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RESEARCH ARTICLE

Out in the cold and sick: low temperatures and fungal infections impair a frog's skin defenses

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ABSTRACT

Amphibians worldwide continue to battle an emerging infectious disease, chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*). Southern leopard frogs, *Rana sphenocephala*, are known to become infected with this pathogen, yet they are considered 'of least concern' for declines due to chytridiomycosis. Previous studies have shown that *R. sphenocephala* secretes four antimicrobial peptides (AMPs) onto their skin which may play an important role in limiting susceptibility to chytridiomycosis. Here, we examined (1) the effects of temperature and AMP depletion on infections with *Bd* and (2) the effects of temperature and *Bd* infection on the capacity to secrete AMPs in juvenile leopard frogs. Pathogen burden and mortality were greater in frogs exposed to *Bd* at low temperature but did not increase following monthly AMP depletion. Both low temperature and *Bd* exposure reduced the capacity of juvenile frogs to restore peptides after monthly depletions. Frogs held at 14°C were poorly able to restore peptides in comparison with those at 26°C. Frogs held at 26°C were better able to restore their peptides, but when exposed to *Bd*, this capacity was significantly reduced. These results strongly support the hypothesis that both colder temperatures and *Bd* infection impair the capacity of juvenile frogs to produce and secrete AMPs, an important component of their innate defense against chytrid fungi and other pathogens. Thus, in the face of unpredictable climate changes and enzootic pathogens, assessments of disease risk should consider the potential for effects of environmental variation and pathogen exposure on the quality of host defenses.

KEY WORDS: Amphibian, Antimicrobial peptides, *Batrachochytrium dendrobatidis*, Chytridiomycosis, *Rana* (*Lithobates*) *sphenocephala*, Climate

INTRODUCTION

Because amphibians are ectotherms, their biological functions, including immune defenses, are affected by temperature. In recent years, the survival of many amphibian populations has been impacted by a sometimes-lethal skin fungus of the phylum Chytridiomycota: *Batrachochytrium dendrobatidis* (*Bd*). *Bd* causes the disease chytridiomycosis (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999) that can disrupt the normal transport of ions across the skin, resulting in death due to a loss of essential sodium and potassium ions (Voyles et al., 2007, 2009, 2012a). Chytridiomycosis has been linked to the recent declines of many amphibian populations and even the extinction of some species (Stuart et al., 2004; Skerratt et al., 2007; reviewed in Fisher et al., 2012; Scheele et al., 2019). Southern leopard frogs, *Rana* (*Lithobates*) *sphenocephala*, are listed as 'least concern' on the IUCN red list, and although *Bd* was detected in this species as early as 1900–1920 and more recently (Talley et al., 2015; Peterson et al., 2007), there have been no reported declines due to chytridiomycosis. However, *Bd* appears to be a particular threat to vulnerable newly metamorphosed juveniles, possibly leading to lower yearly recruitment of young of the year into adult populations (Rachowicz et al., 2006; Wise et al., 2014; Rothermel et al., 2016; Clare et al., 2016).

In Tennessee, breeding of southern leopard frogs generally occurs from late winter through to early spring, and tadpoles metamorphose within 2–7 months (Glorioso, 2011). Newly metamorphosed animals feed continuously until cold temperatures reduce the food supply, at which point they enter overwintering sites (Neill, 1948). Although *Bd* is well adapted to thrive in cold climates (Woodhams et al., 2008; Voyles et al., 2012b), the immune defenses of leopard frogs are impaired by cold temperatures (Green and Cohen, 1977; Maniero and Carey, 1997). One important defense against *Bd* in the skin is the gradual release of antimicrobial peptides (AMPs) from specialized granular glands in the skin (Woodhams et al., 2006a, 2007; Ramsey et al., 2010; Pask et al., 2012; reviewed in Rollins-Smith et al., 2011). In adult northern leopard frogs, *Rana* (*Lithobates*) *pipiens*, AMPs are constitutively produced and released at low but effective antimicrobial concentrations. Their secretion is greatly increased following alarm or injury. After release, the peptides are active on the skin for about 2 h and degrade gradually after that time (Pask et al., 2012, 2013). When skin peptides are experimentally depleted, juvenile northern leopard frogs are much more likely to die following exposure to *Bd* than control animals with intact peptides (Pask et al., 2013).

Southern leopard frogs are known to produce four distinct AMPs (Conlon et al., 1999; Holden et al., 2014, 2015a) brevinin-1Sa, brevinin-1Sb, brevinin-1Sc and temporin-1S (Conlon et al., 1999; Holden et al., 2015a) – and each of these purified peptides is a potent inhibitor of *Bd* growth *in vitro* (Holden et al., 2015a). Prior to the present study, there were only two other reports of temperature having an effect on the production of amphibian AMPs: a study of Australian tree frogs (*Litoria serrata*) showed a positive correlation between production of defensive peptides and stream temperatures (Woodhams et al., 2012) and one on North American wood frogs (*Rana sylvatica*) showed they had no detectable AMPs during hibernation, but produced them after being warmed (Matutte et al., 2000). Because the AMPs are synthesized and stored in granular glands in the skin and *Bd* is a pathogen that disturbs the skin, an active *Bd* infection might also inhibit the function of the granular glands. Thus, the combined effects of temperature and *Bd* infection would have the potential to significantly reduce the capacity of skin

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secretions to inhibit the continuing infection process as new zoospores emerge and re-infect the skin. Here, we examined the separate and combined effects of cold temperature and *Bd* infection on survival and on the synthesis and release of protective AMPs in juvenile southern leopard frogs. Our predictions were that both cold temperature and *Bd* infection would impair the production and release of AMPs, with the potential to impact the survival of this species in the vulnerable post-metamorphic period.

MATERIALS AND METHODS

Animals

Egg masses of *R. sphenocephala* Cope 1886 were collected from water bodies near the Edward J. Meeman Biological Field Station at the University of Memphis Shelby County, TN, USA (35°22′ N/90° 01′ W) in March 2015 and reared in outdoor cattle tanks (diameter 1.83 m) covered with mesh screens as previously described (Holden et al., 2015a). Briefly, the mesocosms were filled with ∼613 l (30.5 cm) of well water and 300 g of dry deciduous (primarily *Quercus* spp.) leaf litter. One 500 ml aliquot of concentrated plankton suspension, originally collected from a nearby pond, was added to each tank. Fiberglass mesh screens (1 mm mesh) were used as lids to provide shading and to prevent colonization of tanks by animals from outside it (e.g. predators or other amphibians). Once the developing frogs reached Gosner stage 25 (Gosner, 1960), the animals were randomly selected and placed in rearing tanks at densities of approximately 30 per tank, and left undisturbed until the tadpoles began to metamorphose, which occurred ∼3 months after eggs were collected. After metamorphosis, they were transported to Tulane University. There they were housed individually for the remainder of the experiment in 22.5×15×16.25 cm plastic terraria with 2.5 cm of filtered tap water and with a small plastic perch which allowed the frogs access to a dry substrate. Terraria were cleaned with 10% bleach solution and fresh water was provided weekly. Upon arrival at Tulane and prior to the start of the experiment (i.e. for ∼3 months after metamorphosis), frogs were housed at 20°C. Acquisition of the animals was authorized under Tennessee State Parks Scientific Research and Collecting Permit number 3800. This study and its methods were approved by the Institutional Animal Care and Use committee (IACUC) at Tulane University (protocols 0391-0391R2).

