Ecological Applications, 23(6), 2013, pp. 1443–1454

Larval exposure to predator cues alters immune function and response to a fungal pathogen in post-metamorphic wood frogs

Maya L. Groner,[1,6] Julia C. Buck,[2] Stephanie Gervasi,[2] Andrew R. Blaustein,[2] Laura K. Reinert,[3] Louise A. Rollins-Smith,[3] Mark E. Bier,[4] John Hempel,[5] and Rick A. Relyea[5]

[1]*Center for Veterinary Epidemiological Research, Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island C1A 4P3 Canada*
[2]*Department of Zoology, Oregon State University, Corvallis, Oregon 97331 USA*
[3]*Departments of Pathology, Microbiology and Immunology and of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232;* and *Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235 USA*
[4]*Center for Molecular Analysis, Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213 USA*
[5]*Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260 USA*

Abstract. For the past several decades, amphibian populations have been decreasing around the globe at an unprecedented rate. *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen that causes chytridiomycosis in amphibians, is contributing to amphibian declines. Natural and anthropogenic environmental factors are hypothesized to contribute to these declines by reducing the immunocompetence of amphibian hosts, making them more susceptible to infection. Antimicrobial peptides (AMPs) produced in the granular glands of a frog's skin are thought to be a key defense against Bd infection. These peptides may be a critical immune defense during metamorphosis because many acquired immune functions are suppressed during this time. To test if stressors alter AMP production and survival of frogs exposed to Bd, we exposed wood frog (*Lithobates sylvaticus*) tadpoles to the presence or absence of dragonfly predator cues crossed with a single exposure to three nominal concentrations of the insecticide malathion (0, 10, or 100 parts per billion [ppb]). We then exposed a subset of post-metamorphic frogs to the presence or absence of Bd zoospores and measured frog survival. Although predator cues and malathion had no effect on survival or size at metamorphosis, predator cues increased the time to metamorphosis by 1.5 days and caused a trend of a 20% decrease in hydrophobic skin peptides. Despite this decrease in peptides determined shortly after metamorphosis, previous exposure to predator cues increased survival in both Bd-exposed and unexposed frogs several weeks after metamorphosis. These results suggest that exposing tadpoles to predator cues confers fitness benefits later in life.

Key words: AchE inhibitor; Batrachochytrium dendrobatidis; *brevinin; chytridiomycosis; disease ecology; emerging infectious disease; immunosuppression; indirect effects;* Lithobates sylvaticus; *temporin.*

Introduction

Emerging infectious diseases (EIDs) are increasing rapidly in number and severity on a global scale, and altered environmental conditions are hypothesized to contribute to these patterns (Jones et al. 2008, Hayes et al. 2010, Martin et al. 2010). The EID chytridiomycosis in amphibians, which results from infection with the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is contributing to amphibian population declines around the world. Bd is found in over 350 species across six continents and appears to be the proximate cause for extinction for some amphibian species (Fisher et al. 2009). At the same time, environmental changes, including habitat degradation, exposure to contaminants, and increased UV-B radiation may also contribute to amphibian population declines (reviewed in Blaustein et al. 2011). It is hypothesized that these environmental stressors may interact with Bd to make amphibians more vulnerable to infection (Carey et al. 1999, Bancroft et al. 2007, Hayes et al. 2010, Blaustein et al. 2011); however, we know very little about what specific stressors are driving these interactions and the mechanisms that underlie them (e.g., Han et al. 2011, Buck et al. 2012).

In this study we focused on the hypothesis that susceptibility to disease increases because exposure to stressors reduces the immunocompetence of the host (Carey et al. 1999). This could occur directly if the stressor inhibits components of the innate or acquired immune system, or indirectly, as a result of altered functioning of the hypothalamo–pituitary–adrenal (HPA) axis or reduced allocation of resources towards immune functions (Carey et al. 1999, Martin et al. 2010). Many anthropogenic stressors, especially contaminants, can be immunosuppressive for amphibians (e.g., Chris-

Manuscript received 13 September 2012; revised 17 December 2012; accepted 28 February 2013. Corresponding Editor: T. W. J. Garner.
[6] E-mail: mgroner@upei.ca

tin et al. 2003, Forson and Storfer 2006, Hayes et al. 2006, Davidson et al. 2007, Rohr et al. 2008); however, less is known about how natural stressors alter amphibian immunocompetence or if multiple stressors have synergistic impacts on immunocompetence. Many pesticides are far more lethal in the presence of a natural stressor (e.g., predator cues; Relyea 2004), but it is unknown if these interactive effects extend to host–pathogen interactions. Moreover, the relationship between altered immunocompetence and changes in disease susceptibility is poorly understood in amphibians (Blaustein et al. 2012).

While both innate and acquired immune functions may be important defenses against Bd infections (e.g., Ramsey et al. 2010, Rollins-Smith et al. 2011), some of the best evidence for an immune defense against Bd are found in the suite of antimicrobial peptides (AMPs) that are released onto the surface of the skin during stressful events (reviewed in Rollins-Smith et al. 2011). Part of the innate immune system, AMPs are thought to only be present on the skin for a brief period (i.e., less than two hours; Pask et al. 2012) following peptide release (reviewed in Rollins-Smith et al. 2011). Releases occur when norepinephrine induces the contraction of the smooth muscles surrounding these glands. Many AMPs inhibit growth of Bd in vitro (reviewed in Rollins-Smith 2009), and production of inhibitory AMPs is correlated with resistance to Bd across species (Woodhams et al. 2006). AMPs are also hypothesized to influence the composition of beneficial bacteria on the skin, which inhibits Bd growth in vivo (Harris et al. 2009).

The role of AMPs in inhibiting Bd may be especially important during metamorphosis. AMPs are first produced in substantial quantities during this time (Bovbjerg 1963), while many components of the acquired immune system (e.g., lymphocyte numbers and viability, mitogen-induced proliferation) are suppressed to prevent autoimmunity against new adult-specific antigens (Rollins-Smith 1998). In many amphibian species, the majority of mortality due to Bd infection occurs during or soon after metamorphosis (e.g., Garner et al. 2009), and Bd infection loads in sampled populations are often the highest at this stage of development (Russell et al. 2010). AMPs and the skin bacteria that they influence may be one of the few defenses against infection available during this critical time.

Environmental stressors including contaminants, predators, and harmful microbes may alter AMPs by changing the development of granular glands where AMPs are stored, AMP synthesis, and the release of AMPs onto the skin (reviewed in Rollins-Smith et al. 2011). Maturation of granular glands in late larval life is dependent on thyroid hormones (Kollros and Kaltenbach 1952, Bovbjerg 1963). Thyroid hormones and glucocorticoids synergize to drive tissue differentiation (Denver 2009); however, excessive glucocorticoids inhibit the maturation of granular glands (Hayes and Gill 1995). In tadpoles, chronic exposure to predators and at least some pesticides elevates the release of glucocorticoids (Denver 2009, McMahon et al. 2011) and may be expected to decrease granular gland development (Simmaco et al. 1997, Romero 2004). Despite compelling immunological and epidemiological arguments, no studies have examined how exposure of amphibians to predators affects AMP production during development.

