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Review

The diversity and evolution of anuran skin peptides

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ABSTRACT

Amphibians exhibit various, characteristic adaptations related to their "incomplete" shift from the aquatic to the terrestrial habitat. In particular, the integument was subject to a number of specialized modifications during the evolution of these animals. In this review, we place special emphasis on endogenous host-defence skin peptides from the cuteanous granular glands anuran amphibians (frogs and toads). The overview on the two broad groups of neuroactive and antimicrobial peptides (AMPs) goes beyond a simple itemization in that we provide a new perspective into the evolution and function of anuran AMPs. Briefly, these cationic, amphipathic and α-helical peptides are traditionally viewed as being part of the innate immune system, protecting the moist skin against invading microorganisms through their cytolytic action. However, the complete record of anuran species investigated to date suggests that AMPs are distributed sporadically (i.e., non-universally) across Anura. Together with the intriguing observation that virtually all anurans known to produce neuropeptides in their granular glands also co-secrete cytolytic peptides, we call the traditional role for AMPs as being purely antimicrobial into question and present an alternative scenario. We hypothesize AMPs to assist neuroactive peptides in their antipredator role through their cytolytic action increasing the delivery of the latter to the endocrine and nervous system of the predator. Thus, AMPs are more accurately viewed as cytolysins and their contribution to the immune system is better regarded as an accessory benefit.

Contents

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"The amphibian skin may be regarded as an enormous storehouse of biogenic amines and active polypeptides. Indeed, no other vertebrate or invertebrate tissue can compare with amphibian cutaneous tissue in regard to variety and concentration of these active compounds."

Roseghini, Erspamer, Endean, 1976

Introduction

Extant amphibians (Lissamphibia) represent the modern descendants of the most ancient tetrapod group, where some well-known members of the amphibian stem group from the Devonian, such as *Ichthyostega*, present some of the first fossil records of terrestrial vertebrates [207]. With a distribution across all continents except Antarctica, lissamphibians are remarkably diverse. Despite the transition from the aquatic environment to a more hostile terrestrial life, lissamphibians have adapted to various habitats ranging from Arctic tundra to arid deserts, and to altitudes ranging from sea level to elevations of 5000 m [80]. Frogs and toads (Anura), in particular, cover the entire width of these habitats. A total of 7302 amphibian species have been described for the three monophyletic amphibian orders, with 88% of these being anurans (Fig. 1) [92].

The shift to terrestriality was associated with drastic changes, particularly many physiological challenges, including maintenance of water balance, readjustment of respiration in response to altered oxygen saturation, and higher fluctuations of the daily environmental temperature. Accordingly, lissamphibians show many characteristic evolutionary adaptations in their morphological and physiological traits, ones that have been conserved throughout the group. Thus, among the most important adaptations for lissamphibians involve those of the skin, which evolved numerous adaptive characters to overcome the radically modified environmental conditions and related physiological issues.

As the immediate interface with the environment, the skin needs to protect the animal from the harmful impacts stated above. Unlike other terrestrial vertebrates (Amniota), lissamphibians lack integumental structures to minimize water loss or facilitate thermoregulation (e.g., scales, feathers, or hair). Instead, the more permeable nature of the amphibian skin increases the risk of desiccation as well as the undesired influx of hostile constituents. Indeed, the latter constitutes a major, current threat with growing environmental pollution caused by human agriculture and industry leading to the recent worldwide phenomenon of amphibian decline. Nevertheless, the amphibian integument is an exquisitely adapted and highly specialized organ that supports physiological functions (e.g., respiration, osmoregulation, thermoregulation) as well as phenotypical color modification by means of chromatophores. The latter may be the result of antipredator adaptations that manifest themselves in different ways, ranging from the inconspicuous appearance of leaf litter frogs (mimicry) to the eye-catching aposematic coloration signaling highly toxic animals (e.g., Dendrobatidae).

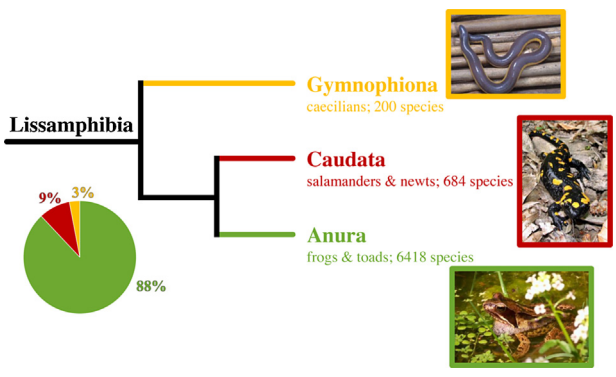

Fig. 1. The three orders of Lissamphibia with the number of species in each. Photos taken from www.amphibiaweb.org.

The amphibian skin is particularly characterized by its remarkable cutaneous exocrine apparatus with numerous granular (serous) and mucous glands [80,254]. These glands are dispersed largely at the dorsum of the animals and communicate directly with the external surface by means of secretory ducts. Whereas the mucous glands constitutively release discrete amounts of mucopolysaccharides to maintain the moist nature of the skin, the discharge of the granular secretions with their venomous and noxious compounds is inducible through various stimuli, one of which is stress (e.g., predatory attack). In addition, clusters of granular glands in exposed parts of the body with high concentrations of venom, called macroglands (e.g., parotoids and inguinal glands), have evolved in several species and reflect an improved defensive mechanism. Interestingly, there are growing indications about intra-individual variation of secretory products between the different granular gland types [120,144], observations that further underpin the remarkable plasticity and adaptive value of the cutaneous organ system in amphibians.

It has been hypothesized that the cutaneous glands of the first amphibians initially maintained homeostasis by producing and releasing key endogenous regulatory molecules as an adaptation to the new terrestrial environmental conditions (e.g., Na^+–K^+ ATPase for sodium and water homeostasis) [73]. Various bioactive substances, such as biogenic amines and neuroactive peptides, which act as hormones, neurotransmitters and neuromodulators [17,83] are still being found in the skin of extant amphibians, particularly in anurans, and have counterparts that occur naturally in the central and peripheral nervous system as well as in the gastrointestinal tract of these animals and other vertebrates (e.g., serotonin, bradykinin). However, given that the amounts of anuran skin amines and peptides often exceed the effective, physiological threshold required for optimal functioning in a regulatory role, it was further hypothesized that the compounds were secondarily recruited for a dose-dependent chemical defence against potential predators [73]. This development, in turn, gave rise to a variety of venomous compounds that are stored in the specialized granular glands, also known as the poison glands.

Although the focus of this review is on the frog skin host-defence peptides, the chemical defence of amphibians goes beyond

these compounds to also include other bioactive constituents like alkaloids [73,75] and steroids [99]. The latter, better known as bufadienolids, occur exclusively in the anuran family Bufonidae, where these cardiotoxic substances are biosynthesized and stored in the prominent parotoid macroglands. By contrast, alkaloids – the main compounds of Dendrobatidae and some other amphibian families (e.g., Bufonidae, Mantellidae, Myobatrachidae, Salamandridae) – are typically sequestered from dietary sources (e.g., arthropods) [46,74] instead of being synthesized by the animals themselves [220]. Most recently, skin alkaloids have been discovered also in an eleutherodactylid frog [194].

Research into the bioactive compounds of amphibian skin secretions has been focussed largely on anurans. As early as 1951, Erspamer and Vialli provided the first evidence for the presence of the biogenic amine serotonin in acetonic extracts of the skin secretion of four amphibian species [88]. This discovery prompted the screening of many additional amphibians for their skin secretions. Subsequent research on hundreds of frog species has unveiled the huge number of natural products, with the broad range of pharmacological properties presented collectively by several of these frog skin peptides heralding the discovery of some novel peptides from the mammalian nervous system [17]. Many of these compounds include neuroactive peptides. The latter and the various peptide families with antimicrobial activity (together, antimicrobial peptides (AMPs) or cytolytic peptides), which represent a second major group and the largest class of frog skin peptides, will be particularly discussed here.

However, at the time of Erspamer's early research with frog skins the analytical tool kit was rather primitive and required the skinning of from several hundred individuals [77]. Besides, it was very time-consuming to determine the structure of the skin compounds. Today the conditions have entirely changed in a dramatic way: the skin secretion obtained by mild electrical stimulation [238] of a single frog yield in sufficient material for analysis in a couple of days – and leaves the animal unharmed; an invaluable ethical aspect in times of the global amphibian decline.

In addition, the recent, rapid developments in molecular biology have considerably contributed to the extensive increase of data in the field of frog skin peptides. For example, modern cloning techniques are increasingly employed for the identification of biosynthetic products expressed in the poison glands of frogs as well as of snakes, scorpions and other venomous animals [37,38] and it is becoming easier to extract the DNA sequence of the entire gene encoding the AMP. Importantly, these latter data may be a useful tool to address evolutionary questions, research that has yet been rarely undertaken [11,20,30,110,118,239] and which forms an important component of our review.

The lack of an evolutionary perspective, in part biased by a research focus on few taxa within the group of "advanced" frogs (Neobatrachia) prompted some of our initial studies to fill this gap [118,121]. Thus, this review intends to follow up on this added perspective using the combination of the immense data set currently available for various anuran species and their different classes of host-defence peptides, piecing together observations from across all Anura to finally propose an alternative scenario explaining the evolution and diversity of this intricate and fascinating adaptive complex.

Methodology of peptide characterization and recent developments

Acquisition of skin secretion

For a long time, most investigators relied upon either methanolic or acetonic skin extracts for peptide isolation. The necessity to sacrifice the specimens, however, makes these an unfavorable mode of sampling, especially in times of an increasing number of endangered species and the global amphibian decline. Instead, a more animal-friendly and ethical manner was established 20 years ago in the form of mild transdermal electrical stimulation of live specimens [238], which induces contraction of the adrenergic myocytes that surround the syncytical granular glands and consequently results in complete discharge of their contents through a holocrine mechanism. In this way, both peptides and mRNA can be isolated from the sample obtained, with the ability to preserve the latter in fresh samples through immediate freeze-drying [37,38]. Indeed, lyophilization of the resultant samples allows their storage for extended periods of up to several years. Importantly, the treatment does not harm the donor amphibians and also provides many scientifically relevant advantages [35] including:

- no sacrifice of specimen
- collection on a regular basis from same individuals (longitudinal sampling)
- applicability for field work
- no contamination (e.g., with blood proteins/peptides from dissected skin)
- ability to generate cDNA libraries

Alternatively, stimulation through hormone injection (e.g., norepinephrine) into the dorsal lymph sacs of the animals is applied in some laboratories, although this represents a more invasive method.

High performance liquid chromatography

Subsequent analysis of the samples by high performance liquid chromatography (HPLC) facilitates separation of the crude skin secretion to obtain the constituent peptides. For amphibian skin secretions, reverse-phase HPLC columns are used. Thorough separation can be achieved by using a slowly increasing gradient of the organic solvent with a likewise slow flow rate (e.g., 1 mL/min). In addition, constant collection in equal time intervals (e.g., 1 min) enables additional HPLC analysis and subsequent screening of each fraction obtained.

Assessing bioactivity

Because the HPLC analyses returns fractions that are only minimally guaranteed to contain peptides, assessing bioactivity of those fractions is essential. For classification into the distinct peptide families, assaying the pharmacological activity of neuropeptides and antimicrobial peptides has been used as diagnostic criterion. Whereas agar diffusion tests enables initial detection of fractions with putative AMPs, a subsequent microtiter dilution test is required to determine the *de facto* potency of the identified AMPs in terms of their minimal inhibitory concentrations (MIC). For the latter assays, synthetic replicates ensure molar accuracy, but require detailed information about the primary structure of the peptide (see section 'Sequence determination').

By contrast, neuroactive peptides often induce signaling pathways that are receptor-mediated and lead to either contraction or relaxation of smooth muscles. Several bioassay systems have been established to determine whether the activity of a certain neuropeptide family resembles that of the known activity of structurally related mammalian counterparts, which were simultaneously used as standards. Classical models involve isolated tissue preparations that are summarized in Table 1. Commonly, a combination of tests is performed to use the resultant activity pattern for characterization of the peptide studied. Indeed, this bioassay model system was used in Erspamer's large scale surveys to

Table 1
Bioassay model systems for anuran skin peptides.

Tissue model	BLP	BRP	Caer	Op	TK	Trp
Blood pressure (dog, rabbit)	+	−−*	−	0	−−−*	?
Rabbit large intestine	+	+	+	0	+++*	?
Guinea pig ileum	Spikes	++*	++	0	+++*	?
Guinea-pig colon	+++*	+	+	0	++	?
Gall bladder (guinea-pig, dog)	+	+	++*	0	+	?
Rat uterus	+++*	+++*	0	0	0	?
Rat urinary bladder	+++*	+	+	0	++	+++
Rat salivary secretion	0	0	0	0	++*	?
Cat/Rat small intestine	+++*	+++*	0	0	+	+++
Mouse vas deferens	0	0	0	−−*	0	?

Adopted from [85].
BLP: bombesins; BRP: bradykinins; CAE: caeruleins; Op: opioids; TK: tachykinins; Trp: tryptophyllins. The most sensitive and characteristic tissue preparation for each peptide family is designated with asterisks. One, two and three symbols mean weak, moderate or strong activity, with + being hypertension or stimulation, − being hypotension or inhibition, and 0 stands for no significant effect. Note that for tryptophyllins bioassays are barely reported (question marks).

verify the presence or absence of a discrete peptide and its related counterparts, respectively. However, as for AMPs, the determination of primary structures of the neuropeptides is essential given that posttranslational modifications (e.g., sulfation, hydroxylation) and amino-acid substitutions can impact on the peptide's potency.

Sequence determination of peptides

A combination of molecular cloning and mass spectrometry (MS) is the way of choice to obtain robust data about the primary structure of an endogenous peptide. Molecular cloning from skin secretion-derived cDNA libraries [37,38] yields complete nucleotide sequence information that can be used for predicting the open-reading frame of the biosynthetic products. Frog skin peptides are classically biosynthesized as prepro-peptides. In amphibian these precursors commonly display a tripartite structure (Fig. 2) comprising

- a signal peptide
- a spacer peptide, and
- a bioactive peptide.

The signal sequence tends to be highly conserved among closely related species, whereas the pro-peptide is hypervariable [239]; a difference related to the respective tasks of the two components. The bioactive peptide varies greatly among anuran species

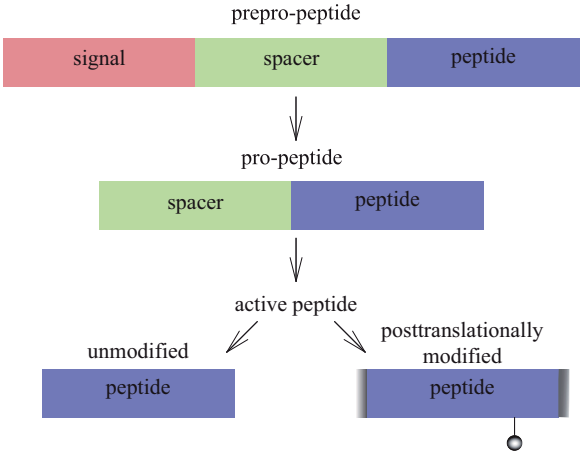

Fig. 2. Biosynthesis of frog skin peptides. Possible posttranslational modifications are N-terminal pyroglutamine and C-terminal amidation (gray shadow at each end) or sulfation and phosphorylation (globe). Also isomers of D-amino acid can be found within the primary structures.

according to the many different functions it possess and/or threats it is designed to counter. The spacer peptide is similarly variable because it maintains the bioactive peptide in an inactive state as a pro-peptide during storage. All bioactive peptides are preceded by two basic residues (e.g., Lys-Arg, Arg-Arg or Lys-Lys) for activation through enzymatic processes (see section 'Protease inhibitors, enzymes and other protein classes').

Frequently, amphibian skin peptides exhibit post-translational modifications. For example, glycinyl residues act as an amide donor for C-terminal amidation. In addition, the conversion of glutamine into pyroglutamine at the N-terminus is very common in neuroactive amphibian peptides to block against potential degradation. By contrast, internal amino acid residues can be either sulphated (Tyr-SO_3H) or appear as D-amino acid isomers (e.g., opioid peptides or bombinin). Such post-translational modifications can be identified by MS, which enables highly sensitive measurements of molecular weights via ionization of a peptide – or any other molecule. Thereby, the observed mass of a peptide obtained from MS can be compared to its calculated mass as deduced from the primary structure obtained from the transcriptome to confirm the identity of the latter.

The diversity of anuran skin peptides

Here, we want to give an overview on the diversity of peptides from the anuran skin secretion with special emphasis on neuroactive and cytolytic peptides. The latter have been often nominated as antimicrobial peptides due to their activity against a wide range of pathogenic microorganisms. Notwithstanding, we prefer the term cytolytic peptide since a number of peptides have proven to be also active as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents [64]. From an evolutionary perspective there are even more sweeping factors that would argue against an exclusive antimicrobial role for these peptides (see section 'Discussion').

However, neuroactive and cytolytic peptides are further grouped into distinct families according to their biological function and structural characters. Peptides with properties on the nervous system are classified as tachykinins, bradykinins, caeruleins, bombesins, opioids, tryptophyllins and miscellaneous peptides (see section 'Neuroactive peptides'), with the latter being a conglomeration of peptides. In general, each of the six main neuropeptide classes tend to be widely distributed across yet investigated anurans, except for opioid peptides that have been isolated only from the skin secretion of hylid frogs from the subfamily Phyllomedusinae (Fig. 3). In contrast, the numerous families of cytolytic peptides are typically shared by closely related frog species (i.e., within the same genus or family). Interestingly, the genes that encode even structurally non-related cytolytic peptides show a highly conserved signal sequence suggesting the physiological active peptides to be under positive selection [235] while the genes share a common evolutionary origin [118,239]. Nonetheless, the evolutionary perspective in the discussion about the anuran chemical defense system remained largely unaddressed. By contrast, the biological properties of the differently acting host-defense peptides have attracted far more attention in part because of the interest of the pharmaceutical industry in using the amphibian skin as a highly abundant source of compounds for drug discovery. Hence, a considerably large – and still increasing – number of studies characterized their biological activities and physiological functions over the last 50 years.

