

Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*)

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Abstract

The mountain yellow-legged frog (*Rana muscosa*) inhabits high elevation lakes in California that are largely undisturbed by human activities. In spite of this habitation in remote sites, populations continue to decline. Although predation by non-native fish is one cause for declines, some isolated populations in fishless lakes are suffering new declines. One possible cause of the current wave of declines is the introduction of the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*) which invades the adult skin to cause chytridiomycosis. In many amphibian species, the skin is protected by antimicrobial peptides secreted into the mucous. Here we show that *R. muscosa* produces three previously unknown antimicrobial peptides belonging to the ranatuerin-2 and temporin-1 families of antimicrobial peptides. These three peptides, along with bradykinin, are the most abundant peptides in the skin secretions detected by mass spectrometry. Natural mixtures of peptides and individual purified peptides strongly inhibit chytrid growth. The concentration of total peptides recovered from the skin of frogs following a mild norepinephrine induction is sufficient to inhibit chytrid growth in vitro. A comparison of the species susceptibility to chytridiomycosis and the antichytrid activity of peptides between *R. muscosa* and *R. pipiens* suggest that although *R. muscosa* produces more total skin peptides, it appears to be more vulnerable to *B. dendrobatidis* in nature. Possible differences in the antimicrobial peptide repertoires and life history traits of the two species that may account for differences in susceptibility are discussed.

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Abbreviations: ATCC, American Type Culture Collection (USA); BCA, Bicinchoninic acid; HPLC, High-pressure liquid chromatography; IUCN, International Union for Conservation of Nature and Natural Resources; MALDI-TOF MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MIC, Minimal inhibitory concentration; NCTC, National Collection of Type Cultures (UK); TFA, Trifluoroacetic acid; UV, Ultraviolet.

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1. Introduction

Mountain yellow-legged frogs (*Rana muscosa*) were once abundant in the Sierra Nevada Mountains of California and the San Gabriel, San Bernardino, and San Jacinto Mountains of southern California, and a few populations were once present in Nevada [1]; however, historical records and museum collections document the dramatic decline in populations of this unique species in the last century [2–5]. Southern populations are now federally listed as endangered, and remaining Sierra Nevada populations will likely also become federally protected soon [6].

R. muscosa occurs mostly at high elevation, and the cool temperature conditions and short summers result in a larval stage that can last as long as 4 years [7]. Because adults are highly aquatic and the larvae require water to survive, the species is dependent on permanent bodies of water for continued survival. Introduced trout are responsible for many of the population extinctions. There is a strong negative correlation between *R. muscosa* and introduced trout [5,6,8–10], and field experiments show that predation drives the pattern and that it can be reversed [6]. However, some declines have occurred in the absence of introduced trout [3], and other possible causes include changes in weather patterns that increase severity of droughts [5], habitat fragmentation [5], air-borne pesticides [11], and disease [rev. 12,13].

Although other pathogens may cause disease, one possible cause of recent population declines in fishless habitats is a newly discovered chytrid fungus, *Batrachochytrium dendrobatidis* [14–16]. This globally emerging pathogen causes the disease chytridiomycosis. The pathogen invades the outermost keratinized layers of the skin and induces hyperkeratosis [14–16]. Although tadpoles of *R. muscosa* [17–19] and other species [14] are infected in the keratinized mouth parts, the pathogen only causes death of newly metamorphosed froglets and adults when zoospores infect the skin [14,19]. The mechanism by which the pathogen causes death is unknown, but it may be due to release of a toxin or disturbance of fluid and ion balance functions of the skin [13,14,16]. Because *B. dendrobatidis* infects the skin, we systematically study the skin defenses of a variety of species that may be exposed to this pathogen. Lack of lymphocytic infiltration in response to the pathogen suggests that adaptive immune responses are not

effective [14,16]. One important innate defense of the skin is the production of antimicrobial peptides in the dermal granular glands. Here we show that *R. muscosa* produces three previously unknown antimicrobial peptides belonging to the ranatuerin-2 and temporin-1 families of antimicrobial peptides. These three peptides, along with bradykinin, are the most abundant peptides in the skin secretions. Their activity in growth inhibition assays against *B. dendrobatidis* suggests that they should provide some protection against infection by chytrid zoospores. In comparison with the antimicrobial peptide defenses of a non-declining species, *R. pipiens*, *R. muscosa* would be predicted to have an adequate antimicrobial peptide defense; however, the cold conditions in which this species survives may allow a cold-adapted pathogen to overcome these limited defenses.

2. Materials and methods

2.1. Animals

Adult mountain yellow-legged frogs (*R. muscosa*) of both sexes were collected by netting in several locations in the Sixty Lake Basin and Milestone Basin of the Sierra Nevada mountains in California, USA by V. Vredenburg and D. Woodhams. They ranged in weight from 10 to 27 g. Adult leopard frogs (*R. pipiens*) were obtained from a commercial supplier in the states of Minnesota and Vermont, or were collected in Van Buren County, Michigan, USA. All animal procedures were approved by Institutional Animal Care and Use Committees at Vanderbilt University Medical Center or the University of California, Berkeley.

