

5-2014

Level of UV-B Radiation Influences the Effects of Glyphosate-Based Herbicide on Fitness of the Spotted Salamander

Nicholas A. Levis

Western Kentucky University, nicholas.levis655@topper.wku.edu

Follow this and additional works at: <http://digitalcommons.wku.edu/theses>

 Part of the [Biodiversity Commons](#), [Biology Commons](#), [Terrestrial and Aquatic Ecology Commons](#), and the [Zoology Commons](#)

Recommended Citation

Levis, Nicholas A., "Level of UV-B Radiation Influences the Effects of Glyphosate-Based Herbicide on Fitness of the Spotted Salamander" (2014). *Masters Theses & Specialist Projects*. Paper 1335.
<http://digitalcommons.wku.edu/theses/1335>

This Thesis is brought to you for free and open access by TopSCHOLAR®. It has been accepted for inclusion in Masters Theses & Specialist Projects by an authorized administrator of TopSCHOLAR®. For more information, please contact topscholar@wku.edu.

LEVEL OF UV-B RADIATION INFLUENCES THE EFFECTS OF GLYPHOSATE-
BASED HERBICIDE ON FITNESS OF THE SPOTTED SALAMANDER

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

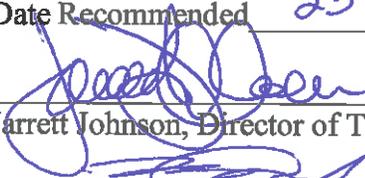
In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

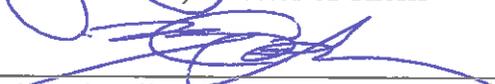
By
Nicholas A. Levis

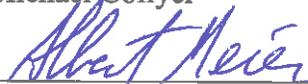
May 2014

LEVEL OF UV-B RADIATION INFLUENCES THE EFFECTS OF GLYPHOSATE-
BASED HERBICIDE ON FITNESS OF THE SPOTTED SALAMANDER

Date Recommended 23 April 2014


Jarrett Johnson, Director of Thesis


Michael Collyer


Albert Meier

 5-5-14
Dean, Graduate Studies and Research Date

I dedicate this thesis to my family for their continued support and encouragement and without whom my passion for science and biology would never have blossomed. I also dedicate this thesis to my fiancée, Alice Reynolds, for contributions at home and in the field that improved my research experience.

ACKNOWLEDGMENTS

This work could not have been completed without the assistance of several people. Thanks are due to Jessica Dunnegan, Alice Reynolds, Cynthia Worcester, and Naomi Rowland for their assistance in administrative and organizational matters. In addition, several field assistants, including Jacob Floyd, Alice Reynolds, Danny Stinson, Andi Esterle, and Kevin Ziegler, contributed to data collection and preparation of the field site. I would like to express gratitude to my committee members, Jarrett Johnson, Michael Collyer, and Albert Meier, for their commitment to professional career and development as a scientist. Furthermore, Michael Collyer provided invaluable assistance with statistical analysis and coding in R. Jarrett Johnson and Ajay Srivastava helped guide my improvement as a teaching assistant. John MacGregor supplied important specimen location information and detailed field notes. The Western Kentucky University Biodiversity Center and Green River Preserve provided valuable resources that allowed this research to be undertaken. Thanks are due to Danny Crain for allowing me to collect eggs from ponds on his property. I would like to thank Western Kentucky University's Biology Department, Ogden College of Science and Engineering, and Graduate School and the Kentucky Academy of Sciences for providing funding for this project. Finally, numerous other faculty and graduate students are deserving of recognition for their friendship, support, and encouragement throughout my tenure at Western Kentucky University. My sincere thanks go to you all.

CONTENTS

List of Figures.....	vi
List of Tables.....	vii
Abstract.....	viii
Introduction.....	1
Materials and Methods.....	4
Results.....	12
Discussion.....	15
References.....	24

LIST OF FIGURES

Figure 1. Set up of film-covered mesocosms.....	33
Figure 2. The two-step experimental design and layout of mesocosms.....	34
Figure 3. Field and experiment UV-B radiation values.....	35
Figure 4. Principal component plot of tail morphologies.....	36
Figure 5. Consensus tail morphologies.....	37
Figure 6. Summary of major findings.....	38

LIST OF TABLES

Table 1. Temperatures associated with each film type.....	39
Table 2. Model comparisons for fitness endpoints.....	40
Table 3. Summary statistics for fitness endpoints.....	41
Table 4. Jackknife classification for morphology (Treatments).....	42
Table 5. Jackknife classification for morphology (Single factors).....	43
Table 6. Pairwise distances of tail morphology centroids.....	44

LEVEL OF UV-B RADIATION INFLUENCES THE EFFECTS OF GLYPHOSATE-BASED HERBICIDE ON FITNESS OF THE SPOTTED SALAMANDER

Nicholas A. Levis

May 2014

44 Pages

Directed by: Jarrett Johnson, Michael Collyer, and Albert Meier

Department of Biology

Western Kentucky University

Numerous causes have been implicated in contributing to amphibian population declines since the 1980's, with habitat modification, ultraviolet radiation (UV-B) and environmental contaminants (such as glyphosate-based herbicide) being among the most common. This study identifies the effects of a generic glyphosate-based herbicide (GLY-4 Plus) on mortality, immune function, body condition, and morphological plasticity of larvae of the spotted salamander (*Ambystoma maculatum*) under conditions that reflect open and closed canopy light regimes. Larval salamander responses to glyphosate-based herbicide varied depending on UV-B conditions. In general, it appears that an open canopy (*i.e.* greater UV-B exposure) may confer fitness benefits. In the presence of herbicide, survival was higher in an open canopy UV-B regime and pooled open canopy survival was higher than that of closed canopy treatments. In the absence of herbicide, body condition and immune function were positively related with amount of UV-B. Finally, herbicide presence appeared to affect morphology under low UV-B conditions. UV-induced breakdown of surfactant or a complex interaction between temperature stratification and trophic relations is potentially responsible for the observed patterns in survival and body condition. However, the mechanistic underpinnings of improved immune function and morphological differences are less clear. As deforestation is likely to continue, amphibians may find themselves in ponds with increasingly open canopies. Combined with the knowledge that some amphibians can become locally adapted to UV

exposure and develop pesticide tolerance, the probability of surviving exposure to this herbicide may be elevated in open canopy ponds. These results emphasize the complexity of natural systems and the importance of including multiple factors in experiments.

Introduction

Amphibian populations are declining worldwide (Houlahan *et al.* 2000; Stuart *et al.* 2004) due in a large part to habitat destruction (Gallant *et al.* 2007). However, a number of other factors have been implicated in contributing to declines (Blaustein *et al.* 2011). Contaminants, such as insecticides and herbicides, and increased ultraviolet-B radiation (UV-B) are among the most widespread.

Extreme use of pesticides is a demonstrated anthropogenic contributor to global amphibian declines (Sparling, Fellers & McConnell 2001). Glyphosate-based herbicides are the most widely applied types of herbicide in the world and can greatly affect amphibians and damage aquatic systems (*e.g.* Govindarajulu 2008; Jones, Hammond & Relyea 2011). Many of these herbicide formulations contain the surfactant polyethoxylated tallowamine (POEA) which has resulted in their classification as moderately to highly toxic from laboratory, experimental mesocosm, and pond enclosure experiments (*e.g.* Bernal, Solomon & Carrasquilla 2009; Jones *et al.* 2011). Glyphosate with POEA can be more lethal when combined with other abiotic and biotic factors such as high pH and predator chemical cues (*e.g.* Wojtaszek *et al.* 2004; Relyea 2005a) and can remain in pond water up to 120 days (Barolo 1993). Because these herbicides are used so widely and can cause dramatic changes to ecosystems, amphibian responses to application of glyphosate-based herbicides warrants continued investigation.

Increased UV-B, a result of ozone depletion, can have significant effects on amphibian populations. The current depletion of stratospheric ozone by production of chlorofluorocarbons (CFCs) and other chemicals has led to long-term increases in ultraviolet radiation (Cockell & Blaustein 2000; Blaustein *et al.* 2003). Ozone depletion

has already resulted in significantly increased levels of UV-B (280-315 nm) in both tropical and temperate regions (*e.g.* Herman *et al.* 1996; Middleton *et al.* 2001). Not only can ultraviolet radiation increase embryonic and larval mortality of amphibians, but UV-B can also slow growth rates, lead to immune dysfunction, and trigger other sublethal damage (*e.g.* Broomhall, Osborne & Cunningham 2000; Tietge *et al.* 2001). Ultraviolet-B radiation is a stressor that can overwhelm an animal's defenses and make it more susceptible to other environmental stressors (Tevini 1993). For example, pesticides, polycyclic aromatic hydrocarbons (PAHS), fertilizers and low pH interact with UV-B to damage developing amphibians (Blaustein *et al.* 2003). Specifically, ultraviolet radiation can break down a contaminant into a more toxic form; ~1.5% of ambient UV-B intensity was sufficient to photoactivate a carbaryl-based insecticide and result in a 10-fold increase in toxicity (Zaga *et al.* 1998). Lund-Høie and Freistad (1986) demonstrated that glyphosate is photolytic and breaks down more rapidly in sunlight, suggesting that high UV-B conditions could potentially confer an advantage against this herbicide. However, a recent study by Puglis and Boone (2011) found little difference in mortality of green frog (*Lithobates clamitans*) tadpoles exposed to glyphosate-based herbicide under UV-B present and absent treatments in the laboratory. Therefore, the interaction among glyphosate-based herbicides and UV-B exposure and potential photoactivation needs further study under more realistic conditions (Govindarajulu 2008).

Amphibians occupy a variety of habitats ranging from flowing streams to small ephemeral pools. The penetration of light into these habitats varies with amount of tree cover and geographic location (Flint & Caldwell 1998; Cordero *et al.* 2013). The light regimes in various habitats likely affect amphibian growth, development, and ultimately

fitness. Given current trends in deforestation and the photolytic nature of glyphosate, there exists the possibility that amphibians occupying habitats with different light regimes will respond differently to the presence of herbicide.