Experimental design

To investigate the effects of temperature and skin peptide depletion on susceptibility to *Bd* infection and to compare the peptides secreted among treatment groups, 32 frogs were randomly selected and their terraria were placed in an environmental chamber (Conviron®, Adaptis, Winnipeg, MB, Canada) at a constant temperature of 14°C, and 32 others were randomly selected and placed in an identical chamber at a constant temperature of 26°C (Fig. 1). Prior to the start of the experiment, frogs in each temperature group were acclimated to their respective temperatures for 7 days and maintained at these temperatures throughout the experiment. Within each group of 32, 16 frogs had their AMPs depleted while the other 16 had their AMPs left intact. Within these two groups of 16, eight frogs were exposed to *Bd* isolate JEL 412 (provided by Joyce Longcore, University of Maine), isolated from a *Sachatamia ilex* frog in Panama in 2005, while the other eight were sham exposed. All 64 frogs were on a 12 h:12 h light:dark cycle and initially housed in identical terraria. However, at the start of week 4, all frogs held at 26°C were moved into larger terraria (35×21.25×23.75 cm with a 2.5 cm depth of filtered tap water) because of their increased size. Frogs were fed to satiation (such that there were always crickets left uneaten) 4 times per week: frogs held at 14°C received five 2 week old crickets per feeding, and frogs held at 26°C received 10 of the same size crickets at each feeding. At the conclusion of testing, all remaining animals were humanely killed.

AMP depletion

AMPs were depleted monthly (days −1, 27, 62) in half of the frogs held at 14°C and half of the frogs held at 26°C according to the experimental design (Fig. 1) by injecting frogs with noradrenaline (norepinephrine; 40 nmol g^{-1}) in amphibian phosphate-buffered saline (APBS) (Ramsey et al., 2010), and frogs with their AMPs left intact were injected with APBS alone (0.01 ml g^{-1} frog body mass) as previously described. This concentration of noradrenaline has been shown to result in nearly complete depletion of the contents of the granular glands, which are the source of the AMPs (Gammill et al., 2012; Pask et al., 2013). After injection, frogs were placed in 25 ml of collection buffer (2.92 g NaCl and 2.05 g sodium acetate in 1 liter of HPLC-grade water) for 15 min. Frogs were then rinsed in fresh water and returned to their terraria. Peptides and collection buffer were poured into sterile 50 ml tubes and stored at −20°C. All surviving frogs were induced to secrete peptides at the end of the experiment on days 90–92.

Experimental exposure to *Bd* zoospores

To prepare zoospores for experimental exposure, *Bd* isolate JEL 412 was grown at 23°C on 1% tryptone agar plates for 7 days. Zoospores

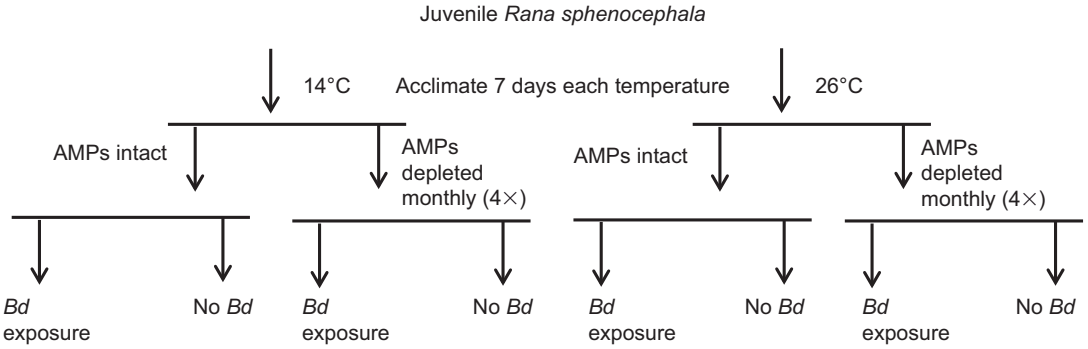

Fig. 1. Experimental design. *Rana sphenocephala* juveniles (*N*=64) at 1 month post-metamorphosis were divided into 8 treatment groups as shown (*N*=8 per group). Half were acclimated to and continuously treated at 14°C and the other half were acclimated to and continuously treated at 26°C. Peptides were collected from antimicrobial peptide (AMP)-depletion groups at days −1, 27 and 62, and from all survivors at days 90–92. Exposure to *Bd* zoospores occurred weekly beginning at day 0.

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were harvested by flooding plates with 5 ml of deionized water. According to the experimental design (Fig. 1), 8 frogs in each of four treatment groups were exposed to the zoospores by individually placing them in a bath of 2×10^6 zoospores suspended in 20 ml of deionized water (10^5 zoospores ml^{-1}) for 12 h. Zoospore concentration was determined by hemocytometer counts. Sham exposures (8 frogs in each of four treatment groups) were carried out in the same way except that 1% tryptone plates without *Bd* were flooded. *Bd* exposures and sham exposures were carried out weekly, starting at day 0. Frogs were monitored daily for clinical signs of chytridiomycosis (lethargy, inappetence, loss of righting reflex, excessive skin sloughing, abnormal posture and cutaneous erythema).

Quantification of pathogen burden

Beginning on day 9 after the first *Bd* exposure, the skin of each frog was swabbed weekly according to a standard protocol (Kriger et al., 2006), and *Bd* load was determined by quantitative (q)PCR. Genomic DNA was extracted from the swabs using the Qiagen DNeasy kit (Germantown, MD, USA) following the protocol for animal tissues with two minor modifications: (1) swabs were incubated for 30 min, vortexed and spun in a centrifuge, and then incubated for an additional 30 min, and (2) samples were eluted twice with 100 µl of elution buffer. qPCR was performed on an Applied Biosystems 7500 system (Foster City, CA, USA) using a 7-fold dilution series of plasmid standards (Pisces Molecular, Boulder, CO, USA) to quantify the number of *Bd* DNA copies found in a 5 µl sample of diluted DNA (1/400th of entire extraction) following the protocol of Boyle et al. (2004), with the following modifications: the samples were diluted 1:10 with molecular grade water, and 0.7 µl of bovine serum albumin (BSA, Applied Biosystems) was added to each well prior to amplification (Boyle et al., 2004; Hyatt et al., 2007; Kreader, 1996). Pathogen burden is presented as the log transformed number of copies of *Bd* DNA detected per 5 µl aliquot of extracted DNA.

