Section I

Reptile Toxinology, Systematics, and Venom Gland Structure

1 The Field of Reptile Toxinology
Snakes, Lizards, and Their Venoms

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CONTENTS

Reptile venoms are typically complex mixtures of primarily peptides and proteins, and the myriad biological effects these molecules produce in envenomated prey and humans are similarly complex and potent. In this book, the many authors discuss the venom apparatus of reptiles, consider the current status of phylogenetic relations of venomous reptiles, explore specific families of venom components, and provide current approaches to the treatment of human envenomations worldwide. In this introduction to the book, variation in venom composition and the factors leading to this variation are discussed. Major patterns of venom compositional trends are identified for the main clades of venomous reptiles, and the identification of novel toxins and interesting structural variants, as well as elucidation of their biological activities and significance, will remain fertile areas of research for many years to come.

I. INTRODUCTION

The production of toxic materials by animals, plants, and microorganisms has fascinated humanity for millennia, for reasons practical, nefarious, and inquisitive. However, only much more recently has the study of these compounds, toxinology, become a formalized discipline. Like many areas of the sciences, toxinology began as a primarily descriptive venture, and technical limitations restrained understanding of the many toxic compounds produced by life-forms. There is still a considerable need for basic descriptive work on venoms and toxins, as the venoms of many species are wholly unknown, and many high-throughput techniques are not yet sufficient at detecting subtle aspects of structure-function differences in many molecules that share a common structural fold but have very different pharmacologies. But as toxinology has moved beyond descriptive work, it has become clear how critical toxins are used as tools for understanding normal homeostatic mechanisms of humans and other animals. Further, study of toxins has contributed greatly to rational drug

design efforts, and many compounds first isolated from natural sources are now used as highly effective drugs for treating human ailments (Opie and Kowolik, 1995; Smith and Vane, 2003; Lewis and Garcia, 2003; Fox and Serrano, 2007). In the last 20 years, particularly with the tremendous advances in genomics and proteomics, we have seen a great increase in the discovery, description, and utilization of purified toxins, and the field of toxinology now includes aspects of virtually all areas of modern life sciences. The use of toxins as "molecular tweezers" has allowed dissection and clarification of numerous important physiological processes, including many aspects of neurotransmission, apoptosis, hemostasis, and signal transduction.

Reptiles include the largest of the venomous vertebrates, and many species produce very large quantities of potent venoms. Envenomations worldwide remain a significant source of morbidity and mortality for humans and their domestic animals in many countries. Species producing venoms are found in several different clades of squamate reptiles, including the snake families Atractaspididae, Elapidae, Viperidae, and the polyphyletic "Colubridae," as well as the lizard family Helodermatidae (Figure 1.1). Within this fascinating and ancient group of animals, there are many interesting and unanswered biological questions, ranging from species diversity and distribution to the ecology and evolution of these (often) highly specialized reptiles. As snakes evolved from a mechanical means of overpowering prey (constriction) to a chemical means (venom injection; Kardong et al.,

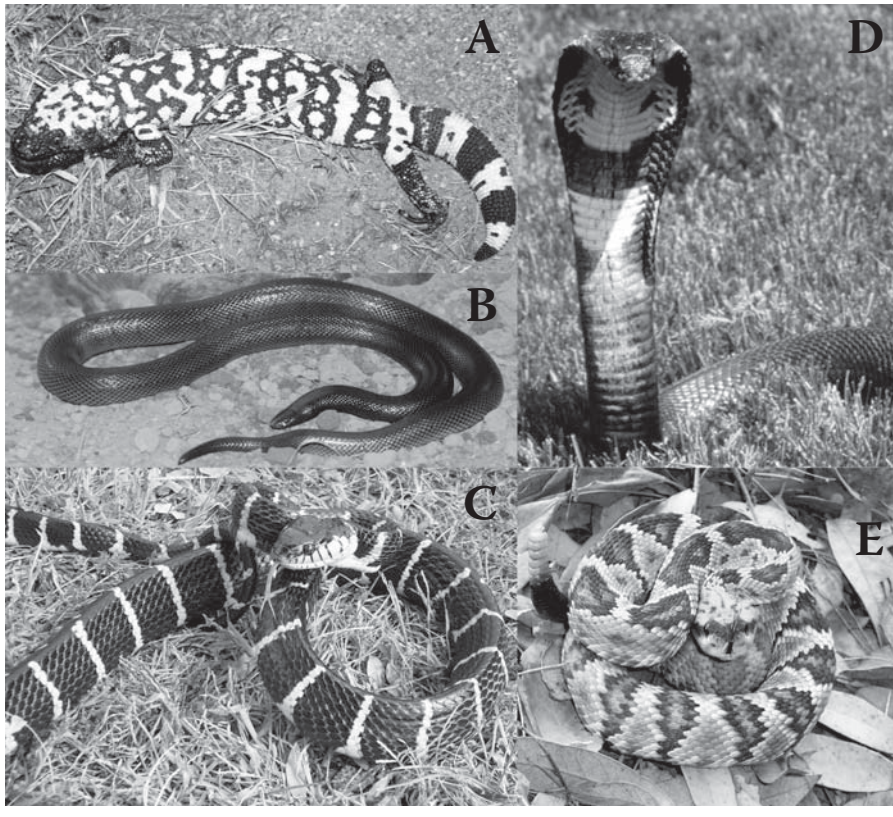


FIGURE 1.1 ***(A color version of this figure follows page 240.)*** Representative examples of venomous squamate reptiles. (A) Gila monster (*Heloderma suspectum*), a member of the family Helodermatidae. (B) Small-scaled burrowing asp (*Atractaspis microlepidota*), family Atractaspididae. (Photograph by Kristen Wiley, courtesy Kentucky Reptile Zoo.) (C) Mangrove catsnake (*Boiga dendrophila*), family Colubridae. (D) Monacled cobra (*Naja kaouthia*), family Elapidae. (E) Northern blacktail rattlesnake (*Crotalus molossus molossus*), family Viperidae.

1997), natural selection has favored fine-tuning of predator-prey interactions, and in the process a wide array of compounds with almost unbelievable specificities have evolved. Although many invertebrates, and a few fish, are also important sources of human envenomations, only venoms and envenomations produced by reptiles will be considered here. Thus, for biologists (in the broadest sense of the word) there is truly something for everyone among venomous reptiles and their venoms.