Exposure to insecticides, may also alter AMP production. For example, exposure to the commonly used insecticide carbaryl diminishes AMP releases in young metamorphs or adults (Davidson et al. 2007, Schadich et al. 2009). Correlational evidence supports the idea that insecticides in particular may be contributing to amphibian population declines by interacting with co-factors; upwind applications of carbamate and organophosphate insecticides that share a mode of action (acetylcholine esterase [AchE] inhibition) have been correlated with amphibian population declines (Davidson 2004, but see Bradford et al. 2011). The pesticides in these ponds are found at concentrations that are too low to be directly lethal to amphibians. This suggests that these pesticides may affect amphibians through indirect effects or by interactions with other stressors.

In this study, we tested the general hypothesis that natural and anthropogenic stressors induce immunosuppression and lead to increased mortality in pathogen-exposed amphibians. We exposed wood frogs (*Lithobates sylvaticus*; see Plate 1) to two different stressors, the AchE inhibiting insecticide, malathion, and predator cues, and subsequently examined susceptibility to Bd in a subset of animals exposed to these stressors. We predicted that: (1) exposing tadpoles to malathion will suppress skin peptide production in metamorphs, (2) exposing tadpoles to predator cues will suppress skin peptide production in metamorphs, (3) the combined effects of malathion and predator cues on skin peptides will be non-additive, and (4) decreased production or release of skin peptides will be associated with decreased survival in post-metamorphic frogs exposed to Bd.

Pesticide Background

Among the hundreds of pesticide active ingredients on the market, malathion may be especially influential to the amphibian immune system. Malathion is an organophosphate insecticide that acts by irreversibly binding to AchE. Organophosphates such as malathion are associated with immunotoxic effects in vertebrates (reviewed in Galloway and Handy 2003). These effects can occur directly, if the contaminant impairs immune functions, and indirectly through modulation of the nervous system or by limiting energy available for developing and maintaining the immune system as a result of lowered metabolism or malnutrition. Malathion suppresses cellular and humoral immune components of amphibians (Kiesecker 2002, Gilbertson et al. 2003)

or increases prevalence of infection in frogs (e.g., lungworms, trematodes and *Aeromonas* bacteria; Kiesecker 2002, Christin et al. 2003, Gilbertson et al. 2003); however, no studies have examined the effects of malathion exposure on AMPs in the skin. In the United States, malathion is the most commonly used insecticide, with 9–11.3 million kg of active ingredient used annually in agriculture and additional millions of kg used in gardening and public health programs (Kiely et al. 2004).

Methods

Experimental design

The study was conducted in two stages. The first stage tested the effects of exposing wood frog tadpoles to predator cues and malathion on the life history traits and release of AMPs onto the skin of newly metamorphosed wood frogs (metamorphs). The second stage used these metamorphosed wood frogs to assess the effects of larval exposure to these stressors on the survival of metamorphs to Bd. The first stage was conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in Linesville, Pennsylvania, USA; the second stage was conducted at Oregon State University in Corvallis, Oregon, USA.

Tadpole exposure to natural and anthropogenic stressors was accomplished using a completely randomized full-factorial design containing three malathion treatments (nominal concentrations of 0, 10, and 100 parts per billion [ppb]) crossed with two predator treatments (predator cues present or absent). The 0 ppb malathion treatments were replicated six times and all other treatments were replicated five times. Experimental units were plastic wading pools (mesocosms) filled with 100-L of well water on 5 April 2009. On 16 April we added 5 g of rabbit chow to serve as a nutrient source, 100 g of leaf litter to serve as a substrate for periphyton, and homogenized aliquots of pond water from three local ponds to establish populations of periphyton and phytoplankton. On 21 April we added zooplankton collected from two local ponds. Thus, the mesocosms contained several components of natural wetlands. Wood frogs from 26 egg masses were collected from three ponds in Crawford County, Pennsylvania, between 20 March and 2 April and raised in predator- and pesticide-free culture pools. All culture pools and mesocosms were covered with mesh lids providing 60% shade to exclude colonization by predators or escape of metamorphs. On 8 May, hereafter "experiment day 1," we added 20 wood frog tadpoles to each mesocosm (mass = 78 ± 4 mg [mean ± SE]; Gosner stage 25; Gosner 1960). A randomly chosen subset of 20 animals was kept in the laboratory after handling (i.e., removing from culture pools and sorting) in order to quantify the effect of handling stress. These animals had a 100% 24-h survival rate.

The predator treatments consisted of exposure to the presence or absence of predator cues. Predators were locally collected larval dragonflies (*Anax junius*). This species has a cosmopolitan distribution throughout North America and is a common predator of wood frogs (Corbet 1999). The dragonflies were caged individually in 200-mL plastic cups (one per mesocosm) with a mesh screen and fed 298 ± 3 mg (mean ± SE) of wood frog tadpoles three times per week. Tadpoles respond to cues from digested conspecifics, so caged predators can induce a response without attacking focal animals (Schoeppner and Relyea 2008). The concentration of cues in this experiment (cues from 3 mg digested tadpoles/L of water) is sufficient to induce the maximum plastic response in wood frogs (Schoeppner and Relyea 2008). Mesocosms assigned the no-predator cue treatment contained empty cages. On feeding days, these empty cages were lifted out of the water and put back to equalize the disturbance across all mesocosms. Predator treatments began on day 4 and continued for 28 d. Dragonflies that died or stopped feeding were replaced with similarly sized dragonflies.

Because we were interested in understanding the effects of pesticides on wood frogs across a developmental transition, we applied pesticide treatments on day 28, which we estimated to be one week before the onset of metamorphosis, based on the developmental stage of tadpoles at that point. The formulation of malathion (Gordon's malathion, 50% active ingredient; PBI/Gordon, Kansas City, Missouri, USA) was diluted to a stock solution of 18.9 g/L active ingredient and we added 0, 51.8, or 518 μL of this stock solution to the appropriate mesocosms to achieve nominal concentrations of 0, 10, and 100 ppb. These concentrations are within the range that has been detected in natural ponds (Odenkirchen and Wente 2007). Pesticides were stirred into each mesocosm with a plastic cup. Two hours after the application, water samples were collected and pooled within pesticide treatments for confirmation of nominal concentrations. Samples were stored at 4°C and then shipped for analysis by high-pressure liquid chromatography (Mississippi State Chemical Laboratory, Mississippi State, Mississippi, USA).

Analyses of the water samples indicated that the actual concentrations of pesticides were 0.24, 2.8, and 32 ppb for the 0, 10, and 100 ppb nominal concentration treatments, respectively (Mississippi State Chemical Laboratory). While the actual concentrations deviated from the nominal concentrations, the deviation was a consistent 68–72% reduction from the targeted concentrations. Variation from nominal concentrations is frequently found in mesocosm experiments (reviewed in Brock et al. 2000) and is thought to arise from precipitation or volatization of pesticides in mesocosms, incomplete mixing of pesticides within mesocosms as a result of binding to substrates or degradation of stored samples before the assay (Brock et al. 2000). The source of trace levels of malathion in the control is unknown.

Stage 1: Response variables

To test the effects of treatments on water quality, we measured dissolved oxygen (DO), temperature, and pH between 13:00 and 15:00 hours on experiment day 32. Temperature and DO were measured with a calibrated MultiLine P4 meter (Wissenschaftlich-Technische Werkstatten, Weilheim, Germany), and pH was measured with a calibrated pH meter (pHTestr 10 double junction; Oakton Instruments, Vernon Hills, Illinois, USA).