2.2. Collection of skin peptides

Secretions containing skin peptides were collected by norepinephrine induction to avoid the more painful electrostimulation procedure. Briefly, *R. muscosa* captured in the field and *R. pipiens* in the laboratory were weighed within 0.1 g and injected with 10 nmol (0.01 ml) per gram body weight (gbw) of norepinephrine (bitartrate salt, Sigma, St. Louis, MO). Animals were placed into 50 ml of collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0) [20] and remained largely submerged for 10–15 min while skin secretions accumulated. Animals were then removed, and the buffer containing peptides was acidified to a

final volume of 1% HCl (Sigma, St. Louis, MO, USA) to inactivate endogenous peptidases [21]. The acidified collection buffer with peptides was passed over C-18 Sep-Pak cartridges, (Waters Corporation, Milford, MA, USA), and the Sep-Paks were stored under moist conditions in a sealed vial containing a small amount of 0.1% HCl briefly until they could be further processed in the Rollins-Smith laboratory [21]. Peptides bound to Sep-Paks were eluted with 70% acetonitrile, 29.9% water, 0.1% trifluoroacetic acid (TFA) (v/v/v) and concentrated to dryness by centrifugation under vacuum. The total concentration of skin peptides recovered after Sep-Pak separation was determined by Micro BCATM (bicinchoninic acid) Assay (Pierce, Rockford, IL, USA) according to manufacturer's directions except that bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve [21,22]. Consequently, concentrations of crude peptide mixtures are expressed as μg equivalents/ml with reference to the bradykinin standard. Dried mixtures of peptides were sent to the Conlon laboratory for further isolation of pure peptides.

2.3. Peptide purification

Skin secretions from five male and five female *R. muscosa*, after partial purification on Sep-Pak cartridges, were re-dissolved in 0.1% (v/v) TFA/water (2 ml), pooled according to sex, and separately injected onto a (2.5 × 25-cm) Vydac 218TP510 (C-18) reverse-phase high-pressure liquid chromatography (HPLC) column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The ability of freeze-dried aliquots (50 μl) of the fractions to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* was determined as described below.

As the elution profiles of the secretions from male and female frogs appeared to be almost identical, corresponding peaks in each chromatogram were pooled and successively chromatographed on a (1 × 25-cm) Vydac 214TP510 (C-4) column and a (1 × 25-cm) Vydac 219TP510 (phenyl) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 49% over 50 min and the flow rate was 2.0 ml/min.

2.4. Antibacterial assays for peptide purification

Skin fractions from the HPLC separation that contained possible antimicrobial peptides were monitored by incubating freeze-dried aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units/ml) from a log-phase culture of *S. aureus* (NCTC 8325) and *E. coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin.

2.5. Chytrid growth inhibition assay

B. dendrobatidis isolate 197 (isolated from the blue poison dart frog, *Dendrobates azureus*) [15] was obtained from Joyce Longcore, and isolate 119 was isolated from *R. muscosa* by the Briggs laboratory and sent to the Rollins-Smith laboratory. Both isolates were maintained in culture as described previously [23,24]. Mature cells or zoospores were plated with or without peptides as described previously [23,24] except that total volume was reduced by half. Briefly, 2.5×10^4 mature cells or 2.5×10^5 zoospores in a volume of 25 μl broth were plated in replicates of five in a 96-well flat bottom microtiter plate (Costar 3596, Corning Inc., Corning, NY, USA) with or without addition of 25 μl serial dilutions of peptides in sterile HPLC-grade water. The plates were covered, wrapped in plastic wrap to limit moisture loss, and incubated at 23 °C. To determine maximal growth (positive control for growth), some wells received 25 μl of HPLC-grade water without peptide. To determine the value for maximal inhibition (negative control for growth) some cultures were treated by temperature shock (60 °C for 10 min) to induce death. Growth at 1–5 days (23 °C) was measured as increased optical density at 492 nm (OD₄₉₂) with a microtiter plate reader. For growth inhibition assays using zoospores, the incubation period was extended up to seven days. Minimal inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was detectable. That is, the OD₄₉₂ was not significantly greater than that observed for negative control wells.

To compare the relative effectiveness of skin peptides from *R. muscosa* and *R. pipiens*, we calculated a measure of the mean MIC equivalents per gram and mean MIC equivalents per cm². An MIC equivalent is the total amount of peptides (μg) recovered from each frog per 1 g of body weight or per 1 cm² of surface area divided by the experimentally determined MIC ($\mu\text{g}/\text{ml}$) for each species [21]. Surface area of the skin in cm² was estimated according to the method of McClanahan and Baldwin [25]. Surface area = 9.9 (weight in grams)^{0.56}.

To estimate the amount of peptides present in the mucous, we assumed the thickness of the mucous layer to be 500 μm . Therefore the volume of mucous covering one cm² of skin would be 50 μl .

