposure scenario—the species and life stages present in the habitat at the time of herbicide application. This information is critical to managing habitat restoration and enhancement activities for listed species.

The Oregon spotted frog (*Rana pretiosa*) is listed as a Federal Proposed Threatened and a Washington State Endangered species. The *Washington Draft Recovery Plan for Oregon Spotted Frogs* [3] includes habitat management as a recovery strategy, and lists several tasks for recovery of the species' populations that also correspond to the potential for exposure to aquatic weed management activities. Included in those tasks are determining essential habitat and connectivity corridors (Priority 1- actions to prevent extinction), monitoring larval development and dispersal (Priority 2- actions to monitor and prevent significant declines), and determining relationships between environmental conditions and surface activity (Priority 3- other actions necessary to meet objectives) [3].

Habitat enhancement for Oregon spotted frogs includes the management of reed canarygrass [Phalaris aurundinacea L., 4] by control or removal. Reed canarygrass is a common issue for wetland restoration in the Pacific Northwest [5]. Control options are limited by the ability of reed canarygrass to spread prolifically by seeds and rhizomes, and effective control

may require the use of herbicide as one of several management tools [6]. Herbicide application after mowing in late summer may provide the most efficacious control with the least amount of chemical [7]. Though habitat enhancement is targeting Oregon spotted frog recovery, other sympatric amphibians may also be impacted by management activities.

A working knowledge of post-breeding habitat associations and phenology of amphibians is key to assessing their potential for exposure to weed management and/or wetland restoration activities. With information about post-breeding phenology, managers may be able to mitigate potential risks to species of concern by timing weed control activities to avoid critical life stages and habitats. Moreover, risk assessment and toxicity data are improved when information is applied to appropriate species and life stages. The purpose of this study is to fill data gaps on Pacific Northwest still-water breeding amphibians during the weed management season. Particular emphasis is placed on species-habitat associations, location of different life stages, and the pattern of phenology of amphibian communities in locations with remnant Oregon spotted frog populations.

MATERIALS AND METHODS

Weekly surveys were conducted late May to early October, 2010 to assess spatial and temporal overlaps of Pacific Northwest amphibian species and life stages with aquatic weed management. Habitats were assessed for vegetation at the start and end of the field season, and hydrology and temperature were recorded in each plot during the surveys. In addition to recording all amphibians detected during surveys, other species including fish, reptiles, and invertebrates were recorded to provide context to the ecological communities at the sites.

Study sites

Surveys were conducted at two field sites in Thurston County, Washington, where remnant breeding populations of Oregon spotted frogs occur. The Beaver Creek wetlands are located at West Rocky Prairie (hereafter WRP), a WDFW property located approximately 19 km south of Olympia, WA. The Forbes locality at Dempsey Creek (hereafter DC) is owned by Port Blakely Tree Farms, LP, and located approximately 16 km southwest of Olympia, WA.

Both sites consist of palustrine wetlands, defined by Cowardin et al. [8] as areas that are permanently to semi-permanently flooded up to 2 m deep and < 8 ha. The WRP wetlands include emergent prairie and scrub wetlands with two deep channels on the east (CHE) and west (CHW) sides, and a dug-out pond (P1) in the southeast. Small elevation changes on the site resulted in a complex of shallow and deeper emergent wetlands with a gradient of hydroperiods. Eight 15 x 30 m plots were already established by WDFW for reed canarygrass manipulation and amphibian egg mass surveys for an associated project. The existing plots were located with four on the east side of CHE, in a shallow emergent area. Four additional plots were located on the north end of the emergent wetland between the two channels (Fig. 1.1). Those plots were placed along a gradient of elevation that resulted in varying depths. All of the existing plots alternated between unmowed (experimental control) and mowed treatments. Mowing was applied the previous fall.

In a stratified random approach, five of the existing plots, representing a gradient of three water depths, were selected for the summer surveys. Two plots, one mowed (T1) and one unmowed (C2), were randomly selected from the shallowest area on the east side of the wetland complex. Three plots were selected from the north side of the complex; two of those plots represented an intermediate water depth, one mowed (T3) and one unmowed (C4). The third was

a deep mowed plot (T4). Two additional, 15 x 30 m plots were added. One was located in a deep area north of the existing plots to serve as a comparable deep unmowed plot (EMN). The second plot (EMS) was located in the southern, shallower area of the wetlands that was dominated by reed canarygrass (Fig. 1.1). The emergent plots EMS, T3, and T1 were oviposition sites for Oregon spotted frog as determined from surveys by WDFW in February and March (personal communication, J. Tyson, WDFW).

The DC site was an active cow pasture adjacent to Dempsey Creek, a tributary of the Black River. No established plots existed on the site. There were two ephemeral channels, one running through the main pasture (CHS) and one north of the farm road around the pasture (CHN); and four ponded areas with varying depths (Fig. 1.2). The CHN bordered an incline leading down from the farm road, and a scrub wetland to the north. One small, shallow pond was located on the west end of the channel (PW), and another small pond was located southeast of the channel (PE). The CHS ran through the main pasture and flowed into an emergent wetland. Oregon spotted frog oviposition was located in the inlet pool by the culvert on the west end of CHS. The emergent wetland had deep (1 m) channels but little to no open water. It intersected the pasture and Dempsey Creek. One small open water area in the south of the emergent wetland accounted for one of the four ponds that were sampled (Lily). A dug-out pond in the southeast of the emergent wetland, adjacent to the terrestrial forest, was also sampled (Overlook).

Vegetation surveys

Vegetation data were collected to provide detail on amphibian association with microhabitats during the weed management season, with particular focus on the potential influence of reed canarygrass on amphibian presence in the plots. Vegetation data were collected from 10 x 10 m plots in May/June and October using the relevè method [9]. At WRP at least one

vegetation survey plot was placed in all of the plots that were surveyed for amphibians. Two vegetation plots were placed in the existing 15 x 30 m amphibian survey plots, one each randomly placed in one half of the 30 m length of the plots. One vegetation plot was placed in the remaining emergent wetland, channel, and pond habitat plots. Vegetation plots were randomly placed along the channels within the amphibian survey area and in a 2 m band around the perimeter of the dug-out pond. At DC, CHS was divided into three sections and randomly placed three vegetation plots along the length of CHS, one in each section. Data were also collected from plots located within the reed canarygrass-dominated wetland between the pasture and the main stem of Dempsey Creek and in the emergent wetland west of the pasture, but both areas were difficult to access and were abandoned for use in amphibian surveys due to logistical constraints. Whenever possible, plants were identified to species on site or fruiting bodies were collected to key-out later using a dissecting scope.

Amphibian surveys

Systematic surveys were conducted approximately once per week, starting 25 May and ending 30 September 2010, with no surveys during the last week of May at DC and first week of June at WRP. The surveys were conducted in 17 and 18 weeks at DC and WRP, respectively. Amphibians observed during vegetation surveys in early May (WRP), early June (DC) and 4 October (both sites) were also recorded for timing of presence. With the vegetation survey day data, a total of 19 (DC) and 21 (WRP) weeks of amphibian presence data was collected.

A combination of visual encounter and dip-net sampling was used to detect amphibians each week. Baitwell nets (Forestry Suppliers), 40.6 cm^2 by 30.5 cm deep with 0.48 cm nylon mesh, were used to collect dips during systematic dip-net sampling and to catch amphibians detected during visual encounter surveys. In an attempt to avoid habitat alteration from frequent

sampling, dips were collected haphazardly by the surveyor standing in the approximate sample location. If no water was available within 1 m of the sample location, a dip was collected from the nearest available water within 3 m, as long as no dip had yet been collected from the available water. When no water was available, observers conducted a spot visual encounter survey at each dip location. Water depth was measured at each systematic dip location to obtain available depth data and from any encounter with amphibians, regardless of survey method, to obtain amphibian habitat-use data.

Visual encounter surveys at WRP were conducted between 0800 and 1700. The plots were sampled in order of accessibility because most plots could not be accessed without crossing, and thus disturbing, another plot. The shallow plots were always sampled first because one had to pass through them to reach the rest of the wetland. The pond followed them, then CHE, the deep and medium depth plots, CHW, and the RCG plot. Visual encounters in the established 15 x 30 m plots involved two observers, at least one wearing polarized glasses, who walked the perimeter of the plots, the second person starting when the first had reached 15 m along one edge. After both surveyors completely circumnavigated the plot, one surveyor dip-netted the plot in 10 sample locations that were oriented along the center of the 30 m length of each plot. Five dip-net sample locations were spaced 6 m apart on each side, approximately 5 m from the plot edge.

A similar systematic sampling methodology was applied to the channel and pond habitats. At WRP, a 30-m section of CHE was randomly selected at the start of the season, and that same section was sampled each week. Visual encounter surveys were conducted with two observers as described for the plots, with the observers walking up one side of CHE, crossing it at the end of 30 m, and walking back down the opposite side of the channel. Observers then

alternated taking dips every 6 m from either side of CHE, starting 3 m offset, so 10 dips were collected in total, 5 dips from each side. The CHW was too deep to cross early in the season and the far edge was not navigable by surveyors, so a 60-m section was randomly selected for the weekly sampling. Observers sampled from the east edge, conducting visual encounter surveys as described, but the second surveyor also collected dips every 6 m, simultaneous with the visual encounter effort. The 10 dips were alternately taken from the shallow bench and the interior depths of the channel until the bench dried in mid-season. When the bench dried, dips were collected alternately from the channel edge and interior.

Surveys in the channels at DC were based on the channels' ephemeral nature. Dips were collected systematically until the channels dried into disconnected pools. At that time, the same channel location was surveyed for visual encounters and dips were collected wherever water was still available along the channel. Multiple dips were often collected in the remaining pools of water.

Surveys in the area around CHN started at PW, where the start and end water and air temperatures were collected. From PW, observers conducted visual encounter surveys while walking east along CHN. At the end of approximately 350 m, the observers moved south through an area dominated by reed canarygrass, to PE. Dips were collected every 3 m around PE. After dipping the pond, the observers returned to CHN and collected 10 dips every 10 m along the channel to PW. Dips were then collected every 3 m around PW.

Surveys in CHS were conducted along approximately 580 m of ephemeral channel that ran between the culvert below the farm road on the west side of the pasture, and the emergent wetland to the east. Oregon spotted frog egg masses were typically located in the ponded area near the culvert. Initially, 30 dips were collected equidistant (approximately every 20 m) along

the length of the channel. As the channel dried into separate pools, dips were collected wherever ponded water was available, and multiple times within larger pools.

Observers circumnavigated the edge of the ponds as described for the plots and channels. After both surveyors returned to the starting location, one person dipped every 3 m around the pond until 10 dips were collected or, if the pond circumference was too small for 10 dips at the 3-m spacing, until circumnavigation was completed. Dips were collected alternately at the shallow bench and the interior of P1 at WRP.

Permanent trap arrays were established at both sites. At WRP, two trap arrays with 5-m drift fences and four funnel traps (two paired on each end) were placed in the southwest and northeast edges of P1. One 10-m array with six funnel traps was placed in CHW, south of the visual encounter/dip sample transect. Two 15-m arrays with eight funnel traps were also deployed. One was located in the deep emergent area in the northwest of the wetlands (EMNW), along the edge of the emergent and scrub habitats. The second was in a channel on the east side of the scrub wetlands (S1). At DC, trap arrays were placed in Dempsey Creek and the two ponds in the emergent wetland, Overlook and Lily. Both ponds were adjacent to the emergent wetland where vegetation data were collected in June but where amphibian surveys were not conducted due to logistical constraints.

Trap arrays were installed by attaching small collapsible minnow traps (Promar, Ranger), 45.7 x 25.4 cm with 1.3 cm opening to drift fences. The drift fences were attached with zip ties to several PVC pipes or fiberglass dowels. Traps were paired on either side of the drift fence, and attached to the fence with zip-ties. Based on water levels at the time of trapping, drift fences and traps were raised and lowered as needed to ensure contact with the surface of the water to prevent drowning of adult amphibians. The traps were used to increase detection rates, sample in

deeper water, sample habitat edges, and sample in areas with woody vegetation or where logistical constraints prevented weekly systematic sampling. Traps were open overnight approximately every 3 weeks. Traps were checked each week to confirm that they were not open when not in use.

All amphibians detected at each site during the course of a survey day were identified to species and life stage whenever possible, and recorded regardless of whether they occurred in an established study area. When amphibians were detected after the start of systematic surveys, the location, general habitat, and water depth were recorded. Juvenile and adult ranids were weighed and shank and snout-vent lengths (SVL) were collected. Juveniles were distinguished from young-of-year (YOY) based on SVL at the time of capture. Date of capture and shank-lengths were later used to confirm and correct coding so that only individuals that metamorphosed in the survey season were coded as YOY. Ranid juveniles and adults were distinguished based on sex and SVL. Female adults were ≥ 60 mm SVL and male adults were ≥ 50 mm SVL, based on the sizes at which individuals are sexually mature [10]. Dorsal photographs were taken for use in measuring ranid tadpoles and salamander larvae. The photographs included a ruler placed at the bottom of the holding dish, and the ImageJ program [11] was later used to measure snout-vent and total lengths. Dorsal photographs were also taken of adult and second-year juvenile ranids. Individual Oregon spotted frogs that were photographically "marked" and recaptured through time were distinguished using dorsal spot pattern recognition from the photographs. Locations of marked individuals through time were assessed for movement patterns. PowerPoint (Microsoft 2003) slides were created for each individual adult and juvenile. Slides were visually searched to identify individuals that were recaptured each week, and new slides were added as new

individuals were caught. Water and air temperatures were recorded at the start and end of all systematic surveys.

Statistical analyses

All amphibian data used for correlation analyses and comparisons among plots were summarized as mean relative abundance over time. Dip, visual encounter, and trap relative abundances were estimated from the sum captures/number of dips, sum of visual encounters/meter, and sum of captures/trap, respectively. Relative abundances from dip and visual encounter data were not normally distributed. Habitat variables and amphibian relative abundances within each plot were assessed using Spearman Rank correlations. Upon visual assessment of the data, differences in amphibian relative abundance among the mowed and unmowed plots at WRP were not statistically assessed due to the confounding factor of the depth gradient among the plots and an insufficient number of plots for paired analyses.

In addition to the amphibian data, water depth and total proportion of dips that were dry were summarized from the systematic dip surveys in each plot. The data were also included in microhabitat assessments, and were used to identify variables that may be autocorrelated with hydrologic data. Because of the strong correlation between depth and proportion dry, and the potential for subtle differences in the way in which amphibians respond to each variable, depth was assessed using the Chi-square test of the distribution of available depths and the observed depths in which amphibians were recorded. The proportion dry was assessed using Spearman Rank correlations with amphibian relative abundance data.

Vegetation habitat variables were quantified for spring and autumn. Species were pooled to create complexes (e.g. *Carex* complex) to account for variability of species among plots. Spearman Rank correlations were used to assess microhabitat relationships among amphibian

data and the proportion of each vegetation plot represented by the dominant habitat variables (those that occurred in five or more plots), including species complexes, reed canarygrass, and open water. Bonferroni corrections were applied to the alpha value for multiple comparisons. All correlations with $\alpha = 0.10$ are presented here to inform future research on areas where biological significance may be indicated, despite a lack of statistical significance after correction for multiple comparisons.

RESULTS

Plot characteristics

Vegetation

Vegetation at Beaver Creek was generally comprised of *Carex obnupta*, *C. utriculata*, *Eleocharis palustris*, *Juncus effusus*, and invading reed canarygrass). North of the emergent wetlands was a scrub area dominated by *Malus fusca* and *Spirea douglasii*. The dug-out pond was bordered by *C. utriculata* and reed canarygrass, and contained *Potamogeton natans* and *Utricularia vulgaris*. Reed canarygrass was the predominant emergent vegetation in many of the shallowest areas of the wetland, generally on the east side of the CHE, and the south side of the emergent wetland area between the channels. In the deeper emergent wetlands, reed canarygrass had been invading and forming raised hummocks, especially along CHE.

Vegetation at DC was predominantly reed canarygrass, *C. obnupta, C. utriculata*, and *J. effusus* along the channels, with *Typha latifolia*, *Sparganium emersum*, *Callitriche heterophylla* and *Nuphar polysepala* occurring in and around the ponded areas. The emergent wetland between the pasture and Dempsey Creek contained a large area dominated by reed canarygrass. The southeast area of the emergent wetland had a more complex vegetative structure, with areas

of predominantly *Potentilla palustris*, *C. obnupta*, and *T. latifolia*, with patches of scrub with *S. douglasii* and *Salix* spp, including at the border of the wetland and Dempsey Creek.

Habitat categories for vegetation and depth data were established for use in correlations with amphibian dip and visual encounter data. The dominant vegetation categories, e.g. those that occurred in five or more plots, were assessed for autocorrelation with other habitat categories. Depth, open water, and proportion dry were considered strong confounding factors for habitat variables, so all habitat categories that were significantly correlated with water variables were removed from analysis with amphibian data (Table 1.1). The remaining habitat variables (those not significantly correlated with water) included median percent cover of Carex from spring and autumn vegetation surveys, and reed canarygrass from autumn.

Hydrology

Several plots at WRP dried at the end of July until it rained again at the end of August (Fig. 1.3). However, the channels, pond, and the deep unmowed plot (EMN) retained water through the summer. By 3 August the shallowest plots were dry. On 10 August searches beneath the organic matter at each dip location in the shallow plots found no amphibians. By 1 July at DC the culvert pool in CHS was disconnected from the rest of the channel. The culvert pool was one of two disconnected pools formed at that time. Over the summer, CHS dried into disconnected small pools until the 29 July survey, when the whole channel was dry. The channel remained dry until the 26 August survey. See Table 1.2 for water depths from all systematically sampled plots, channels, and ponds.

Temperature

Air and water temperatures recorded at WRP during mid-day surveys corresponded respectively to the maximum and mean air temperatures recorded by the Olympia Regional Airport weather station (wunderground.com) (Fig. 1.4). The correlation of mid-day wetland water temperatures to mean daily air temperatures reported by regional weather stations has been observed in other amphibian surveys [12].

Amphibian survey summary

Amphibians were observed more frequently at WRP than at DC (see Table 1.3 for summary of captures and relative abundance). Six species of amphibians were detected in and around the sampled plots at both study sites. At WRP, all of the species were native, lowland amphibians that breed in still water. No western toads (*Anaxyrus boreas*) or non-native American bullfrogs (*Lithobates catesbeianus*) were seen. At DC all but one of the species detected were native, lowland amphibians. No western toads or long-toed salamanders (*Ambystoma macrodactylum*) were observed. One American bullfrog tadpole was caught in Dempsey Creek outside of systematic sampling activities.

The proportion of observations that were detected during systematic dip-net and visual encounter surveys and served as the basis for the relative abundance estimates ranged from 74 percent at WRP (906 observations) to 75 percent at DC (310 observations). The remaining observations were used to inform the timing of presence. They include observations recorded during site visits prior to the start of systematic sampling (WRP: 22 records, DC: 3 records), during the last visit to remove traps from the sites (WRP: 17, DC: 14), detections of frogs by sound only (WRP: 18 and DC: 4), and observations outside of systematic sampling efforts. Data recorded outside of systematic sampling included sightings of amphibians while surveyors were

in-transit between plots, opening or checking traps, or collecting additional dips near traps (WRP: 265, DC: 85).

Individuals that were not identified to species, often because they were detected but not caught, accounted for five and nine percent of the total observations at DC and WRP, respectively. Seven of the unidentified ranids at WRP were detected only by their calls. There were also a few unidentified individuals from trapping, including nine percent (21) of the total amphibians trapped at WRP. One unidentified ranid tadpole was found deceased in a trap at DC. Five of the unidentified individuals from trapping at WRP were deceased due to predation. The remaining unidentified individuals were not identified in the field and identification was not possible from the available photographs. In addition, two frogs at WRP were identified as "hybrid" because they had characteristics of northern red-legged frogs (*Rana aurora*) and Oregon spotted frogs and could not be definitively assigned to one species.

Several fish and predators of amphibians were observed. Olympic mudminnows (*Novumbra hubbsi*) and three-spined sticklebacks (*Gasterosteus aculeatus*) were the most commonly observed fish at both sites and the only fish observed in the wetlands at WRP. Several other fish were recorded at DC, including *Cottus asper*, *C. perplexus*, *Lepomis* sp., *Oncorhynchus kisutch*, and *Rhinichthys osculus*. One lamprey was caught in the traps at DC. Lamprey larvae are difficult to identify to species, but the lamprey could have been one of three species, *Lampetra ayresii*, *L. richardsoni*, or *Entosphenus tridentata*; most likely one of the latter two. The river lamprey (*L. ayresii*) is a Candidate Species on the Washington State Endangered Species list, but the species is not known to travel as far up-stream to spawn as the Dempsey Creek site (M. Hallock, WDFW, personal communication).

Belastomatids and dytiscids were observed at both sites, but were more frequently observed at WRP. Signal crayfish (*Pacifastacus leniusculus*) were observed at DC, not WRP. Two species of garter snakes, *Thamnophis sirtalis* and *T. elegans* were recorded at WRP, but there were 12 visual encounter observations of garter snakes not identified to species that may have included a third species, *T. ordinoides*. All three garter snake species were recorded at DC. Leeches were observed in greater proportions at WRP than DC. Evidence of mammals at WRP included mustelid scat and one observation of a vole, species unknown.

Amphibian life-stage phenology

Amphibians were observed in every week, 6 May to 4 October 2010. There were clear differences in natural history among species based on the life stages and timing of amphibian presence in the wetland. Larvae and tadpoles were observed from all six species detected at each site. Metamorphic individuals of all species were detected, except those for which only one individual was captured at each site. The post-metamorphic young-of-year stage was detected for each of the native frog species recorded. Second-year juvenile northwestern salamanders (*Ambystoma gracile*), northern red-legged frogs, and Oregon spotted frogs were also present.

Northwestern Salamander

Two cohorts of northwestern salamander larvae were identified and separated into first- and second-year larvae (Table 1.4, Fig. 1.5). Second-year larvae were those with SVL > 35 mm. The criterion for larval age was based on a clear break between the sizes of larvae, with none captured between 32 and 36 mm SVL (Fig. 1.5). First-year larvae were caught in every week from 8 June to 28 September at WRP. At DC, first-year larvae were caught 63 percent of the time: in every week from 17 June to 26 August, and one more individual was captured 30

September. Second-year (or older) larvae were caught at least once in every month at both sites; 36 and 42 percent of the time at WRP and DC, respectively. Metamorphs at both sites were in the second-year size class and there was no indication that metamorphosis occurred within the first season (Fig. 1.5). One metamorph was captured at DC 1 July; all others were caught during August.

All second-year northwestern salamander larvae and metamorphic individuals at WRP were <65 mm SVL. Larvae at SVLs 65 mm and above may be considered neotenic (larviform) adults that remain in the water through the summer rather than metamorphosing and moving into the upland, as terrestrial adults do [13]. No neotenic adults were observed at WRP, and the presence of a neotenic population at the site is uncertain. Regardless, first and second-year larva were present in areas that maintain water year-round. In contrast, four larvae > 65 mm SVL were caught at DC; all bore characteristics associated with neotenic adults, including distinct black spots against a green background color (personal observation) and heads shaped like an hour-glass [14]. The presence of a neotenic population at Dempsey Creek was likely because no terrestrial-phase adults were detected in the wetlands during the survey period.

Long-toed Salamander

One long-toed salamander larva was caught at WRP on 13 July. Its SVL (23 mm) was just below the size at which the species is known to metamorphose in King County [12]. The species was not detected at DC.

Rough-skinned Newt

Rough-skinned newts (*Taricha granulosa*) were more frequently detected at DC (63% of the time) than WRP (14%). A pair was observed breeding during a brief site visit to DC in May,

prior to the start of vegetation and systematic surveys. Between mid-June and mid-September, adults were observed 64 percent of the time at DC, in 9 of 14 weeks. The only adult detected at WRP was trapped on 7 July. At WRP, larvae and metamorphs were caught in 2 weeks in August. Late stage [17; 15] larvae were caught 31 August and metamorphs [stages 19 and 20; 15] were caught 17 August. Four of the five larvae were similar in size to the metamorphs caught 2 weeks before (Table 1.4), which may indicate two cohorts from the early summer breeding period. Between mid-June and early September, larvae were caught 46 percent of the time at DC, in 6 of 13 weeks. One metamorph was caught 22 July; it was 4.5 mm larger than the mean SVL of metamorphs caught in August at DC (Fig. 1.6), and may have been an individual that overwintered. Larvae caught in late July and 19 August at DC were near the size of metamorphs caught at WRP (i.e., within physiological range of metamorphosis), and presumably metamorphosed within the survey season. The larva caught 8 September at DC was stage 14 [15] and 5 mm smaller than the average metamorph SVL from both sites (Fig. 1.6). The small size and late date of the last larva and the large size and early date of the first metamorph indicates that there may be at least two cohorts at DC that include a population of overwintering larvae. No larvae were caught after 8 September.

American Bullfrog

The American bullfrog tadpole was caught 29 July at DC. Its size (57 mm SVL) was larger than the expected range of first-year tadpoles from British Columbia, as described by Govindarajulu et al. [16]. The tadpole was removed by WDFW personnel who were on-site.

Pacific Treefrog

At WRP, Pacific treefrog (*Pseudacris regilla*) tadpole captures were highest in the first systematic sample week (25 May) and declined to one capture in each of the first two weeks of July. In contrast, the tadpole captures at DC were highest during the last weeks in which they were observed, until 22 July, likely due to greater detectability in shrinking pool sizes. Pacific treefrog metamorphosis occurred over 6 weeks at WRP. The metamorphic period may have been shorter at DC, with metamorphs observed only in the first 2 weeks of July. The last tadpoles and metamorphs were observed at both sites in mid-July. One post-metamorphic young-of-year was observed within the metamorphic period at each site. Adults were observed in the wetland only in the first week at each site. No other Pacific treefrogs were detected within the wetlands until adults started calling from the trees and uplands in mid-September. Males were heard calling in every week after that to the end of the survey, 4 October.

Northern Red-legged Frog

Northern red-legged frogs were recorded in every sample week at WRP and in 14 of 19 weeks (74%) at DC. The youngest cohort, from the larval to first-year post-metamorphic (young-of-year) stages, was most common at both sites. The last tadpole was captured 15 July at DC and 24 August at WRP. Metamorphs were detected in every week between 6 July and 24 August, and on 9 September at WRP; only one metamorph was observed at DC, 22 July. Metamorphosis was distributed bimodally, with the first peak of metamorph abundance at WRP in mid-July and the second peak in early August (Fig. 1.7). Though metamorphs were not frequently detected at DC, the YOY abundance had a similar bimodal distribution to WRP metamorphs'. Young-of-year were present 13 July to 4 October, but were most abundant between 20 July and 31 August at WRP. At DC, YOY were detected at low levels, 67 percent of the time, starting on 1 July. Young

of year at DC were larger on average than those from WRP (Table 1.5). A decreasing presence of YOY was observed from the end of August through September at WRP (Fig. 1.8). Juveniles (young of the previous year, SVL < 50 mm if male, <60 mm if female) were detected 64 percent of the time at WRP, in every week but one between 6 May and 17 August, and only twice at DC. One adult male was caught at each site; no other adults were detected in the wetlands during the survey period.

Oregon Spotted Frog

Oregon spotted frogs were recorded in each survey week at WRP and all but 1 week at DC. Tadpoles were frequently detected at WRP until 6 July and at DC until 29 July. The last tadpole was observed at WRP on 3 August. Metamorphs were detected in every week between 29 June and 10 August at WRP, with peak detection rates in the second and third weeks of July (Fig. 1.7). The first metamorphs appeared on 15 July at DC, and were observed in 50 percent of the survey weeks between then and 1 September. Post-metamorphic YOY were present in every sample week from 13 July to the end of the survey period at WRP. At DC, YOY were detected in low numbers 56 percent of the time from 5 August to the end of the survey season, 4 October. At WRP, the number of YOY detected was similar in each week starting 31 August until the end of the survey (mean weekly count of YOY=17 ± 5 SD, Fig. 1.8). Juveniles were more frequently detected at WRP (86% of time) than DC (42%). At WRP, one adult was detected 15 June and in each week from 13 July to the end of the survey. Adults were detected 42 percent of the time at DC. Adults were detected in visual encounters when mean water temperatures were ≥ 12°C (Fig. 1.9) and mean air temperatures were ≥ 15°C (Fig. 1.10). A similar temperature relationship was observed with juveniles and YOY (Fig. 1.9), but they were also detected in mean air temperatures as low as 13°C (Fig. 1.10). There was no evidence that adult and juvenile Oregon

spotted frogs were detected at a rate other than expected given the observed frequencies of water (Fig. 1.9) and air temperatures (Fig. 1.10).

Habitat

The distribution of water depths at each site was an important habitat characteristic relative to the distribution of amphibians. Chi-square test results for binned data of available depth and the sum of all amphibians observed in each depth were highly significant for each site (DC, $X^2_{9,0.05} = 61.640$, $p < 0.0001$; WRP, $X^2_{7,0.05} = 37.128$, $p < 0.0001$). The amphibian distribution was shifted to slightly deeper water depths. At both sites the sum of total amphibians observed at each depth peaked between 24 and 28 cm, 4-8 cm deeper than the peak of available water depths (Fig. 1.11).

As expected, differences existed among species and life stages relative to the associated water depths in which they were found (Table 1.6). Generally northwestern salamanders were associated with deeper water, Pacific treefrogs with shallower water, and ranid frogs inhabited the median depth range.

Habitat correlations with amphibian relative abundance were assessed only for those species and life stages for which data existed in five or more plots from each survey method (dip and visual encounter). The proportion of dips that were dry in each plot was an important habitat variable associated with amphibian distributions. It was the only significant variable for northwestern salamanders (1^{st} year larvae $\rho = -0.699$, $p = 0.005$; 2^{nd} year larvae $\rho = -0.697$, $p = 0.006$) and Pacific treefrogs (larvae $\rho = 0.699$, $p = 0.005$).

Few habitat variables were significantly correlated with ranid frog relative abundance. Negative correlations with the proportion of dips that were dry existed for YOY and adult

Oregon spotted frogs and for metamorphic, YOY, and total northern red-legged frogs (Table 1.7). Spring Carex complex cover was the only variable significantly correlated with juvenile Oregon spotted frogs, representing the only plant-related habitat variable that maintained significance with an α corrected for multiple comparisons (0.025). However, that effect was only associated with juvenile Oregon spotted frog observations made while collecting dips; it did not hold for visual encounter data (Table 1.7). Adult Oregon spotted frogs were also positively correlated with spring Carex cover, an effect that approached significance after correction for multiple comparisons (Table 1.7). Metamorphic and juvenile northern red-legged frogs were negatively correlated with spring and autumn Carex cover, an effect that was not significant after correction for multiple comparisons and was not consistent among survey methods (Table 1.7).

Northwestern Salamander

Northwestern salamander larvae were only found in the deepest plots surveyed at WRP, and only in those plots for which the proportion of dips that were dry over the summer did not exceed 32 percent. They were observed in all areas surveyed at DC, including within the culvert leading to the west side of CHS after the channel was dry. At WRP, second-year larvae were observed in CHW and the scrub habitat, but were most abundant in P1. The only metamorphs were also caught in P1. Second-year larvae were not observed in CHS at DC. On average, more larvae were detected in PE than any other plot at DC. It is also where the only metamorphs were found. Three of the neotenic adults at DC were caught in the traps, two in Lily Pond and one in Dempsey Creek. The remaining one was caught in a dip survey of CHN. All three locations were associated with deeper water that was maintained year-round. The surveyed portion of CHN was an off-shoot of a deeper channel that was less accessible for surveying but that was likely the primary habitat for neotenes.

Rough-skinned Newt

Rough-skinned newt larvae were caught in the deepest unmowed plot, EMN, and trapped in the adjacent deep plot, EMNW, at WRP. Metamorphs were also observed in EMN. The only adult caught at WRP was in CHW. At DC, larvae were most abundant in PE, which is also where the only metamorphs were observed. They were also caught in PW and CHS, including in the culvert after the channel dried. Adults were observed in every plot sampled at DC.

Pacific Treefrog

Pacific treefrogs were most abundant in the shallowest plots at WRP, and only found in CHS at DC, including within the culvert. In the deepest (EMN and T4) and shallowest (C2 and T1) plots at WRP, Pacific treefrog larvae were most abundant in the mowed plots (T4 and T1). Metamorphs were only observed in mowed plots, T1 and T4. YOY were observed in CHE, which was adjacent to T1 and T4 as well as the willow clump from which adults were heard calling later in the summer. Pacific treefrogs were not caught in any trapping effort.

Northern Red-legged Frog

Northern red-legged frogs were rare in the shallowest plots at WRP. Tadpoles were most abundant and most frequently observed in the deepest unmowed plots (C4 and EMN) and the channels. They were also observed in the deeper mowed plots (T3, T4) and in P1. Trapping indicated greater abundance in EMNW, an unmowed plot with comparable depth to EMN, and in the scrub habitat, than in P1 and CHW. Tadpoles were only observed in CHS at DC, none were trapped. The relative abundance of metamorphs was highest in the deep unmowed plot (EMN) and the channels at WRP, followed by the deep mowed plot (T4) and the pond. The only metamorph observed at DC was in PE. YOY were most abundant in the pond and channel plots

at WRP, with the most frequent observations occurring in CHW. At DC, YOY were only observed in PW and CHS. More YOY were also trapped in the scrub habitat than in any other trapped plot at WRP.

Juvenile northern red-legged frogs were also observed in the scrub habitat at WRP, though they were not trapped. Relative abundance estimates showed some change in observed locations of juveniles that may indicate shifts in habitat use at WRP over the summer. Early in the season juveniles were observed in the emergent plots across all depths and treatments, in the pond, and in the channels. By the end of June, juveniles were rarely observed in the emergent plots, and observations were concentrated in the channels. The last observations of juveniles were recorded in the channels and near the traps in the scrub habitat in mid-August. At DC, juveniles were only observed in CHS at the end of June and the beginning of October.

At both sites, adult northern red-legged frogs were observed in peripheral habitats. At DC, the only adult was observed in the shallow water trail leading to Lily Pond. At WRP, the only adult was observed in a shallow trail adjacent to CHE.

Oregon Spotted Frog

In spring 2010 at WRP, Oregon spotted frog egg masses were located in mowed plots, T3 (30 masses), T2 (26 masses), and T1 (7 masses), with three masses outlying the plots near EMS and CHW. At the earliest survey on May 6, tadpoles were observed in T3. By mid-May, they were located in P1 and T2, and by the end of May tadpoles were observed in T1, T3, T4, P1, and C4. Tadpoles were observed in CHE in mid-June. On average they were most abundant in P1 and in mowed plots at WRP. Higher abundance in the pond was corroborated in trap surveys; they were also trapped in EMNW and CHW. At DC, tadpoles were most abundant in CHS,

corresponding to the placement of most of the egg masses at the site. Tadpoles were also observed within the culvert at CHS, in Dempsey Creek, and in all of the ponds except Overlook.

At WRP, no Oregon spotted frog tadpoles were trapped in the scrub habitat, but the only metamorphs caught in trap surveys were located there, and YOY were also observed. Metamorphs were most abundant in P1 but they were also observed in all of the mowed plots that were systematically surveyed, both channels, and only the deepest unmowed plots (EMN and EMNW). Metamorphs were rarely detected at DC. Only one was caught in CHS and the remaining individuals were all observed in the Lily pond. Young-of-year were observed in all of the systematically sampled plots at WRP, except the medium-depth mowed, T3. They occurred at low frequencies in all of the unmowed plots, including EMS, the reed canarygrass-dominated plot. They were most abundant in P1 and CHE. At DC, YOY were only observed in CHS, PE, and PW.

Like YOY, juveniles were most abundant in P1 and CHE at WRP. They were observed more frequently in the mowed plots than in unmowed plots, but they were rarely observed in either of the medium- depth plots. Relative abundance estimates from visual encounters indicated movement from the emergent wetland area, where the treatment plots were located, into the channel and pond habitats at the end of June. Juveniles began appearing again in one of the mowed plots at the end of September, though abundance in the pond and channel habitats was still high at that time. At DC, CHN did not appear to be an important habitat for the first-year life stages of Oregon spotted frogs. However, it was perhaps the most important habitat surveyed on the site for juveniles. Juvenile Oregon spotted frogs were most frequently detected in CHN and the ponds associated with the channel, PE and PW. They were also observed in CHS, but only on the last day at the site, 4 October.

Through spot-pattern recognition, 42 individual Oregon spotted frog adults and second-year juveniles were identified at WRP (Table 1.8). The day with the highest number of individuals captured (14 September) indicates a maximum detection rate of 40 percent. On that day, seventeen adult and juvenile Oregon spotted frogs were observed; 12 adults and 3 juveniles were known individuals and two adults were not caught for identification.

Twenty individuals were recaptured at least twice and 14 individuals were recaptured more than twice. Eight female and four male adults were recaptured. Six juvenile females, one juvenile male, and one juvenile too young to determine gender were also recaptured. Five were initially captured in the juvenile size range and their measurements during recaptures revealed a transition into the adult size-class during the survey period.

Most of the individuals were captured at least once in P1 (23 frogs, 55%) and/or CHE (20 frogs, 48%). Movement among plots was determined from 10 of the individuals recaptured. Half of the individuals that moved to a different area were adults. For both juvenile and adult recaptures, four females and one male were recaptured in a different location from a previous capture. Four individuals were recaptured in three different locations. For all of those frogs, the intermediate location was CHE, indicating use of the channel as a corridor for movement. The recapture locations of at least two individuals indicated use of the pond as summer habitat. Each of those individuals was captured in one location outside of the pond early in the summer, followed by four recaptures in the pond, and subsequently recaptured in the original capture location near the end of the summer. Sixteen individuals were captured in the same location two or more consecutive times.