We placed two 30-cm wooden strips into all mesocosms on day 32 to provide a perch for metamorphs. The first tadpoles began to metamorphose on day 33. After day 33, mesocosms were checked for metamorphs daily. Animals with emerged forelimbs were removed from mesocosms and housed in 1-L containers with sphagnum moss until they resorbed their tails to <2 mm (~4 d). At this stage of tail resorption, metamorphosis was considered complete and we weighed the metamorph. After weighing, eight individuals from each treatment were isolated for peptide induction and 12 individuals from each treatment were set aside for a future challenge with Bd. We sorted frogs into the these groups so frogs that metamorphosed early, midway, and late in the experiment were represented in both groups; the 2nd, 5th, 7th, 10th, 13th, 15th, 17th, and 20th frog to emerge were used for peptide induction, while the remainder were in the Bd challenge. Metamorphs used for AMP collection were held individually in 590-mL plastic cups containing sphagnum moss and fed crickets dusted with ReptoCal (TetraFauna, Blacksburg, Virginia, USA) ad libitum for 8 d before peptide collection. Metamorphs used for the Bd challenge were grouped by mesocosm in 14-L tubs containing sphagnum moss and fed crickets dusted with ReptoCal ad libitum. Once all metamorphs had emerged, the animals were overnight-shipped to Oregon State University. Because we waited for all frogs to metamorphose before shipping these animals, the average separation between the collection of peptides from some frogs and the exposure of others to Bd was 22 d.

Antimicrobial peptide collection.—Hydrophobic skin peptides were measured after metamorphosis was completed. While we aimed to collect eight frogs per treatment for skin peptide measurement, mortality pre- and post-metamorphosis reduced our sample size to, on average, six frogs per replicate (31 frogs per treatment). For each frog, we waited 8 d after the completion of metamorphosis before extracting AMPs to allow animals to acclimate to their new environment. These animals were weighed immediately prior to AMP extraction.

AMPs were extracted with a subcutaneous injection of 2 nmol norepinephrine-HCl/g frog. This injection triggers contraction of the smooth muscle surrounding the granular glands that store AMPs, causing them to be released (reviewed in Rollins-Smith et al. 2011). Therefore, measurements of AMPs reflect sensitivity to norepinephrine, as well as AMP storage at the time of release. After injection, frogs were placed into 10 mL of collection buffer (25 mmol/L Na acetate and 25 mmol/L NaCl, pH 7.0; Rollins-Smith et al. 2002*b*). After 10 min, frogs were returned to plastic cups and the collection buffer was acidified with 500 μL of trifluoracetic acid and frozen. The concentration of norephinephrine used in this experiment is thought to be comparable to levels experienced during stressful events in nature. For example, frogs chased by a researcher's hand released the same amount of peptides as frogs injected with 2 nmol norepinephrine-HCl/g frog and significantly more peptides than resting frogs (Ramsey et al. 2010).

After collection, hydrophobic peptides were enriched by passage over C-18 sep-pak cartridges (Rollins-Smith et al. 2002*b*). Peptides were then eluted in 70% acetonitrile, 29.9% water, and 0.1% trifluoracetic acid. Peptide concentrations were determined using the Micro BCA analysis (Pierce, Rockford, Illinois, USA) following the kit's instructions, with the exception that bradykinin was used to establish a standard curve (Rollins-Smith et al. 2002*b*).

Antimicrobial peptide characterization.—Only one AMP has been sequenced for wood frogs (Matutte et al. 2000), and there are no studies of peptides released by wood frog metamorphs. Since many ranids produce a diverse suite of AMPs (Tennessen and Blouin 2007), we examined the skin secretions for novel peptides. Determination of peptide sequences was done using nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF; Waters Corporation, Milford, Massachusetts, USA). Samples from each treatment were combined and loaded onto a 100 μm internal diameter column containing ~10 cm of C-18 packing. Mass spectra were first obtained in the 550–3000 m/z range and promising spectra (with charges >1) were targeted for MS/MS (tandem mass spectrometry). Assignment of sequences was done manually using the methods described in Kinter and Sherman (2000).

Stage 2: Bd challenge

At Oregon State University, metamorphs were placed in 40-L glass terraria (grouped by mesocosm) in a laboratory maintained at a temperature of 21.5°C to 23.3°C with a 13:11 light : dark photoperiod. All individuals were given one week to acclimate to these conditions before being weighed and moved to individual experimental enclosures (plastic petri dishes, 140 mm diameter × 30 mm height, with air holes in the lids). Metamorphs were allowed 2 d to acclimate to their petri dishes, before the experiment was initiated. Petri dishes contained 10 mL of dechlorinated water and 15 mL of Bd inoculate or sham inoculate. The metamorphs were able to partially climb the walls of the petri dish, but could not completely lift themselves off the bottom, keeping them in constant contact with the water. They

were kept in these dishes for the duration of the experiment and fed three pinhead crickets twice a week.

Bd culturing and inoculation.—We infected metamorphs with Bd strain JEL 258, originally isolated from a wood frog from Maine, USA. Bd was cultured on 1% tryptone agar plates that were made 15 d prior to inoculation and held at ~22°C. To harvest Bd from agar plates, we flooded plates with 15 mL dechlorinated water for 5 min to allow for zoospores to be discharged from sporangia. To standardize inoculation dose among Petri dishes, all flooded Bd plates were gently scraped to remove zoospores from the agar and this suspension was pooled to create a single inoculation broth (Searle et al. 2011). The zoospore concentration in the inoculation broth was determined by hemocytometer and diluted to a standard working concentration of $\sim 6.3 \times 10^3$ zoospores/mL. Control animals were given a similar treatment except sterile agar plates were flooded with dechlorinated water. All experimental animals were exposed to the same Bd concentration at each inoculation (~15 ml of inoculate, 9.45×10^4 zoospores/plate). Two experimental inoculations took place over the course of the infection experiment, on experiment day 1 (14 July) and day 9. Water in the petri dishes was changed during reinoculation. Throughout this stage of the study, animals ($n = 245$) were checked twice daily for mortality. The experiment was terminated upon the death of the last animal in the Bd treatment (day 16), and all remaining control animals were humanely euthanized by immersion in MS-222 (tricaine-methanesulfonate; Argent Chemical Labs, Redmond, Washington, USA).

To determine if experimental infection was successful and to test for cross-contamination, several animals from each treatment were preserved in 95% ethanol and the infection status of toe clips from 10 animals from Bd treatments and 10 from Bd controls was determined using quantitative polymerase chain reaction (PCR). We followed the protocol outlined in Boyle et al. (2004), with the exception that toe clips were macerated with a mortar and pestle instead of a bead beater. All samples were run in triplicate. All 10 Bd-exposed individuals tested positive for infection, and none of the 10 control animals tested positive for infection in any of the triplicate wells. The mean infection load for Bd-exposed frogs was 8400 ± 2100 zoospores.

Statistical analyses

For the first stage of the experiment, we tested the effects of malathion and predator exposure on water quality, life history traits of wood frogs, and AMP production in newly metamorphosed wood frogs. For the second stage of the experiment, we tested the effects of Bd exposure, malathion exposure, and predator exposure on the survival of post-metamorphic wood frogs. All statistical analyses for the first stage were run using SPSS (v. 18; SPSS 2009). Analyses for the second stage were run using R (2.15.0; R Development Core Team 2012).

We tested the effects of malathion and predator cues on water quality variables (pH, dissolved oxygen, temperature) using multivariate analysis of variance (MANOVA). All water quality variables met assumptions of equal variances and normality. Because we found no effects of the treatments on water quality variables, we will not discuss them.

