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RESEARCH ARTICLE

Interactive effects of competition and predator cues on immune responses of leopard frogs at metamorphosis

Maya L. Groner[1,*], Louise A. Rollins-Smith[2,3], Laura K. Reinert[2], John Hempel[4], Mark E. Bier[5] and Rick A. Relyea[4]

ABSTRACT

Recent hypotheses suggest that immunosuppression, resulting from altered environmental conditions, may contribute to the increased incidence of amphibian disease around the world. Antimicrobial peptides (AMPs) in amphibian skin are an important innate immune defense against fungal, viral and bacterial pathogens. Their release is tightly coupled with release of the stress hormone noradrenaline (norepinephrine). During metamorphosis, AMPs may constitute the primary immune response in the skin of some species because acquired immune functions are temporarily suppressed in order to prevent autoimmunity against new adult antigens. Suppression of AMPs during this transitional stage may impact disease rates. We exposed leopard frog tadpoles (*Lithobates pipiens*) to a factorial combination of competitor and caged-predator environments and measured their development, growth and production of hydrophobic skin peptides after metamorphosis. In the absence of predator cues, or if the exposure to predator cues was late in ontogeny, competition caused more than a 250% increase in mass-standardized hydrophobic skin peptides. Predator cues caused a decrease in mass-standardized hydrophobic skin peptides when the exposure was late in ontogeny under low competition, but otherwise had no effect. Liquid chromatography tandem mass spectrometry of the skin peptides showed that they include six AMPs in the brevinin and temporin families and at least three of these peptides are previously uncharacterized. Both of these peptide families have previously been shown to inhibit harmful microbes including *Batrachochytrium dendrobatidis*, the fungal pathogen associated with global amphibian declines. Our study shows that amphibians may be able to adjust their skin peptide defenses in response to stressors that are experienced early in ontogeny and that these effects extend through an important life-history transition.

KEY WORDS: Amphibian declines, Antimicrobial peptides, Predators, Competition, Eco-immunology, Disease ecology

INTRODUCTION

Changing environmental conditions are hypothesized to influence the epidemiology of many diseases (e.g. Jones et al., 2008). One mechanism for increased susceptibility to disease is when environmental changes alter critical immune responses against pathogens (Råberg et al., 1998; Carey et al., 1999; Hawley and Altizer, 2011; Raffel et al., 2010; Rollins-Smith et al., 2011). For example, amphibians are declining around the globe, and the emerging infectious diseases caused by ranaviruses and *Batrachochytrium dendrobatidis* (Bd) are contributing to these trends (Daszak et al., 2003). Recent literature suggests that stress-induced immunosuppression may play a role in amphibian declines by increasing the prevalence and virulence of amphibian diseases, but the hypothesis requires a great deal of research to understand when and how it may apply (Carey, 1993; Carey et al., 1999; Daszak et al., 2003; Fisher et al., 2009).

Amphibians have evolved a complex suite of innate and acquired defenses against infections (Clarke, 1997; Richmond et al., 2009; Robert and Ohta, 2009; Rollins-Smith et al., 2011). One important innate immune response is the array of antimicrobial peptides (AMPs) that are released from granular glands onto the skin as part of the sympathetic stress response. Many pathogens that negatively impact amphibian populations can infect the skin, including the bacterium *Aeromonas hydrophila*, the chytrid fungus Bd, the water mold *Saprolegnia ferax* and iridoviruses (Hird et al., 1981; Romansic et al., 2009; Rollins-Smith et al., 2011). AMPs from frogs inhibit the growth of gram-negative and gram-positive bacteria and fungal pathogens including Bd (reviewed in Rollins-Smith and Conlon, 2005; Rollins-Smith et al., 2011). In addition to direct effects on pathogens, it has been hypothesized that AMPs also influence the diversity of beneficial skin bacteria that inhibit pathogen growth (Harris et al., 2009).

The role of AMPs in combating skin infections may be crucial during amphibian metamorphosis (Rollins-Smith et al., 2011). Many components of the acquired immune system (e.g. lymphocyte numbers, lymphocyte viability and mitogen-induced proliferation) are inhibited during metamorphosis to prevent autoimmunity against the new adult-specific antigens produced during this period (Rollins-Smith, 1998). At the same time, AMPs begin to be released in substantial quantities, although the suite of peptides synthesized may not be a full adult set (Bovbjerg, 1963). Therefore, AMPs may be one of the only available defenses against infection during metamorphosis. Substantial mortality and morbidity due to infection often coincides with metamorphosis; however, it is unclear whether this is a result of reduced immune function, increased exposure to pathogens, or some combination of the two (e.g. Green et al., 2002; Garner et al., 2009).

Stress resulting from competition for resources and the threat of predation may have particular relevance for the production and release of AMPs in tadpoles and metamorphs. Both stressors induce behavioral, morphological and life-history responses (e.g. Relyea, 2004; Relyea, 2007), which could result in reduced allocation towards immune function. This hypothesis is further supported by hormonal regulation of life-history traits and AMP functions. The

[1]Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada, C1A 4P3. [2]Departments of Microbiology and Immunology and of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA. [3]Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA. [4]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA. [5]Center for Molecular Analysis, Department of Chemistry, Carnegie Mellon University, Pittsburgh, PA 15213, USA.

*Author for correspondence (mgroner@upei.ca)

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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), yet excessive glucocorticoids inhibit the maturation of granular glands (Hayes and Gill, 1995). Chronic exposure to predators and high competition for resources results in increased concentrations of glucocorticoids in tadpoles (Glennemeier and Denver, 2002; Denver, 2009). Thus, one might expect this to slow the maturation of the granular glands. In contrast, short-duration exposure to predators results in the release of an alarm pheromone that inhibits the hypothalamo-pituitary–adrenal (HPA) axis and reduces glucocorticoids in tadpole tissues (Fraker et al., 2009). This suggests that short durations of predator exposure may not slow development of the granular gland.

