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SYMPOSIUM

Understanding Biological Roles of Venoms Among the Caenophidia: The Importance of Rear-Fanged Snakes

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Synopsis Snake venoms represent an adaptive trophic response to the challenges confronting a limbless predator for overcoming combative prey, and this chemical means of subduing prey shows several dominant phenotypes. Many front-fanged snakes, particularly vipers, feed on various vertebrate and invertebrate prey species, and some of their venom components (e.g., metalloproteinases, cobratoxin) appear to have been selected for "broad-brush" incapacitation of different prey taxa. Using proteomic and genomic techniques, the compositional diversity of front-fanged snakes is becoming well characterized; however, this is not the case for most rear-fanged colubroid snakes. Because these species consume a high diversity of prey, and because venoms are primarily a trophic adaptation, important clues for understanding specific selective pressures favoring venom component composition will be found among rear-fanged snake venoms. Rear-fanged snakes typically (but not always) produce venoms with lower complexity than front-fanged snakes, and there are even fewer dominant (and, arguably, biologically most relevant) venom protein families. We have demonstrated taxon-specific toxic effects, where lizards and birds show high susceptibility while mammals are largely unaffected, for both Old World and New World rear-fanged snakes, strongly indicating a causal link between toxin evolution and prey preference. New data are presented on myotoxin a, showing that the extremely rapid paralysis induced by this rattlesnake toxin is specific for rodents, and that myotoxin a is ineffectual against lizards. Relatively few rear-fanged snake venoms have been characterized, and basic natural history data are largely lacking, but directed sampling of specialized species indicates that novel compounds are likely among these specialists, particularly among those species feeding on invertebrate prey such as scorpions and centipedes. Because many of the more than 2200 species of colubroid snakes are rear-fanged, and many possess a Duvernoy's venom gland, understanding the nature of their venoms is foundational to understanding venom evolution in advanced snakes.

Introduction: the selective advantages of venoms

Critical evolutionary innovation—trophic adaptations facilitating prey handling

Mechanical versus chemically mediated feeding mode

The suborder Serpentes (snakes) consists of more than 3500 species that inhabit terrestrial, arboreal, fossorial, aquatic, and marine environments throughout the world (Greene 1997), and approximately 85% of all extant snakes belong to the superfamily Colubroidea (Lawson et al. 2005; Pyron et al. 2011): the families Homalopsidae (53 species), Lamprophiidae (308 species), Elapidae (361 species), Viperidae (337 species), and Colubridae (1853 species) (reptiledatabase.org; accessed April 2016). Due to this immense diversity, snake evolution has been widely studied (Castoe et al. 2013; Vonk et al. 2013), as has ecology (Wastell and Mackessy 2011, 2016; Barbour and Clark 2012; Shipley et al. 2013; Putman et al. 2016), physiology (Secor 2008; Riquelme et al. 2011; Holding et al. 2014; Lourdais et al. 2015), toxinology (Mackessy 1988, 2008, 2010; Fry et al. 2003a; Calvete et al. 2012; Lomonte et al. 2014; Margres et al. 2014), and behavior (Clark 2004a; Saviola et al. 2011, 2012; Smith et al. 2015). From a trophic standpoint, snakes display extraordinary feeding strategies, and their ability to consume large prey items allows

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them to feed infrequently. Many snakes commonly consume prey weighing 20% of their own body mass, and some venomous snakes have been reported to subdue and consume prey that exceed their own body mass by 50% or more (Greene 1984, 1992). However, swallowing large, potentially dangerous prey necessitates subduing the quarry without sustaining injury in the process, and snakes have evolved various means to facilitate successful prey capture.

The vast majority of non-venomous snakes utilize constriction or body pinning to restrain prey, whereas other species simply employ a jaw-holding technique, or swallow prey whole with little to no body involvement (Greene 1997; Cundall and Greene 2000; Bealor and Saviola 2007). Coachwhips (*Masticophis flagellum*), for example, subdue and consume rodent prey by pinning and swallowing them (Werler and Dixon 2000; Bealor and Saviola 2007); however, this behavior results in significantly longer capture latencies and higher frequencies of prey escape compared to constriction by non-venomous snakes (Bealor and Saviola 2007). In laboratory trials, multiple predatory strikes may be necessary for a non-venomous, non-constricting snake to grasp and subdue prey successfully, but this opportunity is highly unlikely in the wild. Constricting prey is undoubtedly advantageous, as multiple body loops provide more contact with prey and limit the opportunity of prey escape, but it still requires the snake to be in constant contact with prey and increases the likelihood of sustaining injury from the retaliating animal.

Venomous snakes, on the other hand, subdue prey with venom. This complex mixture of proteins and peptides has allowed for the trophic transition from a mechanical (body pinning and constriction) to a chemical (venom) means of immobilizing prey. Venoms exhibit tremendous diversity and may vary significantly based on phylogenetic affinities, geographic localities, snake age, and diet (for reviews see Chippaux et al. 1991; Mackessy 2010). Yet, all venoms fulfill the same basic biological role—facilitating prey acquisition. Unlike non-venomous snakes, many front-fanged venomous species (vipers) do not require constant contact to subdue prey, and during a predatory episode, envenomation occurs via a rapid strike, where the fangs are embedded into prey tissue and a bolus of toxins and enzymes are rapidly delivered. Prey may be released and later relocated, as seen in many viperids (Chiszar et al. 1992), or held and quickly dispatched by fast-acting neurotoxins and other venom compounds, as in many elapids (Mackessy 2010). Rear-fanged venomous snakes often require slightly longer contact time with prey to deliver venom via a low-pressure delivery system (see below); however, the presence of prey-specific toxins in several species (Pawlak et al. 2006, 2009; Heyborne and Mackessy 2013) leads to quick immobilization and debilitation of commonly consumed prey such as lizards and birds.

Venom systems—high versus low pressure systems

To some, the broad classification of ''venomous snake'' might suggest that few differences exist between families and/or species, yet fundamental differences in venom delivery systems, feeding strategies, and venom composition are apparent. In snakes, venoms are produced and stored in paired specialized glands located in the temporal region of the upper jaw, known as either the venom gland in snakes belonging to the families Atractaspididae, Elapidae, and Viperidae (front-fanged venomous snakes; Mackessy and Baxter 2006), or the Duvernoy's venom gland for rear-fanged venomous snakes (Taub 1966; Saviola et al. 2014). Differences in gland structure and size, as well as fang morphology, have resulted in high-pressure and low-pressure venom delivery systems (Kardong and Lavin-Murcio 1993). In front-fanged snakes, the venom gland is relatively large and often contains a basal lumen that is capable of storing sufficient quantities of venom ready for immediate deployment (Kochva et al. 1982; Mackessy 1991; Mackessy and Baxter 2006). In vipers, a primary duct connects the venom gland to an accessory gland (which is lacking in rear-fanged snakes) and a secondary duct connects these glands to the base of a pair of hollow fangs. Further, contraction of a specialized slip of the adductor muscle (compressor glandulae) surrounding the venom gland causes immediate pressurization, allowing venom to be rapidly expelled into prey tissue.

In comparison, the homologous Duvernoy's venom gland in rear-fanged venomous snakes is often smaller, and instead of possessing a basal lumen, venom is stored intracellularly and is exocytosed into secretory tubules that flow into one to several luminal ducts. Rear-fanged venomous snakes also lack a compressor muscle surrounding the gland, and a recent histological analysis of the Duvernoy's gland of the South American *Helicops modestus* indicated that this gland is wrapped in connective tissue (Oliveira et al. 2016), as is observed in front-fanged snake venom glands. Further, instead of possessing hollow fangs as observed in front-fanged snakes, rear-fanged venomous snakes exhibit enlarged and/or grooved posterior maxillary teeth, which are not generally capable of delivering

significant quantities of venom rapidly. Instead, venom is introduced into tissue more slowly, although often at multiple sites, and rear-fanged snakes produce multiple puncture wounds by chewing on prey (see below for feeding strategies). A more recent study, however, has suggested that the grooved enlarged rear maxillary teeth (as seen in many *Boiga* species; Mackessy 2010) are capable of delivering venom effectively and rapidly into tissue (Young et al., 2011). Combined, these features result in a low-pressure venom delivery system that delivers significantly lower quantities of venom when compared to the majority of front-fanged species.

