Rattlesnake venoms consist of a complex mixture of proteins, peptides, carbohydrates, nucleotides, metal ions, and organic acids, but protein/peptide components are generally accepted as the main biologically active components. These specialized toxins are synthesized in secretory epithelial cells, are stably stored under acidic conditions in the presence of low affinity peptide inhibitors, and are spontaneously activated upon injection. Recent advances in genomics, transcriptomics, and especially proteomics, have greatly facilitated the study of venom protein complexity and composition. Many studies have indicated a high level of diversity in venom protein components, with variation in composition existing at the genus, species, population, and even individual levels. However, very recent analyses have suggested that this estimation of vast diversification of components may be somewhat over-estimated, and biologically it is difficult to reconcile why these estimated levels should occur. Furthermore, in the absence of functional data for minor components, which occur at < 0.1%, it is not possible to ascribe biologically relevant roles for many venom protein variants. Here, we highlight the recent advances in our understanding of venom composition and venom evolution, focusing on rattlesnakes. Applications of –omic techniques, together with functional assays to study venom evolution, hold great promise for revealing the sources of variation of venom composition. However, we also stress the caveat that while transcriptomic and proteomic data of venoms has already greatly increased our understanding of compositional trends, caution should be exercised in the interpretation of these data as they pertain to venom evolution.
Introduction to venoms and vipers

Venoms have allowed advanced snakes to utilize a chemical rather than a mechanical means of overcoming fracture and potentially dangerous prey (Kardong, 1980; Kardong et al., 1997). Among the vipers, venoms with rapid-acting effects and component(s) facilitating relocation of prey have allowed the evolution of a strike-and-release mode of predation, further minimizing contact with prey and damage to the snake (Savidis et al., 2013). Among snakes, vipers can also consume the largest prey relative to their own mass (Gren, 1997), introducing potential handling and digestion difficulties, but these appear to be offset in large part by the typically high lytic action of most vipers’ venoms. Venoms clearly have an important trophic role which has influenced the evolution of advanced snakes tremendously, as demonstrated by the prevalence of taxon-specific toxins in several species (Mackessy, 2008; Gibbs and Mackessy, 2009; see Mackessy, 2008). Among species such as Crotalus atrox and Vipera berus, the typically high lytic action of most vipers’ venoms has allowed the evolution of advanced snakes to utilize a chemical rather than a mechanical means of overcoming fracture and potentially dangerous prey (Kardong, 1980; Kardong et al., 1997). Introductions to venoms and vipers

Box 1. Venom – what is it?

For most venomous animals, an inclusive definition of venom is that it is a simple to complex secretion produced in a specialized structure, the venom gland. Reptile venoms consist of 1-10s of individual compounds, commonly referred to as toxins and largely comprised of proteins and peptides. Venom differs from poison because venom must be delivered into tissues via a specialized structure (often hollow, grooved, or bladed maxillary teeth) in order for deleterious effects to occur. Once it is injected, the typically lethal effects occur rapidly, immobilizing prey and essentially eliminating the struggle. Conversely, a poison is typically consumed (or occasionally absorbed) for its effects to occur. Somewhat enigmatically, snake venom can be drunk with impunity, such as serine proteases, phospholipases A2, and three-finger toxins.

Glossary

sequence reads: raw data emerging from sequencing machines are in the form of short (e.g., 100 nucleotides in length) sequencing reads that can be used to estimate a computational reconstruction of a genome or transcriptome, or to measure levels of gene expression.

flow cytometry: a method for measuring the physical characteristics of particles using fluidics and laser technology. Applications to molecular biology include the measurement of cell size and abundance, karyotyping, and genome size estimation.

transcriptomics: the study of RNA produced by a cell or tissue sample at a given time, often focusing on mRNA production exclusively.

differential expression: the observed differences in gene expression often inferred from relative abundances of specific transcripts in transcriptomic datasets or microarrays. Although the genome encodes a myriad of protein-coding genes, expression of these genes (as RNA transcript) is not necessarily constant across tissues, individuals, and time.

neurofunctionalization: the evolution of a new or novel function or biological activity of a gene copy following a gene duplication event. In venoms, this process is believed to be of critical importance to the diversification of toxins within a single protein family.
snake to stab the hollow fangs deep into prey and deliver venom rapidly; the entire strike sequence may last less than 0.5 sec (Kar-dong, 1986).

Venom proteins and other components are synthesized primarily in the secretory cells of the main gland (Figure 2). During a strike, venom within the basal lumen and the ductules is forced out the primary duct and passed through the central duct of an enigmatic structure, the accessory gland. Long hypothesized to be a site of venom activation (e.g., Gennaro et al., 1961; Mackessy, 1991), its role in envenomation is still uncertain. Its cytology is complex, and it is structurally differentiated posterior to anterior (Mackessy, 1991), but it appears to add no significant protein components to the venom bolus expressed from the fang (Mackessy and Baxter, 2006). After leaving the accessory gland, venom passes through a secondary duct, into the hollow fang and out a beveled, hypodermic-like opening into prey tissues.