Pond-breeding amphibians are adapted to highly variable environments (*e.g.* temporary ponds) which promoted the evolution and maintenance of phenotypic plasticity. Phenotypic plasticity is defined as the capacity of a single genotype to exhibit a range of phenotypes in response to variation in the environment (Fordyce 2006) and might serve amphibians well when exposed to variation in anthropogenic stressors. Phenotypic plasticity is favored in heterogeneous environments where selection favors different phenotypes depending on the conditions (Via & Lande 1985) and provides a mechanism by which amphibians can cope with environmental variability (see review by Whitman & Agrawal 2009). However, maintaining an induced phenotype when it is not favored by the environment can have growth and/or developmental costs (Relyea 2002; Auld, Agrawal & Relyea 2010). Recently, Relyea (2012) found that glyphosate-based herbicide (Roundup®) could induce a morphologically plastic response in tadpoles that was similar to predator-induced morphology. In the absence of predators, such induced morphology may result in decreased fitness.

Despite the clear potential for widespread consequences on amphibian populations, UV-B and glyphosate-based herbicide have received little examination together under semi-natural conditions. Furthermore, previous studies have focused primarily on anuran larvae when investigating these stressors and given much less attention to caudates. Since larvae of the spotted salamander, *Ambystoma maculatum*, have a large geographic range (Petranka 1998), demonstrate environmentally-induced

phenotypes (Urban 2008, 2010), and can be found in both open and closed canopy ponds, they serve as an ideal model to investigate potential consequences of glyphosate-based herbicide and UV-B radiation. The aims of this study were to evaluate the independent and interactive effects of glyphosate-based herbicide on a larval salamander under two ecologically relevant UV-B levels. Specifically, I measured 1) mortality, 2) immune function, 3) body condition, and 4) morphological phenotypic plasticity because these endpoints can have important implications for conservation initiatives. I predicted that lower UV-B and the absence of glyphosate-based herbicide would result in the highest salamander fitness.

Materials and methods

Animal Collection and Rearing

I collected eight *A. maculatum* egg masses from a pond near Western Kentucky University's Green River Preserve in Hart County, KY, on March 20-21, 2013. Clutches were held separately in outdoor wading pools covered with a mesh screen. Larvae began hatching on April 20 and all larvae had emerged from eggs by May 1. After hatching, larvae were fed a combination of zooplankton and brine shrimp (*Artemia spp.*) *ad libitum*.

Experimental Design

Experimental set-up was a modified combination of designs used by Relyea (2012) and Searle et al. (2010). Plastic mesocosms (*i.e.* 1200 L cattle tanks) were filled with ~835 L of municipal water, and then 500 g of mixed deciduous leaf litter (*Acer/Quercus spp.*)

and 80 g of rabbit chow were added to serve as initial nutrient sources. An initial 280 mL aliquot of concentrated zooplankton, phytoplankton, and periphyton collected from nearby ponds was added to each tank. Soil was not added to the mesocosms because adding soil has no effect on the toxicity of glyphosate-based herbicide (Roundup®) under mesocosm conditions (Relyea 2005b). I then added a water conditioner (Kordon AmQuel©) to remove chlorine and chloramines that are potentially deadly to aquatic organisms. I subsequently added another 205 mL of concentrated plankton to ponds for a total of 485 mL. After the final aliquot of plankton were added, communities developed for another 19 d and populations were robust at the time larvae were added. To maintain control of algal growth and water clarity, 22 American toad (*Bufo americanus*) tadpoles were added to each tank. By the start of the experiment, all toad tadpoles had metamorphosed and/or perished. On May 8 (defined as day 0), I added 15 larval salamanders (~2wk post-hatch) to each mesocosm and haphazardly selected five individuals to provide a body size estimate. Fifteen larvae were selected as a low density to reduce or eliminate any potential effects of competition on mortality and morphology (Relyea 2004). After approximately two weeks, I added twenty-six *Hyla spp.* tadpoles to each tank to help control algal growth and maintain water clarity.

Larvae were exposed to two levels of ultraviolet-B radiation, UV_{oc} or UV_{cc}, which represented ecologically relevant levels of UV-B in open canopy and closed canopy ponds, respectively. Similar to Searle et al. (2010), UV-B radiation was controlled by covering the mesocosms with 0.127 mm clear Acetate (UV_{oc}) or clear polyester Duralar® (UV_{cc}) film (Grafix Plastics, Cleveland, OH). Blaustein *et al.* (1994) demonstrated that polyester film (Mylar®) transmits 5% ambient UV-B radiation and allows other

wavelengths to pass through and clear acetate films transmit up to 80% of ambient UV-B radiation. I verified the percent transmission of UV-B radiation using a Perkin Elmer Lambda XLS+ spectrophotometer. Acetate film transmitted between 57 and 77 percent of light at 280 and 315 nm, respectively (the extreme ends of the UV-B spectrum). Conversely, the Duralar® film transmitted ~2% of light at 315 nm and attenuated to < 0.3% at 280 nm. Therefore, the amount of UV-B radiation transmitted by these films was comparable to those used in previous studies. To account for potential weathering during experimental duration, I measured the percent UV-B transmission again after the experiment concluded.

Films were attached to a 2.54 cm diameter PVC pipe, the pipe was placed across the center of each tank, and films were then tightened around the outside of each mesocosm and held in place by wooden panels stapled to the film and tanks. In essence, this design resembled a very squat "fly" tent and allowed air circulation in the mesocosms (Fig. 1). UV-B levels outside of eight randomly selected tanks (4 per film type) were measured above the tanks outside the film, just below (≤ 1 cm) the water's surface, and ~40 cm below the water's surface weekly between 1100 and 1300 h using a Solarmeter® model 6.2 UV meter (Solartech Inc., Harrison Twp., MI). Surface temperature and ~40 cm submerged temperatures were also taken in the selected tanks to ensure that only UV-B level differed between film types. For submerged UV-B measurements, the meter was held in a clear Ziplock™ bag. Twenty sample measurements were taken to account for the reduction in percent transmission by the bag compared to ambient levels. Bagged readings were 88 ± 0.6 % of ambient UV and were corrected prior to being reported.

There were two levels of herbicide treatment: added (H+) or not added (H-). The herbicide treatments were applied on May 9 (day 1). I used the most common formulation of glyphosate-based herbicide sold in the area surrounding the study site, GLY-4 Plus™ (Universal Crop Protection Alliance, LLC, Eagan, MN, USA). This formulation contains 356 g/L of the acid glyphosate (480g /L active ingredient) plus POEA. I added 5 mL of the formulated product to each mesocosm in order to attain an acid equivalent (a.e.) concentration within the range of actual worse-case scenarios seen in nature (1.7 to 5.2 mg a.e./L; Edwards, Triplett & Kramer 1980) that can induce morphological changes in some anuran species (Relyea 2012). However, this concentration is lower than laboratory concentrations shown to induce significant (~80%) mortality in this species (Relyea & Jones 2009). The formulated product was added to 25 mL of tank water and then this mixture was distributed across the surface of the mesocosms (Relyea 2012). One hour after the applications, I sampled the middle of the water column and pooled glyphosate treatments (*i.e.* present vs. absent). The water samples were then refrigerated and later shipped for analysis using high-pressure liquid chromatography (National Testing Laboratories, Ltd., Ypsilanti, MI). In addition, glyphosate treatment tanks were also sampled after termination of the experiment to determine the amount of glyphosate degradation under each UV-B regimen. Finally, water samples from the natal pond were analyzed for glyphosate concentration to increase the amount of field data available.

The UV-B and glyphosate conditions described above resulted in four treatments: 1) UV_{oc}/H+, 2) UV_{cc}/H+, 3) UV_{oc}/H-, and 4) UV_{cc}/H-. These treatments were distributed among the eight clutches in a replicated, randomized Graeco-Latin Squares design (Fig.

2). Clutches were not mixed together in mesocosms in order to test for family effects and minimize genetic differences for measures of phenotypic plasticity. Each treatment was replicated eight times (once per clutch) for 32 total experimental units.

Mortality Assessment

After ~5 weeks, I terminated the experiment by removing all water and leaf litter and recovering all surviving salamanders. This duration was selected because it allowed for ~7 weeks for larvae to achieve optimum size for metamorphosis. Survivors were counted and then euthanized in 0.2% MS-222. One limb was removed and preserved in 95% ethanol and the carcasses were fixed in 40% buffered formalin and preserved in 70% ethanol. Additionally, survivors were categorized as larvae or metamorphs based on the size of their gills at the termination of the experiment. Animals with external gills present were considered larvae and salamanders with gills fully absorbed were deemed metamorphs.

Immune Function

Upon termination of the experiment, I haphazardly selected four larvae and four metamorphs for the immune challenge from each tank. However, only tanks with at least four individuals for either stage were used. Immune function was measured according to Seiter (2011) by administering an injection of phytohemagglutinin (PHA; Sigma Aldrich, St. Louis, MO). PHA causes T-lymphocytes to proliferate rapidly *in vivo* and *in vitro* (Smits, Bortolotti & Tella 1999; Martin *et al.* 2006). It causes measurable swelling at the injection site with greater swelling indicating a stronger T-lymphocyte response and,

therefore, stronger immune function. The PHA assay is a commonly used field method for measuring immune response in various vertebrate taxa (*e.g.*; Calsbeek, Bonneaud & Smith 2008; Boughton, Joop & Armitage 2011). Gervasi and Foufopoulos (2008) have also demonstrated its usefulness in measuring amphibian immune response.

The immunoassay was prepared by dissolving 2 mg of PHA in 1 mL of phosphate-buffered saline solution (PBS). Each individual was then injected subdermally at the base of the tail with 15 μ L of the PHA-saline solution using a 0.3-mL, 32-gauge insulin syringe. Skin thickness measurements of each individual were taken before injection, and 24 and 48 h post injection.

Salamanders were removed from tanks and transported to a nearby field station for the PHA assay. To facilitate handling during injection, I anaesthetized the animals using 0.02% MS-222 buffered with NaCO_3 , and before injection, I weighed individuals and measured tail thickness using fine-gauge digital calipers. During the immune challenge, animals were housed individually at the field facility. Immune response was assayed by subtracting the pre-injection tail thickness from the tail thickness at 24 and 48 h (Seiter 2011). After measurement, animals were euthanized in a 0.2% solution of MS-222 and preserved in ethanol as above.

Phenotypic Plasticity

At the conclusion of the experiment, I preserved all remaining individuals in 40% formalin and stored them in 70% ethanol prior to morphology measurements. I then took digital images of the larvae using a Nikon D7000 camera and analyzed them for shape

using the TPS software suite (Rohlf 2001, 2003, 2013). Lateral tail shapes were determined using 34 landmark coordinates.