From 39 observations of Oregon spotted frog juveniles and adults at DC, 21 individuals were identified using spot-pattern recognition. Fifteen individuals were not photographed or the

photographs were not of sufficient resolution to make identification possible. However, four of those were unlikely to have been recaptures based on size at the time of capture and the description of spots included in the field notes. No more than seven individual adults or juveniles were ever observed on one survey day. Of the 21 marked frogs, two were recaptured. One was captured three times in four consecutive weeks. The second was captured twice, at the end of July and the beginning of September. Both frogs were located in CHN each time.

Adults were typically observed at DC as single individuals in available pools of water (ponds or ponded areas along the channels). They were most frequently observed in PW and Overlook. In the week of 26 August, several adults were observed near the traps in Overlook. The only other day on which several adults were observed in one habitat was 4 October, the last day on the site, when five adults were observed in CHS.

DISCUSSION

Native amphibians were present in the wetlands wherever water remained through the weed management season. Hydroperiod and water depth were more important drivers of amphibian distributions than vegetation, but water was a confounding factor in the vegetation data. Depending on species, ontogenetic and seasonal shifts in location were observed, which would result in differential risk of exposure to weed management activities among native amphibian species. Risk assessment for aquatic life stage exposures to herbicides is most appropriate for northwestern salamanders and rough-skinned newts. In contrast, transitional and post-metamorphic anuran life stages are most at risk of exposure to herbicide application for management of reed canarygrass.

Pacific treefrogs and long-toed salamanders are generally associated with shallow, ephemeral water and a life history strategy of metamorphosis early in the growing season. Their

rare occurrence on the sites surveyed is perhaps more indicative of the timing of surveys than the relative abundance of those species at those locations. For those that were observed, the only long-toed salamander was in the shallowest mowed plot. Moreover, Pacific treefrog distribution appeared to have some interaction with depth and the mowed treatment, with a tendency for higher abundance in shallow and mowed plots. A similar pattern was observed at DC with the only Pacific treefrogs occurring in CHS, where cattle grazing maintained shorter vegetation and the channel dried-out.

The locations associated with second-year northwestern salamander larvae are likely to be important for successful recruitment to the adult stage. Although the depths and hydroperiods in CHE and CHW at WRP were similar, portions of CHE outside of the surveyed section did dry during the summer. In contrast, CHW and P1 maintained water throughout the summer, thereby supporting northwestern salamander recruitment into older age classes. At DC, PE was important habitat for northwestern salamanders. The minimal proportion of dips that were dry in PE was not associated with a patchy distribution of pools of water as in other plots, but with the shrinking size of the pond that prevented collection of the full 10 dips in late July/early August. It remained a deep pool of water through the summer despite its shrinking circumference, and was therefore more important to northwestern salamander abundance than the shallower PW.

It is unclear why rough-skinned newts were more abundant at DC than WRP. The sheer abundance of frogs throughout the plots at WRP, including juvenile ranids and adult Oregon spotted frogs, may exert a controlling force on the newts. Predation is likely a factor, possibly from the frogs because the snake abundance did not appear to differ among the sites. The disturbance from cattle at DC may also be a factor in that newts may be more tolerant of it and therefore more abundant on the site in the areas surveyed during the summer.

For both Oregon spotted frogs and northern red-legged frogs, a bimodal peak of metamorphic timing was observed at WRP. The initial peak coincided with a period of high predation pressure at the site. Evidence for heavy predation included the presence of garter snakes and the occurrence of many damaged and dying metamorphic and pre-metamorphic tadpoles in the wetland. Parasitized pre-metamorphic tadpoles were also observed at that time. Injured tadpoles were typically observed ahead of the surveyors, before anyone had walked through the area where the tadpoles were seen.

There are trade-offs involved in early and late metamorphosis, such that populations with metamorphic timing distributed in multiple peaks may take advantage of the benefits while diluting the risks [17]. Though the sample sizes for salamander and newt metamorphs were low, evidence from the size ranges of larvae and metamorphs at different times during the season indicated that they may also exhibit bimodal life event timing [see also 12, 18].

Northern red-legged frogs are thought to move into upland habitats after metamorphosis, but the timing of that movement is not well understood. Post-metamorphic northern red-legged frogs may remain in the wetlands until the fall rains begin (K. Richter, personal communication). Data from WRP indicate that northern red-legged frog YOY may move away from natal wetlands soon after metamorphosis. After the metamorphic peak, Oregon spotted frogs and northern red-legged frogs differed in the abundance of YOY that remained in the wetland. Northern red-legged frog abundance declined at a steady rate after the second peak of metamorphosis (10 August). Oregon spotted frog abundance was relatively stable after the metamorphic period, except in 2 weeks immediately after the peak of northern red-legged frog YOY observations. It cannot be determined from these data whether northern red-legged frog YOY are moving into the uplands or into other, unsurveyed areas in the wetland complex.

However, YOY were observed on the access road through the upland forest above the scrub habitat where traps were located, which suggests that at least some of the population moved upland prior to the onset of fall rains.

Minimum temperatures were identified in association with visual encounters of Oregon spotted frogs. The correspondence of water temperatures in wetlands with mean daily air temperatures may provide a basis for prediction of surface activity of Oregon spotted frogs.

The low detection rates for juvenile and adult Oregon spotted frogs compared to the count of > 200 egg masses at DC [3] indicate that the habitats surveyed were unlikely to be the primary summer habitats for the breeding population. The emergent wetland between the pasture and Dempsey Creek, and the deeper water channels north of CHN are potential summer habitats that were not surveyed in this effort due to logistical constraints. Watson et al [19] found radio-telemetered frogs more frequently in habitats dominated by hardhack (*Spirea douglasii*) and reed canarygrass in the dry season, corresponding to the timing of surveys in the present study. Lily pond, Overlook pond, and Dempsey Creek were the closest locations sampled in the present study to the habitats dominated by hardhack and reed canarygrass at DC. Trapping did not adequately detect Oregon spotted frogs in those locations, though visual encounters during trapping activities did increase detection rates.

Based on the number of egg masses (66) that were recorded in the area surveyed at WRP the spring prior to the survey season, the observation of 11 individual female adults indicates a detection rate of 17 percent of the female population. Although it is possible that our ability to detect frogs was simply low, high recapture rates of individuals in the pond and a maximum detection rate of 40 percent of known individuals on one day suggest that additional, undetected adults who breed on the site are likely using habitats outside of the sampled areas. Because of the

aquatic nature of Oregon spotted frogs and the apparent use of the channels as transition habitats, additional summering habitats likely include sections of the channels that were not surveyed or other deep ponded areas associated with the channels.

Implications for risk assessment

Differences in life stage susceptibilities to contaminants are important for understanding the potential for impacts to the resistance and resilience of exposed non-target populations. Higher susceptibility may occur when the habitats or behaviors of certain life stages place them at greater risk of exposure to a contaminant. Additionally, the potential exists for differences in sensitivity based on life stage. Ecological risk assessment addresses the potential for differences in life stage susceptibility through review of the potential for exposure, and toxicity tests on juveniles and reproduction. Habitats and resources used during the life history of species that occur in areas where contaminants may occur are considered in assessing the potential for exposure, with a particular focus on habitats required for reproduction.

Standard toxicity tests required for pesticide registration include several assessments of toxicity to juvenile life stages. Endpoints in screening-level risk assessments include the lethal concentration in water that results in 50% mortality of a test population (LC50) of juvenile fish and early life-stage invertebrates. Acute dietary tests are used to determine the lowest single oral dose that results in 50% mortality (LD50) of juvenile avian (mallard or quail) test populations. Juvenile test data are used with the assumption that juveniles represent the most sensitive age class of most species [20]. Risk assessments for pesticide registration use information from juvenile toxicity tests as a conservative metric of toxicity to the most-sensitive life stage of the most-sensitive species [20].

Information from toxicity tests is integrated with estimates of exposure to characterize the potential for adverse ecological effects on non-target species [21]. Environmental exposure data are estimated from models that calculate, for example, pesticide run-off and drift into a closed-system farm pond surrounded by treated fields, as a worse-case scenario for aquatic exposure [20]. Terrestrial exposure models incorporate the potential for terrestrial species to ingest pesticides from various vegetation types and consider different feeding-modes among species. However, the available toxicity information may not be applicable to the exposure situation [21].

The relationship between available toxicity information and the exposure situation, as well as the issues with secondary effects, are particularly applicable to issues of life stage susceptibility. For example, in the risk assessment for California red-legged frog exposure to the herbicide, 2,4-D, the standard dietary exposure scenario was used to assess risk to terrestrial-phase frogs [22]. Dietary toxicity data from avian and mammalian toxicity tests for the active ingredient 2,4-D were combined with estimates of dietary exposure to determine risk. However, in a recent review the European Food Safety Authority determined that amphibian feeding patterns are more variable than those of the standard terrestrial test species [23]. Moreover, Brühl et al. [24] suggest there is sufficient evidence that dermal exposure routes are more important for assessing toxicity to terrestrial-phase amphibians. Empirical evidence was also provided in a study designed to test field application scenarios for seven formulated pesticide products, in which caged juvenile frogs were exposed via over-spray to 0.1, 1, and 10 x label-recommended rates [25]. One hundred percent mortality was observed for some products at label-recommended rates after one hour [25]. The use of mammal and avian dietary toxicity data appears to be inconsistent with the exposure scenarios experienced by terrestrial-phase amphibians, and is likely to underestimate risk for that life stage.

Secondary effects may be of greater concern than toxicity when assessing risk of amphibian exposure to aquatic herbicides. Evidence exists that pesticides or pesticide application may disrupt reproductive site selection [26] or nest protection [27], potentially resulting in changes to reproductive success. Risk assessment attempts to address these types of secondary effects through chronic toxicity tests for effects on reproduction in avian, mammal, and invertebrates; although invertebrate reproduction tests are not always required. In addition, habitat requirements for reproduction are included in risk assessment when screening-level assessments indicate a potential for habitat alteration. Unfortunately a lack in understanding of life-history ecology of many species results in large uncertainties in risk assessment for secondary effects associated with life-stage susceptibility. The present study shows that differences in habitat-use exist for species that may be closely related like ranid frogs. The highly aquatic nature of Oregon spotted frogs at all life stages contrasts with the minimal presence of post-metamorphic northern red-legged frogs in wetlands during the aquatic weed management season.

The concern about differences in life stage susceptibility is intrinsically linked to the concern over population viability. Risk assessment at the US Environmental Protection Agency (EPA) extrapolates individual-level toxicity data to population-level effects. This has drawn criticism from investigators who claim that the approach may under- or overestimate toxicity, and is inappropriate for estimating population-level effects [28-30]. In addition, research has demonstrated that differences among life-history strategies result in differences in susceptibility to stressors at a population level [31-33]. Moreover, the importance of each life stage to population viability can vary. For example, most of the information on contaminant toxicity to amphibians is based on tests with early (egg and larvae) life stages [34], yet the survival of post-

metamorphic life stages is critical to population growth rates for many species [35]. Currently, EPA does not consider vital rates of different life stages, or differences among life-history strategies in risk assessment. They also contend that individual-level effects are the best available conservative estimate of population-level effects [20]. However, EPA's policy of including multiple lines-of-evidence and incorporating new information and model approaches when applicable [20, 21] provides an avenue whereby life stage susceptibilities may be incorporated into risk assessment. For example, new approaches to modeling differences in species sensitivities are being incorporated by risk assessors at EPA [20, 36, 37]. Additionally, models [31] and toxicity tests [32] that address life-history strategies and their effects on population-level responses have been developed.

CONCLUSION

The primary goal of this field survey was identify amphibians at risk of exposure to aquatic weed management for the control of reed canarygrass in Oregon spotted frog habitats and other common wetland weeds like purple loosestrife. Baseline data are provided on habitat associations, phenology, and the size ranges of individuals within different life stages that may help to inform toxicity testing and risk assessment, as well as wetland restoration efforts. Moreover, it may serve as a comparison for amphibians at DC and WRP over time, or for those amphibians that are monitored at other sites. A life-stage phenology framework was developed that indicates Oregon spotted frog YOY and northern red-legged frog metamorphs may be the youngest ranid life stages at risk of exposure to aquatic weed control when applications are made later in the summer. Moreover, they exist in habitats where water regimes force them to move to new locations during development. Northwestern salamander and rough-skinned newt larvae are also at risk of exposure to applications made to standing water in wetlands during summer

months. Finally, predators that target all of the native species occur throughout the terrestrial and aquatic habitats. More information on the hazard of aquatic herbicide tank-mixes to native amphibians in relevant life stages is required to fill data gaps in assessing the risks associated with amphibian exposure to herbicides used in wetland restoration. Future research should not only focus on the relevant life stages of amphibians at risk, but the ecological context in which they exist.

REFERENCES

[1] Stuart S, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783-1786.

[2] Simberloff D, Martin J-L, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F, Galil B, García-Berthou E, Pascal M, Pyšek P, Sousa R, Tabacchi E, Vilà M. 2013. Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution* 28:58-66.

[3] Hallock L. 2012. Draft State of Washington Oregon Spotted Frog Recovery Plan. Washington Department of Fish and Wildlife, Olympia, p. 105 + viii.

[4] USDA. ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)* [Online Database]. National Germplasm Resources Laboratory, Beltsville, Maryland. URL: http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?27512 (17 January 2014).

[5] Antieau CJ. 1998. Biology and management of reed canarygrass, and implications for ecological restoration. Washington State Department of Transportation, Seattle, WA. http://www.ser.org/sernw/pdf/RCG_BIO_MGT.pdf Accessed 21 June 2011.

[6] Tu M. 2004. Reed Canarygrass (*Phalaris arundinacea* L.) -- Control & Management in the Pacific Northwest. B Lipinski and J Soll ed. The Nature Conservancy, Wildland Invasive Species Team, Oregon Field Office. 12 pp.

[7] Adams CR, Galatowitsch SM. 2006. Increasing the effectiveness of Reed Canary Grass (*Phalaris arundinacea* L.) control in wet meadow restorations. *Restoration Ecology* 14:441-451.

[8] Cowardin LM, Carter V, Golet FC, LaRoe ET. 1979. Classification of wetlands and deepwater habitats of the United States. FWS/OBS-79/31. In Fish and Wildlife Service US, ed, Online: http://el.erdc.usace.army.mil/emrrp/emris/emrishelp2/cowardin_report.htm. Accessed 16 June 2011 ed. U.S. Dept. of the Interior. Online: http://el.erdc.usace.army.mil/emrrp/emris/emrishelp2/cowardin_report.htm. Accessed 16 June 2011, Washington D.C.

[9] Minnesota County Biological Survey. 2007. *A Handbook for Collecting Vegetation Plot Data in Minnesota : the Releve Method*. Minnesota County Biological Survey, Natural Heritage and Nongame Research Program, Ecological Land Classificiation Program, Dept. of Natural Resources, St. Paul, Minn.

[10] Leonard WP, McAllister K. 2005. Oregon Spotted Frog - *Rana pretiosa*. In Jones LLC, Leonard WP, Olson DH, eds, *Amphibians of the Pacific Northwest*. Seattle Audubon Society, Seattle, WA, pp 210-213.

[11] Rasband WS. 1997-2009. ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA.

[12] Yahnke AE. 2011. Amphibian reproduction and water quality in stormwater ponds. Masters. University of Washington, Seattle, WA.

[13] Nussbaum RA, Brodie ED, Storm RM. 1983. *Amphibians & Reptiles of the Pacific Northwest*. University of Idaho Press, Moscow, ID.

[14] Richter KO. 2005. Northwestern Salamander - *Ambystoma gracile*. In Jones LLC, Leonard WP, Olson DH, eds, *Amphibians of the Pacific Northwest*. Seattle Audubon Society, Seattle, WA, pp 30-33.

[15] Watson S, Russell A. 2000. A posthatching developmental staging table for the long-toed salamander, *Ambystoma macrodactylum krausei*. *Amphibia-Reptilia* 21:143-154.

[16] Govindarajulu P, Price WS, Anholt BR. 2006. Introduced bullfrogs (*Rana catesbeiana*) in Western Canada: Has their ecology diverged? *Journal of Herpetology* 40:249-260.

[17] Wilbur HM, Collins JP. 1973. Ecological aspects of amphibian metamorphosis. *Science* 182:1305-1314.

[18] MacCracken JG. 2008. Biphasic oviposition by Northwestern Salamanders (*Ambystoma gracile*) in the Cascade Mountains of Southern Washington, USA. *Herpetological Review* 39:29.

[19] Watson JW, McAllister KR, Pierce DJ. 2003. Home ranges, movements, and habitat selection of Oregon Spotted Fogs (*Rana pretiosa*). *Journal of Herpetology* 37:292-300.

[20] U.S. EPA. 2004. Overview of the Ecological Risk Assessment Process in the Office of Pesticide Programs, U.S. Environmental Protection Agency: Endangered and Threatened Species Effects Determinations. Office of Prevention, Pesticides, and Toxic Substances; Office of Pesticide Programs, Washington D.C., pp 1-92.

[21] U.S. EPA. 1998. Guidelines for Ecological Risk Assessment. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC, EPA/630/R095/002F.

[22] U. S. EPA. 2009. Risks of 2,4-D Use to the Federally Threatened California Red-legged Frog (*Rana aurora draytonii*) and Alameda Whipsnake (*Masticophis lateralis euryxanthus*) - Pesticide Effects Determination Environmental Fate and Effects Division, Office of Pesticide Programs, U.S. Environmental Protection Agency. Available online:

http://www.epa.gov/oppfead1/endanger/litstatus/effects/redleg-frog/2-4-d/analysis.pdf; accessed January 2013.

[23] Fryday S, Thompson H. 2012. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. EFSA Supporting Publications: EN-343. Food and Environment Research Agency, Sand Hutton, York, UK, p. 348 pp. Available online: http://www.efsa.europa.eu/en/supporting/pub/343e.htm; accessed January 2013.

[24] Brühl CA, Pieper S, Weber B. 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environmental Toxicology and Chemistry* 30:2465-2472.

[25] Brühl CA, Schmidt T, Pieper S, Alscher A. 2013. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? *Scientific Reports* 3. :Available online: http://www.nature.com/srep/2013/130124/srep101135/full/srep101135.html; accessed March 2013.

[26] Vonesh JR, Buck JC. 2007. Pesticide alters oviposition site selection in gray treefrogs. *Oecologia* 154:219-226.

[27] Martin PA, Johnson DL, Forsyth DJ, Hill BD. 1998. Indirect effects of the pyrethroid insecticide deltamethrin on reproductive success of chestnut-collared longspurs. *Ecotoxicology* 7:89-97.

[28] DeMott RP, Balaraman A, Sorensen MT. 2005. The future direction of ecological risk assessment in the United States: Reflecting on the U.S. Environmental Protection Agency's "Examination of risk assessment practices and principles". *Integrated Environmental Assessment and Management* 1:77-82.

[29] Forbes VE, Calow P. 2002. Extrapolation in ecological risk assessment: Balancing pragmatism and precaution in chemical controls legislation. *BioScience* 52:249-257.

[30] Hope BK. 2006. An examination of ecological risk assessment and management practices. *Environment International* 32:983-995.

[31] Spromberg JA, Birge WJ. 2005. Population survivorship index for fish and amphibians: Application to criterion development and risk assessment. *Environmental Toxicology and Chemistry* 24:1541-1547.

[32] Preston BL, Snell TW. 2001. Full life-cycle toxicity assessment using rotifer resting egg production: Implications for ecological risk assessment. *Environmental Pollution* 114:399-406.

[33] Stark JD, Banks JE, Vargas R. 2004. How risky is risk assessment: The role that life history strategies play in susceptibility of species to stress. *Proceedings of the National Academy of Sciences of the United States of America* 101:732-736.

[34] Sparling DW. 2010. Ecotoxicology of organic contaminants to amphibians. In Sparling DW, Linder G, Bishop CA, Krest SK, eds, *Ecotoxicology of Amphibians and Reptiles*, 2nd ed.

CRC Press/Taylor & Francis; Society of Environmental Toxicology and Chemistry (SETAC), Boca Raton; Pensacola, FL, pp 261-288.

[35] Biek R, Funk WC, Maxell BA, Mills LS. 2002. What Is Missing in Amphibian Decline Research: Insights from Ecological Sensitivity Analysis. *Conservation Biology* 16:728-734.

[36] Brix KV, DeForest DK, Adams WJ. 2001. Assessing acute and chronic copper risks to freshwater aquatic life using species sensitivity distributions for different taxonomic groups. *Environmental Toxicology and Chemistry* 20:1846-1856.

[37] Newman MC, Ownby DR, Mézin LCA, Powell DC, Christensen TRL, Lerberg SB, Anderson B-A. 2000. Applying species-sensitivity distributions in ecological risk assessment: Assumptions of distribution type and sufficient numbers of species. *Environmental Toxicology and Chemistry* 19:508-515.

Table 1.1 Spearman Rank correlations for habitat variables that occurred at >0 percent cover in five or more plots. Mean autumn and spring vegetation cover (Carex and rush complexes, reed canarygrass [RCG], and open water [OW]), summer depth, and proportion dry data correlations are reported. Depth and proportion dry data were available for all plots surveyed (n=14). Vegetation data was available for 11 of 14 plots. Bold values are significant at $\alpha \leq 0.05$

		Water depth	% dry	Spring Carex	Spring rush	Spring RCG	Spring OW	Autumn Carex	Autumn rush	Autumn RCG	Autumn OW
Water depth	ρ	1.000	**-.939**	-.110	.546	**-.656**	**.602**	-.023	**.636**	-.352	**.778**
	p	.	.000	.747	.082	.028	.050	.947	.035	.289	.005
% dry	ρ	**-.939**	1.000	.179	**-.632**	**.671**	**-.637**	.037	**-.627**	.390	**-.806**
	p	.000	.	.598	.037	.024	.035	.914	.039	.235	.003
Spring Carex	ρ	-.110	.179	1.000	-.581	-.030	-.428	.538	-.227	-.159	-.267
	p	.747	.598	.	.061	.930	.189	.088	.502	.640	.427
Spring rush	ρ	.546	**-.632**	-.581	1.000	-.413	**.693**	-.279	.592	-.288	.566
	p	.082	.037	.061	.	.207	.018	.406	.055	.390	.069
Spring RCG	ρ	**-.656**	**.671**	-.030	-.413	1.000	**-.622**	-.249	-.351	**.755**	-.394
	p	.028	.024	.930	.207	.	.041	.460	.290	.007	.230
Spring open water	ρ	**.602**	**-.637**	-.428	**.693**	**-.622**	1.000	-.162	**.787**	-.402	.496
	p	.050	.035	.189	.018	.041	.	.635	.004	.221	.120
Autumn Carex	ρ	-.023	.037	.538	-.279	-.249	-.162	1.000	-.048	-.429	-.247
	p	.947	.914	.088	.406	.460	.635	.	.888	.188	.465
Autumn rush	ρ	**.636**	**-.627**	-.227	.592	-.351	**.787**	-.048	1.000	-.274	.494
	p	.035	.039	.502	.055	.290	.004	.888	.	.416	.122
Autumn RCG	ρ	-.352	.390	-.159	-.288	**.755**	-.402	-.429	-.274	1.000	-.428
	p	.289	.235	.640	.390	.007	.221	.188	.416	.	.189
Autumn Open water	ρ	**.778**	**-.806**	-.267	.566	-.394	.496	-.247	.494	-.428	1.000
	p	.005	.003	.427	.069	.230	.120	.465	.122	.189	.

Table 1.2 Total number of dips collected, depth (mean cm ± SD and minimum-maximum), and the proportion of dips that were dry in plots that were systematically sampled each week, 25 May-30 September 2010.

Plots	Treatment	Dips	Depth	Min-max	Proportion dry
WRP					
T1	Mowed	192	6.2 ± 9.4	0-40	0.64
T3	Mowed	181	9.4 ± 10.1	0-32	0.47
T4	Mowed	181	15.9 ± 12.8	0-44	0.31
C2	Unmowed	197	7.5 ± 11.0	0-36	0.64
C4	Unmowed	181	11.3 ± 12.1	0-43	0.47
EMN	Unmowed	183	23.3 ± 16.5	0-69	0.23
EMS	RCG	181	3.4 ± 5.8	0-25	0.70
CHE	Channel	181	33.1 ± 13.0	3-62	0
CHW	Channel	186	33.7 ± 12.7	8-64	0
P1	Pond	191	47.8 ± 21.8	15-150	0
DC					
CHN	Channel	222	35.3 ± 21.2	0-100	0.005
CHS	Channel	714	8.0 ± 10.6	0-42	0.58
PE	Pond	155	31.2 ± 16.0	0-68	0.10
PW	Pond	160	25.1 ± 7.0	0-60	0

Table 1.3 Total number of all amphibian observations and proportion of total represented by each species. Systematic survey data include the number of observations, proportion represented by each species, mean ± SD (*100) relative abundance determined from number of dips collected or number of meters surveyed. Relative abundance not calculated for unidentified (uk) individuals. Data collected May-September 2010 at Dempsey Creek (DC) and West Rocky Prairie (WRP).

	Total observations		Systematic surveys						Traps		
			dip			visual encounter					
	n	%	n	%	mean	n	%	mean	n	%	mean
DC	416		276			34			33		
Northwestern salamander	144	34.6	121	43.8	14.7 ± 41.6	2	5.9	0.1 ± 0.5	16	48.5	31.0 ± 27.0
Rough-skinned newt	70	16.8	53	19.2	16.8 ± 114.3	0	--	--	5	15.2	10.1 ± 15.8
American bullfrog	1	0.2	0	--	--	0	--	--	0	--	--
Pacific treefrog	77	18.5	51	18.5	1.8 ± 9.4	2	5.9	0.01 ± 0.03	0	--	--
Northern red-legged frog	27	6.5	13	4.7	0.8 ± 2.5	9	26.5	0.2 ± 0.6	0	--	--
Oregon spotted frog	75	18.0	29	10.5	2.4 ± 7.3	16	47.1	0.4 ± 0.9	11	33.3	22.3 ± 18.8
uk frog	1	0.2	0	--	--	1	2.9	--	0	--	--
uk ranid	15	3.6	3	1.1	--	4	11.8	--	1	3.0	
uk salamander	6	1.4	6	2.2	--	0	--	--	0	--	--
WRP	1228		414			492			247		
Northwestern salamander	79	6.4	61	14.7	3.4 ± 11.1	13	2.6	0.1 ± 0.5	36	14.6	24.0 ± 15.0
Long-toed salamander	1	0.1	1	0.2	0.1 ± 0.8	0	--	--	0	--	--
Rough-skinned newt	6	0.5	4	1.0	0.2 ± 3.0	2	0.4	0.01 ± 0.2	2	0.8	1.3 ± 1.8
Pacific treefrog	78	6.4	50	12.1	2.8 ± 11.1	7	1.4	0.04 ± 0.3	0	--	--
Northern red-legged frog	507	41.3	190	45.9	10.2 ± 23.5	166	33.7	1.7 ± 5.5	160	64.8	106.7 ± 177.4
Oregon spotted frog	449	36.6	99	23.9	5.5 ± 18.0	235	47.8	2.7 ± 9.3	28	11.3	18.7 ± 31.0
uk frog	5	0.4	0	--	--	5	1.0	--	0	--	--
uk ranid	99	8.1	6	1.4	--	63	12.8	--	15	6.1	--
uk salamander	2	0.2	2	0.5	--	0	--	--	6	2.4	--

Table 1.4 Total length (TL) and snout-vent length (SVL) of salamanders in all life stages caught at West Rocky Prairie (WRP) and Dempsey Creek (DC) May-October 2010. Mean values followed by minimum-maximum in parentheses. All values in mm.

Species	Life stage	n	SVL	min-max	n	TL	min-max
WRP							
Northwestern Salamander	1st-yr larva	81	22.7 ± 4.3	13.6-31.6	81	46.5 ± 9.2	24.7-67.0
	2nd-yr larva	19	48.3 ± 6.7	36.5-62.2	17	99.8 ± 18.5	55.8-136.5
	Metamorphic	3	51.3 ± 2.4	49.6-54.1	3	107.3 ± 6.1	103.3-114.3
Long-toed Salamander	Larva	1	23.0	--	1	43.8	--
Rough-skinned Newt	Larva	5	16.2 ± 3.0	11.0-19.0	5	31.6 ± 6.8	23.0-38.6
	Metamorphic	2	19.4 ± 0.8	19.0-20.0	2	42.6 ± 2.8	40.6-44.6
DC							
Northwestern Salamander	1st-yr larva	122	19.2 ± 3.5	12.0-31.8	122	37.0 ± 7.8	13.7-64.7
	2nd-yr and older larva	26	49.8 ± 11.9	37.6-78.4	25	101.1 ± 21.3	75.9-149.2
	Metamorphic	2	52.4 ± 6.8	47.6-57.2	2	98.8 ± 10.1	91.7-105.9
Rough-skinned Newt	Larva	56	17.5 ± 3.1	7.8-22.5	56	32.1 ± 5.8	13.8-40.7
	Metamorphic	1	23.3	--	1	50.5	--
	Adult	14	72.3 ± 6.9	62.0-82.0	14	157.5 ± 27.8	90.0-195.0

Table 1.5 Sizes of ranid frog tadpoles (Tad), metamorphs (Met), young-of-year (YOY), juveniles (Juv), and adults caught at West Rocky Prairie (WRP) and Dempsey Creek (DC) May-October 2010. Mean values ± standard deviation, minimum-maximum, and number measured (n) for each value reported for snout-vent length (SVL) and shank-length (SL) in mm, and weight (W) in g. Size-range overlaps in adults and juveniles are due to differences in male and female size cut-offs used to determine adult status.

Species	Life stage	n	SVL	min-max	n	S	min-max	n	W	min-max
WRP										
Northern red-legged frog	Tad	220	25.4 ± 3.7	14.2-34.8	1	13.0	--	--		
	Met	85	24.9 ± 2.8	20.1-33.3	54	13.1 ± 1.6	10.6-17.1	68	2.0 ± 0.9	1.1-5.2
	YOY	131	26.1 ± 2.4	20.0-36.0	115	14.0 ± 1.9	7.5-19.5	126	1.7 ± 0.5	0.8-3.3
	Juv	8	43.6 ± 6.1	36.5-52.5	3	29.0 ± 1.8	27.5-31.0	5	6.0 ± 2.8	2.0-9.0
	Adult	1	52.9	--	1	31.5	--	1	14.7	--
Oregon spotted frog	Tad	49	30.7 ± 3.3	19.9-36.3	--			--		
	Met	27	30.5 ± 4.2	20.2-36.0	11	14.7 ± 2.9	9.2-18.2	17	3.9 ± 1.3	1.9-5.7
	YOY	143	34.5 ± 3.6	20.6-48.0	130	16.7 ± 1.5	12.7-20.0	136	4.0 ± 1.0	1.7-6.5
	Juv	64	51.6 ± 5.7	32.8-60.0	47	26.5 ± 2.0	21.0-31.0	55	12.6 ± 3.7	5.5-21.0
	Adult	70	65.1 ± 8.3	50.0-82.5	67	30.9 ± 3.1	25.0-35.6	67	27.4 ± 9.9	12.9-47.8
DC										
Northern red-legged frog	Tad	7	22.3 ± 2.2	18.2-24.9	--			--		
	Met	1	21.2	--	--					
	YOY	11	30.4 ± 2.4	26.5-33.5	9	17.5 ± 2.4	15.0-22.0	11	3.0 ± 1.0	1.6-5.0
	Juv	1	45.8	--				1	9.5	--
	Adult	1	56.0	--	1	29.0	--	1	13.7	--
Oregon spotted frog	Tad	28	27.9 ± 4.4	18.4-35.0	--			--		
	Met	6	32.4 ± 4.9	24.7-40.0	5	13.3 ± 1.0	12.5-15.0	5	3.7 ± 0.9	2.8-5.2
	YOY	3	30.7 ± 2.5	29.0-33.5	3	14.3 ± 0.4	13.9-14.6	3	3.3 ± 0.4	3.0-3.7
	Juv	10	48.7 ± 4.3	44.0-57.7	5	24.1 ± 1.0	25.5-23.0	10	11.0 ± 3.3	6.3-17.0
	Adult	21	65.4 ± 7.3	52.5-74.9	17	30.0 ± 3.2	24.0-35.0	19	33.4 ± 13.8	10.3-54.3

Table 1.6 Water depths (mean ± SD) where amphibians were found at West Rocky Prairie (WRP) and Dempsey Creek (DC), May-October 2010.

Species	Life stage	n	Water Depth	min-max
WRP				
Pacific treefrog	Larva	32	23.6 ± 6.8	9.0-36.0
	Metamorph	5	25.1 ± 5.6	16.5-31.0
	YOY	1	53.0	--
Northern red-legged frog	Larva	93	34.3 ± 17.6	13.0-94.0
	Metamorph	26	27.1 ± 12.9	12.0-76.0
	YOY	36	35.3 ± 22.6	7.5-112.0
	Juvenile	12	23.8 ± 10.2	10.0-43.0
	Adult	1	0.0	--
Oregon spotted frog	Larva	31	30.8 ± 15.7	10.0-88.0
	Metamorph	13	31.8 ± 12.8	14.0-51.0
	YOY	45	40.2 ± 27.8	11.0-150.0
	Juvenile	28	30.1 ± 14.3	13.0-67.0
	Adult	21	41.3 ± 30.8	8.0-127.0
Northwestern salamander	First-year larva	38	40.6 ± 22.0	14.0-89.0
	Second-year larva	10	52.8 ± 30.3	19.0-110.0
	Metamorph	2	60.5 ± 29.0	40.0-81.0
Long-toed salamander	Larva	1	28.0	--
Rough-skinned newt	Larva	1	14.0	--
DC				
Pacific treefrog	Larva	12	21.1 ± 5.1	12.0-30.0
	Metamorph	3	13.3 ± 1.5	12.0-15.0
	YOY & Adult	2	0.0	--
Northern red-legged frog	Larva	8	27.3 ± 7.8	16.0-37.0
	Metamorph	1	31.0	--
	YOY	7	20.1 ± 9.9	9.0-36.0
	Juvenile	1	16.0	--
Oregon spotted frog	Larva	12	26.3 ± 8.1	15.0-43.0
	Metamorph	1	10.0	--
	YOY	2	24.0 ± 5.7	20.0-28.0
	Juvenile	6	38.0 ± 14.5	22.0-60.0
	Adult	13	45.4 ± 33.1	10.0-102.0
Northwestern salamander	First-year larva	39	26.6 ± 9.9	9.0-46.0
	Second-year larva	7	32.0 ± 7.7	23.0-43.0
	Metamorph	2	36.0 ± 8.5	30.0-42.0
Rough-skinned newt	Larva	11	26.1 ± 9.4	10.0-43.0
	Metamorph	1	37.0	--
	Adult	5	34.0 ± 24.6	16.0-77.0

Table 1.7 Spearman rank correlations for habitat and ranid relative abundance from dip and visual encounter surveys. Habitat variables include proportion of dips that were dry (% dry), reed canarygrass (RCG) and Carex complexes from autumn vegetation surveys, and Carex complexes from spring vegetation surveys. Ranid variables include tadpole (Tad), metamorph (Met), young-of-year (YOY), juvenile (Juv), adults, and total across all life stages. Bold values indicate significance (*p)* at $\alpha = 0.10$, asterisks (*) indicate significance after correction for multiple comparisons, $\alpha = 0.025$.

Species	Life stage		% dry	Autumn RCG	Spring Carex	Autumn Carex
Dip						
Northern red-legged frog	Tad	ρ	-0.355	-0.387	-0.060	0.143
		p	0.212	0.240	0.860	0.675
	Met	ρ	**-0.554**	-0.417	**-0.553**	-0.362
		p	0.040	0.202	0.078	0.274
	YOY	ρ	**-0.645***	-0.504	-0.015	-0.077
		p	0.013	0.114	0.966	0.822
	Total	ρ	**-0.501**	-0.400	-0.046	0.208
		p	0.068	0.222	0.893	0.539
Oregon spotted frog	Tad	ρ	-0.046	-0.239	0.446	0.274
		p	0.877	0.479	0.169	0.415
	Met	ρ	0.114	-0.165	0.324	-0.227
		p	0.697	0.628	0.331	0.502
	YOY	ρ	**-0.469**	**-0.525**	-0.035	-0.093
		p	0.091	0.097	0.919	0.786
	Juv	ρ	-0.105	0.078	**0.667***	0.136
		p	0.720	0.820	0.025	0.690
	Total	ρ	-0.232	-0.353	0.402	0.179
		p	0.425	0.287	0.221	0.599
Visual encounter						
Northern red-legged frog	Met	ρ	**-0.495**	-0.010	-0.118	**-0.555**
		p	0.072	0.977	0.730	0.076
	YOY	ρ	**-0.717***	-0.332	-0.111	-0.119
		p	0.004	0.319	0.746	0.727
	Juv	ρ	-0.198	0.335	-0.121	**-0.589**
		p	0.497	0.314	0.724	0.057
	Total	ρ	**-0.570**	-0.129	-0.135	-0.429
		p	0.033	0.705	0.693	0.188
Oregon spotted frog	Met	ρ	-0.271	-0.451	0.043	-0.342
		p	0.348	0.164	0.900	0.303
	YOY	ρ	**-0.478**	-0.012	-0.151	-0.423
		p	0.084	0.973	0.658	0.195
	Juv	ρ	-0.421	-0.126	0.115	-0.358
		p	0.134	0.712	0.736	0.280
	Adult	ρ	**-0.545**	-0.397	**0.660**	0.365
		p	0.044	0.227	0.027	0.270
	Total	ρ	-0.456	-0.025	0.102	-0.351
		p	0.101	0.941	0.766	0.290

Table 1.8 Summary by age and sex of the number of individual Oregon spotted frogs marked by spot-pattern recognition and recaptured at WRP. Undetermined indicates juveniles too young to determine sex.

