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REVIEW

Venom production and secretion in reptiles

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ABSTRACT

The venom glands of reptiles, particularly those of front-fanged advanced snakes, must satisfy conflicting biological demands: rapid synthesis of potentially labile and highly toxic proteins, storage in the gland lumen for long periods, stabilization of the stored secretions, immediate activation of toxins upon deployment and protection of the animal from the toxic effects of its own venom. This dynamic system could serve as a model for the study of a variety of different phenomena involving exocrine gland activation, protein synthesis, stabilization of protein products and secretory mechanisms. However, these studies have been hampered by a lack of a long-term model that can be propagated in the lab (as opposed to whole-animal studies). Numerous attempts have been made to extend the lifetime of venom gland secretory cells, but only recently has an organoid model been shown to have the requisite qualities of recapitulation of the native system, self-propagation and long-term viability (>1 year). A tractable model is now available for myriad cell- and molecular-level studies of venom glands, protein synthesis and secretion. However, venom glands of reptiles are not identical, and many differ very extensively in overall architecture, microanatomy and protein products produced. This Review summarizes the similarities among and differences between venom glands of helodermatid lizards and of rear-fanged and front-fanged snakes, highlighting those areas that are well understood and identifying areas where future studies can fill in significant gaps in knowledge of these ancient, yet fascinating systems.

KEY WORDS: Evolution, Non-model organisms, Organoid, Rear-fanged, Reptile, Secretory epithelium, Toxin

Introduction: chemically mediated trophic adaptations

Chemical weapons and defenses are common across a wide diversity of living taxa, and a bewildering array of toxins is produced by microorganisms, fungi, plants and animals. Venomous snakebite continues to be a source of morbidity and mortality for humans and their domestic animals in many parts of the world (Mukherjee and Mackessy, 2021), and the World Health Organization has recently (again) declared snakebite as a neglected tropical disease (Gutiérrez, 2021; WHO, 2019). Because the toxins that comprise venoms have evolved over tens to hundreds of millions of years, and many are highly specific in their binding targets, animal venoms have also become a fertile arena in the search for therapeutically useful compounds (McCleary and Kini, 2013; Bordon et al., 2020; Herzig et al., 2020; Takacs, 2021). Invertebrate venoms, particularly from spiders and scorpions, are a rich source of specific ion channel-modulating toxins (e.g. Chow et al., 2020; Surm and Moran, 2021), and reptile venoms commonly also contain ligand-directed toxins. This Review will largely be limited to an overview of the venoms and venom apparatus of reptiles, particularly those of snakes.

The taxonomy of venomous reptiles has been contentious, as has the question of what comprises a venomous reptile. Here, I take a conservative approach, considering many advanced snakes and only helodermatid lizards as truly venomous; examples of several of these major groups are shown in Fig. S1. There has been a long-standing and rather antagonistic debate concerning the definition of 'venom' and 'venomous', with some researchers including species of varanid, agamid and anguid lizards, as well as many constricting non-venomous colubrid snakes, among 'venomous' species. However, the presence of transcripts coding for related proteins, and even the presence of some expressed proteins, in the saliva of these reptiles has not been demonstrated to have an obvious trophic role, as is observed for virtually all other venomous reptiles. Instead, the presence of 'venom-like' proteins in harmless species provides even more convincing evidence that venom toxins have indeed evolved from 'normal' housekeeping genes (Fry, 2005; Reyes-Velasco et al., 2015; Junqueira-de-Azevedo et al., 2015).

Regardless of the debates concerning venom definitions and overall occurrence of venoms in reptiles, there are at least three fundamentally different mechanisms of venom deployment seen in extant reptiles. Among the beaded lizards and gila monsters (genus *Heloderma*; Fig. S1A,B), venom is produced in a submandibular gland that lacks skeletal muscle for assisted deployment (via muscular pressure on the gland), and venom is secreted via ducts leading to the base of grooved anterior mandibular teeth (Fig. 1A; venom flow is relatively slow, and extended contact is required for significant venom delivery; Beck, 2005). In large part because of this 'inefficient' delivery system, the observation that human envenomations are often exceptionally painful (nociceptive) but not fatal, and the fact that primary prey consists largely of eggs and nestling birds and mammals, the primary selective driver for this system is believed to be a defensive role in predator deterrence. However, the venom composition of *Heloderma* is complex (Koludarov et al., 2014), containing numerous metalloproteinases, serine proteinases, phospholipase A_2, bioactive peptides (including exenatides) and other proteins (summarized in Beck, 2005), many of which are also found in viper venoms.

Numerous advanced snakes possess a Duvernoy's venom gland (Taub, 1966; Saviola et al., 2014), located in the posterior temporal region and superficially similar to the venom gland of elapid and viperid snakes. Previously, we proposed the use of 'Duvernoy's venom gland', rather than simply venom gland (Saviola et al., 2014), to emphasize the fact that though these glands are homologous structures, there are many structural and functional differences between them. Many species of the families Atractaspididae, Colubridae, Dipsadidae, Homalopsidae, Lamprophiidae, Psammophiidae and Pseudoxyrhophiidae (cf. Uetz et al., 2021, Zaher et al., 2019; formerly synonymized in the paraphyletic family Colubridae) also possess one to several enlarged and/or grooved rear maxillary teeth, hence the collective name 'rear-fanged snakes' (Fig. S1C,D). However, a Duvernoy's