Storage and stabilization of venom
An enigmatic quality of rattlesnake venoms is that they consist of venom components which should be inherently unstable and are toxic, yet must be available for instantaneous deployment. In particular, metalloproteases and phospholipases could potentially be difficult to contain within the gland lumina, because they could be cytotoxic and or autolytic and could cause damage to the gland epithelium or other venom components. Indeed, purified venom metalloproteases are often difficult to work with because they are frequently autolytic, resulting in a loss of structural domains and activity when in solution (Fu-jimura et al., 2000; Moura-da-Silva et al., 2003; Munekiyo and Mackessy, 2005; Peichoto et al., 2010).

Rattlesnakes and other pitvipers have overcome these potential hazards with a combination of redundant mechanisms which protect the snake (and its venom constituents) from damage but which allow full functionality of venom toxins upon injection. It has long been known that at least some venom metalloproteases are synthesized and exported from gland epithelial cells as inactive zymogens (Grams et al., 1993), but once exported into the lumina, metalloproteases are cleaved into their active forms. The metalloproteases, and other enzymes such as the abundant phospholipase A₂, could result in autolytic hydrolysis of venom constituents and gland damage if not properly regulated. Citrate is an endogenous component of venom and is present in millimolar concentrations (Freitas et al., 1992; Fenton et al., 1995; Odell et al., 1998), which can inhibit PLA₂ and metalloproteases, but the major hemorrhagic metalloprotease of Crotalus oreganus venom was not significantly inhibited by citrate at alkaline pH, even at very high (100 mM) concentrations (Mackessy, 1996). We demonstrated some time ago that rattlesnake venom, even from a species with very high metalloprotease activity (e.g., Crotalus molossus), showed exceptional stability under a wide variety of conditions, including 25-fold dilution and storage at 37° C for 7 days (Munekiyo and Baxter, 2006).
Mackessy, 1998). More recently, we showed that two tripeptide inhibitors, pENW and pEQW, are present in many rattlesnake venoms at significant concentrations, and that these peptides inhibit and stabilize venom metalloproteases (Munekiyo and Mackessy, 2005). Therefore, several different mechanisms contribute to storage and stabilization of venoms.

These regulatory conditions may still not be sufficient for efficient protection of venom constituents and snake tissues, and in an excellent demonstration of redundancy of important biological control mechanisms, exocytosed venom is stored in the gland at an acid pH (~5.5). At this pH, most venom enzymes are inactive, and injection into prey tissue (pH ~7.4) results in spontaneous activation. A specific cell type, the mitochondria-rich cell (Figure 3), is responsible for acidifying the venom, and it is hypothesized that these cells store citrate/citric acid (as a buffer) to maintain a pH of 5.5 in the gland lumina (Mackessy and Baxter, 2006). Perhaps telling, at least one species of Bothrops and an Old World viper (Cerastes spp.) also are capable of producing venom at pH 5.5. One can thus envisage a stabilized storage environment which is rapidly reversed following injection: peptide-inhibited venom is injected into a slightly basic milieu, with a concomitant change in pH favoring activity of enzymes, and the low affinity peptide inhibitors dissociate, further activating the venom components. The venom bolus (Figure 4) then diffuses into prey tissues, promoting dysregulation of many systems simultaneously and rapid incapacitation of prey.

Historical approaches to studying venom composition

The history of the study of animal venoms and toxicity in general has largely paralleled advances in protein chemistry. In fact, laboratory research in enzymology has frequently utilized venom-derived enzymes, including L-amino acid oxidase (Zeller, 1944; Tan and Fung, 2010), which gives many venoms their characteristic yellow color, venom exonuclease, and phosphodiesterase (Laskowski, 1980; Mackessy, 1998). As protein chemistry became more sophisticated and analytical, these refinements were turned toward the study of venoms, themselves consisting largely of proteins and peptides. Throughout much of the 1960s and 1970s, most of the advancement in understanding of venom complexity and action was based on isolation and characterization of specific components, and this is a tradition which is still successfully used today. For example, general venom composition can be compared between species or even individuals using various techniques, including SDS-PAGE. When overlain with known protein families, this provides a rapid "molecular fingerprint" comparison of the major proteins present in venom (Figure 5).

Figure 3. Mitochondria-rich cells of the main gland of Crotalus angus. a) TEM, unextracted gland. b) TEM, extracted gland. c) A scanning electron micrograph of cell surface – note that these cells are recessed below the level of secretory cells. d) A drawing of parietal cell of the vertebrate gastric pit (top) and the mitochondria-rich cell of rattlesnake main venom glands, note the highly similar morphology. Reproduced from Mackessy and Baxter (2001).

Figure 4. Hypothetical sequence of events following venom bolus deposition in prey. Reproduced from Mackessy (2008).

Figure 5. Molecular fingerprint of the venoms of taxa from the Western Rattlesnake clade (see Davis, volume 1, Western Complex) using SDS-PAGE under reducing conditions. The protein family (typical activity) of bands with characteristic masses is given on the left, and approximate masses (in kilodaltons) are given on the right. Band intensity is proportional to concentration of a venom; note that for some taxa (C. viridis, C. helleri caliginis, C. abyssus, C. lutosus), both the PIII and PI metalloprotease bands are absent or nearly absent. The following Crotalus species have highly toxic venoms with negligible metalloprotease activity: Ce, C. cerberus; Or, C. oreganus; Vi, C. viridis; Ca, C. helleri caliginis; He, C. helleri; Co, C. concolor; Nu, C. v. nuntius; Ab, C. abyssus; Lu, C. lutosus; Mr, molecular mass standards. Reproduced from Mackessy (2010a).
With advances in molecular biology and our understanding of the genetic underpinnings of cell structure and function, the rapid technical advances in molecular genetics, and more recently genomics, have contributed to replace more classical protein chemistry-based approaches to studying venoms. Obtaining protein sequences of toxins, once laborious, slow and quite costly, can now be provided indirectly by a growing number of PCR-based DNA sequencing techniques which allows amino acid sequences to be inferred from DNA sequences, as a small fraction of the time and cost compared to protein sequencing. In addition, many labs conducted and continue to conduct de-tailed analyses of specific venom components isolated from the venom of a single species. These studies allowed for structure/ function analyses of proteins that demonstrated that specific structural changes can result in profound differences in biological activities.