Venom extraction

The clear distinction between high- and low-pressure venom delivery systems also requires two very different venom extraction methods. Front-fanged venomous snakes, with the compressor glandulae muscle surrounding the venom gland, and a large quantity of venom stored in the basal lumen, can be easily extracted by manual expression of the venom gland. We have found that anesthetizing front-fanged snakes with isoflurane prior to extraction provides an effective way for quickly manipulating snakes and extracting significant quantities of venoms, and this also minimizes the risk to the handler and the snake. However, venom extraction from rear-fanged venomous snakes is more challenging, time-consuming, and generally results in significantly lower venom yields. We routinely anesthetize rear-fanged snakes with ketamine (Hill and Mackessy 1997; Mackessy et al. 2006), although anesthetics such as Zoletile, Tiletamine, and Zolazepam have also been used (Fry et al., 2003a,b). To stimulate salivation once the animal is anesthetized, an injection of pilocarpine is administered, and venoms are obtained by placing glass micropipettes over the animal's posterior maxillary teeth. Extractions may take up to 30 to 60 min, and venom yields are often a fraction of what is obtained from a front-fanged snake. However, these methods, and especially the use of pilocarpine, have been shown to increase venom yields (Hill and Mackessy 1997; Mackessy et al. 2006; Ching et al. 2012), and provide a safe and effective method for venom extraction for both the handler and the snake.

Venom composition

Snake venoms are complex mixtures of proteins and peptides that exhibit a myriad of biological effects (Mackessy 2010). The majority of venomous snakes are found in the families Colubridae, Elapidae, and Viperidae, and venoms from each of these families exhibit dramatically different compositions, while most species within each family share many toxin families. Although a given venom may contain up to 100 different proteins (including isoforms), the vast majority of venom compounds can be classified into approximately 24 distinct protein families (Table 1). Viperid venoms are rich in enzymes such as phospholipase A_2 (PLA_2), serine proteases (SP), metalloproteinases (SVMP), L-amino acid oxidases (LAAO), and phosphodiesterases (PDE), as well as non-enzymatic disintegrins, cysteine-rich secretory proteins (CRISPs) and C-type lectins (Fig. 1A; Calvete et al. 2009; Mackessy 2010). The potent elapid venoms generally exhibit significantly lower concentrations of SVMPs and SPs, lack disintegrins, and are characterized by abundant PLA_2s and fast-acting, highly specific three-finger neurotoxins (Mackessy 2010; Fernández et al. 2015).

Approximately 700 rear-fanged snakes produce venom, but species whose venoms have been investigated often exhibit relatively simple venom composition compared to front-fanged venomous snakes, and coupled with the low yields and lack of significant toxic effects toward humans (Weinstein et al. 2011), the vast majority of rear-fanged venoms are significantly understudied. The few transcriptomic, proteomic, and biochemical analyses conducted on these venoms show that Duvernoy's venoms of some rear-fanged snakes share characteristics with both viperids and elapids (Fig. 1B; see also Hill and Mackessy 2000; Fry 2005; Ching et al. 2012; McGivern et al. 2014; Junqueira-de-Azevedo et al.

Table 1 Major protein families represented in snake venoms

Three-finger toxins	Cysteine-rich secretory proteins
Phospholipases A_2	Myotoxin a/crotamines
Metalloproteinases	Snake venom growth factors – VEGF, NGF
Serine proteinases	Prokineticins/AVIT proteins
Disintegrins	L-amino acid oxidases
Serine proteinase inhibitors	Waprin/Kunitz BPTI
C-type lectins	Hyaluronidases
Dipeptidyl peptidases	Sarafotoxins
Vespryn/ohanin	Acetylcholinesterases
Exonucleases/PDE 5'-nuclotidases	Bradykinin potentiating peptides and natriuretic peptides
Waglerins	Veficolins
Exendins	

Note that a typical venom contains only 10–15 of these.
Underlined, often most abundant proteins in venoms.

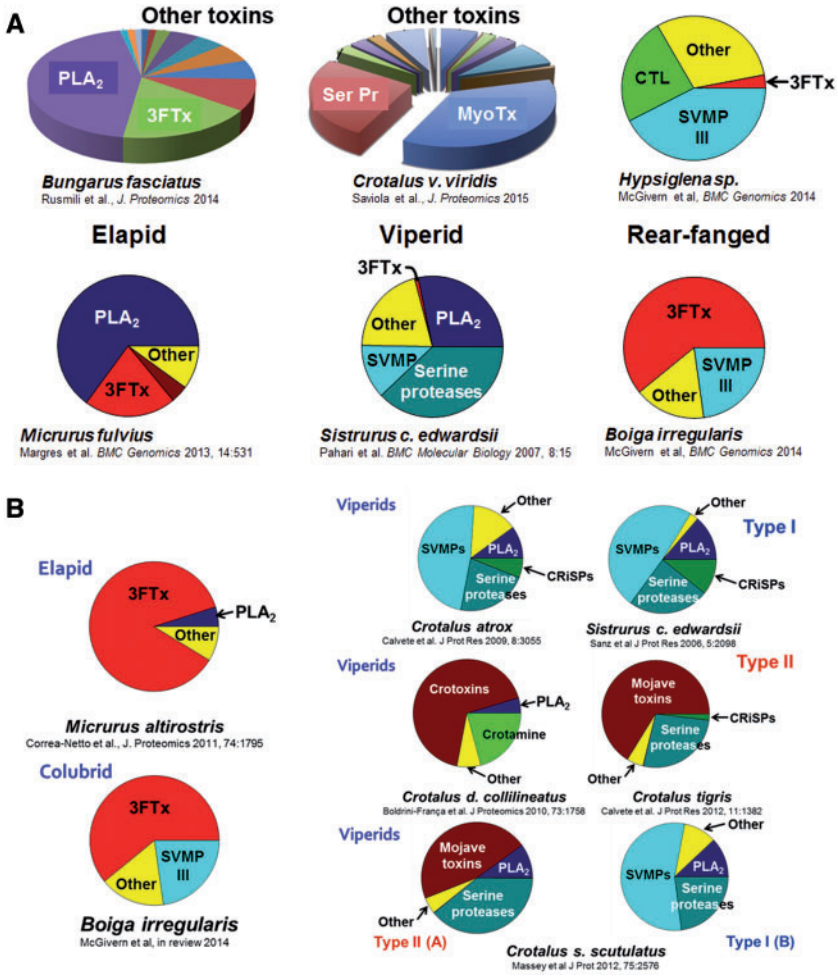

Fig. 1 Simplified comparison of venom composition for colubroid snakes, emphasizing dominant protein families. (**A**) Several examples from major extant clades of snakes. Note that for each species, only a few protein families tend to predominate. (**B**) Variation in venom proteomes between major clades and within the rattlesnakes (*Crotalus* and *Sistrurus*). In the elapid and colubrid examples given, 3FTxs dominate the venom proteome. This toxin family is typically not expressed in viperid venoms. Rattlesnake venoms show two major patterns: type I, dominated by SVMPs and having lower toxicity, and type II, dominated by presynaptic neurotoxins (crotoxin, Mojave toxin, etc.) and having high toxicity. Diagrams derived from sources indicated. CTL, C-type lectin; MyoTx, myotoxin a; PLA_2, phospholipase A_2; SerPr, serine protease; SVMP, snake venom metalloprotease; 3FTx, three-finger toxin.

