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Expression analysis and identification of antimicrobial peptide transcripts from six North American frog species

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ABSTRACT: Frogs secrete antimicrobial peptides onto their skin. We describe an assay to preserve and analyze antimicrobial peptide transcripts from field-collected skin secretions that will complement existing methods for peptide analysis. We collected skin secretions from 4 North American species in the field in California and 2 species in the laboratory. Most frogs appeared healthy after release; however, *Rana boylii* in the Sierra Nevada foothills, but not the Coast Range, showed signs of morbidity and 2 died after handling. The amount of total RNA extracted from skin secretions was higher in *R. boylii* and *R. sierrae* compared to *R. draytonii*, and much higher compared to *Pseudacris regilla*. Interspecies variation in amount of RNA extracted was not explained by size, but for *P. regilla* it depended upon collection site and date. RNA extracted from skin secretions from frogs handled with bare hands had poor quality compared to frogs handled with gloves or plastic bags. Thirty-four putative antimicrobial peptide precursor transcripts were identified. This study demonstrates that RNA extracted from skin secretions collected in the field is of high quality suitable for use in sequencing or quantitative PCR (qPCR). However, some species do not secrete profusely, resulting in very little extracted RNA. The ability to measure transcript abundance of antimicrobial peptides in field-collected skin secretions complements proteomic analyses and may provide insight into transcriptional mechanisms that could affect peptide abundance.

KEY WORDS: Non-lethal collection · Skin secretions · Antimicrobial peptides · Foothill yellow-legged frog · California red-legged frog · Sierra Nevada yellow-legged frog · Pacific treefrog · Real-time PCR

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INTRODUCTION

Amphibians produce a potent arsenal of antimicrobial peptides that are remarkably effective against a wide variety of bacteria (Zasloff 1987, Simmaco et al. 1996, Wegener et al. 1999, Goraya et al. 2000, Mangoni et al. 2003, Apponyi et al. 2004). Some of these peptides (or combinations of peptides) are also effective against some fungi and viruses, including the amphibian chytrid fungus *Batrachochytrium dendrobatidis* and the iridovirus *Frog virus 3* (Simmaco et al. 1996, Goraya et al. 2000, Chinchar et al. 2001, Rollins-Smith et al. 2002a,b,c, 2003, Mangoni et al. 2003, Rollins-Smith & Conlon 2005). Very little is known about the effect of environmental perturbation on the expression and secretion of amphibian antimicrobial peptides; however, exposure to the pesticide carbaryl has been shown to reduce the amount of antimicrobial peptides secreted by foothill yellow-legged frogs *Rana boylii* (Davidson et al. 2007).

Antimicrobial peptides are synthesized as preproproteins and stored in granular glands of the skin in frogs (Rollins-Smith et al. 2005). The antimicrobial

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preproproteins are cleaved by an endopeptidase to release the active peptide at the time of secretion (Resnick et al. 1991). Secretion from the granular glands can be artificially stimulated by electrostimulation or by injection of norepinephrine, neither of which harms the animal (Nutkins & Williams 1989, Tyler et al. 1992). After a full release, the granular gland needs to be totally regenerated before the antimicrobial peptides can be released again, a process that can take longer than 3 wk (Rollins-Smith et al. 2005). Many studies have been done with amphibian antimicrobial peptides isolated from skin secretions collected in the wild (Woodhams et al. 2006, 2010, Tennessen et al. 2009, Melzer & Bishop 2010). However, protein analytical methods can be expensive and only semi-quantitative.

To better understand the effects of environmental disturbance on antimicrobial peptide production, it may be useful to measure the abundance of antimicrobial peptide transcripts, as an intermediate in prepropeptide production. In addition to the peptides and a variety of other bioactive molecules, the granular gland secretions contain poly-adenylated messenger RNAs encoding the antimicrobial preproproteins (Chen et al. 2003). RNA has been isolated from skin secretions collected in the laboratory and immediately preserved, often by snap freezing and lyophilization, for sequencing of antimicrobial peptide precursor transcripts (Chen et al. 2006, Zhou et al. 2006, McCrudden et al. 2007, Bai et al. 2012). Secretion samples collected in the field generally cannot be processed immediately. Here we describe the collection of skin secretions to be used for RNA extraction from 4 anuran species in the field, quantity and quality of RNA extracted from these secretions, and identification of new antimicrobial peptide transcripts from these 4 species, plus 2 species held in the laboratory.

MATERIALS AND METHODS

Laboratory frog care

Frogs maintained in the laboratory were used to develop the secretion collection protocol. Six adult northern leopard frogs *Lithobates pipiens* (formerly *Rana pipiens*) were purchased from Carolina Biological Supply, and 6 adult green frogs *L. clamitans* (formerly *R. clamitans*) were collected as tadpoles from ponds at the Leetown Science Center, West Virginia, USA, in spring 2007 and raised to adults in the laboratory. One adult male Pacific treefrog *Pseudacris regilla* collected at Point Reyes National Seashore, California, USA, on 12 September 2008 and 1 adult male foothill yellow-legged frog *R. boylii* collected at Soda Creek about 85 miles north of Point Reyes National Seashore in December 2009 were shipped to Leetown Science Center. Leopard frogs and green frogs were maintained 2 to 3 frogs tank^{-1}, in black 30.5 cm diameter circular tanks containing spring water to a depth of approximately 10 cm. Each tank contained a Guinea Pig Hut™ (Bio-Serv) to provide both a hiding place and a haul-out area. The Pacific treefrog and yellow-legged frog were each maintained individually in 12 l glass aquaria containing both wet and dry areas and habitat enrichment with branches and rocks.