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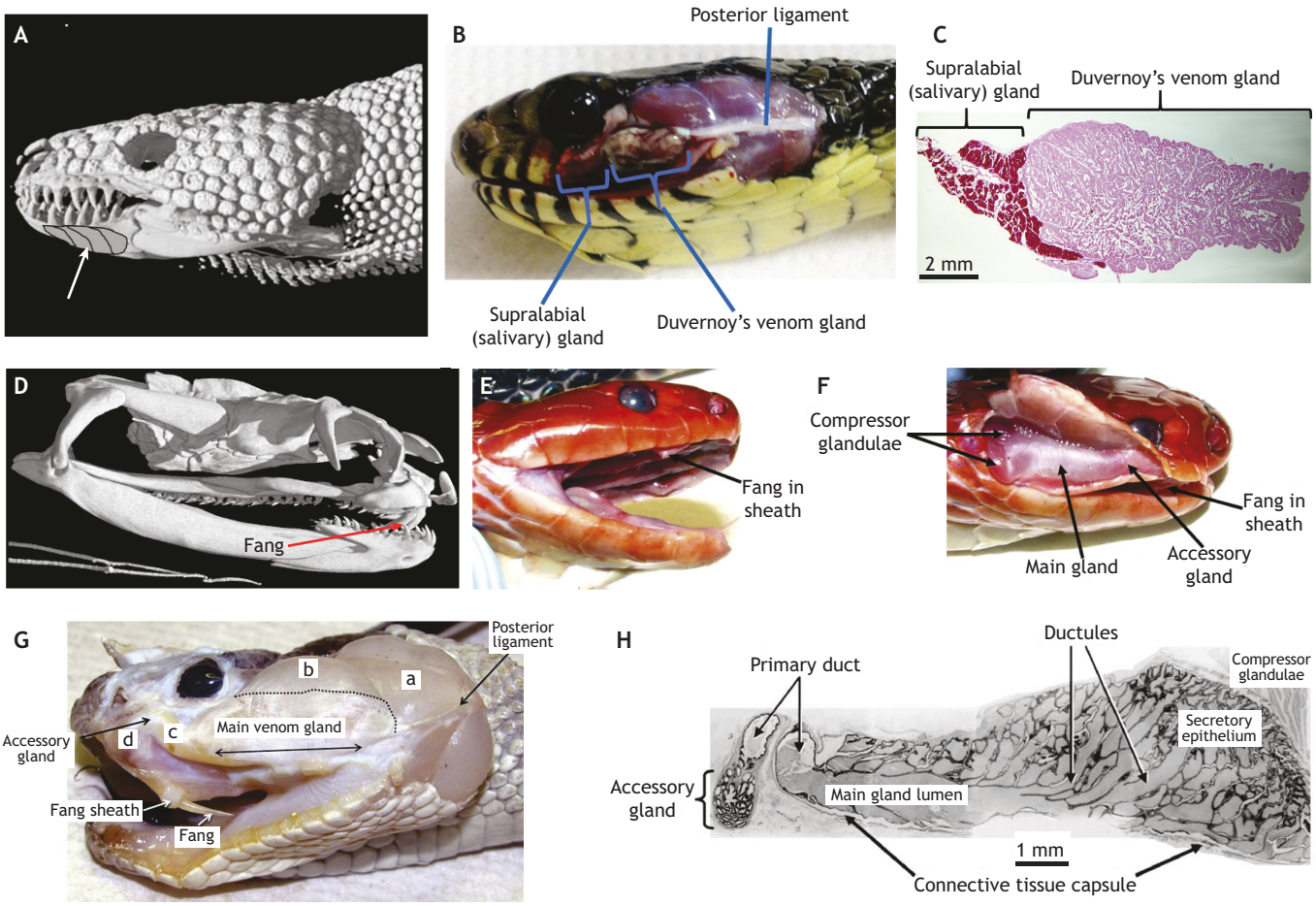

Fig. 1. Mechanisms of venom deployment in extant reptiles. (A) Computed tomography (CT) scan of the head of *Heloderma suspectum* (gila monster). The submandibular venom glands of *Heloderma* sp. are located near the anterolateral side of the mandible (arrow), with ducts associated with each lobule leading to the base of grooved anterior mandibular teeth (used with permission: Dr Jessica A. Maisano, Digital Morphology Group, University of Texas High-Resolution X-ray Computed Tomography Facility; http://www.DigiMorph.org/). (B,C) Colubrid venom apparatus. The Amazon puffing snake, *Spilotes sulfureus*, a tropical rear-fanged colubrid snake from Suriname, showing a close-up of the venom apparatus *in situ* (B) and a sagittal section of the salivary and Duvernoy's venom glands (C), stained with hematoxylin and periodic acid–Schiffs (PASH). (D–F) Elapid venom apparatus. (D) CT scan of the skull of a banded sea krait (*Laticauda colubrina*), a hydrophiine elapid (used with permission: Dr Jessica A. Maisano). The head of red-headed krait (*Bungarus flaviceps*) (E), showing the short fixed fangs and the venom glands (F). (G,H) Venom apparatus of viperid snakes. (G) Venom apparatus of the northern blacktail rattlesnake (*Crotalus molossus molossus*) (adapted from Mackessy and Castoe, 2017). (H) Midsagittal section of the entire venom glands of Northern Pacific rattlesnake (*Crotalus oreganus oreganus*) (from Mackessy, 1991). a, adductor mandibulae; b, modified slip of the adductor mandibulae (the compressor glandulae); c, primary duct; d, secondary duct.

venom gland is also present in numerous species that lack well-differentiated rear maxillary teeth, so this is not a requisite feature for venom production (Young and Kardong, 1996; Fry et al., 2008). In general, virtually all rear-fanged snakes lack differentiated musculature associated with pressurizing the gland (Fig. 1B), so venom release is generally slower and considered to be low pressure (Kardong and Lavin-Murcio, 1993); a more recent analysis of venom delivery by the mangrove snake (*Boiga dendrophila*) indicates that in spite of this mechanical constraint, venom delivery in some species can be reasonably rapid (Young et al., 2011). Additionally, though many protein toxins are shared between front- and rear-fanged snakes (including metalloproteinases, C-type lectins, three-finger toxins, phospholipases A_2 and occasionally L-amino acid oxidase; Junqueira-de-Azevedo et al., 2016), rear-fanged snake venoms commonly lack the complexity of front-fanged snake venoms (with the exception of seasnakes). It should be noted, however, that only a small number of species of rear-fanged snakes have been examined in detail, so it is likely that a multitude of other patterns exist among these diverse snakes.

The remaining group, collectively referred to as front-fanged snakes because of the presence of an enlarged and hollow anterior maxillary tooth, includes a broad assemblage of medically important species in three families: Lamprophiidae (Atractaspidinae only), Elapidae and Viperidae (Fig. S1E–I; Fig. 1D–H). Venoms and venom production by these species have been subject to much more extensive and rigorous analyses (see Mackessy, 2021), because these species are responsible for considerable human morbidity and mortality worldwide (Chippaux, 1998; Gutiérrez, 2021). This Review will necessarily use front-fanged species as prominent examples of the production and secretion of venoms.

Venom versus poison

The unequivocal definition of a system in biology is often challenging, as living organisms exist along a continuum, rarely obeying the simple dichotomies that we humans find most appealing. This difficulty also includes differentiating a venom from a poison, terms used interchangeably in the lay press that are

actually rather distinctively different. In general, a venom is a simple to complex mixture of toxins that are synthesized by a specific organ (the venom gland or cells) of the organism and are introduced into a recipient organism via specialized delivery systems (often including fangs, stinging cells or structures, specialized muscles and other structures); venoms are not orally toxic, and they are used most commonly as an offensive trophic weapon. When injected into tissues, venoms result in cataclysmic loss of homeostasis, including a myriad of effects such as paralysis and other neurotoxic manifestations, tissue necrosis, hemorrhage and/or hemolysis, coagulopathies, renal alterations and other consequences, resulting in prey incapacitation and facilitating prey handling. In contrast, a poison requires ingestion for negative effects to occur, is typically not produced in a specialized organ of the toxic animal and may be obtained/sequestered from an environmental source. Poisons are often used as defensive chemical weapons, and the evolution of aposematic coloration is often associated with poison production (poison dart frogs, monarch butterflies). Virtually all venomous reptiles synthesize venoms in a specialized temporal gland (venom gland or Duvernoy's venom gland in snakes) or in a submandibular gland (helodermatid lizards). The few known examples of poisonous reptiles include several garter snakes (*Thamnophis* sp.) that ingest tetrodotoxin (TTX)-producing newts, resulting in a broad distribution of TTX throughout the snake's body (Brodie and Brodie, 1990), and several species of keel-backed snakes (*Rhabdophis* sp.) that sequester toxins from ingested toads in specialized nuchal sacs (Hutchinson et al., 2007); the latter species are also rear-fanged venomous snakes.

