Deciphering the Evolution of Venom and the Venom Apparatus in Rattlesnakes

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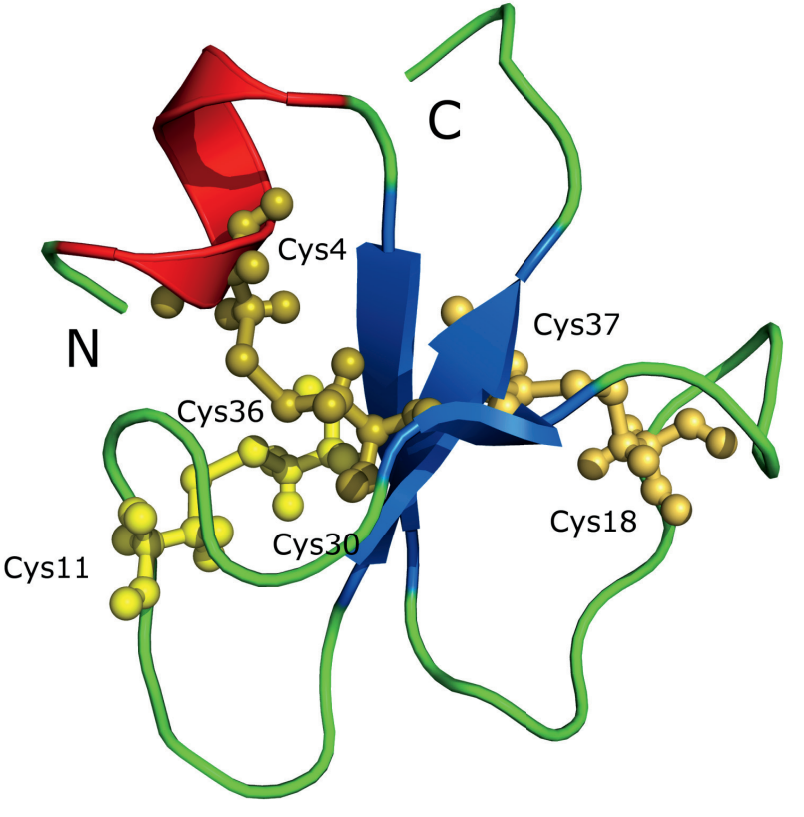


Rattlesnake venoms consist of a complex mixture of proteins, peptides, carbohydrates, nucleotides, metal ions, and organic acids, but protein/peptide components are generally accepted as the main biologically active components. These specialized toxins are synthesized in secretory epithelial cells, are 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 and biological activity.

Introduction to venoms and vipers

Venoms have allowed advanced snakes to utilize a chemical rather than a mechanical means of overcoming fractious 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 for the evolution of a strike-and-release mode of predation, further minimizing contact with prey and damage to the snake (Saviola et al., 2013). Among snakes, vipers can also consume the largest prey relative to their own mass (Greene, 1997), introducing potential handling and digestion difficulties, but these appear to be offset at least in 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 their high levels of toxicity toward mammalian prey (Mackessy, 2008; Gibbs and Mackessy, 2009; see **Box 1** and **Glossary**) and the prevalence of taxon-specific toxins in several species (Mackessy et al., 2006; Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013). However, venom complexity, particularly in vipers, is poorly understood at present, though a potential co-evolutionary arms race between snakes and their prey has been suggested to explain the multiplicity of homologs within a venom protein family and the high degree of venom variation even within species.

At least 15 species of rattlesnakes occur in Arizona (Table 1; see volume 1), more than in any other state in the USA. These species range from broadly-distributed, large-bodied lowland species such as *Crotalus atrox*, to narrowly distributed, small-bodied talus slope specialists such as *C. pricei*. The known basic biology and toxinology of these species also varies widely: for example, a PubMed search (4 May 2014) using these two species' names revealed 433 publications on *C. atrox* but only 1 publication on

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 1–100s 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 specialized structures (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 no injurious effects. On the other hand, poisons are different. Tetrodotoxin, a poison found in puffer fish and newts, is rapidly lethal if consumed. Therefore, route of exposure is critical to venom action, and venoms, which are most diverse and complex in composition among the snakes, have allowed the evolution of a chemical means to dispatch fractious prey with minimal risk from prey retaliation to the snake.

Glossary

Sequencing 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 also 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 transcripts) is not necessarily constant across tissues, individuals, and time.

Neo-functionalization: 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, such as serine proteases, phospholipases A_2, and three-finger toxins.

Crotalus pricei. Similarly, aspects of diet, habits, activity patterns and other aspects of natural history are often species-specific, and so within one state one may encounter nearly the entire known range of variation within rattlesnakes. Venom compositional trends were discussed relatively recently in the entire clade (Mackessy, 2008) and within the *Crotalus viridis* and the Western Group or clade (Mackessy, 2010; see Davis, volume 1, Western Complex), and some of these trends will be summarized below. As an introduction, we describe some aspects of the venom apparatus and overall venom composition.

The venom gland and venom apparatus

Venom gland morphology varies considerably among the major clades of advanced snakes, and that of pitvipers is particularly complex and highly derived (cf. Mackessy, 1991; Zalisko and Kardong, 1992; Mackessy and Baxter 2006; Vonk et al., 2013). Vipers in general and rattlesnakes in particular possess a large gland which lies below and slightly behind the eye, and it is enclosed in a tough capsule of connective tissue with fibers continuous with the posterior ligament (Figure 1). Attaching to the dorsoposterior portion and wrapping around the lateral face of the gland is the compressor glandulae, a differentiated slip of the adductor mandibulae. During venom injection, the contraction of this muscle "wrings out" the gland (with semi-spiral vectors of force), pressurizing the gland and delivering a large bolus of venom under high pressure (Kardong and Lavin-Murcio, 1993). Simultaneously, the maxillae are rotated and the typically long fangs are erected via the strut-like movement of the pterygoid and the palatine bones (Deufel and Cundall, 2006), allowing the

Table 1

General distribution and venom toxicity of rattlesnakes of Arizona.