Included in this book are twenty-four chapters produced by some of the finest researchers in the field. Many chapters focus on specific components of venoms, while others consider the structure and function of the highly specialized venom systems of squamate reptiles, as well as the evolutionary relationships of these animals. The first section of this book places venoms and their study in a broader biological context and will provide source information for toxinologists that is typically omitted from a classical treatment of the field. Specifically, an overview of relationships among venomous animals (systematics) is presented, as well as a summary of the main structural features of the glands producing venoms. Though a structural chemist working in toxinology may have little direct need for such information, it provides a more detailed glimpse into biologically relevant structure-function relationships at levels of organization above the molecular level.

Many different toxins and several new classes of proteins (such as helveprins/cysteine-rich secretory proteins [CRiSPs]) have been described since the last edition of the *Handbook of Natural Toxins* (Tu, 1991), and the increase in the level of sophisticated techniques to reevaluate known toxins and venoms has been impressive. A major section of the book will include a thorough treatment of many enzymatic components found in venoms. Though there are several classes of toxins that have enzymatic activities as well as specific sites of ligand-mediated actions, I believe that it is useful to group those compounds that have catalytic activity (classically, enzymes) vs. ligand-binding mediated activities (classically, toxins). There are many different activities included here, and much new information is presented.

Another major section of the book will include the nonenzymatic proteins and peptides found in venoms. This section will summarize many of the major classes of such toxins. Numerous primary structure and gene sequences for a variety of different toxins are now available via public databases, and these data have greatly increased understanding of gene structure, structure-function relationships among proteins, evolution of toxin families, and generalized patterns of venom protein expression. Some additional venom components that are neither enzymes nor toxins include nonpeptide organic constituents (such as nucleosides), small peptide components of venoms (of both intrinsic and extrinsic action), and inorganic and metal ion constituents of venoms, and these also contribute to the biological potency of venoms.

The last section logically follows the preceding sections and includes chapters on envenomation by reptiles in several different areas of the world, summarizing the significant advances in treatment of the often confusing sequelae of envenomation and identifying problems unique to (and common among) each area. Though antivenins still remain the main course of treatment for envenomations, advances in production and manufacture have increased efficacy and decreased side reactions. Access to health care is still a major concern in many parts of the world, and a contrast between ideal treatment and what is possible in many regions will be apparent. This section has contributions from clinicians/physicians familiar with envenomations as well as from individuals involved in the research, development, and production of antivenoms.

The intent behind this broader treatment of topics within the general field of reptile toxinology is to provide a better context for understanding the complexity of these venoms, which have been shaped by evolutionary and ecological forces. Envenomation is a complex syndrome involving dysregulation of many homeostatic mechanisms simultaneously, and it is hoped that by having a broader understanding of the many factors shaping venoms and envenomation, more effective treatments can be developed. Further, venoms are important natural sources of compounds useful as drugs and

probes of many physiological processes (Fox and Serrano, 2007), and by understanding the context in which venoms evolved, one may be able to exploit novel compound sources more effectively.

II. VENOMS AND TOXINS DEFINED

The definition of venoms has been somewhat contentious, but a venom is here considered to be a simple to complex secretion produced in a specialized gland that is typically delivered via specialized envenomation systems, including a secretory gland, often (but not always) specialized teeth (Vonk et al., 2008), and a suite of specific behaviors allowing delivery of the venom. Further, venoms must be introduced (commonly injected) into recipient tissues in order for deleterious effects to occur, while poisons are typically ingested (Mackessy, 2002a). Thus, reptiles representing an envenomation risk to humans and prey animals are referred to as venomous, not poisonous. Only one species, *Rhabdophis tigrinus*, is known to be both venomous and poisonous, because it possesses a Duvernoy's gland that produces venom (e.g., Sawai et al., 2002) *and* a saccular nuchal gland that sequesters toad toxins, poisonous to potential predators (Akizawa et al., 1985; Hutchinson et al., 2007; Mori and Burghardt, 2008).

In snakes, the venom apparatus consists of bilaterally paired specialized glands (a venom gland or Duvernoy's gland, which are homologous structures) located medial to the upper labial scales, posterior to the nostrils, and behind/below the eyes. In the front-fanged snakes (families Atractaspididae, Elapidae, and Viperidae), this apparatus consists of a large venom gland with a (typically) large basal lumen, allowing for storage of secreted venom for immediate deployment (Mackessy, 1991; Mackessy and Baxter, 2006). There is often a primary duct leading to an accessory gland, and a secondary duct connects the glands to the base of a hollow (and often long) hypodermic fang. Contraction of a specialized compressor muscle pressurizes the gland and delivers a bolus of venom under moderate pressure into recipient tissues. Rear-fanged snakes (the polyphyletic family "Colubridae"; see Chapter 2 for an updated phylogeny) have a somewhat different apparatus. A homologous gland, the Duvernoy's gland, lies in a position similar to that of the front-fanged snakes' venom gland, but it lacks the compressor muscle and a large basal lumen. Instead, the gland is held in place by connective tissue attached to the upper labial scales and a posterior ligament that runs to the rictus of the jaws (Figure 1.2, top); when envenomating prey, jaw adductor muscles pull the ligament posteriorly and labial scales tight, compressing the gland and delivering venom to the base of posterior maxillary teeth with varied morphologies (simple, enlarged, single, multiple, shallowly or deeply grooved, etc.; Figure 1.2, bottom). Venom, which initially was largely stored intracellularly, is then exocytosed and travels through a duct to the rear teeth, where it is introduced into prey tissues. Whereas front-fanged snakes deliver venom rapidly via a pair of enlarged hollow fangs, rear-fanged snakes may introduce venom more slowly (Kardong and Lavín-Murcio, 1993) but at multiple sites via numerous puncture wounds produced as the snake chews on prey. For example, the green vine snake (*Oxybelis fulgidus*), a nonconstricting rear-fanged snake, grasps and holds prey (mouse or lizard) until it becomes quiescent; during this period, obvious adductor muscle contractions without concomitant movement of prey are observed, which could assist venom delivery (unpublished observation). Brown treesnakes (*Boiga irregularis*) use both constriction and venom when subduing prey (Mackessy et al., 2006; personal observation); lizards are held in the jaws without constriction until quiescent, while mice are immediately constricted. Differential behavioral strategies utilized when feeding/biting (e.g., Deufel and Cundall, 2006), as well as differences in venom apparatus architecture and biochemical composition of the venom, can greatly influence the outcome of human envenomations by colubrid snakes, some of which may be quite serious. Whereas a front-fanged snake such as a rattlesnake can initiate and complete a strike in less than 0.5 s (Kardong and Bels, 1998), most colubrid snakes cannot deliver a large bolus of venom rapidly, and contact (bite) time appears to be a significant determinant of severity of envenomation by colubrid snakes (Mackessy, 2002a).