2016). SVMP, SP, and PLA_2 activities have been documented in many rear-fanged snake venoms (Mackessy 2002; Saviola et al. 2014), and several of these enzymes, as well as non-enzymatic CRISPs and 3FTxs, have been isolated and characterized from rear-fanged venoms (Fry et al. 2003a; Pawlak et al. 2006; Peichoto et al. 2007, 2009; Weldon and Mackessy 2012). However, rear-fanged venomous snakes represent very different evolutionary lineages from viperids and elapids (Pyron et al. 2013; Vidal 2002), and they comprise several families, subfamilies, and hundreds of species and subspecies. Advancements in laboratory techniques and continued investigations into rear-fanged venoms may uncover venoms that exhibit a great deal of complexity, and there is the distinct possibility of identifying novel venom compounds that exhibit unique pharmacological activities (Mackessy 2002; Saviola et al. 2014). Therefore, research examining rear-fanged venoms holds significant promise for novel compound discovery and is critical for refining our understanding of the evolutionary origin of venom systems in squamates.

Venom evolution

Much debate centers on the evolutionary origin of venoms and the events that have promoted their toxic effects. It has been suggested that venoms evolved via the recruitment and duplication of genes with normal physiological function expressed elsewhere in the body (Fry 2005). This supposition has been supported in part by evidence of venom

gene homologs expressed in a variety of non-secretory tissues that encode toxins in the non-venomous Burmese python (Reyes-Velasco et al. 2015). Conversely, Hargreaves and colleagues (2014) have recently suggested that snake venoms evolved from pre-existing salivary proteins confined to the venom gland, and that venom toxins did not evolve from body proteins. At present, it appears most likely that "venom protein" homologs are broadly expressed throughout many tissues of both venomous and non-venomous snakes, but that the actual genes of proteins serving as venom toxins are overexpressed in the venom gland only (Reyes-Velasco et al. 2015; Junqueira-de-Azevedo et al. 2015). These data strongly indicate several points: (1) transcript-based sequences of oral gland transcripts/proteins only, derived from a variety of squamate reptiles (including oral gland secretions as venoms), should be interpreted with great caution, as these "venom precursor" genes are likely normal housekeeping genes; and (2) the "raw material" for the evolution of venom toxins are indeed derived from widely distributed genes with normal physiological roles (Fig. 2).

Function versus biological role

The complex nature of venom is reflected in the presence of numerous venom compounds that not only have discrete functionalities, but also can act in concert with other venom components to promote a myriad of biological effects. Approximately 24 different protein families are represented in reptile venoms, with diverse pharmacologies, and approximately one-half of these are expressed in a single venom. It is interesting to note that some toxins within the same family may have widely disparate pharmacologies, as seen with PLA_2s that can exhibit neurotoxic, myotoxic, and anticoagulant effects. Many activities are represented (Table 1), and the end result of prey envenomation is a general and simultaneous dysregulation of numerous systems necessary for basic life functions. However, the biological role of an individual venom compound may significantly differ from its pharmacology, and a clear example of this can be seen with the disintegrin family of snake venom proteins. Disintegrins are small non-enzymatic proteins common in the venoms of viperid snakes (Calvete et al. 2005; Saviola et al. 2015a) that function by selectively blocking integrin receptors present in cell membranes (Eble et al. 2003; Bolás et al. 2014; Saviola et al. 2016). By blocking integrin $\alpha_{IIb}\beta_3$ on platelets, many disintegrins inhibit platelet aggregation and clot formation, complementing thrombin-like serine proteases and hemorrhagic SVMPs to promote the spread of other toxins throughout the envenomated prey. Interestingly, disintegrins also appear to be the "relocator" molecule in venom, allowing rattlesnakes to discriminate between envenomated and non-envenomated chemical cues through vomeronasal chemoreception (Saviola et al. 2013). Although the mechanism facilitating the changes in the chemical odor between envenomated and non-envenomated prey is still unknown, disintegrins clearly indicate how some venom proteins have unexpected, and often surprising, biological roles. In the following section, we discuss the function and biological roles of some common snake venom proteins, focusing on several that serve critical biological roles. Many of these compounds are well studied from the venoms of front-fanged snakes, and we will briefly address current work conducted on rear-fanged snake venoms and specific compounds that have been isolated from these venoms. A recent review presents a catalog of most known components of rear-fanged snake venoms (Junqueira-de-Azevedo et al. 2016).

Snake venom metalloproteinases

It has long been known that many snake venoms exhibit significant proteolytic activity, and the observation that this activity can be abolished by metal chelators such as ethylenediaminetetraacetic acid (EDTA) or *o*-phenanthroline indicated that divalent cations are required for activity, giving rise to the name snake venom metalloproteinase (SVMP) (Satake et al., 1963). Zinc-dependent SVMPs are small to moderate sized enzymes that may consist of multiple domains, and along with ADAMs (a disintegrin and metalloproteinase), they comprise the M12 subfamily of metalloproteinases. SVMPs are thought to have evolved from early recruitment and modification of ADAM-like ancestors similar to ADAM 7, ADAM 28, and ADAMDEC1 (decysin) before the radiation of advanced snakes (Jia et al. 1996; Fry 2005; Casewell 2012). This hypothesis is largely supported by the presence of the largest SVMPs, the P-III class, in the venoms of species belonging to the families Viperidae, Elapidae, Atractaspididae, and Colubridae, although the expression levels of this enzyme can vary significantly between families and species.

SVMPs are classified by the presence of various domain structures (Fox and Serrano 2005), with all SVMPs sharing a metalloproteinase domain that exhibits the canonical HEXXHXXGXXH zinc-binding motif at the catalytic site, followed by a conserved Met turn. The simplest of SVMPs, the P-I class,