The effects of predators and competitors may also be interactive. Exposure to predators alters many plastic traits including life-history responses and inducible defenses (e.g. Relyea, 2004); however, the magnitude of these responses often decreases in highly competitive environments as a result of reduced acquisition of resources, altered allocation of resources and altered neuroendocrine signaling (e.g. Rollins-Smith, 1998; Relyea, 2004). Therefore, we suspect that competition may also modulate the effects of predator exposure on AMP production and release.

The timing of exposure to environmental stressors may also influence AMPs by altering the sensitivity of the HPA axis later in life (Romero, 2004; Denver, 2009, Martin, 2009). Exposure to stressors early in development can have profound and long-lasting effects on the HPA axis. For example, reduced food intake in tadpoles causes increased HPA axis activity in metamorphs (Hu et al., 2008). Similarly, tadpoles injected with stress hormones (e.g. corticosterone) show altered neuroendocrine gene expression post-metamorphosis, suggesting a permanent or lingering effect of stress (Denver, 2009). Collectively, these data suggest that chronic or early exposure to stressors could alter the production of stress hormones, with potential consequences for the development of granular glands and the production and release of AMPs.

We explored how the timing and duration of predator stress under low and high competition during the larval stage affected the amount of peptides released into the skin of northern leopard frogs, *Lithobates pipiens* (Schreber), after metamorphosis. In doing so, we tested four hypotheses: (1) the stress of competitors and predators would reduce skin peptide production, (2) chronic predator stress would reduce skin peptide production more than acute predator stress, (3) acute predator stress early in development would reduce skin peptides more than acute predator stress late in development, and (4) predator and competitor stress would have synergistic effects on skin peptide production.

RESULTS

Life-history traits

The MANOVA on the life-history traits of leopard frogs (survival to metamorphosis, time to metamorphosis and mass at metamorphosis) showed significant effects of competition (Pillai's Trace $F_{3,21}$=92.7, P<0.001) and predator cues (Pillai's Trace $F_{9,69}$=2.4, P=0.022), but not their interaction (Pillai's Trace $F_{9,69}$=0.9, P=0.494).

The multivariate effect of competition was driven by all three response variables (Table 1, Fig. 1). Compared with the low-competition treatments, 35% fewer leopard frogs metamorphosed in the high-competition treatments. Of those animals that did not metamorphose, 55% failed to metamorphose because they died during the experiment while 45% did not metamorphose because they did not develop rapidly enough before the experiment ended. Frogs in the high-competition treatments metamorphosed 15 days later and were 44% smaller than frogs in the low-competition treatments.

The multivariate effect of predator cues was driven by mass at metamorphosis (see Table 1, Fig. 1). Compared with the no-cue treatment, frogs in the early-cue and late-cue treatments were 16% larger (Tukey's honestly significantly difference, HSD, P<0.03). There were no effects of predator cues on survival or time to metamorphosis (Tukey's HSD, all P>0.13).

Skin peptides

There was a significant univariate effect of competition and a marginal competitor-by-predator interaction on mass-standardized skin peptides (Table 2, Fig. 2). Despite having a smaller size, metamorphs raised under conditions of high competition produced, on average, 53% more skin peptides per unit mass than metamorphs raised with low competition. The marginal interaction occurred because the degree to which peptide levels increased as a result of competition varied with predator treatment. In the no-cue treatment, high competition caused a 268% increase in mass-standardized peptide production (Fisher's least significant difference, LSD, P=0.007). In the early-cue and late-cue treatments, competition caused 198% and 564% increases in mass-standardized peptide production, respectively (Fisher's LSD, P=0.052, P<0.001, respectively). In the chronic-cue treatment, however, competition had no effect on skin peptides (Fisher's LSD, P=0.219).

We also examined the effects of predator cues within each competition treatment. Under low competition, mean comparisons indicated that mass-standardized skin peptides were not different from each other (Fisher's LSD, all P>0.16) except for the late-cue treatment, which was 61% lower than the chronic-cue treatment (Fisher's LSD, P=0.04). Under high competition, mean comparisons indicated that mass-standardized peptides did not differ among predator treatments (Fisher's LSD, all P>0.11).

Peptide characterization

Mass spectrometry showed at least six unique *m/z* corresponding to monoisotopic masses of 1427.1, 1875.2, 2569.6, 2593.9, 2623.9 and 2877.0. Tandem mass spectrometry showed that collisional activation of these mass peaks produced fragmentation patterns suggestive of peptides. Only one of these peptides could be fully sequenced (except for isobaric residues of leucine and isoleucine,

Table 1. Results of ANOVA on the effects of competition and predator cues on the survival to metamorphosis, mass at metamorphosis and time to metamorphosis of leopard frogs

Effect	d.f.	Survival to metamorphosis	Mass at metamorphosis	Time to metamorphosis
Model	(7, 24)	6.391 (0.001)	41.263 (0.001)	18.078 (0.001)
Competition	(1, 24)	39.6 (0.001)	274.2 (0.001)	120.7 (0.001)
Predator cues	(3, 24)	1.317 (0.290)	4.082 (0.018)	0.627 (0.590)
Competition×predator cues	(3, 24)	0.388 (0.763)	0.806 (0.503)	1.35 (0.282)

For each response variable, *F*-values are listed first followed by *P*-values in parentheses.