Statistical Analysis

Upon termination of the experiment, each recovered individual was measured for snout-vent length (SVL) and weighed to the nearest 0.1 grams. Body condition (BC) was determined by dividing the mass by SVL. For each treatment, I determined salamander survival, mean BC, percentage of metamorphs, and 48-h immune function.

The relationship among UV, glyphosate, and salamander fitness was evaluated using linear mixed-effects model fitted with restricted maximum likelihood in the lme4 package of R (Bates & Maechler 2009). "Glyphosate" and "UV" were fixed categorical variables and family and block were random effects. Family and block were arbitrary and assumed not to interact with treatments.

To test the effect of treatment on survival and number of metamorphs, I modeled individual survival or stage, respectively, as a categorical variable (*e.g.* alive or dead; larva or metamorph) with a generalized linear mixed-effects model using a logit link and binomial error term. Models were compared using ANOVA after visually assessing assumptions. Data were analyzed using Fisher's exact test with a Bonferroni correction, after the best model was selected using Akaike's Information Criterion (AIC).

Best-fit models for BC and immune function were determined using AIC values as above. Once the best model was determined, a randomized residual permutation procedure (RRPP; Collyer & Adams 2007) was used to calculate effect sizes between groups and to identify between-group differences. Briefly, this procedure extracted the

residuals of a null model and randomly paired them with fitted values. Subsequently, these pseudorandom data were used to calculate pairwise distances using the full model. By repeating this process 10,000 times, I was able to determine the probability of finding random differences as large as or larger than the observed distances between group means (D_{obs}) for multiple comparisons or observed F values for two-model comparisons and thereby assess statistical significance.

Finally, morphology was analyzed from a covariance matrix of the 34 landmark coordinates after Procrustes superimposition using principal component analysis (PCA), jackknife classification, and RRPP with posthoc multiple comparisons on the points from clutch-independent landmark coordinates. PCA performs a rigid rotation of the data space such that the variation explained by two axes is maximized. By color-coding points in the two-dimensional projection, inferences and trends can be made, but no hypotheses are explicitly tested. The further exploratory technique, jackknife classification, was used to determine how well individuals could be placed into their correct treatment. This classification technique involves removal of one subject, calculation of covariation between variables associated with subject differences within groups, then classifying the removed subject. All 58 available dimensions determined from eigenanalysis were used for hypothesis testing. Fifty-eight dimensions were used instead of the possible 68 due to redundancies causing negative eigenvalues for 10 dimensions. The hypothesis that the centroids from each treatment occupied the same position in the morphospace was tested using RRPP with 10,000 iterations and F as the test statistic. The multiple comparisons test followed the same procedure described above in which pairwise distance matrices

were calculated 10,000 times. All analyses were performed using R version 3.0.2 with the probability of type 1 error (α) equal to 0.05.

Results

UV-B and Glyphosate Measurements

UV-B levels varied between UV_{oc} and UV_{cc} treatments at the water's surface (Fig.3), but all UV measurements attenuated to $0\mu\text{W}\cdot\text{cm}^{-2}$ 40 cm below the surface. The surface UV-B levels were within naturally observed values for open and closed canopy ponds (Fig. 3). Conversely, water temperature did not differ much between the two UV treatments, but did vary between the water's surface and the tank bottom (Table 1).

Glyphosate concentration in all H⁺ treatments at the start of the experiment was 2.75 mg/L and decreased to 0.95 and 0.84 mg/L. for UV_{oc} and UV_{cc} treatments, respectively, at the end. No glyphosate was detected in the H⁻ treatments or the natal pond.

Survival

The best predictor of survival was treatment (Table 2); survival significantly varied among groups (Table 3). However, within a given UV-B level there was no difference in survival due to glyphosate presence or absence. Both UV_{oc} treatments had significantly more survivors than the UV_{cc}/H⁺ treatment (UV_{oc}/H⁺ Fisher's exact p value <0.0001; UV_{oc}/H⁻, Fisher's exact p value = 0.0001890).

Immune Function

Immune function was determined by measuring swelling due to T-lymphocyte recruitment following PHA injection and best-fit model was selected using AIC values. No significant effects of treatments were observed over 24 h intervals, but significant effects were observed after 48 h (Table 2). UV_{oc}/H⁻ had greater change in swelling than UV_{oc}/H⁺ and UV_{cc}/H⁻ ($D_{\text{obs}} = 0.2674$, $p = 0.0169$ and $D_{\text{obs}} = 0.4098$, $p = 0.0005$, respectively; Table 3). Thus, immune function was reduced with the addition of herbicide under high UV-B conditions, and it was also reduced with the elimination of UV-B in the absence of herbicide.

Body Condition and Metamorphs

The best predictor of BC for all survivors was treatment (Table 2). UV_{cc}/H⁻ had significantly lower BC than all other treatments ($D_{\text{obs}} = 0.0045$, $D_{\text{obs}} = 0.0065$, $D_{\text{obs}} = 0.0073$, $p = 0.0001$) and UV_{oc}/H⁺ had significantly lower BC than UV_{oc}/H⁻ ($D_{\text{obs}} = 0.0020$, $p = 0.0449$). In other words, under low UV-B, the presence of herbicide yielded increased BC and under higher UV-B, the presence of herbicide decreased BC (Table 3). Developmental stage (larva or metamorph) had no effect on BC ($F = 0.3233$, $p = 0.9993$).

The presence or absence of herbicide best explained the percentage of metamorphs among survivors, with the H⁺ treatments having significantly more metamorphs than the H⁻ treatments ($28 \pm 4\%$ vs. $17 \pm 3\%$; Fisher's exact p value = 0.04)

Morphological Plasticity

The first two principal components accounted for 58% of the total variation for the tail morphology (Fig. 4), with the third and fourth PCs contributing another 21%. This suggests that there is substantial variation not shown in the two-dimensional ordination. Nevertheless, distances calculated in both spaces were correlated ($r = 0.91$). The UV_{oc} treatments tended to have higher PC2 values than the UV_{cc} treatments, but no obviously distinct clusters were observed.

The jackknife classification yielded 47% accuracy in placing individuals into the correct treatment. The majority of misclassifications had at least one of the factors (*i.e.* UV-B or herbicide) classified correctly (Table 4). When classification was run for each factor individually, the success was 58% and 66% for UV-B and herbicide, respectively (Table 5). Expected correct classification by chance for treatment and single factors was 25% and 50%, respectively. Therefore, my individuals were placed into the correct group more often than would occur at random.

Finally, RRPP revealed that there were significant differences (Table 6) in centroid location in the morphospace for $UV_{cc}/H-$ and all other treatments (Fig. 4). The major difference between $UV_{cc}/H-$ animals was in tail arch, and the tail muscle also tended to be shorter and narrower in these larvae compared to the other treatments (Fig. 5). Under open canopy conditions, morphology did not change with herbicide addition, but the addition of herbicide under closed canopy conditions influenced the morphology to converge on that of the open canopy treatments.

Discussion

This study aimed to elucidate differences in how glyphosate-based herbicide affects salamanders under different UV-B conditions. I found evidence to suggest that fitness outcomes vary based on herbicide exposure and UV-B level (Fig. 6). In the presence of glyphosate-based herbicide, UV-B conditions significantly affected the survival of larval salamanders. In addition, UV-B radiation appears to improve survival and immune function. Finally, the interaction between UV-B level and exposure to herbicide can have significant effects on salamander condition and morphology.

Survival

Several mesocosm studies have found that glyphosate-based herbicide both harms amphibians and alters aquatic systems (Howe *et al.* 2004; Relyea, Schoeppner & Hoverman 2005). However, recent studies using natural ponds have found glyphosate-based herbicide to have little effect on amphibians (Edge *et al.* 2012). To my knowledge, all semi-natural and natural studies with glyphosate-based herbicides have had ambient UV-B conditions reflective of an open-canopy pond. The effects of UV-B radiation on aquatic systems is generally negative (Blaustein *et al.* 2011), but depends on the amount of dissolved organic carbon inhibiting light penetration (Crump *et al.* 1999b; Croteau *et al.* 2008). I anticipated that the combined effect of ambient UV-B and glyphosate-based herbicide would overwhelm the salamanders and result in greater mortality. However, my results suggest a potential benefit to higher (near open-canopy ambient) UV-B levels, particularly when exposed to glyphosate-based herbicide. Within a given UV regime, addition of herbicide did not reduce survival. But when herbicide was present, UV_{oc}

ponds had greater survival than UV_{cc} ponds. A number of possible mechanisms could have caused this difference in survival, including UV-induced breakdown of herbicide and complex trophic interactions.

UV-induced Breakdown

The observed difference in survival was not due to differential breakdown of the glyphosate under the two UV-B treatment levels because both ended up with similar concentrations of glyphosate at the experiment's end. Despite several studies showing that the POEA surfactant is significantly more toxic than glyphosate itself (*e.g.* Mann & Bidwell 2001; Tsui & Chu 2003), little is known about the breakdown of the surfactant in natural systems. Additionally, surfactant formulations are often proprietary and may vary among herbicide formulations. Sediment significantly reduces the toxicity and concentration of POEA in microcosms (Wang *et al.* 2005), but no sediment was used in my study. Therefore, POEA concentrations only should have been influenced by time or UV-B treatment. If UV-B radiation breaks down the surfactant, this could explain the difference in survival observed between UV-B levels through two possible mechanisms. First, the surfactant could have directly reduced salamander survival by remaining at higher concentrations in the UV-B_{cc} treatments. Conversely, differential breakdown could have resulted in a bottom up trophic cascade. Exposure to POEA reduces survival of *Daphnia magna* (Brausch, Beall & Smith 2007). This reduced food supply could then decrease the survival of the larval salamanders. Under this scenario, greater breakdown of POEA under UV-B_{oc} conditions would increase the relative food supply of zooplankton and improve salamander survival. Alternatively, high POEA concentrations under the

closed canopy regime would reduce the food supply available and lead to greater salamander mortality. Because knowing precise concentrations of surfactant across herbicide formulations is difficult, comparisons across studies using glyphosate may be misleading. Future studies should emphasize this difference among formulations.