2.6. Structural characterization of peptides

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator. Mass spectral data for purified peptides were obtained on a Bruker Ultraflex ToF/ToF instrument (Bruker Daltonics Inc., Billerica, USA) using alfa-cyano-4-hydroxycinnamic acid as matrix. Spectra were acquired in reflector mode and were internally calibrated using a standard peptide mix. The resulting accuracy is better than 50 ppm. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA).

2.7. MALDI-TOF MS analysis of natural mixtures of peptides

Natural mixtures of skin peptides from 15 males and 15 females of *R. muscosa* were analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. A PerSeptive Biosystems Voyager Elite spectrometer was operated in reflector, delayed extraction, and positive ion mode. Peptide standards included bradykinin fragment 1–7 (monoisotopic mass 754.4) and bovine insulin (average mass 5734.5) (Sigma, St. Louis, MO, USA). Each sample represents the average of 256 laser shots, and data were acquired in the m/z range of 500–7000, then truncated at m/z 3500 and baseline corrected with Data Explorer v4.4 (Applied Biosystems, 2000). The peak values shown represent the monoisotopic mass $[\text{M} + \text{H}]^+$.

2.8. Statistical comparisons

In chytrid growth inhibition assays, each data point represents the mean \pm standard error of five replicate wells. The means were compared by a one-tailed Student's *t*-test. A *p* value of ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. MALDI-TOF MS analysis of natural mixtures of peptides

MALDI-TOF MS analysis of skin peptide mixtures provides a rapid method to assess the relative complexity of a skin peptide mixture. It allows for an estimation of the most abundant peptides, whether or not the peptide pattern is consistent in a number of individuals, and whether there may be gender differences. Analysis of natural skin peptide mixtures from 15 male and 15 female *R. muscosa* after elution from C-18 Sep-Paks revealed a very consistent pattern of peaks (Fig. 1). The first prominent peak of mass 1060.44 was later shown to be bradykinin. The second prominent peak had a mass of 1368.71 and was later shown to be temporin-1M. A third peak of mass 1390.75 is likely to be a sodium adduct $[\text{M} + \text{Na}]^+$ of temporin-1M because it is exactly 22 mass units greater than the peptide. A fourth and a fifth peak with masses of 2929.24 and 3273.42 were later shown to be ranatuerin-2Mb and ranatuerin-2Ma, respectively (Fig. 1).

3.2. Peptide purification

Although, the major peptides expressed by *R. muscosa* could be distinguished by MALDI-TOF MS, it was necessary to isolate the individual peptides in sufficient quantity to characterize them further. The elution profiles on a Vydac C-18 reverse-phase HPLC column of the skin secretions of female frogs is shown in Fig. 2. The chromatogram contained five major UV-absorbing peaks designated 1–5. Growth-inhibitory activity against *E. coli* was associated with peaks 2, 3, and 5 whereas growth-inhibitory activity against *S. aureus* was associated with peak 5 only. There were no major differences in the number, shape, or retention times of the peaks in the chromatogram of secretions from male frogs in comparison with female frogs. The peptides contained in peaks 1–5 from both male and

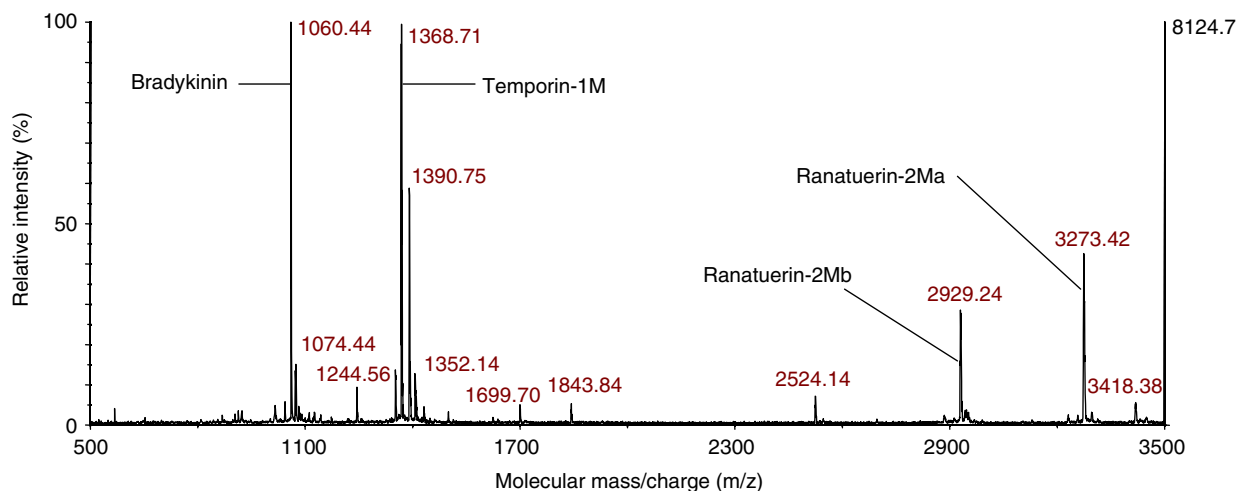


Fig. 1. Profile of a natural mixture of skin peptides secreted by *R. muscosa* determined by MALDI-TOF mass spectrometry. Skin secretions were induced by norepinephrine injection, enriched by passage over C-18 Sep-Paks, and concentrated. Antimicrobial peptides and bradykinin are identified according to their predicted masses determined in this study.