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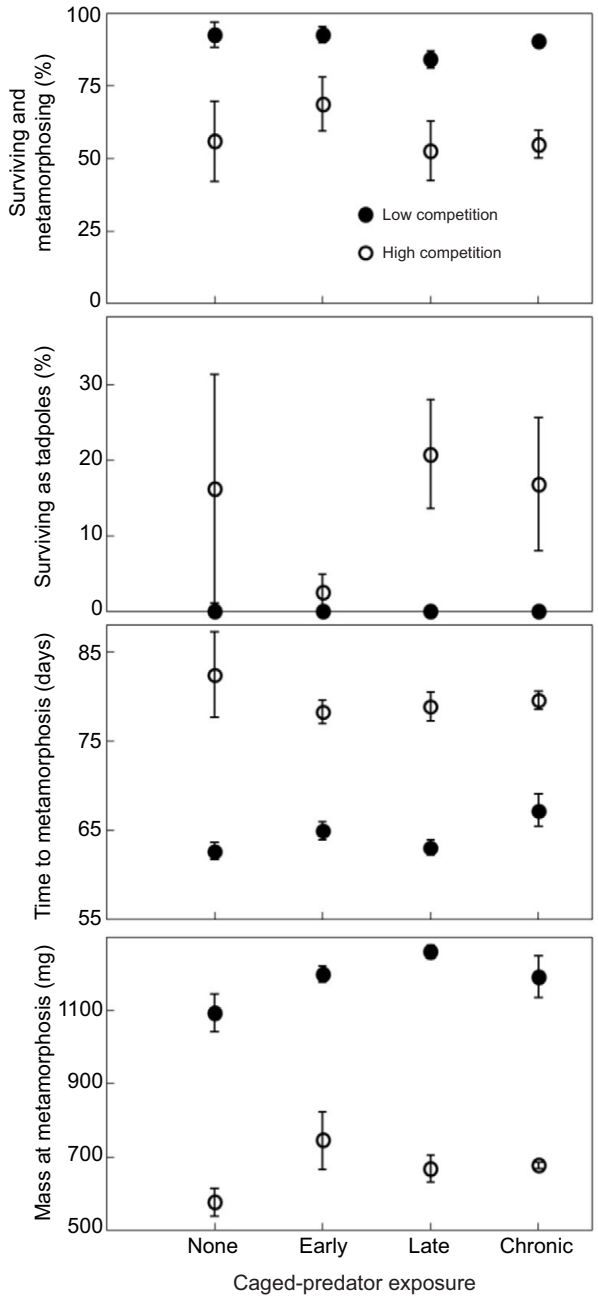

Fig. 1. Effects of competition and predator cue treatments on leopard frog life history and survival. Data are means ± s.e.m. ANOVA showed that higher densities of tadpoles significantly decreased survival, and development and growth rates, while exposure to predators significantly increased mass at metamorphosis relative to animals not exposed to predators.

which were left unidentified) from the spectra that we obtained. In the other cases we suspect that cysteines in the sequence yielded C-terminal cyclization via disulfide bonds, a common structure in AMPs, and such a loop prevented sequencing towards the carboxy-terminal end using our methodology. No reduction of these bonds was attempted. As would be predicted by such bridge structures, MS/MS spectra showed strong sequence signal, followed by an abrupt loss of signal (Kinter and Sherman, 2000). Despite incomplete sequence determination, the sequences obtained were sufficient to categorize these peptides into appropriate families.

Table 2. Results of an ANOVA examining the effects of larval competition and predator cue treatments on the amount of hydrophobic skin peptides produced by leopard frogs 9 days after metamorphosis

Effect	d.f.	Mass-standardized peptides
Model	(7, 24)	5.8 (<0.001)
Competition	(1, 24)	32.3 (<0.001)
Predator cues	(3, 24)	0.008 (0.943)
Competition×predator cues	(3, 24)	3.7 (0.067)

For each response variable, *F*-values are listed first followed by *P*-values in parentheses.

Mass spectrometry led us to conclude that the metamorphs in our study were producing brevinins and temporins (Fig. 3; supplementary material Fig. S1). Currently, there are 13 identified brevinin peptides that leopard frogs produce (supplementary material Table S1). Nearly identical partial sequences and molecular weights with known brevinins suggest that three of these partial sequences are brevinin-1Pd, brevinin-1Pg and brevinin-1Pla. The other two partial sequences do not match the sequences or molecular weights of known brevinins of leopard frogs, suggesting that these may be novel antimicrobial peptides.

We also sequenced an apparently novel temporin with molecular weight 1427.1; temporin-2P (Fig. 3). This is the second temporin that has been identified in leopard frog skin. The mass ion of this peptide is compatible with C-terminal amidation of the imputed structure, which is compatible with known temporins (supplementary material Table S2). Moreover, hydrophobicity and hydrophilicity were nearly homologous (84%) with the published temporin consensus sequence (Wade, 2010).

DISCUSSION

This experiment tested the effects of competition and the timing and duration of exposure to predator cues on life-history traits and the production of skin peptides in leopard frogs. Competition induced more than a 250% increase in mass-standardized skin peptides when predators were absent or after a short exposure to predator cues late in larval development. However, competition had little or no effect when tadpoles were exposed to predator cues chronically or for only a week early in development. This research, which is only the second study of the effects of both competition and predator cues on immune functions in amphibians (Raffel et al., 2010), shows that stress experienced early in development or chronically throughout development can have lasting effects on skin defenses during highly sensitive life-history stages later in life.

