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Authors: Krynak, Katherine L., Burke, David J., and Benard, Michael F.

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Rodeo™ Herbicide Negatively Affects Blanchard's Cricket Frogs (*Acris blanchardi*) Survival and Alters the Skin-Associated Bacterial Community

KATHERINE L. KRYNAK,[1,3] DAVID J. BURKE,[2] AND MICHAEL F. BENARD[1]

[1]*Department of Biology, Case Western Reserve University, Cleveland, Ohio USA*
[2]*Research Department, The Holden Arboretum, Kirtland, Ohio USA*

ABSTRACT.—**Disease-associated mortality is a leading cause of amphibian declines and extinctions worldwide. Understanding the influence of land-management practices, like herbicide use, on amphibian immune defense traits could guide changes to improve conservation outcomes. Amphibians are partially protected from pathogens by two skin-associated immune defense traits: bacterial communities inhabiting their skin, and antimicrobial peptides secreted by the skin. Utilizing the Blanchard's Cricket Frog (*Acris blanchardi*), a declining North American amphibian species, as our model, we manipulated Rodeo™ aquatic herbicide concentration and the life stage at which Rodeo exposure occurred. We assessed juvenile survival, time to metamorphosis, juvenile mass, and skin-associated immune defense traits. We found a 37% decrease in survival of larvae exposed to 2.5 mg a.e. L^{-1} (acid equivalent) compared to controls despite that this commercial herbicide formulation does not contain an added surfactant. Surviving larvae exposed to 2.5 mg a.e. L^{-1} Rodeo had structurally different larval skin bacterial communities compared to controls. Larval Rodeo exposure did not carry over to postmetamorphic traits (juvenile mass, juvenile skin bacterial community, juvenile natural peptide secretions). Rodeo treatments did not affect time to metamorphosis or juvenile survival. Rodeo concentration had marginally significant effects on juvenile mass and the juvenile skin bacterial community. This study suggests glyphosate-based herbicide use may indirectly contribute to disease-related amphibian declines by altering the skin bacterial community that can provide pathogen resistance. Improving our knowledge of the influence of herbicide use on amphibians across life stages provides an opportunity for changes to application strategies to protect amphibian health or at minimum, lessen negative effects of the practice.**

Disease is a leading cause of amphibian declines (Daszak et al., 2003), so understanding how land management practices alter traits central to pathogen resistance is crucial for conservation efforts. Amphibians are protected by two innate immune defense traits: bacterial communities that inhabit their skin and the antimicrobial peptides found within the natural peptide secretions produced by the skin (Harris et al., 2006; Rollins-Smith et al., 2011). Changes to the environment may alter these traits, potentially decreasing disease resistance (Krynak et al., 2015, 2016). Effects of glyphosate-based herbicide use on amphibians are of particular interest because of agricultural and land-management dependence on these chemicals, and the known negative effects of these chemicals on amphibian survival and fitness correlates including growth and development (Howe et al., 2004; Relyea, 2005; Earl and Whiteman, 2015). Even when herbicides do not directly alter amphibian survival, growth, and development, they may still alter amphibian immune defense traits, perhaps leading to decreased fitness and increased risk of local population decline.

Herbicide exposure may depress or stimulate immune function, increasing or decreasing resistance to disease. For example, Davidson et al. (2007) demonstrated that pesticide exposure can depress skin peptide defenses of Foothill Yellow-legged Frogs (*Rana boylii*), which may increase risk of mortality associated with *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen that has caused global amphibian declines; whereas sublethal herbicide concentrations have been shown to decrease Bd-associated mortality in Wood Frogs (*Lithobates sylvaticus*) and Gray Treefrogs (*Hyla versicolor*) (Gahl et al., 2011; Hanlon and Parris, 2014). Herbicide exposure may also alter the amphibian skin bacterial community, in turn affecting protection against Bd or other pathogens.

Like many amphibians, Blanchard's Cricket Frogs (*Acris blanchardi*) have been in precipitous decline in portions of their range over the past several decades (Gray and Brown, 2005; Lehtinen and Skinner, 2006). Anthropogenic environmental factors, including habitat loss, fragmentation, acidification, and chemical contamination, all are hypothesized drivers of this decline (Russell et al., 2002; Lehtinen and Skinner, 2006). Disease outbreaks, including those caused by Bd, have also been suspected contributors to *A. blanchardi* declines (Steiner and Lehtinen, 2008; Gray et al., 2009). Here, we explore the potential role of a commercially available glyphosate-based herbicide (Rodeo™; Dow AgroSciences, LLC, Indianapolis, Indiana, USA) in contributing to *A. blanchardi* decline. Rodeo is commonly used to manage nuisance aquatic plants (e.g., *Typha* spp., *Phragmites* spp.) common in the permanent ponds that *A. blanchardi* inhabit, and therefore, is a reasonable candidate that may be negatively affecting Blanchard's Cricket Frog populations.

Determining the effects of glyphosate-based herbicides on fitness is complicated by the *A. blanchardi* biphasic life cycle; exposure might differentially affect larval and postmetamorphic traits (Edginton et al., 2004; Distel and Boone, 2010). Herbicide exposure alters amphibian hatching success (Bishop et al., 2010), developmental rates (Navarro-Martin et al., 2014), and postmetamorphic mass (Boone and James, 2003), but there is limited evidence on whether herbicide exposure may alter amphibian immune defenses (Davidson et al., 2007; Schadich et al., 2009; Paetow et al., 2012; Rohr et al., 2014), and no studies of which we are aware have examined whether exposure effects carry over across life stages.