Venom apparatus and comparative anatomy of gland structures

Reptiles utilize several specialized adaptations to deliver venom into prey, including the venom gland, complex ducts, an enlarged canaliculated maxillary fang (or grooved or enlarged rear maxillary teeth) and typically a mechanical system (skeletal and muscular) for maximizing the efficiency of venom delivery. Venom glands all contain secretory epithelial cells to produce venom, but the gross anatomy differs significantly between venomous taxa (Helodermatidae, Colubridae and other related families listed above, hereafter collectively referred to as 'rear-fanged snakes'; Elapidae and Viperidae). The evolution of fangs in snakes has been recently reanalyzed, and the authors concluded that the mechanical apparatus has evolved independently at least 4 times in reptiles (Palci et al., 2021). This section will detail some of the major differential patterns observed among extant reptiles. It should be acknowledged here that not all variants will be discussed, including some rather remarkable examples of extreme elongation of venom glands (including several species of *Atractaspis*, *Causus* and *Calliophis*), found in each of the major families of front-fanged venomous snakes.

Lizards of the genus *Heloderma* possess paired submandibular venom glands associated with grooved anterior mandibular teeth (Fig. 1A). Venom is secreted from the glands via ducts to the base of these teeth, and is introduced into tissues slowly and under low pressure because of the lack of associated compressor muscles. The gland is separated into discrete lobes, with each lobe drained by a separate duct (Beck, 2005). Significant envenomation requires extended contact, made possible by the robust jaw adductor muscles (Beck, 2005). Severe human envenomations are uncommon, but it is reported that bites are exceptionally painful (Chippaux and Amri, 2021). Though these lizards have long been known to produce venoms, details of the gland morphology and ultrastructure are scant, and a thorough investigation of this unusual venom system is warranted.

The Duvernoy's venom gland of rear-fanged snakes has similarly been poorly studied, even though these snakes represent the most diverse and widespread clades of venomous advanced snakes (Pyron et al., 2013; Uetz et al., 2021). Though Taub (1966) conducted a comprehensive histological analysis of many species, a similarly comprehensive review of the Duvernoy's venom gland has not been undertaken since that time; a more recent study presented an overview of the gland histology of several rear-fanged snake species (Fry et al., 2008). Nevertheless, a few species have been investigated in detail, including the wandering garter snake (*Thamnophis elegans vagrans*: gland ultrastructure; Kardong and Luchtel, 1986), broadly distributed in northwestern North America, the tiger keelback snake (*Rhabdophis tigrinus*; ultrastructure; Yoshie et al., 1982) and the brown treesnake (*Boiga irregularis*; histology), an invasive species on the island of Guam (Fritts and Rodda, 1998). For *B. irregularis*, fang morphology (Jackson and Fritts, 1995), gland histology and ultrastructure (Zalisko and Kardong, 1992), venom biochemistry (Mackessy et al., 2006; Pawlak et al., 2009), the venom proteome (Pla et al., 2018) and the venom gland transcriptome (McGivern et al., 2014) have been extensively described.

More recently, the venom apparatus, gland histology, venom proteome and venom gland transcriptome were described in the Amazon puffing snake (*Spilotes sulfureus*), a large South American rear-fanged snake (Modahl et al., 2018a). The relatively small Duvernoy's venom gland lies in a typically subdermal location below and posterior to the eye, stabilized at the posterior edge by a ligament extending to the jaw rictus (Fig. 1B). As in other rear-fanged snakes, the Duvernoy's venom gland is a serous secretory gland, histologically distinct from the nearby mucosecretory supralabial (salivary) gland (Fig. 1C). A duct from the gland communicates to the base of three enlarged rear maxillary teeth, and there is no direct insertion of adductor muscles or other specialized muscles on the gland. Venom is presumably expelled from the gland by the compression of the gland against the skin as the jaw adductor muscle contracts. This general pattern is seen in other rear-fanged snakes, including *B. irregularis* (Zalisko and Kardong, 1992), the green parrot snake (*Leptophis ahaetulla marginatus*; Sánchez et al., 2018), and the Aesculapian false coral snake (*Erythrolamprus aesculapii*; Sánchez et al., 2019).

In elapid snakes (cobras, coral snakes, kraits, seasnakes, etc.), a short, hollow fang is present at the anterior end of the elongated maxilla (Fig. 1D–F); in some laticaudine sea snakes, the fangs are exceptionally short (Fig. 1D). Terrestrial elapids, such as the red-headed krait (*Bungarus flaviceps*), also possess short fangs. However, the lateral and medial posterior portion of the venom gland has attached muscle fibers (compressor glandulae) that expel venom from the gland at higher pressures than for rear-fanged snakes; in addition, the anterior portion is differentiated as an accessory gland (Fig. 1F), which at present has no well-defined function. A short duct leads to the base of the hollow maxillary fang.

In viperid snakes (rattlesnakes and other pitvipers, Old World adders and vipers, etc.), a much longer hollow fang is attached to a greatly shortened maxilla, and the fang can be rotated/folded against the oral mucosa at rest (Fig. 1G). An enlarged compressor glandulae muscle attaches to the dorsal, medial and lateral posterior half of the connective tissue capsule enclosing the gland, and a very large basal lumen is present (Fig. 1G,H). The small accessory gland is physically separated by a primary duct of simple columnar epithelium extending from the main gland; distal to the accessory

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gland, a short secondary duct leads to the base of the hollow fang (Fig. 1G,H). This venom apparatus design allows for rapid, large volume and high-pressure injection deep into prey tissues, and the volume of stored venom can be impressive (2 ml or more for large, ∼1–2 m, snakes).