We tested the effects of the malathion and predator cues on metamorph response variables (size at metamorphosis, time to metamorphosis, and survival to metamorphosis) using MANOVA. Whenever a multivariate effect was significant, we conducted ANOVAs on each response variable. The proportion surviving was arcsine square-root transformed to meet the assumptions of normality. These data still did not meet the assumption of equal variances, because the variation in the 10 ppb malathion × predator cue treatment was smaller than other treatments. Therefore, we used Pillai's trace to test for significance, as it is robust to minor violations of this assumption.

We used a nested ANCOVA to test the effects of treatments on AMP production. Since larger animals have a greater skin surface area and more glands, we used mass as a covariate. To control for the non-independence of metamorphs that were reared in the same mesocosm, we included mesocosm as a random nested term within the predator × pesticide interaction. Total skin peptide concentrations were log-transformed to meet the assumption of normality. No assumptions were violated in this test.

We used a Cox's proportional hazards model to test how malathion, predator cues, and Bd affected post-metamorphosis survival. This method allowed us to determine how experimental treatments altered the probability of death relative to the probability of death in a control treatment (e.g., the hazard ratio). Since a proportional hazard model uses individual data and individuals that shared a mesocosm as tadpoles may have correlated responses, we included mesocosm as a random effect nested within treatment. We used forward model selection based on P values and log likelihoods to choose the best model. Our model met the assumption of proportionality.

Results

Stage 1 results

Amphibian growth, development, and survival to metamorphosis.—The analysis of wood frog life history traits detected a multivariate effect of predator cue (Pillai's trace $F_{3,24} = 3.964\ 8$, $P = 0.020$,) but not pesticide (Pillai's trace $F_{6,50} = 1.028\ 8$, $P = 0.418$) or the predator-by-pesticide interaction (Pillai's trace, $F_{6,50} = 0.665\ 8$, $P = 0.678$). Subsequent univariate analyses indicated that the multivariate predator cue effect was driven by an effect of predators on time to metamorphosis ($F_{3,24} = 9.987$, $P = 0.004$), but not on survival

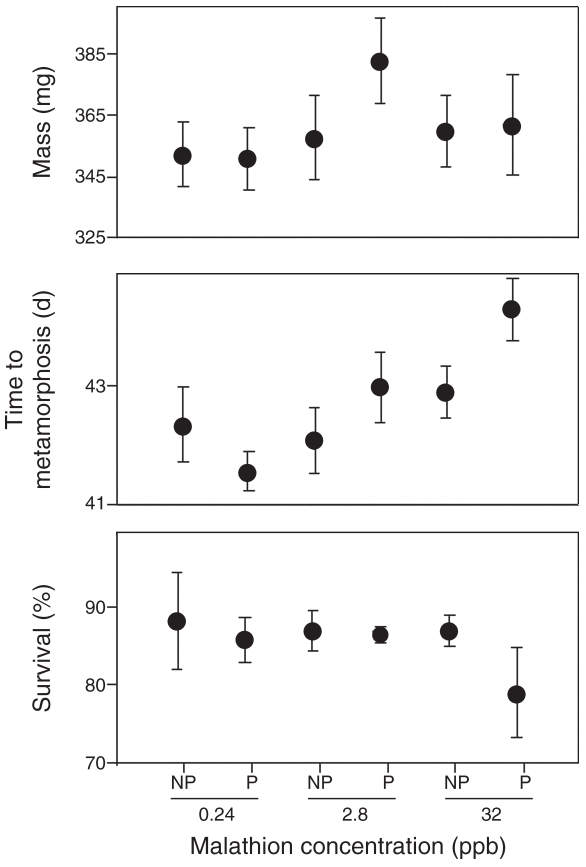

FIG. 1. Effects (means ± SE) of malathion concentration (0.24, 2.8, or 32 parts per billion [ppb]) and predator treatment (predator cues, P; no predator cues, NP) on the mass of wood frogs (*Lithobates sylvaticus*) at metamorphosis, time to metamorphosis, and survival to metamorphosis. Exposure to predator cues caused tadpoles to metamorphose ~1.5 days later than animals exposed to no-predator cues. No other effects were significant.

($F_{3,24} = 1.519$, $P = 0.229$) or mass at metamorphosis ($F_{3,24} = 1.962$, $P = 0.173$). Individuals exposed to predator cues took an average 1.5 days longer to metamorphose than individuals not exposed to predator cues (Fig. 1).

Release of skin peptides.—Averaged across all malathion treatments, skin peptides were 20% lower for animals that were exposed to predators compared to those not exposed to predators (Table 1, Fig. 2), though this effect was not significant. While there was not a significant malathion-by-predator interaction, the effect of predators was stronger in the 0- and 10-ppb treatments. There was also a marginally significant effect of the predator-by-mass interaction and a significant effect of replicate on skin peptides (Table 1). To better understand the predator-by-mass interaction, we examined the effect of the mass covariate within each predator cue treatment. In the no-predator treatments, skin peptides increased by 1.75 μg for every 1 mg increase in mass. This explained about 3% of the variation in peptide production in this treatment ($r^2 = 0.031$). In contrast, there was no correlation between peptide production and mass in the predator treatment ($r^2 = 0.0007$). In short, mass explained very little of the variation in peptides measured within each predator treatment. Malathion exposure had no effect on the production of antimicrobial peptides.

Antimicrobial peptide characterization.—Using mass spectrometry, we detected signals of five peptides in our samples. Of these, nearly complete amino acid sequences could be interpreted for two of them, based on spectra from tandem mass spectrometry (Table 2). The uncertainty occurred because we could not conclusively differentiate leucine from isoleucine. One of these near-complete sequences matched the sequence for brevinin-1SY, which has been previously described in wood frogs by Matutte et al. (2000). While this peptide has been observed in adult wood frogs, this is the first time it has been observed in recently metamorphosed wood frogs.

The other peptide yielded a sequence that has not been previously characterized and which we believe to be a

TABLE 1. Results of a partially nested ANCOVA testing the effects of exposure of wood frog (*Lithobates sylvaticus*) tadpoles to three malathion concentrations (0.24, 2.8, or 32 part per billion [ppb]) and two predator treatments (predator cues or no predator cues) on the production of skin peptides one week after metamorphosis.

Effects	df	*F*	*P*	Partial η^2
Mass	1, 157	0.980	0.324	0.006
Predator	1, 26	3.679	0.097	0.102
Pesticide	2, 26	1.234	0.308	0.087
Replicate (predator × pesticide)	26, 157	1.675	**0.029**	0.217
Predator × pesticide	2, 26	0.179	0.837	0.014
Pesticide × mass	2, 26	1.253	0.302	0.88
Mass × predator	1, 26	4.633	0.065	0.125
Mass × predator × pesticide	2, 26	0.157	0.882	0.10

Notes: In this analysis, mass was used as a covariate and the mesocosm where tadpoles were raised (replicate) was nested as a random effect within fixed treatment effects. All 0.24-ppb malathion treatments were replicated six times each. All other treatments were replicated five times. Partial η^2 approximates the effect size of each factor. Boldface type indicates *P* values that are <0.05.