Species	General distribution[a]	VT (LD_{50}, µg/g)[b,c]	Type[b]
Crotalus abyssus	Grand Canyon and vicinity	2.1	I
Crotalus atrox	S and W Arizona	3.5	I
Crotalus cerastes	SW Arizona	2.4	I
Crotalus cerberus	C Arizona	5.4	I
Crotalus concolor	Extreme NC Arizona	0.4	II
Crotalus lepidus	SE Arizona (Sky Islands)	1.6	I*
Crotalus lutosus	Extreme NW Arizona	2.9	I
Crotalus molossus	C and S Arizona	2.7	I
Crotalus pricei	SE Arizona (Sky Islands)	1.3	I
Crotalus pyrrhus	C and W Arizona	2.5	I
Crotalus scutulatus	W and S Arizona	0.2	II (A)*
Crotalus tigris	SC Arizona	0.07	II
Crotalus viridis	Extreme SW New Mexico	1.6	I
Crotalus viridis	NE Arizona	1.3	I
Crotalus willardi	SE Arizona (Sky Islands)	4.2	I
Sistrurus tergeminus	Extreme SE Arizona grasslands	1.4	I

a, based on individual species accounts, this volume. b, Mackessy (2008, 2010). c, Gibbs and Mackessy (2009). *, some populations show opposite venom type. VT = venom toxicity.

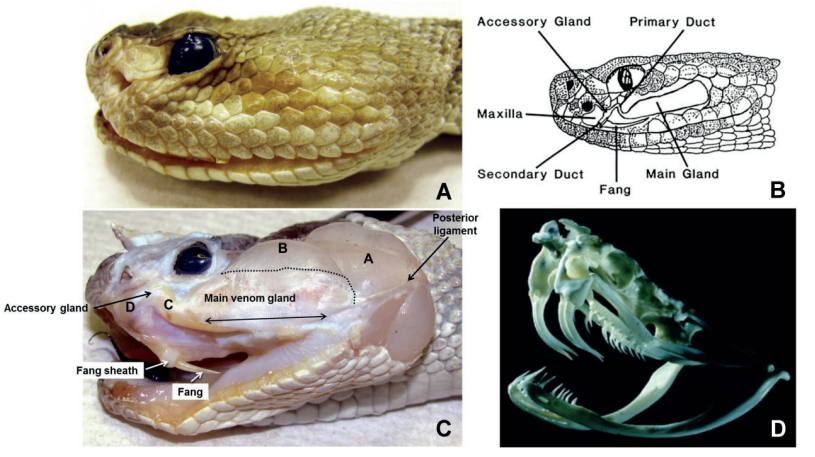


Figure 1. The venom apparatus of rattlesnakes. a) The head of a Black-tailed Rattlesnake (*Crotalus molossus*). b) A drawing of the venom apparatus of Northern Pacific Rattlesnake (*Crotalus oreganus*). c) The venom apparatus of *C. molossus* (same snake as in a). A specialized slip of the adductor mandibulae (A), the compressor glandulae muscle (B), pressurizes venom in the main gland ductules, which passes through the primary duct (C), the accessory gland, the secondary duct (D) and to the base of the long, hollow fang. The junction between the fang basal orifice and the secondary duct is "sealed" by the fang sheath when the fang is erected. d) The skull of an Eastern Diamond-backed Rattlesnake (*Crotalus adamanteus*) showing the primary and replacement fangs attached to the maxillae. B reproduced from Mackessy and Baxter (2006).

snake to stab the hollow fangs deep into prey and deliver venom rapidly; the entire strike sequence may last less than 0.5 sec (Kardong, 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,

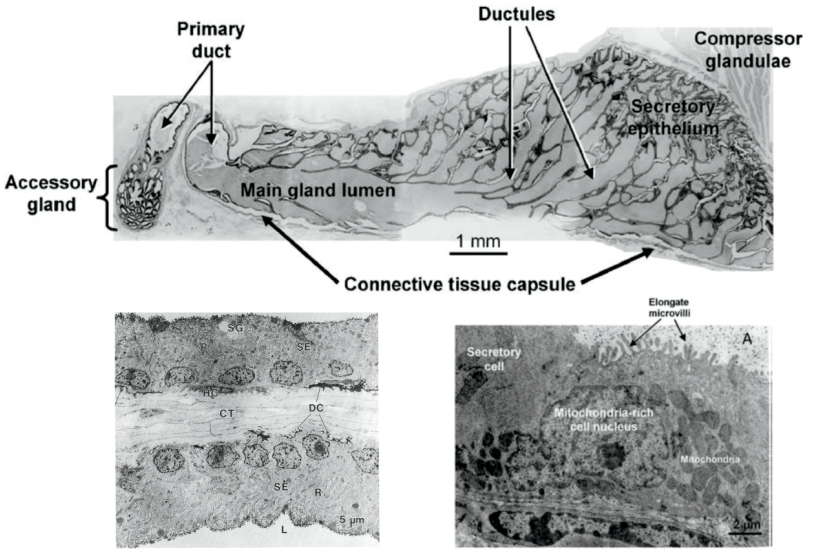


Figure 2. Secretory epithelium of the venom gland apparatus of *Crotalus oreganus*. a) A sagittal section of the entire venom gland. b) A transmission electron micrograph (TEM) of the secretory epithelial cells of the main gland four days post venom extraction; cells are columnar. c) TEM of mitochondria-rich cell of the main gland (unextracted); both mitochondria-rich and secretory cells are cuboidal at this stage of the venom synthetic cycle. Reproduced from Mackessy (1991).