Increasing representation of toxins in DNA and protein sequence databases permitted the generation of hypotheses concerning venom evolution, and the selective pressures which might have favored particular patterns of protein sequences of venoms. However, it is now clear that many factors affect the final composition of proteins, including venoms, produced in many types of tissues. Technological advances in mass spectrometry helped usher in a new era of protein chemistry, now in many types of tissues. Technological advances in mass spectrometry have provided massive increases in the size and comprehensiveness of public databases such as those housed at the NCBI (http://www.ncbi.nlm.nih.gov/). Concomitant advances in bioinformatics also paved the way for massive increases in information availability, and currently the utilization of genomic and proteomic techniques for studying venoms has become routine in many labs.

Proteomes of snake venoms

Proteomics as a discipline aims to catalog and describe the entire protein expression profile of a given tissue type or subtype (Anderson and Anderson, 1998). In an ideal scenario, the entire protein expression potential can be described for a given tissue or cell type. A proteomic approach toward venom analysis began rather modestly, but by the mid-2000s, sequence databases were sufficiently robust to make proteomic analysis of venoms a productive method of obtaining a full description of the venom proteins characteristic of a particular species. Venomics, or the analysis of the venom proteome, was introduced in 2004 in the laboratory of Dr. Juan J. Calvete in Valencia, Spain (Juárez et al., 2004; Box 2), and this approach has been adopted by toxicologists worldwide.

Box 2. Venomics – the venom proteome

Although numerous investigators had been using proteomic methods to investigate venom complexity, the term “venomics” was introduced in a seminal paper by Paula Juárez and colleagues (Juárez et al., 2004), which established a standard- ized approach for analyzing venom complexity. In this method, crude venom is fractionated using reversed phase high pressure liquid chromatography followed by further fractionation of proteins using SDS-PAGE. A portion of the HPLC-fractionated proteins are subjected to mass spectrometric analysis (ESI-LC-MS, etc.) to determine native protein mass. The proteins separated via SDS-PAGE are then cut from the gel and digested with trypsin, producing small peptide fragments which are amenable to analysis via MALDI-TOF-MS (peptide mass fingerprinting). Peptides may also be subjected to nanospray-ionization mass spectrometry techniques to produce amino acid sequence-dependent peptide ion spectra. The highly accurate mass estimates of peptides (or fragmented peptides) are then subjected to analysis via database searches of mass spectra using, for example, Mascot. Peptide mass identification of matching sequences (or CID-derived sequences) is then subjected to BLAST searches for protein homology and identification. This method has many advantages, including providing a high-throughput platform which is amenable to multiple sample analyses, and when it is conducted thoroughly, the entire protein complement of venom (the venom proteome) can be deduced.

Varieties of proteomic approaches

Various proteomic approaches have been utilized in the study of venoms, depending on the types of questions to be answered and the equipment available, but two common methods involve liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS) and a solid phase method, matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS, or simply MALDI). Both of these methods have the potential to provide exceptionally high accuracy mass determinations of parent molecules and tryptic-based digests of proteins, and LC-MS has the advantage of coupling liquid chromatographic fractionation to real-time mass analysis of proteins, thereby simplifying the complex mixtures typically observed in venoms. Both techniques can also be coupled to a second round of mass spectrometry (MS/MS) which is used in protein identification via the analysis of discrete mass peptides and de novo peptide sequencing.

Advantages of venomics

Venomics has allowed for venoms from a large number of species to be analyzed deeply and rapidly, and there is a growing body of literature on this topic. As more species are analyzed using venomic and transcriptomic (see below) methods, a more complete comparison of venoms across taxa, such as rattlesnakes, is becoming possible.

A distinct advantage of mass spectrometry is that small to exceptionally tiny amounts of material (venom) are required for detailed analyses, a complete venomic analysis can be completed...
Disadvantages of proteomic approaches alone

A proteomic approach to venom analysis has many advantages, but there are potential downsides to these methods. Although this has improved somewhat in recent years, in comparison to transcriptomic approaches that may measure levels of abundance accurately over 7-10 orders of magnitude, even the most high-precision proteomic approaches are capable of measuring protein concentrations within only 2-3 orders of magnitude. Less abundant and rare proteins may be missed or over-represent in representations of the venom proteome, depending on how these subsamples are prepared and analyzed. As noted above, most mass spectrometric-based methods are dependent upon a robust database of protein/DNA sequences in order to allow efficient peptide mass fingerprinting (high accuracy mass-based matching of specific peptide fragments) and subsequent protein identification. A detailed proteomic analysis of a venom provides a catalog of venom proteins, but alone, it does not reveal pharmacology/biological activity of specific constituents. Because many (probably most) venom protein families have diversified via gene duplication followed by neo-functionalization (Dolby et al., 2009; Brunt et al., 2013), very similar proteins can have vastly different pharmacological effects, as demonstrated in rattlesnake venoms (Casewell et al., 2013), very similar proteins can have vastly different pharmacological effects, as demonstrated in rattlesnake venoms (Casewell et al., 2013). Such gene duplication allows for the evolution of functions that are specific to venom systems while not interfering with the ancestral gene function, thus allowing a new generation of toxins that can evolve in the study of rattlesnake venoms