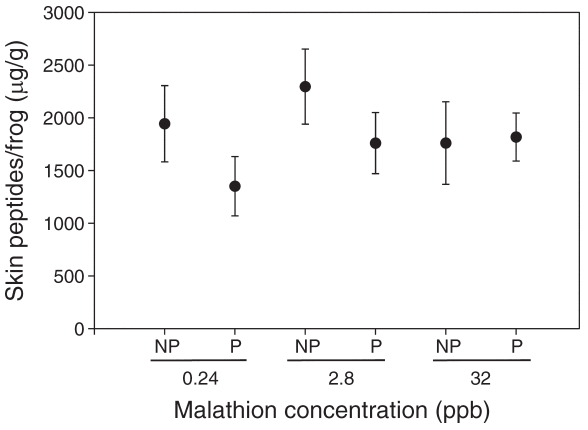

FIG. 2. Effects (means ± SE) of malathion concentration (0.24, 2.8, or 32 ppb) and predator treatment (predator cues, P; no predator cues, NP) on mass-adjusted production of peptides in the skin of wood frogs. To obtain the peptides, frogs were injected with 2 nmol norepinephrine-HCl/g eight days after metamorphosis. A nested ANCOVA showed that prior exposure to predators caused a nonsignificant 20% decrease in total skin peptides. There was no effect of malathion or a malathion × predator cue interaction.

member of the temporin family of peptides (Fig. 3). We have designated this peptide temporin-1SY according to a widely accepted convention for naming new peptides (Conlon 2008). It has a 28% match with a temporin consensus sequence and is highly similar in hydrophobicity (57% for temporin-1SY, 65% for the consensus), length (14 amino acids for temporin-1SY and 13 for the consensus), and charge (+1 for temporin-1SY and +2 average for most temporins) (Wade 2010). Moreover, the y1 series of this peptide was compatible with a 17 dalton increase over the residue mass, suggesting that the C-terminal residue was amidated, as is characteristic of many temporin peptides. The closest matches to temporin-1SY were all temporins from the ranidae family (see Table 2 for similar temporins).

Stage 2 results

Bd challenge.—The best fit proportional hazards model included the terms predator, pesticide, and Bd (Table 3). There were significant effects of Bd and predator cues on survival (Fig. 4). Exposure to Bd increased the risk of mortality nearly eightfold. Exposure to predator cues decreased the risk of mortality in both Bd-exposed and unexposed treatments by nearly 50%. Pesticide exposure and treatment interactions had no effect on the proportional hazards function.

DISCUSSION

This study tested the general hypothesis that predator cues and pesticides can alter the immune defenses and survival of animals exposed to pathogens. It has been proposed that natural and anthropogenic stressors can lead to increased infection prevalence, disease susceptibility, and disease-related population declines because they depress immune functions (Carey et al. 1999, Rollins-Smith 2001, Hayes et al. 2010, Martin et al. 2010). While previous tests of this hypothesis often focused solely on immunocompetence or responses to pathogens (reviewed in Graham et al. 2011), our study focused on both response variables. We examined how stressors experienced during the larval stage affected AMP availability and survival of Bd-exposed frogs post-metamorphosis. This is a life history stage when frogs have a limited immune capacity and often experience high rates of disease mortality in the field (Rollins-Smith 1998, Kiesecker 2002, Garner et al. 2009). We found a trend of reduced amount of hydrophobic skin peptides in wood frogs exposed to predator cues. Moreover, predator cues increased survival of both Bd-exposed and unexposed frogs. There was no effect of a short-term malathion exposure (beginning about five days prior to the beginning of metamorphosis) on the production of skin peptides quantified eight days after metamorphosis or on survival after exposure to Bd. To our knowledge, this is the first study examining the effects of stressor experienced prior to metamorphosis on the production of skin peptides after metamorphosis.

Immunocompetence

Exposure to predator cues during larval life caused a trend of fewer hydrophobic skin peptides released after a mild stimulus with norepinephrine in post-metamorphic

TABLE 2. Peptides found in wood frog metamorphs collected as eggs in Linesville, Pennsylvania, USA.

Peptide name	Organism	Molecular weight (monoisotopic)	Proposed sequence	Source
Brevinin-1SY	*Lithobates sylvaticus*	2440.3	FLPVVAGLAAKVIPSIICAVTKKC	Matutte et al. (2000); our findings
Temporin-1SY	*L. sylvaticus*	1521.8	!!FSA!GNA!SRNF-NH_2	our findings
Peptide A1	*Rana esculenta*	1388.8	FLPAIAGILSQLF-NH_2	Simmaco et al. (1990)
Temporin-Prb	*R. pretiosa*	1391.9	LLPIVGNLLKSLL-NH_2	Simmaco et al. (1996)
Temporin-Sc	*Pelophylax saharica*	1464.8	FLSHIAGFLSNLF-NH_2	Abbassi et al. (2008)
Temporin-1DYa	*R. dybowskii*	1404.8	FIGPIISALASLFG-NH_2	Conlon et al. (2007)
Temporin consensus			FLPILGSLLS(G/K)LL-NH_2	Wade (2010)

Notes: Peptide sequences were assigned manually to spectra collected using nano-flow electrospray liquid chromatography quadrapole time-of-flight tandem mass spectrometry. Since we could not differentiate leucine from isoleucine using these techniques, we marked these amino acids with an exclamation mark (!). The most similar temporins and the temporin consensus sequence are also shown.

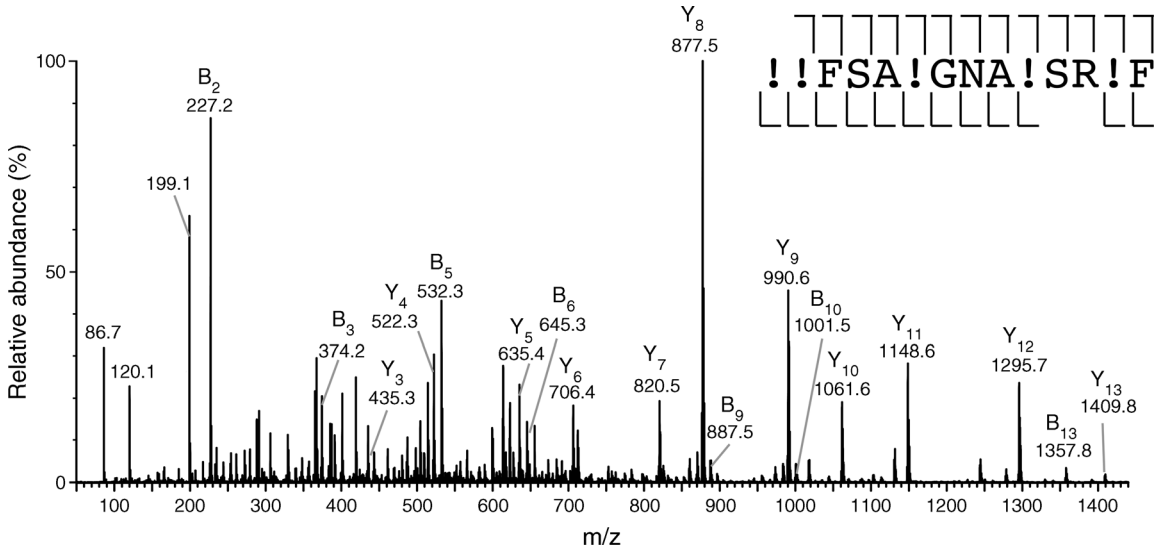

FIG. 3. Tandem mass spectrum for temporin-1SY (molecular weight 1521.8) acquired with nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF; Waters Corporation). Isoleucine and leucine (indicated by exclamation marks [!]) could not be differentiated using this technique. B-ions and Y-ions are indicated with subscripts indicating their order. B-ions extend from the amino terminus, and Y-ions extend from the C-terminus of the peptide.

individuals. While we did not quantify concentrations of specific antimicrobial peptides in different treatments, further research should examine whether small (e.g., 20%) reductions in total skin peptides such as those caused by predators could decrease concentrations of AMPs below levels that inhibit infection (Rollins-Smith et al. 2002*a*). In this experiment, the sample sizes for AMP measurements from each treatment were larger than those typically used in studies of AMP releases (e.g., Davidson et al. 2007, Schadich et al. 2009, Ramsey et al. 2010). The high variation in the amount of skin peptides within treatments suggests that many other unidentified factors may be influencing skin peptide releases.