Age	Male	Female	Undetermined	Recaptures
Adult	12	11	0	12
Juvenile	2	13	4	8

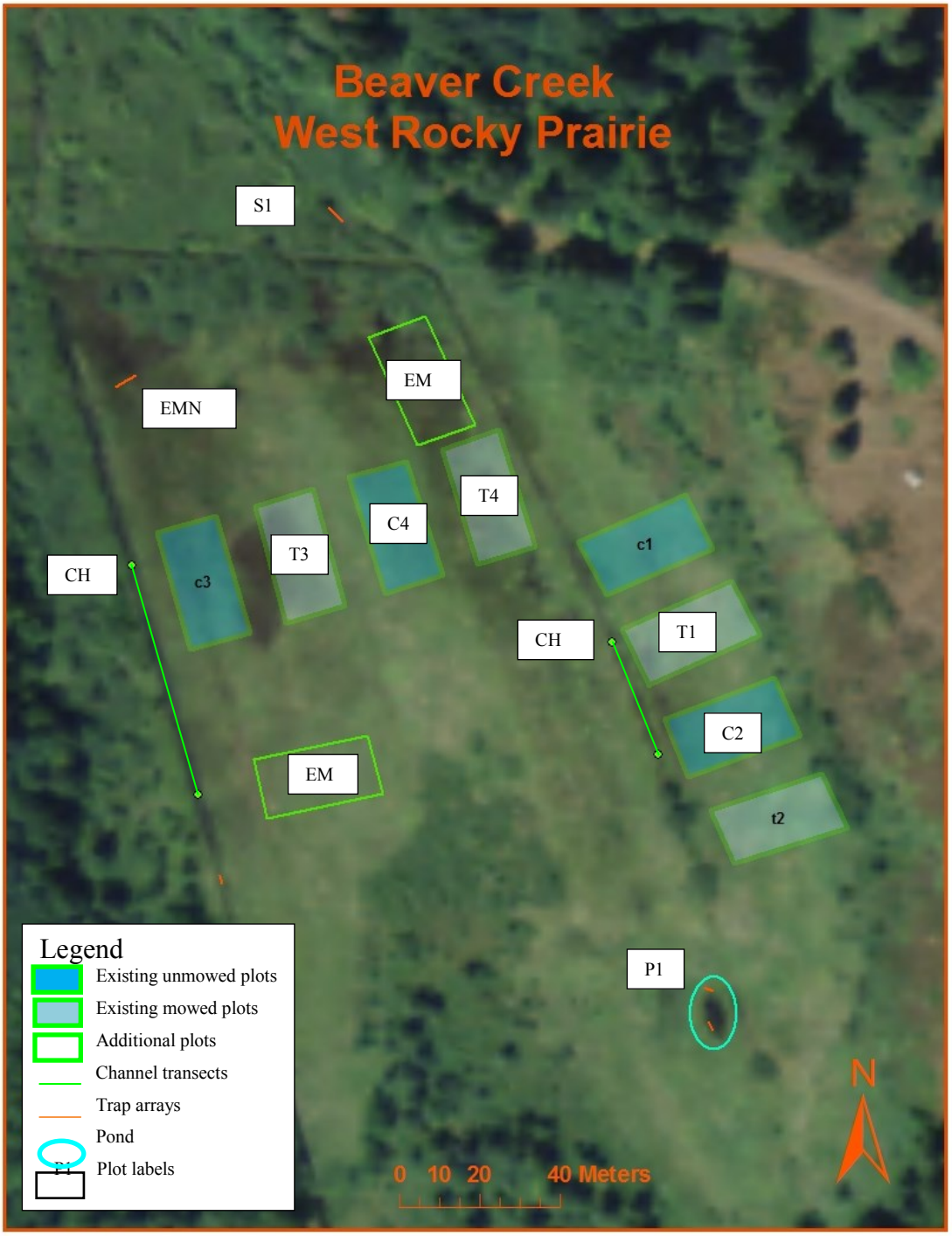


Figure 1.1 West Rocky Prairie plot, transect, and trap array locations for summer amphibian surveys, 2010.

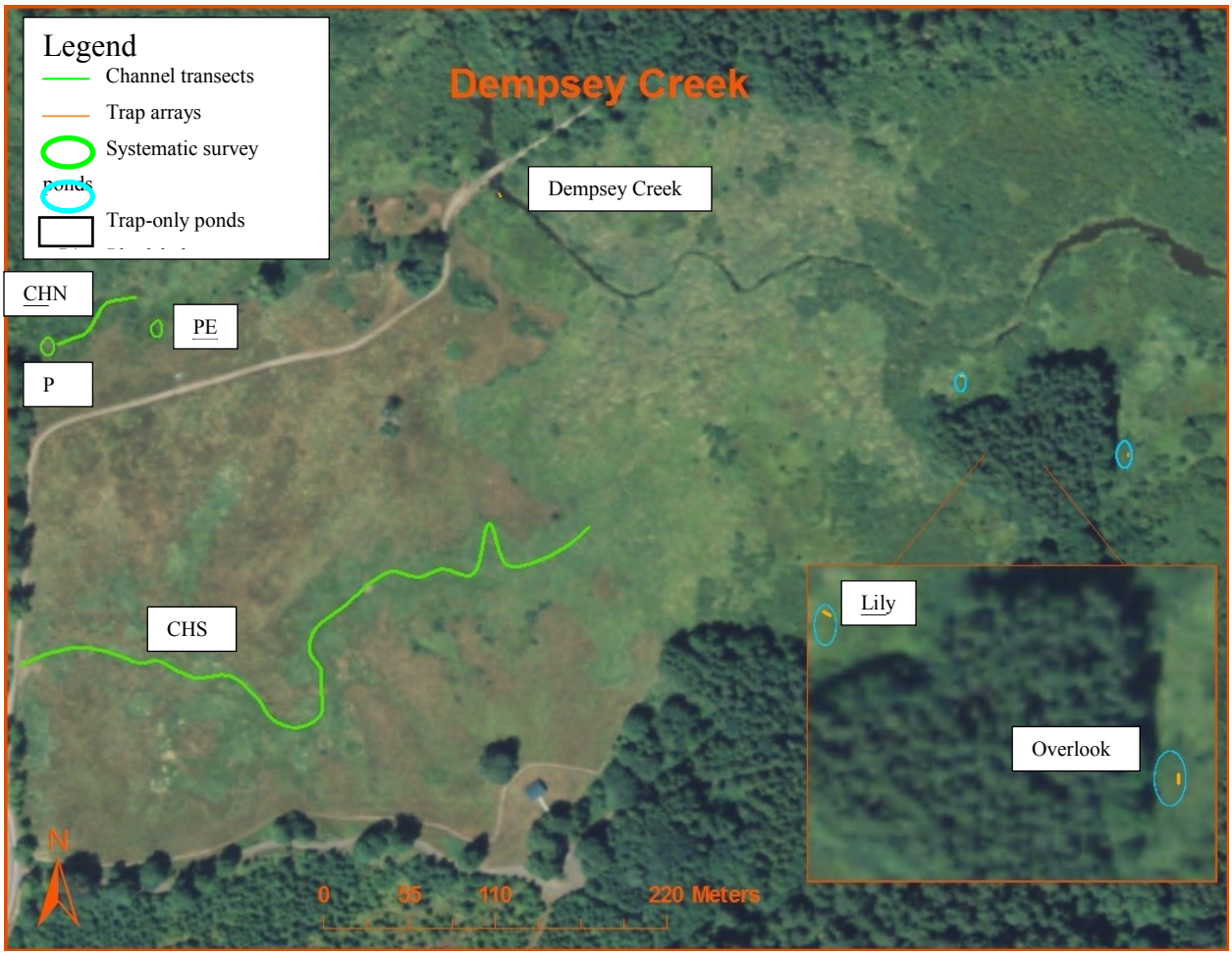


Figure 1.2 Dempsey Creek pond, channel transect, and trap array locations for summer amphibian surveys, 2010.

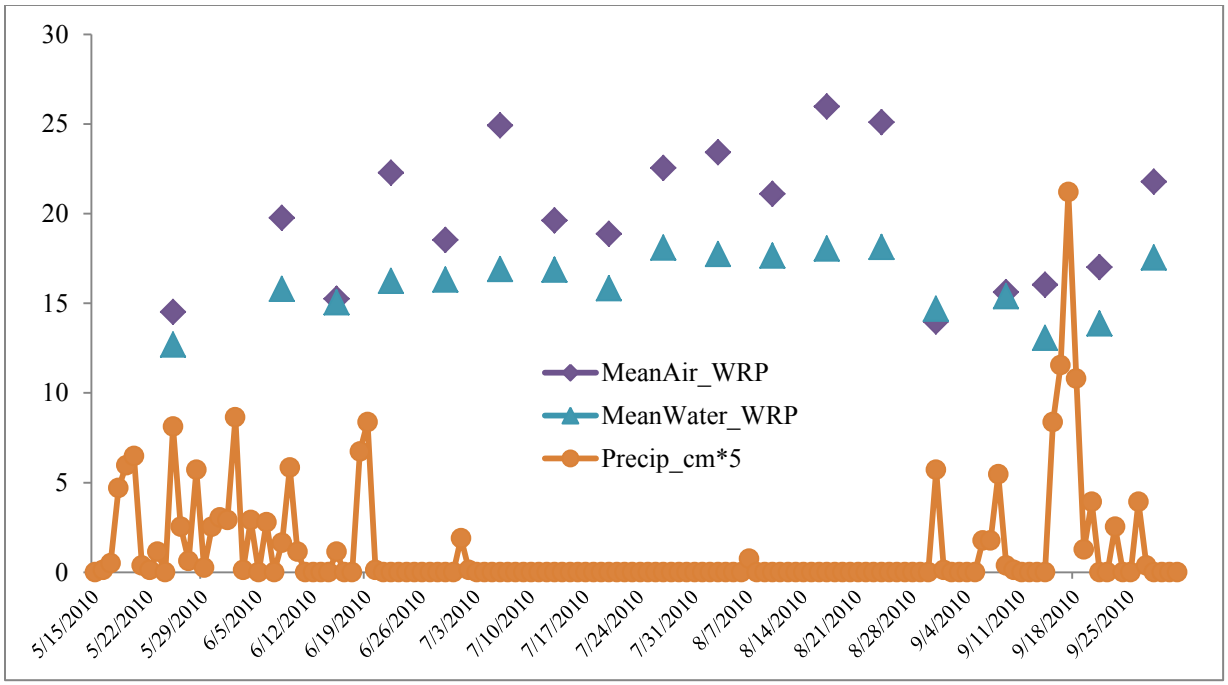


Figure 1.3 Precipitation (cm x5) recorded at Olympia Regional Airport and mean air and water temperatures (°C) recorded in plots at West Rocky Prairie (WRP) during 2010 summer surveys.

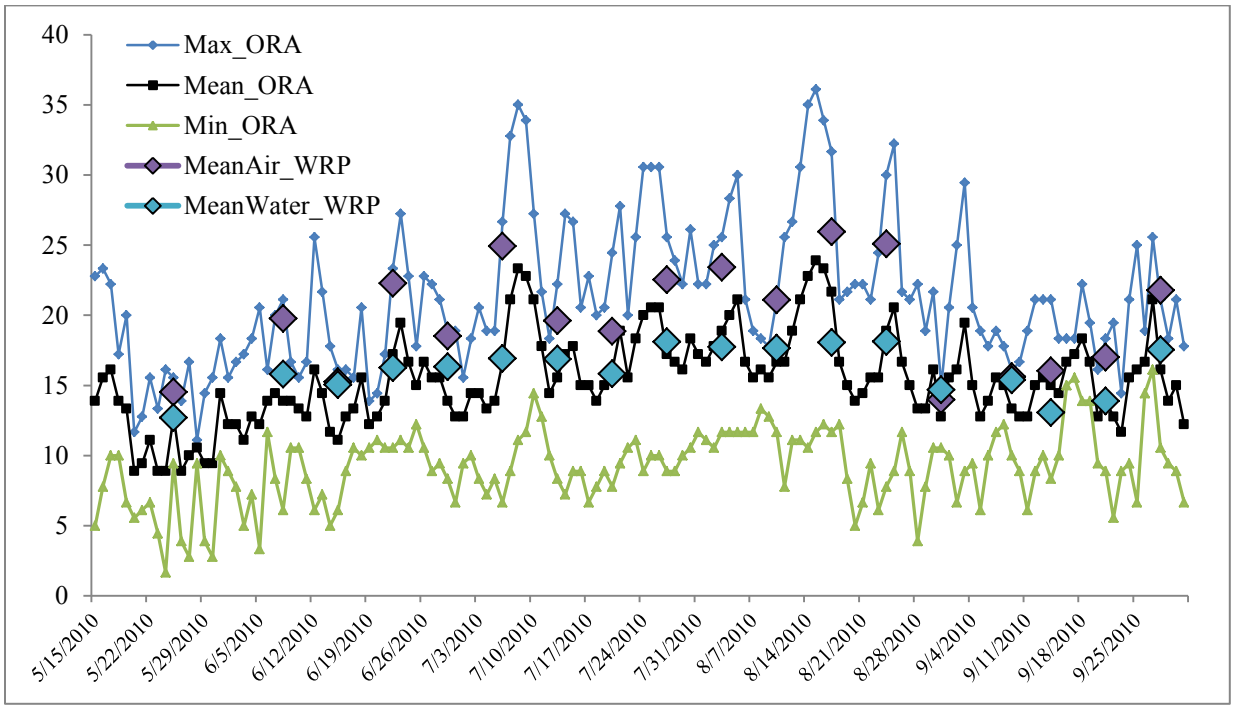


Figure 1.4 Mean daily air and water temperatures recorded at West Rocky Prairie (WRP) and mean, minimum, and maximum daily air temperatures recorded at Olympia Regional Airport (ORA).

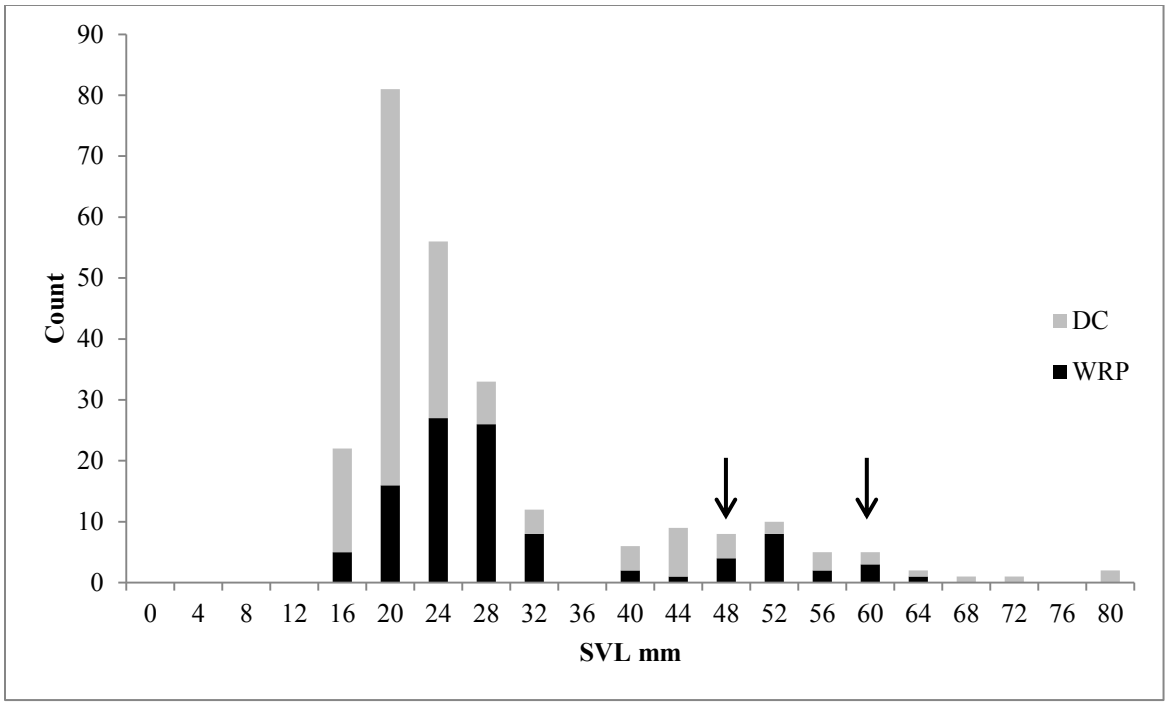


Figure 1.5 Bimodal distribution of northwestern salamander larvae snout-vent lengths (SVL) showing first (16-32 mm) and second-year (≥40 mm) cohorts. Arrows indicate observed size range of metamorphs. Larvae in size classes > 65mm may indicate neotenic (larviform adult) populations.

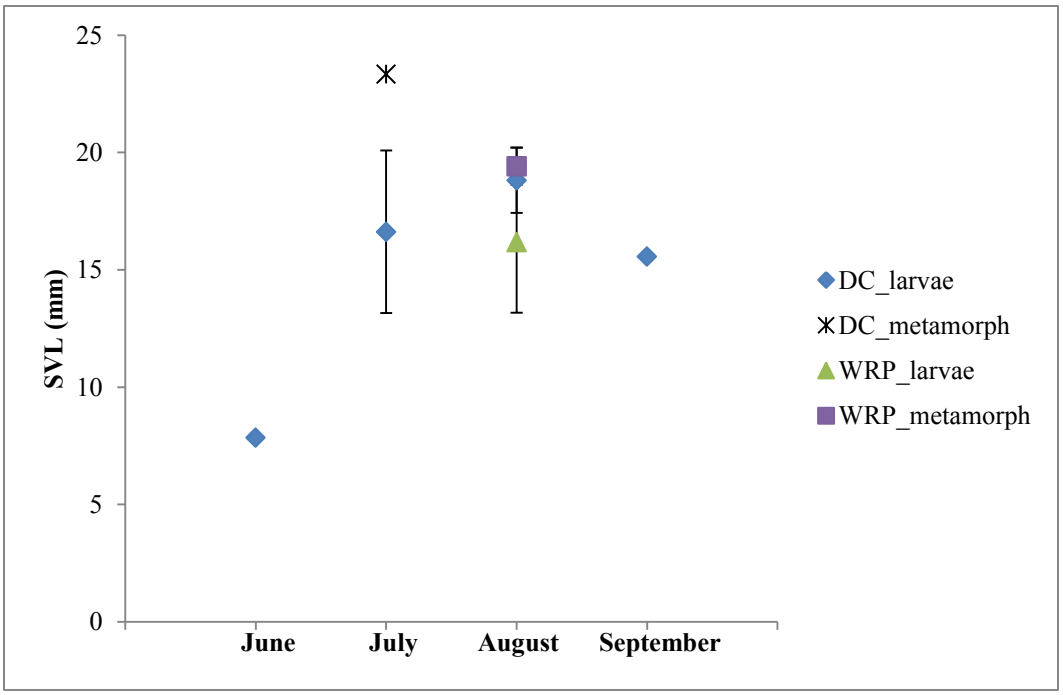


Figure 1.6 Snout-vent lengths (SVL) of rough-skinned newt larvae and metamorphs at Dempsey Creek (DC) and West Rocky Prairie (WRP), June-September 2010

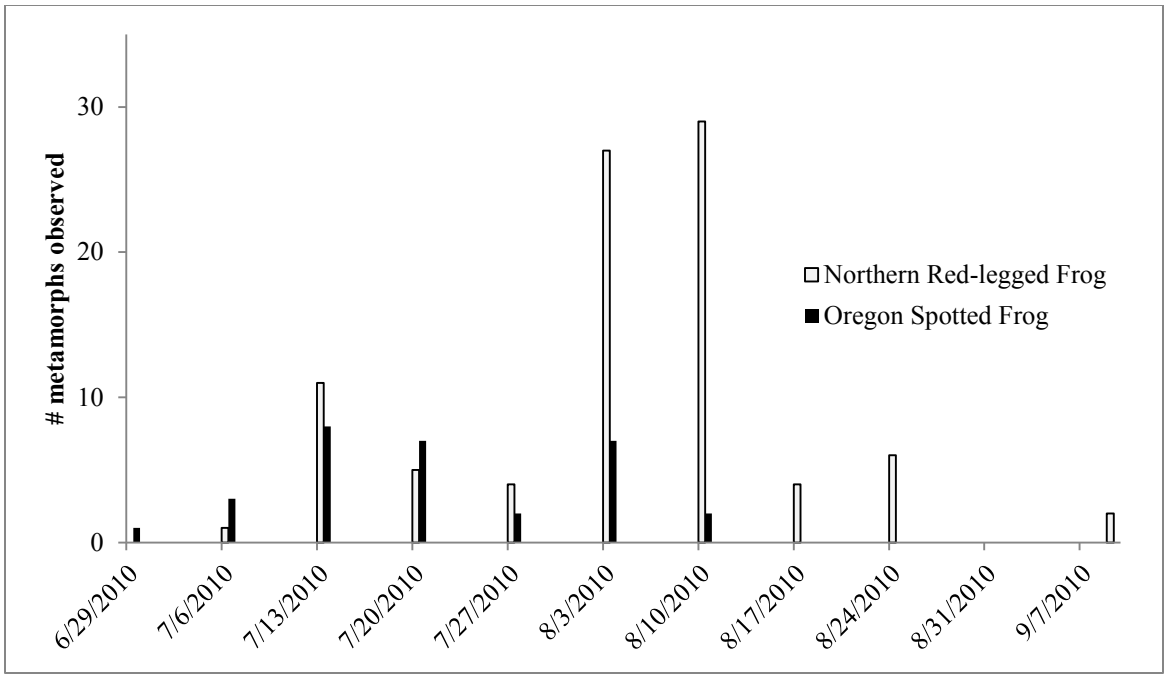


Figure 1.7 Number of metamorphic frogs observed during weekly surveys at West Rocky Prairie. Abundance is from systematic survey days only, and includes systematic survey data and visual encounters during transition between plots.

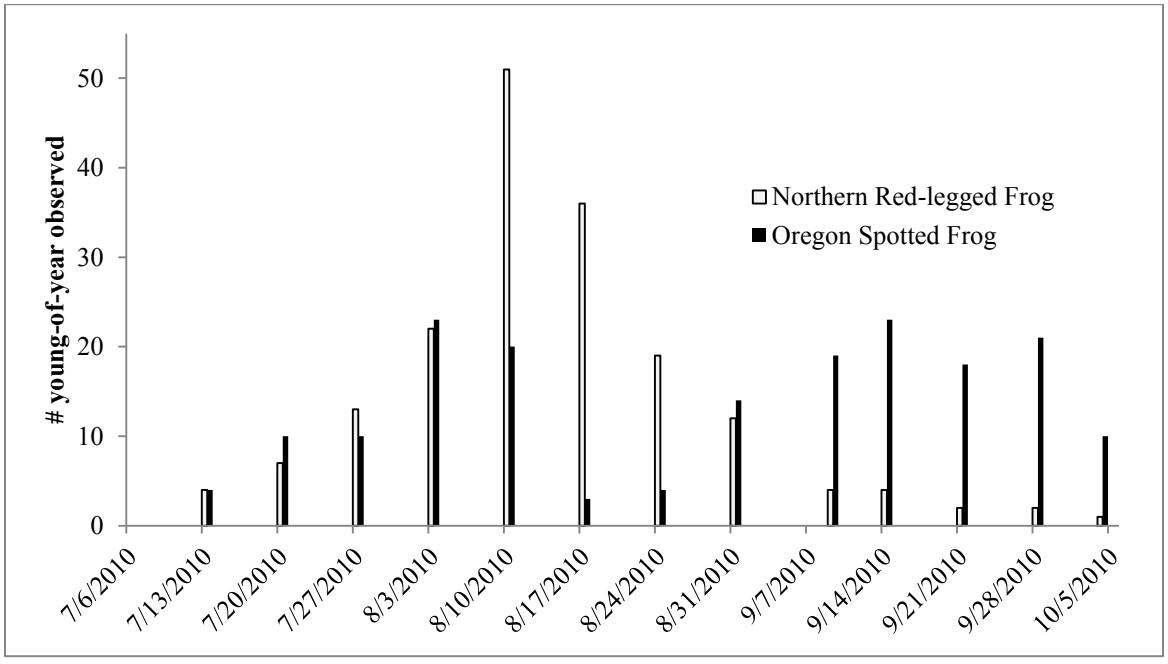


Figure 1.8 Number of post-metamorphic young-of-year juvenile frogs observed during weekly surveys at West Rocky Prairie. Abundance includes systematic survey data and visual encounters during transition between plots.

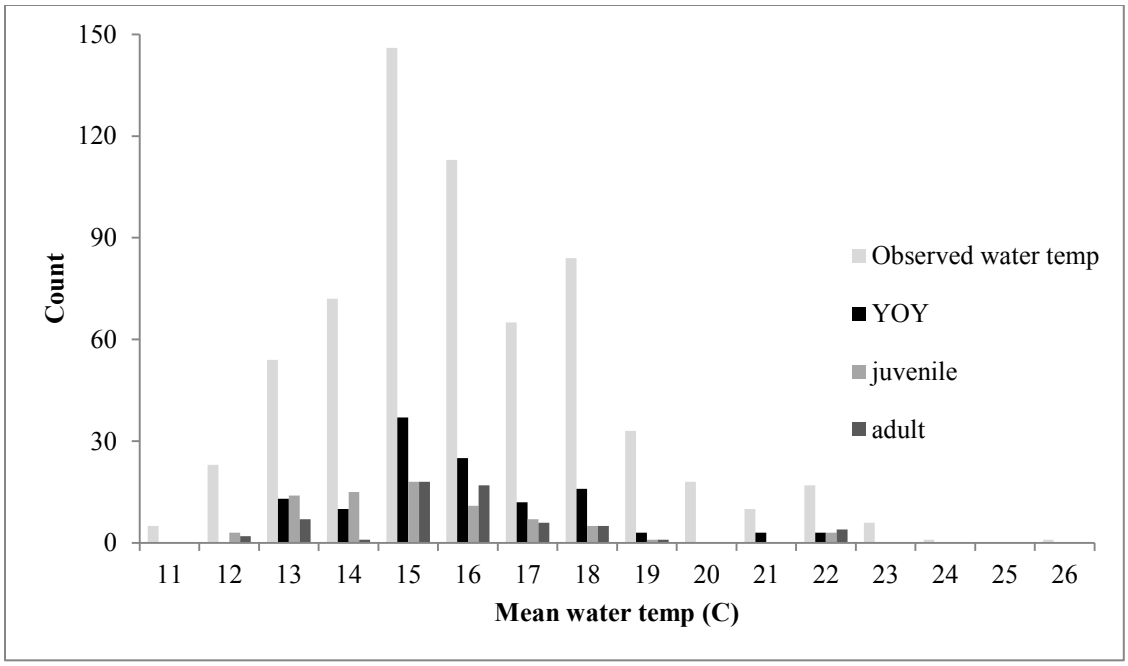


Figure 1.9 Mean water temperatures in which Oregon spotted frogs (OSF) were observed during visual encounter surveys. Three post-metamorphic life stages are represented, including young-of-year (YOY), juvenile, and adult.

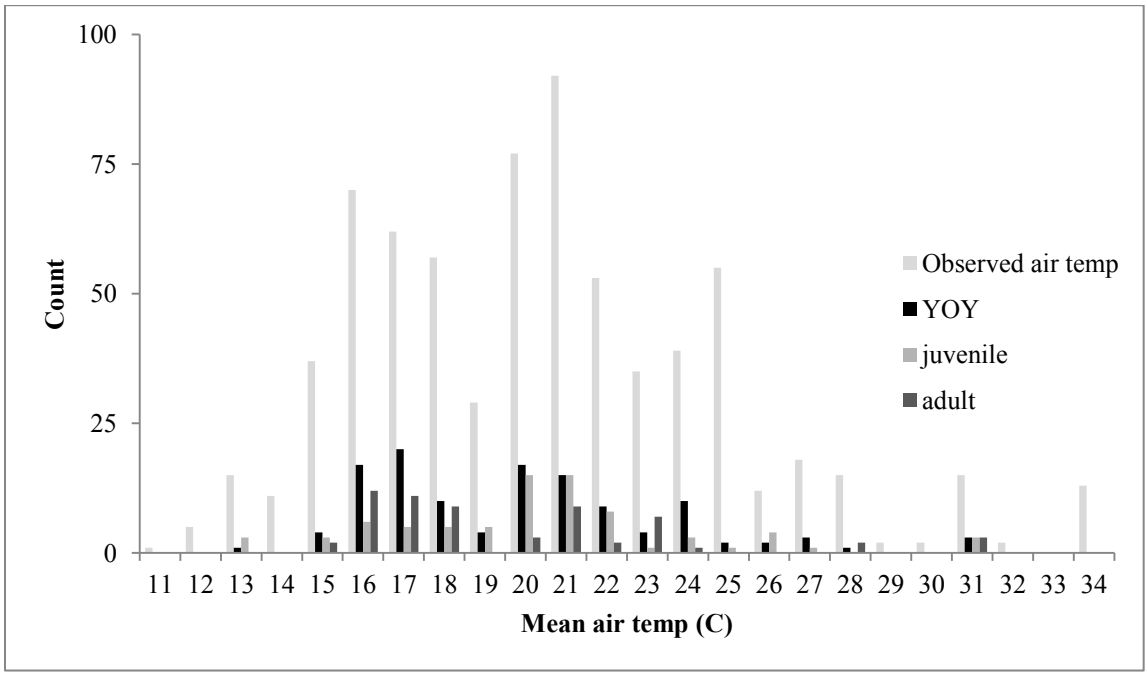
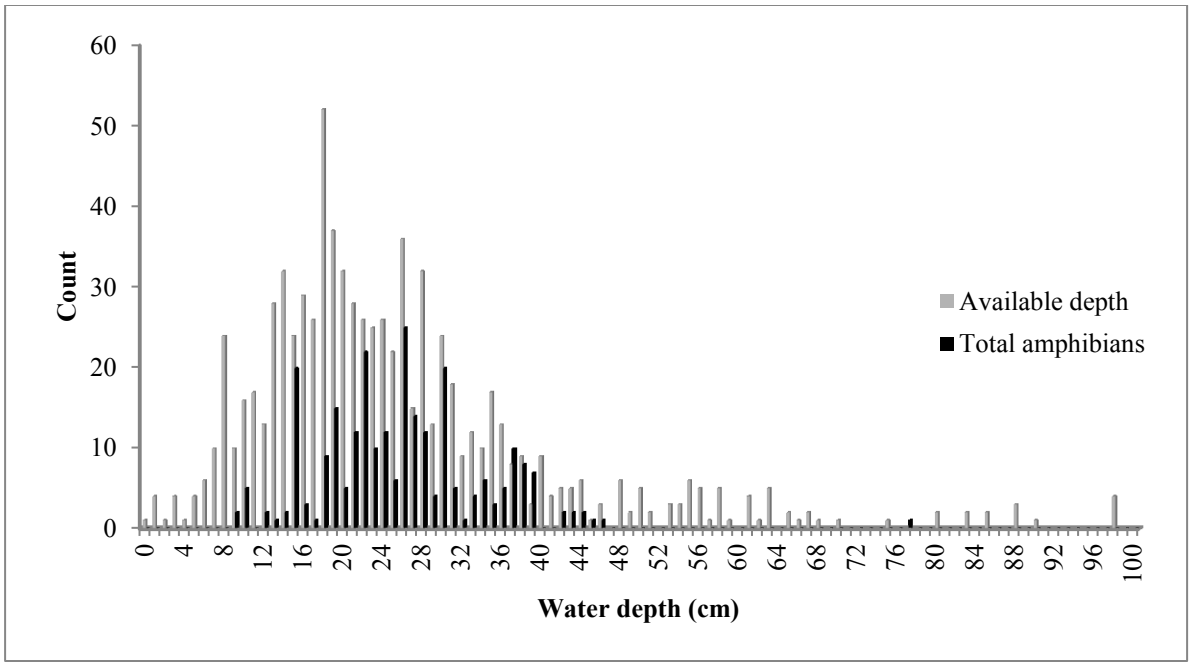


Figure 1.10 Mean air temperatures in which Oregon spotted frogs (OSF) were observed during visual encounter surveys. Three post-metamorphic life stages are represented, including young-of-year (YOY), juvenile, and adult.

A.



B.

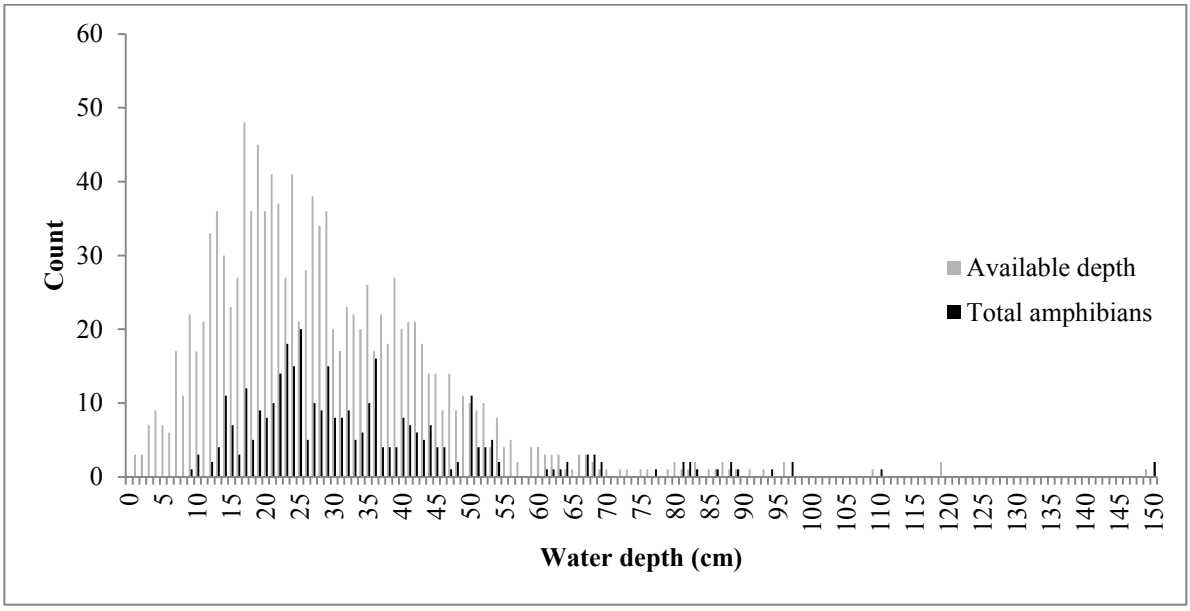


Figure 1.11 Distribution of available water depths from systematic dip-net samples and number of total amphibians observed at each depth at DC (A) and WRP (B).