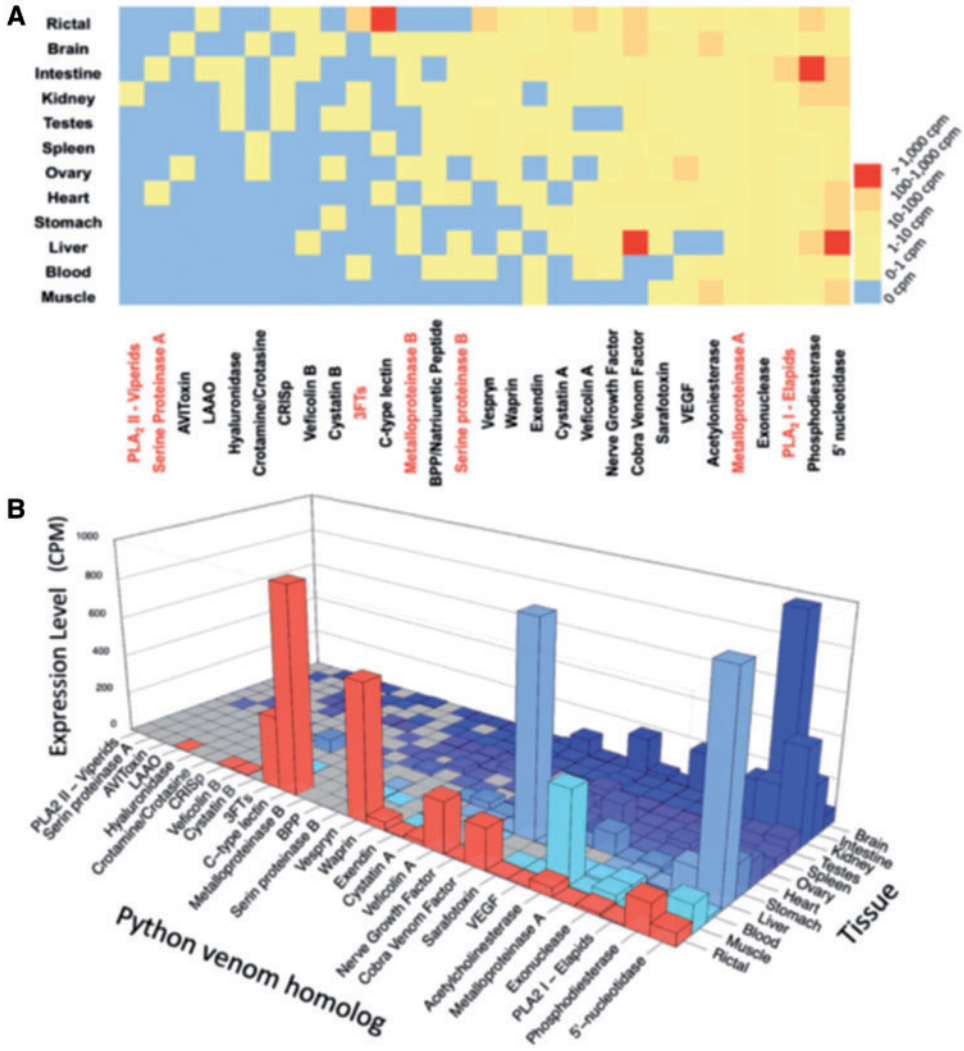

Fig. 2 Venom homolog expression patterns in various tissues of the Burmese Python (*Python molurus bivitattus*). (**A**) Heat map of expression levels (CPM) of venom protein families in various python tissues. (**B**) Expression levels of the same proteins in individual tissues. Modified from Reyes-Velasco et al. (2015).

contains only this metalloproteinase domain. P-II SVMPs express an additional spacer region of amino acids carboxy to the metalloproteinase domain, followed by a disintegrin domain that is often proteolytically processed, liberating the disintegrins observed in viperid venoms. P-I SVMPs, P-II SVMPs and disintegrins apparently occur only in viperid venoms, suggesting an adaptive response to immobilize, pre-digest, and relocate prey (Gutiérrez et al. 2010; Saviola et al. 2013). P-III SVMPs are the largest and most widely distributed of the SVMPs, and comprise a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich domain. These additional domains are postulated to contribute to the potent hemorrhagic activity of P-III SVMPs as compared to the P-I and P-II SVMPs. However, the additional domains are not required for hemorrhagic activity, as all three classes can produce hemorrhage (Fox and Serrano 2005). Post-translational modifications and proteolytic processing of specific domains have contributed to additional subclassifications of P-II and P-III SVMPs, and a more detailed description of SVMP structure and evolution has been reviewed previously (Fox and Serrano 2005, 2008).

SVMPs contribute to the proteolytic degradation of major basement membrane components (primarily type IV collagen and perhaps perlecan; Gutiérrez et al. 2016), and they have been predominantly studied in viperid venoms due to their role in local and systemic pathologies that often manifest in victims following envenomations. In addition, the viperid family exhibits the largest diversity of SVMPs, expressing P-I, P-II, and P-III classes. From a trophic standpoint, SVMPs play a critical role in both prey

immobilization and tissue proteolysis, and they are hypothesized to assist in the pre-digestion of larger, heavy bodied prey items that are commonly consumed by many viperid and some colubrid snakes (Mackessy 1988, 2010). Conversely, elapids feed on more elongate ectothermic prey having higher surface-to-volume ratios, and their venoms typically show low to very low SVMP activity. However, there are conflicting reports on the contribution of venoms in prey digestion (see Thomas and Pough 1979; McCue 2007; Chu et al 2009); unfortunately, these studies used differing methodologies, so comparison across them is difficult.

SVMPs are broadly distributed in venoms of rear-fanged snakes (Hill and Mackessy 2000), and biochemical assays, as well as proteomic and transcriptomic approaches (Ching et al. 2006; Modahl et al. 2016a), demonstrate that SVMPs are one of the most abundant compounds in their venoms. P-III SVMPs were the most abundant and diverse toxin family present in the transcriptome of the Night Snake (*Hypsiglena torquata texana*) (McGivern et al. 2014), and Peichoto et al. (2012) identified SVMPs (based on activity and protein masses) in the venoms of *Philodryas patagoniensis*, *P. baroni*, *P. o. olfersii*, *H. t. texana*, and *Trimorphodon biscutatus lambda*. Individual SVMPs have been characterized from the venoms of *P. olfersii* (Assakura et al. 1994), *Philodryas patagoniensis* (Peichoto et al. 2007), and *Alsophis portoricensis* (Weldon and Mackessy 2012), and all are P-III SVMPs. These isolated enzymes exhibited direct fibrin(ogen)olytic activity, cleaving either the A(α) or B(β)- chains of fibrinogen and/or fibrin; however, three of these SVMPs from the venom of *P. olfersii* lacked the hemorrhagic activity (Assakura et al. 1994) that is common to many rear-fanged snake SVMPs.

Phospholipase A_2

Phospholipase A_2 (PLA_2) enzymes constitute a major snake venom component and often contribute substantially to prey immobilization and capture. To date, PLA_2 activity, as well as isolated PLA_2s, have been identified and characterized from snakes belonging to the families Viperidae, Elapidae, and Colubridae (Kini 1997; Doley et al. 2010). Similar to SVMPs (and the majority of venom compounds), PLA_2 concentrations in venoms show tremendous phylogenetic variation. These enzymes share 40–99% amino acid sequence identity, as well as similarities in their three-dimensional structures, but despite their similarities, the pharmacological properties of individual PLA_2s may differ significantly. Phospholipases A_2 are among the most toxic and pharmacologically active venom compounds, and significant amounts of research have centered on these enzymes. Some elapids, such as the Central American Coral Snake (*Micrurus nigrocinctus*), produce venoms rich in PLA_2s, and approximately 48% of its venom proteome consists of this enzyme (Fernández et al. 2011). Among viperids, the Tiger Rattlesnake (*Crotalus tigris*) exhibits a simple yet extremely toxic venom, and approximately 66% of this venom consists of a homolog of the presynaptic β-neurotoxic PLA_2, crotoxin (Calvete et al. 2012). Similar in structure and function to crotoxin (discussed below), Mojave toxin is the primary toxic component in the venom of the Mojave Rattlesnake (*C. scutulatus scutulatus*) and may comprise 45% of this species' venom in populations in southeastern Arizona, but it is completely absent in nearby north-central Arizona populations (Massey et al. 2012).

To date, little effort has been directed at rear-fanged snake venom PLA_2s; however, activity has been detected in venoms of several species (see Mackessy 2002; Huang and Mackessy 2004; Zelanis et al. 2010; Saviola et al. 2014). Trimorphin, a 13.9 kDa PLA_2 from the venom of the Sonoran Lyre Snake (*Trimorphodon biscutatus lambda*), is the only PLA_2 purified and characterized from a rear-fanged snake venom. Analysis of the first 50 amino acid residues suggest that trimorphin is more closely related to PLA_2s from seas snakes and Australian elapids than to other terrestrial elapid or viperid PLA_2s (Huang and Mackessy 2004). Although the pharmacology of trimorphin has yet to be examined, this PLA_2 did exhibit significant and dose-dependent anti-parasitic (*Leishmania major*) activity *in vitro*, with an IC_{50} of 0.25 μM (Peichoto et al. 2011).