Several physiological mechanisms could explain this trend. The skin peptides available shortly after metamorphosis would have been produced in the granular glands developed in the late stages of prometamorphosis and during climax of metamorphosis (Helff and Stark 1941, Bovbjerg 1963). Extended exposure to predator cues increases the release of glucocorticoids (Denver 2009), and elevated glucocorticoids are associated with the inhibition of AMP synthesis. Thus, reduced skin peptide concentrations in metamorphs that had been previously exposed to predator cues may have resulted from inhibition of antimicrobial peptide synthesis in these animals. Predator cues were administered until day 32 and some tadpoles began metamorphosis at day 33. This means that peptides were measured before frogs could restore depleted peptide stores (which can take as long as 30 days for frogs exposed to 20 nmol norepinephrine/g frog; Ramsey et al. 2010, Pask et al. 2012), if reduced synthesis of peptides had occurred. Alternatively, exposure to chronic stresses such as predators could cause altered sensitivity to stress hormones such as norepinephrine, which are involved in triggering AMP releases (McEwen and Seeman 2006). Further studies of predator effects on AMP production near the time of metamorphosis would assist in parsing apart these explanations.

We hypothesized that exposure to malathion would also reduce skin peptide defenses in wood frogs because malathion alters neuroendocrine signaling, which is necessary to stimulate the release of peptides (Rollins-Smith et al. 2001). We did not find an effect of malathion on the production of skin peptides or survival of recently metamorphosed frogs exposed to Bd. The lack of a malathion effect may have occurred because malathion does not affect skin peptide production or because it does not occur when amphibians are exposed to low concentrations or exposed for short durations.

TABLE 3. Effects on the risk of mortality of exposure of wood frogs as tadpoles to three malathion concentrations (0.24, 2.8, and 32 ppb nominal concentrations) and two predator treatments (predator cues or no predator cues), and effects of exposure of metamorphs to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd).

Fixed effects	Hazard ratio	*Z*	*P*
Predator	0.604	−3.022	**0.0025**
Pesticide	0.921	−0.848	0.40
Bd	7.721	10.325	**>0.0000**

Notes: Results are from a Cox's proportional hazard analysis. The mesocosm that frogs were raised in was included as a random effect in the model (results not shown). Boldface type indicates $P < 0.05$.

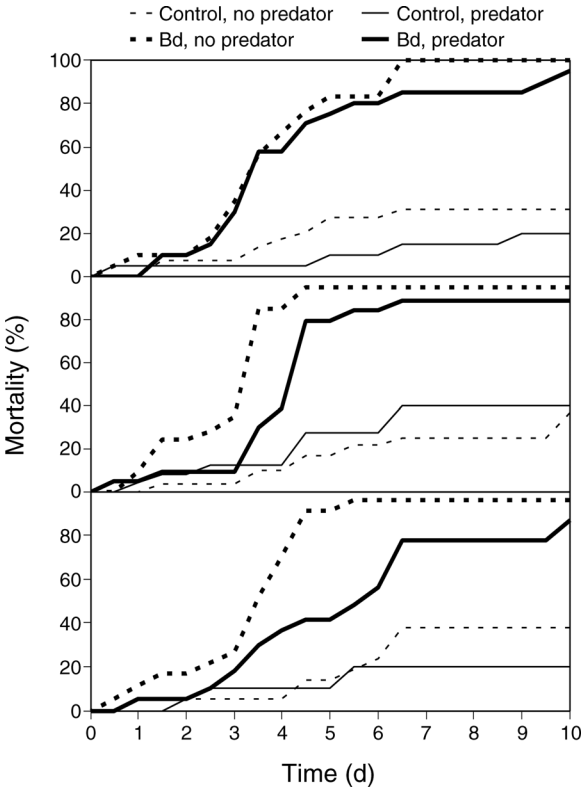

FIG. 4. Effects of exposure of wood frog tadpoles to malathion (0.24, 2.8, or 32 ppb) and caged predators or no predators on the survival of metamorphic wood frogs when exposed to infectious zoospores of the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) or a control broth lacking zoospores ($n = 245$). A Cox's proportional hazards model showed that exposure to *B. dendrobatidis* significantly increased the risk of death by a factor of 8, while exposure to predators decreased the risk of death by nearly 50%.

In contrast to our results, previous studies exploring effects of contaminants on AMPs (Davidson et al. 2007, Schadich et al. 2009) found that exposure to much higher concentrations (~480 ppb) of carbaryl (also an AchE-inhibiting insecticide) reduced AMPs collected from post-metamorphic frogs (*R. boylii* and *Litoria raniformis*) two to three days after exposure. The timing of exposure differed in these experiments. For example, Davidson et al. (2007) exposed metamorphs to the insecticide, whereas we exposed late-stage tadpoles. In the current study, malathion treatments were applied in a manner that would isolate direct effects of malathion on immune functions. Thus, applications were at a much lower concentration than what is lethal to amphibians (e.g., LC50 values range from 1.3 to 5.9 mg/L; Relyea 2004) and they were applied late in development (approximately one week before metamorphosis), so that the wood frogs metamorphosed before indirect effects in the food web could affect life history traits and resource acquisition (e.g., by a trophic cascade). As we expected, this late, brief application had no effect on survival, growth, or development. Though we did not test it here, exposing tadpoles to malathion early enough in their ontogeny to alter growth and development could indirectly lead to altered AMP production by altering acquisition and allocation of resources to life history traits and immune function (Groner and Relyea 2011).

Antimicrobial peptides (AMPs)

The AMPs observed by mass spectrometry in this study have the potential to inhibit Bd. Although the potency of brevinin-1SRY against Bd has not been tested, other brevinins (from other ranid frogs) have varying degrees of antimicrobial activities against pathogens, including Bd (reviewed in Rollins-Smith 2009). The presence of this peptide in metamorphs suggests that they may be able to use AMPs to defend themselves against microbial pathogens during this vulnerable life stage. Temporins can also inhibit Bd growth in vitro (Rollins-Smith et al. 2003). The level of inhibition is dependent upon the ability to attach to the fungal cell membrane, the formation of α-helix after attachment (thought to be necessary for membrane disruption) and resistance against fungal proteases (Rollins-Smith et al. 2003). Temporin-1SY is predicted to form an α-helix, which would yield seven hydrophobic amino acids on a single surface, indicating that it has strong membrane-binding potential (Wang and Wang 2004). Molecular analyses of synonymous mutations of peptide gene sequences suggest that both temporins and brevinins have been under diversifying selection across populations and balancing selection within populations (Duda et al. 2002, Tennessen and Blouin 2007). This suggests that the peptides identified in this study may be adapted to the local microbial environment.