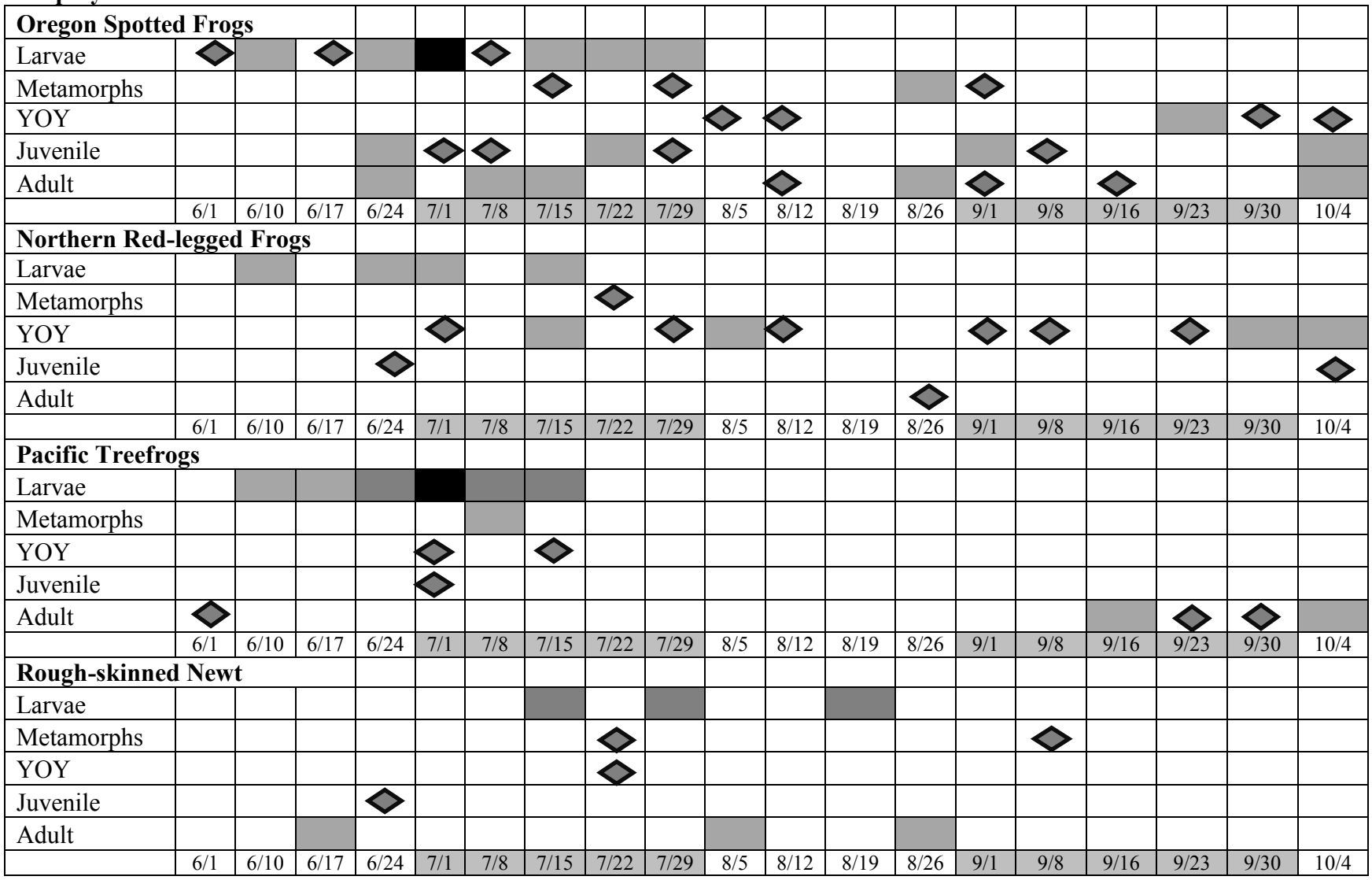
APPENDIX A. AMPHIBIAN OBSERVATIONS DURING SUMMER 2010

	n
◆	1
	≤ 5
	> 5
	Max

West Rocky Prairie

	5/25	6/8	6/15	6/22	6/29	7/6	7/13	7/20	7/27	8/3	8/10	8/17	8/24	8/31	9/9	9/14	9/21	9/28	10/4
Oregon Spotted Frogs																			
Larvae								◆		◆									
Metamorphs					◆														
YOY						◆													
Juvenile																			◆
Adult			◆				◆	◆	◆										◆
Northern Red-legged Frogs																			
Larvae											◆	◆	◆						
Metamorphs						◆													
YOY																			◆
Juvenile			◆		◆	◆	◆												
Adult																	◆		
Pacific Treefrogs																			
Larvae						◆	◆												
Metamorphs					◆	◆													
YOY				◆															
Juvenile																			
Adult																◆			

Dempsey Creek



APPENDIX B. TIMING OF SALAMANDER METAMORPHOSIS IN SUMMER 2010

◆	Metamorphs
▬	Premetamorphic larvae

West Rocky Prairie

Salamander Metamorphosis	5/25	6/8	6/15	6/22	6/29	7/6	7/13	7/20	7/27	8/3	8/10	8/17	8/24	8/31	9/9	9/14	9/21	9/28
Long-toed Salamander				▬														
Northwestern Salamander				▬	▬		▬				◆	◆		▬	◆	▬	▬	▬
Rough-skinned Newt													◆		▬			

Dempsey Creek

Salamander Metamorphosis	6/1	6/10	6/17	6/24	7/1	7/8	7/15	7/22	7/29	8/5	8/12	8/19	8/26	9/1	9/8	9/16	9/23
Northwestern Salamander					▬	◆	▬	▬	▬	▬	◆			▬			▬
Rough-skinned Newt										◆							

CHAPTER 2: EFFECTS OF THE HERBICIDE IMAZAPYR ON JUVENILE OREGON SPOTTED FROGS

Abstract - Conflict between native amphibians and aquatic weed management in the Pacific Northwest is rarely recognized because most native stillwater-breeding amphibian species move upland during summer, when herbicide application to control weeds in aquatic habitats typically occurs. However, aquatic weed management may pose a risk for aquatic species present in wetlands through the summer, such as the Washington State Endangered Oregon Spotted Frog (OSF; *Rana pretiosa*). Acute toxicity of herbicides used to control aquatic weeds tends to be low, but the direct effects of herbicide tank mixes on OSF have remained unexamined. Juvenile OSFs were exposed to tank mixes of the herbicide imazapyr, a surfactant, and marker dye in a 96-h static-renewal test. The tank mix was chosen because of its low toxicity to fishes and its effectiveness in aquatic weed control. Concentrations were those associated with low (3.5 L/ha) and high (7.0 L/ha) volume applications of imazapyr, and a clean-water control. Following exposure, frogs were reared for 2 mo in clean water to identify potential latent effects on growth. Endpoints evaluated included feeding behavior, growth, and body and liver condition indices. No mortalities were recorded and no significant differences were found for any endpoint between the herbicide-exposed and clean-water control frogs. Results suggest that imazapyr use in wetland restoration poses low risk of direct toxic effects on juvenile Oregon Spotted Frogs.

Keywords – Imazapyr, Amphibians, Surfactant, *Rana pretiosa,* Agri-Dex

INTRODUCTION

The leading causes of amphibian decline in the Pacific Northwest are habitat loss and deterioration [1]. The introduction and spread of invasive species can lead to extinctions of native species [2]. Wetlands may be particularly vulnerable to invasion by non-native plant species, especially when surrounding landscape changes alter wetland hydrology and nutrient levels [3]. Invasive plants can alter habitats and reduce abundance and diversity of animal species, alter nutrient cycles, and have the potential to change food-web dynamics [reviewed by 3]. Many invasive wetland plants form monocultures, establishing and maintaining dominance in wetlands through a combination of factors including tolerance to variable hydrologic conditions, high seed production or viability in wet conditions, and ability to spread vegetatively by rhizome expansion or movement of stem or root fragments [3]. The ability of these plants to dominate wetlands coupled with increasing restrictions on chemical control complicate habitat restoration for native species including amphibians.

The Oregon Spotted Frog (OSF; *Rana pretiosa*) is a Federal Candidate for listing under the U.S. Endangered Species Act [4], listed as Vulnerable on the International Union for Conservation of Nature Red List (www.iucnredlist.org, accessed August 2012), and Endangered in Canada (http://www.cosewic.gc.ca/, accessed August 2012) and Washington State (http://wdfw.wa.gov/, accessed August 2012**)**. Habitat loss and degradation are considered among the most likely causes of this species' decline and extirpation from 70-90% of its former range [5]. Loss and alteration of shallow breeding wetlands are of particular concern [5], including degradation caused by invasive reed canarygrass (*Phalaris arundinacea*) [6].

The Washington Department of Fish and Wildlife is responsible for OSF conservation in Washington State. Habitat Enhancement/Recovery is a top priority of the United States Fish and

Wildlife Service for the OSF. Washington State's Wildlife Action Plan makes habitat enhancement/recovery a top priority for at-risk species. Washington State lists wetlands among the Priority Habitats most at risk (wdfw.wa.gov/conservation/cwcs/, accessed August 2012), making them the most deserving of recovery efforts. The relatively recent spread of reed canarygrass into areas formerly occupied by OSF has led to a focus on reed canarygrass control in efforts to restore OSF habitat.

Control of invasive aquatic plants is often difficult because mechanical or manipulative approaches used to date show limited efficacy or are restricted in application because of local conditions. Reed canarygrass is particularly difficult to manage, leading to the establishment of the Reed Canarygrass Working Group within the Northwest Chapter of the Society for Ecological Restoration. A publication from The Nature Conservancy details control options in the Pacific Northwest [7]. Based on available options, effective reed canarygrass control apparently can only be achieved through a long-term commitment using a combination of several different methods, including herbicide application [7].

Few herbicides are approved for use in aquatic habitats in Washington State **(**www.ecy.wa.gov/programs/wq/plants/management/aqua028.html, accessed August 2012**)**. For those that are, data on their effects on native amphibians are lacking. This limitation effectively restricts herbicide use in most habitat enhancement/recovery efforts that could benefit at-risk species. Imazapyr and glyphosate are two non-selective herbicide active ingredients that are recommended for control of emergent aquatic weeds such as reed canarygrass in the Pacific Northwest [8] and allowed for use in aquatic habitats of Washington (www.ecy.wa.gov/ programs/wq/pesticides/regpesticides.html, accessed August 2012**)**. Imazapyr is considered to be among the least toxic herbicides available for use in aquatic environments with a 96-h LC50 >

100 mg/L for fish and aquatic invertebrates, but few data exist on its toxicity to amphibians [9]. Depending on the chemical and life stage, amphibians may be more or less sensitive than fish [10]. The only study available on the toxicity of imazapyr to amphibians is as yet unpublished, but shows very low toxicity (www.cal-ipc.org/symposia/archive/pdf/2008/7Trumbo.pdf, accessed August 2012). The 96-h LC50 of Habitat® (28.7% imazapyr IPA salt) for American Bullfrog (*Lithobates catesbeianus*) tadpoles was 1739 mg/L (95% confidence interval: 990.6 – 2256.7 mg/L). This suggests lower toxicity than triclopyr TEA, the active ingredient of a selective herbicide also allowed for use in aquatic environments (96-h LC50 814.1 mg/L, 95% CI: 769.6 - 847.1 mg/L) (http://www.cal-ipc.org/symposia/archive/pdf/2008/7Trumbo.pdf, accessed August 2012).

Herbicide tank mixes include the formulated product and additional carriers (e.g., water) and may also contain a surfactant and marker dye. Herbicide products labeled for use in aquatic systems are generally formulated without the addition of surfactants that can increase efficacy of the product, but also its toxicity [e.g., glyphosate-based products with the surfactant POEA labeled for terrestrial application, 11]. Surfactants allow the herbicide to penetrate the leaf cuticle, thereby increasing the efficacy of the herbicide. The addition of a surfactant approved for use in aquatic environments is recommended for emergent aquatic weed control. One of the least toxic surfactants (based on LC50s) approved for use in Washington is Agri-Dex®, which consists of a mixture of paraffin-based petroleum oil, polyoxyethylene, and sorbitan fatty acid ester (www.ecy.wa.gov/programs/wq/pesticides/regpesticides.html, accessed August 2012). It represents the surfactant of choice for control of reed canarygrass in OSF habitat, yet no data exist for effects of tank mixes containing imazapyr products + Agri-Dex on amphibians.

A key to understanding the potential effects of herbicides on amphibians is identification of the life stages at risk of exposure to herbicide at the time of weed control. For reed canarygrass, herbicide application in September may achieve the greatest control with the least amount of herbicide [12]. Post-metamorphic juveniles are the youngest OSF life stage (e.g., potentially the most vulnerable to herbicide toxic effects) present in September (A. Yahnke, unpublished data). For management agencies to proceed with reed canarygrass control in OSF habitats, it will be critical to demonstrate that harm to the species targeted for conservation does not occur from habitat restoration efforts.

The present study was designed to examine the acute and latent effects of operational imazapyr tank mixes on juvenile OSFs under laboratory conditions. Frogs were exposed for 96 h and then reared in clean water for 2 mo to assess latent effects on growth. In addition, because physiological effects can manifest through stress in the metabolic or detoxification pathways such as fatty inclusions or enlargement of the liver [13], liver condition indices were compared across treatments. Finally, because of concerns about the potential for endocrine disruption from exposure to environmentally-relevant concentrations of some herbicides [e.g., atrazine, 14], the gonads were visually inspected for gross anomalies and discrepancies between primary and secondary sexual characteristics.

MATERIALS AND METHODS

Experimental design

Post-metamorphic juvenile OSFs were exposed to two tank mixes with different herbicide concentrations associated with low and high volume applications and a clean water control in a 96-h static renewal test. Five aquaria were assigned to each herbicide tank mix and the control. Three frogs were randomly placed in each aquarium. Replicate tanks were randomly

distributed on three sides of a water table (used to maintain constant temperature in the test aquaria), five tanks per side. After the 96-h exposure period the frogs were reared in clean water for 2 mo.

Study animals

Oregon Spotted Frogs were reared from eggs collected from Conboy Lake National Wildlife Refuge, Glenwood, WA. Forty-five juveniles were obtained from the rearing facility (Woodland Park Zoo, Seattle, WA) on 18 August, within 4 wk post-metamorphosis. Prior to transfer, the rearing facility confirmed that the frogs were not infected by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis,* and certified them in good health. At the start of the 96-h exposure, on 23 August, frog masses averaged 3.3 g ± 1.3 SD and body sizes (SVL; snout-vent length) averaged 29.0 mm ± 3.4 SD. Frogs were too young to be sexed at the time of distribution to treatment tanks. At the end of the experiment, gender assessment revealed male to female ratios of 5:10 in the control group, 7:8 in the low group, and 8:7 in the high group.

Animal husbandry

All materials (aquaria, floats, nets, buckets) to which the frogs were exposed were pre-soaked in a buffered, PVP iodine solution (1:200 dilution, Ovadine, Western Chemical, Inc.), then rinsed and soaked in dechlorinated water before use. All nets and any other re-used materials were separated by treatment and held in the same solution of iodine (in separate buckets by treatment) and rinsed in dechlorinated water immediately prior to use.

Frogs were housed in enclosed 37.9-L glass aquaria held in a flow-through water table, with water temperatures maintained at 21.9°C ± 1.0 SD. Light was provided along the edge of one short side of the tanks from 2% UVB fluorescent bulbs (ReptiSun 2.0 UVB, ZooMed)

located within 45 cm of the bottom of the tank and filtered through the nylon mesh screen top [15]. Ultraviolet lights and overhead room lights were synchronized to a 13:11 light:dark cycle, the approximate duration of daylight at the time of the study. Upon receipt from the rearing facility, frogs were acclimated to the aquaria for 3 d prior to the start of the 96-h exposure. During the acclimation and exposure periods, aquaria were filled with 4 kg of clean water or clean water or treatment solution respectively, corresponding to a fluid depth of ca. 2 cm. That depth was sufficient for submersion of the frogs and to allow them to maintain an energy-conserving semi-floating position with hind-limbs contacting the bottom. All water changes used dechlorinated City of Seattle water.

Frogs were each offered five 3-wk-old crickets (Fluker Farms) twice per day, at approximately 0900 h and 1700 h. Frogs were allowed to forage undisturbed for 20 min, after which remaining crickets were counted and removed, along with any other waste, to minimize effects on water quality. During the grow-out in clean water, crickets were dusted with calcium (Tetrafauna Reptocal, Tetra Werke) and vitamins (Reptivite without D_3, Zoo Med Laboratories Inc.) prior to feeding on days when full water changes were scheduled (after feeding) to minimize frog exposure to conductivity changes in the water from the vitamins.

Floats were used as feeding platforms throughout the experiment, and as haul-outs and refugia during grow-out. Floats were constructed from 1.6-cm diameter CPVC pipes and 15 cm^2 × 0.45 cm clear plastic (Lucite International) plates. Fifteen-cm pipes were connected with 90° CPVC elbows using drinking-water grade PVC cement (Rain-R-Shine PVC Cement #30890, Oatey) to make a square float. A 0.45-cm deep feeding well was created by cutting an 11.5-cm diameter hole into the center of the plastic plate and attaching a second plastic plate, 12-cm diameter × 0.20 cm, with aquarium silicone sealant (All-Glass Aquarium Co.). The plastic was

sand-blasted to create a more opaque plate that the frogs could also use as refuge during grow-out. The corners of the plastic plates were attached to the middle of each pipe on the CPVC floats with aquarium silicone (Fig. 2.1). The attachment created holes at the corners of the CPVC pipe squares where frogs could emerge from the opaque refuge while maintaining a sense of cover, thereby providing some habitat complexity in an effort to minimize stress in the laboratory environment. Floats were only provided for 20 min at the 0900-h and 1700-h feedings during the 96-h tank mix exposure, but were placed in aquaria continuously during the 2-mo grow-out.

At the end of the 96-h exposure, frogs from herbicide treatments were transferred to new aquaria and new floats were provided. All aquaria initially received 5.25 L of clean, dechlorinated water. Water volume was increased during the grow-out period to account for frog growth and the associated increase in ammonia levels. Full water changes were made every 2 to 4 d during grow-out, and partial water changes were made as needed based on water quality.

Temperature (°C) in the water bath was monitored daily, with current, minimum, and maximum values recorded approximately every 24 h. Water pH, dissolved oxygen (DO; mg/L), and conductivity (µS/cm) levels were monitored daily during the 96-h exposure, prior to renewal, and at full water changes during the grow-out. Waterproof electronic testers were used to determine instantaneous pH and conductivity (PCTester 35 and ECTester 11, Oakton Instruments) and DO and temperature (HQ-10, Hach). Water quality measurements were collected from mixing buckets prior to distribution of treatment solutions. Ammonia (ppm) levels were monitored daily with API® Freshwater/Saltwater Ammonia Test Kits (Mars Fishcare) during the 96-h exposure and at full and partial water changes during the grow-out.

Frogs were euthanized at the end of the experiment by submersion in MS-222 at 3 g/L with equal sodium bicarbonate for 90 min. Livers were extracted and weighed, and primary sex organs were observed to confirm sex determination from secondary sex characteristics and to check for any overt abnormalities. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington, Protocol 2185-42.

Tank mixes and analytical chemistry

The tank mix included the formulated imazapyr product, Polaris^{TM} AQ (Nufarm Americas Inc.); the surfactant, Agri-Dex (Helena Chemical Company); and marker dye, Hi-Light® (Becker Underwood). Concentrations of tank mixes were determined based on the estimated "worst-case" field exposure scenario for direct over-spray to 2 cm of standing water with no intervening vegetation. The test concentrations of Polaris AQ were based on label recommendations for low-volume, 3.5 L/ha and high-volume, 7.0 L/ha applications (hereafter Low and High). Polaris AQ is formulated with 28.7% isopropyl amine salt of imazapyr (a.i., active ingredient) by weight. Exposure concentrations were 4.8 ppm and 9.7 ppm a.i. for Low and High treatments. These exposure concentrations are 24 to 48 times higher than maximum field concentrations of 0.2 ppm measured 1-h post treatment in Washington (http://www.ecy.wa.gov/programs/wq/pesticides/final_pesticide_permits/noxious/monitoring_data/monitoring_index.html, accessed August 2012). Agri-Dex and Hi-Light were included at equal rates in both treatments. Agri-Dex exposure of 44.4 ppm was based on a calculation of 1% volume/volume and an application rate of 9.4 L/ha. Hi-Light concentration of 11.2 ppm was based on an application rate of 2.3 L/ha.

For the 96-h exposure test, the tank mixes were pre-mixed by weight with deionized water for each treatment in an amber glass stock bottle to make 500 g of stock solution, and

stored at 2-4°C. At each 24-h renewal, fresh treatment solutions were made from 10 mL of each treatment stock mixed with 24 kg dechlorinated water in a 26.5-L plastic bucket with a clean, food-grade plastic liner. The dechlorinated water was the same temperature in which the frogs were maintained. For each replicate tank, renewal treatment solutions were distributed by weight from the stock buckets. Prior to distribution, a sample was collected from the 26.5-L mixing buckets of each treatment solution. Another sample was collected at the time of treatment solution renewal (24, 48, 72, 96 h) from one randomly selected aquaria in each of the High and Low treatments. Two additional samples were collected from High aquaria 24 h after frogs were transferred to clean water at the end of the 96-h exposure period. Samples were held up to 48 h at 2 to 4°C until shipment to Pacific Agricultural Laboratory (Portland, OR) for imazapyr analysis. Samples were analyzed for imazapyr using the American Cyanamid Method (HPLC-MS), with a method reporting limit of 1.0 ppm.

Test endpoints

Endpoints included growth, body condition, behavior, number of crickets consumed, an index of liver to body condition, and sexual traits. Growth was measured as weight (to the nearest 0.01 g) and snout-vent length (SVL: to the nearest 0.5 mm), and was converted to body condition for analysis. Frogs were measured before and after the 96-h exposure test, twice during the 2-mo grow-out, and just prior to sacrifice at the end of the experiment (0, 4, 32, 49, and 60 d after the start of the 96-h exposure).

Body condition was estimated using the scaled mass index (SMI: $\hat{M}_I = M_i[L_0/L_i]^{b}_{SMA}$), which is calculated using the mass (M_i) and SVL of the ith individual (L_i), mean SVL (L_0= an arbitrary value of SVL to standardize the SVL), and slope (b_{SMA}) from the standardized major axis (SMA) regression of linearized mass on SVL [16]. Scaled mass index is preferred over other

measures of body condition because it is insensitive to size differentials that may occur between genders in sexually dimorphic species like OSF [16], and it has been shown to perform better than alternative body condition estimates [17]. The calculation for b_{SMA} was done using the "Software for Reduced Major Axis Regression [synonymous with SMA], JAVA version" (http://www.kimvdlinde.com/professional/rma.html, accessed August 2012). The body condition was also used to estimate an index of liver health by taking the ratio of liver mass (to the nearest 0.001 g) to body condition at the time of sacrifice, 60 d after the start of the 96-h exposure.

Behavior was measured during the 96-h exposure by recording morning feeding activity with a camcorder (Vixia HFS21, Canon) at 24, 48, and 96 h of exposure. Eight min of video were analyzed; starting 1 min after the researchers left the room. The total seconds each individual frog spent in the center circle (11.5 cm diameter) of the float was summed for the duration of time frogs spent on the float per tank. The number of crickets that remained in each aquarium after 20 min of undisturbed feeding was also recorded for both the morning and evening feedings throughout the 96-h test and 2-mo grow-out interval.

Secondary sex characteristics were recorded 44 d after the 96-h exposure, and again with primary sex characteristics during liver extraction after the frogs were euthanized at the end of grow-out. Gonads were visually inspected for gross anomalies, and discrepancies from secondary sex characteristics were recorded.

Statistical analysis

Data were summarized and graphed in Excel (Microsoft, 2010) and explored and analyzed in SPSS (PASW, Version 18). Data were evaluated for departures from normal using Shapiro-Wilk tests [18]. Differences among tanks within treatments and among treatments at the start of the experiment were tested using one-way ANOVAs on initial length, weight, and body

condition for each frog (n =3 per tank, 15 per treatment). Differences in liver condition index among treatments at the end of the experiment were tested using one-way ANOVA [18].

Data were assessed to maximize the strength of the relationship between mass and SVL such that the most reliable estimate of b_{SMA} for calculating body condition was obtained, following Peig and Green [17]. Although use of only control individuals reduced the sample size for the SMA regression used to determine b_{SMA}, the results from the data assessment (not presented) were consistent with Peig and Green's [17] findings that data from reference individuals were more appropriate for estimating b_{SMA} in toxicological studies. Therefore, only data from the control group were used to estimate body condition as SMI for each sample date.

Because individual frogs were not identified over time, tank means were used to evaluate changes in body condition and behavior among treatments across time using linear mixed models (LMM). Frogs were measured at five different times during the experiment, but the sample days were not set at equal intervals. Therefore, the LMM for body condition using sample day as a repeated measure included an unstructured covariance. The cumulative number of crickets consumed per tank was also compared among treatments using LMM. Sample day was included as a random variable because there was no control over the number of crickets that were consumed on a given day, only the number offered. Also, because the frogs could not be sexed when originally distributed in the tanks, the number of males in each tank was included as a random variable during LMM model exploration for the number of crickets consumed. For all models, tank was included as subject, sample day as a covariate with treatment as the factor, and the fixed effects of treatment, sample day, and the interaction of treatment and sample day. Models were estimated using maximum likelihood estimates, and models with random factors

were tested for significant differences from the simple model with no random factors using the difference between -2 log likelihood estimates as the chi-square (X^2) statistic.

RESULTS

At the start of the experiment, no differences existed among tanks within treatments in frog mass, SVL, and body condition as SMI (Table 2.1). No differences were found among tanks within treatments, so all frogs within a treatment were pooled to test for differences among treatments. At the start of the experiment, no differences existed among treatments in body mass ($f_{0.05,4,14} = 0.2$, $p = 0.835$), SVL ($f_{0.05,4,14} < 0.1$, $p = 0.966$), and body condition ($f_{0.05,4,14} = 1.5$, $p = 0.237$). No mortalities occurred during the course of the present study and no overt effects on behavior or general health were observed.

Target concentrations of imazapyr were achieved. Values were 92 to 108 percent of the Low (4.4 to 5.2 ppm) and 91 to 97 percent of the High (8.8 to 9.4 ppm) nominal concentrations. Concentration did not change with time between water exchanges for either the Low (4.6 to 5.0 ppm) or High (9.5 to 9.7 ppm) treatments. Recoveries were 96 to 104 percent at 24 h. No imazapyr was detected in tanks with frogs exposed to High treatments 24-h after placement in clean water at the start of the grow-out.

Water quality

Water quality was similar across treatments in the mixing buckets prior to renewal of treatment water, among treatments in the tanks 24-h after renewal, and among treatments during the grow-out in clean water (Table 2.2). Dissolved oxygen in the tanks was slightly lower in High and Low treatments than in controls, but DO never fell below 6.0 mg/L for any treatment at any time.

Behavior

The relationship between behavior and treatment over time showed significant variance in intercepts across tanks, var = 6453.8, $X^2_{0.05,1} = 5.4$ (the difference between the -2 log likelihood estimates for models without and with random intercepts), $p = 0.020$. A random intercept was included in the final LMM model. No significant effects of treatment ($f_{0.05, 2, 42.884} = 0.359$, $p = 0.701$), sample day ($f_{0.05, 1, 30} = 0.015$, $p = 0.903$), and the interaction of treatment × sample day ($f_{0.05, 2, 30} = 0.504$, $p = 0.609$) were found (Fig. 2.2). Also, no significant relationships existed for any treatment over time, or any interactions between treatment and sample day (Table 2.3).

Visual inspection of graphed data of cumulative crickets consumed over time showed little difference between treatments. Differences existed when data were plotted based on the number of males in each tank (Fig. 2.3), and broad variation existed in the total number of crickets consumed on a daily basis (Fig. 2.4). The number of males in a tank contributed significantly to the variation in the cumulative crickets consumed in each treatment over time, var = 6985.6, $X^2_{0.05,1} = 1121.6$ (difference between -2 log likelihood for models without and with the random factor of number of males), $p < 0.0001$.

The variation over sample day did not follow a consistent pattern, but the number of crickets consumed per day rose and fell consistently among the treatments (Fig. 2.4). Sample day also contributed significant variation to the cumulative crickets consumed in each treatment over time, var = 10.09, $X^2_{0.05,1} = 3330.05$ (difference between -2 log likelihood for models without and with the random factor of sample day), $p = < 0.0001$. The model including both the number of males and the sample day as random factors was further improved over the model without random factors, $var_{males} = 87.6$, $var_{sample\ day} = 9.8$, $X^2_{0.05,2} = 3446.2$, $p < 0.0001$, and was a

significantly better model fit than the model with males only, $X^2_{0.05,1} = 2324.5$, $p = < 0.0001$, and the model with sample day only, $X^2_{0.05,1} = 116.1$, $p < 0.0001$.

In the final model for the cumulative number of crickets consumed over time, the only significant fixed factors were the intercept ($f_{0.05,1,24.8} = 25.592$, $p < 0.0001$) and sample day ($f_{0.05,1,15.0} = 382.204$, $p < 0.0001$); neither significant effects of treatment ($f_{0.05,\,2,\,34.0} = 2.291$, $p = 0.117$) nor treatment × sample day interaction ($f_{0.05,2,15.0} = 0.019$, $p = 0.982$) were found. Significant linear relationships existed between treatment and sample day for control and Low treatments, but not for High, and no significant linear effects of the interactions between individual treatments and sample day were found (Table 2.4).

Body condition

The body condition LMM using sample day as a repeated measure was a significant improvement over the simple model, $X^2_{0.05,\,14} = 52.1$, $p < 0.0001$. Sample day was the only significant predictor for changes in body condition over time, $f_{0.05,\,1,\,15} = 2935.8$, $p < 0.0001$. No significant effects of treatment ($f_{0.05,\,2,\,15} = 3.354$, $p = 0.063$) or the treatment × sample day interaction ($f_{0.05,\,2,\,15} = 1.115$, $p = 0.354$) were found. Significant linear relationships existed between treatment and sample day for the control and High treatments, but not for Low, and no significant effects of the treatment × sample day interactions existed on the linear relationship of body condition over time (Fig. 2.5, Table 2.5).

Liver condition

No differences existed in liver condition among tanks within treatments (ANOVA, control: mean 0.098 ± 0.009 SD, $f_{0.05,\,4,\,14} = 0.6$, $p = 0.641$; Low: mean 0.098 ± 0.007 SD, $f_{0.05,\,4,\,14} = 2.5$, $p = 0.112$; High: mean 0.096 ± 0.010 SD, $f_{0.05,\,4,\,14} = 1.6$, $p = 0.256$) and among

treatments with frogs pooled across tanks ($f_{0.05, 2, 44} = 0.1$, $p = 0.864$). Because frogs within tanks may violate assumptions of independence, liver condition was also compared among treatments using the tank mean values. No differences existed in tank mean liver condition among treatments, $f_{0.05, 2, 14} = 0.1$, $p = 0.898$.

Gonads

No gross anomalies were observed in gonads. Primary and secondary sexual characteristics were consistent for all but one frog. One male frog in the High treatment had well-developed nuptial pads and underdeveloped testes, as compared to the relative primary and secondary sexual characteristics of all other frogs examined.

DISCUSSION

No acute or latent effects of imazapyr tank mixes on OSF juveniles were observed in the present study. Several different end-points were evaluated to establish confidence in our assessment that no differences existed between individuals exposed to imazapyr tank mixes and controls. There was a disconnection between the external and internal states of sexual characteristic development of one male frog relative to other frogs. This was unlikely to be treatment-related because only one individual expressed the condition. It is also not likely to be ecologically important. Presumably gonad development would catch up with the secondary sex characteristic development by the approximate time of the breeding season, 4 mo later.

Treatment effect on body condition approached a significant difference ($p = 0.063$). Due to the randomized distribution of frogs, mean body condition values at the start of the experiment were ranked control > Low > High. At the end of the 96-h exposure period, mean body condition values had converged, such that body condition improved while the frogs were in the tank mix. That may be an effect of feeding during the exposure. Prior to testing, the frogs were reared in

large cattle tanks with many other individuals. The effects of competition during rearing may have resulted in lower body conditions that subsequently improved after frogs were placed in lower densities in the laboratory. It should also be noted that frogs did not restrict foraging to the floats. Crickets that had jumped into the water were also consumed, so frogs in treatment tanks were also ingesting the tank mix, thereby effectively receiving the tank mix through two routes of exposure, absorption through skin and ingestion.

The frogs in this experiment were fed and had no exposure to stressors that can occur in their normal habitats. No determination of imazapyr effects were made on animals under stressful conditions. In this preliminary experiment, the primary concern was with evaluating acute and latent effects of the tank mix itself. In wild populations, several natural stressors can impact the health and survival of individuals. The effects of predation, reduced water levels as ponds and wetlands dry during the summer, and/or high population densities can increase individual stress levels and potentially reduce fitness and survival. The presence of predators alone can increase non-consumptive mortality in some tadpole species, presumably due to increased stress [19, 20]. The toxicity of pesticides may also be altered in the presence of predators. Tadpoles in experimental mesocosm communities with a newt (*Notophthalmus viridescens*) predator experienced higher mortality when a glyphosate product with a toxic surfactant (Roundup with POEA) was added [19, 20].

Pond-drying may also affect individual fitness and survival. Typically pond-drying has been evaluated on larval amphibians, showing that many species have developmental plasticity that allows them to adapt by metamorphosing sooner. This comes at a potential cost to survival later, due to often smaller sizes post-metamorphosis [21]. Introducing a pesticide to amphibian

communities experiencing the effects of pond-drying may alter the "norm of reaction" [as described by 21] to the natural stressor.

For many juvenile frogs, the effects of pond-drying may not have the physiological impact that it has on tadpoles. However, for a more aquatic species like the OSF [6], pond-drying may concentrate individuals into remaining wet habitats, potentially making them more vulnerable to inter- and intra-species predation [22]. Although no treatment-related effects were observed on growth or behavior of OSF juveniles exposed to imazapyr that might indicate a higher vulnerability to predation, no testing was done for responses in natural conditions of pond-drying, concentration of individuals and/or increased competition, or presence of predators [e.g., 23]. Furthermore, no other native species were tested that might be present at the time of herbicide application. Other species present may be predators of OSF juveniles [22]. The disruption of predator-prey dynamics has occurred with exposure of either the predator or the prey to other contaminants [e.g. carbaryl, an acetylcholinesterase-inhibiting insecticide, 24].

A paucity of data exists for direct effects of imazapyr tank mixes on any of the fauna in native wetland communities. This creates uncertainty for land managers who desire to implement its use for habitat restoration in wetlands. In several herbicide studies, the toxicity of the surfactant in the terrestrial formulation is more toxic than the active ingredient [25-27]. Although these data are compelling evidence to support concerns associated with the use of more toxic tank mixes in terrestrial habitats where amphibians occur, they are more difficult to interpret in relation to aquatic herbicide tank mixes used in wetland restoration. In Washington, surfactants are regulated such that only those that have low aquatic toxicity are allowed for use in wetlands. Therefore using an active ingredient with an approved surfactant in Washington should provide

some level of protection against direct toxic effects to fauna. For amphibians, indirect effects may be more important [28].

In amphibian habitats, stressors often occur simultaneously with multiple predator species, the effects of pond drying, and high amphibian densities. Many potential predators of OSF were observed in OSF habitats during the weed management season, and many of them co-occurred in time and location (A. Yahnke, unpublished data, University of Washington, Seattle, Washington, USA). In this case, the indirect effects of herbicide treatments may be of concern in that habitat alteration may increase vulnerability to predation through the removal of plants that provide cover, or change food web dynamics. Future research should focus on ecological interactions with herbicide treatments.

Other aspects of herbicide treatment were outside the scope of this study, but may be important to consider in evaluating herbicides for use in aquatic systems. Latent effects on the reproductive ability or resistance to desiccation of exposed individuals [29] were not evaluated. Nor were effects investigated on a histological or molecular level that may reveal symptoms not visible in the gross examination. More in-depth analyses may be required to identify effects like those that have raised concern with other herbicides, such as effects on endocrine [14] or immune system [30] functions.

Evidence that minimal harm to species targeted for protection will come from habitat management is important in conservation work. There is little information in peer-reviewed literature about the toxicity of imazapyr to amphibians. Documenting the potential for minimal harm is essential to maximizing the effectiveness of habitat restoration tools such as aquatic herbicides. Thus, studies that show no effects of an aquatic herbicide to non-target organisms are a critical contribution to the toxicological literature. Imazapyr is an important and effective tool

in conservation and habitat restoration to manage invasive plants such as reed canarygrass that degrade critical wetland habitat.

REFERENCES

[1] Olson, D.H. 2009. Herpetological conservation in northwestern North America. *Northwest Naturalist* 90:61-96.

[2] Clavero M, García-Berthou E. 2005. Invasive species are a leading cause of animal extinctions. *Trends in Ecology and Evolution* 20:110.

[3] Zedler J, Kercher S. 2004. Causes and consequences of invasive plants in wetlands: Opportunities, opportunists, and outcomes. *Critical Reviews in Plant Sciences* 23:431-452.

[4] U.S. Fish and Wildlife Service. 2010. Endangered and threatened wildlife and plants; Review of native species that are candidates for listing as endangered or threatened; Annual notice of findings on resubmitted petitions; Annual description of progress on listing actions; Proposed rule. Federal Register 75:69222-69294.

[5] McAllister KR, Leonard WP. 1997. Washington State status report for the Oregon Spotted Frog. Washington Department of Fish and Wildlife, Olympia, WA, 38 pp.

[6] Watson JW, McAllister KR, Pierce DJ. 2003. Home ranges, movements, and habitat selection of Oregon Spotted Fogs (*Rana pretiosa*). *Journal of Herpetology* 37:292-300.

[7] Tu M. 2004. Reed Canarygrass (*Phalaris arundinacea* L.) -- Control & Management in the Pacific Northwest. B Lipinski and J Soll ed. The Nature Conservancy, Wildland Invasive Species Team, Oregon Field Office, 12 pp.

[8] Parker B. 2009. Aquatic weed control. In Peachy EO, ed, *Pacific Northwest Weed Management Handbook*. Extension Services of Oregon State University, Washington State University, and University of Idaho, Corvallis, OR. p 267

[9] AMEC Geomatrix Inc. 2009. Human Health and Ecological Effects Risk Assessment - Imazapyr Risk Assessment - Washington State. Washington Department of Agriculture, Lynnwood, WA, 308 pp.

[10] Bridges CM, Dwyer FJ, Hardesty DK, Whites DW. 2002. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? *Bulletin of Environmental Contamination and Toxicology* 69:562-569.

[11] Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* 23:1928-1938.

[12] Adams CR, Galatowitsch SM. 2006. Increasing the effectiveness of Reed Canary Grass (*Phalaris arundinacea* L.) control in wet meadow restorations. *Restoration Ecology* 14:441-451.

[13] Crawshaw GJ, Weinkle TK. 2000. Clinical and pathological aspects of the amphibian liver. *Seminars in Avian and Exotic Pet Medicine* 9:165-173.

[14] Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and field evidence. *Environmental Health Perspectives* 111:568-575.

[15] Adkins E, Driggers T, Ferguson G, Gehrmann W, Gyimesi Z, May E, Ogle M, Owens T. 2003. Roundtable: Ultraviolet light and reptiles, amphibians. Moderator: Klaphake E. *Journal of Herpetological Medicine and Surgery* 13:27-37.

[16] Peig J, Green AJ. 2009. New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos* 118:1883-1891.

[17] Peig J, Green AJ. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. *Functional Ecology* 24:1-27.

[18] Zar JH. 1999. *Biostatistical Analysis*, 4 ed. Prentice-Hall, Upper Saddle River, NJ.