Microanatomy and ultrastructure of secretory and ancillary gland cells and mechanisms of venom secretion

Viperid venom glands have been the subject of numerous studies, so aspects of secretion and the mechanisms of stable venom storage are best known for these snakes. As in other venomous snakes, serous secretory epithelial cells make up the majority of cells in the venom glands of rattlesnakes (Fig. 2A–C,E,F; Mackessy, 1991). In addition to serous secretory epithelial cells, venom glands and ducts may contain an additional six cell types (main gland: mitochondria-rich cells, horizontal cells, 'dark' cells; accessory gland: mucosecretory cells, ciliated mitochondria-rich cells, vesicular mitochondria-rich cells; Mackessy, 1991), some of which act to stabilize venom (the mitochondria-rich cells of the main gland, Fig. 2D,G–I; Mackessy and Baxter, 2006; Perry et al., 2020) or to protect the epithelium against toxic effects during venom expulsion (mucosecretory cells). Structural elements have been revealed by light, confocal and electron microscopy, and neural regulation of secretion via sympathetic autonomic innervation was demonstrated by Kerchove et al. (2004, 2008). Secretory cells in vipers respond to venom use or manual extraction by rapidly increasing in height and changing from a cuboidal to columnar morphology, with a concomitant enlargement of the rough endoplasmic reticulum, a

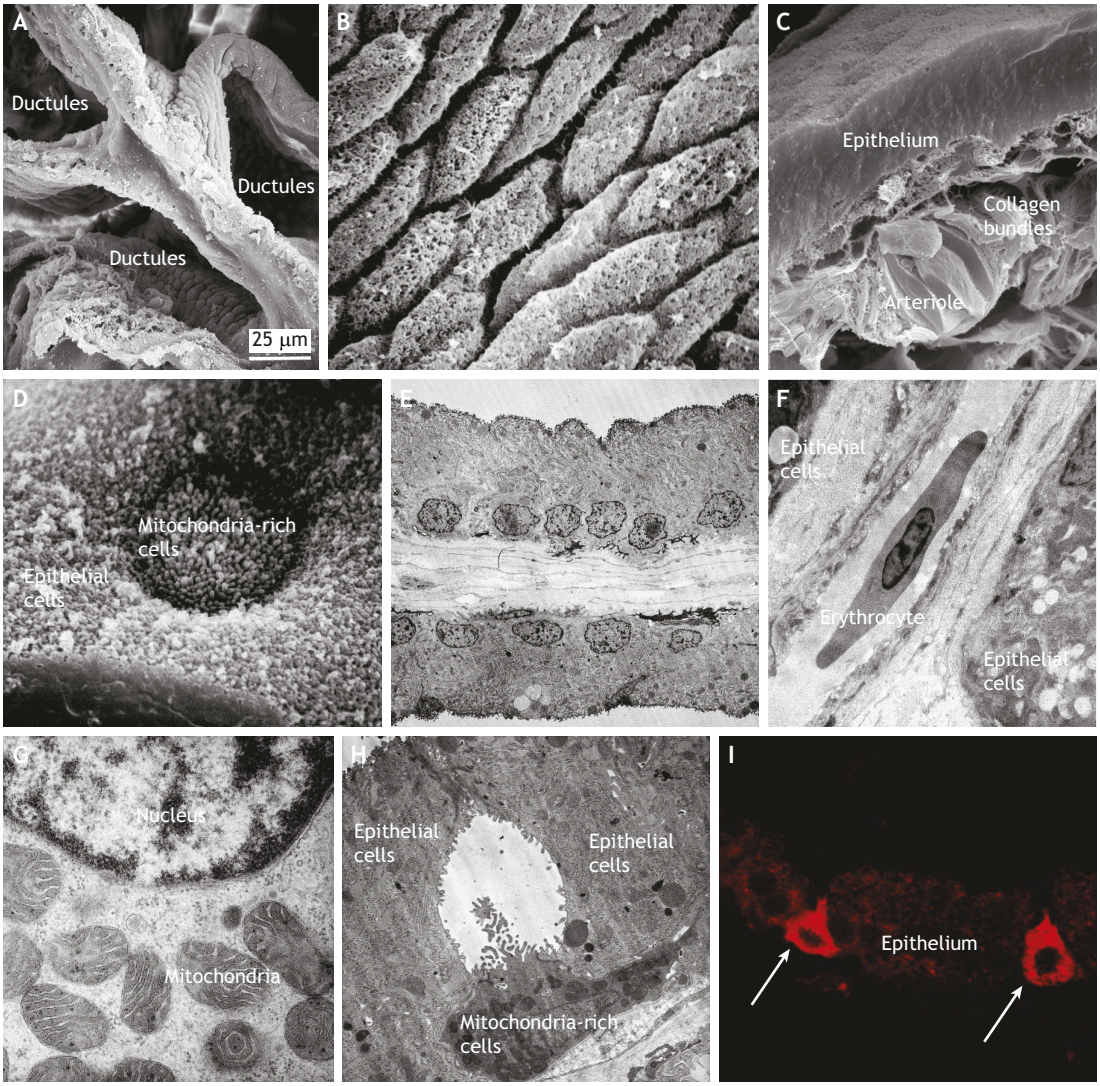

Fig. 2. Northern Pacific rattlesnake (*Crotalus oreganus oreganus*) main venom gland. (A–D) Scanning electron micrograph images. (A) Architecture of the main gland – secretory epithelial cells line the ductules (d) of the gland. (B) Apical surface of the secretory epithelium. (C) Freeze-fracture of the epithelium, showing an arteriole with erythrocytes and collagen bundles (adapted from Mackessy, 1991). (D) Mitochondria-rich cells are recessed below the surface of the surrounding secretory epithelial cells. (E–H) Transmission electron micrograph images. (E) Secretory epithelial cells 4 days post-extraction of venom; cells are columnar (adapted from Mackessy, 1991). (F) Capillary with erythrocyte within connective tissue separating two ductules of epithelial cells. (G) Nucleus and basal mitochondria in a mitochondria-rich cell. (H) Oblique section of a mitochondria-rich cell showing its recessed nature relative to surrounding secretory epithelial cells, elongate microvilli and canaliculate association with secretory cells. (I) Fluorescence micrograph of venom gland secretory epithelium of the prairie rattlesnake (*Crotalus viridis viridis*) (adapted from Perry et al., 2020). The image demonstrates the presence of vacuolar H^+-ATPase in mitochondria-rich cells (arrows), which are hypothesized to titrate citrate buffer in the gland lumina to maintain a pH of 5.4. Polyclonal mouse antibody αH56 designed against the 56 kDa subunit of the vacuolar H^+-ATPase was used as the primary antibody.

large increase in gene expression and mRNA levels and extensive protein synthesis (Rotenberg et al., 1971; Mackessy, 1991; Kerchove et al., 2004; 2008). Recently, this highly dynamic system was shown to involve the differential regulation of thousands of genes, including a multiplicity of regulatory responses that coalesce to secrete venom into the ductules and lumen of the venom gland (Perry et al., 2020). Gene upregulation occurs within 24 h post-extraction of venom and involves cellular stress and unfolded protein response pathways (Perry et al., 2020). Following complete manual depletion of venom, replenishment of the ductule and basal lumen contents takes considerably longer; cell morphology cycles from cuboidal to columnar and then back to cuboidal, following the merocrine secretion of venom into gland ductules. During the peak of the columnar phase (~3–7 days post-extraction), secretory cells are filled with rough endoplasmic reticulum, indicative of a massive protein synthesis response, and proteins are then transported via the Golgi apparatus to secretory granules (Marchi et al., 1978; Oron and Bdolah, 1978). The merocrine release of vesicles into gland ductules occurs shortly thereafter, and cells return to a resting state, often also exhibiting mature intracellular secretory granules, approximately 12–14 days post-extraction.