Frogs were housed at room temperature (19–21°C); spring water, also maintained at room temperature, was changed in tanks and aquaria 3 times wk^{-1}. All frogs kept in the laboratory were fed crickets 3 times wk^{-1}; crickets were fed Fluker's Orange Cube Complete, Fluker's High-Calcium Cricket Feed (Fluker Farms), and Jungle Juice Cricket Formula (Wilcox Pet Supply). In addition to overhead room lighting, frogs were provided 8 h full-spectrum lighting each day. The animal research protocol was approved by the Leetown Science Center Institutional Animal Care and Use Committee.

Both the *Pseudacris regilla* and the *Rana boylii* were alive and healthy 3 and 2 yr, respectively, after secretion collection. The *Lithobates clamitans* and *L. pipiens* were kept in the lab for approximately 6 mo. These frogs were stimulated with norepinephrine approximately once a month; behavior and appearance remained normal throughout this time.

Site selection

Pseudacris regilla were collected from 9 sites in northern California in 2009. Six of those sites were located in an approximately east–west transect, while the other 3 sites and eastern-most of the east–west sites followed a north–south pattern along the crest of the Sierra Nevada. Sites were selected to represent a range of potential exposure to pesticides based on other research (Sparling et al. 2001, Fellers et al. in press). The east–west transect included sites that were just inland from the Pacific Ocean, in the Coast Range, Central Valley, lower foothills of the Sierra Nevada, mid-elevation in the Sierra Nevada, and high elevation in the Sierra Nevada. The north–south transect ran from Lassen Volcanic National Park south to the Lake Tahoe basin, central Sierra Nevada, and southern Sierra Nevada adjacent

to Kings Canyon National Park (Table 1, and see Fig. S1 in Supplement 1; www.int-res.com/articles/suppl/d104p225_supp.pdf). Each site was an isolated pond or a pool in a meadow; streams were not included because we did not want water being flushed out of the ponds the frogs were using.

Because no ranid frog in California is nearly as widespread as *Pseudacris regilla*, it was not possible to sample a species of *Rana* across the same elevational and geographic range. However, we were able to obtain samples from California red-legged frogs *R. draytonii* and *R. boylii* in areas near the coast and in the low to mid-elevations of the Sierra Nevada, and from Sierra Nevada yellow-legged frogs *R. sierrae* in the Sierra Nevada (Table 1, Fig. S1).

Field methods

Frogs were caught using a dip net. Frogs were removed from the net bare-handed, by hand while wearing a vinyl glove, or by using a new Ziploc bag that was inverted over the collector's hand so only the inside of the bag touched the frog. Dip nets were rinsed in the pond after each catch but were not otherwise cleaned between frogs. After capture, frogs were held in their Ziploc bags with a small amount of water.

Table 1. *Pseudacris regilla*, *Rana boylii*, *R. draytonii*, and *R. sierra.* Region (in California, USA) and elevation of each collection site and date (given as mm/dd/yy) and number of frogs captured for collection of skin secretions

Site	Region	Elevation (m)	Collection date	n
P. regilla				
P-084	Coastal	37	02/17/09	13
P-568	Coast Range	282	02/18/09	12
P-780	Central Valley	58	02/19/09	12
P-778	Sierra Foothills	807	03/17/09	12
P-782	Mid-elevation Sierra	2049	05/05/09	15
P-785	High-elevation Sierra	2658	06/09/09	15
L-693	Northern Sierra	2239	06/10/09	15
T-301	Lake Tahoe	2118	05/06/09	14
S-836	South Sierra	2353	05/04/09	15
R. boylii				
Y-806	Coast Range	529	08/30/10 & 08/31/10	14
P-806	Sierra Foothills	398	09/01/10	8
T-016	Sierra Foothills	441	09/02/10	4
R. draytonii				
P-072	Coastal	17	09/01/09	14
P-669	Mid-elevation Sierra	1014	09/08/09	14
R. sierrae				
Y-029	Mid-elevation Sierra	2227	09/03/08 & 09/04/08	15
Y-1025	Mid-elevation Sierra	1633	09/05/08	5
Y-029	Mid-elevation Sierra	2227	09/07/10	12
Y-1025	Mid-elevation Sierra	1633	09/08/10	5

Due to differences in frog behavior, *Rana boylii* and *R. sierrae* were captured during the day, while *Pseudacris regill*a and *R. draytonii* were caught after dark. Depending on the species, skin secretions were collected within a few hours of capture (*R. boylii*, *R. draytonii*, and *R. sierrae*) or the next morning (*P. regilla*). Only male *P. regilla* were used in this study because females were uncommonly encountered. Both sexes were included for all ranid species.

After work at a site had been completed, all equipment that had been in the water (e.g. boots, nets, thermometers) was cleaned of mud and debris, and then dipped in a 10% solution of bleach to kill any amphibian chytrid fungus *Batrachochytrium dendrobatidis* that might be present. This is now a standard procedure for working with anurans, especially in areas like California where the amphibian chytrid fungus is widespread and known to be present at several of our study sites (Fellers et al. 2011).