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 (Fujimura 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 phospholipases A_2, 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 PLA_2s 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

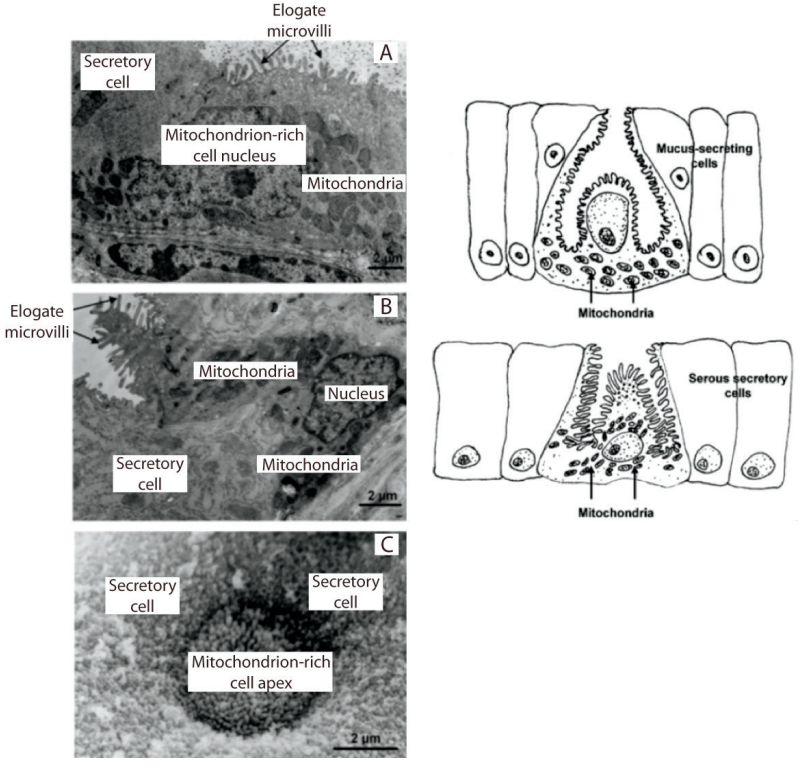


Figure 3. Mitochondria-rich cells of the main gland of *Crotalus oreganus*. a) TEM, unextracted gland. b) TEM, extracted gland. c) A scanning electron micrograph of cell surface – note that these cells are recessed below the level of secretory cells. d) A drawing of parietal cell of the vertebrate gastric pit (top) and the mitochondria-rich cell of rattlesnake main venom glands; note the highly similar morphology. Reproduced from Mackessy and Baxter (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 ~7.3) 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 titrate 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 toxinology in general has largely paralleled advances in protein chemistry. In fact, laboratory research in enzymology has frequently utilized venom-derived enzymes, including venom exonuclease (also known as phosphodiesterase: Laskowski, 1980; Mackessy, 1998) and L-amino acid oxidase (Zeller, 1944; Tan and Fung, 2010), which gives

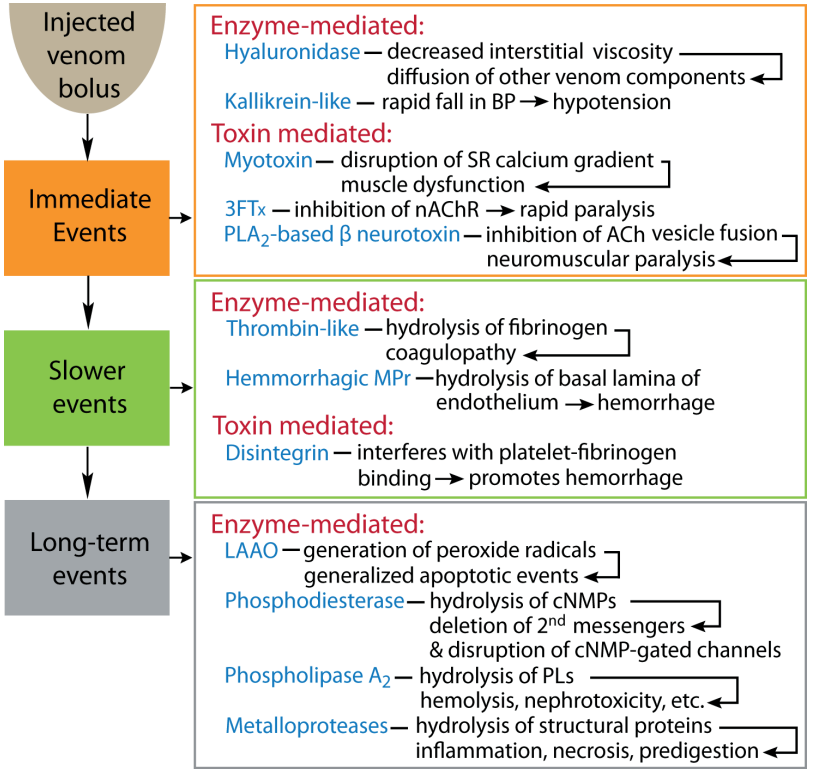


Figure 4. Hypothetical sequence of events following venom bolus deposition in prey. Reproduced from Mackessy (2008).

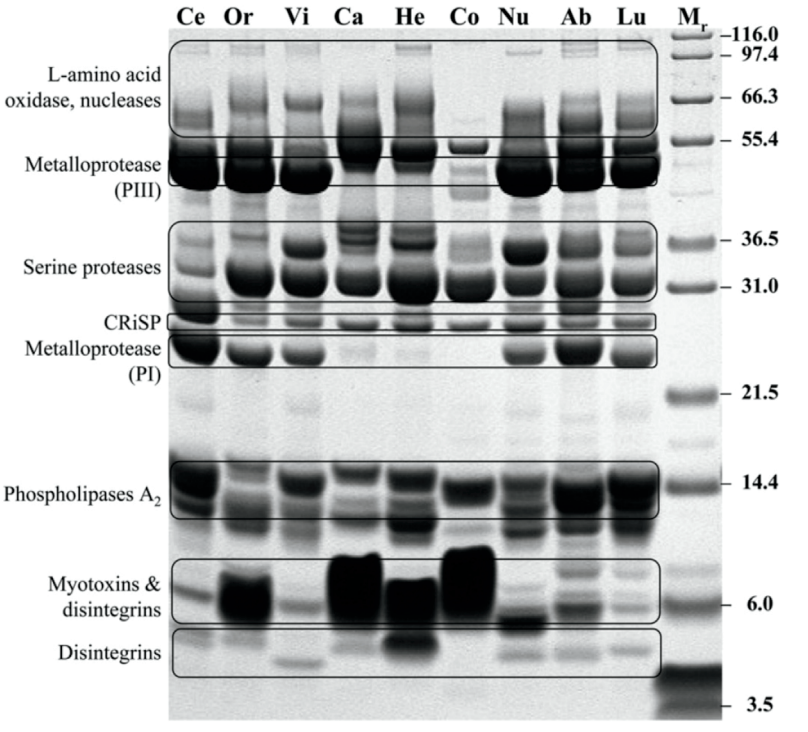


Figure 5. Molecular fingerprint of the venoms of taxa from the Western Rattlesnake clade (see Davis, volume 1, Western Complex) using SDS-PAGE under reducing conditions. The protein family (typical activity) of bands with characteristic masses is given on the left, and approximate masses (in kilodaltons) are given on the right. Band intensity is proportional to concentration of a venom; note that for some taxa (Co), 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).