[19] Relyea RA, Schoeppner NM, Hoverman JT. 2005. Pesticides and amphibians: The importance of community context. *Ecological Applications* 15:1125-1134.

[20] Relyea RA. 2003. Predator cues and pesticides: A double dose of danger for amphibians. *Ecological Applications* 13:1515-1521.

[21] Newman RA. 1992. Adaptive plasticity in amphibian metamorphosis. *Bioscience* 42:671-678.

[22] Licht, L.E. 1974. Survival of embryos, tadpoles, and adults of the frogs *Rana aurora aurora* and *Rana pretiosa pretiosa* sympatric in southwestern British Columbia. *Canadian Journal of Zoology* 82:613-627

[23] Boone MD, James SM. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. *Ecological Applications* 13:829-841.

[24] Bridges C. 1999. Predator-prey interactions between two amphibian species: Effects of insecticide exposure. *Aquatic Ecology* 33:205-211.

[25] Cauble K, Wagner RS. 2005. Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. *Bulletin of Environmental Contamination and Toxicology* 75:429-435.

[26] Relyea RA, Jones DK. 2009. The toxicity of Roundup Original Max® to 13 species of larval amphibians. *Environmental Toxicology and Chemistry* 28:2004-2008.

[27] King JJ, Wagner RS. 2010. Toxic effects of the herbicide Roundup® Regular on Pacific Northwestern amphibians. *Northwest Naturalist* 91:318-324.

[28] Brodman R, Newman WD, Laurie K, Osterfeld S, Lenzo N. 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. *Journal of Herpetology* 44:69-82.

[29] Rohr JR, Palmer BD. 2005. Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. *Environmenta Toxicology and Chemistry* 24:1253-1258.

[30] Kerby JL, Storfer A. 2009. Combined effects of atrazine and chlorpyrifos on susceptibility of the Tiger Salamander to *Ambystoma tigrinum* virus. *EcoHealth* 6:91-98.

Table 2.1 Analysis of variance parameters for frog size and body condition among tanks within treatments at the start of the experiment

	Control		Low		High	
Parameter	f[a]	p	f[a]	p	f[a]	p
SVL (mm)	1.9	0.186	0.4	0.807	1.0	0.443
Mass (g)	2.1	0.159	0.2	0.936	1.8	0.207
Body condition (SMI)	0.5	0.761	0.8	0.526	1.4	0.303

[a] Between groups degrees of freedom = 4; total degrees of freedom = 14
SVL = Snout-vent length

Table 2.2 Water quality (means ± SD) pooled across days from treatment mixing buckets and frog tanks

Parameter	Treatment	Temp (°C)	DO (mg/L)	pH	NH_3 (mg/L)	Conductivity (µS/cm)
Mixing buckets (n=3)	Control	23.2 ± 0.3	8.2 ± 0.2	6.5 ± 0.1	< DL	80.0 ± 0.0
	Low	23.4 ± 0.2	8.1 ± 0.1	6.5 ± 0.0	< DL	80.0 ± 0.0
	High	22.6 ± 0.5	8.4 ± 0.1	6.6 ± 0.1	< DL	80.0 ± 0.0
96-h exposure (n=20)	Control	23.2 ± 0.2	7.9 ± 0.1	6.8 ± 0.1	1.0 ± 0.2	90.5 ± 2.2
	Low	23.3 ± 0.2	6.9 ± 0.4	6.7 ± 0.1	< DL	90.0 ± 0.0
	High	23.2 ± 0.2	7.2 ± 0.6	6.7 ± 0.1	< DL	90.0 ± 0.0
Grow-out (n = 90)	Control	21.5 ± 0.7	7.5 ± 0.2	6.5 ± 0.2	1.6 ± 0.6	95.9 ± 6.5
	Low	21.4 ± 0.7	7.5 ± 0.3	6.6 ± 0.1	1.5 ± 0.6	95.1 ± 5.9
	High	21.4 ± 0.7	7.4 ± 0.4	6.6 ± 0.1	1.6 ± 0.7	95.9 ± 6.3

DO = Dissolved oxygen; DL = Detection limit (1 mg/L)

Table 2.3 Estimates of linear mixed model fixed effects on behavior as the sum of seconds that frogs were on the center of the float

Parameter	Estimate	Standard error	*df*	*t*	*p*	95% Confidence interval
Intercept	182.6	68.2	42.9	2.677	0.010	45.1-320.1
Sample day	-13.6	22	30	-0.619	0.540	-58.3-31.2
High	-45.6	96.4	42.884	-0.473	0.639	-240.1-148.9
Low	-81.5	96.4	42.884	-0.845	0.403	-276-113
Control[a]	0	0	.	.	.	.
High × Sample day	14.3	31	30	0.461	0.648	-49-77.6
Low × Sample day	31.1	31	30	1.003	0.324	-32.2-94.4
Control × Sample day[a]	0	0	.	.	.	.

[a] Control parameters are set to 0 because they are redundant
df = Degrees of freedom

Table 2.4 Estimates of linear mixed model fixed effects on cumulative number of crickets consumed over time

Parameter	Estimate	Standard error	*df*	*t*	*p*	95% Confidence interval
Intercept	7.3	2.6	553.9	2.786	0.006	2.2-12.5
Sample day	15.6	1.4	15	11.143	<0.001	12.6-18.6
High	6.6	6.6	16.9	0.998	0.333	-7.3-20.4
Low	9.1	4.4	293.1	2.078	0.039	0.5-17.8
Control[a]	0.0	0.0	.	.	.	.
High × Sample day	0.2	2.0	15	0.115	0.91	-4.0-4.5
Low × Sample day	0.4	2.0	15	0.192	0.85	-3.8-4.6
Control × Sample day[a]	0.0	0.0	.	.	.	.

[a] Control parameters are set to 0 because they are redundant

Table 2.5 Estimates of linear mixed model fixed effects on body condition over time

Parameter	Estimate	Standard error	t [a]	p	95% Confidence interval
Intercept	3.6	0.1	45.743	<0.001	3.5-3.8
Sample day	0.1	0.004	32.21	<0.001	0.1-0.1
High	-0.3	0.1	-2.585	0.021	-0.5- -0.1
Low	-0.2	0.1	-1.432	0.173	-0.4-0.1
Control[b]	0.0	0.0	.	.	.
High × Sample day	-0.003	0.01	-0.500	0.625	-0.02-0.01
Low × Sample day	-0.01	0.01	-1.469	0.163	-0.02-0.004
Control × Sample day[b]	0.0	0.0	.	.	.

[a] All parameters analyzed with 15 degrees of freedom
[b] Control parameters are set to 0 because they are redundant

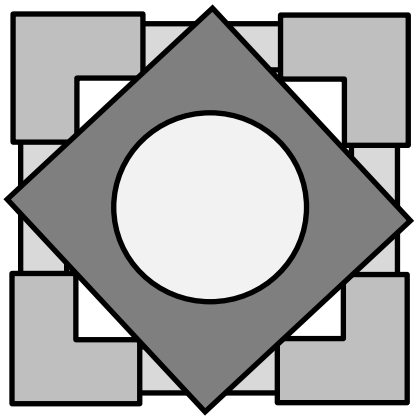


Figure 2.1 Diagram of feeder float. CPVC pipes, 1.5 cm diameter, were connected with 90° CPVC elbows to serve as a float. A 15 cm^2 plastic platform with a 0.45-cm deep, 11.5-cm diameter well was attached to the CPVC pipes. Floats served as feeding and haul-out platforms as well as refuges.

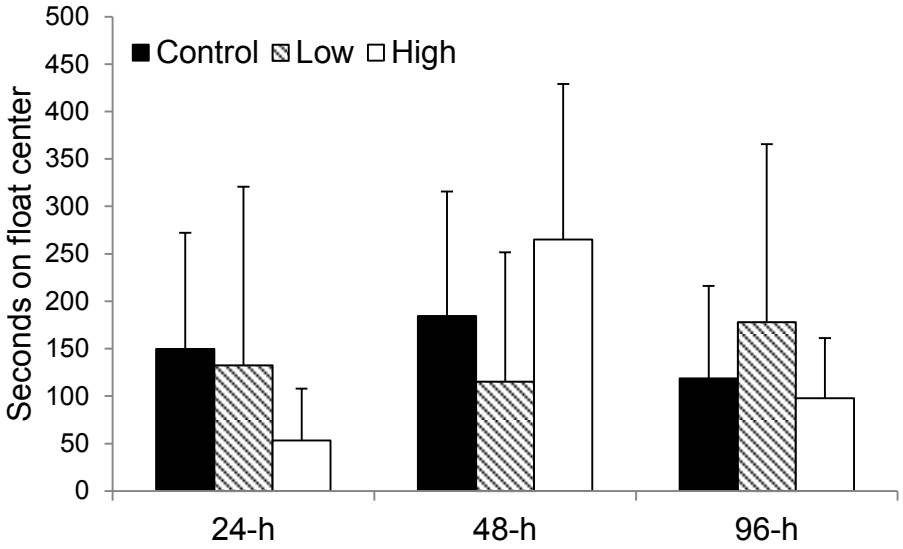


Figure 2.2 Time frogs spent in the center ring of the float/feeding platform during 8 min of the morning feeding at three times during the 96-h exposure. There were no significant effects of treatment, sample day, and the interaction of treatment × sample day. Bars = 1 SD.

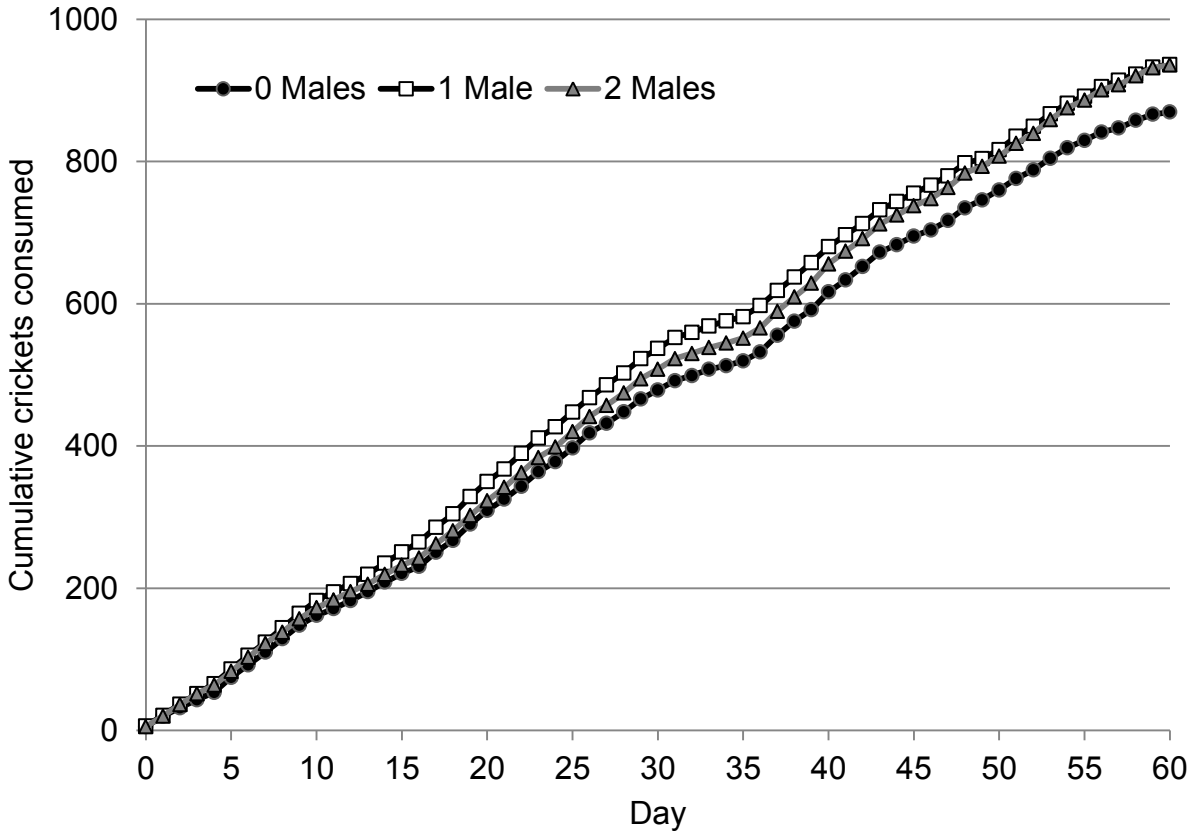


Figure 2.3 Cumulative number of crickets consumed per day by the number of males in a tank. Black circles: none of the three frogs in a tank were male, white squares: one of the three frogs in a tank was a male, gray triangles: two of the three frogs in a tank were male. The number of males in a tank contributed significantly to the variation in crickets consumed over time.

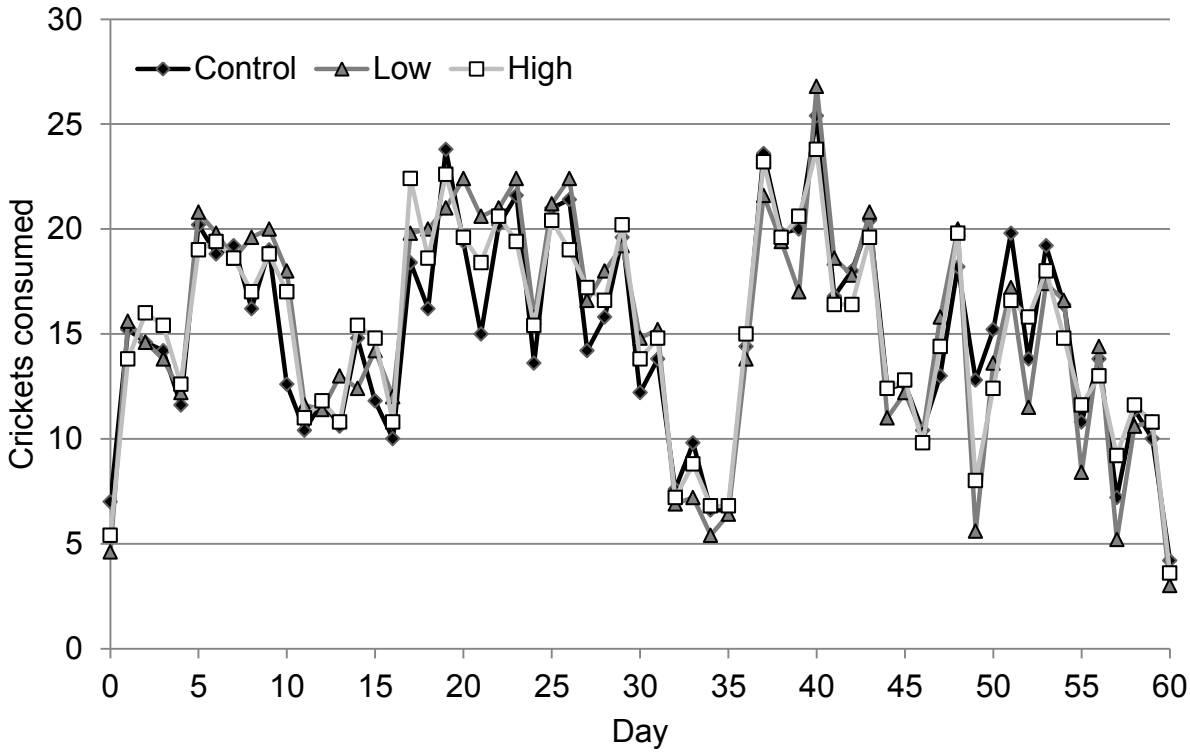


Figure 2.4 Mean crickets consumed per day by treatment. Sample day contributed significantly to the variation in crickets consumed over time, but treatment did not.

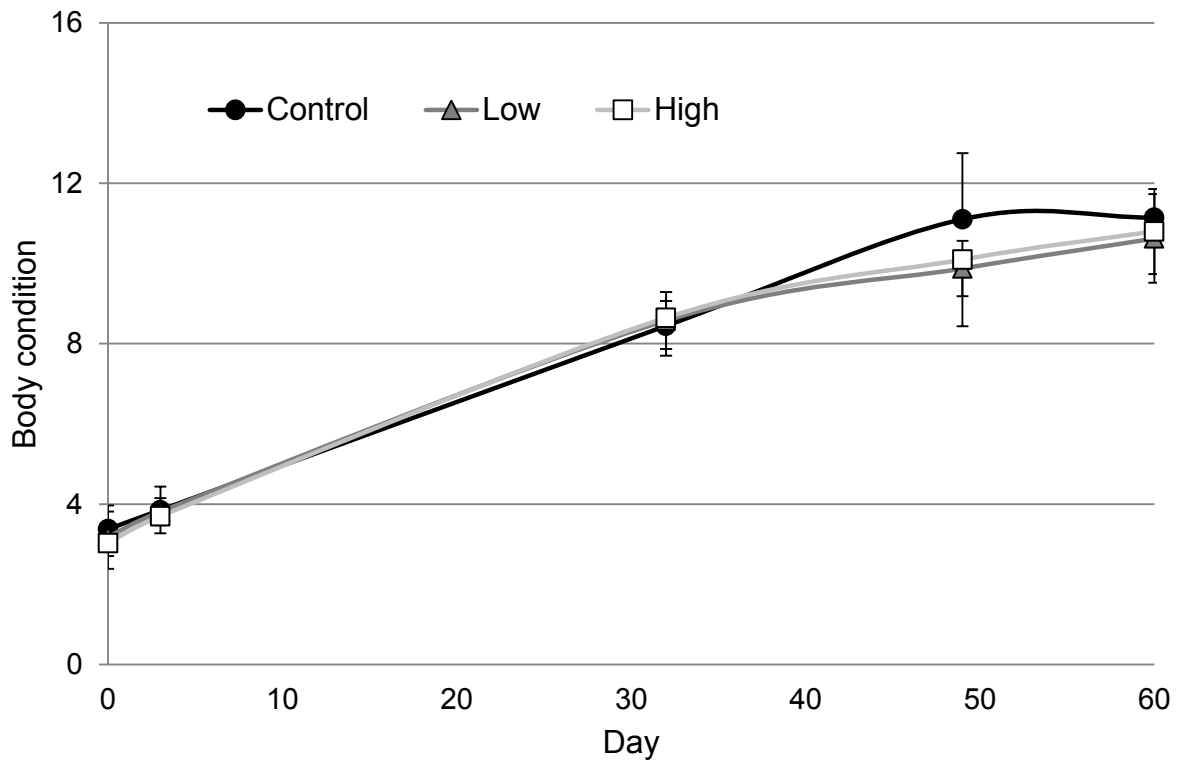


Figure 2.5 Mean body condition per treatment on five sample days. Bars = 1 SD. There were no significant effects of treatment and the interaction of treatment × sample day.

CHAPTER 3: EFFECTS OF THE HERBICIDE TRICLOPYR ON METAMORPHIC NORTHERN RED-LEGGED FROGS

Abstract: Aquatic herbicides are used to manage invasive emergent plants in and around wetlands. Metamorphic frogs that emerge during the aquatic weed management season may be at risk of herbicide exposure. Metamorphic northern red-legged frogs (*Rana aurora*) were exposed to an aquatic triclopyr tank mix used for control of broadleaf emergent aquatic weeds such as invasive purple loosestrife (*Lythrum salicaria*). The tank mix consisted of Renovate® 3 (triclopyr triethylamine salt [TEA] 44.4%), the modified vegetable oil surfactant Competitor® (ethyl oleate, sorbitan alkylpolyethoxylate ester, dialkyl polyoxyethylene glycol; 98%), and marker dye Hi-Light®. Metamorphs were exposed to the tank mix and a clean-water control for 96 h, then reared in clean water for 60 d. Exposure to the tank mix resulted in no treatment-related mortalities, no effects on behavior immediately post-exposure, and no effects on body or liver condition indices. Exposure to the tank mix resulted in a 1-d delay in completion of metamorphosis. The presence of limb deformities was unrelated to exposure but interacted with the tank mix to reduce the number of crickets consumed by deformed frogs, though no overall difference existed between treatments. Observed effects were minimal, but potential may exist for interactions with other environmental stressors to reduce survival.

Keywords – Triclopyr, Amphibian, Surfactant, Competitor, Tank Mix

INTRODUCTION

Integrated pest management (IPM) is usually employed and often required in the control of invasive aquatic plants [1]. Several management tactics are employed when using IPM to manage invasive plants. Herbicides are among the tools of IPM, and may be used to manage invasive plants in wetlands. Concern exists over the potential impacts of herbicides on wetland fauna, especially amphibians, due to the demonstration of impacts from agricultural and other terrestrial-use pesticides [2, 3]. Few data exist, however, for impacts to wetland fauna from the aquatic formulations of herbicides used in wetland restoration.

The United States Environmental Protection Agency (EPA) requires acute toxicity testing of technical-grade active ingredients and "typical end-use products" (formulated product or similar formulated product) on freshwater fish and invertebrates when a product will be applied to water [4]. No requirements for such testing currently exist for amphibians. Herbicide toxicity may vary from that of the active ingredient when combined with surfactants and other inert ingredients that comprise the formulated product [5]. Moreover, considerable variation exists in species [6-9] and life-stage responses to the effects of pesticides [10, 11].

In Washington State, besides the federal requirements, herbicide applications to wetlands require compliance with state permitting and pesticide regulations. Triclopyr triethylamine salt (TEA) is among the selective herbicides currently allowed for use on broadleaf emergent weeds in wetlands. Triclopyr TEA is orders of magnitude less toxic to birds, fish, and aquatic invertebrates than the related triclopyr herbicide compound, triclopyr butoxyethyl ester (BEE) [12]. However, 3,5,6-trichloro-2-pyridinol (TCP), a degradate of triclopyr acid common to both parent chemical formulations, is similar to BEE in toxicity to fish [12, 13]. The toxicity of the

triclopyr degradate reveals uncertainty associated with the use of the aquatic triclopyr formulations and the potential for impacts on wetland fauna.

Amphibians are a major constituent of wetland faunas. Many species depend on wetland habitats for food and refuge; and to support reproduction, early development, metamorphosis, post-metamorphic growth, and over-wintering. Because of the marked variability of amphibian habitat use during the year, Paton and Crouch [14] suggested that amphibian phenology and habitat use should be included in conservation and management of habitats where amphibians occur. Although not all amphibians remain in the wetland throughout the year, many species are present at various life stages during the plant management season of late spring through autumn [15]. Furthermore, the young of many species undergo metamorphosis, a major physiological change, in summer [16], when aquatic weeds are actively growing and herbicide application may be most effective [17].

Amphibians exhibit ontogenetic differences in susceptibility to pesticides, especially for the egg and larval stages. Median lethal concentrations (LC_{50}) of a formulated triclopyr BEE product (Release®) for tadpoles from four genera were lower than those of embryos, suggesting higher sensitivity of tadpoles [10]. Likewise, embryos were more tolerant than metamorphs of exposure to an insecticide and fungicide used in apple orchards [11]. Depending on the formulated product, tank mixes with the same herbicide may increase [18] or decrease [19] time to metamorphosis in amphibians exposed during larval stages, depending on species. Pesticides can also manifest lag effects, such as affecting post-metamorphic stages of amphibians exposed during larval stages [11, 20].

Metamorphosis involves a series of physiological changes and results in increased stress hormones [21]. Amphibians are not known to eat during the metamorphic climax (emergence of

forelimbs) due to the restructuring of the mouth and digestive system at that time [22]. Food availability may ameliorate effects of contaminants. Tadpoles exposed to Release® in low food environments experienced more rapid and greater mortality than those exposed to the same concentrations in high food environments [23]. Without the ability to take in food, the potential exists for greater sensitivity to contaminant exposure during metamorphic climax.

The phenology of wetland species is an important consideration to include in the effective evaluation of the risk of using herbicides to control emergent invasive plants. Results from field studies during summer months indicate that northern red-legged frogs (*Rana aurora*) metamorphose in mid-summer in the Puget Sound region (Yahnke, Chapter 1 in current dissertation). Data are lacking for the effects of contaminants on amphibians exposed at the onset of metamorphic climax. To address that gap, northern red-legged frogs at metamorphic climax were exposed to an aquatic herbicide tank mix at an environmentally relevant concentration. The tank mix consisted of an aquatic triclopyr product used to manage broadleaf weeds in wetlands, a surfactant, and a marker dye. Triclopyr was selected based on its use in wetlands to control emergent broadleaf weeds like purple loosestrife (*Lythrum salicaria*) and a paucity of data for its effects on amphibians. Competitor® was selected based on indications from invasive plant managers that its use was becoming more common (Olympic Knotweed Working Group Meeting, Port Hadlock, Washington, 16 November 2011), and data suggesting it to be more toxic than other aquatic herbicides (K.E Vincent, 2009, MS thesis, San Francisco State University, San Francisco, CA, USA).

MATERIALS AND METHODS

Experimental design

Northern red-legged frogs (*Rana aurora*) were exposed at metamorphic climax to one triclopyr tank mix with an herbicide concentration associated with labeled rates for control of emergent wetland weeds and a clean-water control in a 96-h static renewal test. Fifteen frogs were assigned to each treatment, herbicide tank mix and control, and housed individually in treatment buckets. Replicate buckets were randomly distributed on two shelving units in an environmental chamber. After the 96-h exposure period, frogs were reared in clean water for 2 mo. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington, protocol 2185-42.

Study animals

Eggs were collected from a small pond in Olympia, Washington, on 19 March 2012. Forty-five eggs were gently separated from each of two egg masses for a total of 90 eggs collected. Eggs were further separated into groups of 15 and placed in three net pens in each of two rearing tanks (genetic Group A and B). Each rearing pool was dedicated to the eggs of one egg mass, such that mixing of genetic groups was prevented. Genetic groups were maintained separately throughout the experiment. Water temperature was 10.5° C in the rearing tanks when eggs were introduced. Eggs began hatching in A on 22 March and all were hatched on 25 March, while none had yet hatched from B. Hatching began in B on 25 March and was completed on 27 March. One hatchling was lost due to an abraded yolk sac, which sometimes occurs when rearing in net pens (personal communication, Jon Wittouk, University of Washington Hatchery). One tadpole moribund due to unknown causes was euthanized 16 May.

At the start of the 96-h exposure, the mean weight of metamorphs was 2.9 g ± 0.4 SD, snout-vent length (SVL) averaged 22.3 mm ± 0.5 SD, and total length averaged 67.2 mm ± 3.8 SD. No secondary sex characteristics were visible at that time to determine gender. At the end of the experiment, gender assessment revealed ratios of 9:6 female to male frogs in each treatment.

Animal husbandry

All materials were pre-soaked in a buffered PVP iodine solution (1:200 dilution, Ovadine, Western Chemical, Inc.) and rinsed in dechlorinated water before use. All nets and any other re-used materials were separated by treatment, held in treatment-designated buckets with PVP iodine solution, and rinsed in dechlorinated water before use.

Eggs and tadpoles were reared in net pen nurseries within circular, low-density polyethylene (LDPE) hatchery tanks, 122 cm diameter × 81 cm maximum depth. The tanks were located outdoors at the University of Washington Hatchery. During rearing at the hatchery, the tadpoles were held in aged (at least 3 d), City of Seattle water. Tadpoles were held in the net pens until they were large enough to release into the tank. The nurseries were constructed using monorail net replacement bags, 40.6 × 40.6 cm frame with 15.2 cm depth and 0.8 mm mesh (Aquatic Eco-Systems Inc.). The nets were attached to 46-cm diameter rings of 1.3-cm (0.5 in) cross-linked polyethylene (PEX) pipe (Vanguard Pipe and Fittings, Ltd.) that, with the addition of four black plastic, 13 cm floats (model FL125, Miller Net Company, Inc.), maintained a floating net pen with the lip poised a minimum of 1 cm above the water surface.

Most tadpoles were released from net pens into the tanks 1 June, once they had reached a size large enough to prevent injury during tank maintenance. Tadpoles averaged 16.4 mm snout-vent length (SVL) and 48.4 mm total length when releases began. The seven smallest tadpoles

were maintained in the net pens to reduce risk of injury and increase their opportunity to access food with less competition from conspecifics.

The feeding regime followed that described by Woodland Park Zoo rearing facility personnel who raise the related species, Oregon Spotted Frog (*Rana pretiosa*), from eggs to juveniles for release. Each net pen in A was offered 1/3 of an algal wafer (Hikari ®) on 24 March. Hatchlings clung to the nets with their adhesive glands and did not exhibit directed "searching behavior" for food for several days post-hatching. Hatchlings in A were first seen on wafer debris 28 March. Wafers were first offered to B on 30 March. Net pens also had some established algae growth at the time of hatching in A and B. Tadpoles were offered algal wafers and a mix of wilted kale and romaine lettuce. The greens were blended and frozen in ice cube trays. Cubes of frozen greens were thawed in a cup of tank water before dispersal in the respective tanks.

Water quality was monitored daily for pH, temperature (°C), conductivity (µS), dissolved oxygen (DO; mg/L and % saturation), and ammonia (mg/L). Electronic testers were used for DO and temperature (model 85, YSI Inc.), pH (PCTester 35, Oakton Instruments) and conductivity (ECTester 11, Oakton Instruments). Ammonia was tested in rearing tank and grow-out water using a standard colorimetric aquarium test kit with a detection limit of 0.25 ppm (API® Freshwater/Saltwater Ammonia Test Kits, Mars Fishcare). Water changes began routinely on 30 March, occurring weekly until 18 May, when daily water quality monitoring indicated a need to increase the frequency due to the first detection of ammonia. All water changes replaced approximately two-thirds of the water, using water from tanks identical and adjacent to the rearing tanks. During the water change on 30 May, the tadpoles reacted as if irritated, twitching back and forth when the water was added. Chlorine was detected at 0.08-0.1 mg/L in the water

that had been aged for 3 d, so air stones were added to the aging tanks to improve chlorine removal. City of Seattle water ranges from 0-2 mg/L and may vary over time, such that a higher pulse may have occurred, resulting in more chlorine than previously detected in the tanks.

As tadpoles reached Gosner (1960) Stage 41, beginning 11 July, they were transferred indoors to an environmental chamber (Bally Engineered Structures, Inc.). Temperature within the chamber was set at 18°C to maintain water temperatures within the range of mean temperatures observed in field sites with northern red-legged frog metamorphs (Yahnke, MS thesis, University of Washington, Seattle, Washington, USA; Chapter 1 of current dissertation). Tadpoles were housed in static, dechlorinated City of Seattle water in 189-L plastic tanks until peak of metamorphosis (emergence of first forelimb). Replacement water was held in 208-L lined plastic drums for at least 2 d prior to use. Frog rearing and replacement water were aerated throughout the experiment.

96-h exposure

Rearing tanks were checked daily for metamorphs. Testing began with emergence of the first forelimb, at least 2-d after transfer to the environmental chamber. Because metamorphosis did not occur on the same day for all individuals, the number of metamorphs available on each day was determined and replicates were alternately distributed to tank mix and control treatments. The corresponding number of buckets was then prepared. Controls were distributed first on each day to prevent contamination with the tank mix. Replicate locations were randomized on the shelving unit.

Metamorphs were placed in 7.8-L plastic buckets with 3.785 L of test solution. Buckets were housed in the environmental chamber on two shelves of a metal shelving unit, four buckets to each side of the shelves. Replicates were randomly distributed on the shelves. Each bucket

was fitted with a 21.5 × 9.5-cm clear plexiglass shelf. The length of the shelf matched the bucket diameter. The shelf was placed on plastic brackets 1 cm below the fluid surface. The shelf provided a shallow platform on which metamorphs could rest while remaining submerged, and allowed for metamorphs to be observed when they were below it. Bucket lids were designed to provide access to and refuge from light, which were 2% UVB fluorescent bulbs (ReptiSun 2.0 UVB, ZooMed) positioned within 45 cm of the bucket bottom. Half circles were cut into the standard plastic bucket lid and covered with plastic mesh that was attached with silicone to the opening. Lids were placed such that the straight edge of the half-circle in the middle of the lid was perpendicular to the resting shelf in the bucket (Fig. 3.1). Lights were maintained at a 14:10 light-dark cycle to approximate the daylight duration at the time tadpoles were moved to the environmental chamber.

Dechlorinated City of Seattle water was used for controls and to mix with stock concentrations for the tank mix. Test water was held in a 208-L drum in the environmental chamber where the test buckets were located. The drum was covered when not in use and the water was aerated throughout the test.

Metamorphs were housed in the test solutions for 96 h with 24-h renewal of treatment solutions. The treatment solution renewal involved a series of steps designed to allow for daily measurement of metamorphs with minimal handling and minimal time out of test solutions. New test solution for each replicate was weighed in a clean bucket. Approximately 0.3 L of each subject's pre-weighed solution was collected in a clean 0.5-L plastic cup and distributed to a 7.8-L plastic measurement bucket designated to each treatment. The measurement bucket included a ruler attached to the bottom. Each metamorph was gently netted from its exposure bucket and placed in the measurement bucket with the new solution, during which a dorsal photograph was

collected with a digital camera (PowerShot G6 PC1089, Canon) and the metamorph's test bucket solution was replaced with the remaining pre-weighed new test solution. Metamorphs were returned to their experimental buckets with the remaining volume of new solution in which they were held, and replaced on the shelf. Measurement buckets were rinsed in PVP-ovidine solution and dried with clean paper towels between holding each metamorph.

Metamorphs were observed six times during the 96-h exposure period. Observations were conducted at approximately 0, 6, 24, 48, 72, and 96 h after placement of each metamorph in treatment or control solutions. The data recorded included the location of each metamorph in its bucket (bottom, shelf, water column), body position, and whether it moved during the observation. Body position observations included whether legs were extended or held with knees bent in a typical frog resting position.

Behavior trial

At the end of the 96-h exposure, each metamorph was transferred to a translucent plastic shoebox (33 × 20 × 13 cm; Sterlite Corp.) with black plastic squares attached to the bottom to create a checkerboard pattern. The shoebox was located in an open cooler with white opaque sides and bottom so the clear squares appeared white within the shoebox. Approximately 1 cm of clean water (0.6 kg) was added to the shoebox prior to introducing the metamorph. Metamorphs were transported to the shoebox in an opaque plastic cup with a flat plastic lid. The cup was gently turned upside down and placed in the center of the shoebox, where four alternating black and white squares met. The plastic lid was slid from under the cup and the metamorph was released into the shoebox. The behavior of each metamorph was recorded for 10 min with a camcorder (Vixia HFS21, Canon) located via tripod approximately 1 m above the shoebox. Two shoebox arenas were used, one each for the treatment and controls. Due to an oversight during

arena construction, the alternating checkerboard pattern in the shoeboxes was different: the control box had a black square in the upper left corner, whereas the tank mix box had a white square in that location. The mistake was not noticed until after trials began so data interpretation is presented in the current paper with that in consideration, however it did not appear to influence the results.

Grow-out

After the behavioral trial, metamorphs were returned to their 7.8-L buckets with clean water. Tank-mix exposed metamorphs received new buckets upon placement in clean water. The resting platform was replaced with a float that served as a haul-out and feeding platform. The initial float design was insufficient for metamorphs to successfully haul-out, and resulted in the drowning of 10 individuals from the first group of metamorphs in the test. The floats were made from clear plexiglass plates, 9.5 × 10.5 cm, attached with silicone to sealed, 5-cm long glass vials as floats. The drowning may have been related to the tendency of the metamorphs to try to access the float structure from beneath, but it may also have been an effect of their inability to see the clear plexiglass platform. The lack of any holes to emerge from likely prevented successful access to the platform. To address the issue, water levels were lowered and floats were replaced with a new float designed to allow metamorphs to access the haul-out from below the platform. The new haul-out included a 10-cm square black plexiglass plate attached with silicone to a float made with 1.6 cm (5/8 in) aquarium tubing. Because the metamorphs may not have been able to locate the clear floats, the black plexiglass was used to provide a clear visual cue of structure in the water. The aquarium tubing was connected on each end by a 2-cm section of 1.3-cm (1/2-in) tubing and sealed with silicone to create the float. A 16-cm long strand of plastic aquarium milfoil was attached to the tube with a zip tie to provide a ladder onto the float and underwater

structure. The metamorphs were able to cling to the milfoil and hide in it, as well as use it to access the platform. Individuals lost to drowning were replaced with new metamorphs from the rearing tanks that served as additional replicates in the 96-h exposure and grow-out.

Water was exchanged daily until metamorphosis was complete, using the same measurement bucket transition and dorsal photograph collection methods as described above. The tank mix measurement bucket was replaced with a clean bucket for the grow-out period to avoid re-exposing metamorphs to any residual tank mix chemicals. After metamorphosis, water in the grow-out buckets was exchanged every 2-3 d.

When the tail of metamorphs was resorbed to a length of < 0.25 cm, they were each offered one cricket (Fluker's®), which was left in the containers with the frogs overnight. Beginning three days after completion of metamorphosis (no measurable tail remaining), frogs were offered five crickets overnight. Then at 15 d post-metamorphosis, frogs were offered five crickets for 20 minutes daily. At the end of each feeding period, crickets were counted and removed from the frog buckets.

Metamorphs were measured and weighed five times during this study. Digital dorsal photographs were collected daily from the start of the 96-h exposure to the completion of metamorphosis. Photographs were later assessed in ImageJ [24] to determine snout-vent length (SVL) and tail length (all lengths in mm). Weights (g) were collected at the start and end of the 96-h exposure, at completion of metamorphosis, 45 d post-metamorphosis, and at the end of the 2-mo grow-out, when frogs were sacrificed for liver collection. When metamorphs began to sit-up and dorsal photographs were no longer reliable for SVL and tail measurement, and for all measurements post-metamorphosis, lengths were collected by hand with a ruler.