Partial purification of peptides and mass spectrometry

Skin secretions were lyophilized until dry. Dried material was resuspended in 10 ml of lysis buffer (1 liter distilled water containing 8.77 g NaCl, 1.68 g EDTA, 1.58 g Tris-Cl and 10 ml Triton X-100) with protease inhibitors (1 ml l^{-1} of 5 mmol l^{-1} dithiothreitol, 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride and 5 mmol l^{-1} aminocaproic acid). The 10 ml samples were then spun over 100 kDa molecular weight cutoff Amicon® Ultra-15 spin columns (MilliporeSigma, Burlington, MA, USA) in a swinging bucket rotor at 3000 rpm (1875 ***g***) for 1 h at 4°C. C18 Sep Pak cartridges (Waters Corporation, Millford, MA, USA) were activated with 10 ml of 100% HPLC-grade methanol, then washed with 10 ml Buffer A (0.1% trifluoroacetic acid in HPLC grade water). Lower mass products in the spin column were passed over the activated Sep Pak cartridges, which were then washed again with 10 ml of Buffer A, followed by elution with 11 ml of Buffer B (0.1% trifluoroacetic acid, 70% acetonitrile, 29.9% HPLC-grade water). The eluted material (10 ml) was spun under vacuum until dry, and 1 ml was used for peptide quantification using a Micro BCA assay (Pierce, Rockford, IL, USA) with bradykinin (FW 1060.2, Sigma-Aldrich, St Louis, MO, USA) as a small peptide standard, as previously described (Rollins-Smith et al., 2002). Peptide samples were resuspended at a concentration of 1 mg ml^{-1} and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate at a 1:1 ratio with matrix containing 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Fluka, Sigma-Aldrich), 60% acetonitrile, 39.6% HPLC-grade water and 0.4% trifluoroacetic acid. An Ultraflex III time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) was used and calibrated using the following standards (Sigma-Aldrich): bradykinin fragment 1–7 (*m*/*z* 757.3997), human angiotensin II (*m*/*z* 1046.5423), $P_{14}R$ synthetic peptide (*m*/*z* 1533.8582), adrenocorticotropic hormone fragment 18-39 (*m*/*z* 2464.1989) and bovine oxidized insulin chain B (*m*/*z* 3494.6513). For each peptide standard, peptide signals for 1000 laser shots were collected, and for each frog peptide sample, signals from 250 laser shots were collected (Woodhams et al., 2006b; Tennessen et al., 2009; Holden et al., 2015a). Peptide signals were averaged from 250 consecutive laser shots. Data Explorer™ (v.4.4) (Applied Biosystems) was used for baseline correction, and the peak values represent the monoisotopic mass (Woodhams et al., 2006b; Tennessen et al., 2009; Holden et al., 2015a). Mass spectra were searched manually for expected peptide signals for AMPs previously described for this species: temporin-1S (*m*/*z* 1444), brevinin-1Sa (*m*/*z* 2521), brevinin-1Sb (*m*/*z* 2537) and brevinin-1Sc (*m*/*z* 2612) (Conlon et al., 1999; Holden et al., 2015a). For all peaks in the *m*/*z* range of 1400 to 2650, values for relative intensity of the signals in relation to zero were recorded, giving an approximation of the relative concentration of each peptide in the sample.

Statistics

To test for effects of temperature and *Bd* infection on skin peptide production and growth of these juvenile frogs, we compared the total peptides recovered (per gram body mass), the proportion of frogs expressing brevinin-1Sa signals, the relative intensity of the brevinin-1Sa signal (reported as means±s.e.m.) and body mass under different temperature and exposure conditions. The presence/absence of the brevinin-1Sa signal in skin secretions was compared across temperature groups using either a Fisher's exact test (AMP-intact groups) or a generalized linear mixed model (glmm) to run binary logistic regression (AMP-depleted groups). Total peptides and the log-transformed relative intensities of the brevinin-1Sa signals were compared using either a Mann–Whitney *U*-test (AMP-intact groups) or a linear mixed model (lmm; total peptides) or glmm (brevinin-1Sa) with temperature group, day and their interaction as fixed effects and frog body mass as a random covariate. Frog body mass was compared using lmms with temperature group, exposure group, peptide treatment and their interaction as fixed effects and day as a random covariate.

To test for differences in *Bd* load among *Bd*-exposed treatment groups, we used a glmm with log-transformed DNA copy number estimates from qPCR assays of skin swabs as the dependent variable and temperature and peptide treatments as main effects and frog body mass as a random covariate. To test for differences in survival, we used Cox regressions with temperature, *Bd* exposure and peptide treatment group as categorical covariates.

All analyses were done in SPSS v24. Details of mixed models and results tables can be found in Appendix 1. Error bars shown in all figures represent the standard error of the mean.

RESULTS

Pattern of expression of AMPs

Four typical AMPs have been isolated and characterized in adult southern leopard frogs (Conlon et al., 1999; Holden et al., 2015a). They are brevinin-1Sa (*m*/*z* 2521), brevinin-1Sb (*m*/*z* 2537), brevinin-1Sc (*m*/*z* 2612) and temporin-1S (*m*/*z* 1444). We were able to detect these peptides among the samples that we examined for this study, but not every frog expressed all four of them (Fig. 2). In a previous study, we showed that nearly all frogs greater than 1 year of age from this source population expressed brevinin-1Sa, whereas fewer expressed brevinin-1Sb, brevinin-1Sc or temporin-1S (Holden et al., 2015a).