A specialized venom apparatus, found among lizards only in members of the family Helodermatidae (Figure 1.1A), is both unusual and enigmatic (reviewed in Beck, 2005). Modified

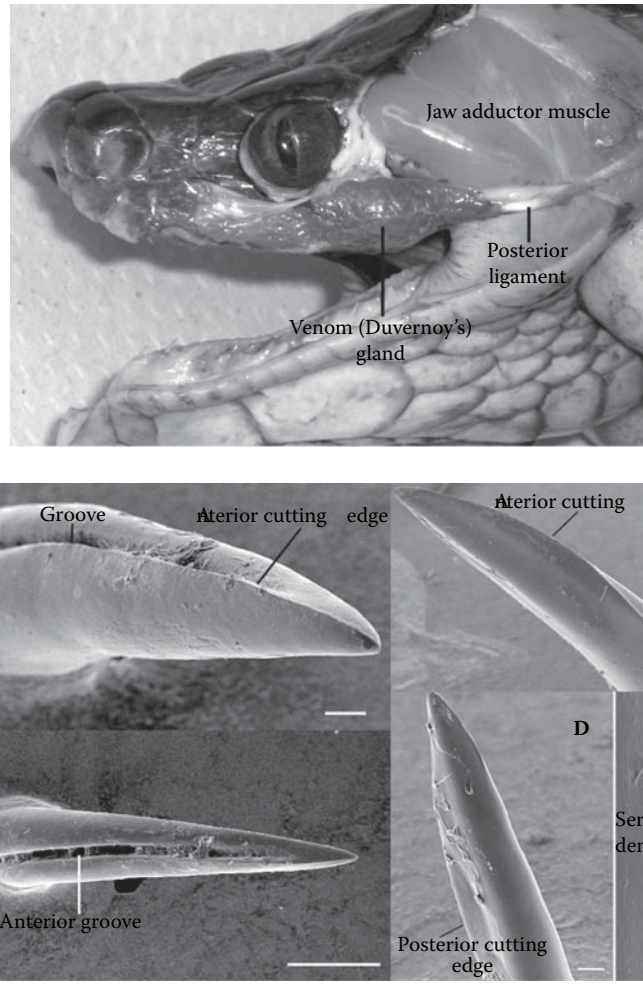


FIGURE 1.2 Reptile glands and teeth. Top: Venom gland of the brown treesnake (*Boiga irregularis*); the skin has been removed from the lateral surface of the head. Muscle fibers do not insert directly on or around the venom gland. (Photograph by C. Rex.) Bottom: Scanning electron micrographs of representative teeth of several squamate reptiles. (A and B) Rear maxillary fang of mangrove catsnake (*Boiga dendrophila*)—note the deep groove, characteristic of many *Boiga* sp. (C) Rear fang of false water cobra (*Hydrodynastes gigas*). (D) Rear fang of night snake (*Hypsiglena torquata*)—note that the cutting edge may be either anterior or posterior in colubrids. (E) Anterior edge of mandibular tooth, crocodile monitor (*Varanus salvadorii*), a large nonvenomous varanoid lizard—note serrated cutting edge, characteristic of most varanids. Scale bars: A, D, and E, 100 μm; B and C, 500 μm.

submandibular glands on the lower jaw produce a complex venom that is released via ducts leading to the base of grooved mandibular teeth. Venom is also primarily stored intracellularly, and as for rear-fanged snakes, delivery of significant volumes of venom requires much longer contact time than is needed by front-fanged snakes. Venoms from helodermatid lizards also contain peptide toxins known as exendins, of which one, Exenatide, has become the "poster child" for development of novel drugs from reptile venom components (e.g., Heine et al., 2005). In order to approach novel venom investigations rationally and effectively, it is important to understand the basics of how venomous organisms use their venoms in a natural predator-prey context. Chapter 3 provides greater detail on comparative aspects of venom apparatus morphology.

III. SOURCES OF VARIATION IN VENOM COMPOSITION

Many reports in the literature have documented different levels of variation in composition of venoms, among major and minor taxonomic groups, between different parts of the same population of one species, during different ages of the animal, and several other factors (see Chippaux et al., 1991, for a review). Venoms can be quite different, at both macro- and microvariation levels, but they also share many compounds across broad taxonomic levels. As venomous reptiles co-opted various regulatory molecules from numerous metabolic pathways and conscripted them as venom constituents (e.g., Fry, 2005), the "evolutionary selection" seems to have been somewhat limited, and venom proteins belong to a relatively small number of protein families (Calvete et al., 2007). However, once conscripted, this limited diversity of proteins has undergone rapid evolution *in situ*, resulting in the production of myriad activities within a single conserved molecular fold. This common motif is seen repeatedly among venom constituents, particularly among the three-finger toxins (3FTXs) (e.g., Kini, 2002; Pawlak et al., 2006, 2009), the phospholipases A_2 (Nakashima et al., 1995; Kini, 1997; Chuman et al., 2000), many serine proteases affecting hemostasis (Deshimaru et al., 1996; Serrano and Maroun, 2005), venom CRISPs (Yamazaki and Morita, 2004), and disintegrins (Juárez et al., 2008).

Venoms can and do vary tremendously in composition, but the absolute mechanisms controlling and producing this variation are poorly understood (but see Earl et al., 2006). Because venoms are trophic adaptations that facilitate handling of prey, their effects on different organisms (including humans) are quite variable, dependent not only on dose but also on the variant molecules contained in a given venom. On the one hand, venoms of some sea snakes (family Elapidae) can be exceedingly simple in composition, containing only two major venom protein families, three-finger α-neurotoxins and phospholipases A_2 (see *Laticauda*, Figure 1.3A). On the other hand, venoms of many front-fanged snakes, such as mambas (family Elapidae) and rattlesnakes (family Viperidae), may contain fifty to one hundred protein and peptide components representing ten to twenty venom protein families (Perkins et al., 1993; Perkins and Tomer, 1995; Sanz et al., 2006). Among the approximately one thousand species of advanced snakes (Caenophidia) that produce venoms, a wide variety of members of these protein families are expressed in the venoms, and many factors interact to determine specific venom composition.