Mitochondria-rich cells, which make up ~2% of the total cell population in *C. o. oreganus* venom glands, also cycle with the secretory cells (Mackessy, 1991). Originally, the function of these cells was unknown, with a hypothesized role in water resorption (Warshawsky et al., 1973). However, it was later shown that mitochondria-rich cells are acid-secreting cells, and their morphology and cytochemistry are very similar to those of the parietal cells of the mammalian gastric pit (Mackessy and Baxter, 2006). Consistent with this hypothesis, venom from different species of vipers, including rattlesnakes, North African *Cerastes* sp. and South American *Bothrops* sp., is stored at a pH of 5.4–5.5, and at this pH, venom lytic enzymes are essentially inactive. Coupled with the presence of low-affinity but high-concentration tripeptide inhibitors (Munekiyo and Mackessy, 2005), low pH allows safe storage of a potentially toxic (and autolytic) venom for long periods of time. Upon deployment, inhibitors dissociate from enzyme toxins and the pH milieu changes by nearly two log steps (~7.4 in prey tissues), resulting in spontaneous activation of enzyme toxins. Recently, the acidification of stored venom was shown to occur via vacuolar ATPases (V_o-ATPase) present in the mitochondria-rich cells that are absent or in very low abundance in surrounding secretory cells (Fig. 2I; Perry et al., 2020). Mitochondria-rich cells react very strongly and specifically with V_o-ATPase antibody probes, and at least 6 V_o-ATPases are upregulated in these cells relative to unextracted glands and non-venom gland tissues (Fig. 2I) (Mackessy, 1991). This is consistent with the observed increase in cell height (mitochondria-rich and secretory cells) seen 3–7 days post-venom extraction, followed by a return to a cuboidal morphology after release of venom-containing granules (Mackessy, 1991). Further, an effective buffer, citrate, is present in low millimolar quantities in stored and secreted venom (Francis et al., 1992), and it appears that mitochondria-rich cell V_o-ATPases titrate this buffer of stored venom to the observed pH (Mackessy and Baxter, 2006; Perry et al., 2020). Citrate may also inhibit venom metalloenzymes via metal chelation (Francis et al., 1992); however, at the pH optimum of a purified metalloproteinase (~pH 9), 100 mmol l^{-1} citrate (approximately 20 times that found in the venom) inhibited activity by <40% (Mackessy, 1996), and physiological levels (~5–10 mmol l^{-1}) had no effect. Secretion of metalloproteinases as zymogens has also been demonstrated (Grams et al., 1993), and this may stabilize venom components intracellularly, but they appear to be processed during secretion into lumina (cf. Portes-Junior et al., 2014), so this does not seem to be an important storage/inhibition mechanism of extracellular venom.

The general histology of the elapid main gland is somewhat different from that of viperid snakes. As noted above, an accessory gland, when present, is seen as a continuous anterior portion of the main gland, rather than as a separate structure connected to the main gland by the primary duct (viperids). Ultrastructural analysis revealed secretory cells as the principal cell type of the Malayan banded coral snake (*Calliophis bivirgata*; formerly *Maticora bivirgata*; Gopalakrishnakone and Kochva, 1990a), Egyptian cobra (*Naja haje*; Kochva et al., 1982) and Hardwicke's sea snake (*Hydrophis curtus*; formerly *Lapemis curtus*; Gopalakrishnakone and Kochva, 1993); these cells contain prominent rough endoplasmic reticulum, secretory granules and a prominent basal nucleus, as in viperids. A study of the general (light micrograph) histology of both elapine (cobras, coral snakes, etc.) and hydrophiine (sea snakes, Australian front-fanged snakes) elapid snakes (23 species) indicated a high degree of similarity in all main venom glands, though the accessory glands were greatly reduced in sea snakes relative to terrestrial elapids (Gopalakrishnakone and Kochva, 1990b). Elapid glands lack the large basal lumen of viperid glands; ductules of secretory epithelia are arranged radially, leading to the central duct that is continuous through the accessary gland and to the base of the fang. Following venom extraction, cells cycle from cuboidal to columnar, with a concomitant increase in rough endoplasmic reticulum and secretory granules; protein biosynthesis peaked at 4–9 days following extraction (Kochva et al., 1982).

Very few species of rear-fanged snakes have been subjected to ultrastructural study, and mechanisms of venom biosynthesis and secretion are essentially unknown. The histology and ultrastructure of the Duvernoy's venom gland of the wandering garter snake (*Thamnophis elegans vagrans*, a natracine colubrid) has been analyzed (Kardong and Luchtel, 1986), and it produces a low-complexity venom (Hill and Mackessy, 2000). *Thamnophis elegans vagrans* venom has only two major protein families, P-III metalloproteinases and cysteine-rich secretory proteins (CRiSPs) (Perry et al., 2018); other colubrids, such as *B. irregularis*, produce venom that is considerably more complex and is dominated by many three-finger toxin isoforms, with metalloproteinases and CRiSPs being significant but less abundant toxins (Pla et al., 2018). In *Boiga irregularis*, *Rhabdophis tigrinus* (tiger keelback snake, also a natricine colubrid) and *Thamnophis elegans vagrans*, the principal cell type of the Duvernoy's venom gland is the serous secretory cell, arranged as simple columnar acinus and surrounding a small central lumen (Yoshie et al., 1982; Kardong and Luchtel, 1986; Zalisko and Kardong, 1992). In *Thamnophis elegans vagrans* and *Rhabdophis tigrinus*, secretory granules stain very intensely, similar to that seen in elapid venom glands; unlike both elapids and especially viperids, only a small amount of venom is stored extracellularly, and venom injected into prey is likely exocytosed during predatory events, thus requiring a longer contact time for sufficient venom delivery, a characteristic of rear-fanged snakes (Kuch and Mebs, 2002; Weinstein and Kardong, 1994).

Comparative venom proteomes: broad phylogenetic patterns

Venoms generally facilitate capture and/or digestion of prey, but the different lineages of reptiles show several distinct patterns of venom proteome composition. In very broad terms, viperid snakes produce venoms rich in enzymes, often including several subclasses of

metalloproteinases, serine proteinases and other hydrolases, whereas elapid snakes produce venoms dominated by non-enzymatic three-finger toxins and phospholipases A_2 (Mackessy, 2021; Tasoulis and Isbister, 2017; Tasoulis et al., 2022). Different species of rear-fanged snakes may follow patterns grossly similar to either elapids or viperids (Modahl et al., 2018a,b); in general, however, their venom proteomes are considerably less complex than those of front-fanged snakes.

