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The importance of antimicrobial peptides (AMPs) in amphibian skin defense

Louise A. Rollins-Smith [a,b,c,*]

[a] Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA
[b] Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, USA
[c] Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

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ABSTRACT

Antimicrobial peptides (AMPs) are produced for defense in nearly all taxa from simple bacteria to complex mammalian species. Some amphibian families have developed this defensive strategy to a high level of sophistication by loading the AMPs into specialized granular glands within the dermis. Enervated by the sympathetic nervous system, the granular glands are poised to deliver an array of AMPs to cleanse the wound and facilitate healing. There have been a number of excellent review publications in recent years that describe amphibian AMPs with an emphasis on their possible uses for human medicine. Instead, my aim here is to review what is known about the nature of amphibian AMPs, the diversity of amphibian AMPs, regulation of their production, and to provide the accumulated evidence that they do, indeed, play an important role in the protection of amphibian skin, vital for survival. While much has been learned about amphibian AMPs, there are still important gaps in our understanding of peptide synthesis, storage, and functions.

1. Introduction

Antimicrobial peptides (AMPs), also called host defense peptides, have been isolated from the skin of many amphibian species over the last approximately forty years. The Antimicrobial Peptide Database (APD, https://aps.unmc.edu/, accessed on 18 January 2023) lists 1196 active peptides from amphibians (1117 from frogs, and 75 from toads). This database maintained at the University of Nebraska Medical Center was manually assembled from the literature and is continually updated. It consists of natural AMPs, which include peptides from the six life kingdoms; predicted peptides, which are predicted by machine learning or other technologies and tested to be active; and synthetic peptides, which are derivatives of natural AMPs. Another very useful database is the Database of Anuran Defense Peptides (DADP, http://split4.pmfst.hr/dadp, accessed on 18 January 2023) (Novković et al., 2012). The entries were also manually selected from the literature and from UniProt entries. It lists 2571 peptides. This database is a useful tool to enable researchers to search for novel peptides within the genomes of amphibians because it lists a large number of signal peptides that are shared by many anuran species.

Another authoritative review publication lists approximately 1900 naturally occurring AMPs and their synthetic analogs and specifies the species of origin and the amino acid sequences (reviewed in Xu and Lai, 2015). The described peptides range in size from about 10 to 50 amino acid residues and are produced by at least 178 amphibian species belonging to about 10 families and 28 genera (reviewed in Xu and Lai, 2015). They are gene-encoded as prepropeptides (reviewed in Nicolas et al., 2003) and synthesized and stored as mature or near-mature peptides (Brunetti et al., 2018) within enveloped granules in the granular glands, also called poison glands. They generally contain a high content of hydrophilic and hydrophobic amino acids, spaced apart, which gives them an amphipathic character (containing both polar and nonpolar regions), and they generally have a net positive charge. Given these rich resources listing AMPs from many amphibian species, have researchers uncovered all or most of the amphibian AMP diversity? That seems unlikely given newly described methods to use machine learning trained on known AMPs to search the genome for new AMPs. One such approach examined the annotated reference transcriptome of the North American bullfrog [*Rana (Lithobates) catesbeiana*] for previously described and novel AMPs. The authors found 16 putative AMPs. Five had been previously described. Of the remaining 11 putative AMPs, only 4 were active against the set of bacterial pathogens chosen for the assays

* Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, A-5119 Medical Center North, 1161 21st Ave South, Nashville, TN, 37232, USA.
E-mail address: louise.rollins-smith@vanderbilt.edu.

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(Li et al., 2022). Some limitations of this approach are the number of AMP sequences used for the training and the limited number of amphibian genomes that have been sequenced. It is, however, an exciting approach to discover new AMPs and learn more about the evolution of AMPs within amphibian groups.