Transcriptomics and genomics in the study of rattlesnake venoms

Major advances in DNA sequencing technology over the past several years have radically increased our ability to collect information rapidly and economically about the genome sequence of an organism, to sequence genes that are expressed (or “turned on”) in a given tissue, and to measure the levels that different genes are being expressed at any given time, in any given tissue and individual. The use of high-throughput technologies for genomic, transcriptomic, and proteomic analysis has indeed greatly improved our understanding of rattlesnake venoms, making venomomics a valid tool for the study of these systems (Casewell et al., 2012). Such gene duplication allows for the evolution of functions that are specific to venom systems while not interfering with the ancestral gene function, which allows a new generation of toxins that can evolve in the study of rattlesnake venoms.

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comparisons of venom composition between individuals and species emerge. In turn, as above, these advances will allow much deeper knowledge of toxins present. As with proteomic methods, these approaches may be notably expanded in rattlesnakes and particularly in C. adamanteus. They also identified an additional 2,879 unique full length ‘non-toxin’ transcripts.

Advantages of transcriptomic and genomic approaches alone

As we have outlined (Box 3), there are a number of challenges in accurately reconstituting venom genes and venomous snake genomes from current next-generation sequencing approaches. All of these challenges are directly related to the problem of trying to reconstruct entities (genes, transcripts, genomes) that contain highly repetitive and highly variable sequences. Because many venom genes families in snakes have experienced relatively recent duplication, these multiple similar copies can be very difficult to discern from one another with the relatively short sequencing reads currently provided by next-generation sequencing. Similary, accurately reconstructing venom transcripts is also difficult, and provides an exhaustive survey of two types of information at once – the sequences of transcripts (from which one may infer the sequences of the encoded proteins) and also the relative levels of different transcripts, which is presumed to largely represent the levels of the protein products of these transcripts.

Disadvantages of transcriptomic and genomic approaches alone

As with proteomic, these methods, these approaches favor a high throughput approach, and as bioinformatics tools capable of handling and processing the huge data streams generated become more efficient and sophisticated, a greater understanding of the heritable genetic material that encodes all aspects of venom systems, including the genes and alleles encoding venom proteins, and the information for directing how, when, and where these genes are transcribed. Transcriptomics has become economical and provides an exhaustive survey of two types of information at once – the sequences of transcripts (from which one may infer the sequences of the encoded proteins) and also the relative levels of different transcripts, which is presumed to largely represent the levels of the protein products of these transcripts.

Box 3. Venom genes and venomous snake genomes are difficult to study

A major challenge posed by current next-generation sequencing technologies is that, while they can produce massive amounts of data, these data come in the form of relatively short fragments or reads, with an upper limit typically between 100-500 bases in length. Rattlesnake genomes are approximately two billion bases in length, most single genes (including protein-coding exons and non-coding introns) may span 10,000 bases or more (Castoe et al., 2015), and even the processed protein-encoding transcripts that have introns removed average around 1,500 bases in length. The challenge in accurately interpreting information from many relatively short reads to make inferences about much longer transcripts, genes, and genomes. Piecing these short reads together accurately is somewhat straightforward if all small pieces of larger genes or genomes are unique and fit together into a perfect puzzle in one way. This, however, is certainly not the case in vertebrate genomes, or in the case of venom genes, in particular. Vertebrate genomes, including snake genomes, may be compiled by 50% or more repeat elements (Castoe et al., 2011, 2013). Venom genes are duplicated in tandem and may occur in many copies (Voink et al., 2013). Therefore, estimating how to put the pieces (i.e., short reads) together into an accurate estimate of venom genes, or genome, is very difficult, and sometimes almost impossible.

Hopefully, in the not-too-distant future, there will be multiple rattlesnake genome references available that may serve as a baseline-reference for interpreting various types of data, including the linking of transcript information to genes, and broadening our understanding of venom gene copy number and allelic variation in the genomes of rattlesnakes. Such resources will immensely increase the ability of researchers to leverage fully various next-generation sequencing approaches for addressing major outstanding questions about rattlesnake venom variation and evolution.

tonic studies is that it typically requires destructive sampling (i.e., removal of the venom gland typically requiring euthanizing the animal), so replicated sampling of the same individual is not possible. Another limitation in using transcriptomic approaches to study venom variation, especially when trying to infer the number of genes and alleles present in an individual snake, is that transcripts are only detected when the gene is “turned on.” Thus, transcriptomic approaches will not detect venom genes present in the genome that are not currently “turned on” at the time the individual was sampled. Lastly, transcripts only show which genes are currently being expressed in the gland, not which proteins are actually made from these transcripts and at what levels and degrees of activity. Mechanisms such as post-transcriptional regulation by small RNAs, and post-translational modifications that
may change protein function, are not captured by transcriptomic approaches, although they may indeed strongly influence the abundance and function of the protein components of venom. In a recent venom transcriptome, a combination of some of these potential limitations of transcriptomic approaches, Nicholsa Casewell and team (Casewell et al., 2014) found that the percentage of venom-toxin-related genomic loci transcribed in the venom gland ranged from 44.12 to 70.15% across six viperid species, whereas the percentage of those genomic loci being translated into secreted venom toxins ranged from 35.29 to 52.88% (Casewell et al., 2014). This example demonstrates that, indeed, transcriptomic approaches may give limited insight into the full capacity of the genome of an individual or species to encode and produce venom proteins.