Though snake venoms are commonly referred to as either 'hemotoxic' or 'neurotoxic', this gross generality greatly oversimplifies actual venom compositional variation and toxicological profiles. A general truism is that snakes have 'opted for' only a few stable molecular scaffolds out of thousands of potential protein structures, resulting in a small number of protein families that dominate the venom proteome. For example, in venoms of numerous rattlesnake species studied, metalloproteinases, serine proteinases, phospholipases A_2 and (often, but not always) peptide myotoxins account for 75–90% of the total venom proteome (e.g. Saviola et al., 2015; Fig. 3A); most viperids are similar. Conversely, in elapid snakes, phospholipases A_2 and three-finger toxins (small non-enzymatic proteins) comprise about the same majority of the proteome (Fig. 3B). Rear-fanged snakes may show either pattern illustrated by front-fanged snakes (Fig. 3C); several species in the family Colubridae produce venoms rich in three-finger neurotoxins (Modahl et al., 2018a; Pla et al., 2018; Mackessy et al., 2020; Heyborne and Mackessy, 2013, 2021), whereas species in the family Dipsadidae often produce venoms rich in metalloproteinases, either snake venom metalloproteinases (SVMPs) (Modahl et al., 2018b) or matrix metalloproteinases (Ching et al., 2012; Junqueira-de-Azevedo et al., 2016; Bayona-Serrano et al., 2020). Relatively few venoms have been deeply characterized, so it is likely that novel compounds are yet to be discovered among rear-fanged snakes; an example of a novel toxin was seen in the discovery of snake venom acid lipase in *Phalotris mertensi* (family Dipsadidae) venom (Campos et al., 2016). The presence of matrix metalloproteinases, another novel toxin family, had been hinted at by earlier studies but these proteins have only more recently been shown as novel components of specific dipsadid rear-fanged snake venoms (summarized in Junqueira-de-Azevedo and Bayona-Serrano, 2021). However, in virtually all snake species studied, each dominant protein family consists of several to many isoforms, often with vastly different pharmacological effects. Rather than recruiting 'new' toxins to the venom arsenal, rapid and extensive gene duplication followed by mutation of toxin genes has led to neofunctionalization within a protein family, resulting in a proliferation of related toxins (Fry, 2005; Fry et al., 2008; Casewell et al., 2011; Tsetlin et al., 2021).

Models for the study of venom production and secretion

Previous studies have detailed the anatomy and timing of venom production (Mackessy, 1991), and the involvement of parasympathetic stimulation in venom synthesis and secretion has been demonstrated (Kerchove et al., 2004, 2008; Luna et al., 2009). Recent application of various -omic techniques has also provided the chromosomal localization of venom genes and an in-depth understanding of many genetic mechanisms involved in venom production (Schield et al., 2019; Perry et al., 2020; Suryamohan et al., 2020). However, the in-depth study of secretory cycling and regulation has been hampered by the lack of a long-lived venom secretory cell culture. Several attempts to culture primary venom gland cells have provided intriguing glimpses into the possibility of

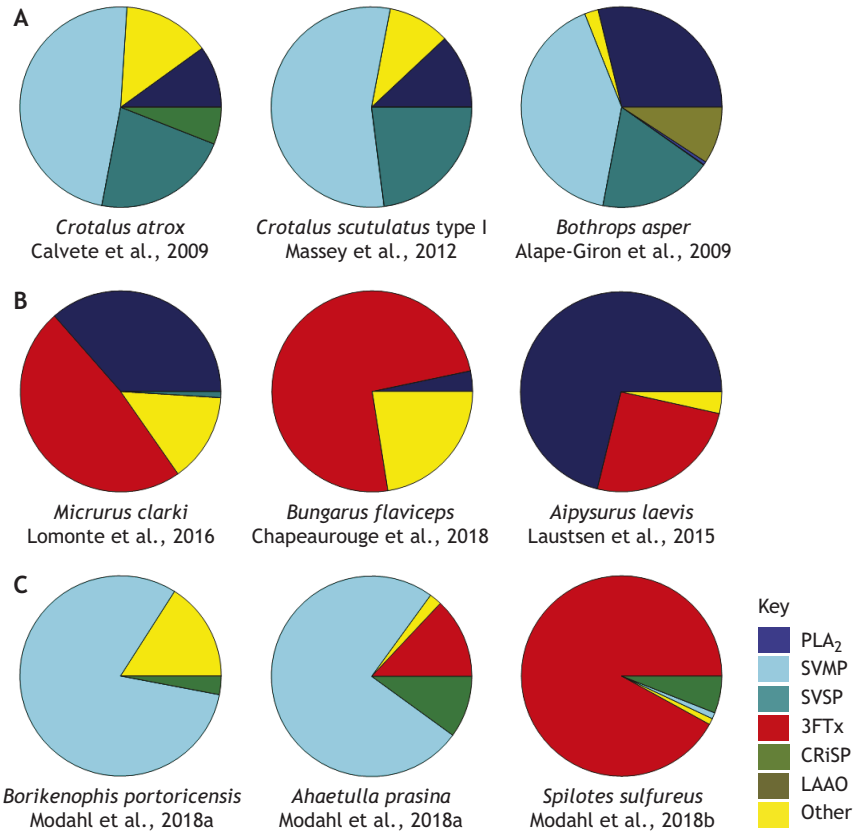

Fig. 3. Comparative simplified venom proteomes, illustrating common compositional patterns. In viperid snakes, enzyme toxins, particularly metalloproteinases, serine proteinases and phospholipases A_2, dominate the proteome. In elapid snakes, non-enzymatic toxins such as three-finger toxins (neurotoxins, cardiotoxins, etc.) and modified phospholipases A_2 (some neurotoxic) are most prevalent. In rear-fanged snakes, either pattern may be seen, with metalloproteinases or three-finger toxins typically dominating the proteome. (A) Crotaline viperid snakes: *Crotalus atrox* (Western diamondback rattlesnake), *Crotalus scutulatus* (Mohave rattlesnake) type I venom and *Bothrops asper* (Terciopelo/Fer-de-Lance). (B) Elapid snakes: *Micrurus clarki* (Clark's coral snake), *Bungarus flaviceps* (red-headed krait) and *Aipysurus laevus* (olive sea snake). (C) Rear-fanged snakes: *Borikenophis portoricensis* (Puerto Rican racer), *Ahaetulla prasina* (Asian vine snake) and *Spilotes sulfureus* (Amazon puffing snake). Note that within each of the protein families listed, several to many isoforms may exist in the proteome. CRiSP, cysteine-rich secretory protein; 3FTx, three-finger toxin; LAAO, L-amino acid oxidase; PLA_2, phospholipase A_2; SVMP, snake venom metalloproteinase; SVSP, snake venom serine proteinase; Other, other protein families (~4% or less each of total proteome). Data from Calvete et al. (2009); Massey et al. (2012); Alape-Girón et al. (2009); Lomonte et al. (2016); Chapeaurouge et al. (2018); Laustsen et al. (2015); Modahl et al. (2018a,b).

creating a cell culture model for studying synthesis and secretion in detail, but these have been stymied by the short (≤30 days) survival time of primary cultures and the lack of replication (Yamanouye et al., 2006). Little progress had been made toward the establishment of an immortal, or at least long-lived, culture of venom secretory cells for many years, when a truly revolutionary approach from the lab of Hans Clevers was recently published (Post et al., 2020). The creation of an organoid model system, with a reported longevity of at least 1 year, has tremendous promise to overcome previous obstacles to the study of venom production in cultured cells.