2. Diversity of amphibian antimicrobial peptides

Depending on the source, amphibian AMPs can be placed into between 40 and 100 different peptide families based on structural similarities (reviewed in Xu and Lai, 2015; reviewed in Wang, 2020). In North American frogs, the AMPs fall into about ten families of related peptides, primarily from ranid family frogs (reviewed in Conlon et al., 2009). Most described AMPs have been isolated from eleven amphibian families: the Hylidae, Ranidae, Pipidae, Liopelmatidae, Alytidae, Bombinatoridae, Myobatrachidae, Leptodactylidae, Microhylidae (suspected), Dicroglossidae, and Hyperoliidae (reviewed in Conlon 2011; reviewed in König et al., 2015). Some individual species of another group of families have been screened, but they appear to lack conventional AMPs (Bufonidae, Ceratophyridae, Eleutherodactylidae, Microhylidae, Pelobatidae, Pyxicephalidae, Rhacophoridae, Scaphiopodidae, Rhinophrynidae, Pelodytidae, Hemiphractidae, Strabomantidae, Craugastoridae, Hemisotidae, Atheroleptidae, Ptychadenidae, Micrixalidae, and Phrynobatrachidae) (reviewed in Conlon, 2011; reviewed in König et al., 2015). Within the Hylidae and Ranidae, the sequences of the AMPs are highly diverse, but the genes encoding the peptides have remarkable similarity in the signal peptide region, the acidic propiece, and in the 5′- and 3′-untranslated regions of the corresponding mRNAs. This suggests that the amazing diversity of amphibian AMPs arose by repeated duplications of an ancestral gene with repeated mutations of the mature peptide domain (Duda et al., 2002; Vanhoye et al., 2003; reviewed in Nicolas and El Amri, 2009). The hypothesized mechanism to generate mature peptide diversity is the possible presence of an error-prone DNA polymerase (Vanhoye et al., 2003). Protection of the conserved prepropeptide region could occur by attachment of specific macromolecules that stop DNA replication and create single strand breaks repaired by a mutagenic polymerase (Conticello et al., 2001; reviewed by Nicolas and El Amri, 2009). It was estimated that this mechanism could result in about 100,000 unique AMPs from about 5000 frog species (Vanhoye et al., 2003). The large number of potentially available AMPs have been positively selected, presumably to provide resistance to specific pathogens encountered by each species (Duda et al., 2002; reviewed in Tennessen, 2005; reviewed in Nicolas and El Amri, 2009). Most species produce a mixture of AMPs that can dramatically synergize with each other (Matsuzaki et al., 1998; Rollins-Smith et al., 2002a; Vanhoye et al., 2003). Thus, the mixtures can protect against different types of pathogens and work together for better inhibition. Because the presence of AMPs is not evenly distributed across anuran species, it has been argued that they evolved to assist the delivery of neuroactive peptides to deter predators and their antimicrobial properties are a secondary benefit (reviewed in König et al., 2015). Regardless, for the species fortunate enough to produce and secrete AMPs, I will argue that they do provide an innate immune system benefit.

2.1. Antibacterial peptides

Most of the described antimicrobial peptides of amphibians were isolated and characterized based on their antibacterial activity. There are no consensus amino acid sequences that are associated with bacterial inhibition, but because the peptides are usually cationic, relatively hydrophobic, and have the propensity to form an amphipathic α-helix in a membrane-mimetic environment, many are potently active against bacteria. Most newly isolated and putative AMPs are tested for their capacity to inhibit growth of reference strains of Gram negative bacteria, such as *Escherichia coli (E.coli*) and Gram positive bacteria, such as *Staphylococcus aureus* (*S. aureus*). Some peptides, such as brevinin-1 and brevinin-2 peptides have broad-spectrum antibacterial activity against Gram positive and Gram negative bacteria (reviewed in Conlon et al., 2004, 2009), whereas other peptides in the ranatuerin-2 family have quite variable amino acid sequences and quite different activities against *E. coli* and Gram positive bacteria (reviewed in Conlon et al., 2004, 2009; reviewed in Xu and Lai, 2015). When tested against known opportunistic bacterial pathogens of amphibians, such as *Klebsiella pneumoniae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila*; peptide mixtures from South African Clawed Frogs (*Xenopus laevis)*, Green and Golden Bell Frogs (*Litoria aurea*), and Southern Bell Frogs (*Litoria raniformis*) inhibited most of the bacterial pathogens (Schadich, 2009; Schadich and Cole, 2010). The exception was the Gram negative bacterium *A. hydrophila*, which was resistant to peptide mixtures and purified peptides from several species (Rollins-Smith et al., 2002a; Tennessen et al., 2009; Schadich, 2009). One possible mechanism of resistance by *A. hydrophila* is the production of bacterial proteases that degrade the peptides (Schadich and Cole, 2009). Because most species that use AMPs for defense produce multiple peptides, it is generally thought that the diverse set of peptides is protective against a wide range of potential bacterial pathogens. However, some pathogens, such as *A. hydrophila*, seem to have evolved mechanisms to resist the AMPs.

2.2. Antiviral peptides

Most amphibian AMPs have not routinely been tested against viruses. However, there have been some studies of the activity of a few amphibian AMPs against the human immunodeficiency virus (HIV) and herpes simplex virus-1 and -2 (HSV-1, HSV-2) (reviewed in Apponyi et al., 2004; reviewed in Conlon et al., 2014; reviewed in Xu and Lai, 2015). Both HIV and HSV are enveloped viruses, and thus the detergent-like activities of amphipathic AMPs can disrupt the envelopes and prevent infection. Multiple peptides in the caerin 1 family of peptides are potent inhibitors of HIV infection of target T cells, and there is a strong correlation between the effectiveness of the peptides in disruption of the viral envelope and their capacity to inhibit infection of T cell targets (VanCompernolle et al., 2005, 2015). Focusing on the role of amphibian AMPs in protection from amphibian viruses, much less is known. Frog virus 3 (FV3) and related ranaviruses infect amphibians and can cause mass mortality events. Infectious particles may be enveloped or “naked” (Chinchar et al., 2017). Susceptible species include the Northern Leopard Frog (*Rana pipiens*) (Echaubard et al., 2010, 2015). When two AMPs from *R. pipiens* were tested against FV3, both were rapidly inhibitory at a concentration of 50 μM (Chinchar et al., 2001). Similar concentrations of four other amphibian AMPs also inhibited FV3 and did so over a wide range of temperatures from 4 °C to 26 °C (Chinchar et al., 2004). Five skin-derived AMPs from another ranid frog, *Rana dybowskii*, were shown to inhibit plaque formation by Rana grylio virus, another iridovirus closely related to FV3 (Zhang et al., 2001; Yang et al., 2012). Following infection of *R. dybowskii* with Rana grylio virus, expression of the AMP genes was rapidly upregulated and viral clearance followed this spike of AMP synthesis (Yang et al., 2012). Collectively, these studies suggest that amphibian AMPs in the skin mucus have the potential to inhibit infection by ranaviruses and other related iridoviruses over a broad range of temperatures if they infect across the skin surface.