Neuroactive peptides

Tachykinins are known to induce fast contraction of intestinal smooth muscles (Greek: *takhus* = swift; *kinein* = to move). In

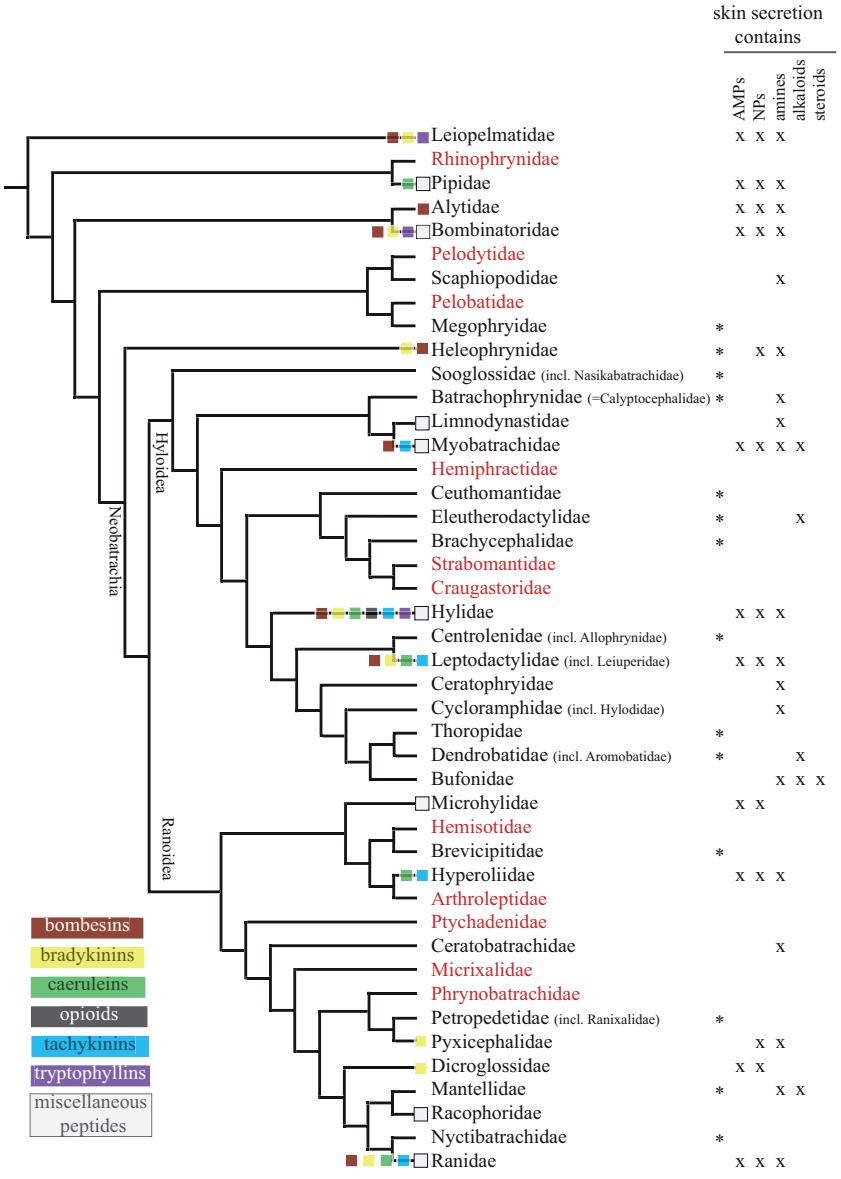

Fig. 3. Phylogeny showing the distribution of frog skin defensive chemicals. NPs: neuroactive peptides; x = compound reported from this family; * = not yet investigated specifically for AMPs. Families in red text have been screened, but apparently lack any compounds.

addition, they cause hypotension through vasodilatation in the peripheral blood vessels, whose endothelial NK1 receptors are directly targeted and cause the release of endogenous relaxing factors [208]. By contrast, blood pressure increases when NK2 receptors are stimulated. More important in terms of their defensive character, tachykinins may enhance the capillary permeability and induce profuse salivation as well as vomiting and diarrhea in an ingesting predator (see [208] for a detailed review).

Tachykinins have been isolated from the nervous system of a wide range of other bilaterian animals (e.g., arthropods, annelids, molluscs, tunicates, branchiostomes) as well as cnidarians [206,209]. As such, tachykinins can be considered to be the most ancient neurotransmitter peptide family in the animal kingdom. Hitherto, they have been identified in the frog skin of five anurans families (SI 1).

The vertebrate peptide hormone **bradykinin** is a cleavage product from the kallikrein kinin system [27,47,48], which is lacking as such in amphibians. Instead, anuran kininogens are expressed exclusively in the exocrine apparatus of the skin and display the typical tripartite gene structure known from cytolytic peptide precursors (Fig. 2), with the spacer-peptide organization present either as single copies (e.g., in *Phyllomedusa, Amolops*, *Limnonectes*) or, more frequently, as consecutively arranged multicopies ([24,32,34,39,40,44,126,136–138,154,219,232,234,237,269,273] and GenBank: ADE41095; FJ842523; HQ172158; EU346894). The canonical bradykinin, a nonapeptide that was first isolated in 1949 from the venom of the brasilian pit viper *Bothrops jararaca* [211] before being identified also from the skin extracts of *Rana temporaria* [7,84], and numerous bradykinin-related peptides (BRPs) have been described from 59 anuran species in eight families (SI 2). Like tachykinins BRPs are myotropic effectors causing either relaxation or contraction of smooth muscles, although they act rather slow (Greek: *bradus* = slow). In addition, bradykinin receptors mediate inflammatory actions in injured tissues and are generally involved in pain response, suggesting BRPs as agonists in those processes [69] through direct or indirect action by releasing other endogenous hormones (e.g., endothelium-derived relaxing factor, nitric oxide). It is noteworthy that each vertebrate group possesses structurally distinct BRPs, with each class having virtually identical counterparts among the anuran skin BRPs (SI 3).

However, the anuran counterparts are often more potent and act longer than their vertebrate analogs due to some N- and C-terminal extensions or post-translational modifications, such as sulfation and hydroxylation [9]. Instead, the natural substitution of the otherwise highly conserved Pro^3 has been shown to demonstrably decrease the potency of two BRPs from the skin secretion of *Bombina variegata* [40]. Evidently, the neuroactive compounds of the anuran skin secretion tend to preserve a minimum of similarity to their vertebrate counterparts in order to turn these agents against potential predators in a defensive manner.

Support for this comes also from **caeruleins**, first isolated from the Australian Tree Frog, *Litoria caerulea* [10], to which the name of the prototype decapeptide is referred. The partial sequence of the heptapeptide caerulein (4–10), which shows the highest similarity to the mammalian cholecystokinin (CCK) 8 and hexagastrin, is essential for the activity of this peptide class, as is the sulphated tyrosine residue at its N-terminus. Accordingly, caeruleins are very potent ligands of CCK receptors involved in cellular pathways that induce acute pancreatitis, vomiting, and diarrhea. Furthermore, the peptide acts as a hunger suppressant by promoting satiety, decreased blood pressure and sedation [21]. Also antinociceptive effects more potent than those of morphine have been demonstrated and hence caerulein has been used clinically as an analgesic and prior to gall bladder surgery [21,83].

Intriguingly, it has been reported that those caeruleins present during the reproductive season of some tree frogs from the genus *Litoria* are converted into less potent desulfated forms that are exclusively expressed during the winter season; in addition, peptides containing a substitution in the third position from the C-terminus (Met into Phe) are also expressed in those frogs [21]. Although these altered peptides show a similar, but reduced smooth muscle bioactivity, the precise adaptive benefit to these variations remains unclear to date.

Currently caeruleins are known from the skin secretions of 46 frog species from five families (SI 4 and SI 5). Most importantly, the first anuran skin peptides to be fully sequenced were caerulein encoding cDNAs from *X. laevis* [104]. Thereafter, the investigators located several putative cleavage sites within the prepro-caeruleins, with the novel peptides from the associated, predicted precursors eventually being found [95]. Interestingly, these caerulein precursor fragments showed high sequence similarity to the hemolytic peptide bombinin isolated from *Bombina bombina* [70], a compound which is now classified as an cytolytic peptide (see section 'Cytolytic peptides from other anurans'). However, subsequent surveys on the exon–intron structure of the caerulein gene in *X. laevis* revealed the presence of at least eight exons [240], with the second exon encoding a signal sequence and a short region of the following pro-peptide. The presence of a similar exon in other *Xenopus* genes encoding skin peptides with different biological activities (e.g., xenopsin, levitide, peptide GLa) raised the hypothesis about an "export exon" (as a form of exon shuffling) that activates those peptides encoded downstream of its insertion site [124].

The evolutionary origins of caerulein, however, is subject to recent discussion and it may well be that they have evolved convergently from the vertebrate hormones CCK and gastrin, distinct genes that apparently originate from an ancient gene duplication event early in the evolution of vertebrates [113]. For instance, although *Litoria splendida* expresses a caerulein peptide that is virtually identical to that from *X. laevis*, Roelants *et al.* [195] showed that each is more closely related to the gastrin and CCK genes, respectively.

As the genetic insights into the caeruleins promoted further understanding of these peptides, the discovery of **bombesin** from *B. bombina* [8] was of similar scientific importance in that its pharmacological characterization yielded the identification of two new mammalian bombesin-like counterparts, the neurotransmitter gastrin-releasing peptide (GRP) [155] and neuromedin B (NMB) [123]. The major function of bombesin is the stimulation of gastrointestinal secretion. Bombesin also acts directly on extravascular smooth muscle to cause potent antidiuretic effects through afferent vasoconstriction [83].

The comprehensive surveys of Erspamer and colleagues [85] suggest both a wide distribution and high diversity of the bombesin-like peptides (BLPs) across Anura (SI 6), culminating in the phylogenetically ancient frog *Ascaphus truei*, where it was suspected to occur, but remains unconfirmed to date. Today, BLPs are subdivided according to their pharmacology (see [83] for details), receptor selectivity and distinct C-terminal tetrapeptides of its members into bombesins (-Gly-His-Leu-Met-NH_2), litorin-ranatensins (-Gly-His-Phe-Met-NH_2), and phyllolitorins (-Gly-Ser-Phe/Leu-Met-NH_2) (SI 7).

While bombesin and litorin-ranatensin peptides can be considered to be the structural counterparts of GRP and NMB, respectively, phyllolitorins have no apparent closely related peptides in mammals. Interestingly, GRP and bombesin are both expressed in the brain and stomach, but are products of different genes arising from a duplicated ancestral gene (i.e., paralogues) [173]. In turn, the frog skin produces only bombesin, again reflecting the exaptation of the expression of neuroactive agents as a defensive weapon.

The most recent discovery of a large novel peptide family in the anuran skin are the **tryptopyllins**, a heterogeneous group that do not have any endogenous counterparts in the nervous system of other metazoans. They are rich in prolyl residues and typically possess a C-terminal tryptophanyl residue. The heterocyclic indole ring structure of the latter fluoresces at a wavelength of 280 nm, a distinctive chemical feature through which tryptophyllins were initially discovered by means of color reaction and conventional paper chromatography. Once again it was Erspamer's group who detected the first member of this peptide family [169], with more than 60 tryptophyllins being isolated from 13 species until today (SI 8). However, their exact distribution among Anura is unknown and understudied, in part due to the poorly understood biological function of these peptides, which are highly diverse in their primary structure despite being very short in length. For example, the actions of tryptophyllin L 1.3 and L 3.1 on smooth muscle preparations is very weak and some orders of magnitude (10,000-fold) lower than that of physalaemin [226]. In addition, bioassays on systemic blood pressure have never met with positive results [83]. Otherwise, PdT-1 obtained from the skin secretion of *Agalychnis* (formerly *Pachymedusa*) *dacnicolor* (please refer to the database of Frost [92] for both current and old taxonomy of amphibian species), and the first amphibian skin tryptophyllin to be pharmacologically (and genetically) characterized in detail, was found to be myoactive and with a higher potency to relax rat arterial smooth muscle than is possessed by bradykinin [33]. Instead, another peptide from *A. dacnicolor*, PdT-2, was found to contract rat urinary bladder smooth muscle [245]. By contrast, earlier studies on a T-1 peptide (Val-Pro-Pro-Leu-Gly-Trp-Met) from the skin secretion of *P. rohdei* showed sedation and sleep inducing effects in pigeon [191], whereas a T-2 peptide (Phe-Pro-Pro-Trp-Met-NH_2) caused an increase in both protein synthesis in the liver and body weight after daily injection in the test animal [114]. The latter observation is consistent with the insulin-releasing effect of GM-14 from *B. variegata* [148], a peptide that is very likely a member of the T-3 subfamily (SI 8).

The sequence similarity between the human opioid peptide hormone endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2), located in the hypothalamus, and two T-2 peptides from *L. rubella* (Phe-Pro-Trp-Leu-NH_2 and Phe-Pro-Trp-Phe-NH_2) provided the impetus for testing them for equivalent bioactivity. Indeed, the T-2 peptides did demonstrate opioid activity using the naxalone antagonist method [109], where, like endomorphin-1, naxalone shows high affinity

with the μ-opioid receptor, which in turn is considered to regulate sedative and arousal behaviors [98]. Furthermore, immunohistochemical studies on rats using radioactive labeled tryptophyllins [191] indicated gonadotrophs in the anterior pituitary, suggesting a function in the central nervous system of mammals.

In contrast to all previous peptides classes, **endogenous opioid peptides** occur exclusively in the skin secretion of South American phyllomedusine species, a group whose skin secretions are renowned for being exceptionally complex and a storehouse for various bioactive peptides [86]. After being first identified in skin extracts of *Phyllomedusa burmeisteri*, *P. rohdei* and *P. sauvagii* [167,168], a total of twelve opioid peptides are currently known (SI 9), most of which have been described from secretion-derived cDNA libraries. All these peptides, which are distinguished into dermorphins and deltorphins, display a typical Tyr-Ala-Phe motif at the N-terminus, with the second amino acid being present in the unusual D-configuration. Apparently, the latter modification is the result of a posttranslational isomerization given that the cDNA sequences contain an ordinary codon for L-amino acids [193], presumedly to confer resistance against enzymatic degradation [162].

Both dermorphins and deltorphins are synthesized as part of a larger precursor comprising the classic tripartite genetic structure (Fig. 2) that shows a high degree of sequence similarity with the dermaseptin precursor of phyllomedusine frogs, one that even extends into the 5′-untranslated region [4]. However, the opioid-encoding precursors differ from prepro-dermaseptin in that they contain a multicopy of five repeated pro-peptide modules [4,193].

The review of Erspamer [82] provides a detailed overview on the biological properties as well as the interaction and selectivity for either μ- or δ-opioid receptors.

A number of anuran skin peptides that cannot be assigned to the families discussed above are summarized as **"miscellaneous" peptides**, which are briefly introduced in the following (names in bold).

Xenopsin was isolated from skin extracts of *X. laevis* [12]. Six amino acids of this octapeptide (pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu,conserved residues underlined) also occur in the same order in neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), a hormone in the mammalian central (e.g., hypothalamus) and peripheral nervous systems (intestinal tract). Although NT itself is not present in amphibian skin and stomach, radioimmunoassays have revealed the presence of another structurally-related tridecapeptide in the brain, small intestine and rectum of the European common frog, *R. temporaria* that shares a conserved C-terminal hexapeptide (pGlu-Ser-His-Ile-Ser-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu) crucial for its biological activity [210]. Xenopsin exhibits similar biological activities to those observed for NT [131]. The most important of these in terms of repelling predators might be induced satiety.

Thyrotropin-releasing hormone is a tripeptide (pGlu-His-Pro-NH_2) that occurs in the hypothalamus of the vertebrate brain. Through stimulation by norepinephrine, it was first isolated from the skin secretion of *B. orientalis* [259] and subsequently from *Pelophylax ridibundus* [190] and *L. pipiens* [107].

Crinia-Angiotensin II (Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe), a N-terminal extended form of the human angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), was first isolated from *Crinia georgiana* and later from other *Crinia* species, all by means of radioimmunoreactivity [87]. As a member of the renin-angiotensin family, this peptide hormone causes vasoconstriction resulting in hypertension. In addition, it stimulates the release of aldosterone in the adrenal cortex, which through the retention of sodium in the distal nephrons enhances the effect of increased blood pressure, thereby causing anadipsia as observed in pigeons [83].

Members of the **prokineticin** family were initially isolated from *B. bombina* and *B. variegata* skin secretions (both termed Bv8) before also being found in *B. orientalis* (Bo8), and *B. maxima* (Bm8a) [38,43,165]; they are also present in fish, reptiles and mammals. Related peptides have been predicted for *R. temporaria* and *Pelophylax esculentus*, but not isolated [177]. Prokineticins tend to be relatively large peptides and have a highly conserved N-terminal hexapeptide sequence (Ala-Val-Ile-Thr-Gly-Cys), possess five disulfide bonds, and bind to G protein-coupled receptors (GPRs) [176]. Interestingly, the frog prokineticin Bv8 shows structural similarity to the black mamba intestinal toxin. Like the latter, the bioactivity of these peptides also includes contraction of smooth muscle in the gastrointestinal tract, but also modulates behavior (feeding, drinking, circadian rhythms) and the release of hypothalamic hormones, as well as being involved in haematopoiesis and in inflammatory and immunomodulatory processes [176,177].

Sauvagine is a 40-mer polypeptide found exclusively in the skin secretion of phyllomedusine frogs. It was initially reported from *P. sauvagii* [170] and shows structural similarity to mammalian corticotrophin-releasing factor and urotensin I from teleost urophysis. Accordingly, its biological activity resembles that of these vertebrate hormones and exhibits both hypotensive and antidiuretic effects in dogs and rats. The peptide also stimulates the release of β-endorphin and adrenocorticotropic hormone. The latter may enhance the antidiuretic effects further given that it activates the adrenal cortex [86].

Recently, some **disulfide-containing peptides** were isolated from three *Crinia* species [111,151,152], but, unlike ranid peptides with a C-terminal disulfide bridge (see section 'Cytolytic peptides from Ranidae'), they do not show any antimicrobial activity. Nevertheless, riparin 1 from both *C. riparia* and *C. deserticola* was shown to function as agonists on CCK_2 receptors in lymphocytes, where they induce cell proliferation [111]. Similarly, disulfide-containing AMPs may both act as immunomodulatory histamine-releasing agents as well as release insulin as has been shown for pipinin-1 from *L. pipiens* [149]. Finally, signiferin 1 from *C. signifera* induces smooth muscle contraction in guinea pig ileum, an effect that is also mediated via the CCK_2 receptor [111]. In general, these peptides appear to be good candidates for having both neuroactive and antimicrobial properties while their secondary structure formed by the disulfide bridges plays an important role in their functioning.

The recent identification of two **FMRF-amide peptides** from *Kassina maculata* and *Phlyctimantis verrucosus* (both Hyperoliidae) [244] may represent one of the most astonishing discoveries of anuran host-defence peptides. Whereas these peptides are a well established invertebrate neuropeptide family and are abundantly present in the endocrine cells of the invertebrate intestinal tract, the anuran counterparts are the first to be found from a vertebrate source. Although the function of these peptides is barely known, even in invertebrates, and the biological activity of the isolated frog peptides was not tested yet, it was proposed that they are used to deter invertebrate predators, such as diving beetles, that co-inhabit the frog's biotope [244], in a similar mode as is suggested for all other host-defence peptides.

Cytolytic peptides

Peptides with antimicrobial activity (AMPs) have been reported from numerous frog species across eleven families (Fig. 3, in Microhylidae presence of AMPs is only suspected) and the total number vastly exceeds that of all previously discussed neuroactive peptides. As such, we do not provide a complete list of all known AMPs here (please refer to the Amphibian Peptide database [243]). Instead, here we present a short recapitulation about the

major AMP families that have been established based on shared structural similarities. Furthermore, emerging issues related to the correct "taxonomic" assignment of existing and future, novel AMPs that largely derive from arbitrarily given names and the lack of a common terminology and evolutionary framework are being addressed. However, it is absolutely essential to illustrate the function of these peptides in terms of their mechanism of action first.

Membranolytic mechanism of action

All cytolytic peptides share some common chemical features needed for their mechanism of action. In addition to possessing several amino acids with hydrophobic side chains, they all have a positive net charge. Positively charged residues (e.g., lysine or arginine) act to bring the peptide in close vicinity to the target membrane. Although cytolytic peptides also show activity against eukaryotic cells (e.g., some fungi, erythrocytes, and protozoans), it is commonly assumed that the electrostatic interaction will be stronger with prokaryotic cell membranes given that they contain comparatively more negatively charged phospholipids at the outer leaflet of the membrane bilayer [263].