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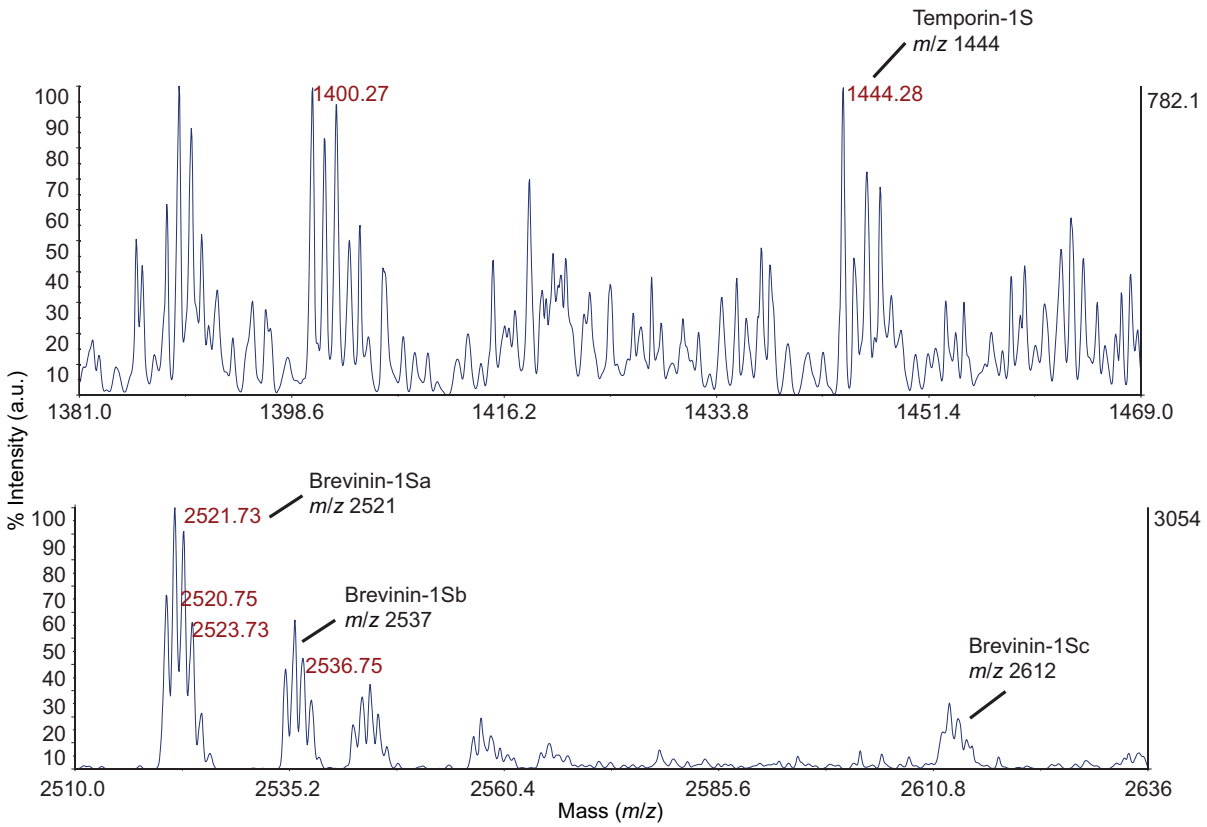

Fig. 2. MALDI-TOF mass spectrometry profile of skin peptides collected from an uninfected juvenile frog. This frog was held at 26°C for 7 days prior to collection of this peptide sample (day −1). Four previously described peptides are evident in this sample: temporin-1S (*m*/*z* 1444), brevinin-1Sa (*m*/*z* 2521), brevinin-1Sb (*m*/*z* 2537) and brevinin-1Sc (*m*/*z* 2612). a.u., arbitrary units.

At 1 month post-metamorphosis, when this study began, newly metamorphosed frogs were just beginning to express their array of AMPs. As we had observed previously, the majority of frogs induced to express their peptides at the beginning of this experiment expressed brevinin-1Sa on day −1 (when the frogs had been acclimated to the test temperature, but prior to *Bd* exposure), and fewer of the frogs expressed the other AMPs (Fig. 3). [Note that in accordance with the design of the experiment, only 32/64 frogs were induced to secrete peptides before *Bd* exposure.] As brevinin-1Sa was the most frequently expressed peptide among these young frogs, we chose to look more carefully at the effects of temperature and *Bd* exposure on expression of this particular peptide. Although our data for the other three AMPs are more limited, they suggest that secretion of these peptides is temperature and (in some cases) infection dependent (see Appendix 2).

Effects of temperature on peptide expression and renewal

Frogs grew faster at 26°C than at 14°C (lmm body mass: $F_{1,212}$=31.566, P<0.001), such that by the end of the experiment, frogs in the 14°C groups had gained, on average, only 1.4 g but frogs in the 26°C groups had gained an average of 12.4 g. We found no evidence for significant differences in body mass due to AMP depletion or *Bd* exposure (lmm body mass: all main and interaction effects P≥0.084; Table S2). Among frogs that were induced to secrete peptides monthly, the mass of peptides expressed at each time point (per gram of frog body mass) showed a significant temperature×exposure interaction (lmm: $F_{1,15.802}$=6.904, P=0.018), where frogs that were exposed to *Bd* at 26°C expressed the least amount of peptides (mean±s.e.m.: 320±53 µg g^{-1}), frogs sham exposed at this same temperature expressed the most (559±53 µg g^{-1}) and frogs at 14°C expressed intermediate amounts (*Bd* exposed: 416±71 µg g^{-1}, sham exposed: 354±51 µg g^{-1}). The total amount of peptides secreted at the end of the study by frogs in our AMP-intact

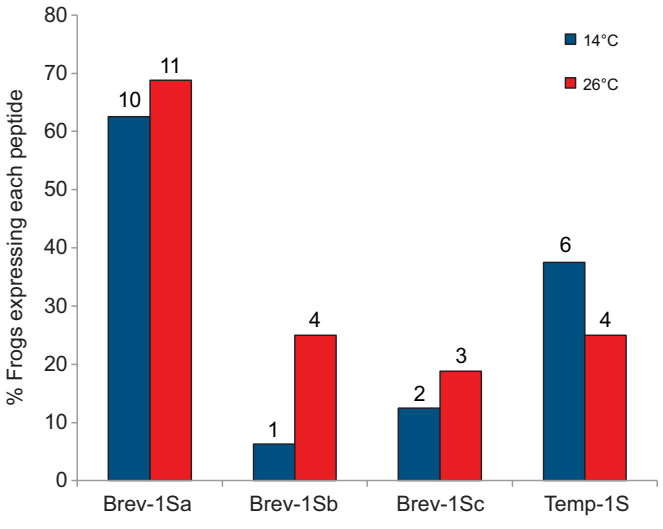

Fig. 3. Percentage of frogs expressing each AMP at day −1. The day −1 samples represent all frogs acclimated to either 14 or 26°C but prior to exposure to *Bd*. Note that the most frequently expressed peptide is brevinin-1Sa. There was no significant difference in peptide expression among the temperature groups (Fisher's exact test: P=0.567). The numbers above each column show the number of frogs expressing each peptide (N=16 per group).

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groups (494±60 µg g^{-1}) did not differ among temperature (Mann–Whitney: P=0.560) or exposure (Mann–Whitney: P=0.452) groups.