2.3. Antifungal peptides

Because amphibian skin is moist and amphibians are generally dependent on water, they are potentially susceptible to fungal infections. Yet, few fungal diseases, with the exception of chytridiomycosis, have been extensively studied. Chytridiomycosis, caused by the two pathogenic chytrid fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*), has been the subject of many studies because of its direct link to global amphibian declines.

Thus, many amphibian AMPs have been tested for their ability to inhibit growth of *Bd*, and a few have been tested for inhibition of *Bsal*. Approximately 66 purified amphibian AMPs from 28 species have been tested (Table 1), and nearly all species tested have at least one AMP that is quite effective (Rollins-Smith et al., 2002a, 2002b, 2002c, 2003, 2005a, 2006; Woodhams et al., 2006a; Davidson et al., 2007; Conlon et al., 2007; Ramsey et al., 2010; Conlon et al., 2013; Holden et al., 2015a; Woodhams et al., 2020; reviewed in Rollins-Smith and Conlon, 2005; reviewed in Rollins-Smith, 2009). In addition to the testing of individual purified peptides for inhibition of *Bd* growth, the enriched hydrophobic peptide mixtures from the skin secretions of many other species have also been tested (Rollins-Smith et al., 2002c, 2006, 2015; Woodhams et al., 2006a, 2006b, 2007a; Davidson et al., 2007; Ramsey et al., 2010; Pask et al., 2012, 2013; Perl et al., 2017; Voyles et al., 2018; Rosa et al., 2022), and they have been shown to be quite inhibitory. Because *Bsal* is thought to be more harmful to salamander species than anuran species (Martel et al., 2014), the skin secretions from a limited number of salamander species have also been tested and found to be active in direct killing or inhibition of *Bsal* zoospores (Smith et al., 2018; Pereira et al., 2018; Carter et al., 2021; Pereira and Woodley, 2021). Six purified AMPs have also been shown to inhibit another fungus associated with amphibians, *Basidiobolus ranarum*. The effective peptides were magainin I and II, CPF, and PGLa from *Xenopus laevis*, dermaseptin from *Phyllomedusa sauvagii*, and ranalexin from *R. catesbeiana* (Rollins-Smith et al., 2002a).

3. What is known about antimicrobial peptide synthesis and storage?

Amphibian antimicrobial peptides are synthesized primarily in the multinucleated granular glands (also called serous glands or poison glands) described by Dockray and Hopkins (1975). The granules are enveloped and appear to contain both full length active peptides as well as inactive peptides that are cleaved rapidly once they are secreted (Giovannini et al., 1987; Magalhães et al., 2008; Brunetti et al., 2018) by specific endopeptidases (Resnick et al., 1991). In recent reports, *in situ* imaging mass spectrometry was used to compare the peptides within intact granules of the granular glands with the secreted products. Some peptides within the granules were identical with those found in skin secretions, whereas others represented longer peptides that were cleaved after the peptides were secreted onto the skin (Magalhães et al., 2008; Brunetti et al., 2018). Once secreted onto the surface of the skin, the AMPs are most active for about 15 min, but they are cleaved and mostly degraded by 2 h after secretion (Pask et al., 2012). Since all the machinery necessary for protein synthesis (e.g., mRNA, ribosomes, enzymes) is present within the syncytium of the multi-nucleated granular glands, it is assumed that peptide synthesis and packaging into membrane-bound glands is continuous. When granular gland contents are secreted, they would be replaced. When secretion is induced by pharmacological concentrations of norepinephrine, complete recovery can be delayed from 6 days (Giovannini et al., 1987) to more than 50 days (Pask et al., 2012). There also appear to be seasonal effects on antimicrobial peptide synthesis or storage in both temperate regions and tropical regions. Greater amounts of peptides in winter and spring of the year were collected in Southern Leopard Frogs (*Rana sphenocephala*), and less was detectable in summer and fall (Le Sage et al., 2021). Fewer detectable peptides in summer may be due to a greater need for use during very active summer periods or, alternatively, due to less synthesis and storage of peptides while resources are devoted to other functions such as breeding or movement. In a tropical setting in Panama, the peptides secreted by Panamanian Rocket Frogs (*Colostethus panamensis*) were less effective in the inhibition of *Bd* when collected in the wet season in comparison with those collected in the dry season (Rosa et al., 2022). The set of peptides detected by mass spectrometry also differed by season in these populations of frogs (Rosa et al., 2022).