Some time ago, it became clear that there was an apparent disconnect between the transcriptome of a venom gland and the proteome of the same venom. For example, a transcriptome analysis of the venom gland of the Desert Massasauga (Sistrurus edwardsii) revealed the presence of low-abundance transcripts coding for three-finger toxins, small toxic proteins which are characteristic of elapid venoms but are absent from viperid venoms (Pahati et al., 2007). However, a proteomic study of venom from the same metapopulation of Sistrurus edwardsii did not reveal 3FTxs in the expressed venom (Sanz et al., 2006), and subsequent searching of many individual venom samples from the same population also failed to show 3FTxs in the expressed venom (Sanz et al., 2006), of venom from the same metapopulation of Sistrurus edwardsii.
om toxins” solely based on evidence for venom gland (or other oral gland) expression and homology to known venom proteins, and against the adaptive and functional relevance of simply observing such transcripts in a given venom-system tissue. These examples indicate the need for a critical re-evaluation of the criteria required to consider a protein a “venom toxin,” and suggest the importance of incorporating more direct evidence for the toxicity or function of venom gland-expressed proteins in prey handling.

The evolution of rattlesnake venoms

The origin of venom and venom delivery systems in reptiles has been hotly debated, but it is clear that venomous forms have been present for a very long time. For example, grooved teeth consistent with a venom delivery system similar to the lizard genus Heloderma (i.e., anterior teeth of the lower jaw, associated in Heloderma with a mandibular venom gland) were described from late Triassic fossil remains dating ~230 million years-before-present (Sues, 1991). Helodermatid fossils of the Cretaceous (~98 million-years-old) also possessed grooved lower teeth (Nydam, 2000), further indicating an early origin of venoms in some squamates. More recently, a single origin of venoms among squamate reptiles was postulated (Fry et al., 2006), and all “venomous” squamates were designated as members of the clade Toxicofera (Fry et al., 2006, 2013). However, there have been numerous objections to this hypothesis (e.g., Gauthier et al., 2012; Losos et al., 2012), in addition to the limitations inherent in drawing conclusions based on transcriptomic data as we have already noted. In spite of these ongoing debates, while venomous reptile lineages undoubtedly have ancient origins, highly toxic venoms and diversification of venom delivery systems most likely evolved independently in several discrete snake lineages (Hargreaves et al., 2014).

Viperid evolutionary origins are considerably more recent, and the oldest known fossils are dated at ~23 million years old, while molecular estimates of divergence times indicate an age of 63 million years ago (Vidal et al., 2009). Rattlesnakes are younger still, with estimated origins of ~10 million years ago (Parmley and Holman, 2007; Cann et al., 2009). The remainder of this section will examine major trends in the evolution of venoms among rattlesnakes, but occasional references will be made to other lineages, as trends seen among rattlesnakes are also reflected in broad venom compositional trends among snake lineages as well. For example, many vipers produce highly enzymatic, tissue-degrading venoms (type I), while many elapids produce venoms rich in post-synaptic neurotoxins which are exceptionally lethal (type II). This generalization holds for many species, and the exceptions are often most informative for answering questions concerning local adaptation and the functional significance of snake venom compositional variation.

Rattlesnake venoms are among the most complex of the snake venoms with regard to number of individual venom components (Table 3), and many species show 100+ protein spots when analyzed on 2-dimensional gel electrophoresis. This complexity results in large part from gene duplication events followed by

<table>
<thead>
<tr>
<th>Component name</th>
<th>Approximate mass (kDa)</th>
<th>Function</th>
<th>Biological activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase</td>
<td>73</td>
<td>Hydrolysis of intercellular hyaluronan</td>
<td>Decreased intercellular viscosity – diffusion of venom components</td>
<td>Tsu and Kuida (2001)</td>
</tr>
<tr>
<td>Lysosomal acid esterase (homologous)</td>
<td>85 – 150</td>
<td>Oxidative deamination of L-lysine acid</td>
<td>Induction of apoptosis, cell death</td>
<td>Tan (1996)</td>
</tr>
<tr>
<td>Snake Venom Metalloproteinase (M2 Matrix Proteases)</td>
<td>48 – 85</td>
<td>Hydrolysis of structural proteins, including tissue plasminogen activator, fibronectin, etc.</td>
<td>Neutrophilic digestion, proteolytic digestion</td>
<td>Fox and Semenza (2000)</td>
</tr>
<tr>
<td>Anginease</td>
<td>25 – 36</td>
<td>Neutrophilic digestion, proteolytic digestion</td>
<td>Neutrophilic digestion of protein</td>
<td>Schwartz and Biber (1965)</td>
</tr>
<tr>
<td>Phospholipase A2 enzymes (Group II)</td>
<td>13 – 15</td>
<td>CO2-dependent hydrolysis of 2-acetyl groups in 3-phospholipids</td>
<td>Hypersensitivity, myonecrosis, rapid membrane damage</td>
<td>Kirk (1997), 2002</td>
</tr>
</tbody>
</table>