Three-finger toxins

Three-finger toxins (3FTxs) were once thought to be unique to elapid venoms; however, the characterization of α-colubritoxin (Fry et al. 2003a), followed by venom protein isolation/characterization (Pawlak et al. 2006, 2009; Heyborne and Mackessy 2013) and transcriptomic and proteomic studies (Junqueira-de-Azevedo et al. 2006; Pahari et al 2007; McGivern et al. 2014; Modahl et al. 2016a), indicate that these toxins are more widely distributed among venoms of advanced snakes. 3FTxs have a highly conserved protein scaffold, with three finger-like loops stabilized by disulfide bonds protruding from their central core (Fig. 3). Differences in non-structural amino acid residues result in a multitude of pharmacological functions with a diversity of

biological roles, in spite of nearly identical crystal structure. Various 3FTxs have been shown to recognize an array of receptors, including nicotinic and muscarinic acetylcholine receptors (Kini and Doley 2010), L-type calcium channels, integrins (Kini 2002), coagulation factor VIIa (Banerjee et al. 2005), and β1/β2-adrenergic receptors (Rajagopalan et al. 2007). Based on these different ligand specificities, venom 3FTxs exhibit diverse biological activities in the form of neurotoxicity, cardiotoxicity, cytotoxicity, and anticoagulation effects (Hegde et al. 2010), some of which are discussed below.

Cysteine-rich secretory proteins

Cysteine-rich secretory proteins (CRISPs) are a family of 20–30 kDa, non-enzymatic proteins widely distributed among reptile venoms. CRISPs display a high degree of conservation in their structure, due to high amino acid sequence similarities and 16 highly conserved cysteine residues that form eight disulfide bonds. Like the 3FTxs, despite their high degree of structural conservation, CRISPs show a variety of pharmacologies (reviewed by Heyborne and Mackessy 2010) including binding to cyclic nucleotide-gated ion channels (Brown et al. 1999; Yamazaki et al. 2002a), blocking vascular smooth muscle contraction (Yamazaki et al. 2002b), inhibiting Ca^{2+} release from the sarcoplasmic reticulum (Morrissette et al. 1995), and blocking calcium currents in neurons (Nobile et al. 1996). CRISPs have been isolated from the venoms of Elapidae (Brown et al. 1999; Yamazaki et al. 2002a), Viperidae (Yamazaki et al. 2002b) and Colubridae (Peichoto et al. 2009), as well as from venom of the Mexican Beaded Lizard (*Heloderma horridum horridum*; Mochca-Morales et al. 1990). A recent evaluation of CRISP molecular evolution suggests that they are under stronger positive selection in snakes than in lizards (Sunagar et al. 2012).

Among rear-fanged venomous snakes, CRISPs appear to be one of the more abundant venom proteins; however, characterization and functional data on these compounds are scarce. Patagonin, a 24.8 kDa CRISP isolated from the venom of *P. patagoniensis*, exhibited unique necrotic activity toward murine gastrocnemius muscle at higher doses, although it did not induce edema or hemorrhage and it had no effect on the aggregation of human platelets or platelet-rich plasma (Peichoto et al. 2009). Patagonin did not exhibit proteolytic activity toward azocoll, azocasein, or fibrinogen. The widespread distribution of CRISPs among reptile venoms suggests a significant trophic role for these proteins, but their biological role(s) in envenomation are far from clear. It is possible that CRISPs may act in concert with other venom compounds to enhance venom lethality, but this possibility has not yet been explored.

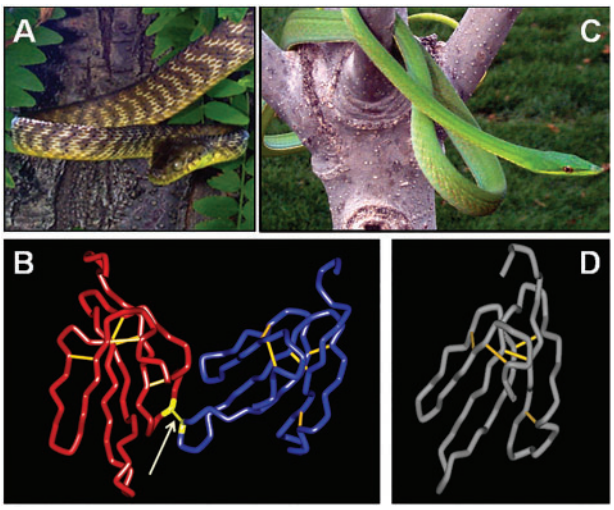

Fig. 3 Taxon-specific three-finger toxins from several species of rear-fanged snakes. Both toxins are potently lethal to lizards, but are non-toxic to mammals. (A) The Brown Treesnake (*Boiga irregularis*), an Old World colubrid, produces a venom containing approximately 10% irditoxin (**B**–backbone structure). (**C**) The Green Vinesnake (*Oxybelis fulgidus*) is a New World colubrid snake; fulgimotoxin (**D**) comprises approximately 35% of this species' venom. (**D**) Adapted from Heyborne and Mackessy (2013).

Feeding strategies of venomous snakes

Although venomous snakes utilize the same fundamental mechanism (envenomation) for limiting prey flight, differential strategies in prey envenomation and handling are seen both ontogenetically and between families. Viperids are generally considered ambush predators, and they use chemical cues in ambush site selection (Duvall et al. 1990; Clark 2004b) and visual-thermal cues to deliver what is most often a single envenomating strike (Hayes and Duvall 1991). Neonate rattlesnakes typically strike-and-hold small ectothermic prey (Mackessy 1988), whereas adults usually strike-and-release larger endothermic prey, returning to a reliance on chemical cues to relocate the envenomated prey that may wander from the attack site (Chiszar et al. 1977; Saviola et al. 2013). The release of prey following the envenomating strike allows snakes to avoid retaliation that may occur from larger, potentially dangerous prey items, yet requires relocating the meal using chemosensory searching once it has succumbed to venom (Chiszar et al. 1977; Parker and Kardong 2005; Saviola et al. 2013).

In contrast, the majority of snakes belonging to the families Colubridae, Elapidae, and Atractaspididae actively forage for prey, although sit-and-wait strategies among specific species are likely not uncommon. However, the mode for venom delivery and prey handling does differ between families. Elapids often strike-and-hold prey, though a few species have been documented to constrict as well (Shine and Schwaner 1985). Multiple predatory strikes have also been observed in elapids (see Radcliffe et al. 1983), possibly due to the presence of shorter fangs (compared to viperids) that may fail to penetrate deeply into prey tissues. A prey capture study with the Egyptian Cobra (*Naja haje*) showed that snakes will quickly release retaliating prey (Kardong 1982), and due to the potent toxicity of elapid venoms, often rich in prey-immobilizing neurotoxins, it is unlikely that prey venture far from the attack site. Mole vipers (*Atractaspis* sp.) feed on neonatal rodents within nests and burrows and use a unilateral slashing behavior to envenomate prey (Deufel and Cundall 2003), so prey escape is unlikely.

With a low-pressure venom delivery system, rear-fanged snakes introduce venom more slowly, seizing prey and using maxillary walking with concomitant "pumping" of venom. This feeding strategy likely introduces multiple envenomation sites without releasing the prey. The Green Vinesnake (*Oxybelis fulgidus*), for example, utilizes this strategy regardless if the snake is fed lizard or small rodent prey (pers. obs.), and the Brown Treesnake (*Boiga irregularis*) also envenomates and subdues lizard and bird prey by firmly grasping them. However, for a few species of rear-fanged snakes, feeding behaviors appear to be prey-dependent. While lizards are never constricted, when offered rodent prey, *B. irregularis* has been observed to use constriction immediately (Mackessy et al. 2006; personal observation), and the Western Terrestrial Garter snake (*Thamnophis elegans*) has also been shown to constrict mice; for the latter species, this behavior rarely occurs when snakes are fed frogs (Bealor et al. 2013). This same study reported that mice exhibited significantly higher struggling intensity compared to frogs, which suggests that Duvernoy's venom toxins of *T. elegans* may have slower or limited effects on mammalian prey.