many venoms their characteristic yellow color. 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 utilized successfully 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).

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, has tended to displace 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 sequence to be inferred from DNA sequences, at a small fraction of the time and cost compared to protein sequencing. In addition, many labs conducted and continue to conduct detailed 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 proliferation of hypotheses concerning venom evolution, and the selective pressures which might have favored particular positions 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 called proteomics. In the late 1990s and early 2000s, mass spectrometric based methods were applied to venoms, but early attempts at protein identification via peptide mass fingerprinting and *de novo* sequencing were often disappointing due to poor representation of venom protein sequences in public and private databases (e.g., Kamiguti et al., 2000). This scenario changed rapidly, however, as proteomics gained rapid utilization in many areas, most notably medical and drug discovery applications. Instrumentation and technical advances followed rapidly as well, as the fields of proteomics, transcriptomics, and genomics 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.

Proteomics 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 (Juarez et al., 2004; **Box 2**), and this approach has been adopted by toxinologists world-wide.

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 Juan J. Calvete and colleagues (Juárez et al., 2004), which established a standardized 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 trypsin-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 or *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

with as little as 2–5 mg of crude venom (Sanz et al., 2006), and using whole venom digests and nanospray LC/MS (Synapt G2 HD Mass Spectrometer), 500 µg or less is sufficient (McGivern et al., 2014). For rapid analysis of crude venom or purified toxins, less than 1 µg is sufficient to provide highly accurate mass estimates using MALDI-TOF-MS (Figure 6). The high sensitivity and minimal material requirement means that venomic approaches have the potential to allow one to catalog the entire proteome of an individual snake's venom. Further, because most steps can be automated, these methods facilitate a move toward a high throughput approach, where many venom samples can be analyzed with a high degree of accuracy and completeness. In

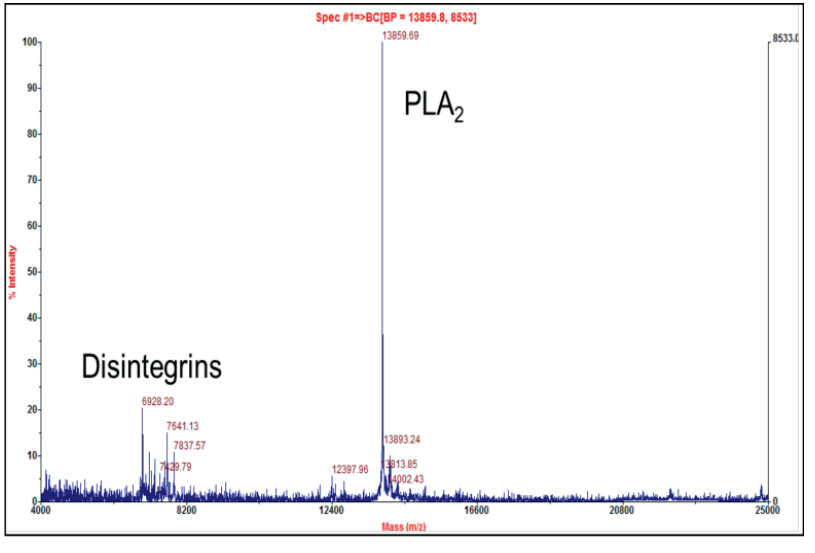


Figure 6. Mass spectrogram (MALDI-TOF-MS) of 1 µg crude venom from *Sistrurus tergeminus edwardsii* using a 4-25 kDa window. The phospholipase A_2 (~14 kDa) and the disintegrin peaks (~6.9 kDa) are labeled.

turn, much deeper comparisons of venom composition are possible, allowing the analysis of individual, population, and species level variation at scales that were previously unattainable.

Disadvantages of proteomic approaches alone

A venomic 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-represented 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 (Doley et al., 2009; Brust et al., 2013), very similar proteins can have vastly different pharmacological effects, as demonstrated in rattlesnake venoms by the diversity of serine proteases (Mackessy, 2010c). Also, the high sensitivity of mass spectrometry-based techniques allows one to detect fine-scale levels of individual variation, but in the

absence of biological/biochemical data, the significance of compositional variation is difficult to interpret. Further, the levels of individual variation which are biologically relevant has not been defined; for example, if numerous proteins are found in venom from individual A but not in individual B, but the total amount of these proteins is less than one percent, is it likely to have a biologically relevant effect on prey during envenomation? Like most new methods in biology, proteomics raises as many questions as it answers, but the potential for this technology to deliver a much deeper understanding of venom evolution is substantial and clear.

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, and snake venomics in general (Pahari et al., 2007; Gibbs and Mackessy, 2009; Vonk et al., 2013; Casewell et. al., 2014). This revolution in high-throughput sequencing technology, also called "next-generation" sequencing, has effectively turned the problem of studying snake venoms upside-down by removing the challenges of collecting massive amounts of information, and replacing this with an equally daunting challenge of accurately interpreting such vast amounts of data.