Of the several models that exist to explain the mechanism of action of these peptides with respect to their potent lytic activity [261], the toroid pore and the carpet-like models are perhaps the most relevant. Generally, both mechanisms result in a displacement of phospholipid head groups in the target cell membrane, thereby causing destabilization and disruption of the lipid bilayer. In the toroid pore model, the peptides are initially oriented parallel to the membrane surface. Their amphipathic character, which results from the adoption of α-helical structures in lipid environments, induces a curvature in the cell membrane that leads to a displacement of the phospholipids. The resultant breaches on the surface causes further destabilization and facilitate the peptide, at a certain threshold, to enter the hydrophobic core of the bilayer. Eventually, the peptides become oriented perpendicular to the membrane causing formation of toroidal pores spanning the membrane bilayer and disrupting its integrity [261]. By contrast, the carpet-like model holds that a high density of peptide monomers can effectively cover most, if not all, of the membrane surface in a carpet-like manner in parallel orientation, leading to a loss of membrane integrity due to unfavorable energetics [261]. It should be realized that these models may not be mutually exclusive in the sense that one or the other might explain the mechanism of action for different AMP families depending on the characteristics of the member peptides (e.g., length, ability to form α-helices).

In addition to their cytolytic activity, some AMPs have demonstrated additional properties. For example, the dermaseptin-related peptide adenoregulin also acts as a modulator of the A_1 adenosine receptor [71]. Moreover, a number of ranid AMPs induce the release of histamine *in vitro* [105]. Finally, some attract macrophages via chemotactile signaling [260] thereby playing an assisting, rather than direct role in immune response. Such assistance for the innate immune system might have been largely underestimated in the past. However, below we will give some more importance to this matter (see section 'Cytolysins: a new hypothesis on the function of AMPs').

Nomenclature of anuran antimicrobial peptides

The names for AMPs, or more accurately cytolytic peptides, historically referred to the species from which the peptide was first isolated. For instance, the name for the families of brevinin-1 and brevinin-2 peptides derives from the species epithet of *Rana brevipoda porsa* [172]. However, the situation has become considerably complicated given the ongoing revision of species taxonomy in anurans, which often leads to potential confusion and makes this system untenable. Hence, we recommend the online reference from Frost [92] for a constantly updated taxonomy, where the accepted name for, in our example, *Rana brevipoda porsa* today is *Pelophylax porosus*. Yet the original peptide name has been retained and would be treated with priority because of the earlier publication date. In addition, because of this naming convention, the peptide names need not reflect any evolutionary or even functional similarities, both within or especially among groups (e.g., palustrin-1 versus -2 versus -3; see section 'Cytolytic peptides from Ranidae'). Together, these deficits have, to some extent, hindered the recognition of meaningful peptide families based on evolutionary origin (i.e., homology in the form of orthologous versus paralogous families).

To partially combat these problems, Simmaco et al. [217] proposed a practical nomenclatural system for AMPs from ranid frogs that has now generally found acceptance. Based on shared primary structures, degree of conservation, and characteristic sequence motifs Simmaco's system applies the name of a previously reported peptide as the "prototype peptide" – in the sense of "first" and not necessarily "typical" – for a respective family, supplemented with the initial letter from the name of the species set in upper case. Isoforms within the same species – evidently paralogs – are assigned with an additional index letter (a–z) set in lower case. Conlon [51] recently revisited this nomenclatural system to address the problem of the growing number of names of species starting with the same letter, and suggested using any two letters of the species name (e.g., brevinin-1PLa from *Lithobates **pa**lustris*). In both cases, however, priority should be given to the names already assigned in earlier publications.

Although neither system explicitly addresses the idea of clustering homologous protein under a single name, the subjugation of species names as an epithet of the main peptide name will help to facilitate meeting this goal. In so doing, an important component in the nomenclatural process is to prevent the proliferation of novel names (synonyms) when peptides can be clearly linked to an established family. This will reduce both the arbitrariness of the names as well as any potential confusion. Thus, upon discovery of a novel peptide, it is highly recommended to also obtain the sequence information underlying it (in the form of either DNA or amino acids) to facilitate searches for structurally related peptides using the Basic Local Alignment Search Tool (BLAST) implemented in GenBank, where sequences and related information should be archived. If these searches do not yield any significant hits, the creation of a new name may then be justified, which may eventually turn into a prototype peptide for a novel family. The procedure is not fool proof, however, given that there will often be cases of high, but not necessarily significant sequence similarity with existing peptides. Here, the experience of the investigator is asked to determine if the differences are large enough to justify erecting a new peptide family. Nevertheless, use of this procedure would have revealed that the amphibian peptides amolopin [141], gaegurin [182], rugosin [233], pelophylaxin [271], grahamin [257], amurin [272], odorranain [135], and chensirin (GenBank ABF13216, ABG66418) can be unambiguously assigned to established AMP families such that the newly created names should not be used.

That being said, we want to give impetus for using a consistent nomenclatural system for cytolytic peptides across all anuran. For example, in the South American subfamily Phyllomedusinae a slightly different system has been adopted consisting of numbers instead of index letters following the species letter [5]. No consistent terminology yet exists for the Australian subfamily Pelodryadinae, with the peptides simply being numbered without reference to the species of origin (e.g., caerin 1.1).

Those among the readers with less experience will find a number of examples in the following that illustrate the issue of a lacking nomenclature, although sequence similarity exists between two

differently named AMPs. In some cases we argue for a reclassification of peptides. Nevertheless, at this stage we still prefer to be prudent in such cases, but with an appealing call to our colleagues to apply at least one of the existing nomenclatural system stated above in combination with a thoroughly performed BLAST before publishing their next future studies.

Cytolytic peptides from Ranidae

The distribution and the prototype peptide of all AMP families from Ranidae are shown in the supplementary information (SI 10 and SI 11, respectively). In the following we introduce each of them compactly to provide some basic information and claim some reclassification where it is evident.

The name brevinin derives from the species *Rana brevipoda porsa* [172], now reclassified as *Pelophylax porosus*, and the family contains many structurally related peptides from both Eurasian and North American ranid frog species that were termed **brevinin-1**. It is noteworthy, however, that the three pipinins isolated from *L. pipiens* have been identified earlier [105] and clearly represent brevinins.

A few exceptional peptides have a deletion of four amino acids, leading them to initially be mistakenly assigned as ranalexins [45], although having an otherwise strong resemblance with brevinin-1 [61]. Additionally, mellitin-related peptides [231] and some acyclic peptides [53,66] have also been classified as brevinin-1. However, because these peptides lack the characteristic disulfide linkage, they may be better reclassified as mellitin-like peptides (see below), which is the first name that appeared in the literature for them [218]. **Brevinin-2** peptides are restricted to European ranid species. However, some truncated peptides from *Lithobates septentrionalis* [16] and *Lithobates virgatipes* [52] may represent exceptional brevinin-2 peptides from North American ranid frogs. The C-terminal disulfide bridge of brevinin-2 peptides is variable in length (four to six residues) making it more heterogeneous than brevinin-1. A global alignment of all ranid AMPs (data not shown) revealed the close structural similarity between brevinin-2 and ranatuerin-2 peptides, the latter frequently showing a C-terminal extension.

The prototype peptides of **esculentin-1 and -2** peptides were isolated from *P. esculentus* [216]. With 46 residues, esculentin-1 peptides are the longest known AMPs among Anura, and more than half of the primary structure appears to be conserved, particularly the basic lysyl residues and the C-terminal cyclic heptapeptide. By contrast, the cyclic heptapeptide is less conserved in esculentin-2 peptides, which are also somewhat shorter, usually containing 37 amino acids.

The skin secretion of *Rana japonica* contains the two peptides **japonicin-1 and -2** [106]. In a recent study, two peptides related to japonicin-1 were isolated from *Nanorana parkeri* [143]. Whereas another study examining *Rana chaochiaoensis* reported four peptides related to japonicin-2 [62], an additional japonicin-1 peptide was isolated from *Rana dybowskii* [112].

As stated above, some brevinin-1 peptides can be reclassified as **mellitin-like peptides** (MLPs), a group of AMPs that was isolated initially from *R. temporaria* [218] and later from five Japanese frogs – *Rana tagoi* [67], *Babina* (formerly *Rana*) *okinavana* [66], *Rana tsushimensis* [53], *Rana sakuraii* [232] and *Rana japonica* [122] – as well as from *Rana arvalis* [205], which is native to Europe and Asia. Altogether these acyclic peptides comprise 21–23 amino acids residues and occur in species from the Eurasian *Rana temporaria* group (brown frogs) [93].

Peptides from the **nigrocin-2** family were first described from *Pelophylax nigromaculatus* [183]. The same study also described nigrocin-1 for another novel peptide in the skin secretion of that species, although its structural similarity with brevinin-2 peptides argues for its inclusion in the latter family. Intriguingly, the prototype peptide from the above species is also present in the skin secretion of *L. palustris*, *Odorrana hejiangensis* and *Odorrana livida* (GenBank CAM57304, CAM35483 and [270], respectively). This shared occurrence of a skin AMP in three different genera is unique among anurans.

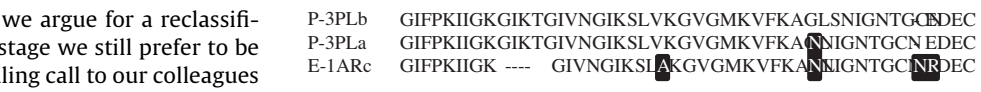

Fig. 4. Alignment of palustrin-3 peptides from *Lithobates palustris* (upper two lines) and esculentin-1ARc from *Lithobates areolatus* (bottom line). Note that P-3VBa from *Odorrana versabilis* is identical to P-3PLb.

Palustrins were isolated initially from *L. palustris* and subdivided into the three classes palustrin-1, -2, and -3 [14]. The palustrin-1 subfamily is characterized by a lysine-rich C-terminal loop. Although a global alignment and phylogenetic analysis of all ranid AMPs reveals a clear cluster for palustrin-2, some peptides from the brevinin-2 family also appear within this cluster (data not shown). A closer look suggests that palustrin-2 may merely represent shorter members of the latter (cf. ranatuerin-2), but with a prolyl-containing C-terminal extension that occurs in virtually all palustrin-2 members. The cyclic heptapeptide before these extensions is generally rich in glycyl residues. Palustrin-3 shows a high degree of sequence identity to esculentin-1ARc from *Lithobates areolatus* (Fig. 4). This suggests that assignment to the esculentin-1 family might be justified, despite esculentin-1ARc differing from palustrin-3 in terms of a four amino acid deletion at the N-terminal end and its disulfide-loop containing one additional residue.

The name **ranacyclin** was first applied to two peptides from *P. esculentus* and *R. temporaria* [146]. However, the actual prototype peptide for the family was described two years earlier in *L. pipiens* and named pLR (peptide leucine arginine) [204]. (This form of nomenclature represents an unbiased alternative in that it simply describes the N- and C-terminal amino acids without implying any relationship to any other peptide through the use of the same name. The latter quality, albeit beset with difficulties, still represents a desirable one for any nomenclatural system with an evolutionary background.) Characteristic for this family is an almost completely conserved region of 13 amino acids that comprises a cyclic undecapeptide formed through a disulfide bridge between Cys^5 and Cys^{15}

Ranatuerin-1 peptides share a characteristic C-terminal Cys-Lys-X-Asn-Lys-Gln-Cys motif and are known from three species of the genus *Lithobates*: *L. catesbeianus*, from which the prototype peptide was obtained [97]; *L. clamitans* and *L. grylio* [100,115]. Based on sequence similarity, ranatuerin-1Ta from *R. temporaria* [215] probably belongs to the brevinin-2 family.

With more than 100 known peptides, **temporins** represent the largest AMP family. They are structurally diverse such that the primary structure of the first two peptides described for this family from the skin secretion of *P. esculentus* [213] are barely adequate to define the group through an N-terminal tripeptide (Phe-Leu/Ile-Pro) that occurs in the majority of temporins. However, two features unite temporins. The first is the complete lack of cysteines and the second is lengths that typically lie between 10–20 residues and which may represent the minimum size for an AMP to be able to span the membrane bilayer of a target cell. However, the heterogeneity within the family precludes any inferences about the relationship among temporins and it cannot be excluded that this family merely represents a collection of the shortest ranid AMPs reported to date. Apart from *Glandirana* and *Babina*, temporins occur in all ranid genera investigated to date. *Odorrana versabilis*, however, is the only species from its genus where evidence for the presence of temporins exists [36].

Cytolytic peptides from Hylidae

Information about the distribution and the sequence of the respective prototype peptide from Hylidae are provided in the supplements SI 12 and SI 13.

Aureins are known exclusively from *Litoria aurea* and *Litoria raniformis* [202], with several being common to both species. Although aureins have been subdivided into aurein 1–3 [202], they do not differ in their primary structures to a degree that would justify such division. Together, aureins comprise the smallest AMPs among Hylidae as well as the smallest peptides with anticancer activity [187] and are good candidates for further research as anticancer agents given a relatively high threshold for haemolysis exceeding 1 mg/ml, which results in low cytotoxic effects against eukaryotic cells. In addition, some apparently biologically inactive aureins exist (classified as aurein 4–5), with aurein-5.2 from this group showing moderate sequence similarity to splendipherin, an aquatic male sex pheromone of *Litoria splendida* [242].

Caerin-1 from *L. caerulea* was the first AMP to have been identified from Australian tree frogs [227], but subsequent studies revealed identical peptides in the skin secretions of *Litoria splendida* [228], *Litoria gilleni* [250] and *Litoria ewingii* [225]. Caerins can be grouped into caerin 1–3 and are known from 16 *Litoria* species so far (SI 12). Caerin-1 peptides have been isolated from seven species and all consist of 23–25 amino acids that adopt two well-defined helices separated by a flexible hinge region from His^{12} to His^{16} [255]. Sixteen positions are highly conserved if not invariable among all caerin-1 members. Caerin-2 peptides are known from three species (*L. caerulea*, *L. gilleni*, and *L. splendida* [228,229,250]) and are more conserved than caerin-1 peptides with only four variable residues across all members and a maximum of two substitutions between any pair of members. We consider the last subfamily, caerin-3, to also contain the caerin-4 subfamily that is recognized by Stone et al. [229], given that the latter differs only slightly from the former in having a C-terminal serinyl residue and three consistent substitutions (Ser^8-Ala^9 and Ala^{14} instead of Asp^8-Lys^9 and Val^{14}).

Citropin-1 peptides have been isolated from *Litoria citropa* [253] and *L. subglandulosa* [26]. Citropin-1 might be closely related to aureins given that the first seven residues of both are virtually identical to one another. Both species also possess citropin-2 and -3 peptides, although only citropin-2.1 appears to be biologically active, presenting a narrow antimicrobial spectrum with moderate activity against the Gram-positive Lactic acid bacteria, *Leuconostoc lactis* [253]. Of the remaining peptides, citropin-2 shows sequence similarity with aurein-5, whereas citropin-3 is similar to aurein-4, both of which are inactive peptides with unknown function.

The N-terminal moiety (Gly^1-Leu-Phe-Asp-Ile-Ile-Lys^7-) of the two identified **dahleins** isolated from *Litoria dahlii* [252] resembles that of both aureins and citropins. Tentatively, they thus have to be reclassified into either of the two latter AMP families and possibly suggest a common origin (and family) for all three groups of peptides.

Dermaseptin-S1 from the skin secretion of *P. sauvagii* was the first AMP isolated from a hylid frog species [171]. With more than 50 characterized peptides, dermaseptins currently represent the largest AMP family of South American Phyllomedusinae. They are heterogenous in length (21–33 amino acid residues) and lack any conserved sites apart from Trp^3 that is universally present except for dermaseptin-A4 (*A. annae*), -C3 (*A. callidryas*) and -S13 (*P. sauvagii*) [133,239,251]. It is noteworthy, however, that these three dermaseptins have not been purified, but only sequenced from cDNA libraries. We propose to divide dermaseptins into two subclasses given that they possess either a highly conserved N-terminal sequence Ala^1-X-Trp-Lys-Y-X-Leu-Lys^8 (Dermaseptin-1, X represents a hydrophobic side chain, and Y a polar side chain or aspartate) or display a Gly^1 followed by an alanine-rich motif in the midregion (-Ala-Ala/Gly-Lys/Gln-Ala-Ala-Leu-Gly/Asn-; Dermaseptin-2).

The skin secretions of five phyllomedusine frogs (*A. annae*, *A. dacnicolor*, *P. bicolor*, *P. sauvagii*, *P. tarsius*) each contain a single **dermatoxin** ([6,42,251]; GenBank P84928), with more than 50% of the 30-mer primary structure being conserved across the family. Interestingly, all sequences obtained from cloning experiments reveal the presence of a C-terminal tripeptide (Gly-Gln-Gly) extension, which could represent a posttranslationally modified derivate of the same gene. However, only traces of this longer peptide (0.2% of the major peptide concentration) have ever been detected in purified samples of *P. sauvagii* and no bioactivity test has been conducted [42].

Phylloseptins were first isolated from *Phyllomedusa hypochondrialis* and *Phyllomedusa oreades* [132] and subsequently identified in five other species of *Phyllomedusa* ([239,264] and GenBank P86282–P86283, P84929–P84931, P85447) and *Agalychnis* (formerly *Hylomantis*) *lemur* [68]. Conserved regions of these peptides are limited to only three residues in the N-terminal region. In our laboratory, we recently identified a second phylloseptin from *P. bicolor*, which is identical to that of *P. sauvagii* [119].

Plasticins comprise peptides from the genera *Phyllomedusa* (2 spp.) and *Agalychnis* (3 spp.) that were previously classified as dermaseptin-related peptides [133,239,251]. The new status and name derive from structural polymorphisms in three of these peptides that could result in distinct biological activities through adopting combinations of different conformations (α-helix, β-sheet, random coil) [81]. However, it has to be stressed that the primary structures of all plasticins, and thus the hypothesized functional plasticity, have been deduced from cDNA sequences only; the predicted secondary structures derive from experiments on synthetic replicates [81]. The natural peptides have not yet been isolated and thus nothing is known about any possible posttranslational modifications, which do have considerable effects on the biological activity of a peptide. Although plasticins share some sequence similarity with dermaseptins, they can be clearly distinguished from the latter through a characteristic richness in Gly and Leu residues that is arranged in serial pentamer motifs Gly-X-X-X-Gly, where at least one X stands for a Leu residue.

Phylloxin was isolated from *P. bicolor* [184] and a structurally related peptide was subsequently reported only from the congeneric *P. sauvagii* [42]. Interestingly, although these peptides do not result in any significant BLAST hits with AMPs of other phyllomedusine frogs, limited similarity with both the levitide-precursor fragments and the XPFs from *X. laevis* [184] is present.

Despite the lack of reliable bioactivity tests for the **hyposins** identified on the basis of MS/MS fragmentation and *de novo* sequencing from *P. azurea* [236] and from *Phasmahyla jandaia* [189], these peptides have been claimed to represent an AMP family [5]. Further, Thompson claims antimicrobial activity for another apparent structural hyposin from *P. hypochondrialis* (GenBank P84524), and proposed that hyposins may target receptors rather than acting cytolytically [236].

Finally, a range of AMPs do not result in any significant BLAST hits with previously characterized AMPs. This includes **fallaxidin-3 and -4** from *L. fallax* [108], **frenatins** from *Litoria infrafrenata* [188], **raniseptins** from *Hypsiboas raniceps* [145], **pseudins** from *Pseudis paradoxa* [180], and **hylins** from *Hypsiboas lundii* [29] and *Hypsiboas albopunctatus* [28], with the latter showing limited sequence similarity with bombinin H isolated from several species of the genus *Bombina* (see section 'Cytolytic peptides from other anurans'). A particular case pose the fallaxidins given that the underlying genetic structure can differ strikingly from the classical neobatrachian pattern of encoding only a single AMP with either a second

or multiple fallaxidin as reported from the precursor [108], the latter two likely representing tryptophyllins (see section 'Neuroactive peptides'). Despite the exceptional nature of the fallaxidin genes to encode both AMPs and putatively neuropeptides, they still possess the highly conserved signal sequence that characterizes virtually all neobatrachian AMP precursors [118,239], leading Jackway et al. [108] to postulate a common origin for prepro-fallaxidins and other (neobatrachian) AMP genes.