Rodeo is a glyphosate-based product approved to control emergent aquatic vegetation because it lacks the surfactant polyoxyethyleneamine (POEA; Dow Agrosciences, 2015) and is considered relatively nontoxic, based on acute exposure studies

[3]Corresponding Author. Present address: Department of Biological and Allied Health Sciences, Ohio Northern University, Ada, Ohio, USA; E-mail: k-krynak@onu.edu
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TABLE 1. Rodeo™ treatment assignments (number of replicates indicated; three animals per replicate). Treatments originally balanced (five replicates per Rodeo concentration/exposure stage combination); however, due to high larval mortality following Rodeo larval treatment, assignments were adjusted to improve ability to assess sublethal effects on low and medium Rodeo concentrations, and the effects of Rodeo exposure timing.

	Rodeo concentrations			
Exposure stage	Control, 0.0 mg a.e. L^{-1} (0.0 mg a.e. L^{-1})	Low, 0.75 mg a.e. L^{-1} (1.01 mg a.e. L^{-1})	Medium, 1.5 mg a.e. L^{-1} (2.02 mg a.e. L^{-1})	High, 2.5 mg a.e. L^{-1} (3.38 mg a.e. L^{-1})
Control (not exposed)	10	–	–	–
Larvae	–	5	5	10
Juvenile	–	5	5	0
Larvae and juvenile	–	5	5	0

that indicate a concentration of >100 mg/L is required to elicit mortality in 50% of the "most sensitive species" used in the studies (LC50/EC50/EE50/LL50; Rode Material Safety Data Sheet, Dow Agrosciences, 2015). Although amphibians often are considered sensitive to environmental pollutants based on their highly permeable skin (Quaranta et al. 2009), amphibians were not included in studies used to assess the safety of Rodeo in aquatic habitats (Rodeo Material Safety Data Sheet; Dow Agrosciences, 2015).

We hypothesized that Rodeo exposure alters *A. blanchardi* traits that are expected to be correlated with amphibian fitness. We assessed the influence of Rodeo exposure on larval and juvenile survival, larval duration, juvenile mass, larval and juvenile skin-associated bacterial communities, the production of natural peptide secretions, and the bioactivity of these secretions against Bd in vitro. We predicted that our environmentally relevant concentrations of Rodeo 1) would alter time to metamorphosis, juvenile mass, and the skin-associated immune defense traits while having no effect on survival; and 2) effects of early life stage (larval) Rodeo exposure would differ from the effects of postmetamorphic (juvenile) Rodeo exposure.

MATERIALS AND METHODS

We obtained larvae from 12 *A. blanchardi* pairs collected from a single pond in Wood County, Ohio, USA. We collected adult males and females and haphazardly paired frogs in 1-gal buckets containing pond water and plastic aquarium plants. Each pair produced 20–100 eggs. Larvae hatched 15–22 June 2013. We randomly assigned larvae to treatments on 27 June 2013. We used four exposure concentrations (control: no Rodeo; and low, medium, and high Rodeo; see details below) and three exposure stages (larval exposure, postmetamorphic juvenile exposure, or exposed as both larvae and juveniles) for a total of 10 treatments. We established five replicates of three larvae each per treatment (i.e., the experimental unit is the replicate; Table 1), but replicate number was reduced by high mortality following high Rodeo larval exposure. To account for this and maximize our ability to detect carryover effects of high Rodeo exposure (Table 1), those replicates originally assigned as high Rodeo™ juvenile became only control replicates, and those replicates originally assigned as high Rodeo larval + juvenile became high Rodeo larval only (i.e., not exposed as juveniles, only exposed as larvae).

We conducted the experiment in an indoor animal facility at Case Western Reserve University maintained at 25.5–27.7°C with a 12 h : 12 h light : dark cycle. Each replicate (three larvae) was a 15-L Sterilite™ (Massillon, Ohio, USA) tank filled with 10 L of dechlorinated water with plastic aquarium plants provided for cover (50 tanks in total). We conducted 50% water/treatment solution changes every other day for the duration of the larval period via static renewal (Relyea, 2004). We fed larvae ad libitum TetraMin™ (Blacksburg, Virginia, USA) sinking tropical tablets daily (0.08 g per tank) and siphoned all uneaten food and solid waste daily. Upon metamorphosis (stage 42; Gosner, 1960), after swabbing for microbial communities (detailed below), we moved these juveniles to ventilated 1-L plastic cups containing 100 mL of dechlorinated water/treatment solution, and plastic aquarium plants. We raised juveniles from the same replicate together, so juvenile group size was 1–3, dependent on larval survival. We performed 100% water changes every other day for juveniles, and fed them ad libitum daily with *Drosophila melanogaster* dusted with RepCal™ (Los Gatos, California, USA) vitamin supplement.

Rodeo treatments (Table 1) included four exposure concentrations (based on milligrams per liter of the acid equivalent, glyphosate) reflecting glyphosate concentrations documented in nature (Feng et al., 1990; Thompson et al., 2004; Relyea, 2005) and below the maximum concentrations expected when spraying emergent aquatic vegetation (>3.7 mg a.i. L^{-1}; Giesy et al., 2000). We report glyphosate concentration as both acid equivalent (a.e.) and active ingredient (a.i.) for comparison with extant literature: control, 0.0 mg a.e. L^{-1} (0.0 mg a.i. L^{-1}); low, 0.75 mg a.e. L^{-1} (1.01 mg a.i. L^{-1}); medium: 1.5 mg a.e. L^{-1} (2.02 mg a.i. L^{-1}); and high, 2.5 mg a.e. L^{-1} (3.38 mg a.i. L^{-1}). We conducted exposures for 12 d, a conservative approach, because glyphosate has a half-life of 12–70 d, depending on the microbial characteristics of the habitat (U.S. Environmental Protection Agency, 1992; Zaranyika and Nyandoro, 1993). We began exposures 1) 6 d after larvae were randomly assigned to replicates (larval; larvae 11–18 d old), 2) 10 d after the final larvae in a replicate reached metamorphosis (juvenile), or 3) during both developmental stages (Fig. 1). Control animals were never exposed. Because of logistical constraints, experiment end-day varied by up to 6 d.