Both competition and predator cues altered life-history traits of leopard frogs. Tadpoles reared in high-competition environments had 19% lower survival, 44% lower growth and took 15 days longer to develop. Much of the mortality occurred in the last 4 weeks of the experiment (i.e. after experiment day 74), so reduced survival changed the competitive environment after the majority of tadpoles in the low-competition environments had metamorphosed. Of the tadpoles surviving in the high-competition treatments, only 80% developed enough to metamorphose before the experiment ended, and those that did were just over half the size of metamorphs raised in the low-competition treatments. These results are consistent with past studies that show negative effects of competition on the life-history traits of frogs (e.g. Relyea and Hoverman, 2003; Relyea, 2004). Decreased growth and development rates in tadpoles are often considered indicators of lower fitness in amphibians because they have been correlated with decreases in adult survival, growth

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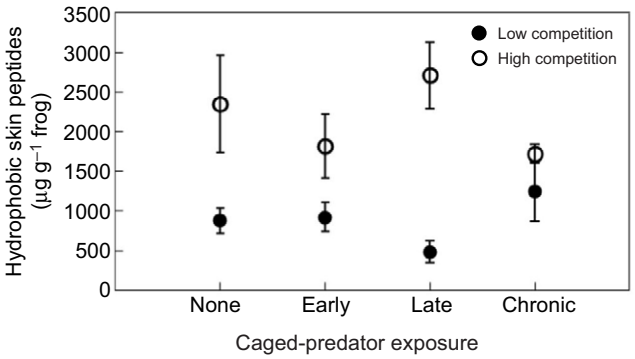

Fig. 2. Effects of competition and predator cue treatments on production of hydrophobic skin peptides by leopard frogs 9 days after metamorphosis. ANOVA showed that high competition significantly increased the production of peptides and that the timing and duration of predator exposure significantly altered the effect of competition.

rates and size at reproduction (e.g. Smith, 1987; Semlitsch et al., 1988; Altwegg and Reyer, 2003).

Exposure to predator cues altered the size of metamorphosing frogs in both the early- and late-cue treatments, but not in the chronic-cue treatment. The lack of an effect of chronic exposure to predator cues on survival, development and growth at metamorphosis is consistent with past studies; meta-analyses by Benard (Benard, 2004) and Relyea (Relyea, 2007) show that while it induces a morphological response in tadpoles, exposure to caged predators rarely causes changes in size or time to metamorphosis. It is interesting that both early-cue and late-cue treatments resulted in larger frogs at metamorphosis whereas the chronic-cue treatment did not. We suspect that faster growth of tadpoles exposed to short pulses of predator cues results from a plastic response that was adapted for a longer exposure to predator cues than these tadpoles experienced.

Competition increased the amount of mass-standardized hydrophobic peptides on amphibian skin. This result is contrary to our prediction that the stress of competition would negatively affect skin peptides as a result of reduced allocation or acquisition of resources or an increase in glucocorticoids. While increases in glucocorticoids, in response to high competition for resources, may have slowed down the development of the granular glands, overall tadpole development was also retarded in these treatments (tadpoles took ~15 days longer to develop on average). Therefore, even though development of the granular glands may have been retarded as a result of excess glucocorticoid production (Hayes and Gill, 1995; Glennemeier and Denver, 2002), they may have still been able develop before metamorphosis, which is driven by both thyroid hormone and glucocorticoids (Denver, 2009). Clearly, measurements of these hormones would be required to test these hypotheses.

Theoretical predictions on the effects of conspecific density on immune function are contradictory. Lochmiller suggested that an increase in the release of stress hormones at high competition should cause immunosuppression (Lochmiller, 1996); however, Svensson and colleagues point out that because density-dependent transmission increases with higher densities, increased allocation to costly immune defenses may provide the greatest fitness benefits at this time (Svensson et al., 2001). The few empirical tests of these hypotheses show mixed results. For example, density-dependent immunosuppression of antibody responsiveness has been found in lizards (Svensson et al., 2001), while density-dependent immune enhancement (in the form of increased melanism) has been found in moths (Hagen et al., 2006). No density-dependent effects on acquired immune functions (leukocyte count and type) were found in a recent study of the effects of competition in American toad tadpoles, *Anaxyrus americanus* (Raffel et al., 2010). Our results support the density-dependent immune enhancement hypothesis; however, further disease challenges are necessary to test the adaptiveness of this response.

The effect of competition on the mass-standardized production of skin peptides depended on the timing and extent of exposure to predators. In the absence of predators, competition caused more than a 250% increase in the mass-standardized production of skin peptides. The effect of competition was reduced in the chronic- and early-cue predator treatments; however, when combined with late cues, high competition caused more than a 500% increase in mass-standardized skin peptides. This was due to a decrease in skin peptides at low competition relative to controls. Observed interactions between predator cues and competition for other amphibian traits (behavior, morphology and physiology) often reveal that predator cues have weaker effects on tadpole phenotypes in high competition environments (Relyea, 2004). This may be because the risk of predation is relatively lower when population sizes are large or because high competition results in limited resources available to induce an anti-predator phenotype. The results of our study also show that immune responses induced by competition are, for chronic exposure, less extreme in the presence of predators.

While interpreting the mechanism underlying these patterns is challenging with only one measurement of mass-standardized skin peptides across time, variation in the effects of these stressors on glucocorticoid production may explain these results. The dampened effect of competition on skin peptides in the chronic predator treatment relative to the no-predator treatment is consistent with previous observations that frequent exposure to predators results in increased glucocorticoid production (Denver, 2009). The lack of dampened effects of high competition in the late-cue and early-cue treatments is consistent with observations that short pulses of predator cues can decrease glucocorticoid production (Hayes and Gill, 1995). As previously stated, measurement of glucocorticoids would be required to test this hypothesis.