Even resting frogs have detectable AMPs on the skin at concentrations that can effectively inhibit survival of *B. dendrobatidis* zoospores (Ramsey et al., 2010; Pask et al., 2012). This suggests that both resting and active frogs have an invisible "mantle" of protective peptides in the mucus being continuously replenished. The peptides constitute not only a protective layer, but the amounts of peptides can be rapidly increased when chased by a potential predator (Ramsey et al., 2010; Pask et al., 2012), thus also providing a potential first aid kit if the skin is injured in the chase (Figs. 1 and 2).

3.1. Regulation of antimicrobial peptide synthesis

While much is known about the mechanisms that induce and regulate production of AMPs in human skin or other mammals, very little information exists for how AMP synthesis may be regulated in amphibian skin. In human skin, commensal microbes induce expression of some AMPs through recognition by toll-like receptor 2 (TLR-2), epidermal growth factor receptor (EGFR), and NF-κB activation (reviewed in Herman and Herman 2019). For amphibian skin, it is likely that microbes or microbial products at the skin surface would be detected by epithelial cell receptors such as toll-like receptors (TLRs) or other pattern recognition receptors and signal to the granular glands for increased synthesis of antimicrobial peptides. Exposure to pathogenic bacteria or yeast resulted in increased synthesis of AMPs in *Rana esculenta* and *Bombina orientalis* (Simmaco et al., 1998; Mangoni et al., 2001). When the skin peptides were depleted by repeated electrostimulation and the frogs were placed in sterile water containing antibiotics, they produced significantly lower amounts of detectable AMPs than control animals maintained in conditions with a natural microbial community (Mangoni et al., 2001). Thus, it seems likely that the commensal microbiome of amphibians helps to maintain the normal constitutive production of AMPs.

The promoter regions of a few amphibian AMPs have been examined. They have been shown to contain putative binding sites for the transcription factors NFκB, NFIL-6 (Miele et al., 2000; Mangoni et al., 2001), GATA-1, and a binding site for the dorsal (dl) transcription factor (Kwon et al., 2000). An in depth study of the genes for AMPs in *Silurana* (*Xenopus*) *tropicalis*, revealed genes encoding up to 19 different peptides. Within the promoter regions of the genes for these peptides, the authors identified conserved cis regulatory elements designated CRE1 and CRE2 that are thought to play important roles in regulation of expression of these genes (Roelants et al., 2013). The CRE1 locus contains two sites with sequence similarity to known binding elements of the transcription factors C-Fos, C-Jun, Fra-1, JunB, JunD, and XBP1. The CRE2 motif was similar to known binding elements for CCAAT/Enhancer binding proteins (C/EBP) and POU class 2 homeobox factor 1(POU2F1), transcription factors associated with the synthesis of AMPs in other species (Roelants et al., 2013).

3.2. Expression of amphibian AMPs in other body tissues

Little is known about AMPs expressed in other tissues of the body beyond the skin of adult amphibians. While there is evidence for their expression in other tissues, there is almost nothing known about their functions outside of the skin mucus. Finding them in other tissues depends on specific antibodies to these small targets or the use of RT-PCR, northern blotting, or *in situ* hybridization with probes for the RNA transcripts. An early study of the expression of AMPs in the stomach of *Xenopus laevis* isolated and characterized nine AMPs. Eight of the nine were members of the magainin family of peptides previously found in skin secretions. The source of the AMPs revealed by immunohistochemistry was a previously undescribed multinucleated granular cell in the gastric mucosa (Moore et al., 1991). Another large granule-filled cell was found in the small intestine with similarity to Paneth cells found in the intestine of mammals (Reilly et al., 1994). Thus, in this setting the AMPs appear to play a role in defense against pathogens in the lumen of the stomach and small intestine. By northern blotting, using probes for

Table 1
Antimicrobial activity (minimal inhibitory concentration, MIC) of purified peptides from amphibians tested against zoospores of *Batrachochytrium dendrobatidis.* This table is an update of two similar previously published tables (Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009).