*Mass in kilodaltons (kDa). Note that this list is not all-inclusive and that masses, functions and activities do not apply to all compounds isolated from all rattlesnake venoms. Specific rattlesnake venoms may not contain all components. (?) – indicates hypothetical function and/or activity. Reproduced from Mackney (2010b); see this paper for references.
neo-functionalization of the duplicated genes; once freed from functional constraints favoring the original activity, the new gene may show mutations in (typically) any non-structural residues. Some of these result in changing specificity pocket configurations (as in serine proteases, common in rattlesnake venoms) such that binding with previous specific ligands no longer occurs, and new substrates are now recognized (Doley et al., 2009; Mackessy, 2010c). These mutations can occur via accelerated segment switch in exons to alter targeting (ASSET) and/or accumulated point mutations.

A major question moving forward is what factors are most important in generating the extensive complexity and functional diversity of venom toxins: having multiple gene copies of venom genes within a gene family, allelic variation, alternative transcription, epigenetic regulation of venom gene expression, or post-translational modifications of venom proteins? Recent work on Old World vipers by Casewell et al. (2014) demonstrates that, indeed, multiple levels of regulation are responsible for generating variation in venom composition between related snake species. This includes differential levels of toxin transcription, translation, and their posttranslational modification, which may all play a deterministic role in the final protein composition of viperid venoms. Perhaps most importantly, the ways in which these different hierarchical modes of regulation modulate venom content and function is not the same across different venom protein families and members of such families. Thus, it seems that the answer to this grand question is quite complex, in that multiple hierarchical mechanisms act together in a diversity of ways to produce the sequence and functional diversity of venom components. These interacting processes also likely contribute to variation in both the toxicity of venoms and the symptoms of snakebite (Casewell et al., 2014).

Evolutionary trends among rattlesnake venoms

Venoms are presumed to have evolved primarily as a mechanism for facilitating prey handling, and as venomous snakes diversified, demands on venoms which helped immobilize fractious prey similarly expanded. Many factors influence venom composition (cf. Mackessy, 2010a), and various resistance mechanisms among prey are assumed to be one factor favoring diversification of the venom proteome. Diet, therefore, should play a major role influencing venom composition, and numerous studies have shown a correlation between diet and the venom proteome (Mackessy, 1988, 1993; Daltry et al., 1996; Mackessy et al., 2003; Barlow et al., 2009). Many rattlesnake species, like other vipers, show age-dependent changes in diet, often favoring lizards, insects, and arthropods as neonates and then switching to mammals as they approach adulthood (Klauber, 1956). Among the diet-related factors influencing the venom proteome, venom ontogeny results in an “adjustment” of composition which is concomitant with dietary changes and which appears to maximize venom effectiveness toward a shift in prey type (Mackessy, 1988). Neonate venoms are more toxic toward prey, but adult venoms contain much greater amounts of metalloproteases, and this ontogenetic shift is associated with a shift to mammalian prey. Initially described in the Southern Pacific Rattlesnake (Crotalus helleri) and Northern Pacific Rattlesnake (Crotalus oreganus), this pattern of venom ontogeny also is characteristic of Crotalus atrox, Crotalus horridus, Crotalus pyrrhus, Crotalus ruber, Crotalus simus and quite pos-

Table 3 Continued

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<thead>
<tr>
<th>Component name</th>
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<th>Function</th>
<th>Biological activity</th>
<th>References</th>
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<tbody>
<tr>
<td>Non-enzymatic proteins/peptides</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich secretory proteins (CRPs)/prekagons</td>
<td>21-29</td>
<td>Possibly block cNTP-gated channels</td>
<td>Induced hypothermia, prey immobilization (1)</td>
<td>Yanagisaki and Morita (2004)</td>
</tr>
<tr>
<td>Nerve growth factors</td>
<td>14-32.5</td>
<td>Promote nerve fiber growth</td>
<td>Unknown, apoptosis (1)</td>
<td>Squires et al. (1987) Kohl et al. (1991)</td>
</tr>
<tr>
<td>PLF2-based proenvenom toxins (2 subunits, acidic and basic)</td>
<td>24</td>
<td>Blocks release of acetylcholine from axon terminus</td>
<td>Potent neurotoxicity, prey immobilization</td>
<td>Ardil et al. (1989) Dacosta et al. (1986) Farrow et al. (1994)</td>
</tr>
<tr>
<td>C-type lectins</td>
<td>27-29</td>
<td>Binds to platelet &amp; collagen receptor</td>
<td>Anticoagulant, platelet modulator</td>
<td>Leitner and Baint (1988)</td>
</tr>
<tr>
<td>Disintegrins</td>
<td>5.2-15</td>
<td>Inhibits binding of integrins to receptors</td>
<td>Plasmin inhibition, prosomatogenic hemorrhage</td>
<td>Calvert et al. (2005)</td>
</tr>
<tr>
<td>Small peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinin-potentiating peptides</td>
<td>1.6-1.5</td>
<td>Increases potency of bradykinin</td>
<td>Pain, hypotension, prey immobilization</td>
<td>Wermelinger et al. (2001)</td>
</tr>
<tr>
<td>Triptopeptide inhibitors</td>
<td>0.44-0.45</td>
<td>Inhibits venom metalloproteinases and other enzymes</td>
<td>Stabilization of venom components</td>
<td>Francis and Kaisar (1983) Munirpollo and Mackessy (2005)</td>
</tr>
<tr>
<td>Small protein-coupled compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purines &amp; purine nucleosides</td>
<td>AMP, 0.54G1</td>
<td>Hypo/excitatory, reverse</td>
<td>Stabilization of venom components</td>
<td>Freilas et al. (1982) Francis et al. (1982)</td>
</tr>
</tbody>
</table>