Venom genes, including the most toxic and medically relevant gene families, often occur in multi-copy tandem arrays, forming large multigene families with multiple protein isoforms (Pahari et al., 2007; Ikeda et al., 2010; Vonk et al., 2013). Generally, many venom genes are thought to be the result of evolutionary duplication of ancestral non-toxic protein coding genes that have been sub-functionalized and or neo-functionalized into venom toxins and expressed primarily in the venom gland (Casewell et al., 2012; Vonk 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 selective advantage and flexibility compared to optimization of a single gene (Casewell et al., 2012).

How many venom genes are there in rattlesnake genomes? Unfortunately, what is currently known about rattlesnake venom genes largely lacks genomic context because it is based on transcripts from venom glands. Such transcript-centric information only provides information about the transcribed exonic and adjacent untranslated transcribed regions of venom genes, making it difficult to relate levels of mRNA transcripts directly to functional venom toxins (the proteins) and directly to the actual toxin-encoding genes. Based on transcriptomic analysis of a single *C. adamanteus* venom gland, Darin Rokyta and his team (Rokyta

et al., 2012) found 123 unique full length 'toxin-encoding' transcripts that cluster into 78 groups, with each group having less than 1% sequence divergence – this was taken as evidence that there may be up to 78 different 'toxin-encoding' genes, though probably less due to single genes producing multiple distinct transcript isoforms. Their data provides some estimation of the extent to which gene duplication has occurred in some toxin families, including their identification of 21 C-type lectin transcript 'clusters,' 16 snake venom metalloproteinase clusters, and 14 serine proteinase clusters, indicating these three gene families 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

Studying the genomes and transcriptomes of rattlesnakes and other species has great potential to reveal all venom toxins which are possible in the venom gland and the genome of snakes. When gland transcriptomic analyses are coupled with next-generation sequencing methods, one can rapidly obtain a "complete" catalog of toxins present. As with proteomic 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 compositional variation at all levels should continue to emerge. In turn, as above, these advances will allow much deeper comparisons of venom composition between individuals and species than are currently possible.

Genomics has the advantage of providing direct information on 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.

Disadvantages of transcriptomic and genomic approaches alone

As we have outlined (**Box 3**), there are a number of challenges in accurately reconstructing 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. Similarly, accurately reconstructing venom transcripts is also difficult, and using transcriptomic data to try to understand how many genes may encode these transcripts is made difficult by the fact that many venom genes likely produce multiple different transcripts, thus the relationship between distinct transcripts and distinct genes is difficult to estimate. A major downside of transcrip-

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., 2013), and even the processed protein-encoding transcripts that have introns removed average around 1,500 bases in length. The challenge in leveraging next-generation data to interpret the sequence and structure of vertebrate genes and genomes (including venom genes) therefore lies 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 only 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 comprised by 50% or more repeat elements (Castoe et al., 2011, 2013). Venom genes are duplicated in tandem and may occur in many copies (Vonk et al., 2013). Therefore, estimating how to put the pieces (i.e., short reads) together into an accurate estimate of venom genes, or genomes, 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 tremendously increase the ability of researchers to leverage fully various next-generation sequencing approaches for addressing major outstanding questions about rattlesnake venom variation and evolution.

tomic 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 (but see Modahl and Mackessy, 2016).

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 example that showcases some of these potential limitations of transcriptomic approaches, Nicholas 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 52.08% (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 tergeminus* "*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 (Pahari et al., 2007). However, a proteomic study of venom from the same metapopulation of *Sistrurus t. 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 3FTx protein expression (S. Mackessy, unpubl. data). A further comparison of the relative levels of specific protein families (Table 2) also showed extensive differences, in particular among metalloproteinases (transcriptome – 12.2%, proteome – 48.6%), serine proteinases (transcriptome – 37.5%, proteome – 24.4%) and phospholipase A_2 (transcriptome – 28%, proteome – 13.7%). Therefore, if one is evaluating relative importance of a protein family to a particular taxon, particularly from an ecological perspective, which should one believe? There have been recent suggestions that because the proteome is closer to the phenotype than is the transcriptome,

Table 2

A comparison of the proteome and transcriptome of the Desert Massasauga (*Sistrurus tergeminus*) in Colorado.

Protein family	Proteome	Transcriptome
Disintegrin	0.9	-
C-type BPP	< 0.1	0.3
Kunitz-type inhibitor	< 0.1	-
DC-fragment	< 0.1	-
Phospholipase A_2	13.7	28.1
Nerve Growth Factor	< 0.1	6.7
CRISP	10.7	6.7
Serine proteinase	24.4	37.5
C-type lectin	< 0.1	1.4
L-amino acid oxidase	2.5	3.6
Zn^{2+}-metalloproteinase	48.6	12.2
Three-finger toxin	-	0.8
Fusion protein (ku-wap-fusin)	-	0.3
Phosphodiesterase	-	0.3

Proteome – Sanz et al. (2006); transcriptome – Pahari et al. (2007); dash – not detected.

proteome-based studies may be informative concerning natural selection and local adaptation (Diz et al., 2012). However, an emerging consensus is that in order to obtain the most complete picture of venom composition, a combination approach is desirable (cf. Aird et al., 2013); a notable feature of this study is that there was good concordance between the proteome and the transcriptome.

Another major drawback of all –omic approaches in general is that such high-throughput approaches do well to identify the composition of venoms, but they fail to provide robust information about what this composition means functionally and practically. Transcriptomic approaches specifically, because they provide information on transcripts and not the proteins they encode or the function of these proteins, require some caution in interpreting, and there are examples in which such approaches have likely been used to over-extend inferences of venom function. The primary issue stems from the default labeling of transcripts (and proteins) that are expressed in venom glands as "venom toxins." It is often assumed that if transcripts for particular proteins are observed in venom glands, then they must be functioning as toxins and or to facilitate prey handling in some way. This is also based on the assumption that proteins expressed in the venom gland are expressed there for adaptive and venom-related functional reasons, and not as a physiological default (e.g., perhaps a particular protein is simply constitutively expressed in all tissues, or in all secretory glands). Also, the identification of transcripts as "venom toxins" is typically based on both the observance of such transcripts in venom glands as well as evidence that the sequence of the encoded protein is somewhat similar to known "venom toxins." Most importantly, such inferences lack experimental evidence that, indeed, transcripts encode proteins that are either toxic, or play some functional role in venom. This problem becomes most pronounced when only venom glands (or oral glands presumed to be venom glands) from a particular species are analyzed (transcriptionally or proteomically), and the presence of transcripts or proteins with sequence similarity to other venoms is interpreted as evidence of the presence of functional venom toxins (Fry et al., 2006).