Vinyl gloves were worn when handling frogs (Cashins et al. 2008), and gloves were changed between each frog. Frogs were weighed, swabbed for a companion study of amphibian chytrid fungus, and then returned to the plastic bag for collection of skin secretions as described below. We then removed the frog to measure its snout–vent length and determine its sex. Frogs were subsequently released at the site of capture. The sample date and number of frogs sampled are listed for each site in Table 1; site locations are shown in Fig. S1. Secretions from 26 *Rana boylii* (collected in 2010), 37 *R. sierrae* (collected in 2008 and 2010), 28 *R. draytonii* (collected in 2009), and 123 *Pseudacris regilla* (collected in 2009) were collected for this study. Twenty *R. sierrae* were caught in 2008; 9 were handled with bare hands and 11 were not. The 9 frogs handled with bare hands were used to assess the effects of this handling on the quality of extracted RNA (Fig. 1), but were excluded from all other analyses and are not included in Table 3 (see 'Results').

Collection of skin secretions

Each frog was stimulated with 0.1 ml of 2.0 mM norepinephrine-HCl dissolved in amphibian phosphate-buffered saline (APBS; 6.6 g NaCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per 1 l distilled water, pH 7.4) per 10 g frog weight (Rollins-Smith et al. 2005). The norepinephrine solution was injected (through the Ziploc

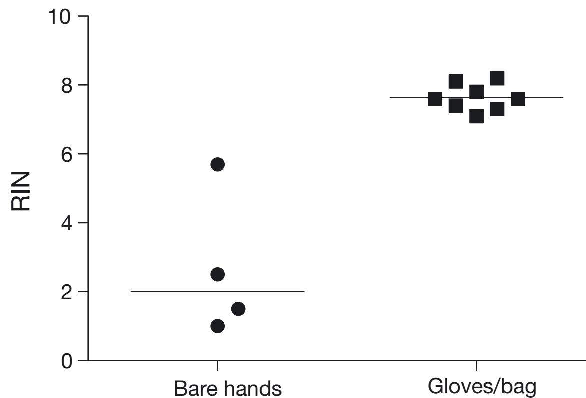

Fig. 1. *Rana sierrae.* Quality of total RNA (reported as the RNA integrity number, RIN) extracted from frogs handled with bare hands compared with frogs handled with gloves or a plastic bag. RNA quality was not measurable in 5 of 9 samples handled with bare hands and in 3 of 11 frogs handled only with gloves or a plastic bag. Median RIN for each sample group is shown as a horizontal line

bag) bilaterally just under the skin on the dorsal side of the frog, using a 1 ml syringe with a 5/8 inch, 25 gauge needle. The frog was removed from the bag after 10 min and the skin secretion collected from the bag. To preserve RNA, the skin secretion was mixed with RNAprotect Saliva Reagent (RPS; Qiagen). When possible, 200 µl of skin secretion were collected from each frog and mixed with 1 ml RPS. If less than 200 µl secretion remained in the bag, then the bag was rinsed with 1 ml RPS to collect all the secretion in the bag. For secretion samples collected from frogs in the laboratory, secretion-RPS mixture was stored at room temperature for 24 h before extracting RNA. For secretion samples collected from frogs in the field, secretion-RPS mixture was kept at ambient temperature for 24 h, and then shipped on ice to Leetown Science Center laboratory for RNA extraction. When necessary, field-collected secretion samples were stored at 4°C before and/or after shipping.

Several other methods to preserve the skin secretion samples for RNA extraction were investigated, including the tissue preservative Ambion RNAlater (Applied Biosystems/Life Technologies). Similar to studies with saliva (Park et al. 2006), skin secretions preserved in RNAProtect Saliva Reagent resulted in higher yield of RNA and better quality RNA (data not shown).

RNA isolation

Total RNA was extracted from skin secretions stabilized with RNAprotect Saliva Reagent using RNeasy Protect Saliva Mini kits (Qiagen), according to the manufacturer's instructions. All RNA extractions included an on-column DNase treatment to remove genomic DNA. RNA was quantified using an ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies/Thermo-Fisher Scientific). As a measure of quality, the RNA integrity number (RIN) was determined for the extracted total RNA using a 2100 Bioanalyzer (Agilent Technologies). RIN is a user-independent measure of RNA quality based on the electrophoretic mobility pattern of the sample (Imbeaud et al. 2005, Schroeder et al. 2006). On a scale of 0 to 10, with 10 representing the highest quality, samples with a RIN of 8 to 10 are considered high quality and not degraded, samples with a RIN of 5 to 7.9 are considered acceptable for some analyses, and samples with a RIN below 5.0 are considered degraded and poor quality.

Sequencing

Skin secretions from frogs held in the laboratory (*Lithobates clamitans*, *L. pipiens*, *Pseudacris regilla*, *Rana boylii*) and skin secretions collected from frogs in the field (*R. draytonii*, *R. sierrae*) were used for sequencing. Total RNA extracted from skin secretions was reverse transcribed and target genes amplified by PCR. Cloned PCR products were sequenced on an ABI3130 (Applied Biosystems/Life Technologies). Sequences were analyzed using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html; Hall 1999).

The primers used to amplify 32 of the antimicrobial peptide precursors, actin, and 18S are listed in Table 2, along with the GenBank accession numbers. Two of the 34 putative antimicrobial peptide precursor cDNA sequences, ranalexin-1C and temporin-1C, were not submitted to GenBank due to short sequence length. Ranalexin-1C and temporin-1C were identified using forward primer LRO-210 and reverse primer LRO-214. The primer sequences used to amplify antimicrobial peptide precursor cDNAs are described in Fig. S2 in Supplement 1; the locations of these primers are illustrated through an alignment to the putative protein coding sequence for *Rana temporaria* mRNA for temporin H precursor (GenBank accession number Y09394; Simmaco et al. 1996). No PCR products were obtained with forward primers LRO-211, LRO-212, or LRO-213 and the reverse primers LRO-215, LRO-216, or LRO-217. An alignment of the predicted antimicrobial peptide precursor sequences is shown in Fig. S3.