Trophic and Spatial Interactions

Since the only food source for salamanders in this study was zooplankton, any effect the treatments had on zooplankton communities could potentially have a strong trophic effect on the salamanders. Storz and Paul (1998) found that *D. magna* reacted to sub-ambient levels (*i.e.* 3×10^6 times lower than sunlight) of ultraviolet light with negative phototaxis, suggesting that they may have moved to deeper water under the UV-B_{oc} condition. In the UV-B_{cc} treatments, salamanders would have had to venture closer to the surface in search of prey items despite their tendency to remain in deeper, cooler waters (Bancroft *et al.* 2008). Since the water surface was warmer than the bottom (Table 1), the herbicide should have stratified and been more concentrated at the water's surface (Jones, Hammond & Relyea 2010). Glyphosate-based herbicide has a greater lethal effect on larval amphibians than on zooplankton communities (Relyea 2005c). Thus, exposure to greater concentrations at the surface due to pursuit of prey items, could account for the differential survival between UV-B levels. The herbicide concentration used in this study was within LC50_{96h} and LC90_{96h} for spotted salamanders raised under laboratory conditions (Relyea & Jones 2009), indicating that most mortality likely occurred early during the study period when the concentration was highest. Because larvae are most vulnerable at early developmental stages (*i.e.* earlier during the study period), exposure to

recently applied, concentrated herbicide at the water's surface could have resulted in high mortality early in the experiment. This expected mortality was mitigated under high UV-B conditions because the phototaxis of zooplankton could have indirectly protected salamanders from stratified herbicide. However, this explanation does not account for the overall difference in survivorship between each UV-B regime.

Benefits of UV-B

My data indicate that elevated UV-B radiation could be beneficial to larval salamander survival in some circumstances. Open canopy conditions resulted in approximately a two-fold increase in survival when herbicide was present. In addition, pooled UV-B_{cc} survival was significantly lower than pooled UV-B_{oc} survival according to Fisher's exact test ($p < 0.00001$). Furthermore, a previous study by Bridges and Boone (2003) found that high ($233\mu\text{W}/\text{cm}^2$) UV-B intensity improved survival of Southern leopard frog (*Lithobates sphenoccephalus*) tadpoles compared to low UV-B levels ($27.4\mu\text{W}/\text{cm}^2$). Bridges and Boone (2003) attributed their outcome to the possibility of their filters eliminating a range of wavelengths critical for vital functions, such as vitamin D production in humans, but the specific function remains unknown. UV-B exposure results in darkened skin in other larval salamander species, but does not improve their UV-B resistance (Belden & Blaustein 2002). Taken together, these results suggest that the larvae receive some benefit from sub-ambient exposure to UV-B.

The immunological benefits of ambient UV-B exposure are equivocal. I found that the combined low UV-B treatments resulted in weaker immune function than the combined high UV-B treatments. This difference in immune function may be related to

vitamin D production. The importance of vitamin D to the immune system of animals has become increasingly appreciated (Priehl *et al.* 2013). Since exposure to UV-B radiation stimulates vitamin D production, animals in the UV_{cc} treatments may have been immunodeficient. Whereas immune function was not affected by the addition of herbicide in UV-B_{cc} conditions, the presence of herbicide reduced immune function in open canopy treatments. This reduced function potentially is due to stress-induced (*i.e.* corticosterone-induced) suppression (Glennemeier & Denver 2002).

Perhaps high UV-B levels are more damaging to potential pathogens (Paul *et al.* 2012) than to larval amphibians. This, coupled with the possibility of a direct immunological benefit from ambient UV-B, may confer improved fitness in open canopy ponds. However, previous studies have found increased susceptibility to pathogens when aquatic organisms were exposed to ambient UV-B or glyphosate-based herbicide (Kreutz *et al.* 2010; Kiesecker 2011). Often water is exposed to UV-B radiation as a sterilization method. By reducing the amount of UV-B radiation in the UV_{cc} treatments, I may be creating an environment in which pathogens could thrive. This possibility could potentially account for differences in survival across UV-B treatments. In nature, the combination of reduced immune function and increased susceptibility to pathogens could decrease population densities. Indeed, more work needs to be done to address the interactions between habitat alteration, UV-B levels, and pathogen exposure.

Body Condition and Metamorphosis

I anticipated that body condition would be reduced with the addition of herbicide under both UV treatments because of stress-induced growth reduction (Denver 2009). As

expected, body condition decreased with addition of herbicide in the UV-B_{oc} treatment. However, body condition increased with the addition of herbicide in the UV-B_{cc} treatment. Exposure to higher temperatures at the water's surface (due to zooplankton phototaxis), coupled with reduced competition (because of increased mortality) could have increased body condition in the presence of herbicide under UV-B_{cc} levels. Similarly, gold-striped salamander (*Chioglossa lusitanica*) embryos exposed to glyphosate-based herbicide (*i.e.* Roundup Plus®) were significantly longer at hatching than controls (Ortiz-Santaliestra *et al.* 2011), but the mechanism is unknown.

There was no difference in body condition between metamorphosed and larval salamanders. Timing of metamorphosis is a phenotypically plastic trait largely controlled by environmental conditions and typically, when salamanders metamorphose to escape a stressful environment (*e.g.* herbicide present), it is the largest (*i.e.* in the best condition) individuals that leave first (Whiteman *et al.* 2012). The salamanders in my study achieved the "optimum" size for metamorphosis for this species (Phillips *et al.* 2002), but the majority of individuals did not metamorphose. Additionally, there was a positive correlation ($R^2 = 0.92$) between the natural log number of metamorphs and total number of survivors in each clutch. However, Fisher's exact test only found significant differences between the highest and three lowest proportions of metamorphs (~45% versus 0-5%). This suggests that intrinsic (*i.e.* genetic) differences rather than treatment effects drove metamorphic timing and that the aquatic conditions may not have been particularly stressful to the salamanders.

Morphology Changes

Interestingly, the morphology of the UV_{cc}/H+ salamanders converged on that of the two UV_{oc} treatments. Contrastingly, the UV_{cc}/H- larvae had tails with a greater downward bend and shorter tail muscles than the other three treatments (Fig. 5). This phenotypic variation may be the result of an interaction between food availability and treatment. It is possible that lack of UV-B exposure or malnutrition could have simply resulted in tail deformity (Jung *et al.* 1978) because the UV_{cc}/H- larvae also had the lowest body condition (Table 3). The presence of glyphosate-based herbicide may counteract this stress-induced phenotype, preventing the deformity in UV_{cc}/H+ larvae. Alternatively, the observed downward, concave tail bend could have been induced to generate greater upward lift (Wilga & Lauder 2001), while conserving energy (Takagi, Tamura & Weihs 2013), to facilitate feeding on zooplankton higher in the water column. Under the closed canopy condition, the presence of glyphosate-based herbicide may have prevented the induction of this tail phenotype for obtaining food near the water's surface. This herbicide-induced phenotype may be beneficial for the horizontal or downward swimming needed under the UV_{oc} treatments (Wilga & Lauder 2001), since food was potentially more available near the bottom in UV_{oc} tanks.

Alternatively, the greater bend in tail morphology for UV_{cc}/H- animals may also be caused by metabolic bone disease (MBD). MBD is common in amphibians that experience low levels of calcium, phosphorus, or vitamin D and results in more fragile and deformed skeletons (Densmore & Green 2007). Among these potential deformities is a curved spine (i.e. scoliosis). Because animals in the UV_{cc}/H- treatment were generally smaller, had lower immune functions, and had a bent morphology, it is plausible that a

vitamin deficiency, caused by lack of UV-B radiation, had an influential role on the phenotype of these animals.

Recently, Relyea (2012) found that a glyphosate-based herbicide, Roundup Original MAX®, could induce a morphological response typically generated by predators in leopard frog (*Lithobates pipiens*) and wood frog (*Lithobates sylvaticus*) tadpoles. Presumably, such induced phenotypes should be maladaptive in the absence of predators. Alton, Wilson and Franklin (2010) found that sub-ambient UV-B levels (*i.e.* ~38 $\mu\text{W}/\text{cm}^2$) inhibited predator-induced morphology. My study suggests that glyphosate-based herbicide can result in morphological plasticity under UV-B_{cc} conditions. Under ambient UV-B levels, there was no difference in morphology between herbicide present and absent groups. The underlying mechanism of herbicide-induced morphology remains unclear. However, the present hypothesis is that interference with stress hormones involved with antipredator defenses causes this maladaptive plasticity (Glennemeier & Denver 2002; Relyea 2012). Delayed UV-mediated effects, free radical formation, or low photolyase activity may also interfere with proper morphological development, cause tissue damage, or prevent repair of UV-B damage (*e.g.* Blaustein & Belden 2003; Blaustein & Bancroft 2007) and lead to altered morphology.

Conclusions & Future Directions

The ecological implications of this study are somewhat hopeful when placed in the context of previous work. Amphibians have demonstrated the ability to locally adapt to UV-B and pesticide use (Marquis *et al.* 2009), to develop cross-tolerance to pesticides with a common mode of action (Hua *et al.* 2013a), and to hormetically respond to

sublethal exposure to pesticides (Hua *et al.* 2013b). Furthermore, my study has demonstrated that UV-B exposure can mitigate population losses due to herbicide exposure and that slightly sub-ambient UV -B levels may confer immunological benefits as well. In addition, larval caudates may be less at risk than larval anurans because they tend to have a higher tolerance to pesticide exposure (Relyea & Jones 2009). Finally, field studies with natural ponds have found that glyphosate-based herbicide alone results in low mortality and that dissolved organic carbon reduces the penetration of UV below the water's surface (*e.g.* Crump *et al.* 1999a; b; Edge *et al.* 2012). Therefore, although these stressors have the potential to considerably affect amphibians, their danger may be overstated in some cases.

Amphibians in open ponds, particularly near agricultural fields, are more likely to be exposed to glyphosate-based herbicides than those in forested ponds. Agricultural use of the herbicide is ~23-fold higher than non-agricultural (*e.g.* silvicultural) use (Grube *et al.* 2011) and agricultural activity occurs in large open areas with plenty of sunlight. Deforestation may decrease the amount of available forested ponds and drive amphibians to open ponds that are more susceptible to herbicide exposure (*e.g.* via drift or run-off). The results of the present study suggest a trade-off between experiencing a high risk of herbicide-related mortality, but a low risk of encountering herbicide in a closed canopy pond (UV_{cc}), and having a low risk of mortality, but a high risk of herbicide exposure in an open canopy pond (UV_{oc}). The UV-B levels used in this study were within naturally occurring ranges for these alternative habitat types and therefore are representative of the possible fitness consequences herbicide exposure can have on larval salamanders. When

considering the anthropogenic causes of amphibian population declines, interactions among stressors need to be addressed, because the outcomes may be unexpected.