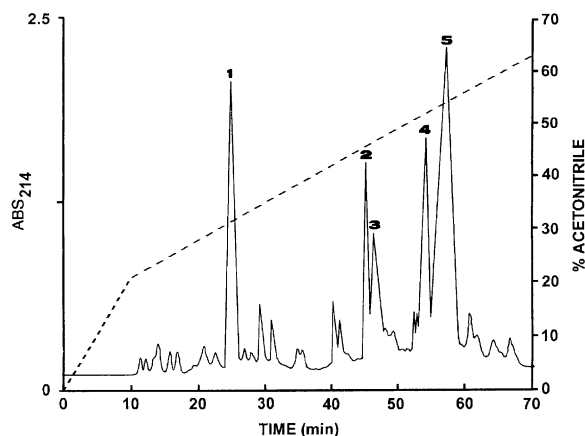


Fig. 2. Reverse-phase HPLC on a preparative Vydac C-18 column of freeze-dried aliquots of skin secretions from female specimens of the frog *R. muscosa*. The dashed line shows the concentration of acetonitrile in the eluting solvent. Peak 1 contained bradykinin, peak 2 ranatuering-2Ma, peak 3 ranatuering-2Mb, peak 4 temporin-1M free acid, and peak 5 temporin-1M.

female frogs were purified to near homogeneity as assessed by symmetrical peak shape and mass spectrometry after further chromatography on Vydac C-4 and phenyl columns. The same procedure was used to purify all peptides and is illustrated by the purification of the peak 2 peptide (subsequently shown to be ranatuering-2Ma) (Fig. 3). The final yield of purified ranatuering-2Ma from skin secretions of both male and female frogs was 170 nmol. Peak 1 was subsequently shown to contain bradykinin (final yield 390 nmol), peak 3

contained ranatuering-2Mb (final yield 45 nmol), peak 4 contained the free acid form of temporin-1M (final yield 90 nmol), and peak 5 contained the C-terminally α -amidated form of temporin-1M (final yield 790 nmol).

3.3. Structural characterization

The primary structures of the five peptides separated by HPLC from the skin secretions of *R. muscosa* were established without ambiguity by automated Edman degradation and are shown in Table 1. The presence of an intramolecular disulfide bridge in the ranatuering-2 peptides was demonstrated by mass spectrometry. The primary structures and amino acid compositions of the temporin peptides from peaks 4 and 5 were identical but the molecular masses differed by 1 amu, consistent with the presence of a C-terminally α -amidated residue in the peptide from peak 5. This conclusion was supported by the fact that the peak 5 temporin displayed growth inhibitory activity against *S. aureus* whereas the peak 4 temporin did not. All previously characterized temporins with antimicrobial activity contain a C-terminally α -amidated residue [26].

3.4. Antichytrid growth inhibition by *R. muscosa* skin peptides

In order to establish whether the peptides of *R. muscosa* may have a protective role against

development of chytridiomycosis, we tested their activity in growth inhibition assays against *B. dendrobatidis*. A natural mixture of skin peptides

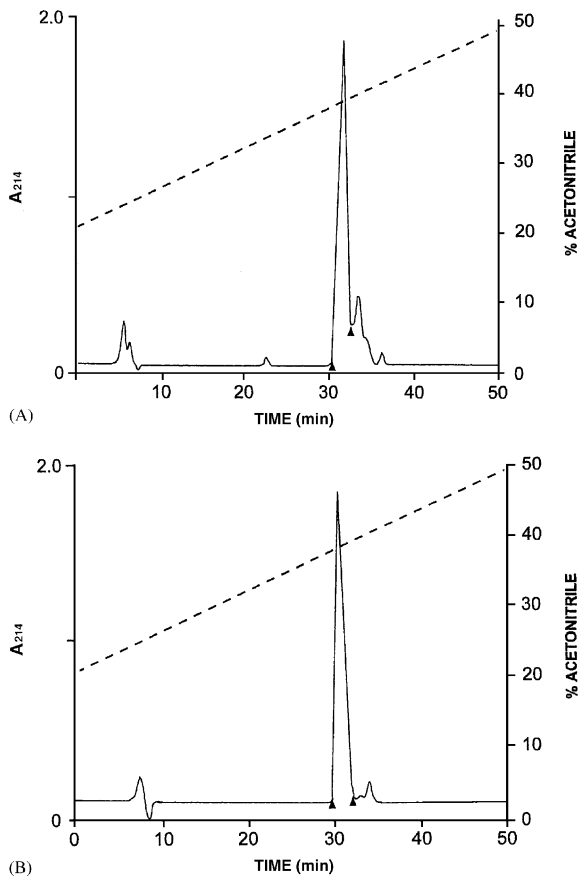


Fig. 3. Purification of ranatuerin-2Ma on (A) Vydac C-4 and (B) Vydac phenyl columns. The arrowheads show where peak collection began and ended.