Table 2. Antimicrobial peptide, actin, and 18S cDNA sequences identified in this study. Species, name of predicted antimicrobial peptide precursor or reference gene encoded by cDNA, GenBank accession number and the primers used to clone cDNA are given for each sequence. Primer sequences for antimicrobial peptide precursor cDNAs are shown in Fig. S2 in Supplement 1 (www.int-res.com/articles/suppl/d104p225_supp.pdf; primer sequences for 18S and actin are described in the 'Materials and methods'

Peptide or gene	Accession no.	Primers
Lithobates clamitans		
Teptide precursor or geneemporin-3C	JQ511827	LRO-208 & 214
18S	JQ511830	LRO-278 & 138
Actin-1	JQ511828	LRO-275 & 276
Actin-2	JQ511829	LRO-272 & 277
Lithobates pipiens		
Brevinin-1Pb	JQ511810	LRO-334 & 332
Esculentin-1Pa	JQ511811	LRO-335 & 331
Esculentin-1Pb	JQ511812	LRO-335 & 331
Esculentin-1Pc	JQ511813	LRO-335 & 331
Odorranain-M-Pa	JQ511814	LRO-208 & 214
Odorranain-M-Pb	JQ511815	LRO-208 & 214
Odorranain-M-Pc	JQ511816	LRO-333 & 330
Ranacyclin-P	JQ511817	LRO-333 & 330
Temporin-1Pb	JQ511818	LRO-333 & 330
18S	JQ511820	LRO-278 & 138
Actin	JQ511819	LRO-275 & 276
Pseudacris regilla		
Odorranain-M-PR	JQ511831	LRO-208 & 214
Ranatuerin-2PRa	JQ511834	LRO-209 & 214
Ranatuerin-2PRb	JQ511835	LRO-209 & 214
Ranatuerin-2PRc	JQ511836	LRO-209 & 214
Temporin-1PR	JQ511832	LRO-209 & 214
Temporin-3PR	JQ511833	LRO-209 & 214
18S	JQ511838	LRO-278 & 280
Actin	JQ511837	LRO-275 & 277
Rana boylii		
Brevinin-1BY	JQ511846	LRO-526 & 214
Esculentin-2BY	JQ511847	LRO-526 & 214
Ranacyclin-1BY	JQ511848	LRO-526 & 214
Ranatuerin-2BYa	JQ511850	LRO-525 & 214
Ranatuerin-2BYb	JQ511849	LRO-526 & 214
Temporin-1BYa	JQ511851	LRO-525 & 214
18S	JQ511853	LRO-278 & 138
Actin	JQ511852	LRO-275 & 277
Rana draytonii		
Brevinin-1DR	JQ511839	LRO-526 & 214
Esculentin-2DR	JQ511840	LRO-525 & 214
Odorranain-M-DR	JQ511841	LRO-525 & 214
Ranatuerin-2DR	JQ511842	LRO-525 & 214
Temporin-1DRa	JQ511844	LRO-526 & 214
Temporin-1DRb	JQ511843	LRO-526 & 214
18S	JQ511845	LRO-278 & 138
Rana sierrae		
Ranatuerin-2SRa	JQ511821	LRO-208 & 214
Ranatuerin-2SRb	JQ511822	LRO-208 & 214
Temporin-1SR	JQ511823	LRO-209 & 214
Temporin-2SR	JQ511824	Sense & antisense[a]
18S	JQ511826	LRO-278 & 138
Actin	JQ511825	LRO-275 & 277

[a]Sense and antisense primers from Chen et al. (2003)

Temporin-2SR was identified using the sense and antisense primers designed to conserved preproregion of ranateurins/brevinins (Fig. S2; Chen et al. 2003). Based on an alignment with other antimicrobial peptide precursor nucleotide sequences, there appear to be 2 nucleotide deletions in the sense primer, resulting in an incorrect predicted sequence for the first 3 amino acids at the amino-terminus of the precursor. These potentially incorrect amino-terminal residues were removed from the temporin-2SR antimicrobial peptide precursor sequence submitted to GenBank and are shown in Fig. S3.

Forward primer LRO-278 (5′-TGG TTG ATC CTG CCA GTA GC-3′) and reverse primer LRO-138 (5′-ACC ACC ACC CAC AGA ATC GAG AAA-3′) were used to amplify an internal fragment of the 18S ribosomal RNA from *Lithobates pipiens*, *L. clamitans*, *Rana sierrae*, *R. draytonii*, and *R. boylii*. No PCR product was amplified with this primer pair from *Pseudacris regilla*; however, a slightly shorter fragment was amplified using the forward primer LRO-278 with the reverse primer LRO-280 (5′-TCG TTT ATG GTC GGA ACT ACG-3′).