Clevers' lab has considerable experience with organoid production, so it is not surprising that his group and colleagues were able to apply this technology toward the successful production of venom gland organoids. A major departure from earlier attempts to culture venom gland epithelial cells was the use of basement membrane extract as a support matrix and the inclusion of numerous components of cell signaling pathways (including Noggin, EGF, R-spondin, TGF beta inhibitor A83-01 and other factors) (Post et al., 2020). Importantly, lower incubator temperatures (32°C) were found to be critical for the survival and expansion of organoids. The subsequent removal of growth factors greatly decreased organoid proliferation but stimulated production of secretory vesicles and secretion of venom from organoid secretory cells. Comparison with a *de novo* transcriptome for the main species analyzed (*Aspidelaps lubricus cowlesi*; Angolan coral snake, an elapid snake) and its previously described venom proteome (Whiteley et al., 2019) demonstrated that the major toxin families were present in the organoid-derived venom (though phospholipase A_2 was notably absent). The biological activity of organoid supernatant (containing venom proteins) was analyzed for acetylcholine receptor blocking activity, and results were similar to those obtained with α-bungarotoxin, demonstrating that the α-neurotoxins expressed were patent and active. Additionally, transmission electron micrographs of organoid secretory cells were remarkably similar to those derived from intact adult venom glands (e.g. Mackessy, 1991); organoid cells in expansion medium (containing growth and signaling factors) appeared columnar, with extensive proliferation of the rough endoplasmic reticulum, whereas cells in differentiation medium (lacking factors) were more cuboidal and showed a preponderance of intracellular secretory granules. These morphological changes in the cells are consistent with previous reports of protein synthesis following venom depletion from intact glands. Gene expression heat maps of *A. lubricus cowlesi* organoids also showed increased expression of toxins in the differentiated organoids (Post et al., 2020). Further, the authors presented evidence that regional differentiation of expression patterns may exist in the glands, such that different toxins, or different suites of toxins, are primarily synthesized and exocytosed from cells in the anterior versus the posterior parts of the venom glands. This conclusion is in contrast to an earlier study indicating that all secretory cells produced all venom constituents, but that different venom components were synthesized and exocytosed at different stages of the secretory cycle (Taylor et al., 1986). In sum, using a variety of different analyses, venom gland organoids were shown to behave very similarly to venom gland secretory epithelial cells. We are still a long way from *ex vivo* commercial-level production of venoms, as the paper intimates, but as an experimental model system, venom gland organoids offer unprecedented opportunities for exploring secretory mechanisms, variable toxin expression and regulation and many other cell- and genome-level phenomena.

Although venom gland tissues derived from late-embryo *A. lubricus cowlesi* were the primary focus of this report (Post et al., 2020), eight additional species, including tissues taken from adult snakes and from both elapid and viperid snakes, were found to yield organoids that could undergo expansion and subsequent passage. This demonstration of general applicability to a variety of species, with very different venom proteomes (see Fig. 3), strongly indicates that the organoid formation method can be applied to many different types of glandular structures and is not dependent on embryonic (and potentially undifferentiated/stem cell) gland tissue. Long-term survival (>1 year) and repeated passage (>18) viability of late-embryo *A. lubricus cowlesi* cells demonstrate that organoid production should be possible for most venomous species, and the production of organoid-based models has tremendous potential for modeling protein synthesis and secretion in a highly controllable system. In particular, the study of invertebrate venom systems, which can be hampered by their small size and a lack of sufficient amounts of secreted venom, could benefit greatly from organoid proliferation of glandular material.

Early in 2021, the Clevers' group published a complete methodology paper on organoid formation and maintenance (Puschhof et al., 2021), with detailed step-by-step instructions for production. Of particular note is the even longer period of propagation of organoids, with some lines having been cultured now for over 2 years. At present, only organoids derived from elapid snakes have been maintained for long periods, and it will be of interest to see whether long-term production of organoids can be maintained with viperid gland tissues. When working with venom glands that produce cytotoxic proteins, extensive washing of tissues was recommended (Puschhof et al., 2021), suggesting that epithelia that produce lytic toxins may be more challenging to maintain as organoids. Additionally, Duvernoy's venom glands from rear-fanged snakes have not yet been subjected to organoid culturing methods, and the detailed exploration of these poorly known venoms and their synthesis mechanisms should be amenable to similar methodological approaches. Venom gland organoid technologies, applied to carefully chosen taxa representing the diversity of venomous vertebrates, including snakes, helodermatid lizards and even monotreme and insectivore mammals, could greatly illuminate our understanding of venom evolution in vertebrates. Further, if regional expression turns out to be a generalized phenomenon in snake venom glands, organoids could be exploited to amplify specific cell lines that express toxin families of interest. However, a potential issue that has not yet been addressed is whether or not specific cellular differentiation cues, either spatial/physical or physiological, might influence secretory behavior of organoid secretory cells in a positive or negative fashion. Growth and signaling factors included in the media appear to be required for competent cell replication and longevity, and these are likely allowing expression of venom in a form similar to that observed in the intact gland. Finally, these studies have demonstrated that organoid models of venom glands can be produced and maintained, in spite of the limitations presented by glands that produce lethally toxic, cytotoxic and autolytic proteins. As organoid technologies become more commonplace, there will likely be applications for the exploration of protein synthesis and gene regulation of these processes in a variety of other tissues in non-model organisms.

New directions for the study of venom protein storage, synthesis and secretion

Tremendous advances in and growth of the various -omics technologies have provided unprecedented insight into the composition of venoms, at exceptional levels of sensitivity and

detection. However, despite the significant level of analyses of the venom proteomes, the mechanisms involved in the production of this labile and toxic secretion remain poorly known. Advances in cell culture and -omics technologies are providing a productive platform from which to approach formerly intractable questions, and the use of venom gland organoids should greatly facilitate progress in these areas.