A. Phylogeny and Taxonomic Relationships as a Source of Variation

Though venom composition varies, often significantly, in composition between species (e.g., Tu, 1982, 1991; Ménez, 2002), more closely related species of reptiles generally tend to have venoms that are more similar in composition than do more distantly related venoms. However, the phylogenetic component of venom variation has only been incompletely explored, and a comprehensive analysis of venom composition and phylogeny, using species representative of the major diverse groups, would be very informative on just how important phylogenetic effects actually are. In general, however, dominance of the major protein families found in venoms follows broad phylogenetic trends; for example, at the family level, elapid venoms share more similarities within the family relative to composition in viperid snake venoms. In elapid venoms, smaller toxins predominate, particularly 3FTXs and phospholipases A_2, whereas in viperid venoms, higher-mass enzymatic toxins are prevalent (Figure 1.3). Venoms of the polyphyletic family "Colubridae" are more variable; some, like several species of *Boiga*, produce venoms rich in 3FTXs, while in other species, such as *Alsophis* and *Lioheterodon*, 3FTXs are apparently absent from the venom (Figure 1.3A). Most "colubrid" venoms assayed contain some enzymatic components, commonly metalloproteases and acetylcholinesterases (Hill and Mackessy, 2000; Mackessy, 2002b); phospholipases A_2 do not appear to be broadly distributed among colubrid venoms (but see Huang and Mackessy, 2004).

Viperid venoms are qualitatively and quantitatively very different than most elapid venoms (Figure 1.3B). The prominence of higher molecular weight components, primarily hydrolytic

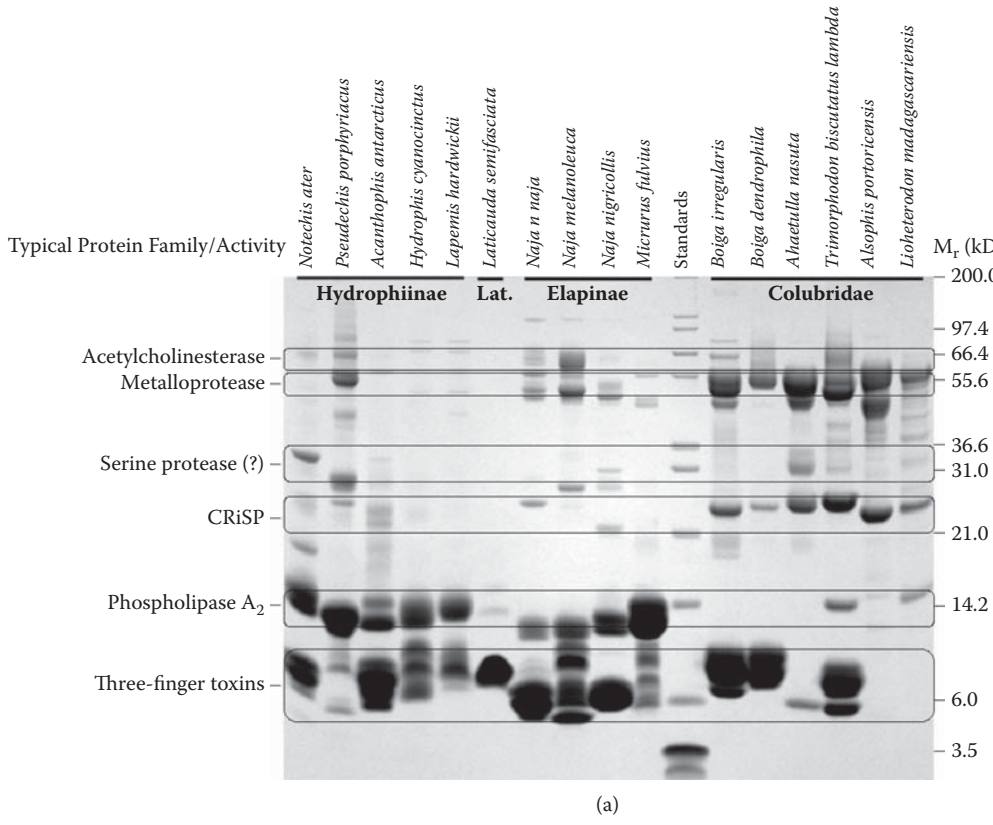
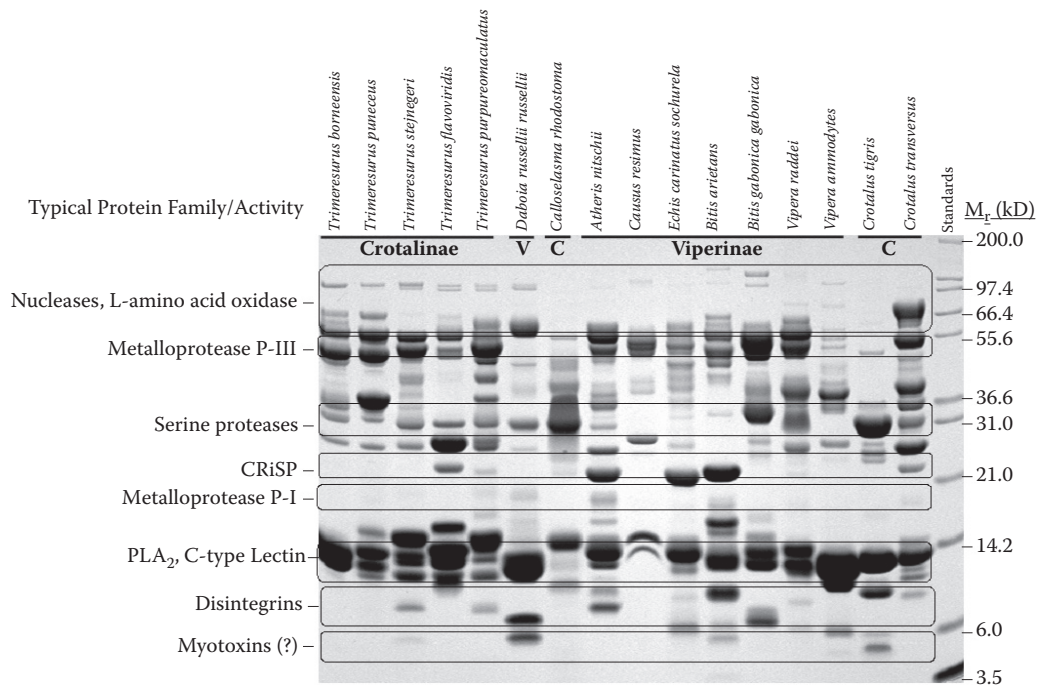