Cytolytic peptides from other anurans

In this section, we outline AMPs from the less investigated anuran taxa in alphabetic order (family level) with their prototype peptides being shown in SI 14.

For the common midwife toad, *Alytes obstetricans* **(Alytidae)**, **alyteserin-1 and -2 peptides** have been reported [57]. Recently, we showed that both AMP families are also present in the skin secretion of the congeneric *Alytes maurus* [121]. Interestingly, alyteserin-1 and -2 peptides show limited similarity to ascaphins and bombinin H peptides, respectively. Moreover, the molecular data provided in our study feeds the hypothesis of a close relationship in terms of the underlying genes encoding bombinin H (and bombinin) and alyteserins [121].

The yellow-bellied toad *B. variegata* **(Bombinatoridae)** is the first anuran from which an AMP (**bombinin**) was isolated [70]. The subsequent identification of its biosynthetic precursors revealed the presence of additional peptides [212] that have been named **bombinin H** to indicate their haemolytic activity [162]. This latter study also showed that bombinin H peptides contain amino acids in D-conformation. Both AMP families are also present in the skin secretion of *B. orientalis* and *B. maxima* [214]. Although the AMPs from *B. maxima* were initially termed maximin and maximin H [129], comparison with bombinin and bombinin H clearly identifies them as being members of the latter. However, additional AMPs are known from *B. maxima* and cannot be assigned to the former classes; they are thus termed **maximin-S** [248].

The short 11/12-mer **tigerinins** isolated from *Hoplobatrachus tigerinus* [203] and *Fejervarya cancrivora* [222] **(Dicroglossidae)** are characterized by having two proline residues within the cyclic nonapeptide formed by a disulfide bridge. This feature is otherwise part of virtually all AMPs known from Ranidae (3.2.3.) and has therefore been referred to as the "rana box" [179]. Noteworthy, both species were classified as ranids until a recent taxonomical revision [93], such that the rana box could reflect a shared primitive traits for ranid and dicroglossid frogs – and possibly others as well given that these two taxa are not immediate sister groups.

Kassinatuerins were isolated initially from *Kassina senegalensis* [153] and later also from *Kassina maculata* [247] **(Hyperoliidae)**. The two peptides from *K. senegalensis* differ slightly in having four variant residues. In addition, kassinatuerin-1Sa has an additional glycinyl residue at the N-terminus. However, kassinatuerin-2Sa lacks antimicrobial activity [153] suggesting the amino acid substitutions to be essential for such. By contrast, the loss of Gly^1 appears to be not as crucial given the absence of this amino acid in the four AMPs isolated from *K. maculata* (named kasseptins), which is evidently due to the loss of its codon that can be seen in all four precursor sequences [247].

A recent study revealed the presence of kassorins in these same species [31]. Like observed in fallaxidins, signiferins and uperins, kassorin S possesses antimicrobial activity whereas kassorin M exhibits smooth muscle activity on guinea pig urinary bladder, despite both kassorins being clearly structurally related to one another with only three invariant positions. In addition, both peptides display mast cell histamine-releasing activity, a property that has also been observed for other AMPs [31].

The extant descendants of the lineage comprising the sister group to all remaining frogs are the two genera *Leiopelma* and *Ascaphus* **(Leiopelmatidae)**. As is apparent from the name, **ascaphins** have been isolated from the two species of the latter genus: ascaphin-1T through -8T from *Ascaphus truei* and ascaphin-1M through -7M from *Ascaphus montanus* [56,65].

Moreover, a recent study on three *Leiopelma* species indicated the presence of peptides with antimicrobial activity also in this genus [159], but no sequences have been reported. Similarly, sequence information for the biosynthetic precursor of all ascaphins is missing.

AMPs from **Leptodactylidae** were isolated initially from *Leptodactylus ocellatus* [175] and later from five other species from the same genus: *L. fallax* [197], *L. pentadactylus* [116], *L. laticeps* [54], *L. syphax* [79] and *L. validus* [117]. The work on *L. ocellatus* was extended [174], yielding additional AMPs from this species, with the family later being named **ocellatins** [50] in line with the nomenclature as described above. Whereas all ocellatins share Asp^4 and Lys^{11}, the variation present in all other positions with a length heterogeneity of 17–25 residues does not appear to alter the chemistry of the peptide.

The 22-mer peptide **leptoglycin** from *L. pentadactylus* cannot be assigned to the ocellatins given a peculiar primary structure constructed from only three different amino acids (59% glycine, 36.5% leucine, 4.5% proline). This peptide was shown to be effective against Gram-negative bacteria, two of which occur naturally on the skin of the frog where they were isolated from [224].

Antibiotic **uperins** have been described from the skin secretions of two species distributed in Australia and New Guinea, including *Uperoleia mjobergii* [23] and *Uperoleia inundata* [22] **(Myobatrachidae)**. Uperins are characterized by a few conserved positions (Gly^1, Asp^4, Lys^7/Arg^7, Asn^{15}) in their primary structure consisting of 17 amino acid residues.

Furthermore, **signiferins** have been isolated from *Crinia signifera* [152] and *Crinia riparia* [151] **(Myobatrachidae)**, whereas the peptide from the latter species is one amino acid longer than the two 16-mers from *C. signifera*. The N- and C-terminal dipeptides as well as Leu^5, Leu^{13} and the tetrapeptide Thr-Ala-Leu-Gly in the midregion of these peptides are conserved.

It has to be stressed that both uperin-1 and signiferin-1 appear to be neuropeptides of the tachykinin family (see section 'Neuroactive peptides'), thus representing yet another example of putatively related peptide-encoding genes yielding functionally different products (cf. fallaxidins from Hylidae).

As stated earlier, investigations of the skin secretion of *X. laevis* **(Pipidae)** were the initial catalyst underlying the field of amphibian skin peptide research and have yielded a large range of AMPs. Although the **magainins** are considered to be the first AMPs that were completely characterized [96,262], some peptides that are derived from caerulein-, xenopsin- and PGLa-precursors were reported earlier [95] and contemporaneously shown to be antimicrobial as well [223]. Recently, Conlon's group has extended research in pipid frogs to seven additional species, namely *X. amieti* [55], *X. andrei* [158], *X. borealis* [156], *X. clivii* [63], *X. muelleri* [157], *Silurana paratropicalis* [158], and *Silurana tropicalis* [3]. The alignment of all pipid AMPs (data not shown) yields distinct clusters for **magainins**, **XPFs, PGLa peptides** and **CPFs**.

Orphan cytolytic peptides across anurans

A considerable number of AMPs with distinct primary structures have been described that are not easily assignable to either of the previously discussed AMP families. Examples include guentherin [268], CPRF peptides [1], dybowskin [112], pleurain [249,258], as well as catesbeianin and lividin (both online submissions) from ranid frogs; maculatin-2.1 and -3.1 [201], maculatin-4.1 [25], uperin-7.1 [225], dermaseptin PD-3-7 and dermaseptin AA-3-4 [251], dermaseptin S9 [133], the heterodimeric distinctin [15] and hylaseptin-P1 [185] from hylid frogs; parkerin [143] and cancrin

[142] from dicroglossid frogs; and leptoglycin from *L. ocellatus* [224]. Because each group is represented by relatively few exemplars or only a single peptide, it seems prudent at this stage to designate these peptides temporarily as "orphan peptides" until more structurally-related peptides are found to facilitate effective application of the current nomenclatural system.

Special mention is required for Pv-1.2 and Pv-1.8 from *Trachycephalus* (formerly *Phrynohyas*) *venulosus* (GenBank AAQ11366 and AAQ11367), which have been predicted to be antimicrobial based on their sequence similarity with other AMP precursors (pers. comm., C. Jared). We successfully cloned a related biosynthetic precursor from a skin secretion derived cDNA library of a closely related species *Trachycephalus resinifictrix* (unpublished data). However, screening for antimicrobial activity has not met with success, suggesting that the encoded peptides have another function that remains to be elucidated.

Enzymes, protease inhibitors and other protein classes

Frog skin secretion also contains a diversity of enzymes, including protease inhibitors. Many of these enzymes serve to catalyse the different postranslational modifications observed in virtually all amphibian skin peptides and which contribute to the high diversity of their biological activity. A very common and important modification is α-amidation of the C-terminal amino acid residue of the skin peptides, which often represents an essential quality to ensure their biological efficacy and potency. This amidation reaction is mediated by a peptidylglycine α-amidating monooxygenase as known from *X. laevis* [163,166].

In addition, the final peptide products are processed from their larger precursor peptides by endoproteolytic enzymes (Fig. 2), with all reported endoproteases having been obtained from the skin secretion of *X. laevis*. Endoproteolytic activity appears to be site-specific and cleavage occurs either adjacent to unique amino residues or in combination with a characteristic, conserved sequence. For example, magainase recognizes α-helical structures at a discrete distance to a lysinyl residue, the latter being the actual cleavage site [192]. Most endopeptidases are site-specific dipeptidyl aminopeptidase and, in the case of amphibian skin peptides, comprise either a basic [76,96,125] or a hydrophobic amino acid residue [78,164] at the active site. However, magainase activity has been shown to result in smaller, inactive peptide fragments within the secretion shortly after discharge, suggesting that it plays also a detoxifying and not soley an activation role to protect the frog from prolonged exposure to its own cytotoxic agents [96]. Such post-secretory, endoproteolytic deactivation is also reported from tree frogs of the genus *Litoria* [253].

Associated with the production of frog skin secretion are integumentary glycoproteins mucins [102,186] and α-galactoside-binding lectins [130]. For the amphibian integument, these secreted proteins act to protect the epithelial layer from various threats like desiccation, physical damage and infection, but also provide for binding and uptake of external substances [230]. Frog skin lectins are likewise secreted onto the skin surface [18,150], but their function is not yet fully understood. However, because lectins are massively secreted along with the compounds of the poisonous granules, they may play a role in chemical defense, analogous to lectins from snake venoms and plant seeds, which also occur in very high concentrations and thereby possibly achieve their toxicity [150].

Protease inhibitors form another class of proteogenous anuran skin compounds. As a group, they are present in a wide range of organisms and are typically classified according to the reactive amino acid residue in the catalytic site that is responsible for the specific mechanism of proteolytic action (e.g., aspartic, cysteine, serine, threonine or metallo-protease inhibitors). Serine protease inhibitors are frequently found in the frog skin and can be subclassified according to the presence of structural motifs (e.g., Bowman-Birk [221], Kazal [94], or Kunitz [59]).

Bombina skin trypsin inhibitor from *B. bombina* [161] was the first member of anuran protease inhibitors to be identified. Subsequently the presence of structural homologs in several congeneric species [41,127,140] as well as other protease inhibitors from the more distantly related neobatrachian frogs *Odorrana versabilis* [221], *O. grahami* [101,134] and *Lithobates areolatus* [2] (all Ranidae), the Giant Monkey Frog *P. sauvagii* (Hylidae) [94], and the Asian Toad *Bufo gargarizans* (Bufonidae) [266,267] have been reported. Most of those species have been reported to produce bioactive peptides in their granular glands, the exception being *B. gargarizans*, which, like other bufonids, instead produces a venom consisting of steroid-like compounds and biogenic amines [83,139]. Otherwise, the co-production of protease inhibitors with other biologically active compounds seems to be the rule with respect to anuran skin secretions. With the exception of Narrowmouth Toads (Microhylidae), bioactive peptides are present in virtually all species that possess protease inhibitors. Nevertheless, trypsin inhibitors have been reported from two microhylid species, *Kaloula pulchra* and *Dyscophus guineti* [59,265].

Generally, the function of the protease inhibitors in the anuran skin secretion is poorly understood. Two main hypotheses that exist in this regard – control mechanism versus supplementary antimicrobial activity [120]. In conclusion, it remains an important task for future studies to completely characterize the enzymatic machinery that is responsible for the processing of anuran host-defence peptides.

Discussion

An evolutionary perspective on frog skin peptides

The highly specialized skin of lissamphibians is a distinctive trait of this group [80,254]. Initial explanations for the extraordinary variety of skin compounds within the group revolve around an increased demand for key regulatory molecules associated with the ancestors of these tetrapods being the first vertebrates to colonize the terrestrial habitat [73]. Although Daly et al. [73] do not specify any physiological functions in particular, these new, hostile conditions and emerging selective pressures triggered the evolution of several molecules to fulfill functions such as thermoregulation (e.g., NMB, BLPs), osmoregulation (e.g., ion channels: Na^+–K^+ ATPase) and vasoconstriction for wound closure (e.g., bradykinin), among others. Indeed, exactly such substances – and many more than are discussed here – are still present in the skin secretion of extant anuran amphibians where they also play an important role in defence. For example, one hypothesis holds that vasoconstriction caused by endogenous peptides (e.g., BRPs, BLPs) protect the frog against hemorrhagic shock and blood loss in case of being seriously injured during a predatory attack [13], thereby stressing the "original" physiological function before the function was exapted for defence.

Defensive compounds

The use of alkaloids represents a "more explicit" defence strategy than is true for host-defence peptides. However, a high degree of toxicity is frequently accompanied with aposematic coloration (e.g., in Denbrobatidae and Mantellidae), although the more cryptically colored Bufonidae demonstrate this is not universally true. As an aside, the latter family is known to produce another class of secondary metabolites in the steroid-like bufadienolids. In addition, bufonid skin secretions often contain endogenous biogenic amines as chemical weapons, a class of compounds known to be present in a wide range of other taxa as well (Fig. 3).

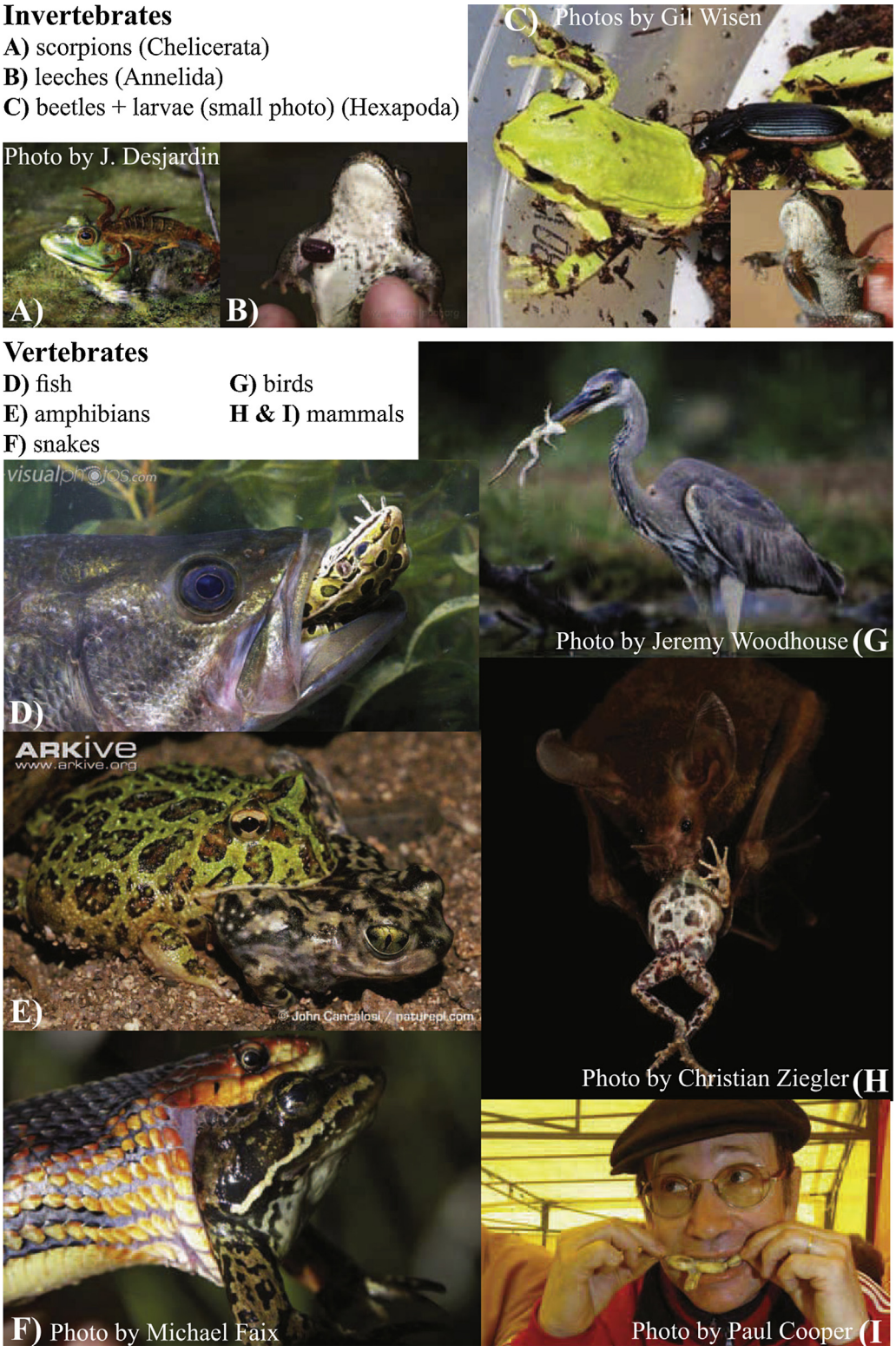

Fig. 5. Range of anuran predators.

More relevant within the context of this study are the endogenous host-defence peptides stored in the exocrine cuteaneous apparatus. Neuroactive peptides are found in relatively high concentrations in the frog skin, thereby evoking a noxious impact despite these substances playing an integral role in the vertebrate central nervous system [17]; some defensive compounds are also known that act on the invertebrate nervous system [244]. Key here is the overproduction of these compounds by the frog, which turned their "original", endogenous regulatory function into an exogenous defensive one. As a consequence, a predator (Fig. 5) ingesting an appropriately armed frog would become overwhelmed by the cocktail of neuroactive peptides and will suffer from the resulting physiological overreactions elicited by them.

Targets of amphibian host-defence peptides

In so doing, the anuran neuropeptides target several organ systems with physiologically different functions: the cardiovascular system (i.e., endothelial cells), the intestinal tract, and the central and peripheral nervous systems. Whereas naturally occurring counterparts regulate functions including blood pressure, gastric secretion and concerted digestion as well as stereotypical behaviors and nociception in these systems, the anuran skin peptides instead provoke disturbance in all these tasks through their chemical mimicry of these substances. For example, BRPs (and their antagonists) naturally act as regulatory peptides that provide an optimal blood pressure following physiological changes (e.g., stress-induced increase of heart frequency, postprandial osmoregulation). The observation that frog BRPs stimulate a vomiting reflex in a predator and thus leads to ejection of the prey unharmed [49] underpins an obvious antipredatory role for these myotropic peptides. In a similar vein, the bombinakinin-gene-associated peptide from *Bombina maxima*, which is coexpressed with the BRP bombinakinin-M suppresses appetite in rats after direct intracerebroventricular injection [128]. However, evoking a similar response in potential anuran predators would require a fast transport to and crossing of the blood–brain-barrier under natural conditions, something that still remains to be studied. By contrast, the results from a range of bioactivity tests on frog skin caeruleins (see section 'Neuroactive peptides') do indicate that they act directly on the digestive tract and in an even more potent manner than their natural counterparts from other vertebrates (CCK and gastrin), suggesting that they could stimulate the vomiting reflex or otherwise induce undesirable movements of the intestinal smooth muscles in the predator that would eventually release the frog. The indirect ability of bombesin to release CCK and gastrin (see section 'Neuroactive peptides') may provoke similar effects through an increased gastric secretion.