Note: Table 3 Continued.
likely other large-bodied Crotalus species. A shift toward dependence on mammalian prey has also been hypothesized as a major driving force in the evolution of Sistrurus venom (Figure 7), and differences in toxicity of venoms toward mice (S. catenatus vs. S. milius) can be extreme (Gibbs and Mackessy, 2009).

Like other snake venoms, rattlesnake venoms tend to show compositional similarities among species which are more closely related (see Figure 5). However, an analysis of C. occidentalis venom (Mackessy et al., 2003) revealed a striking difference from other related Western Group species, as this taxon as an adult produces venom with juvenile-like properties (high toxicity, very low metalloprotease activity). This example is the first description of venom paedomorphosis, which likely occurs in several other species. A comparative analysis of many different rattlesnake venoms suggests that Crotalus durissus terrificus, Crotalus m. mitchelli, Crotalus horridus “attenuatus”, Crotalus tigris, and several populations of Crotalus l polesius clarkii and Crotalus scutulatus may also show venom paedomorphosis (Mackessy, 2010a). This study also established a basic dichotomy in venom composition seen most prominently among rattlesnakes, but also present among many species world-wide, of type I versus type II venoms. As illustrated in Figure 8, type I venoms (adult snakes) contain higher levels of metalloproteases but are less toxic (LD₅₀ > 1.0 µg/g mouse); type II venoms show the opposite (high toxicity, low-no metalloprotease activity). This dichotomy is also apparent in Fig 5; whereas most taxa show prominent PII and PI metalloprotease bands (type I venoms), these are conspicuously absent from C. concolor venom (type II). Venom paedomorphosis was also invoked to explain the trend toward increasing venom neurotoxicity in the C. concolor clade (Calvete et al., 2010). Venom paedomorphosis, which likely occurs in several other species. A phylogenetic hypothesis of Western rattlesnakes (Buhlman and de Queiroz, 2001); note that for C. oreganus, type I venom is a presumed ancestral trait (but see Davis et al., 2016). Reproduced from Mackessy (2010a).

Figure 7. Differences in diet and venom toxicity in the rattlesnake genus Sistrurus. Note that mammals make up the majority of the diet of S. catenatus and S. atrox but are only minor parts of the diets of S. t. atrox and S. milius barbouri. General trends in venom evolution in Sistrurus tend to follow an increasing dependence on mammalian prey. Reproduced from Gibbs and Mackessy (2009).

![Snake diet](image)

**Figure 8.** Venom compositional trends in the Western Rattlesnake clade. a) Metalloprotease activity (responsible for tissue necrosis/predigestion) is high in C. atrox clade and nearly absent in C. concolor clade. See figure 5 for abbreviations. b) Relationship of metalloprotease activity and lethal toxicity. Crotalus atrox shows type I venom, while C. oreganus shows type II venom. 5 A phylogenetic hypothesis of Western rattlesnakes (Buhlman and de Queiroz, 2001); note that for C. oreganus, type I venom is a presumed ancestral trait (but see Davis et al., 2016). Reproduced from Mackessy (2010a).

Venom proteomes (Masey et al., 2012). However, the basic dichotomy of type I (population B) and type II (population A) still holds for the extremes; in fact, C. scutulatus likely represents an important model species for understanding the molecular mechanisms regulating type I and type II differences, as well as for identifying potential geographically regionalized factors leading to local adaptation of these venom types. Adding to the complexity, type II venoms often appear in one taxon of a clade and not others (as in C. concolor), and even in some populations of a single taxon but not others (Rael et al., 1992; French et al., 2004), but no phylogenetic pattern is yet apparent. A key protein differentiating type I and II venoms is crotoxin and its homologs (Meijure toxin, concolor toxin, etc.). These toxins are highly homologous and consist of two protein chains held together by electrostatic and hydrophobic forces; both the A and B subunits are rich in structural motifs common to the cytolytic (the native form of the C. oreganus toxin) or the toxic (the bleached form) enzyme. Both the A and B subunits are rich in structural motifs common to the cytolytic (the native form of the C. oreganus toxin) or the toxic (the bleached form) enzyme.