from *R. muscosa* inhibited growth of zoospores of chytrid isolate 119 (derived from *R. muscosa*) at concentrations of 250 μg equivalents/ml or greater. The MIC was 500 μg equivalents/ml (Fig. 4A). Purified ranatuerin-2Ma inhibited growth of zoospores (isolate 197) at concentrations of 3–50 μM (MIC, 50 μM) (Fig. 4B). Ranatuerin-2Mb had very similar inhibitory capability with an MIC of 25 μM (Fig. 4C). Temporin-1M (α -amidated) inhibited chytrids at concentrations above 6.25 μM with an MIC of 100 μM against zoospores of isolate 197 (Fig. 4D).

3.5. Effectiveness of skin mucosal defenses of *R. muscosa* and *R. pipiens*

The effectiveness of the antimicrobial peptide defenses in the skin mucous depends on two factors: (1) the relative potency of each peptide or peptide mixture against a pathogen; and (2) the total amount of peptides released by actively secreting frogs. To gain some insight into the relative effectiveness of the skin peptide defenses of *R. muscosa* and determine how they compare with those of other ranid species, we compared several parameters of skin peptide production in *R. muscosa*, a declining species, with those of the more common species, *R. pipiens* that is not declining. Total peptide recovery from *R. muscosa* following mild norepinephrine induction (10 nmol/g) was about $1831 \pm 293 \mu\text{g}$ equivalents/g or $521 \pm 63 \mu\text{g}$ equivalents/ cm^2 of skin surface (Table 2). If the thickness of the mucous layer is about 500 μm , then the volume of mucous covering 1 cm^2 of skin would be 50 μl and the amount of total peptides recovered would

Table 1

Primary structures and observed (M_r obs) and calculated (M_r calc) molecular masses of the peptides isolated from *R. muscosa* skin secretions

Peptide	Sequence	M_r (obs)	M_r (calc)
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1059.6	1059.6
Ranatuerin-2Ma	Gly-Leu-Leu-Ser-Ser-Phe-Lys-Gly-Val-Ala-Lys-Gly-Val-Ala-Lys-Asp-Leu-Ala-Gly-Lys-Leu-Glu-Lys-Leu-Lys-Cys-Lys-Ile-Thr-Gly-Cys	3272.9	3272.9
Ranatuerin-2Mb	Gly-Ile-Met-Asp-Ser-Val-Lys-Gly-Val-Ala-Lys-Asn-Leu-Ala-Ala-Lys-Leu-Glu-Lys-Leu-Lys-Cys-Lys-Ile-Thr-Gly-Cys	2928.6	2928.6
Temporin-1M (free acid)	Phe-Leu-Pro-Ile-Val-Gly-Lys-Leu-Leu-Ser-Gly-Leu-Leu	1368.9	1368.9
Temporin-1M	Phe-Leu-Pro-Ile-Val-Gly-Lys-Leu-Leu-Ser-Gly-Leu-Leu.NH ₂	1367.9	1367.9