Family	Species of origin	Peptide	Sequence	MIC (μM)	Ref
Hylidae	*Agalychnis lemur*	dermaseptin-L1	GLWSKIKEAAKAAGKAALNAVTGLVNQGDQPS	>100	1
	A. lemur	phylloseptin-L1	LLGMIPLAISAISALSKL	100	1
	Litoria aurea, L. raniformis	aurein 2.1	GLLDIVKKVVGAFGSL-NH_2	200	2
	L. caerulea, L. splendida	caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	25–50	2
	L. caerulea	caerin 4.1	GLWQKIKSAAGLDLASGIVEGIKS-NH_2	>200	2
	L. chloris	caerin 1.9	GLFGVLGSIAKHVLPHVVPVIAEKL-NH_2	25–50	2
	L. citropa	citropin 1.1 mod 17	GLFAVIKKVAAVIKKL- NH_2	100–200	2
	L. dahlii	dahlein 5.6	GLLASLGKVFGGYLAEKLKPK-OH	200	2
	L. infrarenata	frenatin 3	GLMSVLGHAVGNVLGGFKPKS-OH	100	2
	L. genimaculata	maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2	25–50	2
	L. rubella	tryptophylolin 1.2	FPWL-NH_2	>200	2
	Phyllomedusa sauvagii	dermaseptin	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ.NH_2	23	3
Myobatrachidae	*Uperoleia mjobergii*	uperin 3.6	GVIDAAKKVVNVLKNLF-NH_2	100	2
Leptodactylidae	*Leptodactylus fallax*	fallaxin	GVVDILKGAAKDIAGHLASKVMNKL. NH_2	100	4
Pipidae	*Xenopus laevis*	CPF	GFASFLGKALKAALKIGANLLGGTPQQ-OH	12.5	5
	X. laevis	magainin I	GIGKFLHSAGKFGKAFVGEIMKS	>47	3
	X. laevis	magainin II	GIGKFLHSAKKFGKAFVGEIMNS	162	5
	X. laevis	PGLa	GMASKAGAIAGKIAKVALKAL.NH_2	50	5
Ranidae	*Rana areolata*	esculentin-1A	GLFPKFNKKKVKTGIFDIIKTVGKEAGMDVLRTGIDVIGCKIKGEC	12.5	6
	R. areolata	palustrin-3A	GIFPKIIGKGIKTGIVNGIKSLVKGVGMKVFKAGLNNIGNTGCNEDEC	6.25	6
	R. boylii	brevinin-1BYa	FLPILASLAAKFGPKLFCLVTKKC	12.5	7
	R. boylii	brevinin-1BYc	FLPILASLAATLGPKLLCLITKKC	6.25	7
	R. boylii	ranateurin-2BYa	GILSTFKGLAKGVAKDLAGNLL DKFKCKITGC	25	7
	R. boylii	ranateurin-2BYb	GIMDSVKGLAKNLAGKLLDSLKCKITGC	12.5	7
	R. catesbeiana	ranalexin	FLGGLIKIVPAMICAVTKKC	12.5	6
	R. catesbeiana	ranatuerin-1	SMLSVLKNLGKVGLGFVACKINKQC	12.5	6
	R. catesbeiana	ranatuerin-6	FISAIASMLGKFL.NH_2	>100	8
	R. luteiventris	esculentin-2L	GILSLFTGGIKALGKTLFKMAGKAGAEHLACKATNC	12.5	6
	R. luteiventris	ranateurin-2La	GILDSFKGVAKGVAKDLAGKLLDKLKCKITGC	50	6
	R. ornativentris	brevinin-2Ob	GIFNVFKGALKTAGKHVAGSLLNQLKCKVSGEC	6.25	6
	R. ornativentris	temporin-1Ob	FLPLIGKILGTIL.NH_2	25	6
	R. pipiens	Brevinin-1Pa	FLPIIAGVAAKVFPKIFCAISKKC	12.5	9
	R. pipiens	brevinin-1Pb	FLPIIAGIAAKVFPKIFCAISKKC	6.25	9
	R. pipiens	brevinin-1Pg	FFPIVAGVAGQVLKKIFCTISKKC	12.5	9
	R. pipiens	esculentin-2P	GFSSIFRGVAKFASKGLGKDLARLGVNLVACKISKQC	25	6
	R. pipiens	ranatuerin-2P	GLMDTVKNVAKNLAGHMLDKLKCKITGC	100	6
	R. pipiens	temporin-1P	FLPIVGKLLSGLL	50	8
	R. pipiens, R. palustris	brevinin-1PLa	FFPNVASVPGQVLKKIFCAISKKC	50	9
	R. pretiosa	brevinin-1PRa	FLPVLTGLTPSIVPKLVCLLTKKC	50	10
	R. pretiosa	brevinin-1PRb	FLPVLAGLTPSIVPKLVCLLTKKC	12.5	10
	R. pretiosa	brevinin-1PRc	FFPMLAGVAARVVPKVICLITKKC	6.25	10
	R. pretiosa	brevinin-1PRd	FLPMLAGLAASMVPKLVCLITKKC	12.5	10
	R. pretiosa	esculentin-2PRa	GVFSFLKTGAKLLGSTLLKMAGKAGAEHLACKATNQC	25	10
	R. pretiosa	esculentin-2PRa	GIFSALAAGVKLLGNTLFKMAGKAGAEHLACKATNQC	12.5	10
	R. pretiosa	ranatuerin-2PRa	GILDSFKGVAKGVAKDLAGKLLDKLKCKITGC	25	10
	R. pretiosa	ranatuerin-2PRb	GILDTFKGVAKGVAKDLAVHMLENLKCKMTGC	50	10
	R. pretiosa	ranatuerin-2PRc	GILDSFKDVAKGVATHLLNMAKCKMTGC	100	10
	R. pretiosa	ranatuerin-2PRe	GIMNTVKDVATGVATHLLNMVKCKITGC	100	10
	R. pretiosa	ranatuerin-2PRf	GILDTFKGVAKGVAKDLAVHMLEKLKCKMTGC	25	10
	R. pretiosa	ranatuerin-2PRg	GILSSFKDVAKGVAKNVAAQLLDKLKCKITGC	25	10
	R. pretiosa	ranatuerin-2PRh	GILDTVKGVAKDVAAHLLNMVKCKITGC	50	10
	R. pretiosa	temporin-PRb	FLPIITNLLGKLL.NH_2	100	10
	R. pretiosa	temporin-PRc	NFLDTLINLAKKFI.NH_2	25	10
	R. pretiosa	temporin-PRe	FLPLAMALGKLL.NH_2	>100	10
	R. muscosa[a]	ranateurin-2Ma	GLLSSFKGVAKGVAKNLAGKLLEKLKCKITGC	50	11
	R. muscosa[a]	ranateurin-2Mb	GIMDSVKGVAKNLAAKLLEKLKCKITGC	25	11
	R. muscosa[a]	temporin-1M	FLPIVGKLLSGLL.NH_2	100	11
	R. sierrae[b]	brevinin-1Ma	FLPILAGLAANLVPKLICSITKKC	12.5	12
	R. sphenocephala	brevinin-1Sa	FLPAIVGAAGQFLPKIFCAISKKC	40	13
	R. sphenocephala	brevinin-1Sb	FLPAIVGAAAKFLPKIFCAISKKC	10	13
	R. sphenocephala	brevinin-1Sc	FFPIVAGVAGQVLKKIYCTISKKC	20	13
	R. sphenocephala	bemporin-1S	LLFGKIISRLLGN	250	13
	R. tagoi	melittin-related peptide	AIGSILGALAKGLPTLISWIKNR.NH_2	25	14
	R. tarahumarae	brevinin-1TRa	FLPVIAGIAANVLPKLFCKLTKRC	12.5	15
	R. tarahumarae	ranatuerin-2TRa	GIMDSIKGAAKEIAGHLLDNLKCKITGC	50	15
	R. temporaria	temporin-A	FLPLIGRVLSGIL.NH_2	66	8