![Venom proteome](image)

<table>
<thead>
<tr>
<th>Species</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. c. crotalus</td>
<td>0.23</td>
<td>0.70</td>
</tr>
<tr>
<td>S. c. tremulus</td>
<td>1.48</td>
<td>1.41</td>
</tr>
<tr>
<td>S. c. rutulus</td>
<td>0.60</td>
<td>0.39</td>
</tr>
<tr>
<td>S. m. barbouri</td>
<td>7.19</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Both protein chains are required for potent presynaptic toxicity: the A chain acts as a “chaperone”, helping to direct the toxin to the presynaptic axonal membranes, but the B chain contains the core biological activity. The toxin inhibits acetylcholine release from axonal vesicles, resulting in flaccid paralysis and rapid death. It is the presence of crotoxin homologs in type II venoms which is responsible for the high lethal toxicity of these venoms. Whereas other venomous snake clades (Elapidae, Colubridae) contain species that produce venoms rich in post-synaptic nicotinic receptors (a specific type of three-finger toxin), potent rattlesnake venoms (and venoms of other vipers) contain PLA₂-based protryptic toxins, often at very high concentrations.
An interesting question arising from a consideration of these venom types is, "Which one represents the ancestral condition and which is derived?" Based on venom ontogeny, one might predict that type II venoms are typical of the ancestral condition, but the phylogeny of the Western Group (which includes *C. striatus*) suggests otherwise. In this clade, *C. cerberus*, the basal taxon of the Western Group (see Davis, volume 1, Western Complex) and sister to the remaining taxa of the clade, shows a type I venom, while *C. orinocensis* (a close relative) shows type II venom (Figure 8). Another hypothesis is that type II venoms represent a phenotype of newly colonizing populations (C. Lieb group, 2008).

As more detailed genetic and genomic information concerning regulation of protein expression and post-transcriptional and post-translational processing of transcripts and proteins becomes available, a more satisfactory answer to this question may emerge (cf. Casewell et al., 2014).

While the occurrence of potent neurotoxins or debilitating enzyme toxins in snake venoms makes complete intuitive sense, the presence of other proteins with low or no obvious toxicity in venoms are harder to reconcile with a clear role in feeding. For example, L-amino acid oxidase is present in many venoms, but it is not particularly toxic; a more recent consideration of its activity has demonstrated that LAAOs show apoptotic activity toward a variety of cell types, including immortal (cancer) cell lines (e.g., Lee et al., 2014). Similarly, cysteine-rich secretory proteins (CRiSPs) are found in most venoms, and in colubrid snake venoms as major components (Mackessy, 2002), but though they are hypothesized to play a role in disruption of prey homeostasis (Schnagar et al., 2012), they often show little to no discernible effects on a diversity of prey species. Other proteins, such as natriuretic peptides, C-type lectins, and disintegrins, are also found in many venoms, but a role in prey killing and handling is not established and is unclear at best. However, disintegrins are implicated in tracking down envenomated prey (Saviola et al., 2013).

Pitvipers, and rattlesnakes in particular, are sit-and-wait predators that typically utilize a strike and release pattern of envenomation (see Clark, this volume, Hunting and Feeding Behavior). This mode of chemical predation allows dispatching of prey with minimal contact by the snake, and it largely avoids retaliatory reactions from fractions prey; however, it requires a mechanism of prey relocation, as envenomated prey may travel some distance from the strike site. The exquisite capacity for rattlesnakes to relocate envenomated prey, and to discriminate envenomated prey from non-envenomated animals, was extensively documented over many years in the lab of D. Chiszar and colleagues (e.g., Chiszar et al., 1999, 2008), but the specific protein(s) associated with prey relocation and discrimination remained unknown. Using size exclusion chromatographic fractionation of venom from the Western Diamond-backed Rattlesnake (*Crotalus atrox*) and Chiszar’s bioassay for prey preference, the “relocator protein” was demonstrated to be a disintegrin (crotatroxin), not a toxic enzyme or specific toxin (Saviola et al., 2013). In this example, the biological role of the venom component (disintegrin) which is most relevant is not overt toxicity or due to its pharmacological function as a disruptor of platelet aggregation and cell-cell interactions, but instead due to its trophic role in facilitating strike concentrations (Figure 9). An interesting corollary is that if a venom has high levels of crotoxin homologs, then it does not contain high metalloprotease activity. The opposite condition, type I venom, is characteristic of many species such as *C. atrox*, and the venom proteome of these two types is dominated by the different protein families (Fig. 9).
and release predation. As a colleague once said, we find only what we are looking for – in the case of venomous snakes and their venoms, what appears to be an obvious bias necessity may not be closely related to what its actual central biological role (for the snake) may be. Disintegrins are known to occur only in the venoms of vipers, and this group of snakes shows one of the most advanced modes of predation among vertebrates. No other snakes utilize a strike and release mode of predation, and the presence of free disintegrins in viper venoms likely facilitated the evolution of this trophic adaptation.

Toxins to drugs

The a priori use of venoms or toxins as therapeutics seems counter-intuitive – venoms evolved primarily as an adaptation which facilitates handling of fractious prey, and envenomation often facilitates handling of fractious prey, and envenomation often results in a systemic collapse in the prey. When one considers that the use of toxins or venoms as therapeutics is likely facilitated by the evolution of fractious prey, it seems probable that more human therapeutics will emerge.

Conclusions

Rattlesnake venoms have been the subject of numerous studies, but we still only know the complete proteome of a relatively small number of species, and many of the specialized montane species remain poorly characterized. In addition to the –omic information needed to make sense of the evolution of venoms among rattlesnakes, in order to place this data in a meaningful biological context, there exist equally pressing needs for probing many questions in evolution, at the molecular and organismal levels, and it is clear that there will be many unanticipated adaptations to be found among these magnificent snakes.

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