Such analysis of venom gland transcripts, in the absence of transcriptomes from other non-venom tissues, can lead to a very biased interpretation of "venom presence." For example, recent studies which indicate that venom genes, and genes related to venom genes, may be expressed at moderate to high levels in other non-venom tissues suggests that many genes labeled as "venom toxins" may indeed not be particularly toxic and may also serve diverse functional roles throughout the organism (Hargreaves et al., 2014; Reyes-Velasco et al., 2014). Such results also demonstrate that transcriptomic analysis of venom gland tissue is difficult to interpret fully without the full context of transcriptomic information from other non-venom tissues that serve to highlight what is indeed unique about transcription in venom glands. These findings collectively raise important questions for future research, including: what might be the functional role of all protein components in venom, and do the protein products of venom genes have functional roles outside of venom systems? Such studies also argue strongly against the identification of gene products as "ven-

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 venoms 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 distinct squamate 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; Castoe 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 3

Common components of rattlesnake venoms and general characteristics.

Component name	Approximate mass* (kDa)	Function	Biological activity	References
Enzymes				
Phosphodiesterase	94 -140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and tri-nucleotides; hypotension/shock?	Mackessy (1998); Aird (2002)
5′-nucleotidase	53 - 82	Hydrolysis of 5′-nucleotides	Nucleoside liberation	Rael (1998); Aird (2002)
Alkaline phosphomonoesterase	90 - 110	Hydrolysis of phosphomonoester bonds	Uncertain	Rael (1998); Aird (2002)
Hyaluronidase	73	Hydrolysis of interstitial hyaluronan	Decreased interstitial viscosity – diffusion of venom components	Tu and Kudo (2001)
L-amino acid oxidase (homodimer)	85 - 150	Oxidative deamination of L-amino acids	Induction of apoptosis, cell damage	Tan (1998)
Snake Venom Metalloproteinases: M12 Reprolysins		Hydrolysis of many structural proteins, including basal lamina components, fibrinogen, etc. Some are prothrombin activators (group A & B)	Hemorrhage, myonecrosis, prey predigestion	Fox and Serrano (2005)
P-IV	48 - 85			
P-III	43 - 60			
P-II	25 - 30		Induce DIC, highly toxic	
P-I	20 - 24			
Serine proteases				
Thrombin-like	31 - 36	Catalysis of fibrinogen hydrolysis	Rapid depletion of fibrinogen; hemostasis disruption	Markland (1998); Swensen and Markland (2005); Mackessy (2010)
Kallikrein-like	27 - 34	Release of bradykinin from HMW kininogen; hydrolysis of angiotensin	Induces rapid fall in blood pressure; prey immobilization	Nikai and Komori (1998)
"Arginine esterase"	25 - 36	Peptidase and esterase activity	Uncertain; predigestion of prey (?)	Schwartz and Bieber (1985)
Phospholipase A_2 enzymes (Group II)	13 - 15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage	Kini (1997, 2003)

*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 Mackessy (2010b); see this paper for references.

Table 3 Continued

Component name	Approximate mass (kDa)	Function	Biological activity	References
Non-enzymatic proteins/peptides				
Cysteine-rich secretory proteins CRiSPs)/helveprins	21 - 29	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	Yamazaki and Morita (2004)
Nerve growth factors	14 - 32.5	Promote nerve fiber growth	Unknown; apoptosis (?)	Siigur et al. (1987); Koh et al. (2004)
PLA_2-based presynaptic neurotoxins (2 subunits, acidic and basic)	24	Blocks release of acetylcholine from axon terminus	Potent neurotoxicity; prey immobilization	Aird et al. (1985); Ducancel et al. (1988); Faure et al. (1994)
C-type lectins	27 - 29	Binds to platelet & collagen receptor	Anticoagulant, platelet-modulator	Leduc and Bon (1998)
Disintegrins	5.2 - 15	Inhibit binding of integrins to receptors	Platelet inhibition; promotes hemorrhage	Calvete et al. (2005)
Myotoxins – non-PLA_2	4 - 5.3	Modifies voltage-sensitive Na channels; interacts with lipid membranes	Myonecrosis, analgesia; prey immobilization	Fox et al. (1979); Laure (1975); Bieber and Nedelhov (1997)
Smaller peptides				
Bradykinin-potentiating peptides	1.0 - 1.5	Increases potency of bradykinin	Pain, hypotension; prey immobilization	Wermelinger et al. (2005)
Tripeptide inhibitors	0.43 - 0.45	Inhibit venom metalloproteases and other enzymes	Stabilization of venom components	Francis and Kaiser (1993); Munekiyo and Mackessy (2005)
Smaller organic compounds				
Purines & pyrimidines	AMP = 0.347, Hypoxanthine, Inosine	Broad effects on multiple cell types (?)	Hypotension, paralysis, apoptosis, necrosis (?); prey immobilization	Aird (2002, 2005)
Citrate	0.192	Inhibition of venom enzymes	Stabilization of venom	Freitas et al. (1992); Francis et al. (1992)