This work has set-up several avenues of potentially fruitful exploration. A logical next step would be to obtain more immunological and serological information from the salamanders. Assessing direct amounts of immune cells in the blood and levels of vitamins in the animals would inform the mechanism underlying differences in immune function and potentially morphology. Additionally, analysis of the nutritional components of food sources and determining the effects of different sources would inform whether these animals were experiencing any sort of deficiencies. Furthermore, it would be beneficial to survey the pathogen communities present in the different treatments to determine the effect UV-B regime has on pathogen suppression. Finally, field observations of zooplankton and salamander location more frequently during the study period would be useful to understand the plausibility of the trophic interaction scenario.

References

- Alton, L.A., Wilson, R.S. & Franklin, C.E. (2010) Risk of predation enhances the lethal effects of UV-B in amphibians. *Global Change Biology*, **16**, 538–545.
- Ardia, D. & Clotfelter, E. (2006) The novel application of an immunological technique reveals the immunosuppressive effect of phytoestrogens in *Betta splendens*. *Journal of Fish Biology*, **68**, 144–149.
- Aruoma, D.O.I. (1998) Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists' Society*, **75**, 199–212.
- Auld, J.R., Agrawal, A.A. & Relyea, R.A. (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 503–511.

- Bancroft, B.A., Baker, N.J., Searle, C.L., Garcia, T.S. & Blaustein, A.R. (2008) Larval amphibians seek warm temperatures and do not avoid harmful UVB radiation. *Behavioral Ecology*, **19**, 879–886.
- Barolo, D. (1993) Reregistration eligibility decision for glyphosate. U.S. EPA 738-R-93-014.
- Bates, D. & Maechler, M. (2009) Package “lme4.” URL <http://lme4.r-forge.r-project.org/>.
- Baylis, A.D. (2000) Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science*, **56**, 299–308.
- Belden, L.K. & Blaustein, A.R. (2002) UV-B induced skin darkening in larval salamanders does not prevent sublethal effects of exposure on growth. *Copeia*, **3**, 748–754.
- Bernal, M.H., Solomon, K.R. & Carrasquilla, G. (2009) Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. *Journal of Toxicology and Environmental Health, Part A*, **72**, 966–973.
- Binns, R.M., Licence, S.T. & Peter Wooding, F.B. (1990) Phytohemagglutinin induces major short-term protease-sensitive lymphocyte traffic involving high endothelium venule-like blood vessels in acute delayed-type hypersensitivity-like reactions in skin and other tissues. *European Journal of Immunology*, **20**, 1067–1071.
- Blaustein, A.R. & Bancroft, B.A. (2007) Amphibian population declines: evolutionary considerations. *BioScience*, **57**, 437–444.
- Blaustein, A.R. & Belden, L.K. (2003) Amphibian defenses against ultraviolet-B radiation. *Evolution & Development*, **5**, 89–97.
- Blaustein, A.R., Han, B.A., Relyea, R.A., Johnson, P.T.J., Buck, J.C., Gervasi, S.S. & Kats, L.B. (2011) The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, **1223**, 108–119.
- Blaustein, A.R., Hoffman, P.D., Hokit, D.G., Kiesecker, J.M., Walls, S.C. & Hays, J.B. (1994) UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? *Proceedings of the National Academy of Sciences*, **91**, 1791–1795.
- Blaustein, A.R., Romansic, J.M., Kiesecker, J.M. & Hatch, A.C. (2003) Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions*, **9**, 123–140.

- Boughton, R.K., Joop, G. & Armitage, S.A.O. (2011) Outdoor immunology: methodological considerations for ecologists. *Functional Ecology*, **25**, 81–100.
- Brausch, J.M., Beall, B. & Smith, P.N. (2007) Acute and sub-lethal toxicity of three POEA surfactant formulations to *Daphnia magna*. *Bulletin of Environmental Contamination and Toxicology*, **78**, 510–514.
- Bridges, C.M. & Boone, M.D. (2003) The interactive effects of UV-B and insecticide exposure on tadpole survival, growth and development. *Biological Conservation*, **113**, 49–54.
- Broomhall, S.D., Osborne, W.S. & Cunningham, R.B. (2000) Comparative effects of ambient ultraviolet-B radiation on two sympatric species of Australian frogs. *Conservation Biology*, **14**, 420–427.
- Calsbeek, R., Bonneaud, C. & Smith, T.B. (2008) Differential fitness effects of immunocompetence and neighbourhood density in alternative female lizard morphs. *Journal of Animal Ecology*, **77**, 103–109.
- Chen, C.Y., Hathaway, K.M. & Folt, C.L. (2004) Multiple stress effects of Vision® herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. *Environmental Toxicology and Chemistry*, **23**, 823–831.
- Cockell, C.S. & Blaustein, A.R. (2000) “Ultraviolet spring” and the ecological consequences of catastrophic impacts. *Ecology Letters*, **3**, 77–81.
- Collyer, M.L. & Adams, D.C. (2007) Analysis of two-state multivariate phenotypic change in ecological studies. *Ecology*, **88**, 683–692.
- Cordero, R.R., Seckmeyer, G., Damiani, A., Riechelmann, S., Rayas, J., Labbe, F. & Laroze, D. (2013) The world’s highest levels of surface UV. *Photochemical & Photobiological Sciences*, Epub ahead of print.
- Croteau, M.C., Davidson, M.A., Lean, D.R.S. & Trudeau, V.L. (2008) Global increases in ultraviolet B radiation: potential impacts on amphibian development and metamorphosis. *Physiological and biochemical zoology: PBZ*, **81**, 743–761.
- Crump, D., Berrill, M., Coulson, D., Lean, D., McGillivray, L. & Smith, A. (1999a) Sensitivity of amphibian embryos, tadpoles, and larvae to enhanced UV-B radiation in natural pond conditions. *Canadian Journal of Zoology*, **77**, 1956–1966.
- Crump, D., Lean, D., Berrill, M., Coulson, D. & Toy, L. (1999b) Spectral irradiance in pond water: influence of water chemistry. *Photochemistry and Photobiology*, **70**, 893–901.
- Densmore, C.L. & Green, D.E. (2007) Diseases of amphibians. *ILAR Journal*, **48**, 235–254.

- Denver, R.J. (2009) Stress hormones mediate environment-genotype interactions during amphibian development. *General and Comparative Endocrinology*, **164**, 20–31.
- Edge, C.B., Gahl, M.K., Thompson, D.G. & Houlahan, J.E. (2013) Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*. *Science of The Total Environment*, **444**, 145–152.
- Edge, C.B., Thompson, D.G., Hao, C. & Houlahan, J.E. (2012) A silviculture application of the glyphosate-based herbicide VisionMAX to wetlands has limited direct effects on amphibian larvae. *Environmental Toxicology and Chemistry*, **31**, 2375–2383.
- Edginton, A.N., Sheridan, P.M., Stephenson, G.R., Thompson, D.G. & Boermans, H.J. (2004) Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry*, **23**, 815–822.
- Edwards, W.M., Triplett, G.B. & Kramer, R.M. (1980) A watershed study of glyphosate transport in runoff. *Journal of Environment Quality*, **9**, 661.
- Flint, S.D. & Caldwell, M.M. (1998) Solar UV-B and visible radiation in tropical forest gaps: measurements partitioning direct and diffuse radiation. *Global Change Biology*, **4**, 863–870.
- Fordyce, J.A. (2006) The evolutionary consequences of ecological interactions mediated through phenotypic plasticity. *Journal of Experimental Biology*, **209**, 2377–2383.
- Gallant, A.L., Klaver, R.W., Casper, G.S. & Lannoo, M.J. (2007) Global rates of habitat loss and implications for amphibian conservation. *Copeia*, **2007**, 967–979.
- Gervasi, S.S. & Foufopoulos, J. (2008) Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. *Functional Ecology*, **22**, 100–108.
- Glennemeier, K.A. & Denver, R.J. (2002) Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. *General and Comparative Endocrinology*, **127**, 16–25.
- Govindarajulu, P.P. (2008) Literature Review of Impacts of Glyphosate Herbicide on Amphibians: What Risks Can Silvicultural Use of This Herbicide Pose for Amphibians in B.C.? B.C. Ministry of Environment, Victoria, BC.
- Grube, A., Donaldson, D., Kiely, T. & Wu, L. (2011) Pesticides industry sales and usage: 2006 and 2007 market estimates. United States Environmental Protection Agency, Washington D.C., USA.

- Herman, J.R., Bhartia, P.K., Ziemke, J., Ahmad, Z. & Larko, D. (1996) UV-B increases (1979–1992) from decreases in total ozone. *Geophysical Research Letters*, **23**, 2117–2120.
- Hollán, S. (1995) Free radicals in health and disease. *Haematologia*, **26**, 177–189.
- Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H. & Kuzmin, S.L. (2000) Quantitative evidence for global amphibian population declines. *Nature*, **404**, 752–755.
- Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K. & Veldhoen, N. (2004) Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry*, **23**, 1928–1938.
- Hua, J., Cothran, R., Stoler, A. & Relyea, R. (2013a) Cross-tolerance in amphibians: Wood frog mortality when exposed to three insecticides with a common mode of action. *Environmental Toxicology and Chemistry*, **32**, 932–936.
- Hua, J., Morehouse, N.I. & Relyea, R. (2013b) Pesticide tolerance in amphibians: induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. *Evolutionary Applications*, **6**, 1028–1040.
- Jones, D.K., Hammond, J.I. & Relyea, R.A. (2010) Roundup® and amphibians: The importance of concentration, application time, and stratification. *Environmental Toxicology and Chemistry*, **29**, 2016–2025.
- Jones, D.K., Hammond, J.I. & Relyea, R.A. (2011) Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. *Environmental Toxicology and Chemistry*, **30**, 446–454.
- Jung, R.T., Davie, M., Hunter, J.O. & Chalmers, T.M. (1978) Ultraviolet light: an effective treatment of osteomalacia in malabsorption. *British Medical Journal*, **1**, 1668–1669.
- Kerr, J.B. & McElroy, C.T. (1993) Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science*, **262**, 1032–1034.
- Kiesecker, J.M. (2011) Global stressors and the global decline of amphibians: tipping the stress immunocompetency axis. *Ecological Research*, **26**, 897–908.
- Kreutz, L.C., Gil Barcellos, L.J., Marteninghe, A., Davi dos Santos, E. & Zanatta, R. (2010) Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. *Fish & Shellfish Immunology*, **29**, 694–697.
- Licht, L.E. & Grant, K.P. (1997) The effects of ultraviolet radiation on the biology of amphibians. *American Zoologist*, **37**, 137–145.