Forward primer LRO-275 (5′-ATT GTT GGT CGC CCC AGA CAT C-3′) and reverse primer LRO-276 (5′-TTT AGA AGC ATT TAC GGT GGA C-3′) were used to amplify a fragment of a cytoplasmic actin gene from *Lithobates pipiens* and *L. clamitans*. Forward primer LRO-275 and reverse primer LRO-277 (5′-CTG CTT GCT GAT CCA CAT CTG-3′) were used to amplify a fragment of a cytoplasmic actin gene from *Rana boylii*, *R. sierrae*, and *Pseudacris regilla*. No PCR product was amplified from *R. draytonii* using either pair of primers (LRO-275 and LRO-276 or LRO-275 and LRO-277). A fragment of a second cytoplasmic actin gene was amplified from *L. clamitans* using forward primer LRO-272 (5′-GAC CTG ACA GAC TAC CTC ATG-3′) and reverse primer LRO-277. Potential intron locations were predicted based on alignment to *Xenopus tropicalis* beta-actin (GenBank accession no. NW_003163328; Hellsten et al. 2010). Forward primer LRO-275 is just upstream of the second predicted intron. LRO-276 is downstream of the fifth predicted intron and contains the predicted STOP codon. LRO-277 is located between the fifth predicted intron and the STOP codon.

Real-time PCR

Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies). Real-time PCR was

performed on an Applied Biosystems ABI7900HT Fast Real-time PCR system using SYBR Green technology. Forward primer 5′-GCC CGA CGG TCA AGT CAT-3′ and reverse primer 5′-GGG CCT CTG GAC ACC TGA A-3′ were used to amplify actin, and forward primer 5′-CCT TTT CTT CCT TGG GAC CAT-3′ and reverse primer 5′-TCG TCT TCA TCG GCA TTT CTC-3′ were used to amplify temporin-1BYa from *Rana boylii*. Real-time PCR assays included controls where the reverse transcriptase enzyme was omitted to confirm little or no contamination with genomic DNA. All reactions were performed in triplicate, and standard curves for absolute quantification were included on all plates.

Statistical analyses

Statistical analyses were done using GraphPad Prism 5. Data sets were tested for normality using the Shapiro-Wilk test. Due to non-normal distribution of most data sets, nonparametric statistical tests were used for most comparisons.

RESULTS

Collection of skin secretions

With the exception of several *Rana boylii*, all field-caught frogs (*Pseudacris regilla*, *R. draytonii*, and *R. sierrae*) appeared to be unharmed by handling. The frogs displayed normal stance and righting behavior after handling, and hopped or swam away after release. Fourteen *R. boylii* were collected in the Coast Range and 12 frogs were collected in the Sierra Nevada (2 sites). All of the Coast Range *R. boylii* appeared normal and were handled without mishap. However, 2 of the Sierra Nevada *R. boylii* died immediately after secretion collection, 9 frogs were lethargic and behaved abnormally after release, and only 1 of the 12 frogs appeared normal. Collections were halted at the Sierra Nevada sites because of the signs of ill health after handling. It is not clear whether this abnormal behavior was due to skin secretion collection: the Sierra Nevada *R. boylii* were very easy to catch compared to frogs from other sites, suggesting that they may not have been in good health prior to handling.

The *Rana sierrae* caught at Summit Meadow in Yosemite Park (Site Y-029) belong to a population that has been studied for many years and continues to be surveyed. These frogs were passive integrated transponder (PIT)-tagged (Fellers et al. 2013) and thus can be identified in subsequent years. Of the 15 frogs caught at Site Y-029 in September 2008, 10 frogs were caught again in 2009, 2010, or 2011 (31 total recaptures). Two frogs were caught in all 4 years (2008–2011). This recapture rate of 67% corresponds well with the normal year-to-year survival rate of 72% for *R. sierrae* at this site (Fellers et al. 2013). None of the other field-caught frogs were tagged.

Yield and quality of RNA from skin secretions

Sex, body weight, and amount of total RNA extracted from skin secretions are shown for each species in Table 3. Sex could not be determined for 2 of the smallest *Rana boylii*. Body weight (g) was significantly higher for females than males in *R. boylii* (Mann-Whitney *U*-test, p = 0.0004). Body weight was not significantly different between male and female *R. draytonii* (Mann-Whitney *U*-test, p = 0.0616) or *R. sierrae* (Mann-Whitney *U*-test, p = 0.3012). Only *Pseudacris regilla* males were used in this study; females were not commonly encountered.

Total RNA was extracted from 200 µl skin secretion collected from each frog, when possible. All *Pseudacris regilla* and 1 *Rana sierrae* produced less than

Table 3. *Pseudacris regilla*, *Rana boylii*, *R. draytonii*, and *R. sierra*. Number (n) and body weight of frogs caught in the field and amount of total RNA extracted from skin secretions. Values are shown for all frogs caught, male frogs only, and female frogs only. Median value and inner quartile range shown for body weight and total RNA

Species	n	Body weight (g)	Total RNA (ng)
Total			
P. regilla	123	4.1 (3.4–5.0)	9.0 (0.0–153.2)
R. boylii	26	15.8 (12.0–29.1)	2574 (1677–4814)
R. draytonii	28	93.8 (65.2–115.7)	179.1 (102.3–305.9)
R. sierrae	28	20.5 (14.5–34.8)	728.6 (342.2–4634)
Male			
P. regilla	123	4.1 (3.4–5.0)	9.0 (0.0–153.2)
R. boylii	10	12.1 (10.4–14.1)	2651 (2004–6529)
R. draytonii	19	87.8 (63.0–104.5)	193.8 (100.2–313.2)
R. sierrae	14	19.2 (14.8–27.4)	645.9 (138.9–3303)
Female			
P. regilla	0		
R. boylii	14	25.2 (15.9–37.3)	2277 (1032–3653)
R. draytonii	9	120.0 (78.4–147.1)	169.2 (61.5–259.2)
R. sierrae	14	28.1 (14.3–46.5)	774.6 (380.0–7396)

200 µl skin secretion. Three *R. boylii* produced more than 200 µl secretion; 27 of the 28 *R. draytonii* produced approximately 300 µl of secretion. One *R. sierrae* secreted only foam; the remaining 27 frogs secreted between 200 and 500 µl skin secretion. One *R. sierrae* that was handled with bare hands secreted approximately 800 µl skin secretion.