Water quality was monitored daily in rearing tanks and experimental buckets. Water temperatures in the hatchery rearing tanks were monitored and summarized from samples collected for water quality analysis and thermometers that recorded current, minimum, and maximum temperatures. Experimental buckets were randomly assigned for water quality analysis such that each bucket was tested twice during the 96-h exposure. The same randomized schedule was maintained during the grow-out so that each bucket was tested for water quality approximately twice weekly. It was not possible to accurately read the colorimetric assessment of ammonia in the treatment solution during the 96-h exposure due to the dye in the tank mix, so experimental buckets were not tested for ammonia. All water samples were collected and analyzed in 0.5 L disposable plastic cups to prevent cross-contamination of containers.

Limb and spinal abnormalities were present in the rearing population, and did not correspond to any treatment-related effects. Though scoliosis was apparent in some tadpoles, rearing facilities for Oregon Spotted Frogs in Washington and Oregon have indicated that it was commonly observed at varying rates each year, and that it was no longer apparent after metamorphosis in those individuals that survived (Oregon Spotted Frog Working Group meeting, 16 November 2010). The use of individuals with severe scoliosis was avoided, but numbers were not sufficient to avoid use of all curved individuals. The limb deformities were not apparent at metamorphosis and were unavoidable in the test. When frogs were dissected for the liver weights, spines were also assessed for scoliosis, any limb deformities were recorded, and gonads were assessed for gross anomalies and sex determination. A magnitude was assigned to limb deformities (1-3) based on the number of limbs affected (e.g., 1 = one limb, 3 = three limbs).

Frogs were euthanized at the end of the experiment by submersion in MS-222 at 3 g/L with equal sodium bicarbonate for 90 min. Livers were extracted and weighed, then fixed whole

in 10% buffered formalin. Livers were fixed in formalin for at least 6 mo before being generically coded and sent to the University of Washington Histology and Imaging Core for histological slide preparation. Slides were prepared with standard hematoxylin and eosin staining. Histology was analyzed by Dr. George Sanders at University of Washington Comparative Medicine, who was blind to the nature of the treatments at the time of analysis.

Tank mix and analytical chemistry

Treatment solutions consisted of a clean water control and one concentration of a triclopyr tank mix. The tank mix consisted of Renovate® 3 (triclopyr triethylamine salt [TEA] 44.4%, SePro Corporation), the modified vegetable oil surfactant Competitor® (ethyl oleate, sorbitan alkylpolyethoxylate ester, dialkyl polyoxyethylene glycol; 98%; Wilbur-Ellis), and marker dye Hi-Light® (Becker Underwood Inc.). Concentration of the tank mix was based on a "worse-case" scenario of direct overspray to 2 cm of water without intervening vegetation, and was estimated based on labeled rates for control of purple loosestrife (*Lythrum salicaria*). Exposure concentrations were 47.1 ppm a.i. Renovate3, 41.3 ppm Competitor, and 12.9 ppm Hi-Light.

Four liters of tank mix were pre-mixed by weight with deionized (DI) water in amber glass stock bottles to make 1 L of stock concentration per bottle, and stored at 2-4°C. Stock concentrations were designed such that the volume of stock required in mg was equal to the volume of test solution required in kg. At each 24-h renewal, the tank mix stock solution was distributed by weight to a glass beaker and covered. The solution was transported to the environmental chamber, where it was mixed with the test water and distributed as described to the experimental buckets.

Samples were collected from new test solutions prior to distribution in the buckets for 0-h concentration analyses. Samples for 0-h concentration analyses were spread through time such that 0-h concentrations were evaluated at the start of the test with the first exposure group, in the middle of the test, with the start of replicate numbers 7 and 8, and at the end of the 96-h exposures with the last replicate. Samples were collected from randomly selected replicates at 24-h renewals corresponding to the same start, middle, and end of test period as the 0-h samples. One sample was collected 24-h post placement in clean water. Samples were held at 2-4°C for up to 48 h before being sent to Pacific Agricultural Laboratory (Portland, OR) for triclopyr analysis. Samples were analyzed for triclopyr using Modified EPA 8321B (HPLC-MS) with a quantification limit of 2.0-10.0 ppm (depending on calibration curves for samples analyzed on different days). The sample collected from clean water 24-h into the grow-out was analyzed for triclopyr using the same method, but with a quantification limit of 0.01 ppm. The quantification limit was lower for the clean-water sample because the expected concentration was orders of magnitude below that of the tank mix solution.

Test endpoints

Endpoints included behavior, growth, time to complete metamorphosis, number of crickets consumed post-metamorphosis, liver condition, and liver histology. Liver histology was assessed at 20× magnification. Abnormal cells that occurred within the frame were counted and livers were classified based on acuity, focality, and severity of those cells.

Each frog was observed five times during the 96-h exposure. Behaviors from those observations were summarized as the total number of observations in which a specific behavior was observed for each treatment. From the behavior trials in the checkerboard arenas, 8 min of video were analyzed, starting one minute after the release of the metamorph into the arena.

Different types of movements were identified. Time spent on black and white squares and the number and types of movements were summarized from the videos. The number of metamorphs in each treatment that were on the white or black squares for more than 50% of the time was determined, with the expectation that, at a minimum, controls would spend a greater proportion of time on the black than white squares to maintain crypsis.

Latent effects on foraging behavior were assessed by using the number of crickets consumed over time post-metamorphosis. Growth was measured as SVL (to the nearest 0.5 mm) and mass (to the nearest 0.01 g) and was converted to body condition for analyses [25-27].

Statistical analyses

Data were analyzed in Excel (Microsoft 2010) and SPSS (PASW Version 18). Data were evaluated for departures from normal using Shapiro-Wilk tests. Magnitude of effect for statistically significant results from parametric tests was evaluated using Cohen's *d* [28].

To calculate body condition using the Scaled Mass Index method, data from control individuals may provide the most reliable data to estimate key parameters [26]. When data were assessed for the best fit, data from the control group did not consistently provide the most reliable estimate of bSMA (slope of ordinary least squares [OLS] regression/r) as determined by the OLS R^2 values at each of the different times when the frogs were measured (Table 3.6).

To assess the relative importance of using data from all of the individuals, only the controls, or only tank mix frogs to calculate the body condition, the estimated body condition ($\hat{M}_i$) derived from bSMA estimates was compared from each data set from each period. All body condition data were normally distributed except those associated with the metamorphic period. A one-way ANOVA was used to test the data from the normally-distributed periods, and a Mann-Whitney U test was used to compare the data during metamorphosis.

Liver condition and days to metamorphosis were tested for differences between treatments using standard *t*-tests. Body condition and cricket consumption data were assessed graphically for differences among blocks associated with the bucket location (shelving unit, shelf, and position on shelf), sex, genetic group, limb deformities, and spinal curves. Where differences in body condition were noted graphically, data within each time-point were tested for statistically significant differences using *t*-tests or ANOVAs with an alpha of 0.05.

For data analyses from observations collected during the 96-h exposure, repeated observations of specific behaviors were summarized as the number of times each frog was observed expressing each behavior. The data distributions for each behavior were tested for differences between control and tank-mix exposed frogs using Kolmogorov-Smirnov (K-S) tests. The amount of time metamorphs spent on black or white squares during behavior trials was analyzed using 50% as the null hypothesis for the expected time on either color. The number of metamorphs that spent >50% of their time on white or black squares was summarized in a 2 × 2 contingency table and tested with X_c^2 [29]. The total time (seconds) spent moving was analyzed using a Mann-Whitney U test.

For cricket consumption, the data were summarized as the cumulative number of crickets consumed by each frog, and graphically assessed using only the values between 0-d post-metamorphosis and 53-d post-metamorphosis. Because of differences in date of metamorphosis among frogs, 53-d post metamorphosis was the latest time period when the data from all frogs were available. Differences between the cumulative number of crickets eaten by control and tank-mix exposed frogs at 53-d post-metamorphosis were tested for using a linear mixed model (LMM). Scoliosis, limb deformities, sex, and the interaction of those factors with treatment were random factors that potentially influenced the variance in number of crickets consumed. Data

were explored using LMMs to identify the random factors that contributed most to the variance in cumulative number of crickets eaten. Models were tested for statistically significant improvement from the simple model of the fixed treatment effect (tank mix vs. control) using the difference in -2 Log Likelihood values.

RESULTS

Mortalities and deformities

Ten metamorphs drowned due to a faulty haul-out platform design. Five individuals from each treatment died 3-4 d after the end of their 96-h exposures, corresponding to the approximate time of completed mouth development in the metamorphic process [stages 44-45, 30]. The control metamorphs all died on the same day. One tank-mix exposed metamorph died on the same day as the controls, three died one day later. All except one began the 96-h exposure on the first and second days of the trial. Inadequate replacements were available for in-trial mortalities, so sample sizes were reduced to 15 in both treatments. Because all individuals completed the 96-h exposure and behavior trial, data from all individuals are included in the analyses for body condition from 0-96 h and behavior (n = 20).

At the end of June and prior to the start of exposures, tadpoles in the rearing tanks were assessed for indications of scoliosis, ranging from asymmetric tail musculature to severe curves that extended up the spine anterior to the tail. No evidence of scoliosis was found in 38 percent of frogs in Group A and 27 percent of frogs in Group B. Limb deformities were also observed in the rearing population after metamorphosis, but only in Group B. Of the frogs that completed the 2-month grow-out, 40 percent from each treatment group (control and tank mix) were without any limb or spinal abnormalities. Scoliosis existed in 40 percent of controls and 50 percent of treatments, regardless of the presence of limb deformities. Limb deformities occurred more

frequently in frogs from Group B and the combination of scoliosis and limb deformities only occurred in Group B (Table 3.1). The only deformity observed in Group A was relatively minor. In that frog, the first toe overlaid the second toe of the right foot such that the foot appeared folded over, but the deformity did not appear to hinder mobility. Limb deformities in frogs from Group B were similar to each other, but not to that of the frog from group A. In most cases, the deformity involved missing or fused digits. Limb deformities occurred at a rate of 24 percent in frogs that survived through metamorphosis in Group B (pooled rearing and test populations). No evidence exists that limb deformities or scoliosis were related to exposure to the tank mix.

Analytical chemistry

Target concentrations of triclopyr were 74-83% (35-39 ppm) of nominal at 0 h and 72-79% (34-37 ppm) of nominal at 24 h. Triclopyr concentrations in tank-mix exposed replicates were below detection limits 24-h after metamorphs were placed in clean water at the start of the grow-out. Percent recoveries were 94-108.

Water quality

Water quality was similar among rearing and holding tanks and experimental buckets (Table 3.2). Though mean temperatures in the rearing tanks at the hatchery were below those in the environmental chamber, they were similar to the environmental chamber at the time that tadpoles were transferred inside (10-20 July; Tank A: 18.3°C ± 0.9 SD; Tank B: 18.9°C ± 0.9; n = 36).

Mean DO was lower in tank mix treatments than in controls during the 96-h exposure (Fig. 3.2). Dissolved oxygen values in both samples from the bucket with the last tank-mix exposed individual that died because of limb deformities were identified as outliers and removed

from analyses. Even without the outliers, the dissolved oxygen was statistically lower in tank mix (% saturation: 94.1 ± 2.9 SD; 9.0 mg/L ± 0.3 SD; n = 38) than control (percent saturation: 97.6 ± 2.6 SD; 9.3 mg/L ± 0.2 SD; n = 39) buckets (independent samples t-test; percent saturation: $t_{75} = 5.643$, $p < 0.001$; mg/L: $t_{75} = 6.423$, $p < 0.001$). However, the minimum DO values recorded were 87.2 % saturation and 8.42 mg/L, well above the 60% saturation required for static fish acute toxicity tests [31].

Behavior

During the 96-h exposure, tank-mix exposed metamorphs were more frequently found to be in a sprawled position with hind legs extended. Sixty percent of tank mix metamorphs were sprawled in a total of 22 observations. In contrast, 10 percent of control metamorphs were sprawled in a total of two observations (one each). The difference between the distributions of control and tank mix data from observations of frogs with legs sprawled was statistically significant (K-S $p = 0.013$). Tank-mix exposed metamorphs were also more likely to move (12 observations from 8 metamorphs) than controls (7 observations from 6 metamorphs), but the difference was not statistically significant (K-S $p = 1.000$). No differences were apparent in the recorded locations of individuals within the buckets, with the majority of observations locating control and tank mix metamorphs on the bottom (Table 3.3).

During the behavior trials post-exposure, movement types were described based on the relative speed of movement and body parts involved and compared between control and tank-mix exposed frogs (Table 3.4). The fastest movement was “burst”, a forward propulsion driven mostly by the tail. “Swim” involved the use of the tail and legs, whereas “Crawl” was slower, using legs and forearms without movement of the tail. “In Place” and “Turn” were shifts in position or orientation, without a change in location within the box.

The ratio of control metamorphs that spent greater than 50% of time on black or white squares was 10:10, giving no indication of a preference. A greater number of tank-mix exposed metamorphs spent more than 50% of their time on white squares (n = 12) than black (n = 8), but the effect was not statistically significant ($X_c^2 = 0.10$, $p > 0.75$). Tank mix individuals spent an average of 1.7 sec more time moving in total than controls, but no significant effect existed (Mann-Whitney $U = 98$, $p = 0.257$). The only individual movement type for which a statistical difference existed was crawling (Mann-Whitney $U = 73$, $p = 0.038$). Controls spent more time crawling than tank-mix exposed metamorphs. The tank mix group spent more time swimming on average, but no statistical difference existed, likely due to a high number of zeros in the swimming data (Fig. 3.3). The combination movements (Turn/Crawl and Turn/Swim) were pooled with their respective forward movements (Crawl and Swim) for analyses of the number of movements made by each metamorph. Tank-mix exposed metamorphs exhibited more individual movements than controls overall and for most categories (Fig. 3.4). Control metamorphs executed more bursts and in-place movements on average than tank mix metamorphs. However, no statistical differences existed between treatments in the total number of movements made or the type of movement (Mann-Whitney U, all $p > 0.05$).

Metamorphosis

Completion of metamorphosis was delayed by approximately 1 day in tank-mix exposed frogs. The delay was statistically significant ($t_{28} = -2.26$, $p = 0.031$, Fig. 3.5). The difference represented a small-medium effect size (Cohen's $d = -0.83$, $r = -0.38$) [28].

Cricket consumption

Frogs began feeding on crickets between 0 and 11-d post-metamorphosis, with the majority starting between 3 and 8 d (Fig. 3.6). Fewer cumulative crickets were consumed on average by control frogs (134.8 ± 33.8) than were consumed by tank mix frogs (139.9 ± 34.4) at 53-d post-metamorphosis, but the difference was not statistically significant (t_{28} = -0.404, p = 0.689).

Potential interactions among treatments and deformities were observed when cricket consumption data were graphed, and data from one previous study indicated that sex may be important in cricket consumption patterns of juvenile ranid frogs [27]. Including the random effect of the interaction of limb deformities with treatment was the only LMM with significant improvement over the simple model of the fixed treatment effect (X^2_1 = 3.99, $p < 0.05$). No significant effects of treatment ($f_{0.05,1,29.6}$ = 1.726, p = 0.199) existed. The random effect of the interaction of treatment with limb deformity was assessed using variance components for the covariance structure of the model and found the interaction contributed to 64% of the variance (Table 3.5). Treatment frogs with limb deformities ate fewer crickets on average than treatment frogs without limb deformities and control frogs with and without limb deformities (Fig. 3.7).

Body condition

No statistical differences existed for any time point among body condition indices calculated using the bSMA from all individuals, only control individuals, or only tank mix individuals (all p-values > 0.9). Additionally, no consistent pattern existed in mean $\hat{M}_i$ using the three datasets. At the start of the test (0 d) and 30-d post metamorphosis, $\hat{M}_i$ calculated using the control data was higher than that for the whole dataset and tank mix only. For all other times, $\hat{M}_i$ using the control data was lower than that of the whole dataset and tank mix. The body condition

data were developed using the bSMA from control individuals only to maintain consistency with the previous work with Oregon Spotted Frogs [27].

No difference existed in body condition between control (mean 2.31 ± 0.28, n = 19) and tank-mix exposed (mean 2.27 ± 0.28, n = 20) metamorphs at the start of the 96-h test ($t_{37} = 0.405$, $p = 0.688$). At the end of 96 h, no difference existed between control (mean 2.02 ± 0.24, n = 20) and tank mix (mean 1.89 ± 0.28, n = 20) metamorphs ($t_{0.05,38} = 1.992$, $p = 0.054$). Moreover, no difference existed between treatments in the change (Δ) of body condition from 0 h to 96 h (mean Δ: control = -0.27 ± 0.28 SD, n = 19; tank mix = -0.41± 0.24, n = 20; $t_{37} = 1.622$, $p = 0.113$) A trend towards a significantly lower body condition existed for tank-mix exposed metamorphs at the end of the 96-h exposure, but that trend was lost by the time the frogs completed metamorphosis ($t_{0.05,27} = 0.530$, $p = 0.601$) , on average 8 d later (Fig. 3.8).

Little difference in body condition was detected graphically between controls and the tank-mix exposed frogs when sex, the presence of scoliosis, or limb deformities were considered. The presence of scoliosis was the only variable for which minimal differences were detected in mean body condition over time (Fig. 3.9). The mean body condition was similar for control and tank-mix exposed individuals with and without scoliosis until 30 d post-metamorphosis (42 d in Fig. 3.9). In the last two measurements, the mean body conditions of individuals with spinal curves from both control and tank mix treatments were lower than those without curves (Table 3.7). No statistical differences existed between treatments within the scoliosis groups, so the treatments were pooled to test for differences between frogs with and without scoliosis. The difference was only significant at 42 d ($t_{28} = 3.656$, $p = 0.001$) and not at 66 d ($t_{28} = 1.376$, $p = 0.180$). The effect size for the difference at 42 d was medium (Cohen's $d = 1.34$, $r = 0.56$) [28].

Liver condition

Liver condition indices were similar between control and tank-mix exposed frogs (mean ± SD: control = 0.326 ± 0.008, tank mix = 0.328 ± 0.011). The variance was larger for tank mix frogs (Fig. 3.10), but the difference was not statistically significant (t_{28} = -0.074, p = 0.942).

Liver histology

When histology results were returned from the contract laboratory, one sample each from control and tank mix frogs was missing and unaccounted for, so the N for liver histology became 28. According to the veterinary pathologist who reviewed the histology (Dr. G. Sanders, University of Washington), the only lesions noted within the hepatocytes were characterized by the replacement of normal central cytoplasmic cellular structure with multi-focal to diffuse, brown, non-refractive, granular material (pigment lipofuscin/ melanin, Fig. 3.11). The location of the nucleus moved towards the peripheral edge within the affected cells. This material replaced the normally existing eosinophilic cytoplasmic architecture. The lesions were most likely melanomacrophage cells that comprise a normal component of the frogs' immune systems. No particular distribution pattern was observed within the livers or between the treatment groups (Table 3.8).

DISCUSSION

No direct effect of exposure to a triclopyr + Competitor tank mix on northern red-legged frog survival was observed. Behavior and body condition of tank-mix exposed individuals suggested some level of stress during the 96-h exposure period. However, recovery in activity levels was observed upon placement in clean water. Body condition was similar between tank-mix and control frogs from metamorphosis to the end of the grow-out. An interaction with limb deformities was observed in the number of crickets consumed by tank-mix exposed frogs.

Though limited biological relevance may exist for deformed frogs that are unlikely to survive, the interaction of the tank-mix with an additional stressor is of potential concern.

Mortality of metamorphic and post-metamorphic frogs has been demonstrated with exposure to several different pesticides and pesticide formulations at environmentally-relevant concentrations [2, 11, 32, 33]. In water, triclopyr TEA dissociates rapidly to triclopyr acid and degrades by photolysis to TCP within a day [12]. Though the tank mix was not tested for TCP, the rapid degradation of triclopyr and the persistence of the degradate [12] suggests that it would have been part of the exposure scenario in the present study. Though TCP is more toxic than triclopyr TEA [12], it did not appear to alter the expected toxicity of the tank mix to frog metamorphs.

Metamorphs exposed to the tank mix appeared to be more stressed than control frogs during the 96-h exposure. Exposure to contaminants may induce stress that results in higher respiration rates [34]. Though statistically lower DO was observed in the treatment solutions from tank mix buckets, it is unclear from these data whether the lower DO was due to increased respiration or an inability of the tank mix solution to hold DO. The latter condition has been observed with pesticide tank mixes in previous experiments (C. Grue, unpublished data). Metamorphic frogs respire using a combination of epidermal and developing lung cells [35]. Their capacity for respiration may not be sufficient to alter the observed DO in the tank mix solution. However, the DO was lowest in the tank mix with a metamorph that later exhibited the most severe limb deformities and ultimately drowned.

Metamorphs in tank mix solutions were more likely than controls to be observed in an apparent low-energy body position with legs extended behind, rather than a more typical upright resting position. Lower body conditions in tank-mix exposed metamorphs at the end of the 96 h

are suggestive of higher metabolic rates, another potential result of stress. Growth and development may be reduced with exposure to stressors [36]. If higher metabolic rates were required to maintain homeostasis during the 96-h exposure, that energy tradeoff may have resulted in the observed delay in metamorphosis.

By completion of metamorphosis, no difference existed in the body condition between tank mix and control frogs. This is consistent with another study that showed tadpole recovery from triclopyr exposure after placement in clean water [37]. After time in clean-water during the grow-out, the presence of scoliosis appeared to be more important to body condition than previous exposure to the triclopyr tank mix. The presence of deformities like scoliosis may inhibit mobility, resulting in reduced fitness. No interactions between deformities and herbicide-exposure were observed in body condition through time. The effect of scoliosis on body condition suggests the potential for interactive effects if deformed frogs are exposed to more toxic pesticides or contaminants.

Near the end of the 2-mo grow-out, frogs with limb deformities that were exposed to tank mix treatments had eaten fewer crickets over time than control frogs and treatment frogs without limb deformities. Treatment frogs ate more crickets on average, though the difference from controls was not statistically significant. The effect of the interaction of limb deformities with treatment on cricket consumption suggests the potential for interactions of herbicide tank mixes with other stressors. However, the number of frogs with limb deformities was low compared to those without. Moreover, two of three tank mix frogs with limb deformities also had scoliosis. The unbalanced structure of the data, low sample sizes representing the random effect, and additional confounding factors make it difficult to draw definitive conclusions. Regardless of the statistical significance of the effect of limb deformities with tank-mix exposure on cricket

consumption or the effect of scoliosis on body condition, the biological relevance is ambiguous. Deformed frogs are unlikely to survive for long in pristine or contaminated environments because compromised individuals may be more likely to succumb to predation [but see 38].

Among the most frequently used defensive behaviors of amphibians is the ability to avoid detection. For example, crypsis and immobility are two of the most frequently deployed defense tactics of anurans [39]. In the present study, it was hypothesized that northern red-legged frog metamorphs would preferentially select for dark squares in a behavior arena with black and white checkers. However, metamorphs showed no preference, placing themselves on dark and light squares indiscriminately regardless of treatment or orientation of black and white squares. The assumption was made that the stress of handling and the laboratory environment would provide enough incentive for metamorphs to engage in cryptic behaviors, which may not have been the case. In future tests, it may be desirable to present a threat or challenge in the behavior trial to induce a crypsis-based response.

Movement can increase vulnerability of amphibians to predation. In previous studies with triclopyr, lethargy and reduced response to stimuli have been observed in tadpoles [37]. Recovery in tadpoles exposed to 1.2 ppm of triclopyr generally took 1-3 d [37]. In the present study, northern red-legged frog metamorphs exposed to a triclopyr tank mix were more active than controls immediately upon placement in clean-water behavior arenas. They spent more time moving and employed a swimming movement more frequently than controls. Of all the movement categories, swimming was perhaps the most dangerous movement if employed in the presence of a predator. Crawling was a slow and deliberate action that employed only the legs. Bursts were fast, directional movements to get from one place to another quickly and with only the tail moving for propulsion. In swimming, the use of tail and legs increases the visibly moving

body parts, serving to flag the metamorph's location, and the moderate speed was not cautious and not quick enough to get from point A to B without being noticed. It cannot be determined whether the stimulus for the activity was the placement in clean water (without tank mix smell or taste), placement in clear water (without blue dye), or some effect of the exposure to the tank mix itself (altered neurological state). While in the tank mix, observations indicated that tank-mix exposed metamorphs were no more active than controls. For that reason, it is suspected that the activity has more to do with the change of environment rather than a neurological effect of the tank mix. However, the type of activity once placed in clean water may correspond to increased vulnerability to predation of tank-mix exposed metamorphs. In future studies, it is recommended that the mechanism of the effect be explored with the addition of greater risk using some stimulus like prodding [37] or predator presence to better determine the vulnerability of tank-mix exposed metamorphs.

Northern red-legged frog metamorphs are unlikely to be exposed to triclopyr concentrations as high as that used in this experiment. Though analytical chemistry revealed lower concentrations of triclopyr than the target (47.1 ppm), concentrations remained well above the highest concentration of triclopyr detected in surface waters in Washington (1.3 ppb) (USGS National Water Quality Assessment Warehouse, http://cida.usgs.gov/nawqa_public/apex/f?p=136:7:0; accessed 17 March, 2014). However, environmental conditions and indirect effects of the herbicide on availability and abundance of food and refugia [40] may alter amphibian response to herbicides with relatively low toxicity.

The frogs in this study were reared and exposed in a climate-controlled environment that maintained temperatures at approximately the mean water temperature recorded in field habitats of the species. Temperature was maintained at a constant level, without the characteristic diel

fluctuation. The laboratory environment was also free of predators and sufficient food resources are provided. The potential for pesticide interaction with temperature exists. For example, the insecticide endosulfan and fungicide chlorothalonil were more toxic to tadpoles exposed outside in ambient temperature regimes than those reared inside at standard laboratory temperature regimes [41].

Metamorphs recovered to control levels after placement in clean water, and no effects of treatment were observed during a 2-mo grow-out in clean water. These data suggest that aquatic triclopyr formulations with Competitor as an added surfactant will cause little harm to ranid metamorphs exposed in the field. The interaction of tank-mix exposure with limb deformities demonstrates a potential for herbicide interaction with other stressors. These results warrant caution and indicate a need for more information. It is important to consider confounding factors that may exist in the laboratory when assessing for potential effects that may occur in the field. Limitations on available animals and plasticity of amphibians in laboratory settings may contribute variance that is unlikely to be relevant in the wild. Conversely, environmental and ecological conditions like temperature [41], or the presence of predators may affect amphibians' response to contaminants in ways that cannot be predicted from simplified laboratory studies. Moreover, indirect effects of herbicides on the availability and abundance of food and refugia are likely to be important when considering impacts to amphibians [40, 42]. Opportunity exists to avoid more sensitive life stages in careful planning of pesticide applications around developmental windows. Additional research in a field or mesocosm setting may be important to further our understanding the potential effects of aquatic herbicide tank mixes on metamorphosing amphibians.

REFERENCES

[1] WSDA, WDOE. 2004. Integrated Pest Management Plan for Freshwater Emergent Noxious and Quarantine Listed Weeds. Washington State Departments of Agriculture and Ecology. 105 pp, Olympia, WA.

[2] Relyea RA. 2005. The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecological Applications* 15:1118-1124.

[3] Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker JM, Tsui M. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? *Environmental Health Perspectives* 114:40-50.

[4] US EPA. 2007. 40 CFR 158 - Data Requirements for Pesticides. Vol 158. ecfr.gpoaccess.gov, Electronic Code of Federal Regulations.

[5] Williams BK, Semlitsch RD. 2010. Larval responses of three midwestern anurans to chronic, low-dose exposures of four herbicides. *Archives of Environmental Contamination and Toxicology* 58:819-827.

[6] Bridges CM, Semlitsch RD. 2000. Variation in pesticide tolerance of tadpoles among and within species of ranidae and patterns of amphibian decline. *Conservation Biology* 14:1490-1499.

[7] Bridges CM, Dwyer FJ, Hardesty DK, Whites DW. 2002. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? *Bulletin of Environmental Contamination and Toxicology* 69:562-569.

[8] Relyea RA, Jones DK. 2009. The toxicity of Roundup Original Max(R) to 13 species of larval amphibians. *Environmental Toxicology and Chemistry* 28:2004-2008.

[9] King JJ, Wagner RS. 2010. Toxic effects of the herbicide Roundup® Regular on Pacific Northwestern amphibians. *Northwestern Naturalist* 91:318-324.

[10] Edginton AN, Stephenson GR, Sheridan PM, Thompson DG, Boermans HJ. 2003. Effect of pH and Release on two life stages of four anuran amphibians. *Environmental Toxicology and Chemistry* 22:2673-2678.

[11] Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens* and *Bufo americanus*. *Bulletin of Environmental Contamination and Toxicology* 64:263-270.

[12] US EPA. 1998. Reregistration Eligibility Decision (RED) Triclopyr. Office of Prevention, Pesticides, and Toxic Substances, United States Environmental Protection Agency. 285 pp., Washington D.C.

[13] Wan MT, Moul DJ, Watts RG. 1987. Acute toxicity to juvenile Pacific salmonids of Garlon 3A, Garlon 4, triclopyr, triclopyr ester, and their transformation products: 3,5,6-trichloro-

2-pyridinol and 2-methoxy-3,5,6-trichloropyridine. *Bulletin of Environmental Contamination and Toxicology* 39:721-728.

[14] Paton PWC, Crouch WB. 2002. Using the phenology of pond-breeding amphibians to develop conservation strategies. *Conservation Biology* 16:194-204.

[15] Jones LLC, Leonard WP, Olson DH eds. 2005. Amphibians of the Pacific Northwest. Seattle Audubon Society.

[16] Corkran CC, Thoms C. 1996. *Amphibians of Oregon, Washington and British Columbia: A Field Identification Guide*. Lone Pine Pub., Alberta, Canada.

[17] Parker B. 2009. Aquatic weed control. In Peachy EO, ed, *Pacific Northwest Weed Management Handbook*. Extension Services of Oregon State University, Washington State University, and the University of Idaho, Corvallis, OR. http://pnwhandbooks.org/weed/other-areas/aquatic-weed-contol Accessed 17 June 2011.

[18] Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology & Chemistry* 23:1928-1938.

[19] Cauble K, Wagner RS. 2005. Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. *Bulletin of Environmental Contamination and Toxicology* 75:429-435.

[20] Greulich K, Pflugmacher S. 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquatic Toxicology* 65:329-336.

[21] Glennemeier KA, Denver RJ. 2002. Developmental changes in interrenal responsiveness in anuran amphibians. *Integrative and Comparative Biology* 42:565-573.

[22] Jenssen TA. 1967. Food habits of the Green Frog, *Rana clamitans*, before and during metamorphosis. *Copeia* 1967:214-218.

[23] Chen CY, Hathaway KM, Thompson DG, Folt CL. 2008. Multiple stressor effects of herbicide, pH, and food on wetland zooplankton and a larval amphibian. *Ecotoxicology and Environmental Safety* 71:209-218.

[24] Rasband WS. 1997-2009. ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA.

[25] Peig J, Green AJ. 2009. New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos* 118:1883-1891.

[26] Peig J, Green AJ. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. *Functional Ecology* 24:1-27.

[27] Yahnke AE, Grue CE, Hayes MP, Troiano AT. 2013. Effects of the herbicide imazapyr on juvenile Oregon spotted frogs. *Environmental Toxicology and Chemistry* 32:228-235.

[28] Cohen J. 1988. *Statistical power analysis for the behavioral sciences*. L. Erlbaum Associates, Hillsdale, N.J.

[29] Zar JH. 1999. *Biostatistical Analysis*, 4 ed. Prentice-Hall, Upper Saddle River, NJ.

[30] Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183-190.

[31] American Society of Testing and Materials. 2002. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. *Annual Book of ASTM Standards*. Vol 11.06, Philadelphia, PA, pp 249-269.

[32] Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. *The Science of the Total Environment* 407:1065.

[33] Brühl CA, Schmidt T, Pieper S, Alscher A. 2013. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? *Scientific Reports* 3. Available online: http://www.nature.com/srep/2013/130124/srep101135/full/srep101135.html; accessed March 13, 2013.

[34] Rowe CL, Kinney OM, Nagle RD. 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiological Zoology* 71:27-35.

[35] Viertel B, Richter S. 2000. Anatomy - viscera and endocrines. In McDiarmid RW, Altig R, eds, *Tadpoles: the Biology of Anuran Larvae*. The University of Chicago Press, Chicago, pp 92-148.

[36] Denver RJ. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. *General and Comparative Endocrinology* 164:20-31.

[37] Berrill M, Bertram S, McGillivray L, Kolohon M. 1994. Effects of low concentrations of forest use pesticides on frog embryos and tadpoles. *Environmental Toxicology and Chemistry* 13:657.

[38] Romansic JM, Waggener AA, Bancroft BA, Blaustein AR. 2009. Influence of ultraviolet-B radiation on growth, prevalence of deformities, and susceptibility to predation in Cascades frog (*Rana cascadae*) larvae. *Hydrobiologia* 624:219-233.

[39] Toledo LF, Sazima I, Haddad CFB. 2011. Behavioural defences of anurans: an overview. *Ethology Ecology & Evolution* 23:1-25.

[40] Baker LF, Mudge JF, Houlahan JE, Thompson DG, Kidd KA. 2014. The direct and indirect effects of a glyphosate-based herbicide and nutrients on Chironomidae (Diptera) emerging from small wetlands. *Environmental Toxicology and Chemistry*:n/a-n/a.

[41] Johnson LA, Welch B, Whitfield SM. 2013. Interactive effects of pesticide mixtures, predators, and environmental regimes on the toxicity of two pesticides to red-eyed tree frog larvae. *Environmental Toxicology and Chemistry* 32:2379-2386.

[42] Halstead NT, McMahon TA, Johnson SA, Raffel TR, Romansic JM, Crumrine PW, Rohr JR. 2014. Community ecology theory predicts the effects of agrochemical mixtures on aquatic biodiversity and ecosystem properties. *Ecology Letters*:n/a-n/a.

Table 3.1 Number of frogs by genetic group, sex, and status of deformities that were exposed to a control and tank mix treatment

Treatment	Genetic group	Sex	No deformities	Scoliosis only	Limb deformities only	Scoliosis + limb deformities
Control	A	F	2	1	1	0
		M	1	2	0	0
	B	F	1	2	1	1
		M	2	0	1	0
Tank mix	A	F	3	2	0	0
		M	0	3	0	0
	B	F	1	1	1	1
		M	2	0	0	1

Table 3.2 Water quality means ± SD pooled across days

Parameter	Treatment	Temp (°C)	DO (%sat)	DO (mg/L)	pH	NH_3 (mg/L)	Conductivity (µS/cm)
Rearing tanks[a]	A[b]	13.4 ± 2.5 (599)[c]	102.6 ± 6.7 (111)	10.4 ± 0.9 (111)	6.6 ± 0.2 (112)	0.11 ± 0.03 (118)	65.0 ± 4.2 (112)
	B	13.8 ± 2.5 (599)	102.0 ± 7.1 (111)	10.4 ± 0.9 (111)	6.6 ± 0.3 (112)	0.12 ± 0.04 (118)	63.2 ± 4.7 (112)
Holding tanks[d]	A	17.3 ± 0.4 (121)	100.9 ± 4.8 (121)	9.7 ± 0.5 (121)	7.3 ± 0.4 (121)	0.03 ± 0.08 (120)	74.0 ± 4.6 (121)
	B	17.1 ± 0.4 (121)	102.9 ± 2.4 (121)	9.9 ± 0.2 (121)	7.4 ± 0.4 (121)	0.03 ± 0.08 (120)	72.8 ± 5.6 (121)
Mixing buckets	Control	17.7 ± 0.2 (25)	106.6 ± 2.0 (25)	10.2 ± 0.2 (25)	7.4 ± 0.5 (25)	N/A[e]	66.6 ± 3.5 (25)
	Tank mix	17.5 ± 0.2 (23)	105.3 ± 3.8 (23)	10.1 ± 0.4 (23)	7.3 ± 0.5 (23)	N/A	74.8 ± 2.7 (23)
96-h exposure	Control	17.5 ± 0.3 (39)	97.6 ± 2.6 (39)	9.3 ± 0.2 (39)	7.1 ± 0.4 (39)	N/A	69.2 ± 4.9 (39)
	Tank mix	17.6 ± 0.2 (40)	93.5 ± 4.0 (40)	8.9 ± 0.4 (40)	7.0 ± 0.4 (40)	N/A	75.7 ± 5.5 (40)
Grow-out	Control	17.6 ± 0.3 (225)	97.0 ± 4.6 (226)	9.3 ± 0.4 (226)	7.3 ± 0.2 (226)	0.01 ± 0.04 (225)	71.4 ± 4.1 (226)
	Tank mix	17.6 ± 0.3 (236)	97.4 ± 5.1 (236)	9.3 ±0.5 (236)	7.3 ± 0.3 (236)	0.02 ± 0.07 (233)	71.7 ± 4.1 (234)

[a] Hatchery
[b] Genetic group
[c] Sample size in parentheses
[d] Environmental chamber
[e] Ammonia was not collected because colorimetric analyses were indistinguishable in the tank mix
DO = Dissolved oxygen

Table 3.3 Number of observations of northern red-legged frog metamorphs in different locations within treatment buckets during 96-h exposures to control and triclopyr tank mix

	Bottom	Shelf
Control	80	34
Tank mix	81	38

Table 3.4 Movement behavior categories expressed by northern red-legged frog metamorphs during behavior trials in checkered arenas immediately post-exposure to control or triclopyr tank mix

Movement	Code	Description
Burst	B	Fast, forward movement using mostly tail
Swim	S	Medium speed, forward movement, using a combination of tail and leg
Crawl	C	Slow, forward movement using mostly legs and forearms
In Place	P	Movement of legs in place without forward momentum
Turn	T	Change head/body orientation by ≥ 90°, but not body location within box
Turn/crawl	T/C	Combination of a turn and then a crawl forward when no measureable time passed between two behaviors
Turn/swim	T/S	Combination of a turn and then a swim forward when no measureable time passed between two behaviors

Table 3.5 Estimates of linear mixed model covariance (random) parameters with fixed treatment (control and triclopyr tank mix) effects and random effects of the interaction of treatment with limb deformities.