- Lund-Høie, K. & Friestad, H.O. (1986) Photodegradation of the herbicide glyphosate in water. *Bulletin of Environmental Contamination and Toxicology*, **36**, 723–729.
- Mann, R.M. & Bidwell, J.R. (2001) The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs. *Environmental Pollution*, **114**, 195–205.
- Marquis, O., Miaud, C., Ficetola, G.F., Bocher, A., Mouchet, F., Guittonneau, S. & Devaux, A. (2009) Variation in genotoxic stress tolerance among frog populations exposed to UV and pollutant gradients. *Aquatic Toxicology*, **95**, 152–161.
- Martin, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C. & Wikelski, M. (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoeological technique. *Functional Ecology*, **20**, 290–299.
- Middleton, E.M., Herman, J.R., Celarier, E.A., Wilkinson, J.W., Carey, C. & Rusin, R.J. (2001) Evaluating ultraviolet radiation exposure with satellite data at sites of amphibian declines in Central and South America. *Conservation Biology*, **15**, 914–929.
- Ortiz-Santaliestra, M.E., Fernández-Benítez, M.J., Lizana, M. & Marco, A. (2011) Influence of a combination of agricultural chemicals on embryos of the endangered gold-striped salamander (*Chioglossa lusitanica*). *Archives of Environmental Contamination and Toxicology*, **60**, 672–680.
- Palen, W.J., Schindler, D.E., Adams, M.J., Pearl, C.A., Bury, R.B. & Diamond, S.A. (2002) Optical characteristics of natural waters protect amphibians from UV-B in the U.S. pacific northwest. *Ecology*, **83**, 2951–2957.
- Paul, N.D., Moore, J.P., McPherson, M., Lambourne, C., Croft, P., Heaton, J.C. & Wargent, J.J. (2012) Ecological responses to UV radiation: interactions between the biological effects of UV on plants and on associated organisms. *Physiologia Plantarum*, **145**, 565–581.
- Petranka, J.W. (1998) *Salamanders of the United States and Canada*. Washington : Smithsonian Institution Press.
- Phillips, C.A., Johnson, J.R., Dreslik, M.J. & Petzing, J.E. (2002) Effects of hydroperiod on recruitment of mole salamanders (Genus *Ambystoma*) at a temporary pond in Vermilion County, Illinois. *Transactions of the Illinois State Academy of Science*, **95**, 131–139.
- Priehl, B., Treiber, G., Pieber, T.R. & Amrein, K. (2013) Vitamin D and immune function. *Nutrients*, **5**, 2502–2521.
- Puglis, H.J. & Boone, M.D. (2011) Effects of technical-grade active ingredient vs. commercial formulation of seven pesticides in the presence or absence of UV

- radiation on survival of green frog tadpoles. *Archives of Environmental Contamination and Toxicology*, **60**, 145–155.
- Relyea, R.A. (2002) Costs of phenotypic plasticity. *The American Naturalist*, **159**, 272–282.
- Relyea, R.A. (2004) Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. *Ecology*, **85**, 172–179.
- Relyea, R.A. (2005a) The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. *Archives of Environmental Contamination and Toxicology*, **48**, 351–357.
- Relyea, R.A. (2005b) The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications*, **15**, 618–627.
- Relyea, R.A. (2005c) The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecological Applications*, **15**, 1118–1124.
- Relyea, R.A. (2012) New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecological Applications*, **22**, 634–647.
- Relyea, R.A. & Jones, D.K. (2009) The toxicity of Roundup Original Max® to 13 species of larval amphibians. *Environmental Toxicology and Chemistry*, **28**, 2004–2008.
- Relyea, R.A., Schoeppner, N.M. & Hoverman, J.T. (2005) Pesticides and amphibians: the importance of community context. *Ecological Applications*, **15**, 1125–1134.
- Rohlf, F.J. (2001) TPSSDIG, Version 1.31. Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook, New York, USA.
- Rohlf, F.J. (2003) TPSRELW, Version 1.29. Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook, New York, USA.
- Rohlf, F.J. (2013) TPSUTIL, Version 1.58. Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook, New York, USA.
- Searle, C.L., Belden, L.K., Bancroft, B.A., Han, B.A., Biga, L.M. & Blaustein, A.R. (2010) Experimental examination of the effects of ultraviolet-B radiation in combination with other stressors on frog larvae. *Oecologia*, **162**, 237–245.
- Seiter, S. (2011) Predator presence suppresses immune function in a larval amphibian. *Evolutionary Ecology Research*, **13**, 283–293.
- Smits, J.E., Bortolotti, G.R. & Tella, J.L. (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional Ecology*, **13**, 567–572.

- Sparling, D.W., Fellers, G.M. & McConnell, L.L. (2001) Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry*, **20**, 1591–1595.
- Storz, U.C. & Paul, R.J. (1998) Phototaxis in water fleas (*Daphnia magna*) is differently influenced by visible and UV light. *Journal of Comparative Physiology A*, **183**, 709–717.
- Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L. & Waller, R.W. (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.
- Takagi, T., Tamura, Y. & Weihs, D. (2013) Hydrodynamics and energy-saving swimming techniques of Pacific bluefin tuna. *Journal of Theoretical Biology*, **336**, 158–172.
- Tevini, M. (1993) UV-B Radiation and Ozone Depletion: Effects on Humans, Animals, Plants, Microorganisms and Materials. Lewis Publishers, Boca Raton, FL.
- Tietge, J.E., Diamond, S.A., Ankley, G.T., DeFoe, D.L., Holcombe, G.W., Jensen, K.M., Degitz, S.J., Elonen, G.E. & Hammer, E. (2001) Ambient solar UV radiation causes mortality in larvae of three species of *Rana* under controlled exposure conditions. *Photochemistry and Photobiology*, **74**, 261–268.
- Tsui, M.T.K. & Chu, L.M. (2003) Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere*, **52**, 1189–1197.
- Urban, M.C. (2008) Salamander evolution across a latitudinal cline in gape-limited predation risk. *Oikos*, **117**, 1037–1049.
- Urban, M.C. (2010) Microgeographic adaptations of spotted salamander morphological defenses in response to a predaceous salamander and beetle. *Oikos*, **119**, 646–658.
- Via, S. & Lande, R. (1985) Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, **39**, 505.
- Wang, N., Besser, J.M., Buckler, D.R., Honegger, J.L., Ingersoll, C.G., Johnson, B.T., Kurtzweil, M.L., MacGregor, J. & McKee, M.J. (2005) Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms. *Chemosphere*, **59**, 545–551.
- Whiteman, H.H., Wissinger, S.A., Denoël, M., Mecklin, C.J., Gerlanc, N.M. & Gutrich, J.J. (2012) Larval growth in polyphenic salamanders: making the best of a bad lot. *Oecologia*, **168**, 109–118.

- Whitman, D.W. & Agrawal, A.A. (2009) What Is Phenotypic Plasticity and Why Is It Important? Phenotypic Plasticity of Insects: Mechanisms and Consequences (eds D.W. Whitman & T.N. Ananthakrishnan), pp. 1–63. Science Publishers.
- Wilga, C.D. & Lauder, G.V. (2001) Functional morphology of the pectoral fins in bamboo sharks, *Chiloscyllium plagiosum*: benthic vs. pelagic station-holding. *Journal of Morphology*, **249**, 195–209.
- Wojtaszek, B.F., Staznik, B., Chartrand, D.T., Stephenson, G.R. & Thompson, D.G. (2004) Effects of Vision herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. *Environmental Toxicology and Chemistry*, **23**, 832–842.
- Worrest, R.C. & Kimeldorf, D.J. (1976) Distortions in amphibian development induced by ultraviolet-B enhancement (290–315 Nm) of a simulated solar spectrum. *Photochemistry and Photobiology*, **24**, 377–382.
- Zaga, A., Little, E.E., Rabeni, C.F. & Ellersieck, M.R. (1998) Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. *Environmental Toxicology and Chemistry*, **17**, 2543–2553.

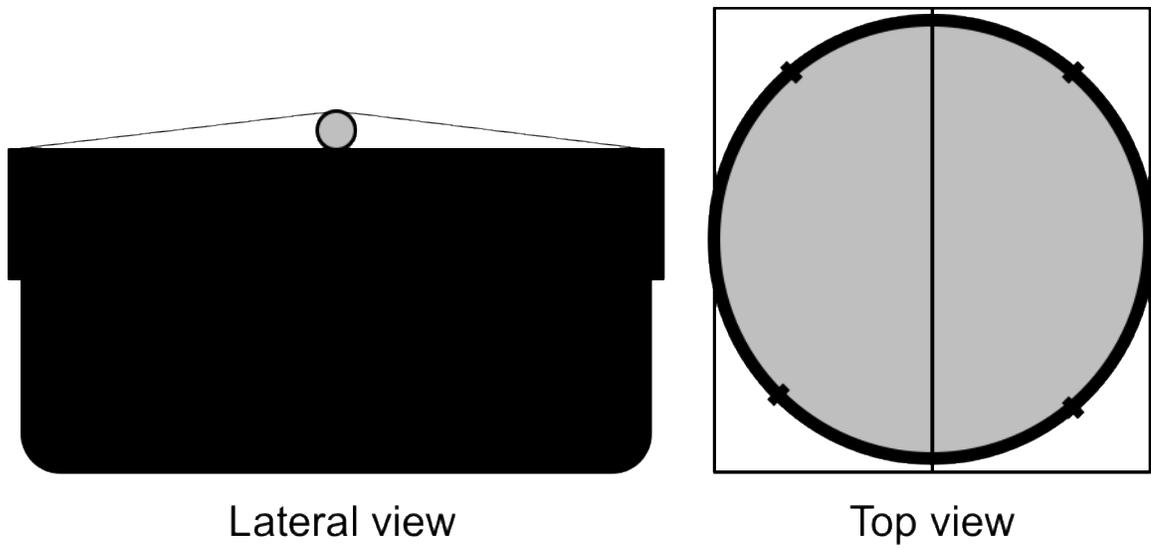
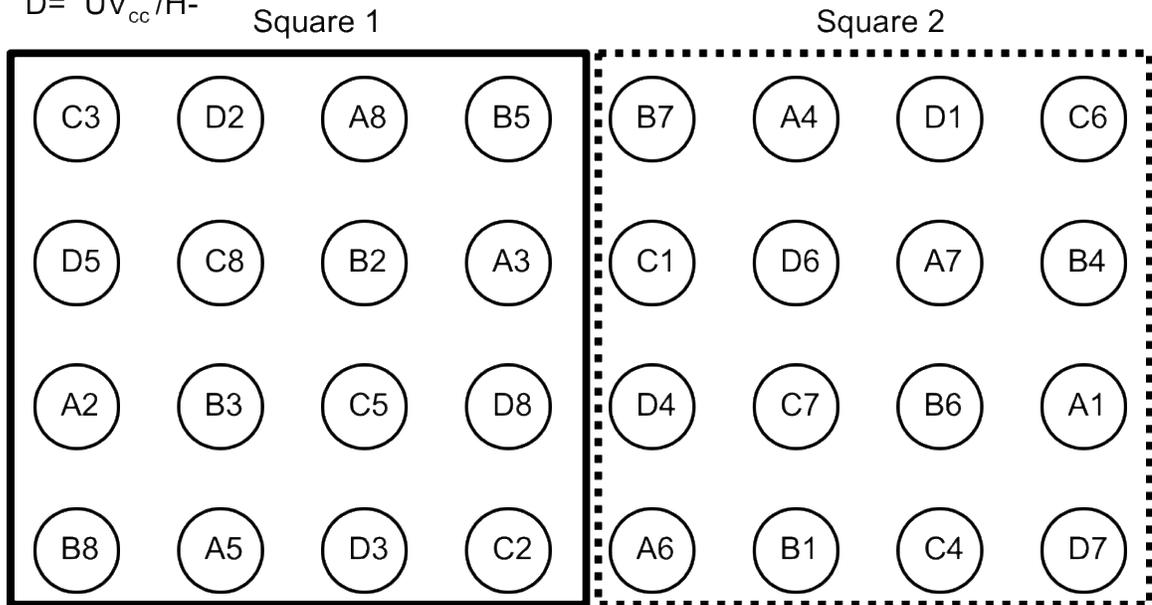