Regardless of temperature, nearly all control frogs (that were not exposed to *Bd* and were not induced to secrete peptides until the end of the experiment) expressed brevinin-1Sa at the conclusion of the experiment (day 92; Fig. 4A). However, among sham-infected frogs that were previously depleted of their peptides at monthly intervals (days −1, 27 and 62), there was a significant effect of temperature; frogs held at 14°C were less likely to express brevinin-1Sa than those held at 26°C (Fig. 4B). The relative intensity of brevinin-1Sa expression for the control (sham-exposed and not previously depleted of AMPs) frogs held at 14°C was not significantly different from that of frogs held at 26°C on day 92 (Fig. 4C). This suggests that at the cooler temperature, the frogs were still able to synthesize and secrete this peptide nearly as well as those held at warmer temperatures if given enough time. In contrast, if the frogs were depleted of peptides monthly, the relative intensity of the brevinin-1Sa signal in the cool frogs was significantly less than that of frogs held at 26°C (Fig. 4D), suggesting they were impaired in their ability to restore the depleted peptide reserves. Additionally, frogs at 26°C secreted more brevinin-1Sa toward the end of the experiment than in the earlier samples, while frogs at 14°C showed no increase in the relative intensity of expression of this peptide over time.

Effects of temperature and *Bd* exposure on survival and brevinin-1Sa peptide renewal

All but two of the *Bd*-exposed frogs (both housed at 26°C) showed evidence of infection (as determined by at least one positive qPCR result), although many frogs at 26°C tested negative for *Bd* for several weeks in a row toward the end of the experiment, suggesting they were able to clear and/or limit infection despite repeated exposures (Fig. 5A). For frogs exposed to *Bd* zoospores weekly, *Bd* load, as determined by qPCR, was greater at 14°C than at 26°C (glmm: $F_{1,6}$=137.151, P<0.001) (Fig. 5A), but there was no

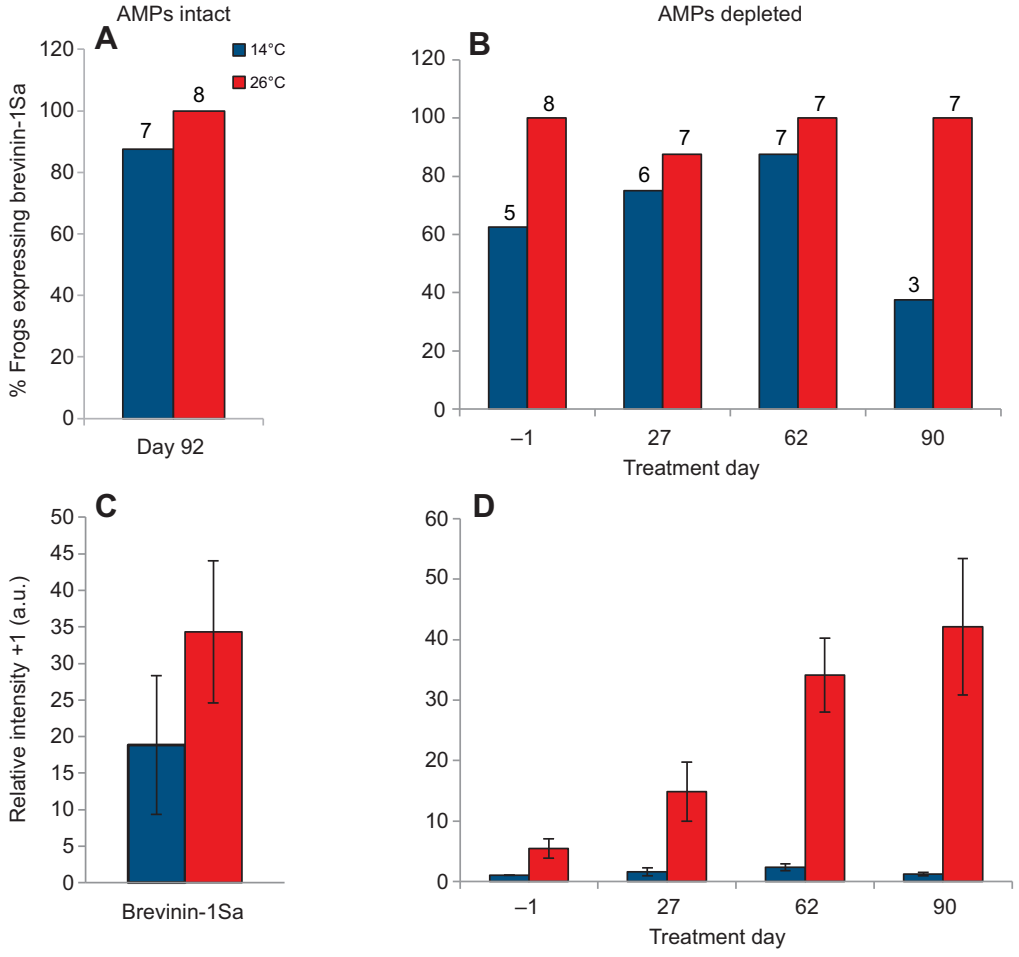

Fig. 4. Effects of temperature on brevinin-1Sa expression and renewal in the absence of *Bd* exposure. (A) Percentage of frogs expressing brevinin-1Sa at day 92 with no prior *Bd* exposure or peptide depletion (N=8 per group). (B) Percentage of frogs expressing brevinin-1Sa with no prior *Bd* exposure and peptides induced monthly on the days shown (N=8 for all groups except treatment day 62 at 26°C, where N=7). Fewer frogs held at 14°C were able to express the peptide in comparison with frogs held at 26°C (glmm, temperature: $F_{1,10}$=9.315, P=0.012). For A and B, numbers above each column show the number of frogs expressing each peptide in each group. (C) Relative intensity of brevinin-1Sa in frogs that were not exposed to *Bd* and had no previous peptide depletion. In the absence of peptide depletion or exposure to *Bd*, there was no significant difference in the relative intensity of this peptide signal at day 90–92 between temperature treatments (Mann–Whitney: P=0.281, N=8 frogs per treatment group). (D) Relative intensity of brevinin-1Sa in frogs with no exposure to *Bd* and peptides depleted monthly (N=7 frogs at 26°C and N=8 frogs at 14°C). Frogs held at 26°C had significantly greater relative intensity of the peptide signal in comparison with those held at 14°C (glmm temperature: $F_{1,5}$=69.266, P<0.001). Frogs at 26°C showed an increase in relative intensity of this peptide over time while frogs at 14°C did not (glmm, temperature×day interaction: $F_{3,54}$=34.698, P<0.001). When a peptide signal either was not detected or was below the threshold for quantification, it was assigned a zero value.