To effect the Rodeo manipulation, we added Rodeo commercially formulated product (53.8% glyphosate, confirmed by Mississippi State Chemical Laboratory) to appropriate tanks on Day 1, bringing concentration to 50% of treatment concentration (8 μL, 16 μL, or 26 μL of Rodeo™ formulated product for low, medium, and high exposures, respectively); we mixed all tanks, including controls, thoroughly. On Day 2, we repeated this process, bringing Rodeo concentrations to prescribed treatment levels: control, low, medium, and high. Beginning on Day 4, we conducted 50% (5 L) water changes via static renewal every other day (Relyea, 2004). On Day 4 (5 July 2013) a mistake was made during the water change that resulted in Rodeo concentrations being elevated temporarily (low, 1.125 mg a.e. L^{-1}; medium; 2.25 mg a.e. L^{-1}, high, 3.75 mg a.e. L^{-1}); this error was caught the next day and remedied with water changes that brought concentrations to the intended levels. This elevated

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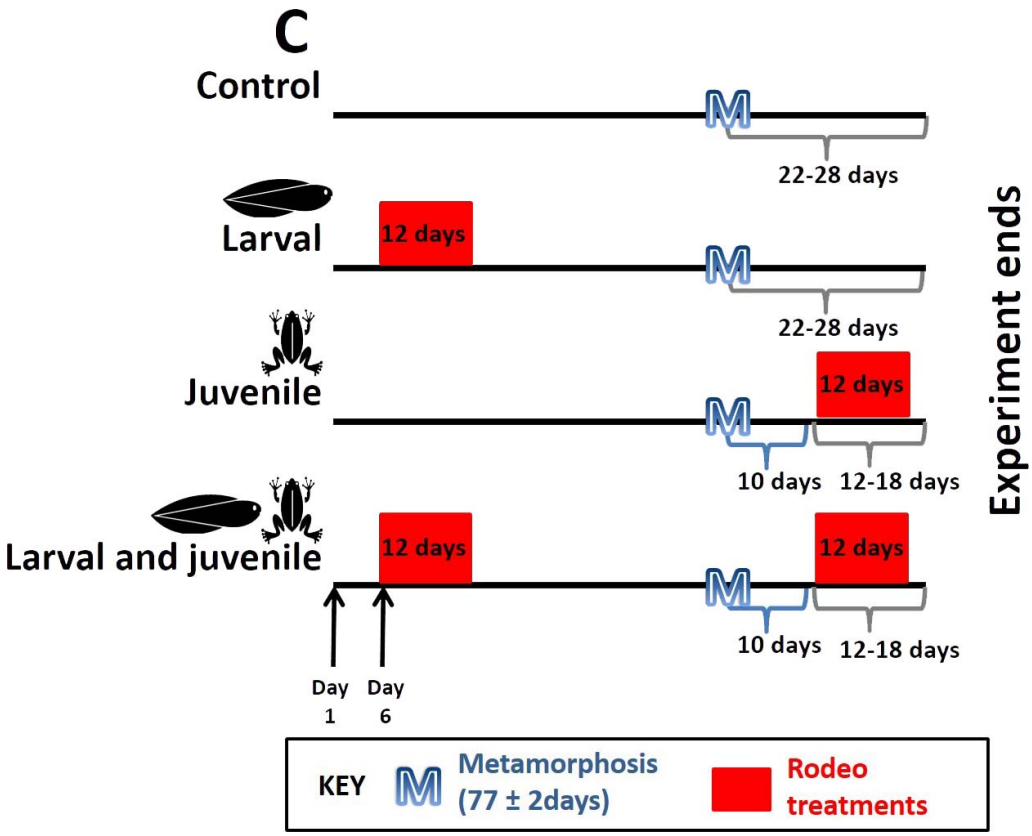


FIG. 1. Experimental methodology. Rodeo™ (Dow AgroSciences, LLC) treatments were conducted at four concentrations: Control, 0.0 mg a.e. L^{-1} (0.0 mg a.i. L^{-1}); low, 0.75 mg a.e. L^{-1}(1.01 mg a.i. L^{-1}); medium, 1.5 mg a.e. L^{-1} (2.02 mg a.i. L^{-1}); and high, 2.5 mg a.e. L^{-1} (3.38 mg a.i. L^{-1}).

glyphosate concentration (highest = 3.75 mg a.e. L^{-1}) is still below the highest concentration expected when spraying aquatic vegetation (Giesy et al., 2000; Relyea, 2005).