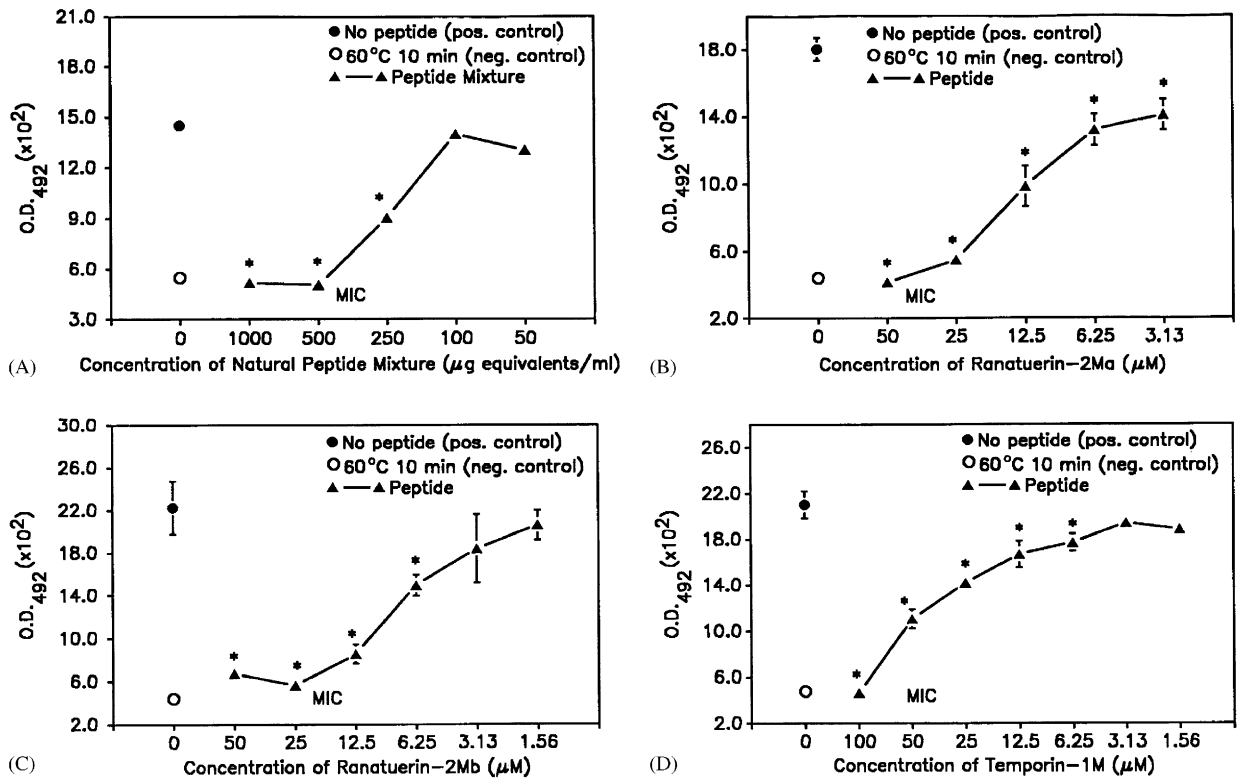


Fig. 4. (A) Growth inhibition of *B. dendrobatidis* zoospores (isolate 119 from *R. muscosa*) at six days of culture by natural mixtures of skin peptides from *R. muscosa* partially purified and concentrated by passage over a C-18 Sep-Pak cartridge. (B) Growth inhibition of zoospores (isolate JEL197) at seven days of culture by ranatuerin-2Ma, (C) by ranatuerin-2Mb, and (D) by temporin-1M. Each data point represents the mean \pm standard error (SE) of five replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. *Significantly less growth than positive controls (no added peptides) by a one-tailed Student's *t*-test; $p \leq 0.05$. MIC is the lowest concentration at which no growth was detected.

Table 2

A comparison of the skin peptide defenses of *R. muscosa* with those of *R. pipiens*

	<i>Rana muscosa</i> ^a	<i>Rana pipiens</i> ^b
Weight	19.2 \pm 0.8	30.7 \pm 0.8
Total peptides ^c recovered (μ g equivalents/g)	1831 \pm 293	383 \pm 45
Total peptides ^c recovered (μ g equivalents/cm ²)	521 \pm 63	180 \pm 21
Total peptides ^c in mucous (μ g equivalents/ml)	10,417 \pm 1260	3425 \pm 445
MIC equivalents/g	3.8 \pm 0.6	0.8 \pm 0.1
MIC equivalents/cm ²	1.0 \pm 0.1	0.4 \pm 0.04

^aThirty individual *R. muscosa* were analyzed.

^bTwenty-five individual *R. pipiens* were analyzed.

^cConcentrations of peptides refer to μ g equivalents expressed relative to a bradykinin standard in a Micro BCA protein assay.

be about 10,417 \pm 1260 μ g equivalents/ml of mucous. The MIC against *B. dendrobatidis* for this natural mixture of peptides was about 500 μ g equivalents/ml. Thus, the amount of peptides available in the mucous following a mild norepinephrine stimulus should be sufficient to destroy zoospores that would contact the

mucous layer. Total peptide recovery from *R. pipiens* induced in the same way as *R. muscosa* was 383 \pm 45 μ g equivalents/g or 180 \pm 21 μ g equivalents/cm². By all measurements (total peptide recovery per gbw or per cm² of skin surface or total peptide recovery per volume of skin mucous), *R. muscosa*

produced about 3–5 fold greater amounts of recoverable peptides (Table 2). The MIC for natural mixtures of skin peptides from each species is the same (500 µg equivalents/ml), and therefore calculated MIC equivalents/g, MIC equivalents/cm², and MIC equivalents/ml of mucous followed the same pattern. That is, the amount of peptides on the skin and their relative effectiveness appeared to be about 3–5 fold greater for *R. muscosa* than for *R. pipiens* (Table 2).

4. Discussion

This study has led to the isolation of three peptides from *R. muscosa* skin secretions with differential growth-inhibitory activity against reference strains of *E. coli* and *S. aureus*. The primary structures of two of the peptides (ranatuerin-2Ma and ranatuerin-2Mb) indicate that they are members of the ranatuerin-2 family, first identified in the skin of the North American bullfrog *R. catesbeiana* [27]. The amino acid sequence of the third peptide (temporin-1M) indicated that it belonged to the temporin family, first identified in the skin of the European frog, *R. temporaria* [28]. The isolation of the free acid form of temporin-1M was an unexpected finding as the presence of such peptides in frog skin secretions has not been reported previously. It is unclear whether the peptide is an authentic secretion product or is derived from temporin-1M by artifactual hydrolysis during the extraction and purification process.