Parameter		Estimate	Std. Error	Wald Z	Sig.	95% Confidence Interval
Residual		832.1	223.4	3.72	0.000	(491.6, 1408.4)
Treatment × limb deformities	Variance	1486.3	1841.0	0.81	0.419	(131.2, 16844.1)

Table 3.6 R^2 values from ordinary least squares regression of ln mass on ln snout-vent length of frogs exposed at metamorphic climax to control or triclopyr tank mix for 96 h and reared in clean water for 2 mo

Time	Control	Tank mix	Pooled data
0 h	0.72	0.37	0.61
96 h	0.74	0.36	0.58
Metamorphosis[a]	0.71	0.61	0.67
30-d post-met[b]	0.87	0.94	0.90
66 d	0.94	0.94	0.93

[a] Metamorphosis occurred 12 d on average after test initiation
[b] 30-d post-metamorphosis occurred 42 d on average after test initiation

Table 3.7 Body condition indices of northern red-legged frogs with and without scoliosis that were exposed at metamorphic climax to a control or triclopyr tank mix for 96 h and reared in clean water for 2 mo

	No scoliosis		Scoliosis	
Time	Control	Tank mix	Control	Tank mix
0 h	2.35 ± 0.28	2.29 ± 0.25	2.31 ± 0.23	2.21 ± 0.30
96 h	2.00 ± 0.24	1.88 ± 0.28	2.10 ± 0.27	1.80 ± 0.26
Metamorphosis	1.42 ± 0.19	1.38 ± 0.17	1.43 ± 0.17	1.42 ± 0.13
30-d post-met	2.31 ± 0.14	2.24 ±0.09	2.08 ± 0.12	2.15 ± 0.11
66 d	3.91 ± 0.18	3.86 ± 0.43	3.75 ± 0.24	3.75 ± 0.22

Table 3.8 Distribution of lesion severity between the livers of control and tank mix exposed frogs

Lesion[a] Severity	#Lesions per 20x field	Control	Tank mix	Total
Mild	1-4	5	5	10
Mild to moderate	1-7	5	6	11
Moderate	5-7	2	0	2
Moderate to severe	7-15	0	2	2
Severe	10-15	2	1	3

[a] Melanomacrophage cells that differed from the normal eosinophilic cell structures

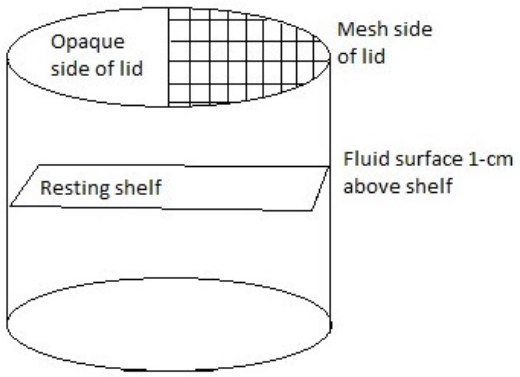


Figure 3.1 Diagram of experimental bucket design. The line between the opaque and mesh sides of the lid was placed perpendicular to the resting shelf to provide shade on one side of the shelf.

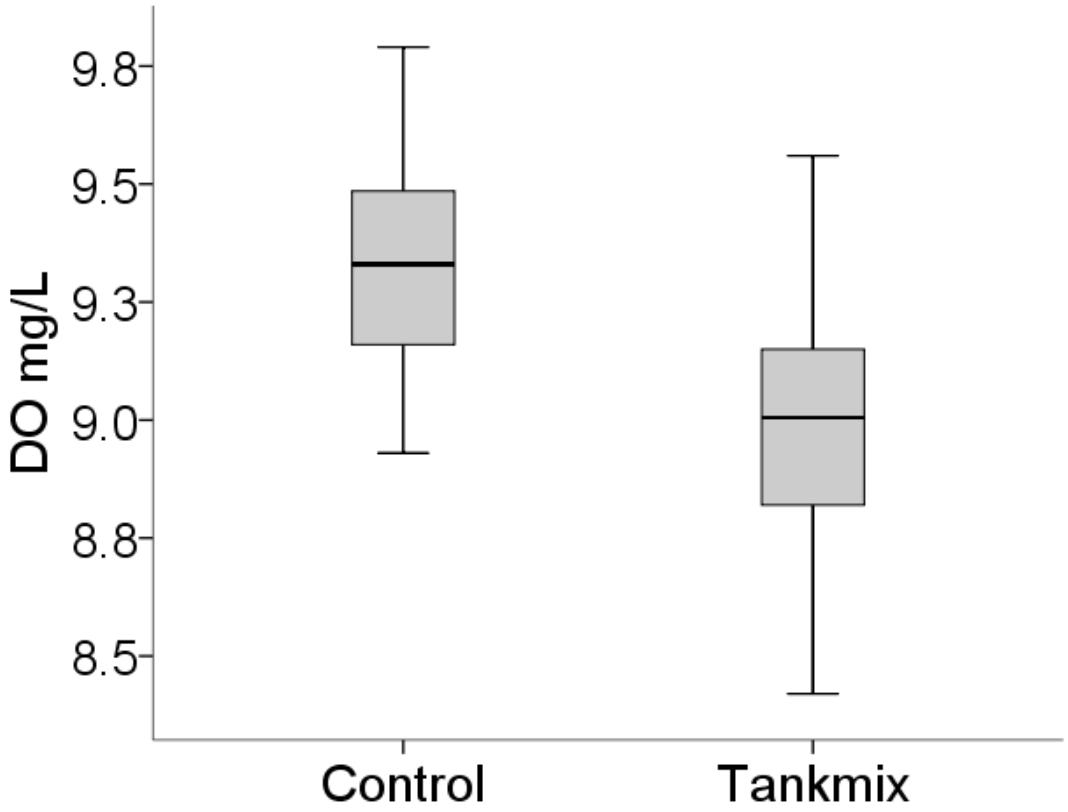


Figure 3.2 Dissolved oxygen (DO; mg/L) collected from control and tank mix buckets during the 96-h exposure. DO within the tank mix was statistically lower than that within the control ($p < 0.001$).

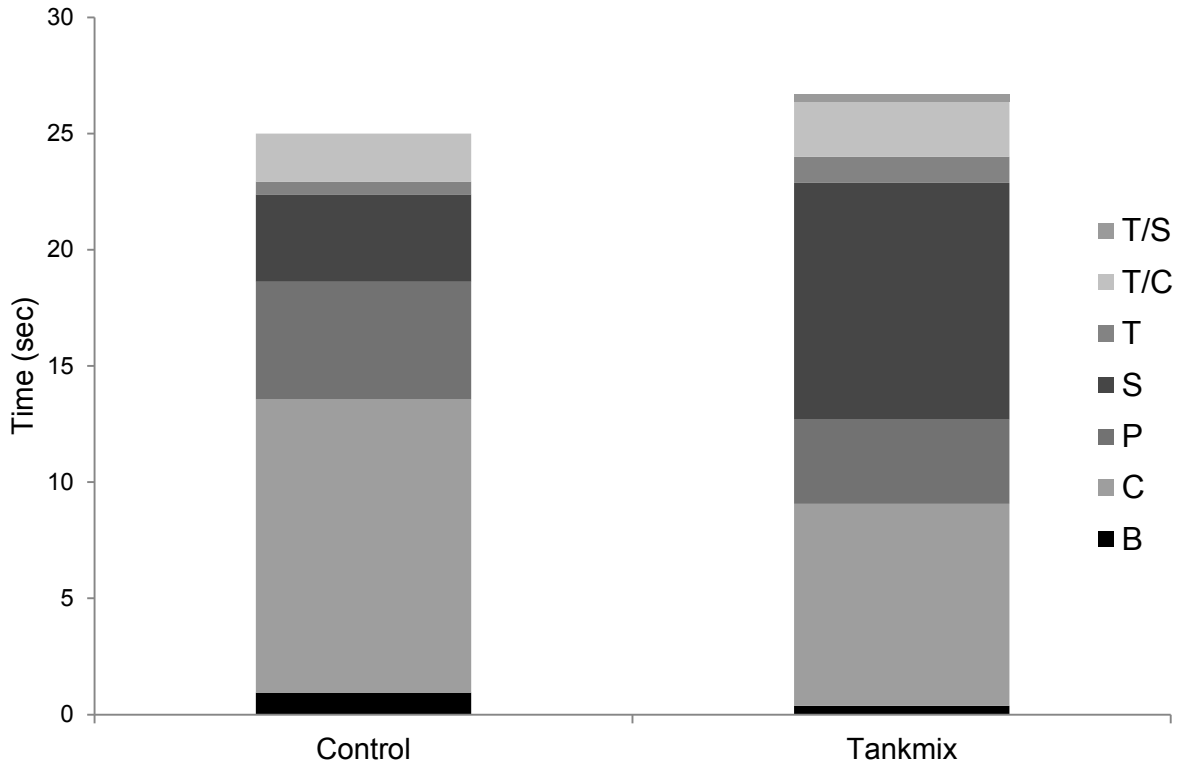


Figure 3.3 Mean time metamorphs spent moving in behavior arenas immediately post-96 h exposure to a control or triclopyr tank mix. Movement categories included burst (B), crawl (C), in-place (P), swim (S), turn (T), and combinations of turn and crawl (T/C) and turn and swim (T/S). Crawl is the only category for which a statistical difference existed.

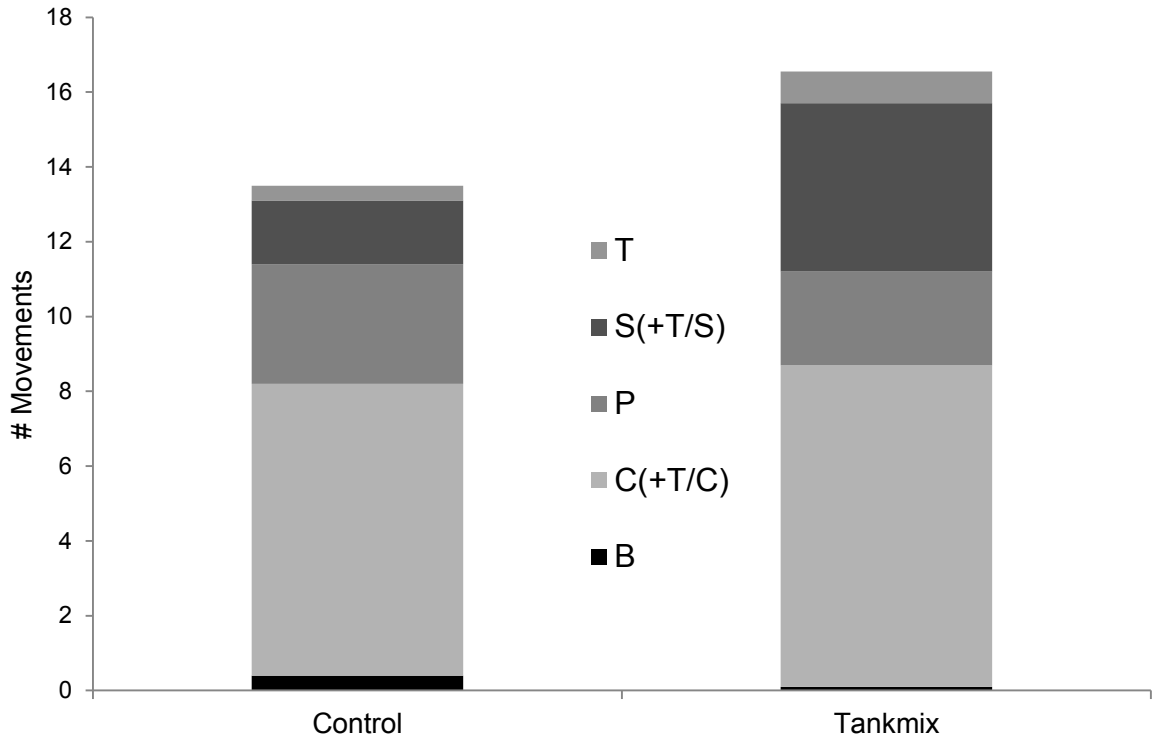


Figure 3.4 Number of distinct movements within each movement category made by northern red-legged frog metamorphs in behavior arenas immediately post-96 h exposure to a control or triclopyr tank mix. Movement categories included burst (B), crawl with turn and crawl [C(+T/C)], in-place (P), swim with turn and swim [S(+T/S)], and turn (T). No statistical differences existed between treatments.

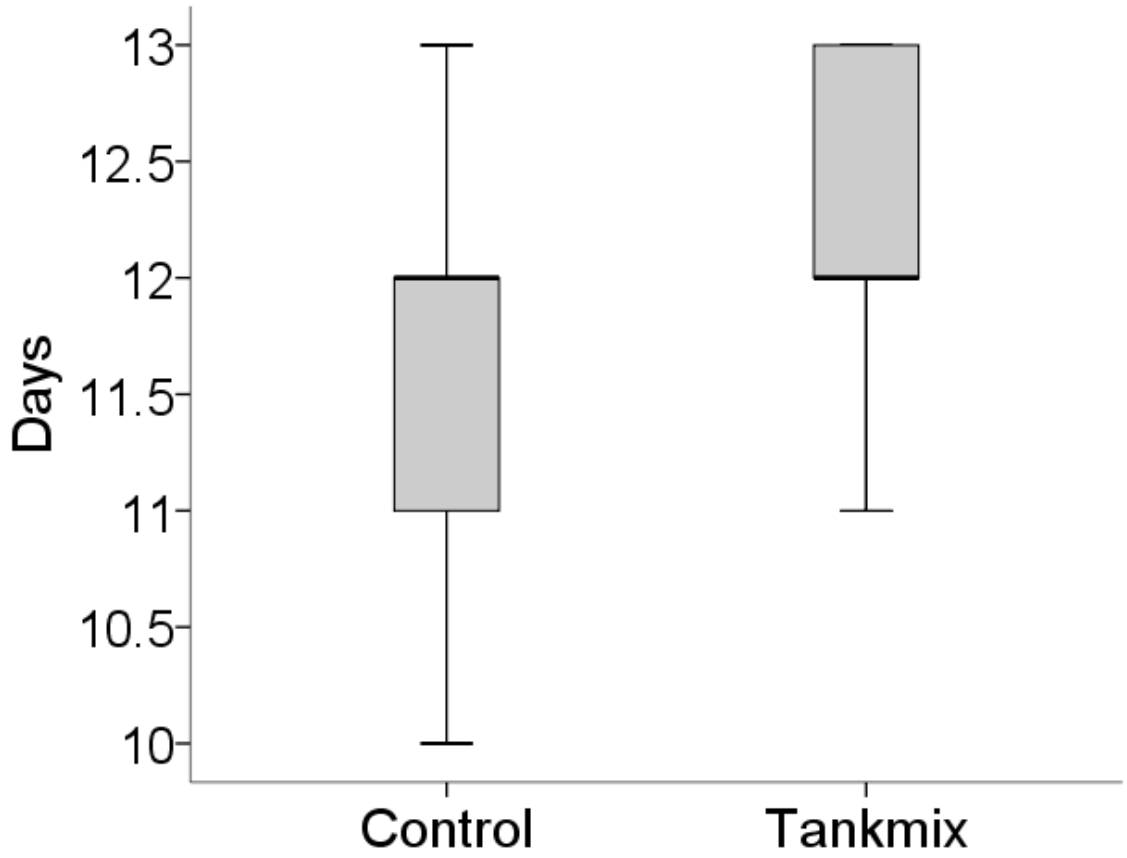


Figure 3.5 Days to complete metamorphosis for control and tank-mix exposed northern red-legged frogs. Completion of metamorphosis was statistically delayed for tank-mix exposed frogs ($p = 0.031$).

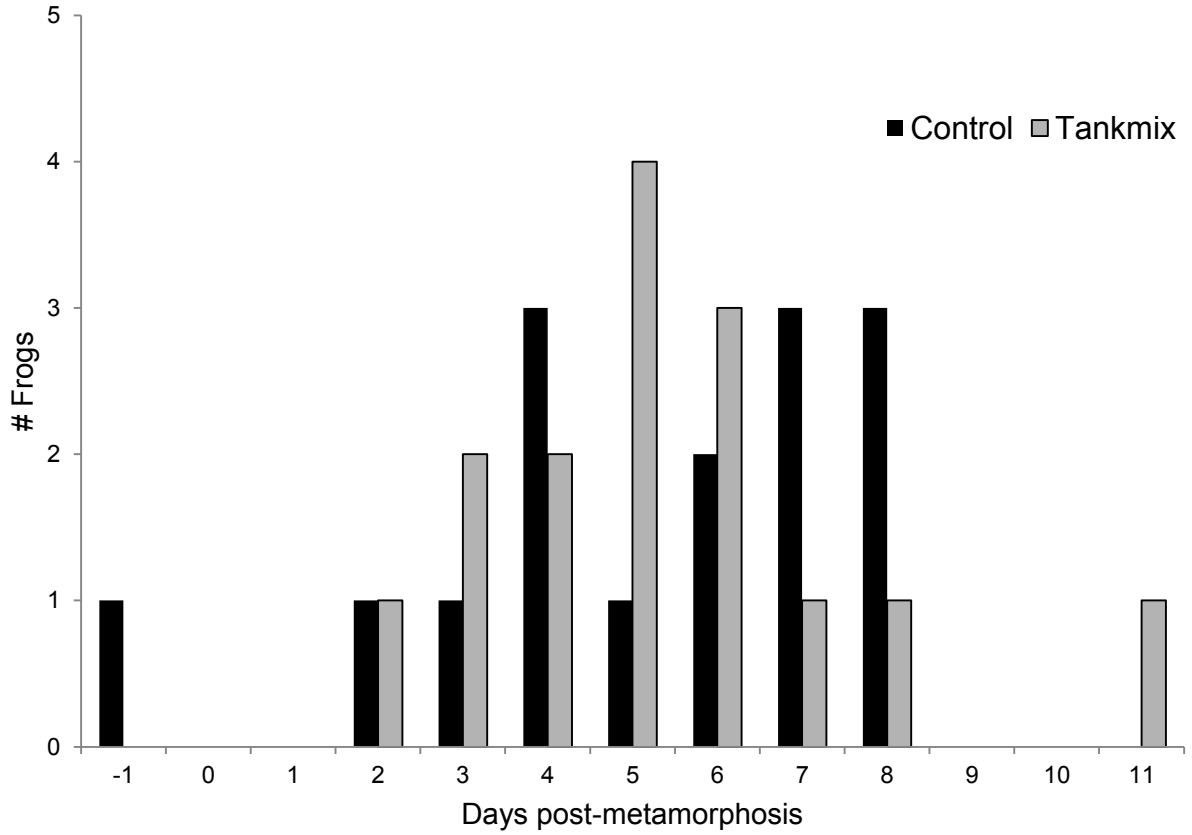


Figure 3.6 Number of frogs that consumed crickets for the first time by the number of days post-metamorphosis on which the crickets were consumed. Cricket consumption started several days after the 96-h exposure to control or triclopyr tank mix solutions, during the clean-water grow out.

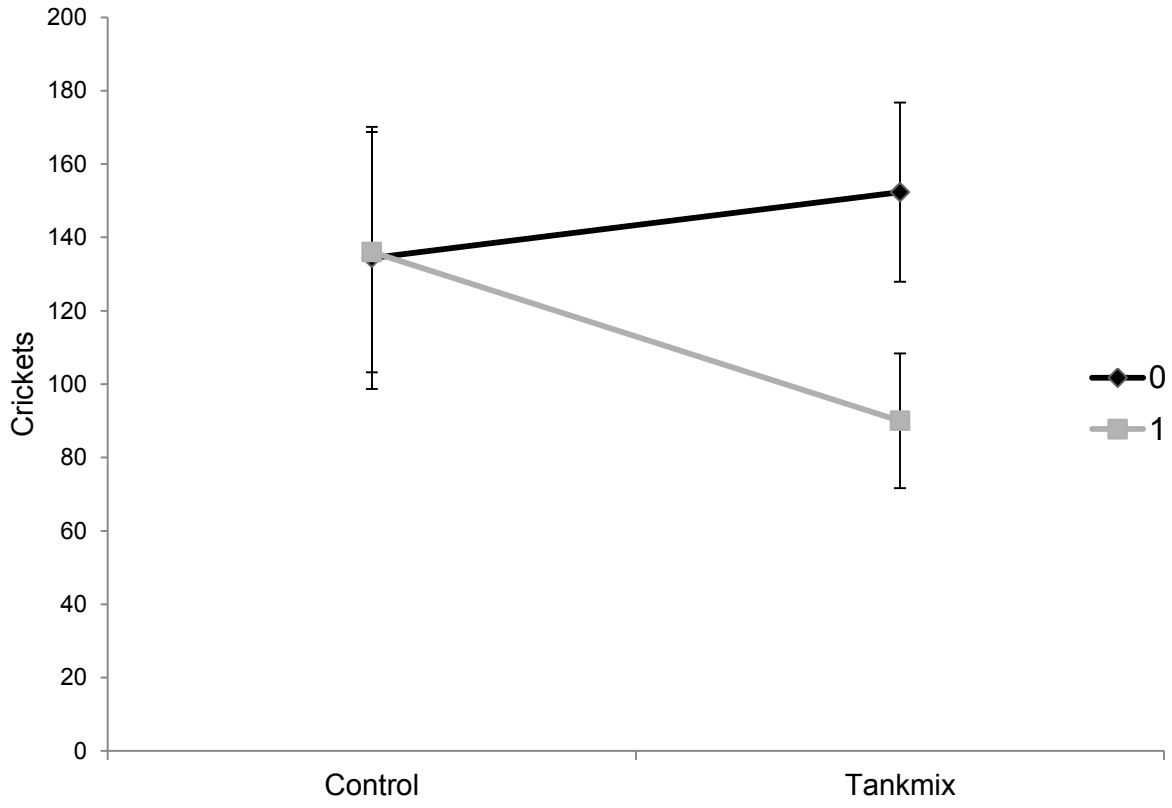


Figure 3.7 Interaction of mean cumulative number of crickets eaten 53-d post-metamorphosis by northern red-legged frogs with and without limb deformities (1 and 0, respectively) that were exposed to a control or triclopyr tank mix for 96-h during metamorphosis.

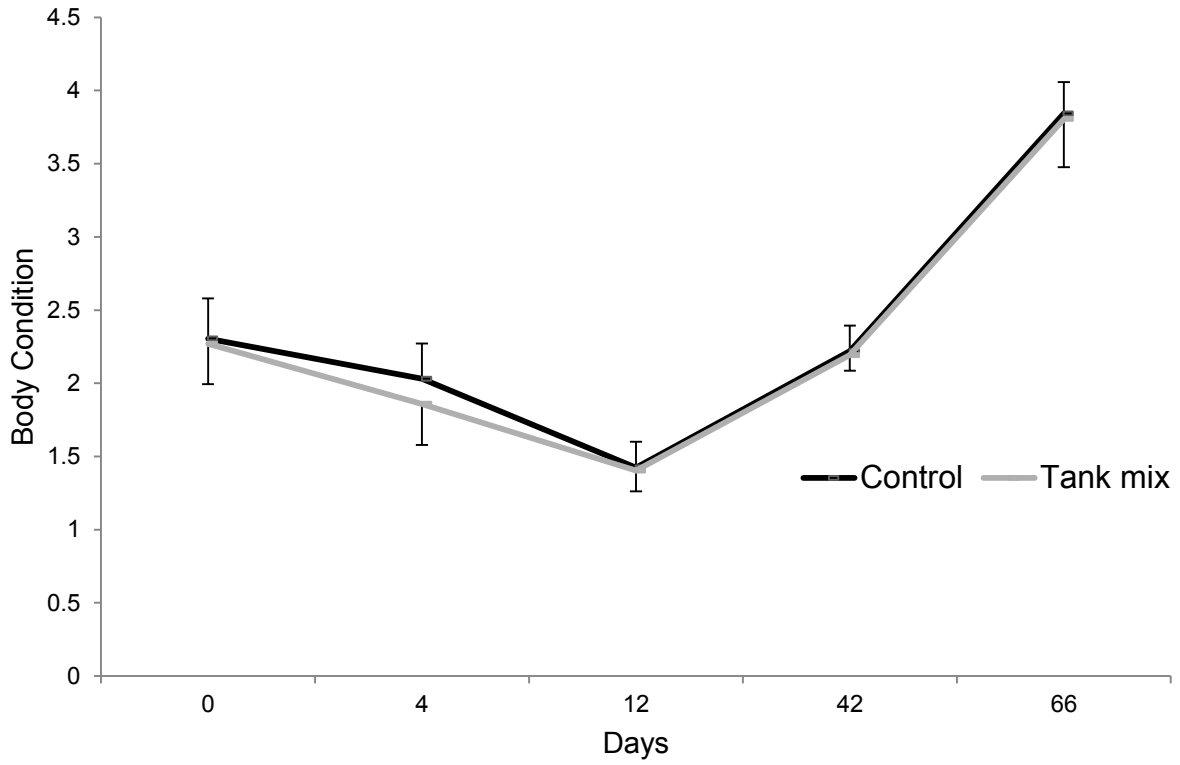


Figure 3.8 Mean body condition ($\hat{M}_i$) over time (days) of northern red-legged frogs that were exposed to a control or triclopyr tank mix (bars = SD, upper for control and lower for tank mix). Measurements were collected at the start (0) and end (4) of a 96-h exposure period, and three more times during a clean-water grow-out. Day 12 represents the mean time at completion of metamorphosis, the growth stage at which the third measurement was collected. Day 42 represents the mean for 30-d post-metamorphosis, when the fourth measurement was collected. The test ended on Day 66.

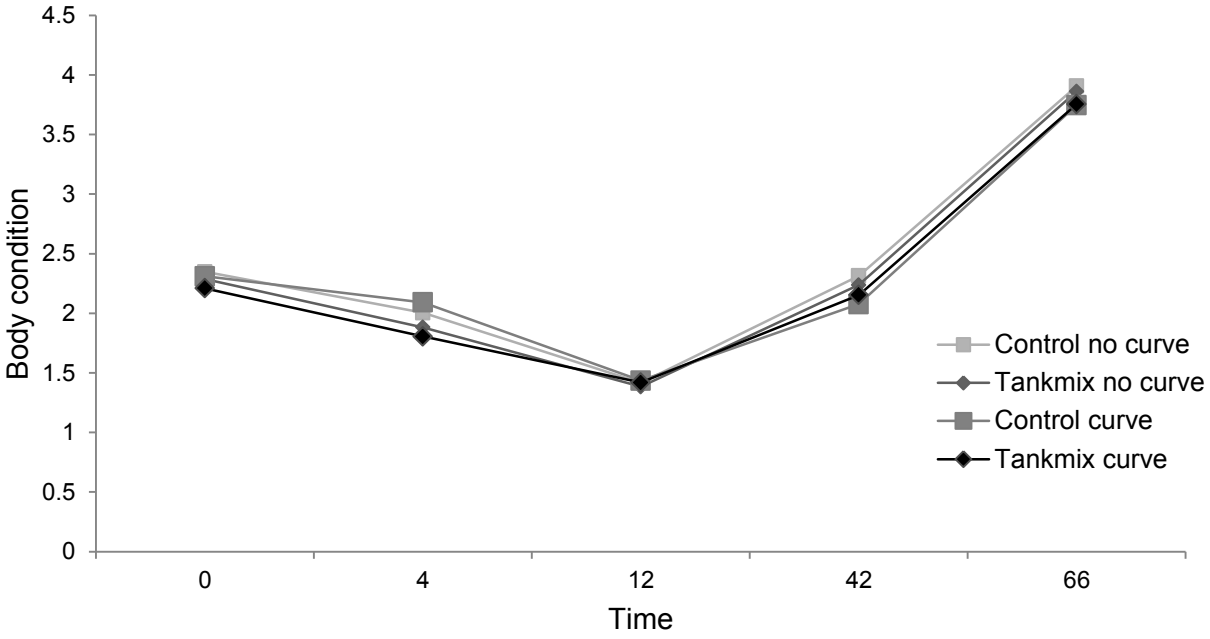


Figure 3.9 Mean body condition ($\hat{M}_i$) over time (days) of northern red-legged frogs with and without spinal curves that were exposed to a control or triclopyr tank mix. Measurements were collected at the start (0) and end (4) of a 96-h exposure period, and three more times during a clean-water grow-out. Day 12 represents the mean time at completion of metamorphosis, the growth stage at which the third measurement was collected. Day 42 represents the mean for 30-d post-metamorphosis, when the fourth measurement was collected. The test ended on Day 66.

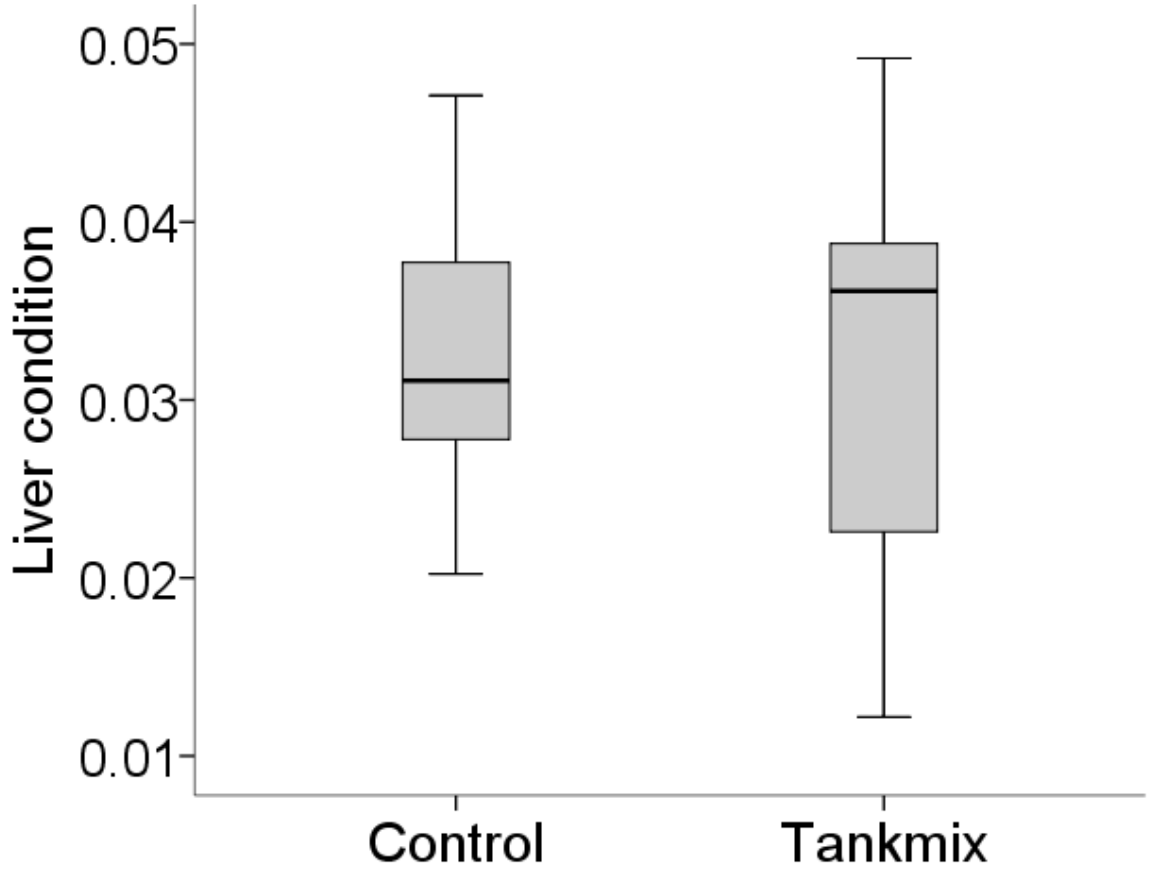


Figure 3.10 Boxplot of liver condition indices of control and tank-mix exposed northern red-legged frogs. Liver condition is derived from liver mass/body condition index.

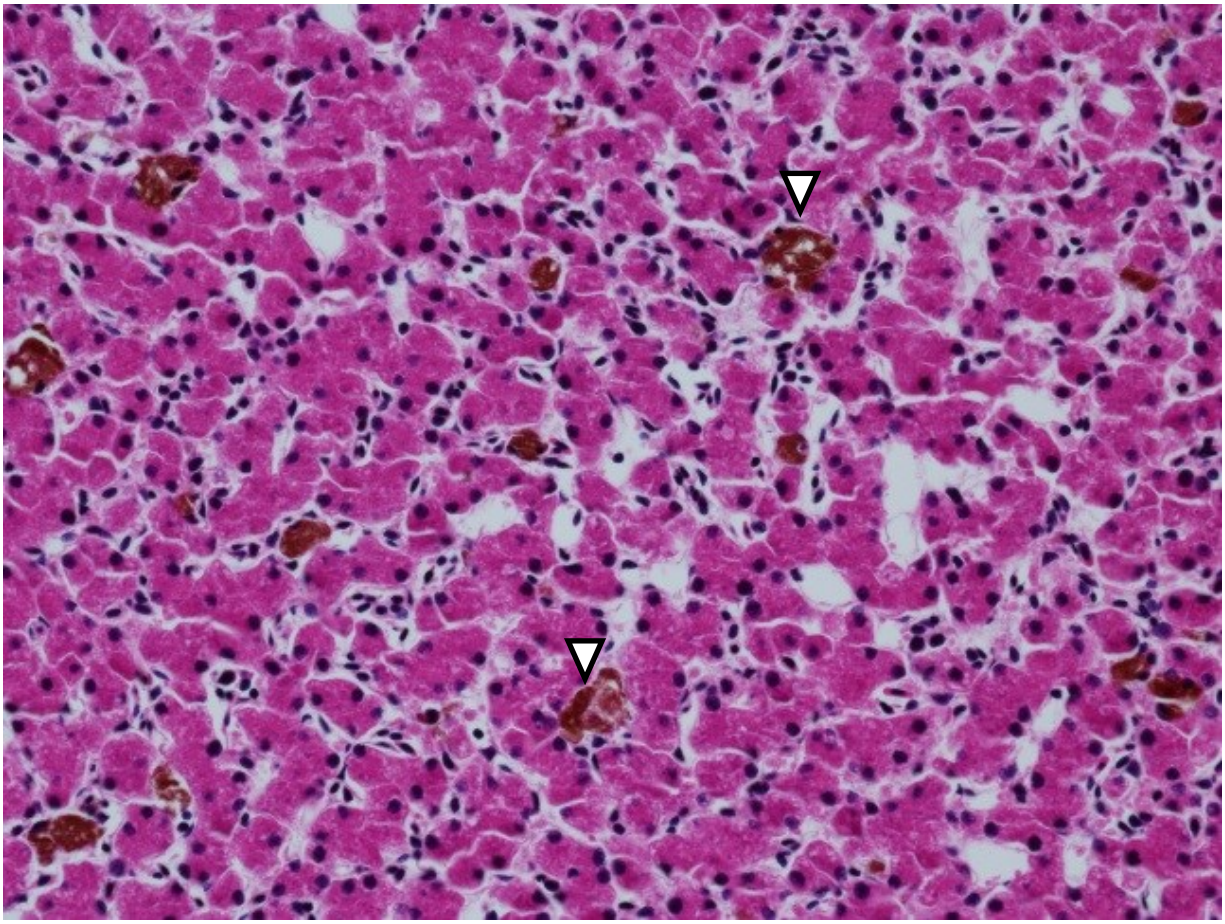


Figure 3.11 Example of melanomacrophage cells (arrows) in northern red-legged frog livers from control and tank-mix exposed individuals. This image is from a tank-mix exposed individual with 10-15 lesions visible at 20x magnification (severe).

CHAPTER 4: SNAKES ON A VISUAL PLANE: ADDING FEAR OF PREDATION TO TOXICITY TESTS WITH ANURANS

Abstract: Recent advances in toxicology have demonstrated that ecological processes may alter pesticide toxicity to amphibians. Most work has demonstrated effects on larval amphibians when chemical cues from predators were combined with exposure to a pesticide. Visual cues may be more important than chemical cues for post-metamorphic amphibians in detecting the presence of predators. Terrestrial-stage amphibians are present when herbicides may be applied to manage invasive aquatic plants. The potential interaction of pesticides and stress associated with the threat of predation detected through visual cues has not been examined. To address that gap, a toxicity test for anuran exposure to pesticides in the presence a snake predator was developed. The proof-of-concept was tested by holding northern red-legged frogs (*Rana aurora*) in clean-water using a two by two design with two substrates, shallow water and damp terrestrial, and exposure to presence of a common garter snake (*Thamnophis sirtalis*) or a control with no exposure to the snake. The test was designed to identify behaviors associated with frog exposure to the visual cue of snake presence, and to determine sample sizes required for toxicity tests with pesticides. Frogs exposed to snakes expressed a range of behaviors associated with crypsis and flight, regardless of substrate. Sample sizes were large for some metrics, but the number of crickets consumed by the frogs after exposure to the snakes and at least one behavior returned sample sizes below 25. The addition of stress associated with fear of predation to toxicity tests for post-metamorphic anurans will improve the tests' ecological accuracy. It will also contribute to more appropriate risk assessments for anuran exposure to pesticides.