References (Ref): [1]Conlon et al. (2007); [2]Woodhams et al., 2006a; [3]Rollins-Smith et al. (2002a); [4]Rollins-Smith et al. (2005a); [5]Ramsey et al. (2010); [6]Rollins-Smith et al. (2002b); [7]Davidson et al. (2007); [8]Rollins-Smith et al. (2003); [9]Tennessen et al. (2009); [10]Conlon et al. (2013); [11]Rollins-Smith et al. (2006); [12]Woodhams et al. (2020); [13]Holden et al. (2015a); [14]Rollins-Smith and Conlon, unpublished; [15]Rollins-Smith et al. (2002c).

[a] These peptides were identified as being secreted by *Rana muscosa*, but location of collection would suggest they may have come from *Rana sierrae*.

[b] Also inhibited *Bsal* at 12.5 μM.

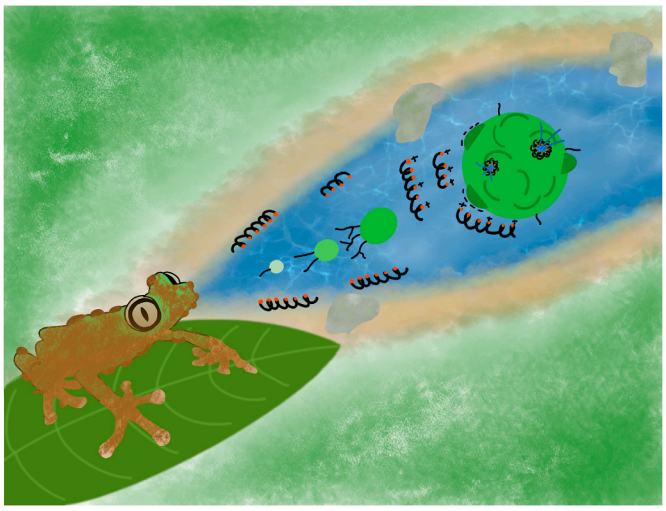

Fig. 1. An artist's depiction of the Lemur leaf frog (*Agalychnis lemur)* of Panama and Costa Rica infected with *Batrachochytrium dendrobatidis* (dark patches on skin) releasing two antimicrobial peptides capable of killing the chytrid zoospores on the skin. Art work by Hyoungjin (John)Lee.

the peptides magainin and PGLa, signals were detected in all portions of the gastrointestinal tract, the skin, and small amounts in muscle and lung, but none in blood, liver, brain, oviduct, or testis (Reilly et al., 1994). Another study of the distribution of brevinin-1SY mRNA in tissues of adult wood frogs (*Rana sylvatica*) found expression in skin, lung, stomach, large intestine, and small intestine (Katzenback et al., 2014).