Analysis of the antimicrobial peptide sequences provides information for phylogenetic comparisons. *R. muscosa* is generally classified with frogs of the Amerana group [29] (also known as the *R. boylii* species group) which include *R. aurora*, *R. boylii*, *R. cascadae*, *R. luteiventris*, and *R. pretiosa*. On the basis of the nucleotide sequence of several mitochondrial genes, it was concluded the Amerana species group is monophyletic and approximately 8 million years old [30]. More recent phylogenetic analysis using nucleotide sequences from the mitochondrial genome suggests that *R. muscosa* is the sister-group to a clade comprising *R. aurora aurora* and *R. cascadae* [31]. The primary structures of the antimicrobial peptides isolated in this and in earlier related studies [32,33, rev. 26] support the hypothesis that *R. muscosa*, *R. boylii*, *R. aurora*, and *R. luteiventris* share a close phylogenetic relationship. As shown in Fig. 5, amino acid sequence identities between corresponding peptides of the

ranatuerin-2 and temporin families are between 75% and 91% despite the fact that the primary structures of members of these two families have generally been poorly conserved during the evolution of the ranid frogs [26]. For example, sequence identity of ranatuerin-2Ma with ranatuerin-2 from *R. catesbeiana* (Aquarana species group) is 56% and for the orthologous temporins only 23–31%.

One of the most abundant peptides in the skin secretions of *R. muscosa* is bradykinin. The biological role of bradykinin in skin secretions is unknown, but it has been suggested that it acts as a deterrent to ingestion by predators [34]. A bradykinin-like ability to contract rat uterine and gastrointestinal smooth muscle has been demonstrated in extracts of the skins of a wide range of frogs from Africa [35], America [36], Australia and Papua New Guinea [37], and Europe [38] by Erspamer and co-workers [rev. 39]. In particular, the skins of certain ranid frogs are associated with very high concentrations of such bradykinin-like bioactivity, but the species distribution is sporadic. For example, the skin of the European common frog *R. temporaria* contains a very high concentration (200–250 µg/g tissue) of a peptide that was identical to mammalian bradykinin [40,41], but bradykinin was undetectable in the skins of *R. dalmatina*, *R. graeca*, and *R. latastei* [38]. Similarly, the present study has demonstrated that skin secretions of *R. muscosa* contain a high concentration of bradykinin, but the peptide is either absent from the secretions of the other members of the Amerana species group (*R. boylii* [42] and *R. aurora* [32]), or present only in very low concentration. The procedure for stimulating secretions and purifying the peptides was the same for each of the three species.

R. muscosa populations are in serious decline. This species is currently listed as “vulnerable” in the IUCN Red list scheme [6,43]. In comparison, the common leopard frog, *R. pipiens*, is relatively abundant and listed in the category of “least concern” in the IUCN Red list scheme [43]. Both *R. muscosa* and *R. pipiens* can be infected by *B. dendrobatidis* [12,18,19,44,45], but the outcome appears to be quite different in each species. About 10% of *R. pipiens* specimens collected in Quebec between 1960 and 2001 were infected with *B. dendrobatidis*, and the incidence of infection in 12 common species from Canada and the United States was not different in the period 1990–2001 as compared with the period 1960–1969. Thus,

Ranatuerin-2		
<i>R. muscosa</i>	GLL SSFKGVAKGVAKDLAGKLLLEKLEKCKITGC	
<i>R. aurora</i>	GIL SSFKGVAKGVAKNLAGKLLDELKCKITGC	(88%)
<i>R. boyliei</i>	GIL STFKGLAKGVAKDLAGNLLDKFKCKITGC	(81%)
<i>R. luteiventris</i>	GIL DSFKGVAKGVAKDLAGKLLDKLCKITGC	(91%)
<i>R. luteiventris</i>	GIL SSIKGVAKGVAKNVAQQLLDTLKCKITGC	(75%)
<i>R. catesbeiana</i>	GLFLDTLKGAAKDVA***GKL*EGLKCKITGCKLP	(56%)
<i>R. muscosa</i>	GIMDSV***KGVAKNLAALKLEKLEKCKITGC	
<i>R. boyliei</i>	GIMDSV***KGLAKNLAGKLLDSLKCKITGC	(86%)
Temporin		
<i>R. muscosa</i>	FLPIVGKLLSGLL.NH ₂	
<i>R. aurora</i>	FLPIIGQLLSGLL.NH ₂	(85%)
<i>R. boyliei</i>	FLPIIAKVLSSGLL.NH ₂	(77%)
<i>R. catesbeiana</i>	FISAIASMLGKFL.NH ₂	(23%)
<i>R. catesbeiana</i>	FLSAIASMLGKFL.NH ₂	(31%)
<i>R. catesbeiana</i>	FISAIASFLGKFL.NH ₂	(23%)

Fig. 5. A comparison of the primary structures of the ranatuerin-2 and temporin peptides isolated from *R. muscosa* with corresponding peptides from other frogs of the *R. boyliei* species group (*R. aurora*, *R. boyliei*, *R. luteiventris*). Peptides from the bullfrog, *R. catesbeiana* are shown for comparison. The values in parentheses show % amino acid sequence identity. Gaps in the sequences denoted by (*) have been inserted in order to maximize sequence similarity.