Response to Bd exposure

We hypothesized that a decrease in the amount of skin peptides would be correlated with increased mortality in Bd treatments. Contrary to our expectation, we found that animals exposed to predator cues survived longer in both the Bd and Bd-free treatments. This suggests that the early exposure to predators primed these frogs to be better equipped to respond to pathogens and other stressors later in life. One possible reason for the apparent discrepancy between the trend of decreased peptides and the increased survival in predator-exposed animals is the time lag between these measurements. While this 22-day difference was necessary to allow all frogs to metamorphose and acclimate to a new laboratory environment before the Bd challenge, adult amphibians have been shown to recover peptide storage after release due to norepinephrine induction after about 30 days (Ramsey et al. 2010). Understanding the mechanism and conditionality behind beneficial responses to stressors earlier in life will aid in interpreting how early and chronic exposure to stressors and allostatic loads manifest later in life.

Exposure to malathion did not alter survival of frogs exposed to Bd. This result is not surprising given that

PLATE 1. Adult wood frog (*Lithobates sylvaticus*). Photo credit: María Forzán.

malathion did not alter skin peptide production; however, mortality from Bd exposure occurred so rapidly in this study that we did not test for effects of exposure on pathogen load, growth, or behavior. Thus, we cannot rule out the possibility that malathion could alter responses to a lower dose of infectious zoospores or less virulent strains of Bd.

Conclusions

Understanding how multiple stressors interact to affect survival and physiology is an important goal of ecology. This is an especially timely challenge in the case of emerging infectious diseases (EIDs), where there is a pressing need to understand how natural and anthropogenic stressors in the environment alter immune function and disease rates. This experiment found that exposure of wood frog tadpoles to predator cues tended to reduce the production of skin peptides in newly emerged metamorphs. However, this reduction was not associated with increased mortality in predator-exposed animals. Instead, predator stress increased survival of both Bd-exposed and Bd-free animals. Positive effects of predator stress on animals later in life suggests that stresses experienced early in development may prime individuals to tolerate stress later in life. We did not find any effects of exposure to malathion on production of skin peptides or survival after pathogen exposure. In contrast, other experiments testing effects of malathion exposure on cellular and humoral immune responses in adult amphibians have found strong negative effects; however, they have not examined delayed effects of stress exposure across life history stages. We encourage more exploration to reveal the mechanisms underlying variation in effects of stressors on immune functions, disease susceptibility and acclimation to future stress.

ACKNOWLEDGMENTS

Abhinav Mithal, Heather Shaffery, and Caroline Scott helped in the execution of this experiment. David Wade and Dick Zimmer provided useful communications about peptide characterization. Henrik Stryhn, Jill Anderson, and Steven Tonsor provided useful advice about the data analysis. Funding was provided by a Pape grant, Chicago Herpetological Society Grant, Sigma Xi GIAR, and a Gaige Award to M. L. Groner, NSF graduate research fellowships to J. C. Buck and M. L. Groner, NSF grants O619536 and 0843207 to L. A. Rollins-Smith, and NSF grant 05-18250 to R. A. Relyea. Funding for the Waters QTOF2 mass spectrometer and nanoflow ESI equipment was from Carnegie Mellon Biotechnology Initiative Fund and a Faculty Development Fund, respectively, awarded to M. E. Bier.

LITERATURE CITED

Abbassi, F., B. Oury, T. Blasco, D. Sereno, G. Bolbach, P. Nicolas, K. Hani, M. Amiche, and A. Ladram. 2008. Isolation, characterization and molecular cloning of new temporins from the skin of the North African ranid *Pelophylax saharica*. Peptides 29:1526–1533.

Bancroft, B. A., N. J. Baker, and A. R. Blaustein. 2007. A meta-analysis of the effects of ultraviolet B radiation and its synergistic interactions with pH, contaminants, and disease on amphibian survival. Conservation Biology 22:987–996.

Blaustein, A. R., S. S. Gervasi, P. T. J. Johnson, J. T. Hoverman, L. K. Belden, P. W. Bradley, and G. Y. Xie. 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B 367:1688–1707.

Blaustein, A. R., B. A. Han, R. A. Relyea, P. T. J. Johnson, J. C. Buck, S. S. Gervasi, and L. B. Kats. 2011. The complexity of amphibian population declines: Understanding the role of co-factors in driving amphibian losses. Annals of the New York Academy of Sciences 1223:108–119.

Bovbjerg, A. M. 1963. Development of the glands of the dermal plicae in *Rana pipiens*. Journal of Morphology 113:321–243.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141–148.

Bradford, D. F., R. A. Knapp, D. W. Sparling, M. S. Nash, K. A. Stanley, N. G. Tallent-Halsell, L. L. McConnell, and S. M. Simonich. 2011. Pesticide distributions and population declines of California, USA, alpine frogs, *Rana muscosa* and *Rana sierrae*. Environmental Toxicology and Chemistry 30:682–691.

Brock, T. C. M., R. P. A. Van Wijngaarden, and G. Van Geest. 2000. Ecological risks of pesticides in freshwater ecosystems. Part 2: Insecticides. Report 089. Alterra, Green World Research, Wageningen, The Netherlands.

Buck, J. C., E. A. Scheessele, R. A. Relyea, and A. R. Blaustein. 2012. The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians. Freshwater Biology 57:61–73.

Carey, C., N. Cohen, and L. A. Rollins-Smith. 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23:459–472.

Christin, M., A. D. Gendron, P. Brousseau, L. Ménard, D. J. Marcogliese, R. S. Cyr, and M. Fournier. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and its resistance to parasitic infection. Environmental Toxicology and Chemistry 22:1127–1133.

Conlon, J. M. 2008. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. Peptides 29:1815–1819.

Conlon, J. M., J. Kolodzieiek, N. Nowotny, J. Leprince, H. Vaurdy, L. Coquet, T. Jouenne, and S. Iwamuro. 2007. Cytolytic peptides belonging to the brevinin-1 and brevinin-2 families isolated from the skin of the Japanese brown frog, *Rana dybowskii*. Toxicon 50:746–756.

Corbet, P. S. 1999. Dragonflies: behaviour and ecology of Odonata. Harley Books, Colchester, UK.

Davidson, C. 2004. Declining downwind: amphibian population declines in California and historic pesticide use. Ecological Applications 14:1892–1902.

Davidson, C., M. Benard, H. B. Shaffer, J. Parker, C. O'Leary, J. M. Conlon, and L. A. Rollins Smith. 2007. Effects of chytrid infection and carbaryl exposure on survival, growth and antimicrobial peptide defenses in foothill yellow-legged frogs (*Rana boylii*). Environmental Science and Technology 41:1771–1776.

Denver, R. J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. General and Comparative Endocrinology 164:20–31.

Duda, T. F., Jr., D. Vanhoye, and P. Nicolas. 2002. Roles of diversifying selection and coordinated evolution in the evolution of amphibian antimicrobial peptides. Molecular Biology and Evolution 19:858–864.

Fisher, M. C., T. W. J. Garner, and S. F. Walker. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual Review of Microbiology 63:291–310.

Forson, D. D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecological Applications 16:2325–2332.

Galloway, T., and R. Handy. 2003. Immunotoxicity of organophosphorous pesticides. Ecotoxicology 12:345–363.

Garner, T. W. J., S. Walker, J. Bosch, S. Leech, J. M. Rowcliffe, A. A. Cunningham, and M. C. Fisher. 2009. Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. Oikos 118:783–791.

Gilbertson, M., G. D. Haffner, K. G. Drouillard, A. Albert, and B. Dixon. 2003. Immunosuppression in the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. Environmental Toxicology and Chemistry 22:110–110.