Another recent study of AMP transcripts in premetamorphic tadpoles of North American bullfrogs (*R. catesbeiana*) showed a surprising diversity of AMP transcripts in back skin, liver, olfactory epithelium, and tail fin tissues (Helbing et al., 2019). A previous study of the expressed and secreted AMPs in bullfrog tadpoles demonstrated some of the same AMPs as adults (Woodhams et al., 2016a). Thus, at least two recent studies suggest that this species, with its long-lived tadpole stage, makes and expresses AMPs in the skin and other tissues even in larval stages. Almost nothing is known about the cell types that produce the peptides or their role in development or immune defenses. This is surely an exciting area of research deserving more attention with techniques such as single-cell RNAseq.

4. Skin peptide depletion studies

Because the granular glands are innervated by sympathetic nerves (Sjoberg and Flock, 1976), frogs can be induced to secrete the contents of the granular glands by injection of norepinephrine (Benson and Hadley, 1969; Holmes and Balls, 1978) or repeated electrostimulation (Mangoni et al., 2001). The amount of total hydrophobic peptides recovered is dependent on the amount of norepinephrine injected and is maximal at concentrations of about 40–80 nmol/g (Rollins-Smith et al., 2005b; Ramsey et al., 2010; Pask et al., 2013). For nearly complete depletion of peptides from granular glands, two injections within two days are required (Gammill et al., 2012). When the granular glands were fully depleted in this way, the recovery of peptide secretions to initial levels took many weeks to months (Ramsey et al., 2010; Pask et al., 2012).

4.1. Antimicrobial peptide depletion and susceptibility to pathogens

Exposure to *B. dendrobatidis* following granular gland depletion resulted in increased pathogen burdens in both young *X. laevis* (Ramsey et al., 2010) and juvenile northern leopard frogs (*Rana pipiens*) (Pask et al., 2013). Although there was no difference in survival of *X. laevis*, peptide-depleted Northern Leopard Frogs (*R. pipiens*) were more likely to die when exposed to *Bd* in comparison with controls that retained intact granular glands (Pask et al., 2013). Granular gland development occurs only in mature tadpoles near the time of metamorphosis, and in juvenile Southern Leopard Frogs (*Rana sphenocephala*), development of the full complement of expected AMPs took at least four weeks (Robak et al., 2019) and up to 12 weeks (Holden et al., 2015a). Both cold temperature (14 °C) and *Bd* infections impaired the renewal of AMPs after peptide depletion. Peptide-depletion in this study did not result in higher *Bd* pathogen burdens or mortality (Robak et al., 2019). Although these laboratory studies showed that AMPs are protective from experimental infections, it was not clear to what extent they may play a role in a natural setting. To examine this question, Panamanian Rocket Frogs (*C. panamensis*) were depleted of their peptides by norepinephrine injection and released to be recaptured at 36 h and 8, 15, or 21 days later. Those frogs that were released with reduced stores of skin peptides had a higher prevalence of *Bd* and greater *Bd* pathogen burdens when recaptured in comparison with those that retained a normal complement of skin peptides (Rosa et al., 2022).

5. Interactions of antimicrobial peptides with the bacterial skin community

Recent studies of the microbial communities in amphibian skin mucus have demonstrated a great diversity of culturable and transmissible bacterial species that can inhibit the growth of *Bd* (Harris et al., 2006, 2009; Lauer et al., 2007, 2008; Woodhams et al., 2007b; Walke et al., 2011, 2015; Bell et al., 2013; Becker et al., 2015; Holden et al., 2015b; Medina et al., 2017; reviewed in Walke and Belden, 2016; Woodhams et al., 2016b). The growing number of such bacteria comprises the antifungal isolates database of amphibian skin-associated bacteria (Woodhams et al., 2015). Secretion of AMPs into the skin mucus has the potential to disturb the community of protective bacteria

Fig. 2. What do antimicrobial peptides do for amphibians? The abundant peptides secreted continuously may serve as a protective mantle against pathogens that enter the mucus (left panel). Alternatively, they may provide a first aid kit to cleanse a wound and aid in healing if the amphibian skin is injured (right panel).

(Woodhams et al., 2007c; Holden et al., 2015a), and the community of bacteria may be especially important in protection from fungal pathogens in juvenile frogs until the AMP repertoire matures (Holden et al., 2015a). However, it should be noted that potentially protective bacterial species, such as the odor-producer *Pseudomonas* sp. MPFS (related to *Pseudomonas fluorescens*) appear to have evolved mechanisms to resist destruction by AMPs (Brunetti et al., 2022). Modest concentrations of a mixture of skin peptides from Mountain Yellow Legged Frogs (*Rana muscosa*) also did not inhibit the growth of a potentially protective species of *Pseudomonas fluorescens* (Myers et al., 2012). *Pseudomonas fluorescens* produces the metabolite 2,4-diacetylphloroglucinol (2, 4-DAPG), one of four known bacterial metabolites that inhibit growth of *Bd* at relatively low micromolar concentrations (Brucker et al., 2008a, 2008b). The other known bacterially derived anti-*Bd* metabolites are indole-3-carboxaldehyde, violacein (Brucker et al., 2008a, 2008b) and viscosin (Martin et al., 2019). When 2,4-DAPG was used in combination with *R. muscosa* skin peptides, the amounts of peptides required to inhibit chytrid growth was reduced suggesting a natural synergy of host-derived AMPs and bacterially derived antifungal metabolites (Myers et al., 2012). This suggests that many members of the normal amphibian skin microbiome may be relatively resistant to the natural concentrations of AMPs in the skin mucus, and the bacterial metabolites may play an important role in protection from some skin pathogens such as *Bd*. In this issue, Woodhams et al. (2023) describe the nature of an adaptive microbiome in which an ongoing infection can select for enrichment of microbes that can inhibit the pathogen and reduce the effects of a secondary infection.