Again, most of the putative defensive benefits ascribed to endogenous frog skin peptides, which regulate many physiological processes in both the frog and potential predators, derive from conclusions based on bioassays on isolated tissue preparations. Observations under natural conditions are largely inexistent. The most complete study in the latter regard is that of Barthalmus [13], who demonstrated that the caerulein containing skin secretion of *X. laevis* induces oral dyskinesias in a water snake and thereby allows the frog to escape. More, albeit still indirect, support for an adaptive, defensive function of peptides may come from ligand-receptor interactions that mediate also the "original" regulatory function. Although each vertebrate group – as well as potential invertebrate predators (e.g., arthropods) – share common types of receptors (e.g., GPRs) with subtypes for certain ligands (e.g., tachykinins, bradykinins), differences in the primary structure of the receptors do exist, especially between phylogenetically distant animals. These accumulated changes in the receptor binding site may be driven by minute mutations of the actual agonist (or vice versa), which requires adaptation via (positive) natural selection to restore optimal affinity with the ligand. In a similar fashion, positive selection has likely helped shape the defensive arsenal of anurans through a form of chemical mimicry. In particular, the relatively short sequences of diverse frog skin bradykinins strongly resemble those of naturally occurring counterparts from a wide range of other vertebrates (see SI 2 and SI 3) and the primary structures of individual BRPs typically mirror those of the species-specific spectrum of predominant predators in the natural habitat of the frog [39].

In contrast to the neuroactive peptides, the similarly endogenous AMPs have arguably garnered more research, with hundreds having already been documented among anurans [243]. The common explanation for the remarkable diversity of frog skin AMPs is a defensive function aimed at invading microbes and pathogens as part of the innate immune system [178,198,256]. This hypothesis is consistent with the moist skins of amphibians and the humid environments they tend to favor, both being factors that promote microbial growth. Although most investigators do indeed argue AMPs to be a defensive adaptation to the variety of microorganisms present in the anuran environment, it must be noted that the spectrum of experimentally tested microorganisms in AMP bioassays rarely includes natural micropredators of amphibians, such as the Gram-negative bacteria *Pseudomonas aeruginosa* and *Citrobacter freundii* [224] or the deadly chytrid fungus *Batrachochytrium dendrobatidis* (reviewed in [196]). Instead, scientists have focused exclusively on pathogenic strains that are associated with human diseases given the huge therapeutic potential of AMPs [19,60,64].

A challenge to the antimicrobial role of AMPs

Although the potential medical impact of AMPs is clear, the lack of research in a natural setting is curious. AMPs undoubtedly possess antimicrobial activity because of fundamental physicochemical properties (see section 'Membranolytic mechanism of action'). However, the specific amino acid sequence seems secondary, if at all relevant. Essentially, there are strong arguments against an exclusive antimicrobial function for these peptides, especially from an evolutionary point of view. In particular, arguments for a purely antimicrobial role for AMPs appear to be rather dogmatic and cannot explain the following observations:

Firstly, in virtually all studies, the skin secretions have been collected from the dorsal skin of the animal. Indeed, upon inducing stress (without electrical stimulation), the back of a frog apparently produces much more secretion than the ventral side (own observation). Hence, the repelling agents are directed toward the side of attack and we would expect that the number of granular glands containing the defensive compounds should be higher on this side of the organism. Thus, because microorganisms are much more abundant in the soil and other natural substrates (e.g., leaf, perch), the ventral side of most frog species should exhibit a higher number of granular glands to provide sufficient amounts of antimicrobial agents. Instead, in the majority of the cases this side is apparently the dorsum, although most morphological studies tend to exclusively use dorsal skin strips for their investigations (G. Delfino, pers. comm.) and thus cannot substantiate whether the abundance of granular glands is truly related to the body part that is prone to predator attack. Interestingly, however, the aquatic *X. laevis* covers its entire body with a predator-repelling secretion ([13]; and own observations) apparently because predators can potentially approach the frog from all directions in the water.

Secondly, and more strikingly, is that a considerable number of species appear to lack AMPs entirely (SI 15). However, if AMPs function exclusively as part of the innate immune system against microbial pathogens, this deficiency would reduce the fitness of these species decisively given the lack of protections against the broad spectrum of fast evolving microorganisms present in virtually all habitats. Indeed, a reasonably high number of species exist for which the absence of AMPs and any other skin peptides cannot be easily explained, especially given the presence of toxic and noxious skin secretions in their close relatives. Although there is likely to be a degree of underreporting in the literature of "negative results", our list (SI 15) likely overestimates the number of species lacking any biologically active skin peptides given that their absence was frequently inferred from the lack of apparent bioactivity. However, because of the use of different methods, inadequate bioassays (cf. tryptophyllins), non-systematic sampling, and examining for a specific class of biomolecules only (i.e., AMPs or neuroactive peptides, but not both), negative results in detecting peptides do not necessarily mean the absence of any defensive compounds in the focal species whatsoever. Systematic re-investigation of the same species using the modern methods and technology

would undoubtedly provide positive results in at least some of these candidates. For example, although no peptides were detected initially in *Pseudis paradoxa* and *Hypsiboas albopunctatus* [85], peptides termed pseudin [180] and hylin-a1 [28], respectively, were later reported from the same species. Additionally, subsequent studies aiming to show for instance the presence of biogenic amines [83] often met with positive results in the same species where nothing was detected previously [85] (SI 15).

Cytolysins: a new hypothesis on the function of AMPs

Thus, although AMPs do fulfill an antimicrobial function in nature, the complete lack of AMPs in some frog species would indicate that the general threat posed to the frog by these microorganisms must be overstated or of secondary importance. Accordingly, the term AMP is somewhat misleading because it suggests a general *antimicrobial* function, whereas the above arguments speak against the exclusive immune function. Consequently, we have recently proposed the term cytolysin for this class of host-defence peptides [121].

A productive course of research would be to consider other potential functions of cytolysins, which might give a hint to an alternative (primary) role in anurans. In this, a global view of all host-defence peptides across Anura offers interesting insights into a potential additional role for cytolysins. Importantly, virtually all frog species show a common pattern in the composition of their skin secretions, where neuroactive peptides and cytolytic AMPs tend to co-occur within the same species (Fig. 3), suggesting a strong (functional) correlation between these fundamentally different acting compounds. As pointed out above, the function of neuropeptides is mediated via receptors, most of which are activated (or de-activated) by their ligands that act in a hormone-like manner in the endocrine system. Therefore, in adopting a defensive, anti-predator role, a key consideration becomes the effective delivery of these neuropeptide toxins to the nervous system of the attacking predator, a function well suited to the cytolytic nature of AMPs. Our hypothetical scenario involves the membranes of the epithelial cells forming the mucous layer in the buccal cavity of an ingesting predator being disrupted by an armada of cytolysins, thereby becoming permeable for other compounds that can then access the proximal blood circuit immediately behind this layer and thereby elicit their effects in the endocrine system of the predator (Fig. 6). Importantly, the cytolytic mechanism of action for AMPs is also effective against eukaryotic cells, albeit to a lesser degree than against prokaryotic ones [121,263].

Indirect support for the co-occurrence hypothesis comes from the honey bee, which produces large amounts of mellitin (>50%), one of the strongest AMPs known from nature, in combination with other toxins of lower concentration (e.g., apamin, ca. 2%) in the venom glands of its sting apparatus [103]. For these insects, an immune function for mellitin can definitely be excluded given that honey bees die shortly after stinging, such that AMPs would represent a costly and needless investment. Analogous to the proposed hypothesis on the function in anurans, mellitin in the bee venom serves to improve delivery of the toxins by locally disrupting the tissues of the predator. Therefore, the matter of co-occurrence of differently acting venom compounds deserves more attention in future studies and can be expanded also to other organisms than anuran amphibians.

Under this scenario, the primary role of frog skin cytolysins is as part of an anti-macropredator response, with the origin and evolution of this system being facilitated by the presence of a cutaneous exocrine apparatus regulating physiological functions. Therefore, the extraordinarily high diversity of cytolysins does not so much reflect the broad spectrum of microorganisms faced by the frog, but rather their unspecific mechanism of cytolytic action. Moreover, the relatively high concentrations of most AMPs compensate for

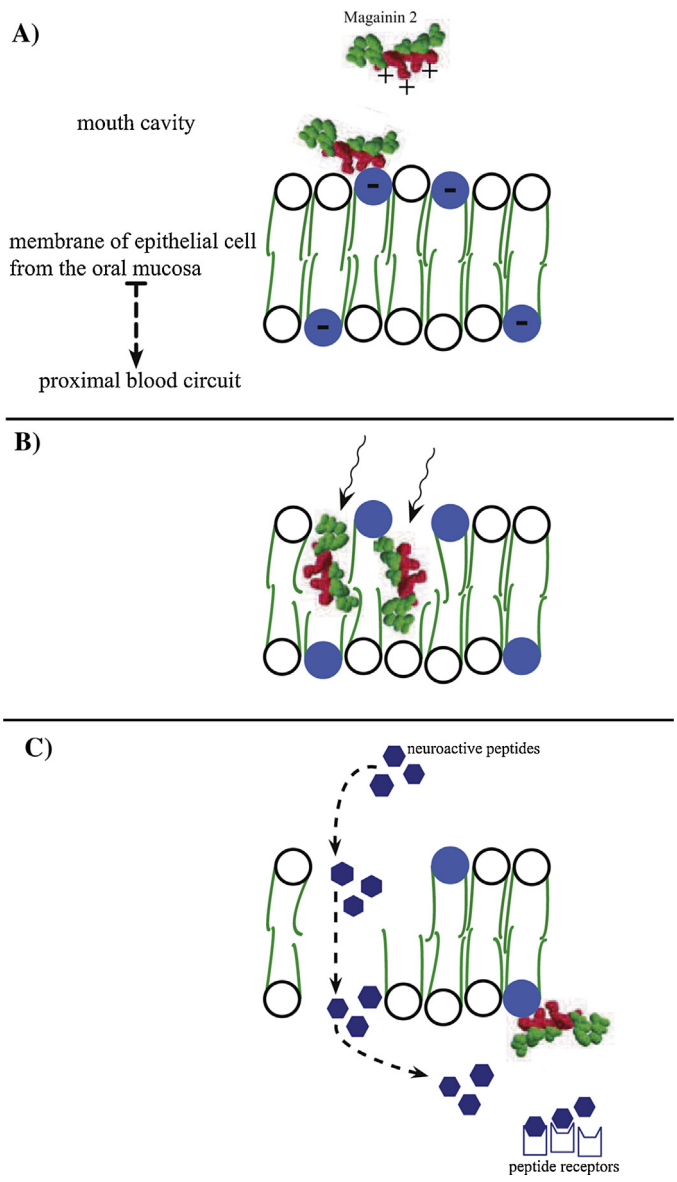

Fig. 6. Hypothetical mechanism of action for cytolysins. In the initiation phase (A), the positively charged cytolysin (here represented by magainin 2; image taken from Zasloff [263]) approaches the negatively charged cell membrane. The resulting electrostatic charge interaction leads to disruption of the membrane in the penetration phase (B) allowing subsequent entry of the neuroactive peptides in the assault phase (C). Note that the cytolysins can also enter the proximal cells to potentially disrupt other cells in the deeper tissue layers (C).

their lesser efficacy against eukaryotic cell membranes compared to prokaryotic cell membranes [263]. Finally, the use of a combination of different cytolysins, which appears to be the rule in most frog species, might reflect important synergistic effects through an increased potency and accordingly a decreased minimal inhibitory concentration that have been reported to show up [147,200].

Important to this discussion is the realization that numerous species of anurans exist that apparently rely on strategies other than chemical defence to protect themselves against predatory threats. These strategies include camouflage, life-histories with cryptic living in the leaf litter or burrowing, persistence with short active periods during reproduction (e.g., Pelobatidae, Limnodynastidae), or highly effective escape mechanisms like gliding as in Racophoridae. Included in this list are also those amphibian species that still use their skin secretion to repel predators by using repulsive odors or secreting adhesive exudates [89]. For example,

the massive discharges of glue-like secretions can act to adhere a predator either to the substrate or to itself and can even yield immobilization of the jaw, head and body of the attacking animal [89]. Altogether, additional defensive attributes such as these help to explain the apparent lack of bioactive compounds in these species, especially if we assume an additional defensive role for AMPs outside the innate immune response.

The origin of the peptide-based defence system

Although the underlying molecular data are not yet available, the presence of cytolysins, bradykinins and tryptophyllins in the skin secretion of *Ascaphus truei* [56,58,65] suggests the presence of a set of peptide-encoding genes in the common ancestor of all crown-group anurans. Together with additional indications for the presence of (uncharacterized) peptides with antifungal activity in *Leiopelma* [159], these observations suggest the chemical defence system to have arisen early in the evolution of Anura. It cannot be excluded, however, that specific defensive peptides might have arisen convergently within Anura and some evidence in this regard exists for AMPs [118], caeruleins [195] and tachykinins [246].

However, to provide definitive statements regarding evolutionary scenarios of potential gene loss or convergent evolution of defensive peptides within Anura (or more broadly within Amphibia), the major quest for the future should include more thorough screening of different frogs species (and those outside of Neobatrachia in particular) using the combination of modern methods (see section 'Methodology of peptide characterization and recent developments') to facilitate a more exact identification of homologous peptides on the genetic level. As was shown by Roelants et al. [195], chromosomal location can be an additional source of information when looking at adjacent sequences of host-defence peptide-encoded genes, while considering chromosomal rearrangements (e.g., recombination, cross-over, fissions and fusions) that have occurred in the genome during the 350 MY since the first appearance of amphibians [181].

In potentially explaining the evolution of all host-defence peptides in frogs, it is important to consider the hypothesis of Kreil and colleagues [124] about a sort of "export exon". For instance, despite the higher number of exons in the caerulein gene of *X. laevis*, the genetic organization between *Bombina orientalis* [160] and *P. bicolor* [90,241] appears to be comparable in that the first exon encodes the signal sequence plus the first three codons of the spacer peptide, whereas the following exon comprises the variable region of the gene. By contrast, the "export exon" in *X. laevis* is the second out of a total of eight exons [240]. However, the different signal sequence motifs [118] that are present among the AMP precursors from these three species suggest the presence of several such "export exons", each defining a particular gene subfamily. Hypothetically, the sequence conservation of any given "export exon" might be explained through its mediating role associated with exon shuffling and thus activation and recruitment of new genes. The adaptive benefit is obvious and natural selection would maintain the functional parts almost unchanged. Gene duplication after the insertion of the "export exon" would then underlie the evolution of paralogues to yield functionally different peptide products. Support for this hypothesis comes from neuropeptide-encoding precursors that appear to share the same signal sequence with an AMP precursor (e.g., phyllokinin and phylloseptin; [119]). At the same time, this method of skin peptide recruitment ensures immediate co-expression of AMPs and neuroactive compounds, which, subject to improved, systematic testing in species where it has not been documented (SI 15 and Fig. 3), we assume to be a widespread if not virtually universal phenomenon among frogs. Indeed, several other neuropeptide families have precursors with a signal sequence that shows a high degree of similarity to that found in AMP precursors (e.g., tachykinins, bradykinins, opioids, tryptophyllins). Thus, a purely transcriptomic approach appears sufficient for the assignment to gene subfamilies that are defined by distinct signal sequence motifs and the structural architecture of the precursor [118] and not necessarily by the function of the actual peptide encoded.

By contrast, co-occurring AMPs and neuroactive peptides can use different signal sequence motifs, as we reported for the AMPs and the BLP alytesin identified in *A. maurus* [121]. Thus, overlooking signal sequence motifs can lead to an incomplete picture about the extent of "export exons" that are actually present in the anuran (and more broadly in the amphibian) gene pool. Altogether these issues highlight the importance of both systematic sampling as well as obtaining more complete information about the DNA sequences underlying the peptides under investigation, and then analysing these data in an explicit evolutionary framework. In so doing, it will become possible to distinguish between different molecular mechanisms (e.g., exon shuffling, convergence, gene duplication, or frame-shift mutations) that have helped shape the evolution of the anuran host-defence peptide system.

Concluding remarks

Altogether skin secretions from at least 545 amphibian species (Dendrobatidae and Mantellidae with their alkaloid-containing skin secretions not included: see [72] and related references for species list) have been screened for bioactive compounds by the end of 2012. The order Gymnophiona remains largely uninvestigated in this regard and data exist for only 20 species of Caudata, where only three species have been found to produce biogenic amines and/or alkaloids [88,199]. In addition, the presence of a skin peptide was reported from the salamander species *Plethodon cinereus*, although its primary structure remains to be elucidated [91].

By contrast, the overwhelming majority of data derive from Anura, where bioactive skin compounds have been detected and isolated from more than 400 anuran species. Although impressive, this number still represents only a small fraction of the 6418 described anuran species [92]. Nevertheless, as shown here, the diversity of peptides from the skin secretions of even this minute proportion of anurans is extraordinarily high and it is intriguing that no two species share exactly the same cocktail of host-defence peptides. Hence, the discovery of further novel biomolecules from this remarkably rich natural source seems almost assured given that more than 90% of frog species still await screening.

In addition to not having investigated all species, another clear gap exists related to how all the compounds that have been sampled are related to one another. To close this gap, it is necessary to study the evolutionary history of host-defense peptides, which in turn holds the potential to find promising candidate species for the discovery of new natural products. In addition, although it is possible to distinguish all the peptide families discussed here based on structural characters and distinct motifs in their primary structure, an evolutionary analysis of their apparent similarities will help to identify supergene families (also between AMPs and neuroactive peptides!) that must be present. However, in reconstructing host-defense peptide evolution, it is important to go beyond peptide sequences to also include information from the underlying genes. On the one hand, this will help to distinguish orthologous from paralogous peptide products that have arisen through the many gene duplication events that are apparent during anuran evolution. In addition, there is a clear potential for convergence within the system (e.g., caerulein [195]; the evolution of the AMP system [118]), meaning that other instances of peptide similarity need not be indicative of a close evolutionary origin. On the other hand, dissimilarity among peptide sequences could also be misleading, with

frame-shift mutations in the underlying genes potentially leading to highly divergent gene products although being homologous. This latter possibility remains virtually uninvestigated to our knowledge.

A final problem involves nomenclature. Although frameworks have been suggested to optimize the naming of the vast number of AMPs, it is unclear what should be done when a single peptide has multiple functions or a single gene encodes different products with different functions (e.g., a cytolytic and neuroactive function as for fallaxidins). In the former case, the lack of testing of peptides for both functions means that the potential for this phenomenon is likely underestimated and there may be instances of the same peptide being known by different names. However, it is clear that identical names have to be used for the products that evidently derive from the same gene and likewise for peptides from orthologous genes to avoid confusion. By contrast, a more prudent way should be applied when gentic data is lacking given that the same function of two peptides does not necessarily reflect their evolutionary origin (and vice versa). Altogether, these issues that we have identified here highlight the need for both increased sampling (and especially for non-anuran amphibians) as well as the application of an evolutionary framework to obtain a more comprehensive understanding of the peptide-based defence system in amphibians.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2014.11.003.