On Day 15, prior to the water change, we tested pH and ammonia levels in all larval tanks. Rodeo addition significantly decreased water pH, but this small difference is unlikely to be biologically important (mean ± SE: control = 7.61 ± 0.01, low = 7.54 ± 0.01, medium = 7.52 ± 0.02, high = 7.51 ± 0.01; $F_{3,46}$ = 14.15, $P < 0.0001$; Pierce, 1985). Ammonia levels were significantly higher in medium and high Rodeo larval tanks compared to controls (control, 0.34 ± 0.03 mg L^{-1}; low, 0.40 ± 0.04 mg L^{-1}, $Z = -1.35$, $P = 0.18$; medium, 0.50 ± 0.00 mg L^{-1}, $Z = -3.5073$, $P = 0.0004$; high, 0.50 ± 0.00 mg L^{-1}, $Z = -3.5073$, $P = 0.0004$ Kruskal-Wallis test; for three comparisons, the Bonferroni corrected $\alpha = 0.017$), but again these levels are unlikely to negatively affect larval survival (Jofre and Karasov, 1999). Juvenile treatments were conducted with identical concentrations also for 12 d. We conducted 100% water changes on juvenile cups every other day (100 ml cup^{-1}). Juveniles had the opportunity to climb out of direct contact with the treatment solutions, as would be the case in the natural environment.

We collected skin-associated bacterial community samples from each metamorphosing larvae during the transfer to juvenile housing and we collected juvenile bacterial community samples immediately prior to collection of natural peptide secretions at the experiment's end (22–28 d after the last larvae metamorphosed in a replicate). We collected bacterial community samples (skin swabs) and natural peptide secretions following Krynak et al. (2015). Our unit of analysis was the replicate (1–3 individuals): natural peptide secretions were collected simultaneously from all individuals in the replicate, and we collected mass on all individuals subsequent to euthanasia (MS-222) that immediately followed peptide collection. We collected the mass data with an analytical scale after blotting the MS-222 solution from the carcasses of the frogs. We averaged time to metamorphosis (days from assignment to replicate to reaching Gosner stage 42) and mass by replicate.

We extracted bacterial DNA from skin swabs, pooling swabs by tank, using a bead beating and phenol chloroform extraction method (Burke et al., 2006, 2008). We amplified bacterial DNA with the use of 16S rRNA gene primers: 338f and 926r, following Carrino-Kyker et al. (2012). With the use of terminal restriction fragment length polymorphism profiling (TRFLP), we examined bacterial community structure across treatments (Krynak et al., 2015, 2016). TRFLP profiles were processed using the TRFLPR package (Petersen et al., 2015; R Core Team, 2013). We used nonmetric multidimensional scaling analyses (NMDS) to assess bacterial community structure across treatments in PC-ORD (Version 5.0; Bruce McCune and MJM Software, 1999). We used axis scores from the resulting NMDS ordination solution to assess influence of treatments on the variation across each NMDS axis independently to maximize statistical power (see statistical analysis description below). Differences in NMDS axis scores indicate differences in the taxonomic composition of the bacterial community on the amphibians' skin.

We processed natural peptide secretion samples as in Krynak et al. (2015), and utilized a Micro BCA Protein Assay Kit (product 23235; Rockford, Illinois, USA) for analysis of total protein concentration, standardizing for total frog biomass per tank (Krynak et al., 2015). We measured natural peptide secretion bioactivity by determining pathogen growth rate in culture when challenged by natural peptide secretions from

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frogs following Krynak et al. (2015). We read optical density (OD; BioRad Imark, Hercules, California, USA) at 490 nm on Day 0 (immediately after plating), Day 1 (13 h postplating), Days 2–9. We fit a logistic growth model to data using a self-starting *nls* logistic model function (R Development Core version 3.0.2, 'stats' package, José Pinheiro and Douglas Bates), and Bd growth rate (r) was determined (Krynak et al., 2015). We used Bd growth rate (r) as our proxy for bioactivity of the natural peptide secretions; rapid growth rate indicated natural peptide secretions with reduced bioactivity against Bd.

Statistical Analysis.—We tested if our treatments affected larval and juvenile percent survival per tank utilizing a Kruskal-Wallis test for multiple comparisons. We compared survival in each treatment to survival in the control group. To account for multiple comparisons, we applied Bonferroni correction (Bonferroni corrected $\alpha = 0.05/n$, n = number of comparisons). We used analysis of variance (ANOVA) to test if Rodeo treatments applied during the larval stage affected larval duration or any of the three axes of the NMDS ordination of the larval bacterial community. In these models, each response variable was analyzed with a single predictor variable (larval Rodeo concentration) with four levels (control, low, medium, and high) via ANOVA. We included replicates that underwent postmetamorphic (juvenile) treatments in these analyses of larval traits; replicates that received juvenile-only Rodeo exposures were incorporated into the control group, and replicates that received both larval and juvenile Rodeo exposures were incorporated into the larval groups. We also tested whether larval-stage Rodeo exposure (four levels: control, low, medium, and high) carried over to affect postmetamorphic juvenile traits (average juvenile mass, log-transformed natural peptide secretion production, log-transformed bioactivity of the natural peptide secretions, and each of the three NMDS ordination axes describing juvenile bacterial community structure) using analysis of covariance (ANCOVA). Average age (in days) postmetamorphosis was included as a covariate in each model to account for the possible confounding factor of age at time of juvenile sampling. Finally, we used ANCOVA to test if Rodeo treatments affected postmetamorphic juvenile traits, including average age postmetamorphosis as a covariate in each model. In these ANCOVA models we assessed each of the responses (average juvenile mass, log-transformed natural peptide secretion production, log-transformed bioactivity of the natural peptide secretions, and each of the three NMDS ordination axes describing juvenile bacterial community structure) as a function of the stage at which the animals were exposed to Rodeo (three levels: larval exposure, juvenile exposure, or both larval and juvenile exposure) and the concentration of Rodeo to which they were exposed (two levels: low or medium). Control and high Rodeo concentration treatments were excluded in these particular analyses to create a balanced design and meet the assumptions of our model. We included age postmetamorphosis as a covariate in each model. Interactions were not included because of low statistical power associated with small sample size. We utilized Type III sums of squares for all ANOVA/ANCOVA analyses. Planned contrasts were used to compare treatment means in all ANOVA/ANCOVA models.