Fig. 1. Set up of film covered mesocosms. Two sheets of film were attached to a PVC pipe that spanned the diameter of the mesocosm and were glued and stapled to the rim of the tank. A mesh lid was then placed over this design to prevent colonization of predators (*e.g.*, dragonflies).

A = UV_{oc} /H+
 B = UV_{cc} /H+
 C = UV_{oc} /H-
 D = UV_{cc} /H-

Replicated Graeco-Latin Squares



Randomized Replicated Graeco-Latin Squares

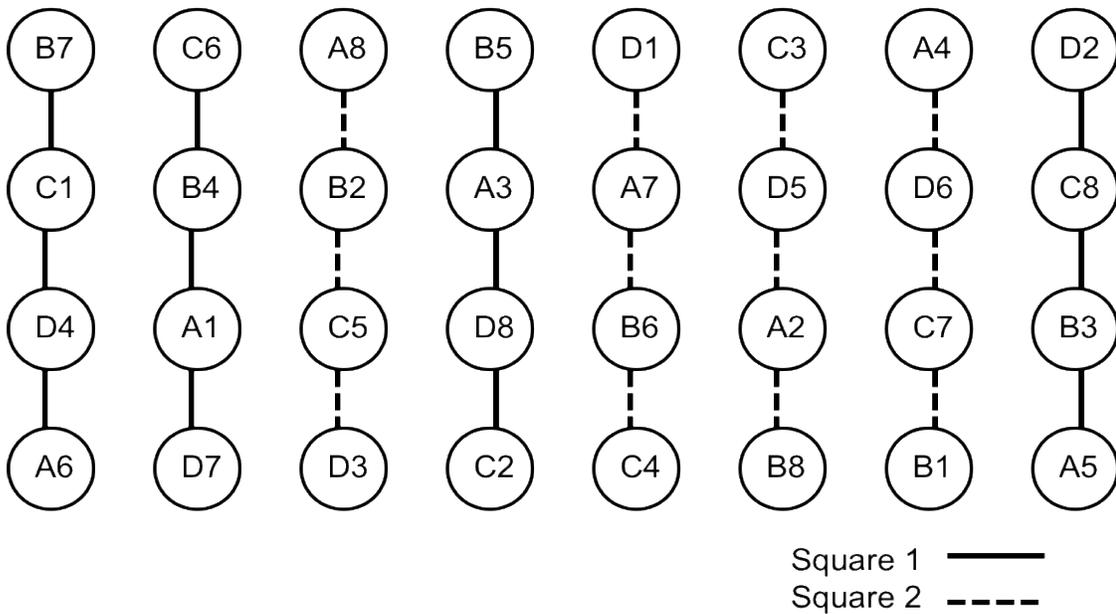


Fig. 2. The two-step experimental design and layout of mesocosms. First, two Graeco-Latin Squares were made using treatment and clutch. Both the starting treatment and clutch were randomly selected for each square. After both squares were designed, the order of each column was then randomized to remove potential bias in mesocosm location.

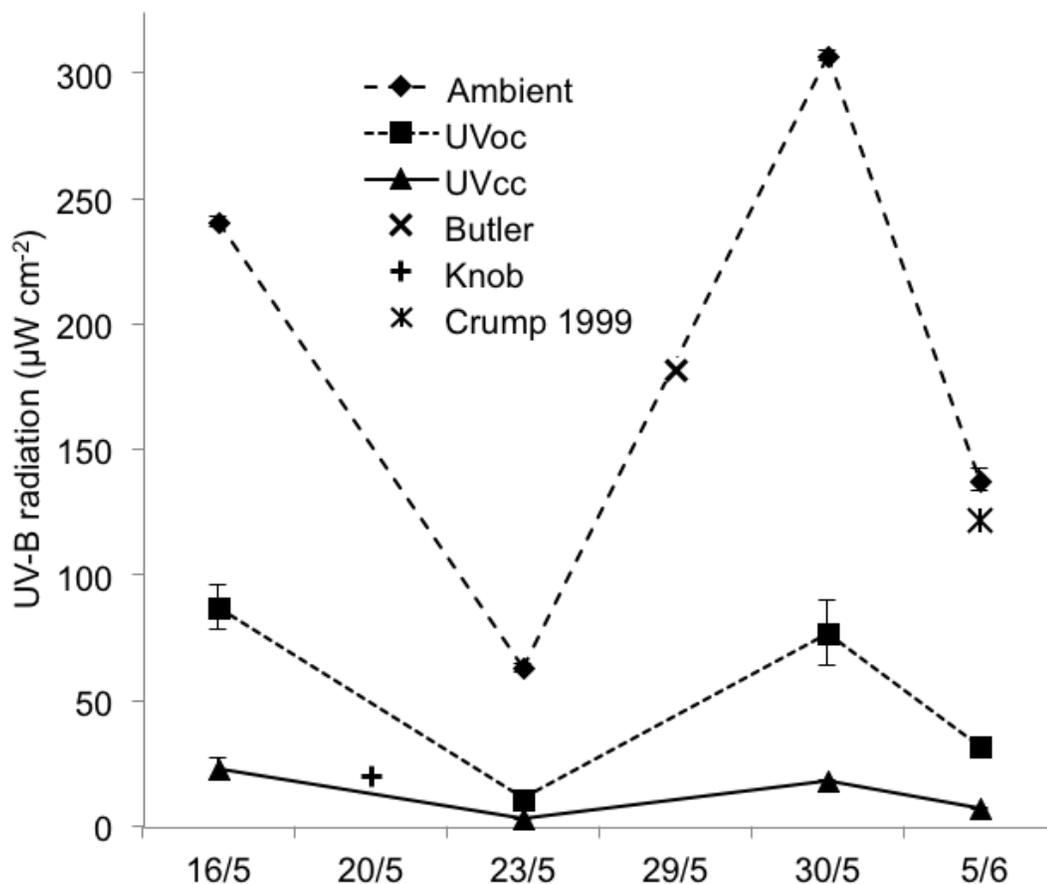


Fig. 3. UV-B radiation values (\pm s.e.m.) for the two UV-B treatment types, two natural ponds with different canopy characteristics, and a previous study with an open canopy pond. On 23 May, the sky was overcast and very cloudy such that little UV-B reached the tanks. Butler and Knob ponds were open and closed canopy ponds, respectively, that were used as natural comparisons for UV_{oc} and UV_{cc}, respectively. Measurements for this study were taken between 1100 and 1400 h at the water's surface, unless otherwise noted. From Crump *et al.* (1999a), "Experiments were conducted in an area of the marsh without emergent vegetation or tree cover, where differential shading could be avoided." My experimental treatments were somewhat conservative in their variation, but were within the range of UV-B levels encountered in nearby natural ponds.