References

[1] Ali MF, Knoop FC, Vaudry H, Conlon JM. Characterization of novel antimicrobial peptides from the skins of frogs of the *Rana esculenta* complex. Peptides 2003;24:955–61.
[2] Ali MF, Lips KR, Knoop FC, Fritzsch B, Miller C, Conlon JM. Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, *Rana areolata*. BBA-Proteins Proteom 2002;1601:55–63.
[3] Ali MF, Soto A, Knoop FC, Conlon JM. Antimicrobial peptides isolated from skin secretions of the diploid frog, *Xenopus tropicalis* (Pipidae). BBA-Protein Struct Mol Enzym 2001;1550:81–9.
[4] Amiche M, Ducancel F, Mor A, Boulain J, Menez A, Nicolas P. Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. J Biol Chem 1994;269:17847–52.
[5] Amiche M, Ladram A, Nicolas P. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. Peptides 2008;29:2074–82.
[6] Amiche M, Seon AA, Wroblewski H, Nicolas P. Isolation of dermatoxin from frog skin, an antibacterial peptide encoded by a novel member of the dermaseptin genes family. Eur J Biochem 2000;267:4583–92.
[7] Anastasi Erspamer V, Bertaccini G. Occurrence of bradykinin in the skin of *Rana temporaria*. Comp Biochem Physiol 1965;14:43–52.
[8] Anastasi A. Alytesin and Bombesin, two peptide analogs from amphibian skin. Naunyn-Schmiedeberg's Arch Pharmacol 1971;269:135–9.
[9] Anastasi A, Bertacci G, Erspamer V. Pharmacological data on phyllokinin (bradykinyl-isoleucyl-tyrosine O-sulphate) and bradykinyl-isoleucyl-tyrosine. Br J Pharmacol Chemother 1966;27:479–85.
[10] Anastasi A, Erspamer V, Endean R. Isolation and structure of caerulein, an active decapeptide from the skin *Hyla caerulea*. Cell Mol Life Sci 1967;23:699–700.
[11] Apponyi MA, Pukala TL, Brinkworth CS, Maselli VA, Bowie JH, Tyler MJ, et al. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. Peptides 2004;25:1035–54.
[12] Araki K, Tachiban S, Uchiyama M, Nakajima T, Yasuhara T. Isolation and structure of a new active peptide Xenopsin on the smooth muscle, especially on a strip of fundus from a rat stomach, from the skin of *Xenopus laevis*. Chem Pharm Bull 1973;21:2801–4.
[13] Barthalmus GT. Biological roles of amphibian skin secretions. In: Heatwole H, editor. Amphibian biology. Surrey Beatty and Sons; 1994. p. 382–410.
[14] Basir YJ, Knoop FC, Dulka J, Conlon JM. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, *Rana palustris*. BBA-Protein Struct Mol Enzym 2000;1543:95–105.
[15] Batista CVF, Scaloni A, Rigden DJ, Silva LR, Romero AR, Dukor R, et al. A novel heterodimeric antimicrobial peptide from the tree-frog *Phyllomedusa distincta*. FEBS Lett 2001;494:85–9.
[16] Bevier CR, Sonnevend A, Kolodziejek J, Nowotny N, Nielsen PF, Conlon JM. Purification and characterization of antimicrobial peptides from the skin secretions of the mink frog (*Rana septentrionalis*). Comp Biochem Physiol C 2004;139:31–8.
[17] Bevins CL, Zasloff M. Peptides from frog-skin. Annu Rev Biochem 1990;59:395–414.
[18] Bols NC, Roberson MM, Haywoodreid PL, Cerra RF, Barondes SH. Secretion of a cytoplasmic lectin from *Xenopus laevis* skin. J Cell Biol 1986;102:492–9.
[19] Boman HG. Antibacterial peptides: basic facts and emerging concepts. J Intern Med 2003;254:197–215.
[20] Bowie JH, Separovic F, Tyler MJ. Host-defense peptides of Australian anurans. Part 2. Structure, activity, mechanism of action, and evolutionary significance. Peptides 2012;37:174–88.
[21] Bowie JH, Tyler MJ. Host defense peptides from Australian amphibians: caerulein and other neuropeptides. In: Kastin JA, editor. Handbook of biologically active peptides. Amsterdam: Academic Press; 2006. p. 283–9.
[22] Bradford A, Raftery M, Bowie J, Tyler M, Wallace J, Adams G, et al. Novel Uperin peptides from the dorsal glands of the Australian Floodplain Toadlet *Uperoleia inundata*. Aust J Chem 1996;49:475–84.
[23] Bradford AM, Bowie JH, Tyler MJ, Wallace JC. New antibiotic uperin peptides from the dorsal glands of the Australian toadlet *Uperoleia mjobergii*. Aust J Chem 1996;49:1325–31.
[24] Brand GD, Krause FC, Silva LP, Leite JRSA, Melo JAT, Prates MV, et al. Bradykinin-related peptides from *Phyllomedusa hypochondrialis*. Peptides 2006;27:2137–46.
[25] Brinkworth CS, Bowie JH, Tyler MJ, Wallace JC. A comparison of the antimicrobial skin peptides of the new guinea tree frog (*Litoria genimaculata*) and the fringed tree frog (*Litoria eucnemis*). Aust J Chem 2002;55:605–10.
[26] Brinkworth CS, Pukala TL, Bowie JH, Tyler MJ. Host defence peptides from the skin glands of Australian amphibians Caerulein neuropeptides and antimicrobial, anticancer, and nNOS inhibiting citropins from the glandular frog *Litoria subglandulosa*. Aust J Chem 2004;57:693–701.
[27] Campbell DJ. Bradykinin and its related peptides. In: Kastin JA, editor. Handbook of biologically active peptides. 1st ed. Amsterdam: Academic Press; 2006. p. 1175–9.
[28] Castro MS, Ferreira TCG, Cilli EM, Crusca Jr E, Mendes-Giannini MJS, Sebben A, et al. Hylin a1, the first cytolytic peptide isolated from the arboreal South American frog *Hypsiboas albopunctatus* (spotted treefrog). Peptides 2009;30:291–6.
[29] Castro MS, Matsushita RH, Sebben A, Sousa MV, Fontes W. Hylins: bombinins H structurally related peptides from the skin secretion of the Brazilian tree-frog *Hyla biobeba*. Protein Pept Lett 2005;12:89–93.
[30] Cei JM. Taxonomic and evolutionary significance of peptides in amphibian skin. Peptides 1985;6:13–6.
[31] Chen H, Wang L, Zeller M, Hornshaw M, Wu YJ, Zhou M, et al. Kassorins: novel innate immune system peptides from skin secretions of the African hyperoliid frogs, *Kassina maculata* and *Kassina senegalensis*. Mol Immunol 2011;48:442–51.
[32] Chen T, Bjourson AJ, McClean S, Orr DF, O'Kane EJ, Rao P, et al. Cloning of maximakinin precursor cDNAs from Chinese toad, *Bombina maxima*, venom. Peptides 2003;24:853–61.
[33] Chen T, Orr DF, O'Rourke M, McLynn C, Bjourson AJ, McClean S, et al. *Pachymedusa dacnicolor* tryptophyllin-1: structural characterization, pharmacological activity and cloning of precursor cDNA. Regul Pept 2004;117:25–32.
[34] Chen T, Shaw C. Cloning of the (Thr6)-phyllokinin precursor from *Phyllomedusa sauvagei* skin confirms a non-consensus tyrosine O-sulfation motif. Peptides 2003;24:1123–30.
[35] Chen T, Zhou M, Gagliardo R, Walker B, Shaw C. Elements of the granular gland peptidome and transcriptome persist in air-dried skin of the South American orange-legged leaf frog, *Phyllomedusa hypochondrialis*. Peptides 2006;27:2129–36.
[36] Chen T, Zhou M, Rao P, Walker B, Shaw C. The Chinese bamboo leaf odorous frog (*Rana* (*Odorrana*) *versabilis*) and North American *Rana* frogs share the same families of skin antimicrobial peptides. Peptides 2006;27:1738–44.
[37] Chen TB, Bjourson AJ, Orr DF, Kwok H, Rao PF, Ivanyi C, et al. Unmasking venom gland transcriptomes in reptile venoms. Anal Biochem 2002;311:152–6.

[38] Chen TB, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. Granular gland transcriptomes in stimulated amphibian skin secretions. Biochem J 2003;371:125–30.
[39] Chen TB, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Bradykinins and their precursor cDNAs from the skin of the fire-bellied toad (*Bombina orientalis*). Peptides 2002;23:1547–55.
[40] Chen TB, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin. Eur J Biochem 2002;269:4693–700.
[41] Chen TB, Shaw C. Identification and molecular cloning of novel trypsin inhibitor analogs from the dermal venom of the Oriental fire-bellied toad (*Bombina orientalis*) and the European yellow-bellied toad (*Bombina variegata*). Peptides 2003;24:873–80.
[42] Chen TB, Walker B, Zhou M, Shaw C. Dermatoxin and phylloxin from the waxy monkey frog, *Phyllomedusa sauvagei*: cloning of precursor cDNAs and structural characterization from lyophilized skin secretion. Regul Pept 2005;129:103–8.
[43] Chen TB, Xue YZ, Zhou M, Shaw C. Molecular cloning of mRNA from toad granular gland secretion and lyophilized skin: identification of Bo8 – a novel prokineticin from *Bombina orientalis*. Peptides 2005;26:377–83.
[44] Chen XL, Wang L, Wang H, Chen H, Zhou M, Chen TB, et al. A fish bradykinin (Arg(0), Trp(5), Leu(8)-bradykinin) from the defensive skin secretion of the European edible frog *Pelophylax kl. esculentus*: structural characterization; molecular cloning of skin kininogen cDNA and pharmacological effects on mammalian smooth muscle. Peptides 2011;32:26–30.
[45] Clark DP, Durell S, Maloy WL, Zasloff M. Ranalexin – a novel antimicrobial peptide from Bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. J Biol Chem 1994;269:10849–55.
[46] Clark VC, Harinantenaina L, Zeller M, Ronto W, Rocca J, Dossey AT, et al. An endogenous bile acid and dietary sucrose from skin secretions of alkaloid-sequestering poison frogs. J Nat Prod 2012;75:473–8.
[47] Conlon JM. The kallikrein-kinin system: evolution of form and function. In: Vaudry H, Tonon MC, Roubos EW, deLoof A, editors. Trends in comparative endocrinology and neurobiology: from molecular to integrative biology. New York: New York Academy of Sciences; 1998. p. 1–8.
[48] Conlon JM. Bradykinin and its receptors in non-mammalian vertebrates. Regul Pept 1999;79:71–81.
[49] Conlon JM. Bradykinin-related peptides from frog skin. In: Kastin JA, editor. Handbook of biologically active peptides. 1st ed. Amsterdam: Academic Press; 2006. p. 291–4.
[50] Conlon JM. A proposed nomenclature for antimicrobial peptides from frogs of the genus *Leptodactylus*. Peptides 2008;29:1631–2.
[51] Conlon JM. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. Peptides 2008;29:1815–9.
[52] Conlon JM, Abraham B, Sonnevend A, Jouenne T, Cosette P, Leprince J, et al. Purification and characterization of antimicrobial peptides from the skin secretions of the carpenter frog *Rana virgatipes* (Ranidae, Aquarana). Regul Pept 2005;131:38–45.
[53] Conlon JM, Al-Ghaferi N, Abraham B, Sonnevend A, Coquet L, Leprince J, et al. Antimicrobial peptides from the skin of the Tsushima brown frog *Rana tsushimensis*. Comp Biochem Physiol C 2006;143:42–9.
[54] Conlon JM, Al-Ghaferi N, Abraham B, Sonnevend A, King JD, Nielsen PF. Purification and properties of laticeptin, an antimicrobial peptide from skin secretions of the South American frog *Leptodactylus laticeps*. Protein Pept Lett 2006;13:411–5.
[55] Conlon JM, Al-Ghaferi N, Ahmed E, Meetani MA, Leprince J, Nielsen PF. Orthologs of magainin, PGLa, procaerulein-derived, and proxenopsin-derived peptides from skin secretions of the octoploid frog *Xenopus amieti* (Pipidae). Peptides 2010;31:989–94.
[56] Conlon JM, Bevier CR, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Peptidomic analysis of skin secretions supports separate species status for the tailed frogs, *Ascaphus truei* and *Ascaphus montanus*. Comp Biochem Phys D 2007;2:121–5.
[57] Conlon JM, Demandt A, Nielsen PF, Leprince J, Vaudry H, Woodhams DC. The alyteserins: two families of antimicrobial peptides from the skin secretions of the midwife toad *Alytes obstetricans* (Alytidae). Peptides 2009;30:1069–73.
[58] Conlon JM, Jouenne T, Cosette P, Cosquer D, Vaudry H, Taylor CK, et al. Bradykinin-related peptides and tryptophyllins in the skin secretions of the most primitive extant frog, *Ascaphus truei*. Gen Comp Endocrinol 2005;143:193–9.
[59] Conlon JM, Kim JB. A protease inhibitor of the Kunitz family from skin secretions of the tomato frog, *Dyscophus guineti* (Microhylidae). Biochem Biophys Res Commun 2000;279:961–4.
[60] Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. BBA-Proteins Proteom 2004;1696:1–14.
[61] Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from the skins of North American frogs. BBA-Biomembranes 2009;1788:1556–63.
[62] Conlon JM, Leprince J, Vaudry H, Jiansheng H, Nielsen PF. A family of antimicrobial peptides related to japonicin-2 isolated from the skin of the chaochiao brown frog *Rana chaochiaoensis*. Comp Biochem Physiol C 2006;144:101–5.
[63] Conlon JM, Mechkarska M, Ahmed E, Leprince J, Vaudry H, King JD, et al. Purification and properties of antimicrobial peptides from skin secretions of the Eritrea clawed frog *Xenopus clivii* (Pipidae). Comp Biochem Physiol C 2011;153:350–4.
[64] Conlon JM, Mechkarska M, Lukic ML, Flatt PR. Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anticancer, anti-viral, immunomodulatory, and anti-diabetic agents. Peptides 2014;57:67–77.
[65] Conlon JM, Sonnevend A, Davidson C, Smith DD, Nielsen PF. The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, *Ascaphus truei*. Biochem Biophys Res Commun 2004;320:170–5.
[66] Conlon JM, Sonnevend A, Jouenne T, Coquet L, Cosquer D, Vaudry H, et al. A family of acyclic brevinin-1 peptides from the skin of the Ryukyu brown frog *Rana okinavana*. Peptides 2005;26:185–90.
[67] Conlon JM, Sonnevend A, Patel M, Camasamudram V, Nowotny N, Zilahi E, et al. A melittin-related peptide from the skin of the Japanese frog, *Rana tagoi*, with antimicrobial and cytolytic properties. Biochem Biophys Res Commun 2003;306:496–500.
[68] Conlon JM, Woodhams DC, Raza H, Coquet L, Leprince J, Jouenne T, et al. Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (Hylidae: Phyllomedusinae). Toxicon 2007;50:498–506.
[69] Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammation. Eur J Pharmacol 2001;429:161–76.
[70] Csordas A, Michl H. Isolation and structure of an hemolytic polypeptide from defensive secretion of European *Bombina* species. Monatsh Chem 1970;101:182–9.
[71] Daly JW, Caceres J, Moni RW, Gusovsky F, Moos M, Seamon KB, et al. Frog secretions and hunting magic in the upper Amazon – identification of a peptide that interacts with an adenosine receptor. Proc Natl Acad Sci U S A 1992;89:10960–3.
[72] Daly JW, Garraffo HM, Spande TF. Alkaloids from amphibian skins. In: Pelletier SW, editor. Alkaloids: chemical and biological perspectives. Amsterdam/Lausanne/New York/Oxford/Shannon/Singapore/Tokyo: Pergamon; 1999. p. 1–161.
[73] Daly JW, Myers CW, Whittaker N. Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic noxious substances in the amphibia. Toxicon 1987;25:1023–95.
[74] Daly JW, Secunda SI, Garraffo HM, Spande TF, Wisnieski A, Cover JF. An uptake system for dietary alkaloids in poison frogs (Dendrobatidae). Toxicon 1994;32:657–63.
[75] Daly JW, Spande TF, Garraffo HM. Alkaloids from amphibian skin: a tabulation of over eight-hundred compounds. J Nat Prod 2005;68:1556–75.
[76] Darby NJ, Lackey DB, Smyth DG. Purification of a cysteine endopeptidase which is secreted with bioactive peptides from the epidermal glands of *Xenopus laevis*. Eur J Biochem 1991;195:65–70.
[77] De Caro G, Endean R, Erspamer V, Roseghini M. Occurence of caerulein in extracts of the skin of *Hyla caerulea* and other Australian hylids. Br J Pharmacol Chemother 1968;33:48–58.
[78] Dex Morais Carvalho K, Joudiou C, Boussetta H, Leseney AM, Cohen P. A peptide-hormone-inactivating endopeptidase in *Xenopus laevis* skin secretion. Proc Natl Acad Sci U S A 1992;89:84–8.
[79] Dourado FS, Leite JRSA, Silva LP, Melo JAT, Bloch Jr C, Schwartz EF. Antimicrobial peptide from the skin secretion of the frog *Leptodactylus syphax*. Toxicon 2007;50:572–80.
[80] Duellman WE, Trueb L. Biology of amphibians. 3rd ed. Baltimore/London: John Hopkins University Press; 1994.
[81] El Amri C, Lacombe C, Zimmerman K, Ladram A, Amiche M, Nicolas P, et al. The plasticins: membrane adsorption, lipid disorders, and biological activity. Biochemistry-US 2006;45:14285–97.
[82] Erspamer V. The opioid-peptides of the amphibian skin. Int J Dev Neurosci 1992;10:3–30.
[83] Erspamer V. Bioactive secretions of the amphibian integument. In: Heatwole H, Barthalmus GT, editors. Amphibian biology: the integument, vol. 1. Chipping Norton: Surrey Beatty and Sons; 1994. p. 178–350.
[84] Erspamer V, Cei JM, Bertaccini G. Occurence of bradykinin-like substances in amphibian skin. Experientia 1962;18:563–4.
[85] Erspamer V, Erspamer GF, Cei JM. Active peptides in the skins of two hundred and thirty American amphibian species. Comp Biochem Physiol C 1986;85:125–37.
[86] Erspamer V, Melchiorri P, Erspamer GF, Montecucchi PC, Decastiglione R. Phyllomedusa skin – a huge factory and store-house of a variety of active peptides. Peptides 1985;6:7–12.
[87] Erspamer V, Melchiorri P, Nakajima T, Yasuhara T, Endean R. Amino acid composition and sequence of crinia-angiotensin, an angiotensin II-like endecapeptide from the skin of the Australian frog *Crinia georgiana*. Cell Mol Life Sci 1979;35:1132–3.
[88] Erspamer V, Vialli M. Presence of enteramine in the skin of amphibia. Nature 1951;167:1033.
[89] Evans CM, Brodie ED. Adhesive strength of amphibian skin secretions. J Herpetol 1994;28:499–502.
[90] Fleury Y, Vouille V, Beven L, Amiche M, Wroblewski H, Delfour A, et al. Synthesis, antimicrobial activity and gene structure of a novel member of the dermaseptin B family. BBA-Gene Struct Expr 1998;1396:228–36.
[91] Fredericks LP, Dankert SR. Antibacterial and hemolytic activity of the skin of the terrestrial salamander *Plethodon cinereus*. J Exp Zool 2000;287:340–5.
[92] Frost DR. Amphibian species of the world: an online reference. Version 6.0 (2 October, 2014). New York, USA: American Museum of Natural History; 2014. Electronic database accessible at: http://research.amnh.org/vz/herpetology/amphibia/index.html