While predator cues modulated the effect of competition, there were few detectable effects of predators on mass-standardized skin peptides when controlling for competition. The negative effect of late exposure to predator cues on mass-standardized skin peptides is consistent with a previous study on the effects of predator cues on mass-standardized skin peptides in wood frogs, which showed a trend of reduced skin peptides in the presence of predators (Groner et al., 2013). While the exposure of wood frogs was not late in their development, this species develops more rapidly than leopard frogs. Therefore, these wood frogs may have been similarly limited in their ability to adjust their physiology in order to compensate for the effects of this stressor.

Chemical analysis of hydrophobic peptides produced by our leopard frog metamorphs suggests that they expressed one new temporin, temporin-2P, and five brevinins: brevinin-1Pd, brevinin-1Pg, brevinin-Pla and two additional unique brevinins, which could not be matched to known brevinins or sequenced completely. It is likely that the majority of these brevinins have important antimicrobial properties against amphibian pathogens. A high level of variation in brevinins has previously been documented in leopard frogs; analysis of the brevinin gene in leopard frogs provides evidence for several gene duplication events resulting in at least five loci encoding at least 13 different brevinins (reviewed in Tennessen

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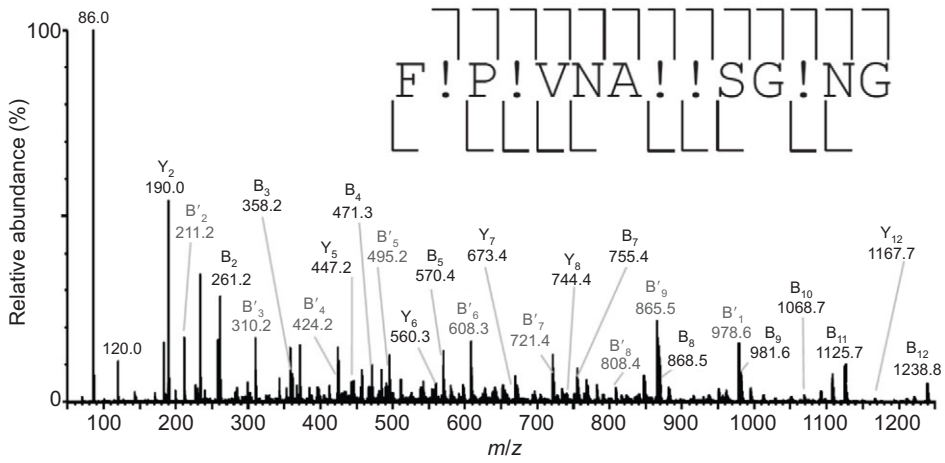

Fig. 3. Product mass spectrum of temporin-2P from leopard frogs acquired with nano-flow electrospray liquid chromatography tandem mass spectrometry (ESI QTOF2). The sequence interpreted is shown with bars above indicating support for characterization from B-series ions and bars below indicating support for characterization from Y-series ions. Proline at the third position afforded preferential cleavage before that residue, resulting in two peptides fragmenting independently and yielding two B-series, one starting with the B2 ion corresponding to the dipeptide from the first two residues and continuing, and the other beginning with a B2 ion corresponding to the third and fourth residues and continuing. The latter B-series is shown in gray. These methods do not differentiate leucine from isoleucine (denoted as '!').

and Blouin, 2007; Tennessen et al., 2009). Brevinin-1Pg was found to have the highest allele frequency in leopard frog populations in the eastern United States (Tennessen et al., 2009), while the other two brevinins sequenced are present at lower frequency. This suggests that there may be more variation in brevinin alleles than was previously detected in the eastern United States. High rates of synonymous substitutions but low allelic diversity relative to interspecies allelic differences suggest that that these genes have undergone recent positive selective sweeps (Tennessen and Blouin, 2007). Brevinin peptides in leopard frogs vary regionally, suggesting that they may be adapted to local microbial environments (Tennessen et al., 2009). This is further supported by growth inhibition studies showing that different brevinins vary in their ability to inhibit Bd and the gram-positive bacteria *Staphylococcus epidermidis* (Tennessen et al., 2009). Collectively, these data suggest that these peptides may offer important fitness benefits to leopard frog metamorphs.

Temporins have also been shown to inhibit harmful microbes including Bd (Rollins-Smith et al., 2003). The level of inhibition is dependent upon the ability to attach to the fungal cell membrane, the formation of an α-helix after attachment (thought to be necessary for membrane disruption), and resistance against fungal proteases (Rollins-Smith et al., 2003). Temporin-2P is predicted to form an α-helix, which would yield six hydrophobic amino acids on the same surface, providing a strong membrane binding potential (Wang and Wang, 2004). This is only the second temporin identified for leopard frogs. Further analysis of variation in the production of both temporins across geographic ranges and ontogeny may yield key insights into their function and the potential for local adaptation. Given the abundance of new suspected AMPs identified in this study, we suspect that more local variation in AMP diversity awaits discovery.

This study did not examine the effects of competition and predator stress on disease dynamics per se, and caution should be used in extrapolating effects of altered immune responses to disease resistance (Hawley and Altizer, 2011). For example, altered behavior may compensate for an altered immune response. Moreover, patterns of reduced or enhanced immune responses may not always intersect with thresholds that determine whether an immune response is successful. Finally, increased immune responses may ultimately cause more damage as a result of auto-immunity and build-up of allostatic load (Lochmiller and Deerenberg, 2000). However, in our study organism, many components of the acquired immune system are temporarily unavailable during metamorphosis, and the concentrations of peptides on amphibians during this time are generally within the range that can inhibit skin infections (e.g. Ramsey et al., 2010; Pask et al., 2012). Therefore, if the alterations of skin peptides found in this study as a result of competition and predator stress are due to changes in AMPs, this may have important consequences for the transmission and virulence of amphibian pathogens and their effects on host populations. This suggests that tadpoles can respond to increased disease threats in high-density environments by increasing allocation to immune function; however, chronic or early exposure to predators may dampen these effects.