Results

Larval Traits.—Survival to metamorphosis in the high Rodeo treatment was 37% lower than in controls (Fig. 2; $Z = 2.688$, $P = 0.007$; for three comparisons, the Bonferroni corrected $\alpha = 0.017$), but larval survival did not differ between control and low ($Z = 0.284$, $P = 0.776$) or medium ($Z = -0.174$, $P = 0.862$) Rodeo treatments (Fig. 2).

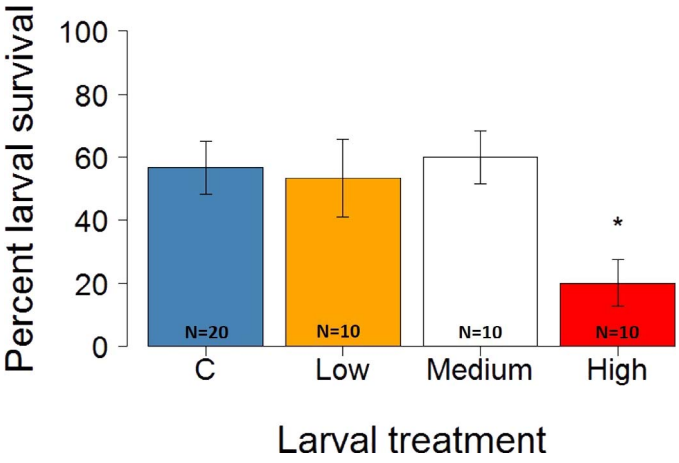


FIG. 2. Larval *Acris blanchardi* survival in response to Rodeo™ (Dow AgroSciences, LLC) concentration. High Rodeo concentration for a period of 12 d reduced survival by 36.67% compared to control (Kruskal-Wallis test with Bonferroni correction for multiple comparisons; $Z = 2.69$, $P = 0.0017$). N = number of replicates at beginning of the experiment.

The larval bacterial community was marginally affected by larval Rodeo concentrations along NMDS Axis 2 (Axis 1: $F_{3,33} = 1.63$, $P = 0.20$; Axis 2: $F_{3,33} = 2.63$, $P = 0.07$; Axis 3: $F_{3,33} = 0.41$, $P = 0.75$; Fig. 3A). Post hoc planned contrasts indicated a significant difference in the larval bacterial community between high Rodeo (2.5 mg a.e. L^{-1}) and control (Axis 2: $T = 2.8$, $P = 0.009$; Fig. 3A) treatments, but there were no differences between low and medium Rodeo treatments and controls (Fig. 3A). Time to metamorphosis was not affected by exposure to Rodeo (77.29 ± 2.27 d; Rodeo concentration: $F_{3,33} = 0.16$, $P = 0.92$).

Postmetamorphic (Juvenile) Traits.—Survival from metamorphosis to the end of the experiment did not significantly differ between control and any Rodeo treatment (low larval exposure: $Z = 0.053$, $P = 0.958$; low juvenile exposure: $Z = -0.472$, $P = 0.637$; low larval + juvenile exposure : $Z = -0.479$, $P = 0.632$; medium larval exposure: $Z = 0.217$, $P = 0.828$; medium juvenile exposure: $Z = 0.840$, $P = 0.401$; medium larval and juvenile exposure: $Z = -1.461$, $P = 0.1440$; high larval exposure: $Z = -1.461$, $P = 0.144$; for seven comparisons, the Bonferroni corrected $\alpha = 0.007$).

We found no evidence of carryover effects of larval Rodeo on juvenile mass, natural peptide secretion production, bioactivity of the natural peptide secretions, or any of the juvenile bacterial community NMDS ordination axes in ANCOVA models (Appendix 1; Fig. 3B). When examining possible additive effects of treatments, we found a marginal effect of Rodeo concentration on juvenile mass; however, if controlling for multiple comparisons, the effect was not significant. Juveniles were larger in the medium Rodeo treatment than in the low treatment (low: 0.30 ± 0.02 g; medium: 0.38 ± 0.02 g; $F_{1,19} = 4.43$, $P = 0.05$; Appendix 3). We did not find significant effects of Rodeo concentration or the timing of Rodeo exposure on natural peptide secretion production or bioactivity (natural peptide secretion production: 252.84 ± 30.24 µg/mL per gram body weight; bioactivity: 1.00 ± 0.05; Appendix 3). We did not find evidence of a strong effect of Rodeo™ concentration or life stage of exposure on the juvenile bacterial community (Appendix 3), but we did find a marginal effect of Rodeo concentration on the