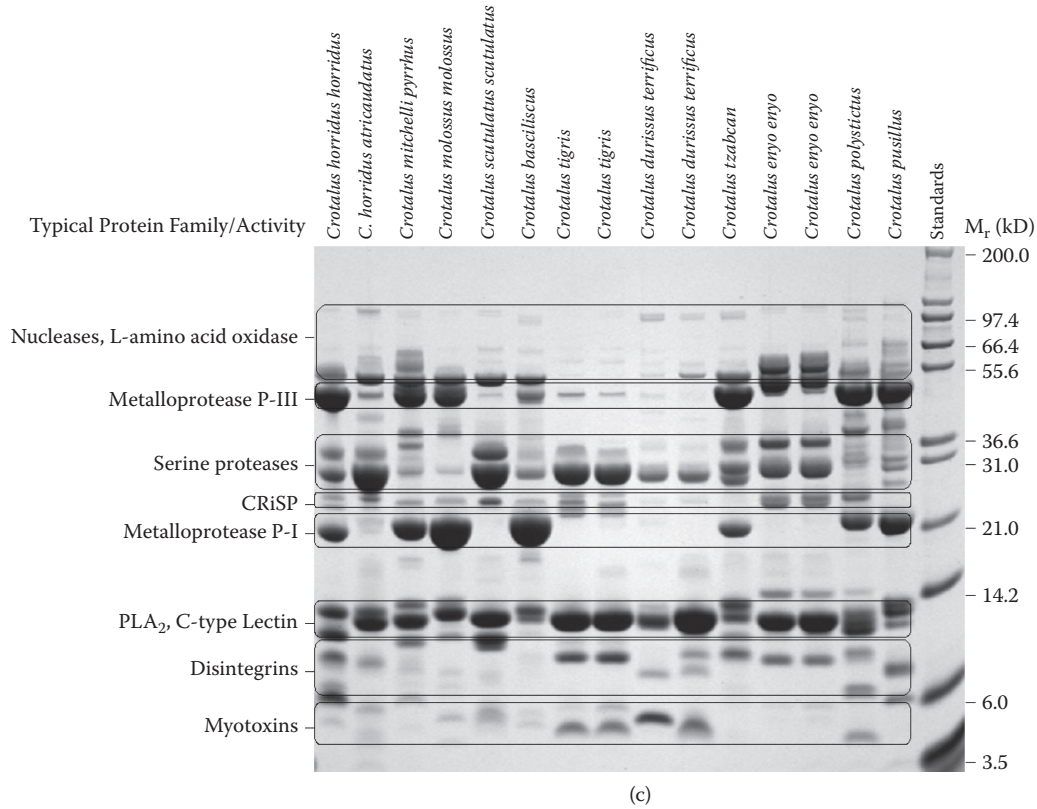
FIGURE 1.3 SDS-PAGE comparison of major venom components in the main clades of venomous snakes. (a) Representatives of the families Elapidae, subfamilies Elapinae, Laticaudinae (Lat.), and Hydrophiinae, and the "Colubridae." (b) Family Viperidae, subfamilies Crotalinae (C) and Viperinae (V). (c) Family Viperidae, subfamily Crotalinae—rattlesnakes. Each lane contains 24 μg venom; 12% acrylamide NuPage gels and MES (2-(*N*-morpholino)ethanesulfonic acid) running buffer (Invitrogen) were used. Major protein families are given on the left, and relative molecular masses (M_r) are on the right of each gel. Ovals enclose bands that are typical of protein families indicated, based on published masses; however, not all bands within a given oval are representatives of indicated families, some protein families are not indicated, and not all bands are identified. Gel C is from Mackessy (2008). See text for discussion of differences.

enzymes, is apparent, and serine proteases (thrombin-like, kallikrein-like, arginine esterase, etc.) dominate the mid-mass ranges (~28–36 kDa), which are typically missing from elapid and colubrid venoms. In general, what one notices is that the pattern of mass distributions within a family is more similar than between families. This predominance of enzymatic components in viperid venoms is strongly supported by many proteomic studies as well (e.g., Nawarak et al., 2003; Li et al., 2004; Serrano et al., 2005; Sanz et al., 2006, 2008; Angulo et al., 2008). A comparison of the families of proteins present in the major taxa of venomous reptiles highlights these trends noted above (Tables 1.1–1.4), and it is apparent that though distinct differences occur between species and families, there are many venom components that are broadly shared, indicating that evolution of venoms among reptile lineages has not been completely random or unrelated.

But phylogenetic consistency is only part of the overall pattern. Venom composition within a well-defined evolutionary lineage, the rattlesnakes (family Viperidae, *Crotalus* and *Sistrurus*), does *not* strictly follow phylogeny but instead appears to follow one of two specific trends, which may be mutually incompatible, independent of close phylogenetic relatedness (Mackessy, 2008). Type I venoms showed high levels of P-I and P-III metalloproteases and were less toxic than Type II venoms,



(b)



(c)

FIGURE 1.3 (continued).

TABLE 1.1
Some Common Components of *Heloderma* Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity
		Enzymes	
Hyaluronidase	73	Hydrolysis of interstitial hyaluronan	Decreased interstitial viscosity
Serine proteases	28–63	Kallikrein-like	
Gilatoxin/horridum toxin	31–33	Kallikrein-like; releases bradykinin	Induces rapid hypotension
Phospholipase A_2 enzymes (Group III)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage
		Nonenzymatic Proteins/Peptides	
CRISP—helothermine	25	May induce hypothermia	Lethargy, paralysis; role in prey capture (?)
Nerve growth factor		Stimulates neuron growth	Unknown
Exendins 1–4	3.5–4.0	Bind to VIP receptors, GLP-1 receptors; stimulate amylase/insulin release, hypotension, etc.	Envenomation role unclear—relation to periodic fasting (?)
Gilatide (Exendin 4 fragment)		Binds to GLP-1 receptor; improves memory	Role in predator avoidance conditioning (?)
		Smaller Organic Compounds	
Serotonin		Neurotransmitter	Mediates inflammation, vasodilation, etc.