[93] Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, et al. The amphibian tree of life. Bull Am Mus Nat Hist 2006:8–371.
[94] Gebhard LG, Carrizo FU, Stern AL, Burgardt NI, Faivovich J, Lavilla E, et al. A Kazal prolyl endopeptidase inhibitor isolated from the skin of *Phyllomedusa sauvagii*. Eur J Biochem 2004;271:2117–26.
[95] Gibson BW, Poulter L, Williams DH, Maggio JE. Novel peptide-fragments originating from PGLa and the caerulein and xenopsin precursors from *Xenopus laevis*. J Biol Chem 1986;261:5341–9.
[96] Giovannini MG, Poulter L, Gibson BW, Williams DH. Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones. Biochem J 1987;243:113–20.
[97] Goraya J, Knoop FC, Conlon JM. Ranatuerins antimicrobial peptides isolated from the skin of the American bullfrog *Rana catesbeiana*. Biochem Biophys Res Commun 1998;250:589–92.
[98] Greco MA, Fuller PM, Jhou TC, Martin-Schild S, Zadina JE, Hue Z, et al. Opioidergic projections to sleep-active neurons in the ventrolateral preoptic nucleus. Brain Res 2008;1245:96–107.
[99] Habermehl GG, Hammann PE, Krebs HC. 2. Steroide und 3. Biogene Amine und Alkaloide. 2nd ed. Berlin: Springer; 1992.
[100] Halverson T, Basir YJ, Knoop FC, Conlon JM. Purification and characterization of antimicrobial peptides from the skin of the North American green frog *Rana clamitans*. Peptides 2000;21:469–76.
[101] Han Y, Yu H, Yang X, Rees HH, Liu J, Lai R. A serine proteinase inhibitor from frog eggs with bacteriostatic activity. Comp Biochem Physiol B 2008;149:58–62.
[102] Hauser F, Hoffmann W. P-domains as shuffled cysteine-rich modules in integumentary mucin C.1 (FIM-C.1) from *Xenopus laevis*. Polydispersity and genetic polymorphism. J Biol Chem 1992;267:24620–4.
[103] Haux P, Sawerthal H, Habermann E. Sequence analysis of bee venom neurotoxin (apamine) from its tryptic and chymotryptic cleavage products. Hoppe-Seyler's Z Physiol Chem 1967;348:737–8.
[104] Hoffmann W, Bach TC, Seliger H, Kreil G. Biosynthesis of caerulein in the skin of *Xenopus laevis* – partial sequences of precursors as deduced from cDNA clones. EMBO J 1983;2:111–4.
[105] Horikawa R, Parker DS, Herring PL, Pisano JJ. Pipinins – new mast-cell degranulating peptides from *Rana pipiens*. Fed Proc 1985;44:695.
[106] Isaacson T, Soto A, Iwamuro S, Knoop FC, Conlon JM. Antimicrobial peptides with atypical structural features from the skin of the Japanese brown frog *Rana japonica*. Peptides 2002;23:419–25.
[107] Jackson IMD, Reichlin S. Thyrotropin-releasing hormone abundance in the skin of the frog *Rana pipiens*. Science 1977;198:414–5.
[108] Jackway RJ, Bowie JH, Bilusich D, Musgrave IF, Surinya-Johnson KH, Tyler MJ, et al. The fallaxidin peptides from the skin secretion of the Eastern Dwarf Tree Frog *Litoria fallax* Sequence determination by positive and negative ion electrospray mass spectrometry: antimicrobial activity and cDNA cloning of the fallaxidins. Rapid Commun Mass Spectrom 2008;22:3207–16.
[109] Jackway RJ, Maselli VM, Musgrave IF, Maclean MJ, Tyler MJ, Bowie JH. Skin peptides from anurans of the *Litoria rubella* Group: sequence determination using electrospray mass spectrometry Opioid activity of two major peptides. Rapid Commun Mass Spectrom 2009;23:1189–95.
[110] Jackway RJ, Pukala TL, Donnellan SC, Sherman PJ, Tyler MJ, Bowie JH. Skin peptide and cDNA profiling of Australian anurans: genus and species identification and evolutionary trends. Peptides 2011;32:161–72.
[111] Jackway RJ, Pukala TL, Maselli VM, Musgrave IF, Bowie JH, Liu YQ, et al. Disulfide-containing peptides from the glandular skin secretions of froglets of the genus *Crinia*: structure, activity and evolutionary trends. Regul Pept 2008;151:80–7.
[112] Jin L-L, Li Q, Song S-S, Feng K, Zhang D-B, Wang Q-Y, et al. Characterization of antimicrobial peptides isolated from the skin of the Chinese frog, *Rana dybowskii*. Comp Biochem Physiol B 2009;154:174–8.
[113] Johnsen AH, Jønson L, Rourke IJ, Rehfeld JF. Elasmobranchs express separate cholecystokinin and gastrin genes. Proc Natl Acad Sci U S A 1997;94:10221–6.
[114] Kaemmerer K, Fink J, Kietzmann M. Peptidi come promotori di crescita. Riv Zootec Vet 1985;13:3–12.
[115] Kim JB, Halverson T, Basir YJ, Dulka J, Knoop FC, Abel PW, et al. Purification and characterization of antimicrobial and vasorelaxant peptides from skin extracts and skin secretions of the North American pig frog *Rana grylio*. Regul Pept 2000;90:53–60.
[116] King JD, Al-Ghaferi N, Abraham B, Sonnevend A, Leprince J, Nielsen PF, et al. Pentadactylin: an antimicrobial peptide from the skin secretions of the South American bullfrog *Leptodactylus pentadactylus*. Comp Biochem Physiol C 2005;141:393–7.
[117] King JD, Leprince J, Vaudry H, Coquet L, Jouenne T, Conlon JM. Purification and characterization of antimicrobial peptides from the Caribbean frog, *Leptodactylus validus* (Anura: Leptodactylidae). Peptides 2008;29:1287–92.
[118] König E, Bininda-Emonds ORP. Evidence for convergent evolution in the antimicrobial peptide system in anuran amphibians. Peptides 2011;32:20–5.
[119] König E, Clark VC, Shaw C, Bininda-Emonds ORP. Molecular cloning of skin peptide precursor-encoding cDNAs from tibial gland secretion of the Giant Monkey Frog, *Phyllomedusa bicolor*. Peptides 2012;38:371–6.
[120] König E, Wesse C, Murphy AC, Zhou M, Wang L, Chen T, et al. Molecular cloning of the trypsin inhibitor from the skin secretion of the Madagascan Tomato Frog, *Dyscophus guineti* (Microhylidae), and insights into its potential defensive role. Org Divers Evol 2013:1–9.
[121] König E, Zhou M, Wang L, Chen T, Bininda-Emonds ORP, Shaw C. Antimicrobial peptides and alytesin are co-secreted from the venom of the Midwife toad, *Alytes maurus* (Alytidae, Anura): implications for the evolution of frog skin defensive secretions. Toxicon 2012;60:967–81.
[122] Koyama T, Iwamuro S. Molecular cloning of a cDNA encoding atypical antimicrobial and cytotoxic Brevinin-1Ja from the skin of the Japanese brown frog, *Rana japonica*. Zool Sci 2008;25:487–91.
[123] Krane IM, Naylor SL, Helin-Davis D, Chin WW, Spindel ER. Molecular cloning of cDNAs encoding the human bombesin-like peptide neuromedin B. Chromosomal localization and comparison to cDNAs encoding its amphibian homolog ranatensin. J Biol Chem 1988;263:13317–23.
[124] Kuchler K, Kreil G, Sures I. The genes for the frog-skin peptides GLa, xenopsin, levitide and caerulein contain a homologous export exon encoding a signal sequence and part of an amphiphilic peptide. Eur J Biochem 1989;179:281–5.
[125] Kuks PFM, Creminon C, Leseney AM, Bourdais J, Morel A, Cohen P. Xenopus laevis skin Arg-Xaa-Val-Arg-Gly-Endoprotease – a highly specific protease cleaving after a single arginine of a consensus sequence of peptide-hormone precursors. J Biol Chem 1989;264:14609–12.
[126] Lai R, Liu H, Lee WH, Zhang Y. A novel bradykinin-related peptide from skin secretions of toad *Bombina maxima* and its precursor containing six identical copies of the final product. Biochem Biophys Res Commun 2001;286:259–63.
[127] Lai R, Liu H, Lee WH, Zhang Y. Identification and cloning of a trypsin inhibitor from skin secretions of Chinese red-belly toad *Bombina maxima*. Comp Biochem Physiol B 2002;131:47–53.
[128] Lai R, Liu H, Lee WH, Zhang Y, Bombinakinin. M gene associated peptide, a novel bioactive peptide from skin secretions of the toad *Bombina maxima*. Peptides 2003;24:199–204.
[129] Lai R, Zheng YT, Shen JH, Liu GJ, Liu H, Lee WH, et al. Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*. Peptides 2002;23:427–35.
[130] Lazarus LH, Attila M. The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 1993;41:473–507.
[131] Leeman SE, Carraway RE. Neurotensin: discovery, isolation, characterization, synthesis and possible physiological roles. Ann N Y Acad Sci 1982;400:1–16.
[132] Leite J, Silva LP, Rodrigues MIS, Prates MV, Brand GD, Lacava BM, et al. Phylloseptins: a novel class of anti-bacterial and anti-protozoan peptides from the *Phyllomedusa* genus. Peptides 2005;26:565–73.
[133] Lequin O, Ladram A, Chabbert L, Bruston F, Convert O, Vanhoye D, et al. Dermaseptin S9, an alpha-helical antimicrobial peptide with a hydrophobic core and cationic termini. Biochemistry-US 2006;45:468–80.
[134] Li JX, Wu J, Wang YP, Xu XQ, Liu TG, Lai R, et al. A small trypsin inhibitor from the frog of *Odorrana grahami*. Biochimie 2008;90:1356–61.
[135] Li JX, Xu XQ, Xu CH, Zhou WP, Zhang KY, Yu HN, et al. Anti-infection peptidomics of amphibian skin. Mol Cell Proteomics 2007;6:882–94.
[136] Li L, Bjourson AJ, He JY, Cai GX, Rao PF, Shaw C. Bradykinins and their cDNA from piebald odorous frog, *Odorrana schmackeri*, skin. Peptides 2003;24:863–72.
[137] Liang JG, Han YP, Li JX, Xu XQ, Rees HH, Lai R. A novel bradykinin-like peptide from skin secretion of rufous-spotted torrent frog, *Amolops lolensis*. Peptides 2006;27:2683–7.
[138] Liu XH, You DW, Chen LH, Wang X, Zhang KY, Lai R. A novel bradykinin-like peptide from skin secretions of the frog, *Rana nigrovittata*. J Pept Sci 2008;14:626–30.
[139] Liu Y, Feng J, Xiao Y, Guo Z, Zhang J, Xue X, et al. Purification of active bufadienolides from toad skin by preparative reversed-phase liquid chromatography coupled with hydrophilic interaction chromatography. J Sep Sci 2010;33:1487–94.
[140] Lu XY, Ma YF, Wu J, Lai R. Two serine protease inhibitors from the skin secretions of the toad, *Bombina microdeladigitora*. Comp Biochem Physiol B 2008;149:608–12.
[141] Lu Y, Li J, Yu H, Xu X, Liang J, Tian Y, et al. Two families of antimicrobial peptides with multiple functions from skin of rufous-spotted torrent frog, *Amolops loloensis*. Peptides 2006;27:3085–91.
[142] Lu Y, Ma Y, Wang X, Liang J, Zhang C, Zhang K, et al. The first antimicrobial peptide from sea amphibian. Mol Immunol 2008;45:678–81.
[143] Lu ZK, Zhai L, Wang H, Che QL, Wang D, Feng FF, et al. Novel families of antimicrobial peptides with multiple functions from skin of Xizang plateau frog, *Nanorana parkeri*. Biochimie 2010;92:475–81.
[144] Maciel NM, Schwartz CA, Pires OR, Sebben A, Castro MS, Sousa MV, et al. Composition of indolealkylamines of *Bufo rubescens* cutaneous secretions compared to six other Brazilian bufonids with phylogenetic implications. Comp Biochem Physiol B 2003;134:641–9.
[145] Magalhães BS, Melo JAT, Leite JRSA, Silva LP, Prates MV, Vinecky F, et al. Post-secretory events alter the peptide content of the skin secretion of *Hypsiboas raniceps*. Biochem Biophys Res Commun 2008;377:1057–61.
[146] Mangoni ML, Papo N, Mignogna G, Andreu D, Shai Y, Barra D, et al. Ranacyclins, a new family of short cyclic antimicrobial peptides: biological function, mode of action, and parameters involved in target specificity. Biochemistry-US 2003;42:14023–35.
[147] Mangoni ML, Shai Y. Temporins and their synergism against Gram-negative bacteria and in lipopolysaccharide detoxification. BBA-Biomembranes 2009;1788:1610–9.
[148] Marenah L, Flatt PR, Orr DF, McClean S, Shaw C, Abdel-Wahab YHA. Skin secretion of the toad *Bombina variegata* contains multiple insulin-releasing peptides including bombesin and entirely novel insulinotropic structures. Biol Chem 2004;385:315–21.

[149] Marenah L, Flatt PR, Orr DF, Shaw C, Abdel-Wahab YHA. Characterization of naturally occurring peptides in the skin secretion of *Rana pipiens* frog reveal pipinin-1 as the novel insulin-releasing agent. J Pept Res 2005;66:204–10.
[150] Marschal P, Herrmann J, Leffler H, Barondes SH, Cooper DNW. Sequence and specificity of a soluble lactose-binding lectin from *Xenopus laevis* skin. J Biol Chem 1992;267:12942–9.
[151] Maselli V, Bilusich D, Bowie J, Tyler M. Host-defence skin peptides of the Australian Streambank Froglet *Crinia riparia*: isolation and sequence determination by positive and negative ion electrospray mass spectrometry. Rapid Commun Mass Spectrom 2006;20:797–803.
[152] Maselli VM, Brinkworth CS, Bowie JH, Tyler MJ. Host-defence skin peptides of the Australian Common Froglet *Crinia signifera*: sequence determination using positive and negative ion electrospray mass spectra. Rapid Commun Mass Spectrom 2004;18:2155–61.
[153] Mattute B, Knoop FC, Conlon JM. Kassinatuerin-1: a peptide with broad-spectrum antimicrobial activity isolated from the skin of the hyperoliid frog, *Kassina senegalensis*. Biochem Biophys Res Commun 2000;268:433–6.
[154] McCrudden CM, Zhou M, Chen T, O'Rorke M, Walker B, Hirst D, et al. The complex array of bradykinin-related peptides (BRPs) in the peptidome of pickerel frog (*Rana palustris*) skin secretion is the product of transcriptional economy. Peptides 2007;28:1275–81.
[155] McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, et al. Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochem Biophys Res Commun 1979;90:227–33.
[156] Mechkarska M, Ahmed E, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae). Comp Biochem Physiol C 2010;152:467–72.
[157] Mechkarska M, Ahmed E, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Peptidomic analysis of skin secretions demonstrates that the allopatric populations of *Xenopus muelleri* (Pipidae) are not conspecific. Peptides 2011;32:1502–8.
[158] Mechkarska M, Eman A, Coquet L, Jérôme L, Jouenne T, Vaudry H, et al. Genome duplications within the Xenopodinae do not increase the multiplicity of antimicrobial peptides in *Silurana paratropicalis* and *Xenopus andrei* skin secretions. Comp Biochem Phys D 2011;6:206–12.
[159] Melzer S, Bishop PJ. Skin peptide defences of New Zealand frogs against chytridiomycosis. Anim Conserv 2010;13:44–52.
[160] Miele R, Ponti D, Boman HG, Barra D, Simmaco M. Molecular cloning of a bombinin gene from *Bombina orientalis*: detection of NF-[kappa]B and NF-IL6 binding sites in its promoter. FEBS Lett 1998;431:23–8.
[161] Mignogna G, Pascarella S, Wechselberger C, Hinterleitner C, Mollay C, Amiconi G, et al. BSTI, a trypsin inhibitor from skin secretions of *Bombina bombina* related to protease inhibitors of nematodes. Protein Sci 1996;5:357–62.
[162] Mignogna G, Simmaco M, Kreil G, Barra D. Antibacterial and hemolytic peptides containing D-alloisoleucine from the skin of *Bombina variegata*. EMBO J 1993;12:4829–32.
[163] Mizuno K, Ohsuye K, Wada Y, Fuchimura K, Tanaka S, Matsuo H. Cloning and sequence of cDNA encoding a peptide C-terminal α-amidating enzyme from *Xenopus laevis*. Biochem Biophys Res Commun 1987;148:546–52.
[164] Mollay C, Vilas U, Hutticher A, Kreil G. Isolation of a dipeptidyl aminopeptidase, a putative processing enzyme, from skin secretion of *Xenopus laevis*. Eur J Biochem 1986;160:31–5.
[165] Mollay C, Wechselberger C, Mignogna G, Negri L, Melchiorri P, Barra D, et al. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. Eur J Pharmacol 1999;374:189–96.
[166] Mollay C, Wichta J, Kreil G. Detection and partial characterization of an amidating enzyme in skin secretion of *Xenopus laevis*. FEBS Lett 1986;202:251–4.
[167] Montecucchi PC, Decastiglione R, Erspamer V. Identification of dermorphin and Hyp6-dermorphin in skin extracts of the Brazilian frog *Phyllomedusa rhodei*. Int J Pept Protein Res 1981;17:316–21.
[168] Montecucchi PC, Decastiglione R, Piani S, Gozzini L, Erspamer V. Amino acid composition and sequence of dermorphin, a novel opiate-like peptide from the skin of *Phyllomedusa sauvagei*. Int J Pept Protein Res 1981;17:275–83.
[169] Montecucchi PC, Gozzini L, Erspamer V, Melchiorri P. Primary structure of tryptophan-containing peptides from skin extracts of *Phyllomedusa rhodei*. Int J Pept Protein Res 1984;23:276–81.
[170] Montecucchi PC, Henschen A, Erspamer V. Structure of sauvagine, a vasoactive peptide from the skin of a frog. Hoppe-Seyler's Z Physiol Chem 1979;360:1178.
[171] Mor A, Nguyen VH, Delfour A, Miglioresamour D, Nicolas P. Isolation, amino-acid-sequence, and synthesis of Dermaseptin, a novel antimicrobial peptide of amphibian skin. Biochemistry-US 1991;30:8824–30.
[172] Morikawa N, Hagiwara K, Nakajima T. Brevinin-1 and Brevinin-2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*. Biochem Biophys Res Commun 1992;189:184–90.
[173] Nagalla SR, Gibson BW, Tang DZ, Reeve JR, Spindel ER. Gastrin-releasing peptide (GRP) is not mammalian bombesin – identification and molecular-cloning of a true amphibian GRP distinct from amphibian bombesin in *Bombina orientalis*. J Biol Chem 1992;267:6916–22.
[174] Nascimento A, Chapeaurouge A, Perales J, Sebben A, Sousa MV, Fontes W, et al. Purification, characterization and homology analysis of ocellatin 4, a cytolytic peptide from the skin secretion of the frog *Leptodactylus ocellatus*. Toxicon 2007;50:1095–104.
[175] Nascimento ACC, Zanotta LC, Kyaw CM, Schwartz ENF, Schwartz CA, Sebben A, et al. Ocellatins: new antimicrobial peptides from the skin secretion of the South American frog *Leptodactylus ocellatus* (Anura: Leptodactylidae). Protein J 2004;23:501–8.
[176] Negri L, Lattanzi R, Giannini E, Melchiorri P. Bv8/prokineticin proteins and their receptors. Life Sci 2007;81:1103–16.
[177] Negri L, Melchiorri P. Opioid peptides from frog skin and Bv8-related peptides. In: Kastin JA, editor. Handbook of biologically active peptides. 1st ed. Amsterdam: Academic Press; 2006. p. 269–75.
[178] Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu Rev Microbiol 1995;49:277–304.
[179] Nissen-Meyer J, Nes IF. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch Microbiol 1997;167:67–77.
[180] Olson L, Soto AM, Knoop FC, Conlon JM. Pseudin-2: an antimicrobial peptide with low hemolytic activity from the skin of the paradoxical frog. Biochem Biophys Res Commun 2001;288:1001–5.
[181] Otto SP, Whitton J. Polyploid incidence and evolution. Annu Rev Genet 2000;34:401–37.
[182] Park JM, Jung JE, Lee BJ. Antimicrobial peptides from the skin of a Korean Frog, *Rana rugosa*. Biochem Biophys Res Commun 1994;205:948–54.
[183] Park S, Park SH, Ahn HC, Kim S, Kim SS, Lee BJ. Structural study of novel antimicrobial peptides, nigrocins, isolated from *Rana nigromaculata*. FEBS Lett 2001;507:95–100.
[184] Pierre TN, Seon AA, Amiche M, Nicolas P. Phylloxin, a novel peptide antibiotic of the dermaseptin family of antimicrobial/opioid peptide precursors. Eur J Biochem 2000;267:370–8.
[185] Prates MV, Sforca ML, Regis WCB, Leite J, Silva LP, Pertinhez TA, et al. The NMR-derived solution structure of a new cationic antimicrobial peptide from the skin secretion of the anuran *Hyla punctata*. J Biol Chem 2004;279:13018–26.
[186] Probst JC, Gertzen EM, Hoffmann W. An integumentary mucin (FIM-B.1) from *Xenopus laevis* homologous with the von Willebrand factor. Biochemistry-US 1990;29:6240–4.
[187] Pukala TL, Bowie JH, Maselli VM, Musgrave IF, Tyler MJ. Host-defence peptides from the glandular secretions of amphibians: structure and activity. Nat Prod Rep 2006;23:368–93.
[188] Raftery MJ, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. The structures of the frenatin peptides from the skin secretion of the giant tree frog *Litoria infrafrenata*. J Pept Sci 1996;2:117–24.
[189] Rates B, Silva LP, Ireno IC, Leite FSF, Borges MH, Bloch C, et al. Peptidomic dissection of the skin secretion of *Phasmahyla jandaia* (Bokermann and Sazima, 1978) (Anura, Hylidae, Phyllomedusinae). Toxicon 2011;57:35–52.
[190] Ravazzola M, Brown D, Leppaluoto J, Orci L. Localisation by immunofluorescence of thyrotropin-releasing hormone in the cutaneous glands of the frog, *Rana ridibunda*. Life Sci 1979;25:1331–4.
[191] Renda T, Deste L, Buffa R, Usellini L, Capella C, Vaccaro R, et al. Tryptophyllin-like immunoreactivity in rat adenohypophysis. Peptides 1985;6:197–202.
[192] Resnick NM, Maloy WL, Guy HR, Zasloff M. A novel endopeptidase from *Xenopus* that recognizes α-helical secondary structure. Cell 1991;66:541–54.
[193] Richter K, Egger R, Kreil G. D-Alanine in the frog-skin peptide dermorphin is derived from L-alanine in the precursor. Science 1987;238:200–2.
[194] Rodríguez A, Poth D, Schulz S, Vences M. Discovery of skin alkaloids in a miniaturized eleutherodactylid frog from Cuba. Biol Lett-UK 2011;7:414–8.
[195] Roelants K, Fry BG, Norman JA, Clynen E, Schoofs L, Bossuyt F. Identical skin toxins by convergent molecular adaptation in frogs. Curr Biol 2010;20:125–30.
[196] Rollins-Smith LA. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. BBA-Biomembranes 2009;1788:1593–9.
[197] Rollins-Smith LA, King JD, Nielsen PF, Sonnevend A, Conlon JM. An antimicrobial peptide from the skin secretions of the mountain chicken frog *Leptodactylus fallax* (Anura: Leptodactylidae). Regul Pept 2005;124:173–8.
[198] Rollins-Smith LA, Reinert LK, O'Leary CJ, Houston LE, Woodhams DC. Antimicrobial peptide defenses in amphibian skin. Integr Comp Biol 2005;45:137–42.
[199] Roseghini M, Erspamer GF, Severini C, Simmaco M. Biogenic amines and active peptides in extracts of the skin of thirty-two European amphibian species. Comp Biochem Physiol C 1989;94:455–60.
[200] Rosenfeld Y, Barra D, Simmaco M, Shai Y, Mangoni ML. A synergism between temporins toward Gram-negative bacteria overcomes resistance imposed by the lipopolysaccharide protective layer. J Biol Chem 2006;281:28565–74.
[201] Rozek T, Waugh RJ, Steinborner ST, Bowie JH, Tyler MJ, Wallace JC. The maculatin peptides from the skin glands of the tree frog *Litoria genimaculata*: a comparison of the structures and antibacterial activities of maculatin 1.1 and caerin 1.1. J Pept Sci 1998;4:111–5.
[202] Rozek T, Wegener KL, Bowie JH, Olver IN, Carver JA, Wallace JC, et al. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis* – the solution structure of aurein 1 2. Eur J Biochem 2000;267:5330–41.
[203] Sai KP, Jagannadham MV, Vairamani M, Raju NP, Devi AS, Nagaraj R, et al. Tigerinins: novel antimicrobial peptides from the Indian frog *Rana tigerina*. J Biol Chem 2001;276:2701–7.
[204] Salmon AL, Cross LJM, Irvine AE, Lappin TRJ, Dathe M, Krause G, et al. Peptide leucine arginine, a potent immunomodulatory peptide isolated and structurally characterized from the skin of the Northern Leopard frog, *Rana pipiens*. J Biol Chem 2001;276:10145–52.
[205] Samgina TY, Artemenko KA, Gorshkov VA, Ogourtsov SV, Zubarev RA, Lebedev AT. Mass spectrometric study of peptides secreted by the skin glands of