MATERIALS AND METHODS

We conducted the experiment in the spring and summer of 2009 at the Pymatuning Laboratory of Ecology (Linesville, PA, USA). We used a completely randomized design composed of a factorial combination of four predator cue treatments and two competition treatments. The four predator cue treatments consisted of no-predator cues, an early exposure to predator cues, a late exposure to predator cues and a chronic exposure to predator cues throughout the larval period. The two competition treatments consisted of low and high tadpole densities.

The eight treatments were replicated four times for a total of 32 experimental units. Each experimental unit consisted of an 800 l polypropylene mesocosm filled with 600 l well water (pH 8), 15 g of rabbit chow to serve as a nutrient source and 200 g of dry leaf litter (primarily *Quercus* spp.) to serve as a substrate for algal growth. We also added ~0.5 l pond water, collected from several local sites, to serve as a source of microbiota and plankton. These additions were made on 16 April. On 21 April and 2 May, we added additional water collected from several local wetlands to each mesocosm to further bolster microbial populations. Mesocosms were covered with shadecloth to prevent colonization by flying insects and escape of metamorphosing frogs.

We collected eight leopard frog egg masses from a local pond and hatched them in outdoor mesocosms until they reached Gosner stage 25 (Gosner, 1960) (mean mass ± 1 s.e.m., 32.0±1.7 mg). At this point they were added to the experimental mesocosms (5 May). Unfortunately, some wood frog tadpoles, *L. sylvaticus* (LeConte), were accidentally mixed with the leopard

frog tadpoles. As a result, wood frogs comprised 8.6±1.2% (mean ± 1 s.e.m.) of the individuals in each tank. Statistical tests (described below) indicated that these additions did not alter the results of the experiment.

The four predator treatments were designed to vary both the timing and duration of exposure to predator cues relative to tadpole ontogeny. The early-cue treatment was a 1 week pulsed exposure to caged predators during week 1 of the experiment. The late-cue treatment was a 1 week pulsed exposure to caged predators during week 5 of the experiment. The chronic-cue treatment was an exposure to caged predators until the tadpoles metamorphosed (from week 1 until halfway through week 7). The no-cue treatment was an exposure to empty predator cages. Hereafter, these treatments will be referred to as the early-cue, late-cue, chronic-cue and no-cue treatments.

We collected green darner dragonfly larvae, *Anax junius* (Drury), from local ponds to create predator cues. These dragonflies have a cosmopolitan distribution in the United States and commonly consume leopard frog tadpoles (Corbet, 1999). Each mesocosm contained two predator cages that were constructed of 800 ml plastic drainpipes secured with mesh screens on each end, held in place with rubber bands. These cages were suspended at the water surface and dragonfly larvae were held in each container during the relevant exposure periods for each treatment. Cages were opened to feed leopard frog tadpoles (300±10 mg each) to the predators three times per week. Dragonfly larvae that died or were not feeding were replaced with new dragonflies at the next feeding. Cages in treatments without predators were similarly disturbed. Previous experimental work shows that the concentration of predator cue used in this experiment is sufficient to induce plastic responses (Schoeppner and Relyea, 2008). The first predator feeding was on 11 May (defined as experiment day 1) and the last feeding was on day 45, just before leopard frog tadpoles began to metamorphose.

Intraspecific competition was manipulated by altering tadpole densities. Low-competition treatments contained 15 tadpoles whereas high-competition treatments contained 30 tadpoles.

Leopard frogs began to metamorphose on day 46. After the first metamorphs appeared, tanks were checked daily. Individuals with four emerged limbs were collected and held in 1 l containers with sphagnum moss until metamorphosis was complete. We considered metamorphosis to be complete when the tail was resorbed to less than 2 mm. At this point, we took the mass of each individual. After metamorphosis was complete, individuals were weighed and placed in 600 ml cups with mesh lids containing wet sphagnum moss. Each metamorph received two, ~6 mm crickets that were dusted with the calcium and vitamin supplement Reptocal (TetraFauna, Blacksburg, VA, USA).

Skin peptide collection

We measured hydrophobic skin peptides on leopard frogs ~9 days after metamorphosis was complete. We attempted to collect skin peptides from eight frogs per replicate; however, we averaged five frogs per replicate because not enough animals survived or metamorphosed before the experiment was terminated. As we did not measure skin peptides from all frogs, we chose animals that represented early-, mid- and late-developers from each experimental unit.

We induced the release of skin peptides from the granular glands using subcutaneous injections of 20 nmol g^{-1} frog noradrenaline hydrochloride salt dissolved in amphibian phosphate-buffered saline (APBS). Noradrenaline induces the contraction of the smooth muscles surrounding the granular glands, releasing stored peptides onto the skin (reviewed in Rollins-Smith and Conlon, 2005). The concentration of noradrenaline used in this experiment results in AMP releases that are somewhat higher than levels released by frogs during stressful events. For example, frogs chased by a researcher's hand released the same amount of peptides as frogs injected with 2 nmol noradrenaline g^{-1} frog and significantly more peptides than resting frogs (Ramsey et al., 2010; Pask et al., 2012). Maximum peptide release is achieved with an injection of 40 nmol noradrenaline g^{-1} frog for this species (Pask et al., 2013). As metamorphs do not release as many peptides as adult frogs (L.A.R.-S., unpublished data), we used 20 nmol g^{-1} frog of noradrenaline to allow the release of a detectable amount of peptides without approaching a maximum level of release (Ramsey et al., 2010; Pask et al., 2013).