As the primary function of venom is to facilitate prey capture, it is not surprising that diet is a major driving force of venom evolution (Mackessy 1988; Barlow et al. 2009; Gibbs and Mackessy 2009), and different feeding strategies among species are largely driven by the presence of specific venom compounds that target and exhibit greater toxicity toward distinct prey types. In addition, venoms have been shown to undergo ontogenetic shifts in composition (Mackessy 1988; Alape-Girón et al. 2008; Saviola et al. 2015b), and these changes are correlated with prey type most commonly consumed at specific life stages. Snakes are gape-limited and can only consume prey that they can swallow whole; therefore, shifts in prey preferences associated with snake age (and size) have been documented in many species. In some rattlesnake species, for example, neonates consume small ectothermic prey, and they shift to larger, more energetically favorable endothermic prey as adults. This coincides with a shift in venom composition from a more toxic venom with less enzymatic (SVMP) activity in neonates, to a venom that is less toxic but with elevated enzymatic activity in adults. Mackessy (2008) classified rattlesnake venoms into type I venoms that contain higher SVMP activity with lower toxicities ($LD_{50} > 1.0$ μg/g), or type II venoms which exhibit low SVMP activity but higher toxicity ($LD_{50} < 1.0$ μg/g) toward rodents (*Mus musculus*). The commonly observed ontogenetic shift in venom composition in rattlesnakes, as well as many other vipers, is from a more type II-like venom in neonates to a type I venom in adults. Recently, we identified an opposite trend in venom composition from a population of Prairie Rattlesnakes (*Crotalus viridis viridis*) in eastern Colorado, with venoms shifting from higher SVMP activity and lower myotoxin a concentration in neonates, to lower SVMP activity and higher myotoxin a concentration in adults (Saviola et al. 2015b). The increased concentration of SVMPs, which may assist in the pre-digestion of larger, heavy-bodied prey, should not be as essential for smaller ectothermic prey items, which are commonly consumed by neonatal snakes, and the significance of this trend at a trophic level still needs to be addressed. However, the increased concentration of myotoxin a in adult venoms does correlate with a diet consisting predominately of mammals. Further, the astonishing speed at which myotoxin a acts may reduce the utility of SVMPs in prey immobilization, relegating them to a secondary digestive role.

Taxon-specific toxins in snake venoms

Monomeric and dimeric three-finger toxins

Identification and characterization of prey-specific toxins further demonstrate that venom compounds have evolved under positive selection, favoring structural variants that target receptors of prey frequently consumed by the snake. Crude venom of adult *B.*

irregularis also demonstrates prey-specific lethality, with venoms being quite toxic toward chicken (*Gallus domesticus*; LD_{50} = 1.75 μg/g) and lizard (*Hemidactylus* geckos; LD_{50} = 2.5 μg/g and *Carlia* skinks LD_{50} = 4.5 μg/g) prey, whereas toxicity toward rodent prey (*Mus musculus*) was significantly lower (LD_{50} = 31 μg/g; Mackessy et al. 2006). Irditoxin, a 17-kDa covalently linked heterodimeric 3FTx present in the venom of *B. irregularis*, appears to be primarily responsible for toxic effects, and the purified toxin had LD_{50} values of 0.22 μg/g and 0.55 μg/g for chicks and lizards, respectively, whereas mice showed no effects at doses up to 25 μg/g (Fig. 3A and B; Pawlak et al. 2009). Denmotoxin, an 8.5 kDa 3FTx from the venom of *B. dendrophilia*, exhibits irreversible postsynaptic neuromuscular activity against chicken tissues, but this activity is readily reversible in rodent neuromuscular preparations (Pawlak et al. 2006). Fulgimotoxin, a 3FTx from the venom of the Green Vinesnake (*Oxybelis fulgidus*), showed potent toxicity towards *Anolis* (LD_{50} = 0.28 μg/g) but was non-toxic to mice at doses more than fifteen times greater (Fig. 3C and D; Heyborne and Mackessy 2013). These toxins are all very closely related at the x-ray crystal structural level to α-cobratoxin (Fig. 4), and the taxon-specific effects of the colubrid venom 3FTxs are hypothesized to reside in highly conserved sequence motifs found in loop II of taxon-specific toxins, but not other 3FTxs (Heyborne and Mackessy, 2013).

Myotoxin a—a mammal-specific toxin

The taxon-specific effects of rear-fanged snake venom α-neurotoxins prompted us to test toxins from several front-fanged species. One of these, myotoxin a, from *C. v. viridis* venom (Fig. 5), is a well-known homolog of crotamine (*C. durissus terrificus* venom). These toxins, recently suggested to be homologs of the β-defensins (Zhu et al. 2014; Sunagar et al. 2014), produce rapid tetanic contraction of skeletal muscle and rapid immobilization of mice (Bieber and Nedelkov 1997). In our hands, myotoxin produced rapid lethal effects in NSA mice, with an LD_{50} of 2.0 μg/g, whereas no effects were observed in either *Sceloporus* or *Hemidactylus* lizards at concentrations up to 25 μg/g. To the best of our knowledge, this toxin represents the first-known example of a mammal-specific toxin in snake venoms.

Potential taxon-specific toxins

Many elapids, including several *Naja* and *Bungarus* species, produce venoms containing non-conventional α-neurotoxins, also known as "weak" neurotoxins because of their typically high to very high LD_{50}s (Hegde et al. 2010). This subfamily of three-finger toxins is characterized structurally by the presence of a fifth disulfide linkage in loop I of the toxin. Interestingly, this structural feature is also present in the colubrid 3FTxs, suggesting that the elapid toxins may also show taxon-specific effects. However, this was recently demonstrated to be *not* the case, as several non-conventional 3FTxs from *Naja kaouthia* venom showed no toxicity toward lizards or mice (Modahl et al. 2016b). The role of these apparently non-toxic "toxins" in elapid venoms is enigmatic, though they may represent an example of "evolutionary tinkering" (*sensu* Vidal 2002) of a 3FTx, repurposed for a different biological role.

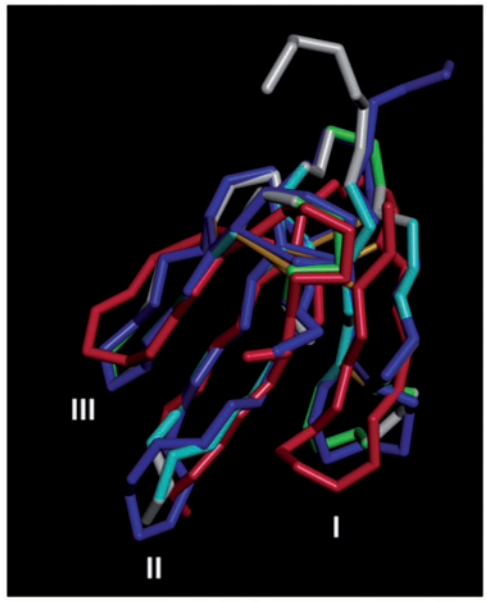

Fig. 4 The conserved molecular fold of snake venom 3FTxs. This overlay of the protein backbone structures of α-cobratoxin, fulgimotoxin, and denmotoxin demonstrates the high degree of structural conservation of 3FTxs. α-cobratoxin is highly toxic to both lizards and mammals, whereas fulgimotoxin and denmotoxin (and the dimeric irditoxin) are highly lethal to lizards but harmless to mammals. Reproduced from Heyborne and Mackessy (2013).