Note: 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 venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

Source: Based on Beck (2005).

which were the most toxic rattlesnake venoms and which had low to no metalloprotease activity. These gross differences in venom composition can be seen following one-dimensional SDS-PAGE (Figure 1.3C): the highly hemorrhagic and tissue-damaging venoms of *C. atrox*, *C. molossus*, and *C. ruber* (Type I) show prominent P-I and P-III bands, and the highly toxic venoms of *C. tigris* and *C. durissus terrificus* (Type II) lack these bands. To an extent, this Type I/Type II dichotomy also occurs globally, as elapid venoms typically are quite toxic and rich in smaller toxins but poor in metalloproteases (and other larger enzymatic components), while the converse is generally true for viperid venoms. What these broad patterns of venom composition variation indicate is that there are other factors that may be more important determinants of absolute venom composition and specific venom gland gene expression than phylogeny. In fact, the presence of genes encoding 3FTXs in venom gland transcriptomes from viperids (Junqueira-de-Azevedo et al., 2006; Pahari et al., 2007), but not the translated toxin in the proteome of the same species (Sanz et al., 2006), suggests that there is a potential for much greater genetic identity of the venom gland genome among venomous species than has been previously acknowledged. That the proteome of venomous reptiles can vary so significantly indicates that many other factors determine which venom genes are translated into the final product utilized by the snake or lizard.

B. Age as a Source of Variation

Age affects several parameters of venom, most obviously overall yield. Volume and total dry weight of venom produced increase exponentially with age/size in several species (Klauber, 1956;

TABLE 1.2
Some Common Components of Colubrid Snake Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
		Enzymes		
Phosphodiesterase (low activity)	94–140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and trinucleotides; hypotension/shock (?)	Mackessy, 1998, 2002; Aird, 2002
Acetylcholinesterase	55–60	Hydrolysis of acetylcholine	Depletion of neurotransmitter; tetanic paralysis (?)	Broaders and Ryan, 1997; Hill and Mackessy, 2000
Snake venom metalloproteinases: M12 reprolysins		Hydrolysis of many structural proteins, including basal lamina components	Hemorrhage, myonecrosis, prey predigestion	Hill and Mackessy, 2000; Kamiguti et al., 2000; Komori et al., 2006; Peichoto et al., 2007
P-III	48–55			
P-II (?)	38			
Serine proteases	36	Hydrolysis of fibrinogen (α and β subunits)	Hemostasis disruption (?)	Assakura et al., 1994
Phospholipase A_2 enzymes (Group I)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage	Hill and Mackessy, 2000; Huang and Mackessy, 2004
		Nonenzymatic Proteins/Peptides		
Cysteine-rich secretory proteins (CRiSPs)/helveprins	21–29	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	Yamazaki and Morita, 2004
Dimeric three-finger toxins	17	Potent inhibitor of neuromuscular transmission; show taxon-specific effects	Rapid immobilization of prey, paralysis, death	Pawlak et al., 2009
Three-finger toxins, α-neurotoxins	6–9	Potent inhibitors of neuromuscular transmission; may show taxon-specific effects	Rapid immobilization of prey, paralysis, death	Fry et al., 2003; Lumsden et al., 2005; Kini, 2002; Pawlak et al., 2006

Note: 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 colubrid venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

Mackessy, 1985, 1988; Mirtschin et al., 2002; Mackessy et al., 2003, 2006). Because head size (and gland volume) increase with age, this general trend is expected for essentially all venomous reptiles, and yields of adult snakes may be one to two orders of magnitude greater that those of neonates. Protein concentration may also vary with age, and lyophilized *Boiga irregularis* venom from neonate snakes had approximately one-half the protein content (w/w) of venoms from adult snakes (Mackessy et al., 2006). However, in addition to allometric increases in overall venom quantity, venom may also vary ontogenetically in composition. For many rattlesnakes (Mackessy, 1985, 1988, 1993, 1996, 2008; Gutiérrez et al., 1991; Mackessy et al., 2003, 2006) and Latin American pit vipers (e.g., *Bothrops atrox*: Guércio et al., 2006), this results in venoms with very different biochemical composition and pharmacology at different times in the life history of an individual snake.

Venom ontogeny has been noted for several species, with lower protease activity noted in venoms from neonate/juvenile snakes, and age-related differences in composition are apparently more

TABLE 1.3
Some Common Components of Elapid Snake 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 trinucleotides; 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
Acetylcholinesterase	55–60		Anderson and Dufton, 1998	
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
Prothrombin activators				
Group C	>250	Activate factor VII or factor X	Induce DIC, highly toxic	Rosing and Tans, 1991, 1992
Group D	45–58	Activate factor X		
(Group A)	~45	Activates factor X		Gao et al., 2002
Snake venom metalloproteinases: M12 reprolysins		Hydrolysis of many structural proteins, including basal lamina components	Hemorrhage, myonecrosis, prey predigestion	Fox and Serrano, 2005
P-III	43–60			
Phospholipase A_2 enzymes (Group I)	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides, fibrinogen, etc.	Myotoxicity, myonecrosis, lipid membrane damage	Kini, 1997, 2003
		Nonenzymatic 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 (?)	Hogue-Angeletti et al., 1976; Siigur et al., 1987; Koh et al., 2004
PLA_2-based presynaptic neurotoxins (monomeric to tetrameric)	13.5–80	Blocks release of acetylcholine from axon terminus	Potent neurotoxicity; prey immobilization	Bon, 1997

(continued on next page)

TABLE 1.3 (continued)
Some Common Components of Elapid Snake Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
Three-finger toxins, α-neurotoxins, cardiotoxins, fasciculins, etc.	6–9	Potent inhibitors of neuromuscular transmission, cardiac function, acetylcholinesterase, etc.	Rapid immobilization of prey, paralysis, death	Nirthanan and Gwee, 2004; Kini, 2002; Doley et al., 2008
		Smaller Organic Compounds		
Purines and pyrimidines	AMP = 0.347, hypoxanthine, inosine	Broad effects on multiple cell types (?)	Hypotension, paralysis, apoptosis, necrosis (?); prey immobilization	Aird, 2002, 2005