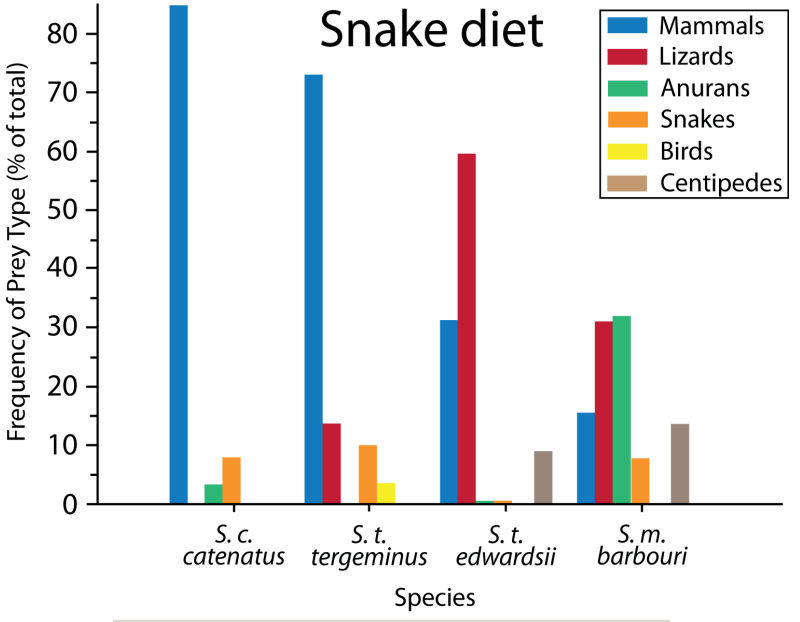
neo-functionalization of the duplicated genes; once freed from functional constraints favoring the original activity, the new gene may show mutations in (typically) any non-structural residues. Some of these result in changing specificity pocket configurations (as in serine proteases, common in rattlesnake 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 post-translational 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 overall 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-

sibly 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* venoms (Figure 7), and differences in toxicity of venoms toward mice (*S. catenatus* vs. *S. miliarius*) can be extreme (Gibbs and Mackessy, 2009).



Venom toxicity:	mice	lizards
S. c. catenatus	0.23	0.70
S. t. tergeminus	1.48	1.41
S. t. edwardsii	0.60	0.39
S. m. barbouri	7.19	0.66

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. t. tergeminus* but are only minor parts of the diets of *S. t. edwardsii* and *S. miliarius barbouri*. General trends in venom evolution in *Sistrurus* tend to follow an increasing dependence on mammalian prey. Reproduced from 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. concolor* 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. mitchellii*, *Crotalus horridus* "*atricaudatus*," *Crotalus tigris*, and several populations of *Crotalus lepidus klauberi* 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 worldwide, 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 (LD_{50} >1.0 µg/g mouse); type II venoms show the opposite (high toxicity, low-no metalloprotease activity). This dichotomy is also apparent in Figure 5: whereas most taxa show prominent PIII 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. simus* + *C. durissus* clade of Central and South America (Calvete et al., 2010).

Biological phenomena are rarely encapsulated by simple dichotomies, and a recent analysis of venoms from *C. scutulatus* in Arizona identified a much more complex series of venom

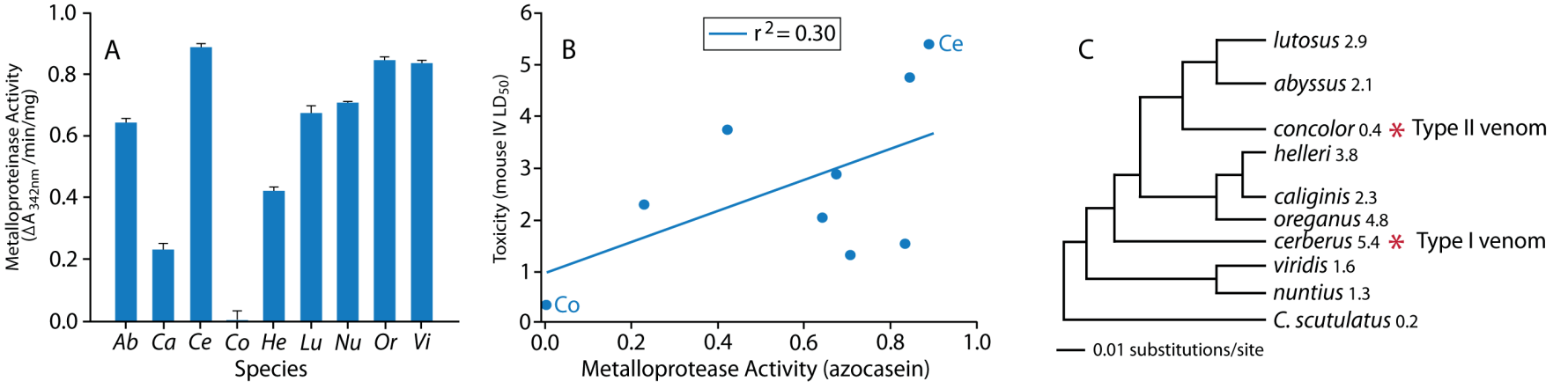


Figure 8. Venom compositional trends in the Western Rattlesnake clade. a) Metalloprotease activity (responsible for tissue necrosis/prey predigestion) is highest in *Crotalus cerberus* and nearly absent in *Crotalus concolor*. See Figure 5 for abbreviations. b) Relationship of metalloprotease activity and lethal toxicity. *Crotalus cerberus* show type I venom, while *C. concolor* shows type II venom. c) A phylogenetic hypothesis of Western rattlesnakes (Ashton 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).

proteomes (Massey 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 (Mojave 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 based on PLA_2 scaffolds (Faure et al., 1994). 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 may produce venoms rich in post-synaptic α-neurotoxins (a specific type of three-finger toxin), potent rattlesnake venoms (and venoms of other viperids) contain PLA_2-based presynaptic toxins, often at very high