"Generalist" toxins in snake venoms

α-Cobratoxin

The above examples clearly demonstrate that several venom components show profound taxon-specific activity, leading to rapid immobilization and death of prey species commonly consumed by these snakes. However, many venom compounds appear to lack specificity for particular prey taxa and instead act as "generalist" toxins. α-cobratoxin (Fig. 6), a

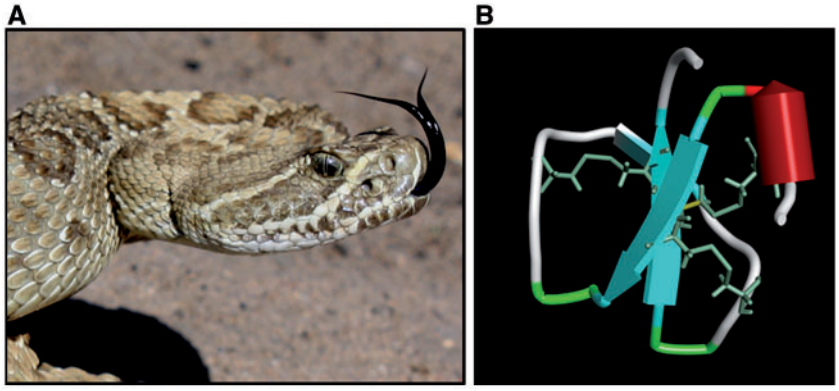

Fig. 5 Rattlesnake mammal-specific toxin. The Prairie Rattlesnake (*Crotalus viridis viridis*; (**A**) produces a venom which may consist of up to 37% myotoxin a (**B**), a mammal-specific toxin that produces extremely rapid tetanic paralysis in rodents, but is harmless to lizards.

canonical example of an elapid post-synaptic α-neurotoxin, binds very tightly to the nicotinic acetylcholine receptor α subunit of the vertebrate skeletal muscle motor endplate, resulting in rapid blockade of the ion channel and producing flaccid paralysis of prey (Nirthanan and Gwee 2004). It is present in venoms of many species of *Naja*, and similar homologs are found in many elapid venoms (Doley et al. 2008). Unlike the rear-fanged venom 3FTxs mentioned above, α-cobratoxin isolated from *Naja kaouthia* venom has recently been shown to be equally toxic toward both lizard and mouse models, with an LD_{50} of ~0.1 μg/g toward both *Hemidactylus* geckos and NSA mice (Modahl et al. 2016b).

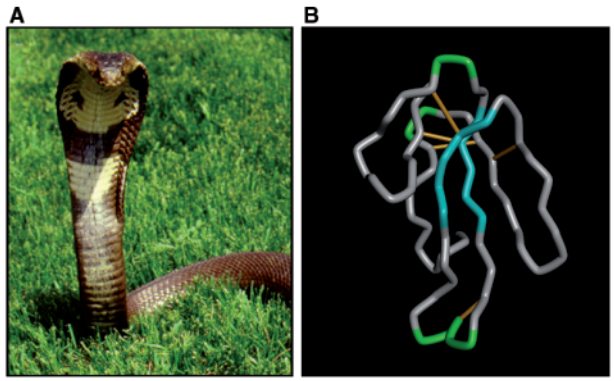

Fig. 6 "Generalized" snake venom toxins: Elapidae. The Monocled Cobra (*Naja kaouthia*; (**A**) produces a venom rich in 3FTxs, including several non-conventional 3FTxs structurally similar to colubrid toxins, but only α-cobratoxin (**B**) is highly lethal to vertebrate prey.

Crotoxin and homologs

Crotoxin is a presynaptic neurotoxin first isolated from the venom of *C. d. terrificus* (Horst et al. 1972), and homologs have been isolated from many rattlesnakes (Aird and Kaiser 1985; Aird et al. 1985) as well as other pitviper species (Lomonte et al. 2015). The presence of these toxins in rattlesnake venoms led to the recognition of type I/type II venoms in vipers (Mackessy 2008, 2010), and those species with venoms containing significant amounts of the toxin are the most toxic (type II venoms). These toxins are non-covalent heterodimers composed of an acidic, non-toxic, non-enzymatic 9.4 kDa subunit, and a basic, moderate toxicity 14.2 kDa PLA_2 subunit (Fig. 7); the complex is quite potent toward mice (LD_{50} ~0.03 μg/g) (Aird and Kaiser 1985). These subunits can be separated under extremely acidic conditions (pH < 2) or when incubated with 6 M urea. When separated, the basic PLA_2 unit exhibits weak toxicity, but in its native form the complex is at least one order of magnitude more toxic. The acidic subunit, on the other hand, does not exhibit biological activity and appears to act as a chaperone protein for the basic subunit, targeting the PLA_2 to axonal membranes and blocking non-specific binding of the PLA_2 subunit to other cell membranes (Délot and Bon 1993; Krizaj et al. 1997). The high degree of similarity between A or B subunits from different crotoxin homologs was first demonstrated by substitution experiments (Aird and Kaiser 1985; Aird et al. 1989), which showed that the A subunit of toxin 1 could substitute for the A subunit of toxin 2 (or B for B), with no loss of lethal toxicity of the heterologous complex.

The sequence of concolor toxin from *C. oreganus concolor* (Midget Faded Rattlesnake) was recently determined from mRNA transcripts isolated from the venom of the snake (Modahl and Mackessy 2016), and its sequence is nearly identical (>98%) to that of crotoxin, Mojave toxin and other crotoxin homologs. Similar to results obtained with α-cobratoxin (Fig. 6), concolor toxin was equally lethal toward NSA mice and *Hemidactylus* geckos, with an LD_{50} of 0.06 μg/g. Both concolor toxin and α-cobratoxin

therefore appear to act as generalist toxins, potently lethal to divergent taxa of commonly utilized vertebrate prey.

Venom metalloproteinases

Snake venom metalloproteinases (SVMPs) are found in the venoms of nearly all snakes, both front-fanged and rear-fanged, but some broad, global patterns of occurrence are observed. Vipers typically (but not always) have venoms rich in SVMPs, with 2–3 subtypes (PI-PIII) in a given venom, while elapids, and specifically marine species, typically (but not always) lack significant levels of these enzymes; they are also common to nearly all known rear-fanged snake venoms as well (Mackessy 2010). These enzymatic toxins catalyze the hydrolysis of many different proteins, particularly structural elements like collagen, elastin, and many proteins comprising connective tissues and the basal lamina/basement membranes of blood vessels and other epithelia (Fox and Serrano 2008; Oliveira et al. 2010). In this sense, their actions can be considered generalized, and structural degradation of prey tissues is a common manifestation of SVMP activity, in prey as diverse as crickets (Munekiyo and Mackessy 1998), lizards (Weldon and Mackessy 2012), and mammals (Moura-da-Silva et al 1996). However, a recent study of the specificity of PI, PII and PIII SVMPs toward basement membrane components, and proteomic analysis of wound exudates generated by individual SVMPs, indicated that although type IV collagen was a substrate common to all three subtypes, other structural elements were differentially affected (Herrera et al. 2015). Whether these specific actions apply to non-mammalian prey is unknown at present, but the three subtypes of SVMPs do appear to catalyze different types of structural damage.

Summary and conclusions

Why study rear-fanged snake venoms?

As venoms from more species of rear-fanged snakes are investigated, it is becoming apparent that many venom toxin families are shared among the advanced snakes, and some species show levels of venom complexity similar to those seen in venoms of elapids and viperids. However, relatively few rear-fanged snake species have been investigated, and sampling has often relied on specimen availability, rather than phylogenetically-directed sampling that is needed to encompass the diversity of venoms likely present across the clades comprising the rear-fanged snakes. In addition, phenomena such as taxon-specific effects were first noted among rear-fanged snake venoms (Mackessy et al. 2006), leading to a search for toxins with similar specificities in other venoms.