Gosner, K. L. 1960. A simple table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:83–190.

Graham, A. L., D. M. Shuker, L. C. Pollitt, S. K. J. R. Auld, A. J. Wilson, and T. J. Little. 2011. Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Functional Ecology 25:5–17.

Groner, M. L., and R. A. Relyea. 2011. A tale of two pesticides: how common insecticides affect aquatic communities. Freshwater Biology 56:2391–2404.

Han, B. A., C. L. Searle, and A. R. Blaustein. 2011. Effects of an infectious fungus, *Batrachochytrium dendrobatidis*, on amphibian predator-prey interactions. PLoS ONE 6:e16675.

Harris, R. N., et al. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Hayes, T. B., et al. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environmental Health Perspectives 114:40–50.

Hayes, T. B., P. Falso, S. Gallipeau, and M. Stice. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology 213:921–933.

Hayes, T. B., and T. N. Gill. 1995. Hormonal regulation of skin gland development in the toad (*Bufo boreas*): the role of the thyroid hormones and corticosterone. General and Comparative Endocrinology 99:161–168.

Helff, O. M., and W. Stark. 1941. Studies on amphibian metamorphosis. XVIII. The development of structures in the dermal plicae of *Rana sylvatica*. Journal of Morphology 68:303–329.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. Global trends in emerging infectious diseases. Nature 451:990–993.

Kiely, T., D. Donaldson, and A. Grube. 2004. Pesticide industry sales and usage: 2000 and 2001 market estimates. US EPA, Washington, D.C., USA.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences USA 99:9900–9904.

Kinter, M., and N. E. Sherman. 2000. Protein sequencing and identification using tandem mass spectrometry. Wiley and Sons, New York, New York, USA.

Kollros, J. J., and J. C. Kaltenbach. 1952. Local metamorphosis of larval skin in *Rana pipiens*. Physiological Zoology 25:163–170.

Martin, L. B., W. A. Hopkins, L. D. Mydlarz, and J. R. Rohr. 2010. The effects of anthropogenic global change on immune functions and disease resistance. Annals of the New York Academy of Sciences 1195:129–148.

Matutte, B., K. B. Storey, F. C. Knoop, and J. M. Conlon. 2000. Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica*, in

response to environmental stimuli. FEBS Letters 483:135–138.

McEwen, B. S., and T. Seeman. 2006. Protective and damaging effects of mediators of stress: elaborating and testing the concepts of allostasis and allostatic load. Annals of the New York Academy of Sciences 896:30–47.

McMahon, T., P. Crumrine, N. Halstead, S. Johnson, T. R. Rattel, J. Romansic, and J. R. Rohr. 2011. Chlorothalonil: a deadly, immunomodulatory, and corticosterone-inducing fungicide to amphibians. Environmental Health Perspectives 119:1098–1103.

Odenkirchen, E., and S. P. Wente. 2007. Risks of malathion use to the federally listed California red-legged frog (*Rana aurora draytonii*). U.S. EPA Environmental Fate and Effects Division, Washington, D.C., USA.

Pask, J. D., D. C. Woodhams, and L. A. Rollins-Smith. 2012. The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. Global Change Biology 18:1231–1238.

R Development Core Team. 2012. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramsey, J. P., L. K. Reinert, L. K. Harper, D. C. Woodhams, and L. A. Rollins-Smith, LA. 2010. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. Infection and Immunity 78:3981–3992.

Relyea, R. A. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environmental Toxicology and Chemistry 23:1080–1084.

Rohr, J. R., T. R. Raffel, S. K. Sessions, and P. J. Hudson. 2008. Understanding the net effects of pesticides on amphibian trematode infections. Ecological Applications 18:1743–1753.

Rollins-Smith, L. A. 1998. Metamorphosis and the amphibian immune system. Immunological Review 166:221–230.

Rollins-Smith, L. A. 2001. Neuroendocrine-immune system interactions in amphibians: implications for understanding global amphibian declines. Immunologic Research 23:273–280.

Rollins-Smith, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochimica et Biophysica Acta 1788:1593–1599.

Rollins-Smith, L. A., C. Carey, J. M. Conlon, L. K. Reinert, J. K. Doersam, T. Bergman, J. Silberring, H. Lankinen, and D. Wade. 2003. Activities of temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. Antimicrobial Agents and Chemotherapy 47:1157–1160.

Rollins-Smith, L. A., J. K. Doersam, J. E. Longcore, S. K. Taylor, J. C. Shamblin, C. Carey, and M. A. Zasloff. 2002*a*. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Developmental and Comparative Immunology 26:63–72.

Rollins-Smith, L. A., J. P. Ramsey, J. D. Pask, L. K. Reinert, and D. C. Woodhams. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integrative and Comparative Biology 51:552–562.

Rollins-Smith, L. A., L. K. Reinert, V. Miera, and J. M. Conlon. 2002*b*. Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. Biochemical and Biophysical Research Communications 297:361–367.

Romero, L. M. 2004. Physiological stress in Ecology: lessons from biomedical research. Trends in Ecology and Evolution 19:249–255.

Russell, D. M., C. S. Coldberg, L. P. Walts, and E. B. Rosenblum. 2010. *Batrachochytrium dendrobatidis* infection dynamics in the Columbia spotted frog, *Rana luteiventris*, in north Idaho, USA. Diseases of Aquatic Organisms 92:223–230.

Schadich, E., A. L. J. Cole, D. Mason, and M. Squire. 2009. Effect of the pesticide carbaryl on the production of the skin peptides of *Litoria raniformis* frogs. Australasian Journal of Ecotoxicology 15:17–24.

Schoeppner, N. M., and R. A. Relyea. 2008. Detecting small environmental differences: Risk-response curves for predator-induced behavior and morphology. Oecologia 154:743–754.

Searle, C. L., S. S. Gervasi, J. Hua, J. I. Hammond, R. A. Relyea, D. Olson, and A. R. Blaustein. 2011. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. Conservation Biology 25:965–974.

Simmaco, M., A. Boman, M. L. Mangoni, G. Mignongna, R. Miele, D. Barra, and H. G. Boman. 1997. Effect of glucocorticoids on the synthesis of antimicrobial peptides in the skin. FEBS Letters 416:273–275.

Simmaco, M., D. de Biase, C. Severini, M. Alta, G. F. Erspamer, D. Barra, and F. Bossa. 1990. Purification and characterization of bioactive peptides from skin extracts of *Rana esculenta*. Biochimica et Biophysica Acta 1033:318–323.

Simmaco, M., G. Mignogna, S. Canofeni, R. Miele, M. L. Mangoni, and D. Barra. 1996. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. European Journal of Biochemistry 242:788–792.

SPSS. 2009. PASW statistics for Windows. Version 18.0. SPSS, Chicago, Illinois, USA.

Tennessen, J. A., and M. S. Blouin. 2007. Selection for antimicrobial peptide diversity in frogs leads to gene duplication and low allelic variation. Journal of Molecular Evolution 65:605–615.

Wade, D. 2010. Preview: Temporin-/Vespid chemotactic peptide- (or T/V-) like peptides and their consensus sequences. Wade Research Foundation Reports 5:1.

Wang, Z., and G. Wang. 2004. APD: the Antimicrobial Peptide Database. Nucleic Acids Research 32:D590–D592.

Woodhams, D. C., L. A. Rollins-Smith, C. Carey, L. K. Reinert, M. J. Tyler, and R. A. Alford. 2006. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531–540.