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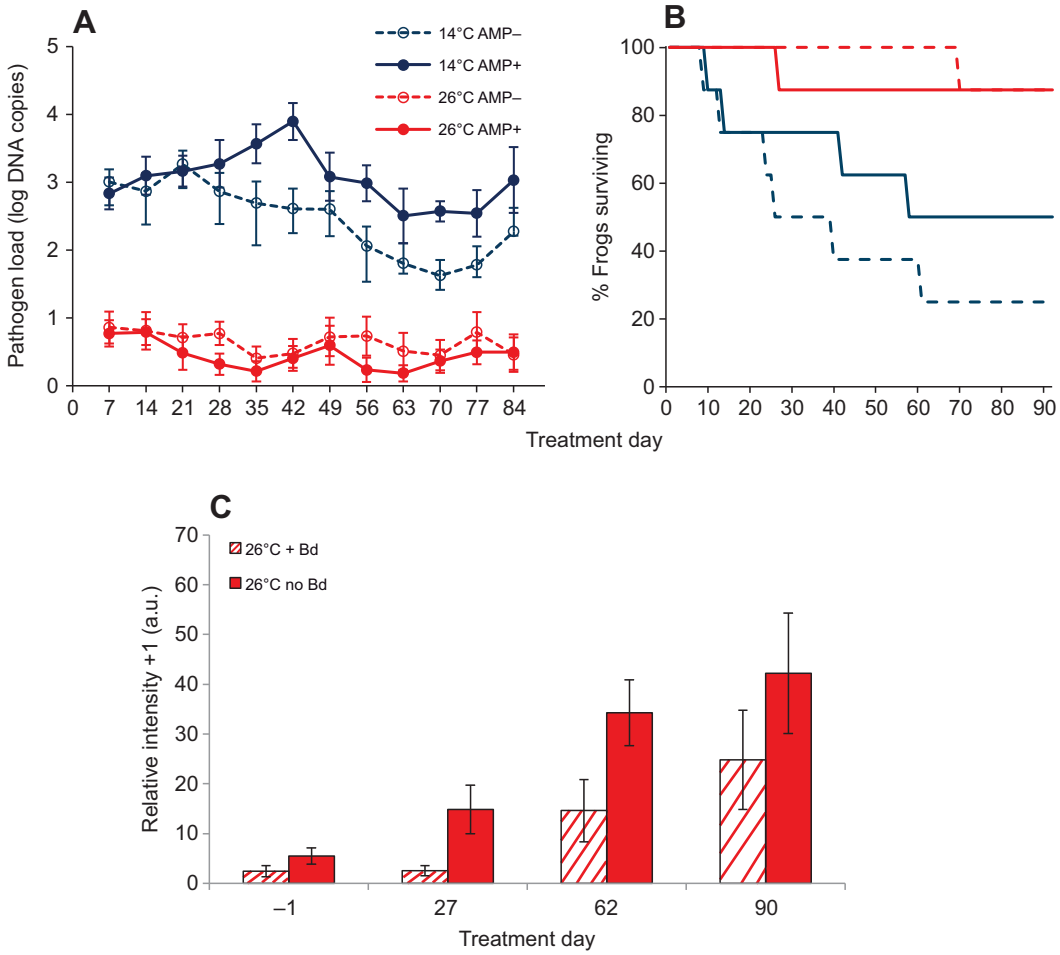

Fig. 5. Effects of temperature and *Bd* exposure on survival and brevinin-1Sa peptide renewal. (A) *Bd* load per quantitative (q)PCR reaction (1/40th of whole swab extraction) over time at each temperature for all surviving frogs regardless of treatment at each temperature. (B) Survival of *Bd*-exposed frogs held at 14°C (blue) or 26°C (red) with (dashed) or without (solid) AMPs depleted monthly. (C) Relative intensity of brevinin-1Sa expression in frogs held at 26°C with AMPS depleted monthly and with or without exposure to *Bd*. When a peptide signal either was not detected or was below the threshold for quantification, it was assigned a zero value. Infection with *Bd* significantly impaired the capacity to renew depleted peptides (glmm, exposure group: $F_{1,52}$=8.249, P=0.006). N=8 frogs sampled at each time point except for days 62 and 90; at day 62, one in the no *Bd* group was dead and at day 90 two frogs in the *Bd* group were dead.

significant main effect of AMP treatment (glmm: $F_{1,6}$=0.492, P=0.509) and no significant interaction between AMP treatment and temperature (glmm: $F_{1,6}$=1.235, P=0.309) on *Bd* load. All but one of the sham-exposed frogs survived until the end of the experiment and survival of animals in *Bd*-exposed groups was significantly lower (Cox regression: χ^2_1=6.959, P=0.008). The interaction between *Bd* exposure and temperature was significant (Cox regression: χ^2_1=10.332, P=0.001), such that frogs held at 14°C suffered greater mortality than those held at 26°C only when *Bd* exposed. However, temperature, on its own, was not a significant predictor of survival (Cox regression: χ^2_1=0.135, P=0.713). While fewer *Bd*-exposed frogs in the AMP-depletion group survived to the end of the experiment at 14°C, the difference in survival between AMP treatment groups was not significant at either temperature (Cox regression: $\chi^2_1 \leq 0.932$, $P \geq 0.334$; Fig. 5B). This suggests that the cooler temperature impaired the capacity of *Bd*-exposed frogs to control the infection and survive. In contrast, if frogs were exposed to *Bd* and held at 26°C, nearly all that were depleted of their peptides monthly and exposed to *Bd* were able to survive to day 90 (Fig. 5B).

Because so few frogs held at 14°C, exposed to *Bd* and induced to secrete peptides monthly survived, it was not possible to examine the effects of *Bd* exposure on the capacity to restore secretion of brevinin-1Sa signals after each round of peptide depletion. However, most of the frogs induced to secrete peptides monthly and held at 26°C survived to the end of the experiment, regardless of whether or not they were exposed to *Bd*. In these frogs, it was possible to gauge the effects of *Bd* infection on peptide synthesis and secretion. Here, we detected brevinin-1Sa more often in sham-treated than in *Bd*-exposed frogs toward the end of the experiment (glmm, exposure×day interaction: $F_{3,57}$=3.108, P=0.033) and the relative intensity of the brevinin-1Sa peptide signals in frogs exposed to *Bd* was significantly lower than in those frogs that were never exposed to *Bd* throughout the experiment (Fig. 5C). In the frogs held at 26°C that were not induced to secrete peptides until the end of the experiment (day 92), there was no effect of *Bd* exposure on whether or not they expressed brevinin-1Sa (5 of 6 exposed to *Bd* versus 7 of 8 not exposed to *Bd*) or the relative intensity of the brevinin-1Sa signal (34.4±13.8 a.u. for those exposed to *Bd* versus 33.3±9.7 a.u. for those not exposed to *Bd*). Thus, regardless of whether or not they were exposed to *Bd*, they were able to synthesize and secrete AMPs over this long period in which their AMP reserves were not disturbed.

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DISCUSSION

Skin defenses, including AMPs, provide the first line of defense against *Bd* and other pathogens.

Our results show that both temperature and *Bd* exposure had significant effects on the capacity of juvenile leopard frogs to restore peptides after monthly depletion. This suggests that at certain times of the year and in certain climates, frogs may be more vulnerable to infection.