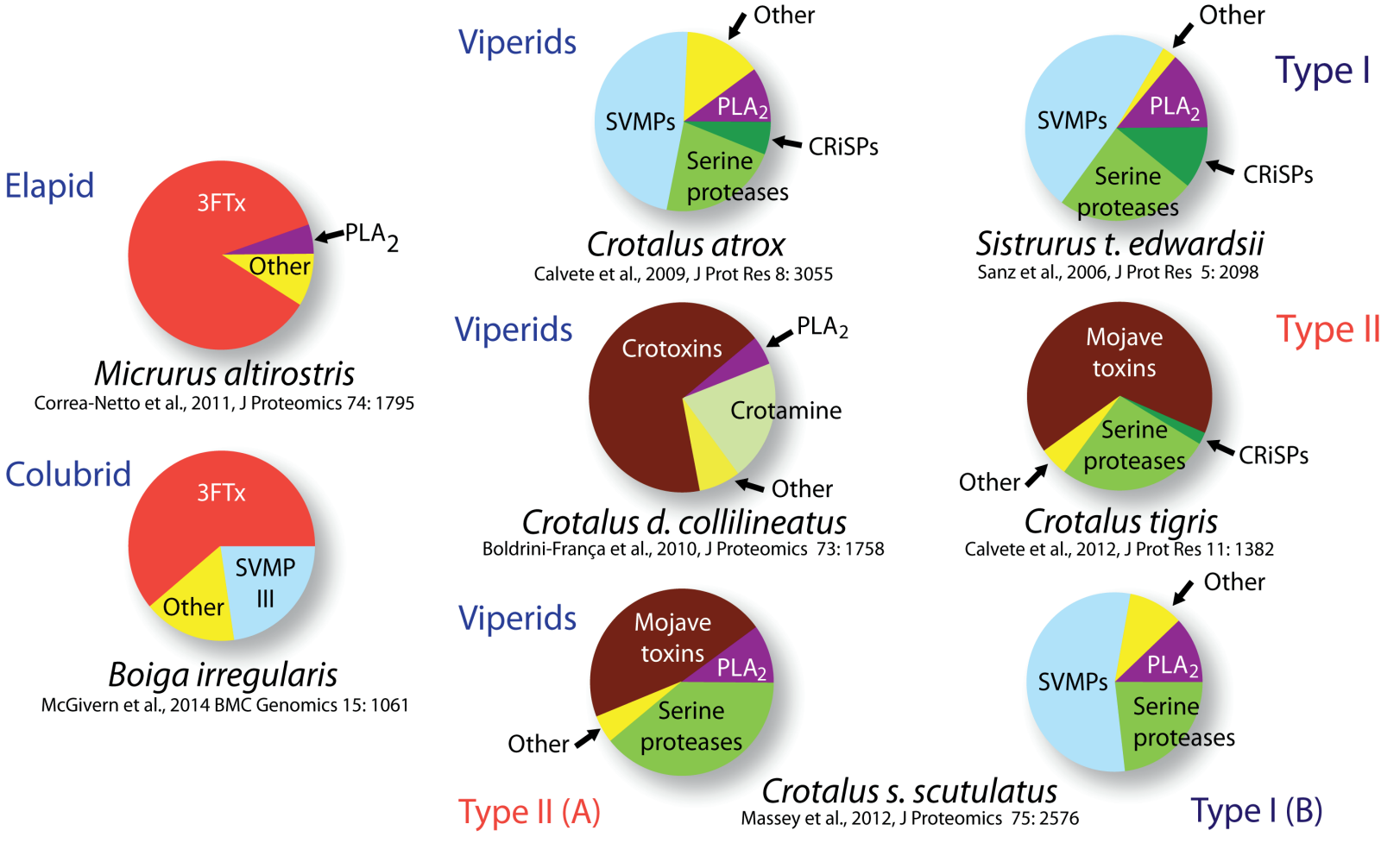


Figure 9. Simplified venom proteomes of an elapid, a colubrid, and five species of rattlesnakes. Note that in the elapid and colubrid examples, three-finger toxins (3FTx) predominate; these toxins include the highly potent, post-synaptic α-neurotoxins. In type I rattlesnake venoms, snake venom metalloproteases are dominant components; these proteases are responsible for tissue necrosis and prey tissue degradation. In type II rattlesnake venoms, crotoxins and homologs (e.g., Mojave toxins) predominate, and metalloproteases, if present, are very minor components. Different populations of *Crotalus scutulatus* may show either type: the A populations show type II venoms, while the B populations show type I venoms.

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 (Figure 9).

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. viridis*) 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. oreganus* (a close relative) shows type II venom (Figure 8). Another hypothesis is that type II venoms represent a phenotype of newly colonizing populations (Powell and Lieb, 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 (Sunagar 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, although 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 retaliation from fractious 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 Diamondback 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 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 bioactivity 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 results in rapid death. However, venom toxins are homologs of "normal" regulatory proteins, and they can be simplistically thought of as co-opted from other tissues. When injected into prey, these "regulators" are introduced into tissues at levels many orders of magnitude greater than normal and at inappropriate times, resulting in a systemic collapse in the prey. When one considers them as derived from regulatory compounds, the potential use of toxins as drugs seems much more plausible.

Many cultures throughout history have used rattlesnake venoms as a therapeutic, and the practice continues today, for example, in numerous parts of Mexico. However, the rational scientific use of venom proteins as drugs is relatively recent. In fact, one of the first and most successful drugs (captopril) based on animal venoms was derived from a peptide in the venom of a highly dangerous Brazilian viperid, *Bothrops jararaca* (Cushman and Ondetti, 1999). At least 8 drugs currently used as human therapeutics are derived from pitviper venoms, and many more venom-derived toxins are used as clinical diagnostic tools (Takacs and Nathan, 2014). There is a growing literature on the use of natural toxins in the development of drugs (e.g., Fox and Serrano, 2007; Koh and Kini, 2012; Minea et al., 2012; Takacs and Nathan, 2014), and as more diverse species' venoms are evaluated, 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 meaning biological context, there exist equally pressing needs for information on the natural history and ecology of species to understand the evolutionary and functional interaction between their ecology and venom. Basic information such as distribution, activity patterns, and diet are poorly known for many species, but these factors may have a profound influence on the evolution of rattlesnake venoms. This means that there are ample opportunities for important contributions to understanding venom evolution at a variety of levels, from

next generation sequencing of genomes and transcriptomes, to functional characterizations of the multitude of proteins that comprise venoms, to detailed field and museum-based studies.

One of the major challenges facing rattlesnake biologists in the near future is the thorough integration of the tremendous amounts of data from –omic studies with the natural history/ecology of the animals themselves to arrive at a more robust and holistic understanding of venom function and evolution. Rattlesnakes, therefore, represent an excellent model organism 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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