The amount of total RNA (ng) extracted from 200 µl skin secretion (or less in *Pseudacris regilla*) g^{-1} body weight (yield) is shown in Fig. 2 for each species. Yield was significantly different among species (Kruskal-Wallis, $p < 0.001$). Yield was higher in *Rana boylii* (median 166.8 ng RNA) and *R. sierrae* (median 43.5 ng RNA) compared with *R. draytonii* (median 2.45 ng RNA) and *P. regilla* (median 2.3 ng RNA; Dunn's multiple comparison post test). *R. draytonii* (Table 3; median body weight 93.8 g) were bigger than *R. sierrae* (median body weight 20.5 g) and *R. boylii* (median body weight 15.8 g), and much bigger than *P. regilla* (median body weight 4.1 g); however, *R. draytonii* did not secrete more than *R. sierrae* or *R. boylii*, and little total RNA was extracted from the secretions. The total RNA extracted from 200 µl skin secretion was not significantly different between males and females for *R. boylii* (Mann-Whitney *U*-test, $p = 0.3340$), *R. draytonii* (Mann-Whitney *U*-test, $p = 0.4606$), or *R. sierrae* (Mann-Whitney *U*-test, $p = 0.5351$).

Skin secretions were collected from *Pseudacris regilla* at sites that ranged from west to east across north-central California and from north to south in the Sierra Nevada. Male frogs were collected at the time of breeding. Due to seasonal differences in site locations, breeding occurred in late winter and early spring at the coast, Central Valley (February), and Sierra Nevada foothill (March) sites but occurred in late spring and early summer at the mid- to high elevation sites in the Sierra Nevada (May to June). The amount of RNA extracted from secretions was significantly different among sites and collection dates (Fig. 3; Kruskal-Wallis $p < 0.0001$), with higher amounts of RNA extracted from secretions collected on earlier dates at more western sites (Dunn's multiple comparison post test).

Of the 20 *Rana sierrae* caught in the Sierra Nevada in September 2008, 9 were handled with bare hands; the remaining 11 frogs were not. RNA quality was not measurable in 5 of 9 samples handled with bare hands and in 3 of 11 frogs handled with gloves or a plastic bag, due to aberrant electrophoretic patterns in the samples. The RNA extracted from frogs caught with bare hands was of poor quality (lower RIN) compared to RNA extracted from frogs handled only within a plastic bag or with gloved hands (Fig. 1).

RNA quality was assessed for all *Rana boylii* samples for which more than 1 µg RNA was extracted from the skin secretion (n = 21). RNA quality was

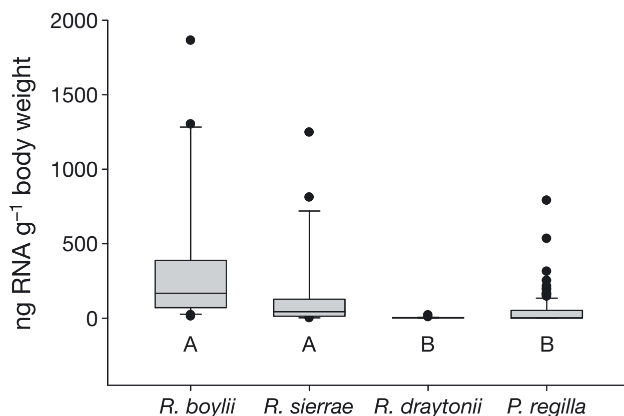

Fig. 2. *Rana boylii*, *R. sierrae*, *R. draytonii*, and *Pseudacris regilla*. Total RNA (ng) extracted from 200 µl secretion g^{-1} body weight of frog, by species. Box plots show lower quartile, median, and upper quartile; whiskers indicate 10th and 90th percentiles. Skin secretions were collected from 26 *R. boylii*, 28 *R. sierrae*, 28 *R. draytonii*, and 123 *P. regilla*. Statistical significance was assessed by a Kruskal-Wallis test ($p < 0.0001$) with Dunn's multiple comparison post hoc pairwise comparisons. Dunn's pairwise comparisons ($p < 0.05$ significance level) are marked by letters below the box plots

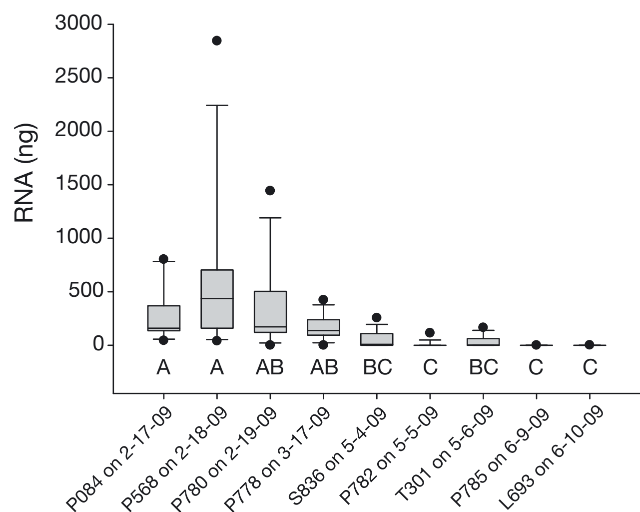

Fig. 3. *Pseudacris regilla.* Total RNA (ng) extracted from skin secretions by date (mm-dd-yy) and site where frogs were collected. Box plots show lower quartile, median, and upper quartile for amount of RNA extracted per frog; whiskers indicate 10th and 90th percentiles. Secretions were collected from 12 to 15 frogs at each site. Statistical significance was assessed by a Kruskal-Wallis test ($p < 0.0001$) with Dunn's multiple comparison post hoc pairwise comparisons. Dunn's pairwise comparisons ($p < 0.05$ significance level) are marked by letters below the box plots