Effects of temperature and AMP depletion on infection

Frogs held at 14°C were poorly able to restore peptides in comparison with those held at 26°C (Fig. 4D). While frogs held at 26°C were better able to restore their peptides, if they were exposed to *Bd*, this capacity was significantly reduced (Fig. 5C). Pathogen burden and mortality were greater in frogs held at 14°C in comparison with those at 26°C. This could be due to low temperature interfering with peptide renewal and/or favoring the survival and reproduction of *Bd* at 14°C. There is very little information in the literature about the effects of temperature on the synthesis and release of AMPs in frogs. One previous study of the production of brevinin-1SY by wood frogs (*Rana sylvatica*) showed that cold frogs at 5°C had no detectable brevinin-1SY. However, when the frogs were warmed to 30°C for 3 weeks, the peptide was detectable (Matutte et al., 2000). In green-eyed tree frogs (*Litoria serrata*), increased production of defensive skin peptides was correlated with elevated stream temperatures (Woodhams et al., 2012). Our current study provides more support for the hypothesis that cold temperatures would slow peptide synthesis and renewal.

Our analysis did not support a role for decreased AMPs at lower temperature as the best explanation for greater *Bd* loads. Contrary to our predictions based on the previously established link between AMPs and *Bd* susceptibility in other amphibian species (Woodhams et al., 2006a,b, 2007; Pask et al., 2013), pathogen burden was no greater in our peptide-depleted groups than in our peptide-intact groups at either temperature. Survival was lowest in the group of frogs exposed to *Bd* at 14°C that had been depleted of their peptides monthly, although perhaps due to small sample sizes, survival in this group was not statistically lower than that in the analogous group with intact peptides. If the difference in survival we saw between these two groups is indicative of a true survival cost for infected frogs with depleted AMPs, it is interesting to note that their pathogen burden was similar, suggesting tolerance of heavy pathogen burden rather than resistance to infection as a potential role of skin peptides in susceptibility to chytridiomycosis. The same pattern, i.e. similar *Bd* load but reduced mortality in frogs with intact (versus depleted) skin defenses, was seen when northern cricket frogs (*Acris crepitans*) with experimentally altered skin microbial communities were challenged with *Bd* at different temperatures. Taken together, these studies suggest that rather than preventing infection (i.e. conferring resistance), skin defenses may play an important role in a frog's ability to survive (i.e. tolerate) heavy *Bd* loads.

At 26°C, a temperature at which the growth of *Bd* is not optimal in culture (Piotrowski et al., 2004), most frogs survived until the end of our experiment. Although we cannot separate effects of temperature on *Bd* growth from those on amphibian immune defenses with our study design, the pattern of infection over time suggests that frogs at 26°C were successfully resisting infection. Many frogs at this temperature became infected after the initial exposures but were uninfected for several weeks toward the end of the study. In a previous study of juveniles of this species, we also observed decreased pathogen burden after repeated *Bd* exposure (Holden et al., 2015b). At 26°C, we saw no difference in pathogen burden or survival between frogs with intact and depleted peptides, but we cannot rule out the action of other defenses, such as an adaptive immune response (Ramsey et al., 2010), as causes of the low pathogen burden and pattern of infection clearance we observed.

Effects of *Bd* infection on AMP production

Although the presence of a well-developed repertoire of AMPs is associated with lower susceptibility to chytridiomycosis in anuran species (Woodhams et al., 2006a, 2007), few studies have examined whether the presence of an infection with *Bd* in the skin actually affects the synthesis and secretion of AMPs in the granular glands. There is a correlation among frogs sampled in nature showing that those with a higher burden of infection produce a lower amount of total hydrophobic peptides and, specifically, two of the characterized anuran AMPs (maculatin 1.1 and maculatin 2.1; Woodhams et al., 2010). Our study is the first to show experimentally that *Bd* infection impairs the renewal of peptides after their depletion. The repeated injections of noradrenaline in our study were given to cause the release of peptides and a significant depletion of the stored peptide reserves (Gammill et al., 2012; Pask et al., 2013). However, the depleted glands are still intact and, as predicted, our data show that the injected animals rapidly began to synthesize new peptides to replace those that were secreted. We did not investigate the mechanisms by which *Bd* infection in the skin might alter peptide synthesis or release. We can speculate that because AMP synthesis in the granular glands is an ongoing process throughout many areas of the skin (Woodhams et al., 2016) and *Bd* infections disrupt the skin in the areas very close to the underlying granular glands (Berger et al., 1998), the disruption of the epidermal layer near granular glands by *Bd* might interfere with the discharge process. Another alternative explanation is that the chytrid infection is a stressor that results in elevated corticosteroid hormones (Kindermann et al., 2012; Peterson et al., 2013; Gabor et al., 2015; reviewed in Rollins-Smith, 2017) and experimentally elevated corticosteroids can inhibit AMP production (Simmaco et al., 1997).

We speculate that the reduced AMP repertoire of recently metamorphosed juvenile southern leopard frogs, combined with effects of colder temperatures experienced during the autumn, winter and spring of the first year on AMP production, would predispose these animals to be more susceptible to infection and development of chytridiomycosis. The peptide reserves of these young frogs may be initially limited as a result of energy resources being devoted to other functions and can be further depleted through expression in response to natural events such as escape from a predator (Pask et al., 2013). If so, these young frogs would be less able to avoid or clear infections and survive to become sexually reproductive adults. Our results suggest a mechanism by which populations may fail in the recruitment of new individuals from the young of the year in the presence of ongoing chytridiomycosis (Rachowicz et al., 2006; Wise et al., 2014; Rothermel et al., 2016; Clare et al., 2016). While our results do not address population-level effects, they do point to the need for a clearer understanding of the effects of interactions between environmental and biotic stressors (such as pathogen exposure) on immune defenses in order to predict how susceptibility of wildlife to emerging pathogens may vary in space and time.