Note: 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 elapid venoms. Specific venoms may not contain all components. (?) indicates hypothetical function or activity.

pronounced among viperids than among elapids. Two prominent changes that occur involve overall toxicity of venom to prey and total metalloproteinase content of neonate vs. adult venoms, with neonate venoms being more toxic but showing much lower levels of metalloproteinase activity. It was proposed that these biochemical differences were related to changes in prey (both taxonomic differences and physical parameters, such as bulkiness), with venoms acting optimally on prey utilized preferentially by a specific age class (Mackessy, 1988). It was also noted some time ago that among northern Pacific rattlesnakes (*Crotalus oreganus oreganus*), this shift in composition included a change from production of higher-mass metalloproteinases (P-III/P-IV) by neonate snakes to a predominance of lower molecular mass metalloproteinases (P-III, P-II, and P-I) in venoms from adult snakes (Mackessy, 1993). This same shift in composition has recently been confirmed in *Bothrops atrox* and *B. asper* by several proteomic studies (Guércio et al., 2006; Alape-Girón et al., 2008), and so it appears that this ontogenetic shift in composition may occur broadly among viperid snakes.

However, not all rattlesnakes or Latin American vipers show this same pattern of ontogenetic variation in metalloproteinase content, and this age-related shift is associated with the production of Type I venoms (see above) but not with Type II venoms. Examples of this lack of gross change in metalloproteinase production have been noted in *C. o. concolor* (Mackessy et al., 2003) and *C. durissus terrificus* (Gutiérrez et al., 1991). In both species, both juvenile and adult snakes produce very toxic venoms. In *C. o. concolor*, this constraint on venom composition may limit prey selection and breadth of foraging activity.

C. Geography as a Source of Variation

Venoms may also vary in composition as a function of geographic location. The biological significance of these differences is not clear, but they may result from the occurrence of one of two (or more) mutually exclusive evolutionary "strategies" similar to the Type I/II dichotomy noted above. Clinically, these geographic differences can have profound impacts, as the venom of the same subspecies of snake (such as *C. s. scutulatus*) from different localities may be very different in toxicity and metalloproteinase activity (Glenn and Straight, 1978; Glenn et al., 1983), resulting in very different patient presentations following envenomation. A similar difference in composition with locality

TABLE 1.4
Some Common Components of Viperid 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 trinucleotides; 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
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 (groups A and B)	Hemorrhage, myonecrosis, prey predigestion	Fox and Serrano, 2005, 2008
P-III	43–85			
P-II	25–30			
P-I	20–24			
Serine proteases				
Thrombin-like	31–36	Catalysis of fibrinogen hydrolysis	Rapid depletion of fibrinogen; hemostasis disruption	Markland, 1998; Swenson and Markland, 2005
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
		Nonenzymatic 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

(continued on next page)

TABLE 1.4 (continued)
Some Common Components of Viperid Venoms and General Characteristics

Component Name	Approximate Mass (kDa)	Function	Biological Activity	References
PLA_2-based presynaptic neurotoxins (2 subunits, acidic and basic)	24	Blocks release of acetylcholine from axon terminus	Potent neurotoxicity; prey immobilization	Aird and Kaiser, 1985; Ducancel et al., 1988; Faure et al., 1994
C-type lectins	27–29	Binds to platelet and 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 and 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

Note: 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 or activity.

has been observed for the southern Pacific rattlesnake (*C. o. helleri*), and for both *C. s. scutulatus* and *C. o. helleri*, the high toxicity of venoms from some snakes is due to the expression of Mojave toxin genes and a concomitant high level of Mojave toxin and homologs in the venom (Wooldridge et al., 2001; French et al., 2004). Variation in composition in Latin American viperids has also been long noted (e.g., Jiminez-Porras, 1964), and J. M. Gutiérrez and colleagues have since greatly extended these studies (i.e., Saravia et al., 2002; Alape-Girón et al., 2008; Angulo et al., 2008).

Regional variation in phospholipase A_2 and peptide myotoxin components has also been noted for several viperids. Creer et al. (2003) noted significant variation in phospholipase A_2 (PLA_2) isoforms in the venoms of *Trimeresurus stejnegeri* from Taiwan and nearby islands, and these geographic differences were ascribed to differences in prey taken. While this geographic difference in PLA_2 isoform content may in fact be prey driven, one shortcoming of this and most studies is that there are no data indicating whether specific isoforms might have greater effects against particular prey types (see below). Geographic variation in α-neurotoxin isoform type and overall content was recorded for *Naja atra* and *Naja kaouthia* from China, Thailand, and Taiwan (Wei et al., 2003). This variation did not appear to be phylogenetically or clinally based, and the authors suggested

that variation may be associated with differences in prey or habitat. Similar to the study with venom PLA_2 isoform variation, the biological significance of this variation is unclear.

D. Diet and Venom Compositional Variation

As trophic adaptations, it is expected that venom composition would be related to some aspects of diets, because different species of prey animals are differentially sensitive to various types of toxins. Further, if venomous reptiles are significant predators in a given ecosystem, one might expect some type of coevolutionary adjustments between predator and prey species, perhaps leading to an "arms race" among the interacting species. Venoms of reptiles should be subject to selective forces shaping effectiveness toward particular prey, and the result of selection may manifest as taxon-specific toxicity of venoms or venom components.

Correlations between age-related changes in diet and venom composition have been inferred for many species (i.e., Daltry et al., 1996a), but the causal link between these features that support such claims is for the most part weak. The correlation in Pacific rattlesnakes (*C. o. helleri* and *C. o. oreganus*) was considerably strengthened by the demonstration of greater toxicity of the neonate venoms toward preferred prey, in this case lizards (Mackessy, 1988), which accompanied concomitant changes in venom composition. A similar relationship between venom toxicity and preferred prey was observed for numerous species of South American coral snakes (*Micrurus*: Jorge da Silva and Aird, 2001) and *Micrurus nigrocinctus* from Costa Rica (Urdaneta et al., 2004). In both studies, venoms were most effective against the preferred (ectothermic) prey.