the brown frog *Rana arvalis* from the Moscow region. Rapid Commun Mass Spectrom 2009;23:1241–8.
[206] Satake H, Kawada T, Nomoto K, Minakata H. Insight into tachykinin-related peptides, their receptors, and invertebrate tachykinins: a review. Zool Sci 2003;20:533–49.
[207] Säve-Söderbergh G. Preliminary note on Devonian Stegocephalians from East Greenland. Medd Gron 1932;94:1–107.
[208] Severini C, Improta G. Amphibian tachykinins. In: Kastin JA, editor. Handbook of biologically active peptides. Amsterdam: Academic Press; 2006. p. 261–8.
[209] Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V. The tachykinin peptide family. Pharmacol Rev 2002;54:285–322.
[210] Shaw C, McKay DM, Halton DW, Thim L, Buchanan KD. Isolation and primary structure of an amphibian neurotensin. Regul Pept 1992;38:23–31.
[211] Silva MRE, Beraldo WT, Rosenfeld G. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasmaglobulin by snake venoms and by trypsin. Am J Physiol 1949;156:261–73.
[212] Simmaco M, Barra D, Chiarini F, Noviello L, Melchiorri P, Kreil G, et al. A family of bombinin-related peptides from the skin of *Bombina variegata*. Eur J Biochem 1991;199:217–22.
[213] Simmaco M, Debiase D, Severini C, Aita M, Erspamer GF, Barra D, et al. Purification and characterization of bioactive peptides from skin extracts of *Rana esculenta*. Biochim Biophys Acta 1990;1033:318–23.
[214] Simmaco M, Kreil G, Barra D. Bombinins, antimicrobial peptides from *Bombina* species. BBA-Biomembranes 2009;1788:1551–5.
[215] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us. Biopolymers 1998;47:435–50.
[216] Simmaco M, Mignogna G, Barra D, Bossa F. Novel antimicrobial peptides from skin secretion of the European frog *Rana esculenta*. FEBS Lett 1993;324:159–61.
[217] Simmaco M, Mignogna G, Barra D, Bossa F. Antimicrobial peptides from skin secretions of *Rana esculenta* – molecular cloning of cDNAs encoding and isolation of new active peptides. J Biol Chem 1994;269:11956–61.
[218] Simmaco M, Mignogna G, Canofeni S, Miele R, Mangoni ML, Barra D. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. Eur J Biochem 1996;242:788–92.
[219] Sin YT, Zhou M, Chen W, Wang L, Chen TB, Walker B, et al. Skin bradykinin-related peptides (BRPs) and their biosynthetic precursors (kininogens): comparisons between various taxa of Chinese and North American ranid frogs. Peptides 2008;29:393–403.
[220] Smith BP, Tyler MJ, Kaneko T, Garraffo HM, Spande TF, Daly JW. Evidence for biosynthesis of pseudophrynamine alkaloids by an Australian myobatrachid frog (*Pseudophryne*) and for sequestration of dietary pumiliotoxins. J Nat Prod 2002;65:439–47.
[221] Song GH, Zhou M, Chen W, Chen TB, Walker B, Shaw C. HV-BBI-A novel amphibian skin Bowman-Birk-like trypsin inhibitor. Biochem Biophys Res Commun 2008;372:191–6.
[222] Song YZ, Lu Y, Wang LJ, Yang HL, Zhang KY, Lai R. Purification, characterization and cloning of two novel tigerinin-like peptides from skin secretions of *Fejervarya cancrivora*. Peptides 2009;30:1228–32.
[223] Soravia E, Martini G, Zasloff M. Antimicrobial properties of peptides from *Xenopus* granular gland secretions. FEBS Lett 1988;228:337–40.
[224] Sousa JC, Berto RF, Gois EA, Fontenele-Cardi NC, Honório-Júnior JER, Konno K, et al. Leptoglycin: a new glycine/leucine-rich antimicrobial peptide isolated from the skin secretion of the South American frog *Leptodactylus pentadactylus* (Leptodactylidae). Toxicon 2009;54:23–32.
[225] Steinborner ST, Bowie JH, Tyler MJ, Wallace JC. An unusual combination of peptides from the skin glands of Ewing's tree frog Litoria ewingi. Sequence determination and antimicrobial activity. Aust J Chem 1997;50:889–94.
[226] Steinborner ST, Wabnitz PA, Waugh RJ, Bowie JH, Gao CW, Tyler MJ, et al. The structures of new peptides from the Australian red tree frog '*Litoria rubella*'. The skin peptide profile as a probe for the study of evolutionary trends of amphibians. Aust J Chem 1996;49:955–63.
[227] Stone DJM, Bowie JH, Tyler MJ, Wallace JC. The structure of Caerin 1.1, a novel antibiotic peptide from Australian tree frogs. J Chem Soc Chem Commun 1992:1224–5.
[228] Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs. Structures of the caerins and caeridin 1 from *Litoria splendida*. J Chem Soc Perkin Trans 1 1992;1:3173–8.
[229] Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian Frogs – the structures of the Caerins from *Litoria caerulea*. J Chem Res-S 1993;138:910–36.
[230] Strous GJ, Dekker J. Mucin-type glycoproteins. Crit Rev Biochem Mol 1992;27:57–92.
[231] Suzuki H, Conlon JM, Iwamuro S. Evidence that the genes encoding the melittin-related peptides in the skins of the Japanese frogs *Rana sakuraii* and *Rana tagoi* are not orthologous to bee venom melittin genes: developmental- and tissue-dependent gene expression. Peptides 2007;28:2061–8.
[232] Suzuki H, Iwamuro S, Ohnuma A, Coquet L, Leprince J, Jouenne T, et al. Expression of genes encoding antimicrobial and bradykinin-related peptides in skin of the stream brown frog *Rana sakuraii*. Peptides 2007;28:505–14.
[233] Suzuki S, Ohe Y, Okubo T, Kakegawa T, Tatemoto K. Isolation and characterization of novel antimicrobial peptides, rugosins A, B, and C, from the skin of the frog, *Rana rugosa*. Biochem Biophys Res Commun 1995;212:249–54.
[234] Tazato S, Conlon JM, Iwamuro S. Cloning and expression of genes enocoding antimicrobial peptides and bradykinin from the skin and brain of Oki Tago's brown frog, *Rana tagoi okiensis*. Peptides 2010;31:1480–7.
[235] Tennessen JA. Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. J Evol Biol 2005;18:1387–94.
[236] Thompson AH, Bjourson AJ, Orr DF, Shaw C, McClean S. Amphibian skin secretomics: application of parallel quadrupole time-of-flight mass spectrometry and peptide precursor cDNA cloning to rapidly characterize the skin secretory peptidome of *Phyllomedusa hypochondrialis azurea*: discovery of a novel peptide family, the hyposins. J Proteome Res 2007;6:3604–13.
[237] Thompson AH, Bjourson AJ, Shaw C, McClean S. Bradykinin-related peptides from *Phyllomedusa hypochondrialis azurea*: mass spectrometric structural characterisation and cloning of precursor cDNAs. Rapid Commun Mass Spectrom 2006;20:3780–8.
[238] Tyler MJ, Stone DJM, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. J Pharmacol Toxicol 1992;28:199–200.
[239] Vanhoye D, Bruston F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. Eur J Biochem 2003;270:2068–81.
[240] Vlasak R, Wiborg O, Richter K, Burgschwaiger S, Vuust J, Kreil G. Conserved exon–intron organization in two different caerulein precursor genes of *Xenopus laevis* – additional detection of an exon potentially coding for a new peptide. Eur J Biochem 1987;169:53–8.
[241] Vouille V, Amiche M, Nicolas P. Structure of genes for dermaseptins B, antimicrobial peptides from frog skin-exon 1-encoded prepropeptide is conserved in genes for peptides of highly different structures and activities. FEBS Lett 1997;414:27–32.
[242] Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. Animal behaviour: aquatic sex pheromone from a male tree frog. Nature 1999;401:444–5.
[243] Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;37. D933-D7.
[244] Wang L, Smyth A, Johnsen AH, Zhou M, Chen TB, Walker B, et al. FMRFamide-related peptides (FaRPs): a new family of peptides from amphibian defensive skin secretions. Biochem Biophys Res Commun 2009;383:314–9.
[245] Wang L, Zhou M, Chen TB, Walker B, Shaw C. PdT-2: a novel myotropic Type-2 tryptophyllin from the skin secretion of the Mexican giant leaf frog, *Pachymedusa dacnicolor*. Peptides 2009;30:1557–61.
[246] Wang L, Zhou M, Lynch L, Chen TB, Walker B, Shaw C. *Kassina senegalensis* skin tachykinins: molecular cloning of kassinin and (Thr(2), Ile(9))-kassinin biosynthetic precursor cDNAs and comparative bioactivity of mature tachykinins on the smooth muscle of rat urinary bladder. Biochimie 2009;91:613–9.
[247] Wang L, Zhou M, McGrath S, Chen T, Gorman SP, Walker B, et al. A family of kassinatuerin-2 related peptides from the skin secretion of the African hyperoliid frog, *Kassina maculata*. Peptides 2009;30:1428–33.
[248] Wang T, Zhang J, Shen JH, Jin Y, Lee WH, Zhang Y, Maximins S. a novel group of antimicrobial peptides from toad *Bombina maxima*. Biochem Biophys Res Commun 2005;327:945–51.
[249] Wang X, Song Y, Li J, Liu H, Xu X, Lai R, et al. A new family of antimicrobial peptides from skin secretions of *Rana pleuraden*. Peptides 2007;28:2069–74.
[250] Waugh RJ, Stone DJM, Bowie JH, Wallace JC, Tyler MJ. Peptides from australian frogs – the structures of the caerins and caeridins from *Litoria gilleni*. J Chem Res-S 1993:139.
[251] Wechselberger C. Cloning of cDNAs encoding new peptides of the dermaseptin-family. BBA-Protein Struct Mol Enzym 1998;1388:279–83.
[252] Wegener KL, Brinkworth CS, Bowie JH, Wallace JC, Tyler MJ. Bioactive dahlein peptides from the skin secretions of the Australian aquatic frog *Litoria dahlii*: sequence determination by electrospray mass spectrometry. Rapid Commun Mass Spectrom 2001;15:1726–34.
[253] Wegener KL, Wabnitz PA, Carver JA, Bowie JH, Chia BCS, Wallace JC, et al. Host defence peptides from the skin glands of the Australian Blue Mountains tree-frog *Litoria citropa* – solution structure of the antibacterial peptide citropin 1.1. Eur J Biochem 1999;265:627–37.
[254] Wells KD. The ecology and behaviour of amphibians. Chicago: University of Chicago Press; 2007.
[255] Wong H, Bowie JH, Carver JA. The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian Green Tree Frog, *Litoria splendida*. Eur J Biochem 1997;247:545–57.
[256] Woodhams DC, Rollins-Smith LA, Alford RA, Simon MA, Harris RN. Innate immune defenses of amphibian skin: antimicrobial peptides and more. Anim Conserv 2007;10:425–8.
[257] Xu XQ, Li JX, Han YP, Yang HL, Liang JG, Lu QM, et al. Two antimicrobial peptides from skin secretions of *Rana grahami*. Toxicon 2006;47:459–64.
[258] Yang HL, Wang X, Liu XH, Wu J, Liu CB, Gong WM, et al. Antioxidant peptidomics reveals novel skin antioxidant system. Mol Cell Proteomics 2009;8:571–83.
[259] Yasuhara T, Nakajima T. Occurrence of Pyr-His-Pro-NH2 in the frog skin. Chem Pharm Bull 1975;23:3301–3.
[260] Yasuhara T, Nakajima T, Erspamer V, Falconieri-Erspamer G, Tukamoto Y, Mori M. Isolation and sequential analysis of peptides in *Rana erythraea* skin. In: Kiso Y, editor. Peptide chemistry 1985. Osaka: Protein Research Foundation; 1986. p. 363–8.
[261] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27–55.
[262] Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin – isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 1987;84:5449–53.

[263] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95.
[264] Zhang RW, Zhou M, Wang L, McGrath S, Chen TB, Chen XH, et al. Phylloseptin-1 (PSN-1) from *Phyllomedusa sauvagei* skin secretion: a novel broad-spectrum antimicrobial peptide with antibiofilm activity. Mol Immunol 2010;47:2030–7.
[265] Zhang YX, Wang M, Wei SS. Isolation and characterization of a trypsin inhibitor from the skin secretions of *Kaloula pulchra hainana*. Toxicon 2010;56:502–7.
[266] Zhao Y, Jin Y, Lee WH, Zhang Y. Isolation and preliminary characterization of a 22-kDa protein with trypsin inhibitory activity from toad *Bufo andrewsi* skin. Toxicon 2005;46:277–81.
[267] Zhao Y, Jin Y, Wei SS, Lee WH, Zhang Y. Purification and characterization of an irreversible serine protease inhibitor from skin secretions of *Bufo andrewsi*. Toxicon 2005;46:635–40.
[268] Zhou J, McClean S, Thompson A, Zhang Y, Shaw C, Rao P, et al. Purification and characterization of novel antimicrobial peptides from the skin secretion of *Hylarana guentheri*. Peptides 2006;27:3077–84.
[269] Zhou JW, Bjourson AJ, Coulter DJM, Chen TB, Shaw C, O'Rourke M, et al. Bradykinin-related peptides, including a novel structural variant, (Val(1))-bradykinin, from the skin secretion of Guenther's frog, *Hylarana guentheri* and their molecular precursors. Peptides 2007;28:781–9.
[270] Zhou M, Chen T, Walker B, Shaw C. Lividins: novel antimicrobial peptide homologs from the skin secretion of the Chinese Large Odorous frog, *Rana* (*Odorrana*) *livida* – identification by shotgun cDNA cloning and sequence analysis. Peptides 2006;27:2118–23.
[271] Zhou M, Chen TB, Walker B, Shaw C. Pelophylaxins novel antimicrobial peptide homologs from the skin secretion of the fukien gold-striped pond frog *Pelophylax plancyi fukienensis* – identification by shotgun cDNA cloning and sequence analysis. Peptides 2006;27:36–41.
[272] Zhou M, Liu Y, Chen T, Fang X, Walker B, Shaw C. Components of the peptidome and transcriptome persist in lin wa pi: the dried skin of the Heilongjiang brown frog (*Rana amurensis*) as used in traditional Chinese medicine. Peptides 2006;27:2688–94.
[273] Zhou X, Wang L, Zhou M, Chen T, Ding A, Rao P, et al. Amolopkinins W1 and W2 – novel bradykinin-related peptides (BRPs) from the skin of the Chinese torrent frog, *Amolops wuyiensis*: antagonists of bradykinin-induced smooth muscle contraction of the rat ileum. Peptides 2009;30:893–900.