high for all of these samples, and RIN ranged from 8.5 to 9.5. RNA quality was not assessed for *R. draytonii* or *Pseudacris regilla* samples because low amounts of total RNA were extracted from the skin secretions and because this RNA was used for quantification and sequencing.

Identification of putative antimicrobial peptide precursor and reference genes from skin secretions

Transcripts of 34 antimicrobial peptide precursors were sequenced: 6 from *Rana boylii*, 6 from *R. draytonii*, 4 from *R. sierrae*, 6 from *Pseudacris regilla*, 3 from *Lithobates clamitans*, and 9 from *L. pipiens*. An alignment of the predicted antimicrobial peptide precursors is shown in Fig. S3, grouped by the predicted mature peptide. Novel sequences were named based on sequence similarity to other mature peptides and according to the nomenclature conventions suggested by Conlon (2008).

Partial sequences for 18S were obtained for *Lithobates clamitans*, *L. pipiens*, *Pseudacris regilla*, *Rana boylii*, *R. draytonii*, and *R. sierrae*. Fragments encoding partial predicted coding sequence for actin were identified and sequenced from *L. clamitans*, *L. pipiens*, *P. regilla*, *R. boylii*, and *R. sierrae*; no actin sequence was amplified from *R. draytonii*. Two different actin genes were identified from *L. clamitans*. The nucleotide sequences for these 2 actin gene fragments from *L. clamitans* were 81% identical over 531 bases; however, the predicted protein sequences were 99% identical over the encoded 177 amino acids. The 18S and actin sequences were identified for use as possible reference genes for real-time PCR.

Quantification of antimicrobial peptide expression by real-time PCR

The mRNA abundance of actin and temporin-1BYa and the normalized abundance of temporin-1BYa were not different between the *Rana boylii* collected in the Coast Range and those collected in the Sierra Nevada. When both sites were grouped together, the mRNA abundance of temporin-1BYa was significantly higher in male than in female frogs (*t*-test, $p = 0.0191$); normalized abundance of temporin-1BYa was not higher in males than females (Fig. S4). The amount of total RNA (ng) and quality of the extracted RNA (RIN) were not significantly different between male and female frogs and were not significantly different between Coast Range and Sierra Nevada frogs.

DISCUSSION

We developed a field assay for collection of skin secretions for RNA extraction. This assay allowed identification of putative antimicrobial peptide precursor genes and development of real-time PCR assays to measure expression of these putative antimicrobial peptide precursors in rare or declining frog species in the wild. A large amount of skin secretion was collected (200 µl) in the field and did not require freezing, which can be difficult at field sites. Skin secretions were collected from 4 species in the field: *Pseudacris regilla*, *Rana boylii*, *R. draytonii*, and *R. sierrae*. The assays we used were generally non-lethal in both the laboratory and the field and in multiple frog species. Our methods were easy to perform in the field and did not require specialized equipment.

Regeneration of antimicrobial peptides after a full release of the granular glands can be a lengthy process, potentially leaving the frog vulnerable to skin pathogens (Rollins-Smith et al. 2005). The concentration of norepinephrine used in this study results in a moderate, but not complete, secretion of peptides (Rollins-Smith et al. 2005). Frogs were not rinsed for collection of skin secretions. While rinsing the frog would allow a more complete collection of the skin secretion, rinsing also generates a large sample volume that is difficult to process in the field. Because the frogs were not rinsed, some secretion remained on frog skin, potentially protecting the frog for a short time after release.

Wearing gloves while handling amphibians has been recommended to avoid potential transfer of the amphibian chytrid fungus and other microbial pathogens (Phillott et al. 2010). As noted by Skerratt et al. (2011), wearing gloves while handling amphibians may also improve specificity of diagnostic quantitative PCR (qPCR) tests used to identify infection. However, there are reasons to avoid gloves while catching and handling frogs. Gloves are extremely slippery when wet, which makes catching frogs in aquatic environments difficult. More importantly, handling frogs with gloves may be harmful to the health of the frog. While there are no published reports of lethal effects due to handling adult frogs with gloves, handling tadpoles with gloves, even for very short amounts of time, can be lethal (Cashins et al. 2008). Our study found that handling frogs with bare hands resulted in poor quality RNA extracted from skin secretions, compared with RNA collected from frogs that were never directly touched. Handling frogs with gloves or plastic bags may avoid contami-

nation of skin tissue or skin secretions with RNases from the researcher's hands. The quality of RNA is important for gene expression analyses: decreased mRNA abundance may be measured in RNA samples of lesser quality (Imbeaud et al. 2005, Fleige & Pfaffl 2006, Fleige et al. 2006, Schroeder et al. 2006, Robertson et al. 2012). Skin secretion samples collected in the field yielded high-quality RNA that was suitable for downstream applications. However, very little RNA was extracted from the secretions of some species (*Pseudacris regilla* and *Rana draytonii*).