6. Skin peptides, pain, healing, and immune response

One important aspect of amphibian defense that may be overlooked is the capacity of an injured amphibian to continue to function and heal. In this regard it is notable that the synthesis and release of skin peptides with the capacity to bind to opioid receptors has evolved several times within groups of frogs (reviewed in Xu and Lai, 2015). Species of the Phyllomedusinae subfamily of the family Hylidae (e.g., *Phyllomedusa bicolor*) synthesize and store the peptide opioids termed dermorphins and deltorphins in the granular glands (Montecucchi et al., 1981; Kreil et al., 1989; Lacombe et al., 2000) with specificity for the μ- or δ-opioid receptors. Approximately twenty naturally occurring opioid peptides in the dermorphin and deltorphin families have been described in the genera *Phyllomedusa*, *Agalychnis*, and *Phasmahyla*, (reviewed in Xu and Lai 2015). It is tempting to speculate that these opioid peptides might be retained as natural pain-killers for the species that produce them. However, a careful study of the binding of deltorphins to the amphibian or human δ-opioid receptors showed that although the human receptors were strongly activated, the amphibian receptors were essentially unresponsive to the frog opioid peptides (Vardy et al., 2015). Other skin peptides may play a role in wound healing (reviewed in Xu and Lai, 2015; reviewed in Wang 2020). A study of the effects of the peptide bombesin in a model of human keratinocyte wound healing showed that a very low concentration of bombesin (10 nM) increased cell proliferation, cell migration, and the rate of experimental wound closure (Baroni et al., 2008). Another peptide from the skin of *Phyllomedusa bicolor* contains a calcitonin gene-related peptide designated pbCGRP (Seon et al., 2000). Related peptides have been shown to promote proliferation of keratinocytes in a murine model of skin injury (Seike et al., 2002). Another amphibian-derived peptide, cathelicidin-DM from the Chinese frog *Duttaphrynus melanostictus* showed activity in a murine wound-healing assay (Shi et al., 2020). These studies suggest that some amphibian skin peptides can play an important role in wound healing of the amphibian following injury.

In addition to their direct roles as antimicrobial agents, some amphibian skin peptides also have the capacity to attract and engage leukocytes to promote an immune response in injured skin. Peptides that can induce macrophage, lymphocyte, and neutrophil migration include members of the temporin family, temporin A, T1P, and Rana 6 (Chen et al., 2004) and members of the dermaseptin family, Drs-S1 and Drs-S9, (Ammar et al., 1998; Lequin et al., 2006; reviewed in Nicolas and El Amri, 2009). A number of other amphibian skin peptides have been reported to either stimulate or inhibit the release of cytokines by mammalian leukocytes (reviewed in Conlon et al., 2014; reviewed in Xu and Lai, 2015). Thus, it seems likely that following skin injury that results in breakage of the skin, the peptides released at the skin surface would likely attract macrophages, neutrophils, and lymphocytes to respond to possible pathogen derived molecules and to clear any resulting infections and allow for wound healing.

7. Concluding Remarks

The granular glands in the skin of many, but not all, amphibian families synthesize, sequester, and release a diverse array of antimicrobial peptides that have inhibitory activity against known amphibian viral, bacterial, and fungal pathogens. The peptides vary widely by species, and over time, they have been positively selected, most likely to protect against specific pathogens. The host-derived peptides are individually quite effective, but they also synergize with each other and with microbiome-derived antimicrobial factors. The long-term retention and evolution of these peptide arsenals argues strongly that they are beneficial for the survival of the species that make them. Further support for this argument emerges from studies in which disease is more likely to develop when the skin peptides are pharmacologically depleted. Thus, although many amphibian AMPs have been isolated and screened for their potential applications in human medicine, they should continue to be considered as a first line defense of the skin of the amphibians that produce them.

Current gaps in our knowledge of amphibian AMPs include: What is the mechanism by which novel peptides emerge? How rarely or commonly do new AMP variants occur within a large population of frogs? What is the function of the AMPs expressed in tadpole tissues? Do they function to recruit leukocytes to remodel tissues during development? What signaling pathways induce new rounds of synthesis of AMPs, and what explains seasonal variation in their production and function? Amphibian AMPs have fascinated chemists and biologists for multiple decades, and studies about their natural functions will continue to be an important area of research.

Data availability

No data was used for the research described in the article.

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