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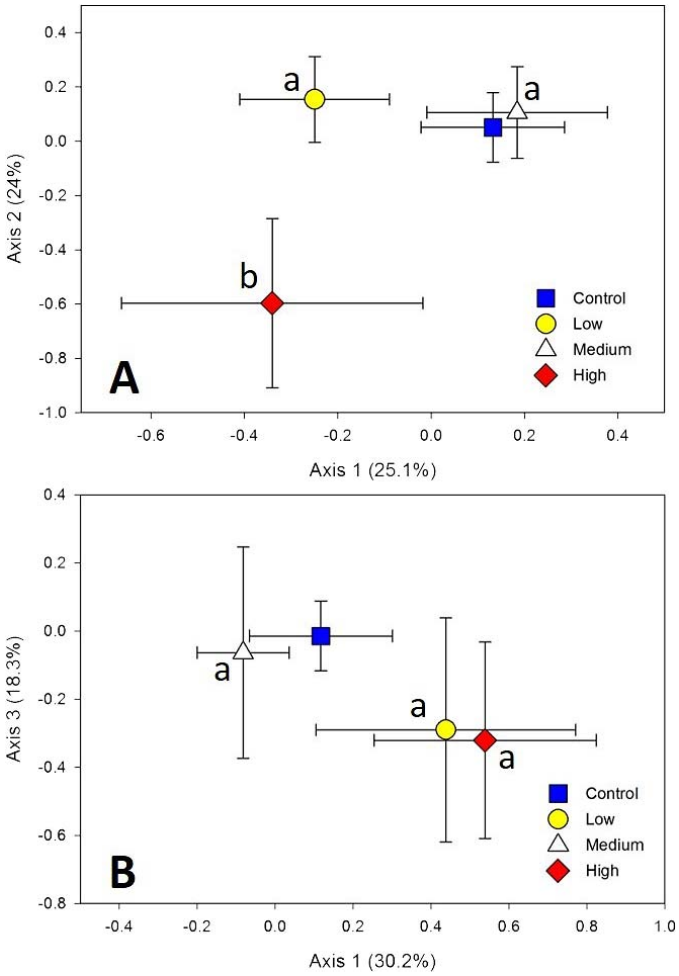


FIG. 3. (A) Larval microbiome nonmetric multidimensional scaling analyses (NMDS) ordination (3D solution stress = 15.87%; Axis 3 not shown) as influenced by larval Rodeo™ (Dow AgroSciences, LLC) concentration (mean and standard error shown; control$_{n = 14}$, 0.0 mg a.e. L^{-1}; low$_{n = 8}$, 0.75 mg a.e. L^{-1}; medium$_{n = 10}$, 1.5 mg a.e. L^{-1}; high$_{n = 5}$, 2.5 mg a.e. L^{-1}). Rodeo concentration altered larval microbial community structure along NMDS Axis 2 ($F_{3,33} = 2.632$, $P = 0.07$). Post hoc planned contrasts: a = not significantly different from control; b = $P < 0.009$ compared to control. (B) Juvenile microbiome NMDS ordination (3D solution stress = 11.2%; Axis 2 not shown) as a function of larval Rodeo concentration (mean and standard error shown; control$_{n = 6}$, 0.0 mg a.e. L^{-1}; low$_{n = 2}$, 0.75 mg a.e. L^{-1}; medium$_{n = 3}$, 1.5 mg a.e. L^{-1}; high$_{n = 5}$, 2.5 mg a.e. L^{-1}). Larval Rodeo concentration did not carry over to affect the juvenile microbiome (excluded replicates with postmetamorphic treatments, i.e., replicates exposed as juveniles only as well as replicates exposed as both larvae and juveniles). Post hoc planned contrasts: a = not significantly different from control.

juvenile bacterial community along NMDS Axis 3 (Axis 3: $F_{1,19} = 4.24$, $P = 0.06$).

DISCUSSION

In light of rapid disease-related amphibian declines across the globe (Berger et al., 1998; Daszak et al., 2003), assessing factors in the environment that inhibit pathogen resistance is important. The influence of herbicide exposure on amphibian immune defense traits merits particular attention, because these chemicals are widely used in agriculture and land management and are likely to be frequently encountered by amphibians (Moyer et al., 1994; Robles et al., 2010; Linz and Homan, 2011). We used environmentally relevant concentrations of glyphosate from a commercial formulation (Relyea, 2005) administered to *A. blanchardi* for a conservative duration (Colombo and Masini, 2014), and found a 37% decrease in survival of larvae exposed to 2.5 mg a.e. L^{-1} compared to controls, an effect not predicted by the results of acute analyses of glyphosate toxicity (Dow Agrosciences, 2015). Furthermore, we found that the skin bacterial communities of surviving larvae of the 2.5 mg a.e. L^{-1} treatment were significantly altered compared to controls (NMDS Axis 2; Fig. 3A). We did not find effects of larval Rodeo concentration on time to metamorphosis.

Effects on postmetamorphic juvenile traits were less dramatic, highlighting the importance of understanding the effects of exposure across life stages. Rodeo treatments did not decrease juvenile survival. Similarly, we found no evidence of carryover effects of Rodeo concentration on postmetamorphic mass, peptide production, and bioactivity. We did not find main effects of Rodeo concentration or the timing of exposure across developmental stages on the natural peptide secretions; however, we did find some evidence that skin-associated bacterial communities of juveniles were altered by Rodeo concentration. The frogs in low and medium Rodeo treatments were marginally significantly different from one another in juvenile skin bacterial community composition (NMDS Axis 3). Given the potential role of the bacterial community in disease resistance, this suggests that disease resistance could be affected when amphibians are exposed to Rodeo herbicide at concentrations recommended by the manufacturer (Dow Agrosciences, 2013). We also found that medium Rodeo concentration marginally differed from low Rodeo concentration in terms of effects on juvenile mass.