chytridiomycosis appears to be enzootic in the northern leopard frog [45]. Yet this species is not declining. In contrast, infection became detectable in postmetamorphic pathogen-free *R. muscosa* exposed to infected tadpoles within 18 days, and they died within 50 days [19]. Furthermore, most post-metamorphic juveniles of *R. muscosa* die when experimentally exposed to a high number of *B. dendrobatidis* zoospores (M. Stice and C. Briggs, unpublished data) while *R. pipiens* adults are relatively resistant (C. Carey, personal communication). Thus, we expected that the antimicrobial skin defenses of *R. muscosa* against *B. dendrobatidis* might be inferior to those of *R. pipiens*. *R. muscosa* has only three described antimicrobial peptides (Table 1), whereas eight antimicrobial peptides have been isolated from the skin of *R. pipiens* [32]. The peptide repertoire of *R. pipiens* contains two additional families of antimicrobial peptides (brevinin-1 and esculentin-2) that are absent from *R. muscosa*. Although purified brevinin-1 peptides from *R. pipiens* have not yet been tested for antichytrid activity,

brevinin-1 peptides from three other ranid species were more effective than ranatuerin-2 peptides [22,24, rev. 46]. Two of the other purified peptides from *R. pipiens* were tested for antichytrid activity. One peptide (esculentin-2P) had an MIC of 25 μ M and the second one (ranatuerin-2P) had an MIC of 100 μ M [24]. Both ranatuerin-2 family members from *R. muscosa* were more effective than the ranatuerin-2 expressed by *R. pipiens* against *B. dendrobatidis* (Figs. 4B and C). When a natural mixture of *R. pipiens* skin peptides was tested for activity against *B. dendrobatidis*, the MIC was 500 μ g/ml (L. Rollins-Smith and D. Woodhams, unpublished data) similar to that of *R. muscosa* (Fig. 4A). Thus, it appears that *R. muscosa* has relatively robust skin peptide defenses (Table 2), but the brevinin-1 peptides are the dominant family of peptides in the skin secretions of *R. pipiens*, and they may be more effective than the ranatuerin-2 family members found in *R. muscosa*.

The difference in susceptibility of the two species to chytridiomycosis may also relate to differences in

the life history traits of the two species. *R. muscosa* is highly aquatic with adults spending most of their time in the water or very close to the shoreline of high elevation lakes that are home to this species. A previous study of ecological traits that may predict amphibian population declines showed that lifetime aquatic index (a measure of the time spent by each species in the water) was the best predictor of species susceptibility to declines in Central America [47]. The alpine lakes inhabited by *R. muscosa* are permanently cold, and the cold delays the growth and metamorphosis of tadpoles such that they do not complete metamorphosis within one season [7]. The mouthparts support chytrid growth, but the tadpole skin is free of infection, and tadpoles do not die from the infection [17–19]. Thus, tadpoles may constitute a permanent reservoir of infected individuals continuously releasing infectious zoospores [17,19]. Adults are continuously exposed to infectious zoospores under cold conditions, and postmetamorphic juveniles can be infected by exposure to infected tadpoles under these cold conditions [19]. We did not test the activity of the antimicrobial peptides from *R. muscosa* at cold temperatures; however, two antimicrobial peptides from *R. pipiens* function very well at cold temperatures (4–14 °C) [24,48,49], and it seems likely that the peptides isolated from *R. muscosa* would also function well at these temperatures. It is possible, however, that peptide renewal following discharge might be very slow at cold temperatures. Furthermore, development of effective antibody or T-cell mediated defenses of amphibians is inhibited in the cold [50–52]. *B. dendrobatidis* is very well adapted to cold conditions. It survives for 6 months at 4 °C [53]. At low temperatures, rate of growth is slowed, but fecundity (number of zoospores produced) is increased (D. Woodhams, unpublished data). In a natural setting, the prevalence of chytrid infections was increased among amphibians collected in Quebec in the cooler spring and fall months in comparison with mid-summer months [45]. In contrast, *R. pipiens* is a wide-ranging species, and adults spend little time directly in the water after they disperse from the breeding ponds and lakes in the spring time. Tadpoles metamorphose within one season, and it is assumed that juveniles with heavy chytrid infections would die prior to entering the over-wintering lakes. Thus, tadpoles are not a permanent reservoir for chytrids in this species, and adults appear to have effective skin peptide

defenses [24,32]. Whether adult *R. pipiens* can develop effective antibody and T-cell mediated defenses against this pathogen is currently being studied.

Other factors, such as pesticide exposure, may also inhibit immune defenses allowing a controlled infection to become lethal [rev. 54]. There is a clear correlation between population declines of four amphibian species, including *R. muscosa*, in the Sierra Nevada and predicted pesticide drift [11]. The interaction of pesticides with the immune system and the impact on disease development is an important area for future research.

In conclusion, although antimicrobial peptide defenses in *R. muscosa* may provide some protection from infection by zoospores of *B. dendrobatidis*, life-history characteristics and continuous exposure to infectious zoospores may limit the effectiveness of this innate defense. The ability to develop an effective adaptive immune response may also be inhibited by chronic cold conditions.

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