The amount of RNA extracted from *Pseudacris regilla* skin secretions varied with the date or site of collection. These differences were not due to breeding season or air temperature. Secretions were collected from calling adult male *P. regilla* collected during the breeding season. The timing of the breeding season varied between sites, although the air temperature did not vary among collection sites or dates (data not shown). However, the location, surrounding land use (protected national park, pasture, or intensive agriculture), winter weather (extent of winter snow pack), prevailing wind pattern, and exposure to airborne contaminants varied among sites.

The assay described here measures transcript abundance for antimicrobial peptide precursors, not abundance of the antimicrobial peptide, which is the final functional product. A chromatography-based method (Rollins-Smith et al. 2002c) to partially purify antimicrobial peptides from amphibian skin secretions, followed by mass spectrometry to identify specific peptides, has been extremely useful for numerous studies both in the laboratory and in the field. However, these chromatography methods are only semi-quantitative; mass spectrometry and protein purification methods are laborious and expensive. Quantitative real-time RT-PCR is a widely available and relatively inexpensive technique that can accurately measure very small amounts of mRNA (Higuchi et al. 1993) over a 100 000-fold range (Morrison et al. 1998, Palmer et al. 2003), requires small amounts of starting material (Wang & Brown 1999, Malinen et al. 2003, Czechowski et al. 2004), and can distinguish between almost identical sequences, which is important for the analysis of multi-gene families. The global variation between transcriptome (the population of transcripts weighted for abundance) and translatome (the population of proteins weighted for abundance) can be large, and mRNA abundance does not allow a simple calculation of protein abundance (Gygi et al. 1999, Greenbaum et al. 2002, Vogel & Marcotte 2012). However, transcription is a required intermediate step in protein production, and changes in transcript abundance can affect protein abundance.

The production of antimicrobial peptides is regulated in amphibians, and this regulation may occur at the transcriptional level. For example, antimicrobial peptides are produced by wood frogs at warm temperatures, but not by fasting, cold-acclimated frogs, and both peptide production and regeneration of the granular gland are stimulated by microorganisms (Matutte et al. 2000, Mangoni et al. 2001). In *Drosophila*, transcriptional regulation of the antimicrobial genes involves toll or toll-like receptors and the transcription factor NF-kappa B (or Rel family protein; Lemaitre et al. 1996). Antimicrobial peptide genes from pigs, striped bass, and silkworm contain NF-kappa B binding site motifs in their promoters (Zhao et al. 1995, Ponnuvel & Yamakawa 2002, Shike et al. 2002). Functional NF-kappa B transcription factor binding motifs have also been identified in the promoter of the bombinin gene of *Bombina orientalis* (Miele et al. 1998, 2001).

The antimicrobial peptide genes from hylid and ranid frogs compose a family of genes that encode preproproteins with an extremely variable antimicrobial peptide domain and a region that contains a highly conserved signal peptide and acidic region (Duda et al. 2002, Vanhoye et al. 2003). Transcripts of 34 antimicrobial peptide precursors were sequenced using primers designed to conserved regions around the predicted start codon, signal peptide, and a conserved region downstream of the predicted stop codon: 6 from *Rana boylii*, 6 from *R. draytonii*, 4 from *R. sierrae*, 6 from *Pseudacris regilla*, 3 from *Lithobates clamitans*, and 9 from *L. pipiens*. Two of the predicted mature peptides encoded by transcripts identified in this study for *R. boylii* (temporin-1BYa and brevinin-1BY) are identical to mature peptides identified by Conlon et al. (2003); the other 4 are novel. Three of the 6 *R. draytonii* predicted mature peptides identified in this study have been previously identified (Conlon et al. 2006); 3 are novel: esculentin-2DR, temporin-1DRa, and odorranain-M-DR. Two of the predicted mature peptides encoded by transcripts identified in this study for *L. clamitans* (ranalexin-1C and temporin-1C) are identical to previously identified peptides (Halverson et al. 2000); temporin-3C has not been previously identified. Two of the mature peptides encoded by transcripts identified in this study from *L. pipiens* skin secretions are identical to the previously identified peptides brevinin-1Pb and ranacyclin-P, which is also called peptide leucine arginine (pLR). No antimicrobial peptides have been identified previously for *P. regilla* or *R. sierrae*.

This study identified antimicrobial peptide precursor transcripts for novel antimicrobial peptides that have not been identified by peptide-based methods, in species where the antimicrobial peptides have been investigated. It is possible that these novel transcripts are not translated and processed into functional antimicrobial peptides. However, it is also possible that these precursors do encode functional peptides. The array of antimicrobial peptides produced can differ between populations, due to allelic differences (Tennessen et al. 2009). In addition, expression of distinct antimicrobial peptides may be differentially regulated, depending upon the gender, physiological status, or microhabitat of the individual. As shown in Fig. S4 in Supplement 1, the expression of temporin may be regulated by sex in *Rana boylii*. The ability to preserve skin secretions collected in the field for RNA analysis will complement proteomic analyses in the identification of novel antimicrobial peptide precursors, determination of the full complement of antimicrobial peptides produced by an individual, population, or species, and evaluation of antimicrobial peptide abundance. These techniques will also enable investigation into the potential transcriptional regulation of antimicrobial peptide gene expression and possible effects of environmental perturbation on the transcription of these important components of the amphibian immune system.

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