The finding that high Rodeo concentration (2.5 mg a.e. L^{-1}) reduced larval survival was counter to what would be expected from the product's environmental safety sheet (Dow Agrosciences, 2013). Acute toxicity studies that report a LC50 of >100 mg L^{-1} suggested to us that the concentrations used in this study would not affect survival (Dow Agrosciences, 2015). The increased mortality in our highest Rodeo concentration was consistent with other studies, however, demonstrating reduced survival in several amphibian species at environmentally relevant concentrations of glyphosate-based herbicides (Relyea, 2005; Relyea and Hoverman, 2006). Furthermore, environmental factors such as competitors, predators, and temperature, may exacerbate negative effects of glyphosate on amphibian survival across life stages (Relyea et al., 2005; Wagner et al., 2013; Lötters et al., 2014). *Acris blanchardi* have a central North American distribution (Gamble et al., 2008) and habitats vary in numerous biotic and abiotic ways, so some populations may be more sensitive to glyphosate exposure than others. Furthermore, as *A. blanchardi* is a declining species that largely is annual (estimated complete population turnover = 16 mo; Burkett, 1984), conservation efforts rest critically on a thorough examination of potential mortality effects associated with land management practices. Our results suggest that a single early-season (spring) Rodeo treatment (*A. blanchardi* larval stage) could severely decrease local population size.

Rodeo exposure did not alter larval duration, but marginally affected juvenile mass. Previous studies have found that these measures are affected by other forms of glyphosate-based herbicides. Round-up Original™ (Monsanto Co., St. Louis, Missouri, USA) decreased growth and development in the Northern Leopard Frogs, *Lithobates pipiens* (Howe et al., 2004), VisionMax™ (Monsanto) slows developmental rates in Wood

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Frogs, *Lithobates sylvaticus*, possibly by altering the expression of genes involved in development (Navarro-Martin et al., 2014) and Round-up WeatherMax™ (Monsanto) may alter development by disrupting hormonal pathways in *L. sylvaticus* (Lanctot et al., 2013). Shifts in larval duration can negatively affect amphibians by increasing desiccation risk in seasonally drying ponds and/or increased predation or competition via changes in densities or size of cohabiting species over time (Van Buskirk and Saxer, 2001; Bridges, 2002). Juvenile mass is often correlated with amphibian survival to reproduction (Earl and Whiteman, 2015) and therefore careful consideration should be given to potential effects on juvenile mass found here. Surprisingly, juveniles exposed to our medium Rodeo concentration were larger than those exposed to our low Rodeo concentration, suggesting that the herbicide may increase growth. This is consistent with the finding by Lanctot et al. (2014) that sublethal exposure to Round-up WeatherMax and Vision increased larval body condition in *L. sylvaticus*. Increased mass following herbicide exposure may indicate a compensatory effect such as increased mass counterbalancing depressed immune function on fitness, or may result from metabolic changes due to increased Rodeo concentration (Salbego et al., 2010). This, in conjunction with the finding that Rodeo did not affect *A. blanchardi* juvenile survival, suggests that delaying applications of glyphosate-based herbicide products until after metamorphosis could increase *A. blanchardi* fitness, but additional studies are needed to support this prediction.

When assessing effects of glyphosate-based herbicides on amphibians, one also must consider the additives in each formulation. Round-up and Vision products differ from Rodeo in one key aspect: they contain a surfactant (either as an undisclosed proprietary formula or polyethoxylatedamine, POEA) that is commonly thought to be the driver of effects on amphibians (Annett et al., 2014; Mann and Bidwell, 1999). Because glyphosate formulations labeled as safe for use in and around aquatic habitats do not contain surfactants, the negative effects of herbicide treatment may not be as pronounced in aquatic formulations; however, Dow Agrosciences (2013) recommends mixing Rodeo with a nonionic surfactant to improve efficacy. Although our study did not assess the addition of a surfactant to the Rodeo formula, future studies should also examine surfactant effects on amphibian traits correlated with fitness, including skin-associated immune defense traits (Mann and Bidwell, 1999; Trumbo, 2005; Puglis and Boone, 2010).

We did not find effects of Rodeo on the amount of *A. blanchardi* natural peptide secretions, or their ability to inhibit Bd in vitro. Wild *A. blanchardi* populations in Ohio and Michigan (USA) differ in the amount of natural peptide secretions they produce, and variation in peptide secretions is correlated with environmental characteristics including land use and water quality (Krynak et al., 2016). Here we examined a single Ohio population, but Rodeo could alter natural peptide secretions in other *A. blanchardi* populations. Consistent with previous work, we found that the natural peptide secretions from *A. blanchardi* were not bioactive against Bd (Conlon, 2011; Krynak et al., 2016). Pathogens not tested in this study, such as iridoviruses or *Batrachochytrium salamandrivorans* (Bsal), may be inhibited by *A. blanchardi* natural peptide secretions and such inhibition may be altered by herbicide exposure (Forson and Storfer, 2006; Martel et al., 2013). Therefore, although our results indicated that Rodeo herbicide may not affect the efficacy of peptides against Bd, investigation of interactions between exposure to Rodeo™ and exposure to pathogens other than Bd across populations is warranted.