After injection with noradrenaline, the frogs were immediately immersed in 45 ml of collection buffer (25 mmol l^{-1} sodium acetate and 25 mmol l^{-1} NaCl, pH 7.0) for 10 min. The resulting buffer was acidified with 450 µl of trifluoroacetic acid (TFA) and stored at −20°C (Ramsey et al., 2010). Peptides break down rapidly and are present at maximum concentrations on the skin only for the period immediately following peptide release (reviewed in Rollins-Smith and Conlon, 2005). Therefore, measurements of skin peptides reflect the most recent peptide release, which is influenced by sensitivity to noradrenaline as well as by the amount of peptides that are stored in the glands.

Hydrophobic peptides were collected onto C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA) (Ramsey et al., 2010) and then eluted in elution buffer containing 70% acetonitrile, 29.9% water and 0.1% TFA (Rollins-Smith et al., 2006). Concentrations of peptides were determined using Micro BCA analysis (Pierce, Rockford, IL, USA) following the manufacturer's instructions, with the exception that bradykinin was used to establish a standard curve (Rollins-Smith et al., 2006). This method of isolation enriches for hydrophobic peptides, which include AMPs, but because other hydrophobic peptides that are not conventional cationic antimicrobial peptides may also be enriched, we refer to the collected peptides as skin peptides. Peptide concentrations were then standardized for mass by dividing by the mass of the frog (e.g. Davidson et al., 2007). While standardizing for cutaneous surface area would be ideal, and can be estimated using an allometric scaling equation (e.g. McClanahan and Baldwin, 1969), we did not believe this would be appropriate in our study. Both competition and predator stress in tadpoles have been shown to induce different morphologies in juvenile frogs (Relyea, 2001; Relyea and Hoverman, 2003). As an allometric scaling equation would not take these differences into account, we used mass as the most direct measurement of amphibian size.

Because of the large quantity of peptide samples that we had to process, some samples were processed immediately after elution, while others were stored at –80°C and processed several days later. Peptide samples that had been frozen at –80°C were statistically different from all other samples after controlling for treatment effects (using ANOVA). As these samples were all of substantially lower concentration, we suspect that they had degraded as a result of freezing and thawing and we excluded these samples from the analysis. This reduced our sample size by 26%.

Peptide characterization

Initial attempts to identify peptides were conducted using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager DE-STR, Applied Biosystems Corporation, Foster City, CA, USA). However, inhibition of desorption/ionization was suspected and confirmed when addition of our samples to known standards prevented detection of a signal. This has been previously observed (Groner et al., 2013), suggesting that metamorphic frogs release the inhibiting compound. Ultimately, determination of peptide sequences was done using capillary liquid chromatography (Michrom BioResourses Corporation, Auburn, CA, USA) electrospray ionization (ESI) MS/MS. The mass spectrometer used was a hybrid quadrupole mass filter followed by a time-of-flight mass analyzer (Q-TOF2) (Waters Corporation), which allowed the collection of MS/MS product ion scans. Samples were combined across treatments and loaded onto a homemade fused silica capillary column (100 µm i.d.), which incorporated a pulled ESI tip packed to a length of ~10 cm with 10 µm particles of R10 Poros C-18 beads (Applied Biosystems Corporation). Mass spectra were first obtained in the 550–3000 *m*/*z* range and promising ions with +2 or +3 charge states were targeted for MS/MS analysis. Amino acid sequences were assigned using methods described previously (Kinter and Sherman, 2000).

Statistical analysis of life-history traits

We used a multivariate analysis of variance (MANOVA) to test the effects of competition and predator cues on metamorphosis response variables (size at metamorphosis, time to metamorphosis and survival at metamorphosis). When MANOVA were significant, we conducted ANOVA on significant variables. Pairwise comparisons among the four predator treatments were made with Tukey's HSD test.

Tadpole survival and mass at metamorphosis violated the assumption of sphericity (Box's $M_{42,952.633}$=0.007); however, this assumption was met in MANOVA that tested the effects of either competition or predator cues, suggesting that the violation was fairly minor. We used Pillai's trace, which is more robust to violations of sphericity, to calculate *P*-values [see p. 434 of Quinn and Keough (Quinn and Keough, 2002)]. To meet the assumption of normality, data on time to metamorphosis were square-root transformed and data on the proportion of tadpoles surviving to metamorphosis were arcsine square-root transformed.

To test whether the accidental inclusion of wood frogs in treatments affected these response variables, we included the number of individuals that were accidentally added to each tank as a covariate. This term was not significant (Pillai's trace $F_{3,21}$=0.6, P=0.981), so we excluded it from the final model.

Statistical analysis of skin peptides

We used an ANOVA to test the effects of treatments on the tank means of mass-standardized antimicrobial peptides (μg AMP g^{-1} of frog). Mass-standardized antimicrobial peptides were log-transformed to meet the assumption of normality. Pairwise comparisons were made with Fisher's LSD test. Because we had specific *a priori* hypotheses about the effects of predator treatments and competition on skin peptide production, we did not adjust for multiple comparisons [see p. 49 of Quinn et al. (Quinn et al., 2002)]. No assumptions were violated in these tests.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.L.G., R.A.R. and L.A.R.-S. conceived and designed the experiment. M.L.G. conducted the mesocosm experiment. M.L.G., L.K.R. and L.A.R.-S. isolated and quantified hydrophobic skin peptides, while M.L.G., J.H. and M.E.B. performed mass spectrometric analyses of isolated skin peptides. M.L.G. performed statistical analyses. All authors were involved in writing the paper.