Conversely, many New World Colubridae and Dipsadidae produce venoms with apparently very low complexity, with proteomes and transcriptomes often dominated by 3FTxs. For example, over 61% of total toxin reads for *B. irregularis* venom gland were for 3FTxs (McGivern et al. 2014), and the exceptionally low complexity venom of *O. fulgidus* is dominated by a single 3FTx isoform (~35%; Heyborne and Mackessy 2013). *Hypsiglena* also produces a very simple venom (Hill and Mackessy 2000), but this venom is dominated by PIII SVMPs, which comprise greater than 68% of total toxin reads (McGivern et al. 2014). Why some venoms are dominated by neurotoxins, while others consist largely of lytic enzymes, is unclear at present, but these compositional motifs likely reflect feeding ecology.

Diets of many rear-fanged snakes are often highly specialized, and many smaller species prey upon potentially dangerous animals such as scorpions, spiders and centipedes. Coupled with low complexity venoms, investigations of venom composition and its relation to feeding patterns may be more tractable in rear-fanged snakes, allowing us to more readily discern the roles of specific toxins. Front-fanged snakes, such as *Crotalus* and *Dendroaspis*, may have over 100 protein/peptide components in the venom of a single snake (e.g., Mackessy 2010), making it difficult to assign a specific trophic role to a particular toxin. *Hypsiglena*, *Thamnophis elegans vagrans*, as well as several other rear-fanged species, lack this complexity, and PIII SVMPs, a CRISP and a C-type lectin bands are the only proteins discernible following 1D SDS PAGE (Hill and Mackessy 2000), strongly indicating SVMPs as the most important components of the venom. *Hypsiglena* envenomation of other snakes resulted in severe localized hemorrhage and necrosis, further implicating SVMPs as the causative agent.

Venom evolution has had major adaptive significance for the Colubroidea by facilitating prey handling and reducing the need for mechanical manipulation of prey (Savitsky, 1980). Minimizing the need for more robust body musculature to subdue prey has in turn removed constraints on body form, and venoms may have allowed for the diversification of arboreal forms (e.g., *Ahaetulla, Boiga, Oxybelis, Thelotornis*), aerial forms (*Chrysopelea*), and even brackish/aquatic forms (*Cerberus*).

Snake venoms are complex secretions with many different activities and biological roles, and the diversity of diets, habits, ecology, and venom

biochemistry of rear-fanged snakes reflects the complex evolutionary history of these species. Venoms have evolved from an apparently small selection of possible protein precursors (approximately 24 protein families out of thousands known: Wu et al. 2003; Bateman 2014), suggesting that venoms may have evolved only once in squamate reptiles. However, the disparate morphology of venom glands, the broad occurrence of venom "homologs" in non-oral gland tissues, different trends in venom composition among major clades and the long evolutionary history of squamates suggest that it is more parsimonious that venoms and venom delivery systems evolved several times in disparate squamate clades (Fig. 8).

It is important to keep in mind that we presently possess only a fragmentary understanding of the diversity of venoms and venom systems among rear-fanged snake; therefore, broad generalizations concerning their evolution and biological roles must be taken as working hypotheses. On the other hand, there are abundant opportunities for important contributions at many different levels, from many different parts of the world, because rear-fanged species are found nearly everywhere snakes are found. The implications for venom studies are that there are local species for toxinologists, evolutionary biologists, etc., to explore, particularly in speciose areas of the Neotropics and tropical Africa and Asia. Future prospects for unraveling the evolutionary history and biological significance of venoms in advanced snakes are excellent, as proteomic and genomic technologies become more cost-effective and generally available. It is important to keep in mind, however, that venoms evolved in a biological context, shaped by natural selection for adaptive traits, and so exploration of the natural history and ecology of venomous species is critical for assessing the biological roles provided by these potent and diverse compounds.

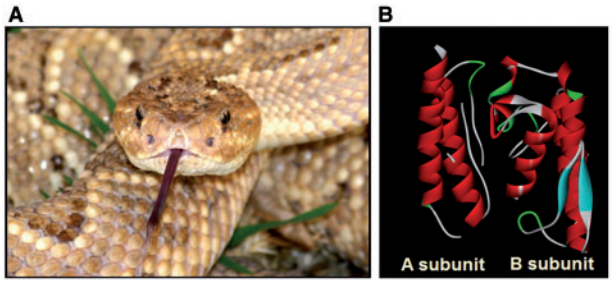

Fig. 7 "Generalized" snake venom toxins: Viperidae. The Aruba Island Rattlesnake (*Crotalus* (*durissus*) *unicolor*; (**A**) possesses a PLA_2-based complex (crotoxin; **B**) that is lethal toward a wide variety of vertebrate prey. Homologs of this toxin are found in all *Crotalus* venoms with LD_{50}s less than 1.0 μg/g.

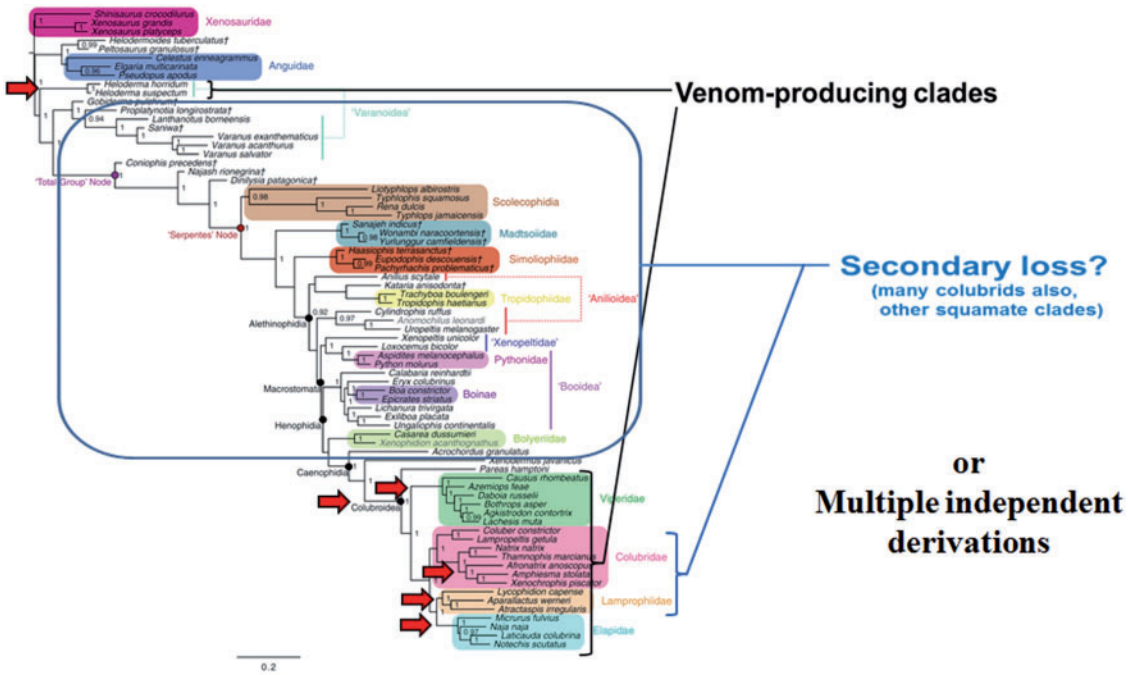

Fig. 8 Alternative hypotheses for the evolution of venoms in squamate reptiles. A single origin would require loss of expression of multiple genes in a diverse array of squamate taxa. Multiple independent derivations of venoms may have occurred numerous times (arrows). Phylogenetic hypothesis modified from Hsiang et al. (2015).

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