Although we did not find effects of Rodeo on *A. blanchardi* natural peptide secretions, we did find effects on the other important component of the amphibian innate immune system: the skin-associated bacterial community. The larval skin bacterial community of *A. blanchardi* was altered by our high Rodeo treatment, but this effect did not carry over to alter the juvenile bacterial community. Additionally, the bacterial community of postmetamorphic juveniles showed a trend suggesting Rodeo concentration altered juvenile bacterial community structure; bacterial communities of juveniles exposed to medium Rodeo marginally differed from those exposed to low Rodeo. Together these results suggest that early-season Rodeo treatment of habitats might have more severe consequences for *A. blanchardi* disease resistance than late season treatment. This is particularly important due to the commonly used regime of Cattail (*Typha angustifolia)* Rodeo treatment in the spring when *A. blanchardi* larvae are present and Common Reed (*Phragmites australis*) Rodeo treatment in the late summer when larvae are metamorphosing (Wright and Wright, 1949; Dow Agrosciences, 2013). Particular bacteria found on amphibian skin are capable of producing metabolites that suppress pathogen infection (Harris et al., 2006; Becker et al., 2009), but if bacterial communities are disrupted, such function may not be possible. Alternatively, the changes to the *A. blanchardi* skin bacterial communities caused by Rodeo may not result in functional changes if structurally different communities are functionally redundant (Kung et al., 2014; Lear et al., 2014). Additional studies are needed to determine the functional capacity of the *A. blanchardi* bacterial community in response to Rodeo exposure.

In agreement with other studies of glyphosate-based herbicide effects on amphibians, we conclude that Rodeo exposure at field-relevant concentrations can increase mortality in *A. blanchardi*, a species already suffering population declines and extirpations in the northern portions of its range (Gray and Brown, 2005; Lehtinen and Skinner, 2006; Gamble et al., 2008). Additionally, we showed that Rodeo could change the skin-associated bacterial community structure, which may indirectly decrease amphibian fitness. Improving our knowledge of the influence herbicide use has on amphibians across life stages provides an opportunity for changes to application strategies to protect amphibian health or, at minimum, lessen negative effects of the practice.

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APPENDIX 1.

Description as to how Rodeo™ concentrations were achieved.

Rodeo formulated product (stock solution) is at a concentration of 4 lb/gal glyphosate acid (479,305 mg a.e. L^{-1}).

To achieve the desired concentrations for a 10-L water volume, please see below:

Low Rodeo

(0.75 mg a.e. L^{-1})(10 water volume)/479305.7137 mg L^{-1} glyphosate = 0.000016 L, or 16 μL

Medium Rodeo

(1.5 mg a.e. L^{-1})(10 water volume)/479305.7137 mg L^{-1} glyphosate = 0.000032 L, or 32 μL

High Rodeo

(2.5 mg a.e. L^{-1})(10 water volume)/479305.7137 mg L^{-1} glyphosate = 0.000052 L, or 52 μL

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APPENDIX 2. ANCOVA analysis of larval Rodeo™ (Dow AgroSciences, LLC) concentration effects on juvenile *Acris blanchardi* traits (carryover effects). Excluded replicates with postmetamorphic treatments because of the unbalanced design, the result of larval mortality. Average days postmetamorphosis was included as a covariate in the model.

Response	Treatment	df	*F*	*P*
Juvenile mass (g)	Rodeo concentration	3,12	0.18	0.91
	Average days Postmetamorphosis	1,12	1.24	0.29
Natural peptide secretion production (μg/mL gbw^{-1})	Rodeo concentration	3,12	0.42	0.74
	Average days Postmetamorphosis	1,12	1.18	0.30
Natural peptide secretion bioactivity (*Batrachochytrium dendrobatidis* growth rate *r*)	Rodeo concentration	3,12	0.60	0.63
	Average days Postmetamorphosis	1,12	0.04	0.86
Juvenile bacterial community, NMDS[a] Axis 1	Rodeo concentration	3,12	0.47	0.71
	Average days Postmetamorphosis	1,12	0.03	0.86
Juvenile bacterial community, NMDS Axis 2	Rodeo concentration	3,12	0.34	0.80
	Average days Postmetamorphosis	1,12	1.77	0.21
Juvenile bacterial community, NMDS Axis 3	Rodeo concentration	3,12	0.02	1.00
	Average days Postmetamorphosis	1,12	1.44	0.26

[a] Nonmetric multidimensional scaling analyses.

APPENDIX 3. ANCOVA analysis of Rodeo™ (Dow AgroSciences, LLC) treatment effects on *Acris blanchardi* juvenile traits. Treatments consisted of combinations between two exposure concentrations (low and medium Rodeo) and three Rodeo exposure stages (larval, juvenile, or both: larval and juvenile Rodeo exposure). Average days postmetamorphosis was included as a covariate in the model.

Response	Treatment	df	*F*	*P*
Juvenile mass (g)	Rodeo concentration	1,19	4.43	0.05
	Exposure stage	2,19	0.50	0.61
	Average days postmetamorphosis	1,19	1.30	0.27
Natural peptide secretion production (μg/mL gbw^{-1})	Rodeo concentration	1,19	0.33	0.57
	Exposure stage	2,19	0.26	0.77
	Average days postmetamorphosis	1,19	1.97	0.18
Natural peptide secretion bioactivity (*Batrachochytrium dendrobatidis* growth rate *r*)	Rodeo concentration	1,18	0.21	0.65
	Exposure stage	2,18	0.38	0.69
	Average days postmetamorphosis	1,18	2.35	0.14
Juvenile bacterial community NMDS[a] Axis 1	Rodeo concentration	1,19	2.28	0.15
	Exposure stage	2,19	2.51	0.11
	Average days postmetamorphosis	1,19	0.84	0.37
Juvenile bacterial community NMDS Axis 2	Rodeo concentration	1,19	1.24	0.28
	Exposure stage	2,19	0.59	0.57
	Average days postmetamorphosis	1,19	0.69	0.42
Juvenile bacterial community NMDS Axis 3	Rodeo concentration	1,19	4.24	0.06
	Exposure stage	2,19	2.13	0.15
	Average days postmetamorphosis	1,19	1.05	0.32

[a] Nonmetric multidimensional scaling analyses.

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