However, some species of rattlesnakes, such as *C. o. concolor* (Mackessy et al., 2003) and *C. d. terrificus* (Gutiérrez et al., 1991), do not show an age-related difference in toxicity and metalloproteinase activity level as do related conspecifics, even though diet changes with age. For *C. o. concolor*, which occurs in a rather harsh temperate climate (southern Wyoming), the lack of significant metalloproteinase activity in venoms may limit the size of prey taken, a hypothesis supported by diet data (Mackessy et al., 2003). Interestingly, differences in myotoxin-a homolog levels do vary ontogenetically, and neonate snakes, which feed nearly exclusively on lizards, produce very low levels of myotoxins in their venoms. It is unknown whether peptide myotoxins are more effective on specific prey taxa.

The most striking examples of taxon-specific differences in susceptibility to venoms and venom toxins, which are almost certainly tied to diet, occur among colubrid snakes. The brown treesnake (*Boiga irregularis*) is an arboreal snake that largely feeds on birds and lizards, although mammals are taken opportunistically as well. However, as noted above, the way prey is handled varies by taxon, and mammals are typically killed via constriction. Analysis of the crude venom showed that toxicity (IP LD_{50}) to birds and lizards was very different than for mammals, and venom from adult snakes was ~15 times more toxic to birds and lizards than to mice (Mackessy et al., 2006). The venom of *B. irregularis* contains a plethora of low-mass (7–10 kDa) proteins, which were suspected to be neurotoxins (or other 3FTXs), and monomeric 3FTXs occur in the venom of the related *B. dendrophila* (Lumsden et al., 2005; Pawlak et al., 2009). In a very recent study, irditoxin (*B. irregularis* dimeric toxin), the first described member of a covalently linked dimer subfamily of 3FTXs, was shown to explain this taxon specificity (Pawlak et al., 2009). Irditoxin, which accounts for ~10% of the total venom protein content (w/w), was rapidly lethal to birds (0.22 μg/g IP) and lizards (0.55 μg/g IP) but was nontoxic to mice at doses up to 25 μg/g (highest dose tested). Venom yields from large snakes commonly exceeded 20 mg, and based on the action of irditoxin alone, this amount of crude venom could kill 9 kg-equivalents of bird (domestic chicken). It should be clear that during a predatory strike on a native bird, in which much less than 20 mg venom is expected to be expended, the potency of this venom is more than sufficient to immobilize or kill prey rapidly. It is likely that several other species of *Boiga*, as well as other rear-fanged colubrids, produce venoms with homologous dimeric 3FTXs. Further, because of the lower complexity of the venom proteome of most colubrids relative to viperids and elapids, as well as the dependency of many species on

ectothermic vertebrates or invertebrates as prey, colubrid venoms should serve as convenient models for assessing the relationship of venom composition to diet.

E. Other Possible Sources of Variation

1. Seasonal Variation

Although seasonal variation has been suggested to occur in composition of venom from several species, the evidence suggesting this supposition is lacking. In fact, one study using isoelectric focusing of venoms, which should be sensitive enough to detect minor differences, suggested just the opposite. For three species of rattlesnakes (*Crotalus atrox*, *C. molossus*, and *C. oreganus* (formerly *viridis*) *helleri*), no differences were seen in protein banding patterns of samples collected from the same snake over a period of 20 months (Gregory-Dwyer et al., 1986). This seasonal constancy in venom composition is consistent with observations on venoms from *C. viridis viridis* from Weld Co., Colorado, as well as observations on composition of venoms taken from a single adult individual (many different species) in captivity over several years (Mackessy, unpublished observation). Though there is a general belief that venoms do vary seasonally, the available evidence is scant. An earlier report suggested that venoms from *Vipera ammodytes* showed differences between summer- and winter-obtained venoms, with summer venoms containing two additional bands (lethal proteins) that were missing from samples collected from captive snakes in winter (Gubenšek et al., 1974). Seasonality as a source of compositional variation is a factor that requires further study.

2. Sex-Based Variation

Results of earlier studies have suggested that little to no differences in venom composition occur between the sexes of the same species (see Chippaux et al., 1991, for references). An isoelectric focusing study of venoms from a large number of *Calloselasma rhodostoma* noted that one band was present in venoms from females but absent from male venoms (Daltry et al., 1996b), but this band was not identified. However, recent studies using a proteomics approach (two-dimensional electrophoresis, mass spectrometry) indicate that at least subtle differences in venom composition exist between male and female *Bothrops jararaca* (Menezes et al., 2006; Pimenta et al., 2007). Using SDS-PAGE, sex-specific bands were noted, with male snakes only producing venoms with a 100 kDa protein, and female snakes' venom contained a gelatin-degrading component (likely a metalloproteinase) of ~25 kDa that was absent from male snake venoms. Following two-dimensional electrophoresis, significant differences between male and female venoms in spot intensities were also noted for several different protein groups (not identified), with female venoms generally showing more intense spots. Differences in crude venom activities toward several protein and peptide substrates were somewhat variable, but male venoms were less active toward casein and more active toward D-Val-Leu-Lys-pNA, while female venoms showed the opposite trend (Menezes et al., 2006). A MALDI-TOF-MS study of bradykinin-potentiating peptides (BPPs) identified significant individual variation in numbers and levels of this peptide, and four peptides were found only in female snake venoms (Pimenta et al., 2007). These four novel peptides were found to be cleaved BPPs that lacked the C-terminal portion (Gln-Iso-Pro-Pro), and they are apparently inactive BPPs. The biological significance (if any) of these sex-based differences in venom composition are unclear, but it is apparent that at least some sex-based differences may be expected in venoms from other species of reptiles.

IV. CONCLUSIONS

Toxinology as a field of study has grown tremendously over the last 10 to 20 years, in large part driven by the technical advances in genomics and proteomics. As these tools are utilized to probe venoms from more species in ever-increasing detail, it is important to keep in mind that these venoms and the toxins comprising them have evolved in a specific biological context, largely dominated by numerous trophic and predator-prey interactions. It is now feasible to expect full proteome and

venom gland genome catalogs to be produced for many species of venomous reptiles within the next 10 years, and these complete descriptions of venom compositional diversity will contribute greatly to our understanding of the mechanisms favoring the evolution of specific venom profiles among specific taxa. The evaluation of the biological activities of the many isoforms, presently known and yet to be described, remains a daunting task, but this information is necessary to identify the biological roles of specific components and to place venom compositional diversity into a more meaningful biological context. One of the wonderful aspects of toxinology is that there is no limit to the number of interesting questions concerning venomous reptiles and their venoms. Our job is to pose and pursue those questions, and it is hoped that this book will contribute to that pursuit in some small way.

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