Appendix 1

Details of statistical models

glmms for detection of less-common peptides by temperature, pathogen exposure and date

To test for effects of temperature and pathogen exposure on the detection (presence/absence) of the less-commonly expressed AMPs (brevinin-1Sb, brevinine-1Sc and temporin-1S) via MALDI mass

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spectrometry in frogs that were induced to secrete peptides monthly (AMP-depleted group), we used glmms with individual frogs as subjects and day as a repeated measure. The models had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. We ran this like a binomial logistic regression, using a binomial distribution with a logit link function, and used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were temperature group, exposure group, day, and all two- and three-way interactions among these variables. Frog body mass was included as a random covariate. Fixed effects results for each peptide can be found in Table S1.

lmm for frog body mass by temperature, peptide treatment and exposure group

To test for differences in body mass among frogs in our treatment groups, we used a lmm with individual frogs as subjects and day (ordinal) as a repeated random covariate. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. The fixed effects were temperature group, peptide group (depleted or intact) and exposure group (*Bd* or sham). We used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects results for this model can be found in Table S2.

lmm for total peptides expressed by temperature and exposure group

To test for differences in the mass of total peptides expressed following noradrenaline injection per gram of frog body mass by frogs in different temperature and exposure groups, we used a lmm with individual frogs as subjects and day (ordinal) as a repeated random covariate. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. The fixed effects were temperature group, exposure group (*Bd* or sham) and the interaction between temperature and exposure, and day was included as a random covariate. We used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects results for this model can be found in Table S3.

glmm for detection of brevinin-1Sa in sham-infected frogs by temperature and date

To test for effects of temperature and day on the detection (presence/absence) of brevinin-1Sa via MALDI mass spectrometry in frogs that were induced to secrete peptides monthly (AMP depleted) but not exposed to *Bd* (see Fig. 4B), we used a glmm with individual frogs as subjects and day (ordinal) as a repeated measure. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. We ran this like a binomial logistic regression, using a binomial distribution with a logit link function, and used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were temperature group, day and the interaction between temperature and day, and frog body mass was included as a random covariate. The fixed effects results for this model can be found in Table S4.

glmm for relative intensity of brevinin-1Sa in sham-infected frogs by temperature and date

To compare the log-transformed relative intensities of brevinin-1Sa expression in frogs that were induced to secrete peptides monthly (AMP depleted) across sampling days and temperature groups (see Fig. 4D), we used a glmm with individual frogs as subjects and day (ordinal) as a repeated measure. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. As the data are continuous values but zero-inflated, we used a normal distribution with an identity link function but used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were temperature group, day and the interaction between temperature group and day, and frog body mass was included as a random covariate. The fixed effects results for this model can be found in Table S5.

glmm for *Bd* load among temperature and peptide treatment groups

To compare pathogen burden across *Bd*-exposed frogs in our different temperature and peptide treatment groups, we used a glmm with individual frogs as subjects and day (ordinal) as a repeated measure. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. As the data are continuous values but zero-inflated, we used a normal distribution with an identity link function but used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were temperature group and peptide group (induced or sham-induced monthly) and body mass and day were included as random effects. The fixed effects results for this model can be found in Table S6.

glmm for detection of brevinin-1Sa in peptide-depleted frogs at 26°C by exposure group

To test for effects of pathogen exposure and day on the detection (presence/absence) of brevinin-1Sa via MALDI mass spectrometry in frogs that were induced to secrete peptides monthly and held at 26°C, we used a glmm with individual frogs as subjects and day (ordinal) as a repeated measure. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. We ran this like a binomial logistic regression, using a binomial distribution with a logit link function, and used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were exposure (*Bd* or sham) group, day and the interaction between exposure group and day. Frog body mass was included as a random covariate. The fixed effects results for this model can be found in Table S7.

glmm for relative intensity of brevinin-1Sa in peptide-depleted frogs at 26°C by exposure group

To compare the log-transformed relative intensity of brevinin-1Sa expression across sampling days and exposure groups (*Bd* versus sham) (see Fig. 5C), we used a glmm with individual frogs as subjects and day (ordinal) as a repeated measure. The model had an autoregressive-1 (AR1) covariance structure and used restricted maximum likelihood. As the data are continuous values but zero inflated, we used a normal distribution with an identity link function but used a Satterthwaite approximation for degrees of freedom and a robust (Huber–White sandwich) method to estimate the covariance matrix. The fixed effects were exposure group, day and the interaction between exposure group and day. Frog body mass was included as a random covariate. The fixed effects results for this model can be found in Table S8.

Appendix 2

Variation in the less-commonly expressed AMPs with temperature and infection

We detected brevinin-1Sb, brevinin-1Sc and temporin-1S less frequently than brevinin-1Sa in juvenile *R. sphenocephala*. Among

frogs that were induced to secrete peptides monthly, we were unable to obtain enough data to compare the relative intensities of the less-commonly expressed peptides across treatment groups, although we did test for differences in detection (presence/absence) of these peptides in our MALDI data across temperature and *Bd*-exposure groups.

As with brevinin-1Sa, we detected brevinin-1Sb (glmm, temperature: $F_{1,63}$=5.912, P=0.018) and brevinin-1Sc (glmm, temperature: $F_{1,26}$=32.873, P<0.001) more often when frogs were held at 26°C than at 14°C. There was also a significant interaction between temperature and day of sampling that indicates that frogs at the higher temperature produced detectable amounts of these peptides more often as the experiment progressed (glmm, temperature×day: brevinin-1Sb $F_{3,36}$=3.431, P=0.032; brevinin-1Sc $F_{3,15}$=101.598, P<0.001). Evidence of an effect of *Bd* exposure on brevinin-1Sb was limited to a significant three-way interaction between exposure group, temperature group and day of sampling (glmm: $F_{3,26}$=7.117, P<0.001, P≥0.206 for all other effects involving exposure group) that was difficult to interpret. However, for brevinin-1Sc there was a clear difference among exposure groups (glmm, P<0.001 for main and interactive effects of exposure group). With one exception (a frog in our 26°C *Bd*-exposure group with no previous peptide depletions on day 92), we did not detect this peptide in frogs exposed to *Bd*. The 11 other frogs that secreted detectable amounts of this peptide were from sham-exposure groups. This suggests that infection with *Bd* limited the expression of this peptide by juvenile *R. sphenocephala*.

The pattern of detection for temporin-1S was quite different from that of the brevinin peptides, although it also showed a pattern of temperature dependence. We detected temporin-1S more often in frogs held at 14°C (glmm, temperature: $F_{1,56}$=4.461, P=0.039), and frogs produced detectable amounts of it more often early on than later in the experiment (glmm, day: $F_{3,50}$=2.950, P=0.042), especially at low temperature (glmm, temperature×day: $F_{3,50}$=2.958, P=0.041). We found no evidence for an effect of *Bd* exposure on production of this peptide (glmm, P≥0.125 for all effects involving exposure group).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.J.R., C.L.R.-Z.; Methodology: L.K.R., L.A.R.-S., C.L.R.-Z.; Validation: L.K.R., L.A.R.-S., C.L.R.-Z.; Formal analysis: M.J.R., L.K.R., L.A.R.-S., C.L.R.-Z.; Investigation: M.J.R., C.L.R.-Z.; Resources: L.A.R.-S., C.L.R.-Z.; Data curation: C.L.R.-Z.; Writing - original draft: M.J.R., L.A.R.-S.; Writing - review & editing: L.A.R.-S., C.L.R.-Z.; Supervision: L.A.R.-S., C.L.R.-Z.; Project administration: C.L.R.-Z.; Funding acquisition: C.L.R.-Z.

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Data availability
Data are available from the Dryad digital repository (Robak et al., 2019): dryad.rd736bd

Supplementary information
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