Keywords – Predator Stress, Visual Cues, Amphibian, Snake

INTRODUCTION

Recent advances in toxicology have demonstrated that ecological processes may alter pesticide toxicity to amphibians. For example, the addition of cues from predators in toxicity trials with tadpoles caused mortality at pesticide concentrations that were not lethal without the predator cues [1]. The mechanism for increased toxicity of pesticides in the presence of predators is unclear, however. Predator stress may potentiate genotoxic effects of some pesticides at lower concentrations [2]. Currently, no requirement exists for toxicological studies that incorporate ecological stressors when pesticides are evaluated for registration (www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series850.htm). Moreover, few data exist for effects of pesticides to post-metamorphic amphibians [3], and none address the potential for interactions with fear of predation at that life stage.

Several studies have demonstrated that many aquatic prey species detect and respond to chemical cues indicating the presence of a predator [reviewed by 4]. Visual cues are also important for many prey species, but few data exist relating to the importance of visual cues in amphibian detection of predators. Recently, Hettyey et al. [5] demonstrated that anuran tadpoles respond to visual cues from different predators, suggesting that aquatic-phase amphibians can use visual cues to detect predator presence. Juvenile eastern American toads (*Anaxyrus a. americanus*) responded by crouching or fleeing when eastern garter snakes (*Thamnophis s. sirtalis*) were visible on the opposite side of a terrarium that was divided by a screen [6]. Toad behavior did not differ from controls when only chemical cues from the snakes were present on the opposite side. Though Hayes [6] did not control for chemical cues when the snakes were present, his work is one of the first to demonstrate that visual detection of a predator is an important component to elicit defensive behaviors from post-metamorphic anuran prey. More

recently, Narayan et al [7] demonstrated that stress hormone (corticosterone) levels increased in adult Fijian ground frogs (*Platymantis vitiana*) with duration of exposure to predatory cane toads (*Rhinella marina*) that were visible on the opposite side of a terrarium from which the ground frogs were separated by mesh. Though neither study controlled for chemical cues when the predators were visible through the screens, they each demonstrate that visual cues are an important component of post-metamorphic anuran detection of predators.

Metamorphic and post-metamorphic amphibians are heavily preyed on by snakes. In a review of literature summarizing vertebrate predation of anurans, snakes were the most common predator [8]. Snakes were represented in 45% of the reports of predation [8]. In North America, garter snakes (*Thamnophis* spp.) become frequent in wetlands when amphibians are metamorphosing. Metamorphosing anurans have been found in garter snake diets at disproportionately higher rates than the metamorphs occur on the landscape [9].

Snakes are likely to be present in amphibian habitats during the growing season, when pesticides may be applied to manage invasive plant species or mosquitoes. Snakes are a primary predator of amphibians and metamorphosing and post-metamorphic amphibians may detect snake presence through visual cues. Because predator cues are known to alter the effects of pesticides on amphibians, and snakes are the predominant predator of post-metamorphic frogs, a test to measure the effects of pesticides on post-metamorphic frogs with the presence of a snake predator is needed. To meet that need, a 96-h toxicity test protocol was developed whereby northern red-legged frog (*Rana aurora*) juveniles could be exposed to a chemical and the presence of a snake predator. For the current proof-of-concept experiment, the frogs were not exposed to any chemicals, but to clean-water substrates only. The experiment was designed to address the following questions: 1) Does exposure to a visual cue of snake presence induce

measureable behavioral responses in juvenile frogs? 2) Are different behaviors associated with different substrates? 3) What sample sizes are required to detect statistically significant differences in frog behavior when exposed to snakes?

MATERIALS AND METHODS

Experimental design

Juvenile northern red-legged frogs were held in two clean-water substrates and exposed to snakes three times, at 24, 48, and 72 h. Control frogs were not exposed to snakes but were housed in arenas adjacent to the treatment frogs on shelves that were divided by an opaque plastic screen. Exposure arenas consisted of one plastic box where the frogs were individually housed within a large plastic bin, into which the snake was released. Six frogs each were assigned to four treatment categories (total frogs = 24): control (no snake) and snake-exposed frogs in one of two substrates, shallow water or damp terrestrial (Table 4.1). To manage for space and time limitations, the frogs were removed from the trial after exposure to the snake at 72 h. Frogs would not have been exposed to the snakes at 96 h, the last day of a standard 96-h toxicity trial, so that extended exposure time could not be part of this evaluation.

Study animals

Northern red-legged frog juveniles were reared from eggs as previously described (Yahnke, Chapter 3 in current dissertation). Briefly, eggs were collected from a pond in Olympia, Washington, USA on 19 March 2012. Tadpoles were reared to metamorphosis and trials began approximately 48 days after all had completed metamorphosis, on 20 October 2012. Prior to the start of the trials, the frogs were sampled for infection by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. Samples were collected and stored using the sample collection kits provided by a contract analytical laboratory (Pisces Molecular, LLC, Boulder CO,

USA). Sixteen juveniles were swabbed and the samples were stored in capped tubes of premeasured 70% ethanol. Samples were pooled in two groups of eight. Pooled samples were sent to the laboratory for polymerase chain reaction (PCR) analyses. Tests confirmed that the frogs were not infected with chytrid fungus. At the start of the trials, frog masses averaged 7.6 g (± 1.2 standard deviation [SD]) and snout-vent lengths (SVL) averaged 39.1 mm (± 2.9 SD).

Three wild common garter snakes (*Thamnophis sirtalis*) were caught 9 July 2012 at the West Rocky Prairie Wildlife Area, a Washington Department of Fish and Wildlife managed preserve near Tenino, Washington, USA. At the start of the trials, snakes averaged 68.1 g (± 11.5 SD) and 650.8 mm (± 19.8 SD) total length.

Animal husbandry

The details on tadpole rearing and frog maintenance are described in Chapter 3. During maintenance and trials, all frogs were offered 2-week old crickets at a rate of five per frog per day.

Snakes were housed in a separate room from the frogs, at room temperature (approximately 22 °C). Snakes were maintained individually in 37.8 L glass aquaria with screened tops and approximately 5 cm depth of Carefresh® Natural ™ pet bedding. A gradient of daytime temperature was created using under-tank heating pads (ReptiTherm® U.T.H., 15.2 x 20.3 cm; Zoo Med Laboratories Inc.) and 100-watt basking lights (Repti Basking Spot Lamp; Zoo Med Laboratories Inc.) that were hung in dome fixtures above each tank. The heating pads and basking lamps were placed at one end of each tank to provide a warmer spot. The lights and heaters were connected to a timer on a 13:11 light-dark cycle to allow for diel temperature fluctuations. Tanks were monitored daily at the warmest location for current (24.5°C ± 1.9 SD), maximum (27.2 °C ± 2.8 SD), and minimum (19.4 °C ± 1.3 SD) temperatures. Each snake tank

was provided with an empty cardboard paper towel tube shelter and a food-grade plastic bowl of dechlorinated City of Seattle water. The water was placed at the opposite end of the tank from the sources of heat. Snakes were offered earthworms (*Lumbricus* spp.) and/or thawed frozen silversides (Ocean Nutrition, no species identified by producer) every three to four days during maintenance. During the trials, snakes were offered food immediately after each trial session (see below for details), every three days. Water was exchanged at each feeding or more frequently as needed.

Frogs were housed in clear plastic "critter carrier" boxes (30 cm x 20 cm x 20 cm, medium Pet Keeper, PetCo) that allowed visual but no physical contact with the snake during trials. The frog boxes were placed within a larger, outer plastic bin (67.9 cm x 42.5 cm x 16.5 cm) that served as the snake arena for snake treatments. The arenas were translucent but not clear. Trials were held in an environmental chamber (Bally Engineered Structures, Inc.) maintained at 18°C.

The lids were modified on the frog boxes and the snake arenas to allow for viewing of the frogs and snakes from directly above. For each frog box, the top of the lid was removed above the vent holes on the side and replaced with a clear piece of Plexiglas cut to fit. The Plexiglas was attached to the lid with clear, aquarium-grade silicone. The modification of the frog box lids allowed for viewing from above without removing the lid. The lids of the outer container of the snake arena were modified in a similar way with Plexiglas. For the snake entrance to the arena, a hole was cut to fit a 2.5-cm threaded PVC pipe nipple with a PVC ball valve. The entrance hole was centered on one short side of the arena (front), 1-cm above the bottom. The entrance hole valves remained closed at all times except when the snakes were introduced to the arenas.

Pet Bedding (virgin wood pulp, Healthy Pet, Absorption Corp) at 0.65 kg was added to the snake arenas and tamped down to approximately 2.5-cm depth. At the start of each 72-h trial, terrestrial frog boxes contained 0.05 kg of the same bedding material with 0.2 kg dechlorinated water. Frog boxes in the water substrate treatment contained 0.5 kg dechlorinated water from an aerated holding tank that maintained the water temperature at that of the environmental chamber. Each frog box was placed 12 cm from the back and side edges of the snake arena, and 31 cm from the front (Fig. 1).

One control and one treatment arena was placed on the lowest shelf of a 160-cm tall wire rack. The racks were surrounded by opaque plastic sheeting to minimize disturbance. A thin, opaque PVC sheet was hung between control and treatment arenas to prevent control frogs from seeing the snake movements in the treatment arenas. Space existed for four trial arenas at a time, two arenas per shelf. The same substrate pairs (control and snake-exposed in water or terrestrial) were maintained on a shelf for each trial, but substrate and treatment locations were rotated on the shelves between each trial. The top shelf was covered by a thin white shower curtain to minimize glare from the lights above. Fluorescent lights were hung on each side of the rack next to the arenas but below their tops to allow for diffuse light in the arena but limit glare.

Frogs were acclimated in the frog boxes for 24-h before snake trials began. After each snake trial, frog boxes were removed from the arena so that water could be exchanged in the water treatments and fecal matter could be removed from terrestrial treatments. The water removed from the frog boxes was sampled for water quality [pH, dissolved oxygen (mg/L and % saturation), temperature (°C), conductivity (μS) and ammonia (mg/L)] and fresh water (0.05 kg) was added to each terrestrial frog box at that time. Frog boxes were returned to the arenas, and frogs were offered five crickets each. Remaining crickets were counted the following day.

At 72 h, frogs were removed from the frog boxes and housed in a 37.8 L glass aquarium in groups of up to 10 frogs. Frogs were maintained in the aquaria on pre-soaked coir substrate (Eco Earth Compressed Coconut Fiber, ZooMed^{TM}) and provided with 0.7 L food-grade plastic containers (Newspring Packaging) containing dechlorinated water until all trials were completed. After completion of the trials, all the frogs were returned to the original site of egg collection.

Trials

Prior to each trial, 25 ml of water from the frog-rearing tanks was pipetted onto the snake arena substrate along the front edge of each frog box. The placement of the water was intended to provide the scent of frogs to entice the snakes to interact with the frog box in the snake treatments.

Trials were recorded with a camcorder (Vixia HFS21; Canon) set on a shelf directly above the control and snake arenas. The shelf was set at the highest level available on the metal rack to allow for simultaneous viewing of control and snake arenas. Frogs were recorded for a total of 6 min, 3 min before introducing the snakes to the arenas of treatment frogs to measure pre-snake behaviors, and 3 min with the snakes. Frogs were not recorded for latent behaviors after removal of the snakes because the arena design did not allow for removal of the snake without lifting the arena lid and disturbing the frogs with the activity and presence of the observers.

Control and snake treatment valves were each opened when introducing snakes to the snake treatment arenas to maintain consistency among treatments. The valves were closed once the snakes entered the arenas and their tails were free of the ball valve. After 3 min, snakes were removed from the arenas.

Due to the limited number of snakes, one snake was used for the SW and ST treatments on each trial day. The treatment that would be exposed to the snake first was randomly selected. This allowed for rotation of the snakes by day so that each snake had 3 d of rest between each trial day. Food was offered to the snakes immediately after each trial so that the snakes were fasted for 3 d before each trial.

Endpoints

From the videos, the number of movements, type of movements, and time spent moving were recorded for each frog pre- and post-snake exposure. Control frogs were not exposed to snakes, but their movements were recorded during the same time frame, as were the snake-exposed frog behaviors. Snake behavior at the time of each frog movement was also recorded and summarized as the percent of frog movements that occurred when the snake was facing towards the frog, moving, and touching the frog box. The number of crickets consumed was also recorded.

Statistical analyses

Emphasis was placed on power and sample size analyses because the current experiment was intended for proof of concept and sample sizes were generally too low for standard hypothesis testing. The proportion of the most common movements expressed by frogs in each treatment was calculated as the pooled number of movements among 6 frogs per treatment/18 (6 frogs times 3 snake exposure days). The proportion of movements was used to estimate the power and sample sizes required to detect differences at different power and confidence levels. Sample sizes were estimated for confidence levels of 0.90 and 0.95 with power of 0.80 and a confidence level of 0.90 with power of 0.75. Sample sizes were calculated using the online

calculator from www.select-statistics.co.uk/sample-size-calculator-two-proportions. The formula for sample size calculation was $n = [(Z_{\alpha/2}+Z_{\beta})^2(p_1[1-p_1]+p_2[1-p_2])]/(p_1-p_2)^2$, where $Z_{\alpha/2}$ is the critical value of the normal distribution at $\alpha/2$, Z_{β} is the critical value of the normal distribution at β, and p_1 and p_2 were the expected sample proportions of two treatment groups based on the data from the most common behaviors.

The mean cumulative number of crickets that were consumed by the frogs over the course of the experiment was also used to calculate sample sizes and minimum detectable differences. An iterative approach was used to determine sample size given a minimum detectable difference of three or five crickets. This represented suppression of the frogs' appetites for one day, given their tendency to consume most of the crickets offered each day. Power and sample sizes were estimated for two-sample *t*-tests for differences between control and snake-exposed treatments within substrates and between substrates for snake treatments [10]. The calculator at http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html was used to estimate the power of detecting differences within the existing data.

Several behaviors were identified and quantified by the number of frogs and the sum of the number of times each movement was expressed by each frog before and after snakes were introduced to the treatment arenas. The number of movements within each behavior expressed by snake-exposed frogs were pooled and tested for differences between terrestrial and water substrates. The procedure for comparison of two Poisson counts described by Zar [10] was used with the one-tailed hypothesis that frogs in terrestrial substrates would express fewer movements and rely on more cryptic behaviors. Given a high number of zeroes and variability in pre- and post-snake movement data across behaviors, data were pooled among days for analyses.

RESULTS

Water quality

Water quality was similar between snake-exposed and control treatments (Table 4.2). Water temperatures were slightly warmer than the fresh replacement water after 24-h in the frog box.

Behavior

Several behaviors were identified based on different types of movements (Table 4.3). Nine of 12 control frogs and five of 12 snake-exposed frogs expressed no movements before the snakes entered the snake arenas (Table 4.4). During snake trials, one control frog in the terrestrial substrate expressed three movements (hop, shift, and turn). Two control frogs in the water substrate shifted and one turned. The remaining control frogs remained motionless in each substrate. In contrast, all snake-exposed frogs expressed at least one movement. All frogs in the water substrate and five of six in the terrestrial substrate expressed a combination of at least two movements (Table 4.4, Fig.4.2).

The most common movements of snake-exposed frogs were crouch, jump, and shift (Fig. 4.2). Frogs in the water substrate also swam. Swim and jump were similar in that they were rapid, seemingly frantic movements suggestive of escape behavior. Because swim was similar to jump, but not a behavior that could be expressed in the terrestrial environments, these two behaviors were pooled in the water substrate treatment for comparison with jump in the terrestrial treatment. No statistical difference existed in number of movements among snake treatments for crouch (Z=0.94, p>0.10), jump (Z=-0.96, p>0.10), and with swim and jump pooled (Z=0.17, p>0.25). The shift behavior was not tested for differences among treatments because of complications in interpretation. Frogs in the water substrate shifted more often to

reinforce a crouch position under water, but that aspect of the behavior is indistinguishable in the data from shifts that were not associated with reinforcing a defensive position in water (e.g., shifting weight). Therefore, the shift expressed by snake-exposed frogs in water substrates was not directly comparable to other treatments.

Frogs in terrestrial substrates were more likely to jump when the snakes were facing toward them and were positioned near the frog box (Table 4.5). Most of the frog movements in both substrates occurred while snakes were moving. The frogs tended to crouch more when snakes were not facing them and positioned away from them.

Sample sizes

The sample sizes required to distinguish between different sample populations were calculated using the crouch, jump, and jump+swim data from snake-exposed frogs in each substrate. The rate of events per observation was calculated as the total sum of movements observed/total number of observations for each movement and substrate. For example, crouch was observed 11 times in total from 6 frogs in the terrestrial substrate during 3 observations (18 total observations) for a rate of 0.61 crouches per observation (Table 4.6). Jump showed the greatest difference in observation rate. Therefore, sample sizes were also calculated for the jump observation rates rounded to the nearest tenth (Table 4.6).

Cricket consumption was similar among all treatments except snake-exposed frogs in terrestrial substrates (Fig. 4.3). Little power existed to detect differences among terrestrial (β = 0.09), water (β = 0.06) and snake (β = 0.10) treatments at alpha 0.05 with the sample size of six used in the current pilot experiment. Sample sizes required to detect minimum differences of five crickets ranged from 3, when comparing control and snake treatments in water, to 14, when comparing control and snake treatments in terrestrial substrates (Table 4.7). Sample sizes to

detect a minimum difference of three crickets were larger, requiring up to 36 frogs when comparing snake and control treatments in terrestrial substrates (Table 4.7).

DISCUSSION

Northern red-legged frogs reacted to the predominantly visual cue of snake presence by expressing behaviors consistent with attempts to flee (jump, swim), reducing detection (crouch), and/or reducing the snakes' ability to successfully capture and swallow the frog (crouch, with arms out and/or puffed body). Subtle differences were observed between substrates, but the sample sizes were too small to detect statistical differences.

In a review of the defensive postures expressed by anurans, crouch was the most commonly expressed defense mechanism employed across anuran taxa [14]. Variations observed in the crouch behavior of the frogs in the current study included chin pressed low to ground, forelimbs in front of head with palms facing out, and occasional puffing or inflation of the body. Those behaviors may serve to make the frogs more difficult to handle and swallow during a predation attempt [14]. Most of the frogs that tried to flee by jumping or swimming landed in a crouch posture. Many of the frogs employed the full range of tactics when exposed to snakes, including attempts to flee by swimming (67%) or jumping (83%), followed by sustained crouches (83-100%).

Prey may assess a certain level of threat and alter behavior accordingly [16]. The behavioral tactics described in the present study were similar to those observed by others. For example, Hayes [6] observed that eastern American toads crouched or fled in the presence of garter snakes. However, the toads' responses and corresponding encounter survival rates depended on the position of the snake [6]. Toads were more likely to survive the encounter with a snake if they remained immobile when contacted by the body of the snake or attempted to flee

when contacted by the head [6]. In the present study, subtle differences were manifest in response to snake position between frogs in the different substrates. Frogs in terrestrial substrates were more likely to attempt to flee when snakes were facing them and near the box, a situation like that of the toads that fled when contacted by the heads of snakes. All other movements by frogs in the current study were more likely to occur when the snakes faced away and were positioned away from the frogs. Appropriate behavior when threatened can increase the probability of survival for individuals. The frogs in the present study crouched to reduce detection by the snakes and fled when danger was imminent.

Frogs in terrestrial substrates crouched and jumped more frequently than frogs in water when exposed to snakes. But when the jump behavior was pooled with swimming as behaviors indicative of flight for frogs in water substrate, the numbers were similar to that of jumps expressed by frogs in terrestrial substrates. Sample sizes required to detect differences in specific behaviors were large. The lowest sample sizes were those associated with jump (without swim), in which the difference in detection rates between treatments was approximately 0.30. When behaviors were very similar between treatments, the power to detect statistical differences with smaller sample sizes was diminished. Duration of behavior was not a useful metric in this experiment, but potential exists for that metric in situations where exposure to a contaminant increases excitability and induces over-responsive behaviors, e.g., the duration of jumping may be extended.

Sample size estimates were also large for detecting differences between treatments in the number of crickets consumed. The cricket consumption data were also similar when comparing between substrates and between snake and non-snake exposed frogs. Moreover, frogs housed in terrestrial substrates and exposed to snakes ate fewer crickets on average, but with high variation,

as indicated by the error bars in Figure 3. Because the terrestrial data were more variable, larger sample sizes will be required to detect minimum differences of three or five crickets between treatments using a terrestrial substrate. How exposure to different contaminants may influence consumption rates of juvenile frogs is unclear. However, data from juvenile Oregon spotted frog exposure to imazapyr showed no effect of the herbicide on cricket consumption (Chapter 2).

Exposure to a contaminant may alter behavior of prey in a way that makes them more vulnerable to predators. Prey may be more active or overreact upon detection of a predator, they may be lethargic or underreact, or they may express inappropriate behaviors that increase vulnerability [11]. Each has the potential to impact survival. Change in response may be a result of the suppression of prey's ability to perceive a predator [12], interference with metabolic or cell-signaling processes, neurological damage, or other mechanisms [11].

Stress hormones (corticosterone) can induce either stimulatory or suppressive responses [13]. Adult Fijian ground frogs (*Platymantis vitiana*) showed a suppressive response when exposed to the presence of a predator [7]. The frogs maintained tonic immobility longer when flipped on their backs after exposure to the predator [7]. Differences in metabolic responses with crouching or fleeing may be important in assessing toxicodynamics of pesticide effects when combined with stress from predation threats. Frogs that crouch may be suppressing metabolic activity, which may result in reduced uptake or prolonged exposure to a contaminant. Frogs that flee may increase metabolism, which may result in higher contaminant exposure by increased consumption of contaminated air or water or transformation of contaminants to more toxic metabolites. Thus the response of frogs exposed to predators may rely on mechanisms that suppress and stimulate activity dynamically with changes in the perceived threat level.

CONCLUSION

The complexities of toxicodynamics and behavior make predicting responses to contaminant exposure in the presence of predators difficult with standard risk assessment procedures. A need exists for toxicity tests to incorporate greater ecological complexity to better estimate potential effects in the field [15]. In the current study, a protocol was developed by which the toxicity of contaminants to post-metamorphic anurans may be tested in the presence of the visual cue of a snake predator. Sample sizes required to detect statistical differences among treatments were prohibitively large for some metrics. The similarity of responses observed in the current proof of concept experiment contributed to large sample size estimates. However, sample sizes were attainable when considering potential effects on appetite with the number of crickets consumed. Other metrics to consider include duration of response. Physiological metrics like stress hormone levels may also be informative but were outside the scope of the current experiment. Results from this proof of concept experiment can be used to design future laboratory studies that incorporate the ecological stress of predation into toxicity tests.

REFERENCES

[1] Relyea RA. 2003. Predator cues and pesticides: A double dose of danger for amphibians. *Ecological Applications* 13:1515-1521.

[2] Giri A, Yadav SS, Giri S, Sharma GD. 2012. Effect of predator stress and malathion on tadpoles of Indian skittering frog. *Aquatic Toxicology* 106-107:157-163.

[3] Brühl CA, Pieper S, Weber B. 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environmental Toxicology and Chemistry* 30:2465-2472.

[4] Ferrari MCO, Wisenden BD, Chivers DP. 2010. Chemical ecology of predator-prey interactions in aquatic ecosystems: A review and prospectus. *Canadian Journal of Zoology* 88:698-724.

[5] Hettyey A, Rolli F, Thurlimann N, Zurcher AC, Van Buskirk J. 2012. Visual cues contribute to predator detection in anuran larvae. *Biological Journal of the Linnean Society* 106:820-827.

[6] Hayes FE. 1989. Antipredator behavior of recently metamorphosed toads (*Bufo a. americanus*) during encounters with garter snakes (*Thamnophis s. sirtalis*). *Copeia* 1989:1011-1015.

[7] Narayan EJ, Cockrem JF, Hero J-M. 2013. Sight of a predator induces a corticosterone stress response and generates fear in an amphibian. *PLoS ONE* 8:e73564.

[8] Toledo LF, Ribeiro RS, Haddad CFB. 2007. Anurans as prey: an exploratory analysis and size relationships between predators and their prey. *Journal of Zoology* 271:170-177.

[9] Arnold SJ, Wassersug RJ. 1978. Differential predation on metamorphic anurans by Garter Snakes (*Thamnophis*): Social behavior as a possible defense. *Ecology* 59:1014-1022.

[10] Zar JH. 1999. *Biostatistical Analysis*, 4 ed. Prentice-Hall, Upper Saddle River, NJ.

[11] Grue CE, Gardner SC, Gibert PL. 2002. On the significance of pollutant-induced alterations in the behaviour of fish and wildlife. In Dell'Omo G, ed, *Behavioural Ecotoxicology*. John Wiley & Sons, Ltd., West Sussex, UK, pp 1-90.

[12] McIntyre JK, Baldwin DH, Beauchamp DA, Scholz NL. 2012. Low-level copper exposures increase visibility and vulnerability of juvenile coho salmon to cutthroat trout predators. *Ecological Applications* 22:1460-1471.

[13] Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* 21:55-89.

[14] Toledo LF, Sazima I, Haddad CFB. 2011. Behavioural defences of anurans: An overview. *Ethology Ecology & Evolution* 23:1-25.

[15] Relyea R, Hoverman JT. 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters* 9:1157-1171.

[16] Ferrari MCO, Chivers DP. 2009. Temporal variability, threat sensitivity and conflicting information about the nature of risk: understanding the dynamics of tadpole antipredator behaviour. *Animal Behaviour* 78:11-16.

Table 4.1 Experimental design showing the code for each treatment combination of snake or no snake (control) and substrate.

Treatment	Substrate	
	Terrestrial	Water
Control	CT	CW
Snake	ST	SW

Table 4.2 Water quality from frog boxes in control (CW) and snake (SW) treatments at the time of water exchanges, 24-h after the last exchange and from the exchange water (Fresh water)

Treatment	n	Temp	DO % sat	DO mg/L	pH	Ammonia	Conductivity
CW	12	17.7 ± 0.4	93.6 ± 6.1	8.9 ± 0.6	7.0 ± 0.1	0.3 ± 0.1	76.3 ± 2.8
SW	13	18.0 ± 0.6	94.7 ± 3.4	9.0 ± 0.4	7.0 ± 0.2	0.3 ± 0.1	78.7 ± 4.4
Fresh water	6	17.3 ± 0.1	105.2 ± 0.8	10.1 ± 0.1	7.2 ± 0.2	N/A	75.2 ± 1.5

Table 4.3 Frog behaviors observed during snake trials. Behaviors were recorded for control and snake-exposed frogs before and during presence of the snake.

Behavior	Description
Crouch	Body flattened to substrate or under water, often with forelimbs out, palms facing outward and bodies sometimes puffed [see Toledo et al. (13)]
Dig	Slow scrabble (see below) with forearms
Hop	A single jump to a different location
Jump	A series of rapid jumping movements with pauses no longer than 1 sec
None	No visible movement; immobility evoking anti-predator crypsis indistinguishable from lack of movement unlinked to a response
Scrabble	A quick digging motion using forelimbs at the side of the container
Shift	A single movement to shift position while maintaining the same location (as in shifting weight to a different leg)
Strike	Striking at side of container, like motion used in foraging
Swim	A rapid series of movements using forelimbs and legs while remaining low in the water (water substrate only)
Turn	A change in orientation while maintaining the same location

Table 4.4 Number of frogs (n=6) expressing behaviors in each treatment before snakes were introduced to the snake arenas. Treatments are control (C) or snake-exposed (S) in water (W) or terrestrial (T) substrates.

Time	Treatment	Crouch	Dig	Hop	Jump	None	Scrabble	Shift	Strike	Swim	Turn
Pre-snake	CT	0	1	0	0	4	0	2	1	NA	0
	CW	0	0	0	0	5	0	1	0	0	0
	ST	0	0	1	0	2	0	3	0	NA	1
	SW	1	0	1	0	3	0	0	0	0	1
Post-snake	CT	0	0	1	0	3	0	1	0	NA	1
	CW	0	0	0	0	3	0	2	0	0	1
	ST	6	0	1	5	0	0	3	0	NA	0
	SW	5	0	0	5	0	2	3	0	4	0

Table 4.5 Proportion of total number of frog movements across trials within different substrates that occurred during specific snake behaviors. Snake movements may occur simultaneously, so proportions do not total to 100 percent.

Substrate	Frog movement	Total # frog movements	Snake position		
			Facing frog	Moving	Near frog box
Terrestrial	crouch	11	0.45	0.82	0.36
	jump	16	0.69	0.88	0.81
	shift	4	0.25	1.00	0.25
Water	crouch	7	0.43	1.00	0.29
	jump+swim	17	0.47	0.88	0.35
	shift	7	0.29	0.43	0.14

Table 4.6 Sample sizes required for different power (β) and confidence levels (α) given sum of movements, number of observations, and rate of movement observations from snake-exposed frogs in terrestrial and water substrates. Sample sizes were also determined for jump rates rounded to the nearest tenth (in parentheses).

Parameter	Crouch	Jump	Jump+swim
Sum of movements (from 18 total observations per substrate)			
Terrestrial	11	16	16
Water	7	11	17
Rate			
Terrestrial	0.61	0.89 (0.9)	0.89
Water	0.39	0.61 (0.6)	0.94
Sample size (α, β)			
0.10, 0.75	53	24 (20)	320
0.10, 0.80	61	27 (23)	368
0.05, 0.80	78	35 (29)	467

Table 4.7 Sample sizes required to detect differences in mean crickets consumed between substrate (control vs. snake-exposed in water or terrestrial) and snake (in water vs. terrestrial substrate) treatments with alpha of 0.05 and power of 0.90.

Minimum detectable difference (number of crickets)	Terrestrial	Water	Snake
3	36	7	35
5	14	3	14

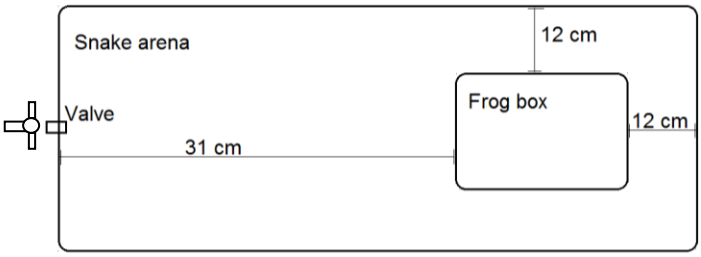


Figure 4.1 Diagram of snake arena containing the frog box. Snakes entered the arena through the valve.

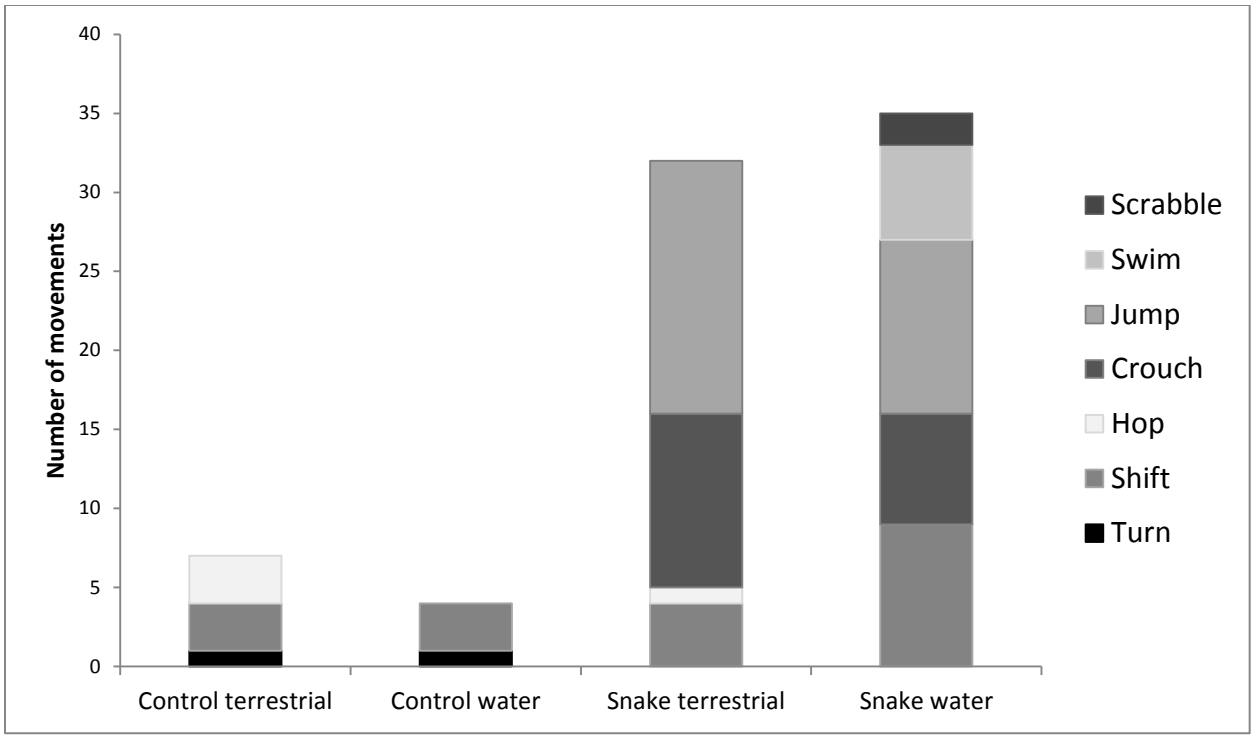


Figure 4.2 Pooled number of movements expressed by control and snake-exposed frogs in terrestrial and water substrates during 3 min of exposure to snakes. Order of behavior key applies to bars. Scrabble, swim, and jump were associated with flight behaviors; crouch was an expression of crypsis, the remaining behaviors, hop, shift, and turn, were not associated with flight or crypsis.

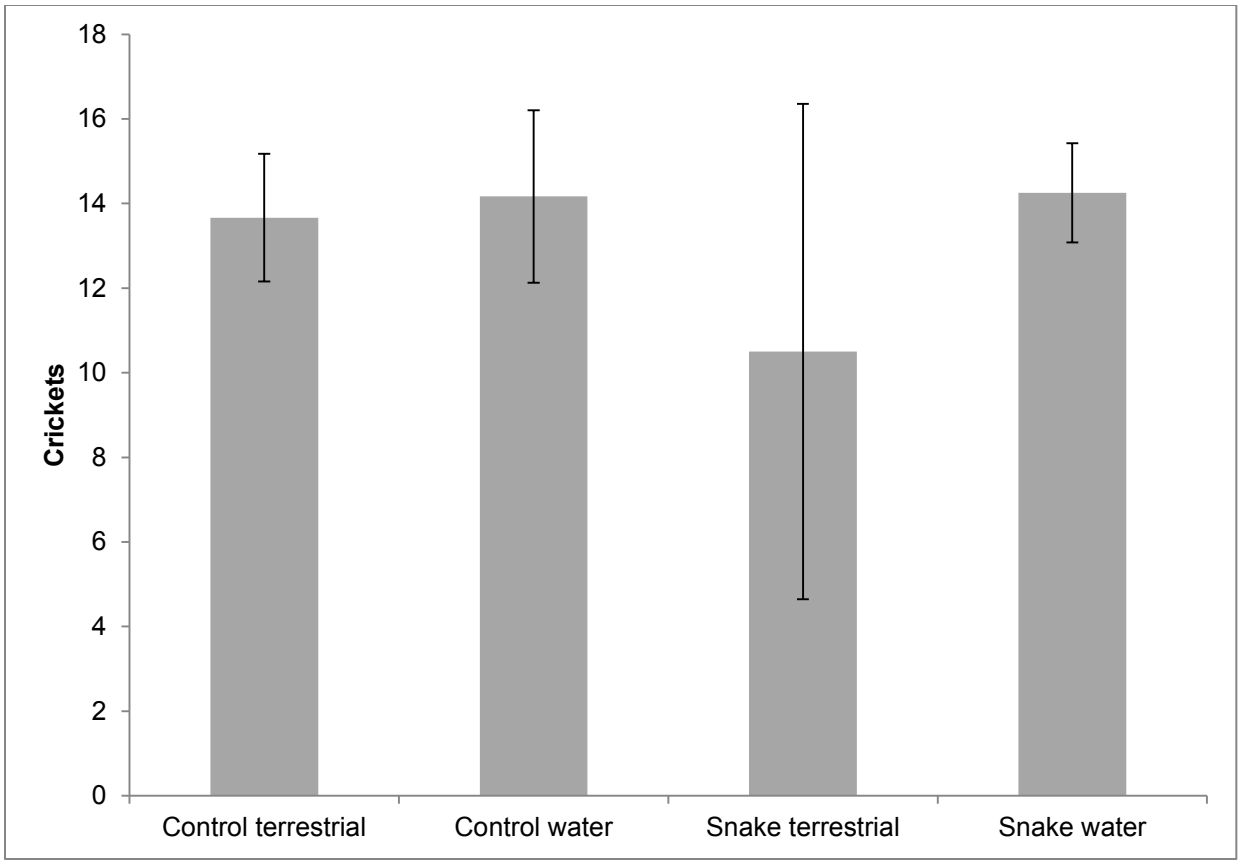


Figure 4.3 Mean number of crickets consumed by control and snake-exposed frogs in terrestrial and water substrates. Bars = standard deviation.