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Supplementary material

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Fig. S1. Product mass spectrum of brevinins of leopard frogs (*L. pipiens*) acquired with nano-flow electrospray liquid chromatography tandem mass spectrometry (ESI QTOF2, Waters Corporation). Peptide molecular weights are indicated and the sequence interpreted is shown with bars above indicating support for characterization from B-series ions and bars below indicating support for characterization from y-series ions. Proline at the third position afforded preferential cleavage before that residue, resulting in two peptides fragmenting independently and yielding two B-series, one starting with the B2 ion corresponding to the dipeptide from the first two residues and continuing, and the other beginning with a B2 ion corresponding to the third and fourth residues and continuing. The latter B-series is shown in grey. These methods do not differentiate leucine from isoleucine (denoted as '!').

Download Fig. S1

Table S1. Partial sequences for brevinins found in this paper and brevinins known to be found in leopard frogs (*Lithobates pipiens*)

Sequence name	Monoisotopic molecular weight Experimental (Theoretical)	Sequence	Citations
Likely New	1875.2	F!P!VV!VPF!!. . .	This study
Likely Brevinin-1Pd	2569.6	F!P!!ASVA. . .	This study
Likely Brevinin-1Pg	2593.9	FFP!VAGVA. . .	This study
Likely Brevinin-1-Pla	2623.9	FFPNVASV. . .	This study
Likely new	2877.0	FFP!VAA. . .	This study
Brevinin-1Pa*	2561.0 (2561.5)	FLPIIAGVAAKVFPKIFCAISKKC	Horikawa et al., 1985; Goraya et al., 2000; Tennessen and Blouin, 2007, 2008
Brevinin-1Pb*	2575.1 (2575.5)	FLPIIAGIAAKVFPKIFCAISKKC	Horikawa et al., 1985; Goraya et al., 2000; Tennessen and Blouin, 2007
Brevinin-1Pc*	2581.0 (2581.5)	FLPIIASVAAKVFSKIFCAISKKC	Horikawa et al., 1985; Goraya et al., 2000

Brevinin-1Pd*	2566.6 (2567.4)	FLPIIASVAANVFSKIFCAISKKC	Goraya et al., 2000
Brevinin-1Pe*	2591.3 (2591.5)	FLPIIASVAAKVFPKIFCAISKKC	Goraya et al., 2000; Tennessen and Blouin, 2007
Brevinin-1Pf	(2591.5)	FLPIIAGIAAKFLPKIFCAISKKC	Tennessen and Blouin, 2007
Brevinin-1Pg*	2594.5 (2594.5)	FFPIVAGVAGQVLKKIFCTISKKC	Tennessen and Blouin, 2007, 2008
Brevinin-1Ph	(2543.3)	GIPLLPGLAANLCRPIYCTITKNC	Tennessen and Blouin, 2007
Brevinin-1Pi	(1834.0)	GIPLLPGLAANLCRPINC	Tennessen and Blouin, 2007
Brevinin-1Pj	(2651.5)	FFPNVASVPGQVLRKIFCAISKKC	Tennessen and Blouin, 2008
Brevinin-1Pk	(2593.5)	FLPIIAGVAAKVFPKIFCTISKKC	Tennessen and Blouin, 2008
Brevinin-1Pl*	2607.4 (2607.4)	FLPIIAGMAAKFLPKIFCAISKKC	Tennesseen et al., 2009
Brevinin-1Pla*	2623.2 (2621.4)	FFPNVASVPGQVLKKIFCAISKKC	Basir et al., 2000, Tennessen and Blouin, 2008

*The monoisotopic molecular weights listed are for the disulfide bridged peptides.
Both experimental and theoretical molecular weights are shown. All experimental values were determined using tandem mass spectrometry except Brevinin-1Pla which was determined using Edman degradation. In cases where peptide sequences are based on genetic information, only theoretical molecular weights are shown. Because we could not differentiate between leucine and isoleucine, we marked these amino acids with an '!'. The mass accuracy of our work was ±0.5 Da. Known cysteine bridges are shown.

Table S2. Temporins closely resembling the temporin found in this paper

Sequence Name	Monoisotopic Molecular Weight Experimental (Theoretical)	Species	Sequence	Citations
Temporin-2P	1427.1 (1426.8)	*Rana pipiens*	F!P!VNA!!SG!NG	This paper
Temporin-CPb	1395.8 (1395.9)	*Lithobates capito*	FLPIVGRLISGIL	Conlon et al., 2009
Temporin-1ARa	1397 (1395.9)	*Rana aerolata*	FLPIVGRLISGLL	Ali et al., 2002
Temporin-1VE	1370.0 (1367.9)	*Rana versabilis*	FLPLVGKILSGLI	Chen et al., 2006
Temporin-1PLa	1368.8 (1367.9)	*Rana palustris*	FLPLVGKILSGLI	Basir et al., 2000
Temporin-1P	1368.0 (1367.9)	*Rana pipiens*	FLPIVGKLLSGLL	Goraya et al., 2000
Temporin-1M	1367.9 (1367.9)	*Rana muscosa*	FLPIVGKLLSGLL	Rollins-Smith et al., 2006

The other temporin identified in leopard frogs (*L. pipiens*) is also shown. Because we could not differentiate between leucine and isoleucine, we marked these amino acids with an '!'. The mass accuracy of our work was ±0.5 Da.

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