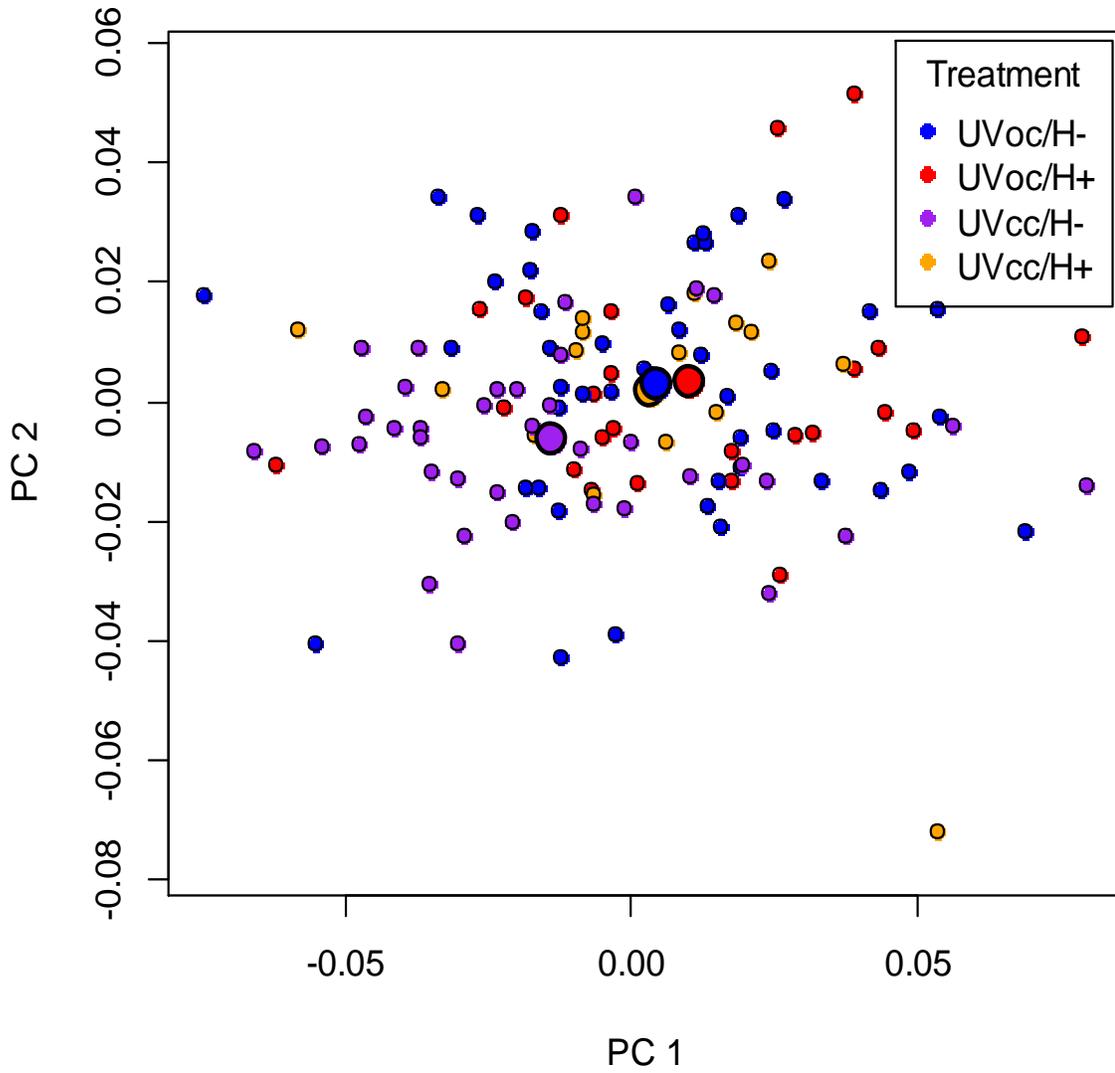


Fig. 4. Clutch-independent principal component ordination plots for tail morphology based on covariance matrices. PC1 and PC2 explain ~58% of the variation in eigenvalues. In addition, there was a 0.91 correlation between two dimensional distances and those of the full data space. The large dots denote the centroid of each treatment.



Fig. 5. Consensus tail morphologies for A) $UV_{oc}/H+$, $UV_{cc}/H+$, $UV_{oc}/H-$, and B) $UV_{cc}/H-$. The primary difference between these two morphologies is the "bend" in the tail. Additionally, the distance from landmarks 5 and 30 to landmark 4 (*i.e.*, tail muscle length) is shorter in B.

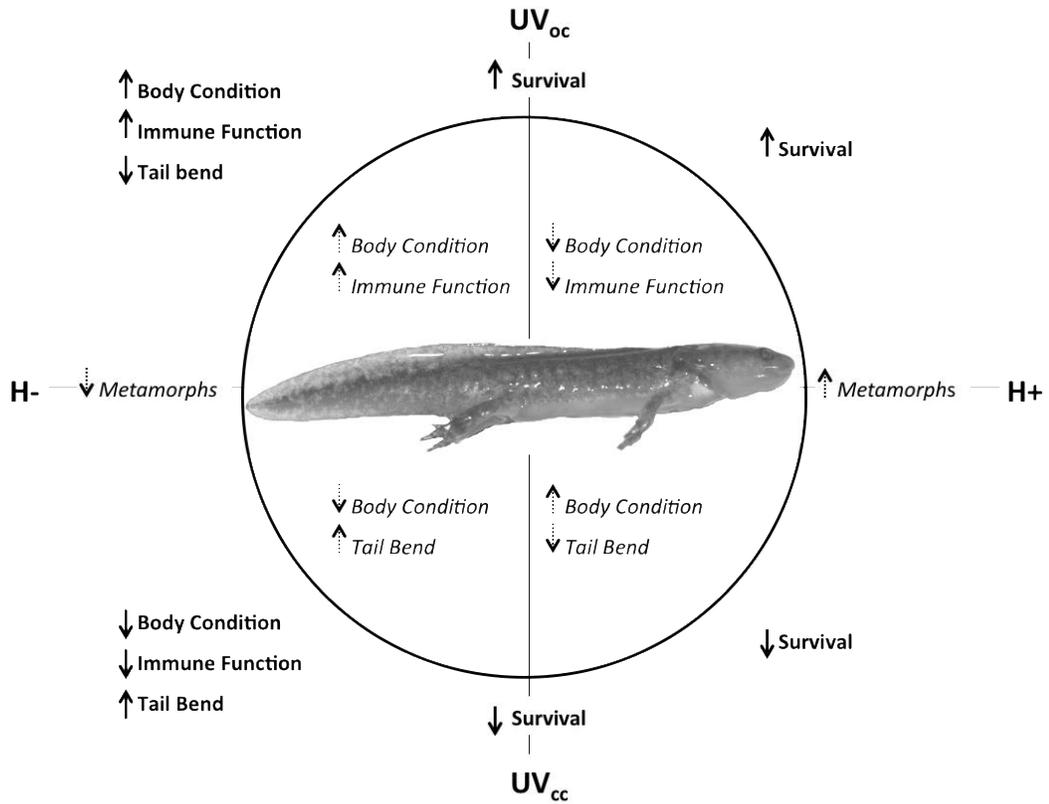


Fig. 6. Summary of major results found in this study. Italicized changes within the circle are due to the addition or removal of herbicide within a given UV-B regime. Bolded changes outside of the circle are due to alterations of UV-B level within each herbicide treatment. Changes on each axis result from changes in only that factor (*i.e.*, herbicide or UV level). Arrows represent the direction change from the other condition. Dotted arrows indicate changes between herbicide conditions and bold arrows indicate changes between UV-B conditions.

Table 1. Temperatures above and below the two film types. For a given measurement location, there were no differences in temperature across the treatments. Under both films, water surface temperature was significantly higher than ambient temperature above the film and water temperature at the bottom of the tank. Different letters indicate significant differences in mean value. Values presented as mean \pm s.e.m. and $\alpha = 0.05$.

	T_{UVoc} ($^{\circ}C$)	T_{UVcc} ($^{\circ}C$)
Ambient	27.68 ± 1.08^a	27.65 ± 1.26^a
Surface	30.52 ± 0.62^b	30.99 ± 0.62^b
Bottom	27.43 ± 0.49^a	27.64 ± 0.50^a

Table 2. Model comparison for survival, number of metamorphs, body condition (BC), and swelling after 48 h. Best-fit models are denoted with *** in the Δ AIC column and were used for analysis. In most cases, the interaction of glyphosate and UV-B was commensurate with Treatment. All models contained clutch and block as random effects.

A. Model	d.f.	Survival				Metamorphs				
		Δ AIC	log Like	X ²	P	d.f.	Δ AIC	log Like	X ²	P
Null	3	26.60	-308.92	---	---	3	2.64	-134.77	---	---
Herbicide	4	28.40	-308.82	0.20	0.654	4	***	-132.45	4.64	0.031
UV-B	4	0.58	-294.91	27.83	1.20E-07	4	4.29	-134.59	0.00	0.552
Herb + UV-B	5	2.44	-294.84	0.13	0.716	5	1.19	-132.04	5.10	0.368
Herbi * UV-B	6	4.12	-294.62	0.32	0.570	6	1.47	-131.18	1.72	0.189
Treatment	6	***	-292.62	4.12	<2.2E-16	6	1.47	-131.18	0.00	<2.2E-16

B. Model	d.f.	BC				Swelling _{Δ48}				
		Δ AIC	log Like	X ²	P	d.f.	Δ AIC	log Like	X ²	P
Null	4	37.80	987.83	---	---	4	6.91	-63.722	---	---
Herbicide	5	35.10	990.17	4.68	0.0304	5	8.43	-63.484	0.48	0.489
UV-B	5	31.60	991.91	3.48	0.0043	5	4.21	-61.385	4.22	0.030
Herb + UV-B	6	30.60	993.42	3.01	0.0827	6	5.40	-60.98	0.81	0.367
Herb * UV-B	7	0.00	1009.72	32.60	1.13E-08	7	0.00	-57.28	7.40	0.006
Treatment	7	***	1009.72	0.00	<2.2E-16	7	***	-57.28	0.00	1.000

d.f. indicates degrees of freedom, Δ AIC is the change from lowest AIC value, log Like is log-likelihood

Table 3. Summary statistics for various fitness endpoints in this study. Different letters indicate significant differences according RRPP with $\alpha = 0.05$.

Treatment	<u>Salamander survival (%)</u>		<u>BC (g mm⁻¹)</u>		<u>Swelling₀(mm)</u>		<u>Swelling_{Δ48} (mm)</u>	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
UV _{oc} /H+	69.17 ^a	4.23	0.0435 ^a	0.001	3.76 ^a	0.07	0.590 ^a	0.04
UV _{oc} /H-	66.67 ^a	4.32	0.0455 ^b	0.001	3.71 ^a	0.06	0.860 ^b	0.03
UV _{cc} /H+	39.17 ^b	4.47	0.0463 ^{a,b}	0.002	3.91 ^a	0.10	0.670 ^{a,b}	0.06
UV _{cc} /H-	52.50 ^{a,b}	4.57	0.0390 ^c	0.001	3.49 ^b	0.08	0.440 ^a	0.03

BC= body condition, s.e.m. = standard error of the mean

Table 4. Jackknife (leave one out cross-validation) classification table for individuals based on tail morphology. Posterior probabilities were used to determine into which treatment an individual was classified. Forty-seven percent of individuals (bold values) were classified correctly.

	UVoc/H-	UVoc/H+	UVcc/H-	UVcc/H+
UVoc/H-	21	11	9	4
UVoc/H+	9	9	4	4
UVcc/H-	10	4	19	0
UVcc/H+	3	4	0	10

Table 5. Jackknife (leave one out cross-validation) classification table for individuals based on tail morphology. Posterior probabilities were used to determine into which treatment an individual was classified. Fifty-eight percent and 66% of individuals (bold values) were classified correctly.

	UVoc	UVcc
UVoc	45	26
UVcc	27	28
	H-	H+
H-	58	25
H+	18	25

Table 6. P values for pairwise distances among treatments for centroid location in the 58 dimensional morphospace. Bold values are significant with $\alpha = 0.05$.

Treatment	UV _{oc} /H+	UV _{oc} /H-	UV _{cc} /H+
UV _{oc} /H-	0.2685	---	---
UV _{cc} /H+	0.1883	0.0738	---
UV _{cc} /H-	0.0010	0.0021	0.0013