In spite of their abundance, broad global distribution and tremendous diversity, relatively little is known about the secretory cycles of epithelial cells of colubroid rear-fanged snake venom glands. Transcriptomic protocols have assumed that secretory cycles of rear-fanged snake venom glands mirror those of front-fanged snakes, with mRNA levels reaching a maximum at 3–4 days post-extraction (but with no supportive evidence), but even among front-fanged snakes, only a handful of studies have been conducted, most of these prior to the advent of -omic technologies (i.e. Rotenberg et al., 1971; Kochva et al., 1982). Thus, tremendous opportunity lies ahead for the investigation of most rear-fanged snake species, at every technical level currently possible. Their venom proteomes can vary greatly between species (i.e. Modahl et al., 2018a,b; see also Fig. 3), so the events leading to the regulation of synthesis, maintenance and deployment of venoms may also show significant variation. Bioprospecting, the investigation of natural sources for the development of therapeutics and other useful compounds, could also greatly benefit from a better understanding of the diversity of venoms and venom secretion mechanisms seen among these species.

Snakes have slowly been gaining traction as highly productive non-model species for investigating novel twists on such commonplace functions as mitochondrial respiration (Castoe et al., 2008, 2009), gastrointestinal responses upon feeding (Riquelme et al., 2011; Perry et al., 2019) and social interactions/parental care (Lind et al., 2017). In each case, snakes have provided unique examples that differ significantly from common patterns seen in related vertebrates, often also providing clues for the adaptive management of prominent human pathological conditions (e.g. Riquelme et al., 2011). Consider the venom gland: this relatively small structure inducibly synthesizes toxins that generally have deleterious effects on vertebrates and other animals, potentially also on the possessor of the gland. Mechanisms have evolved (in at least some species) that allow for the synthesis, storage and effective deployment of this biological weapon; venoms also may be stored for very long periods of time, requiring that the venom in gland lumina be maintained in an inactive state, but ready for immediate use (see above). In addition, protein structures and activities must be maintained in a viable state, for months or perhaps years, and some of the larger enzyme toxins (L-amino acid oxidases, P-III metalloproteases etc.) are notoriously unstable once purified. These observations imply that endogenous stabilization mechanisms must exist, not only to prevent autolysis but also to promote the stable storage of labile components. Further, snake venoms contain small amounts of nucleic acids, including mRNAs, that appear to be stable for long periods of time (see Modahl and Mackessy, 2016; Smith et al., 2018). The thorough investigation of these mechanisms may be applicable to the production and storage of nucleic acid-, protein- and peptide-based therapeutics in the future or currently in development. These examples indicate clearly that non-model organisms, such as venomous snakes, can provide unique answers to difficult or longstanding questions in biology, and future studies will undoubtedly reveal additional novel and informative processes among these animals.

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Fig. 1A,D 3-dimensional renderings are from the University of Texas Digital Morphology web site (DigiMorph.org), courtesy of Dr Jessica A. Maisano.

Competing interests

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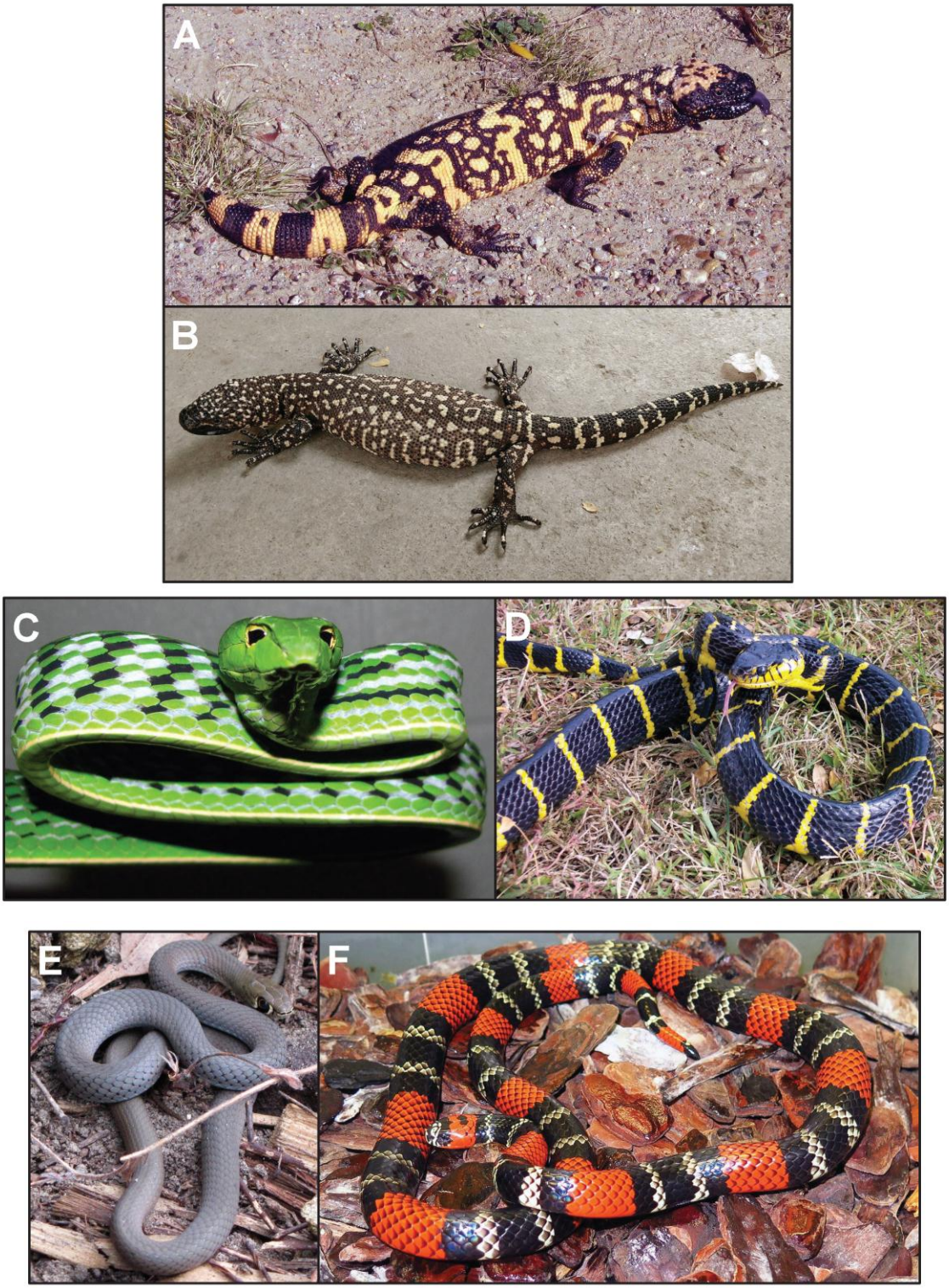

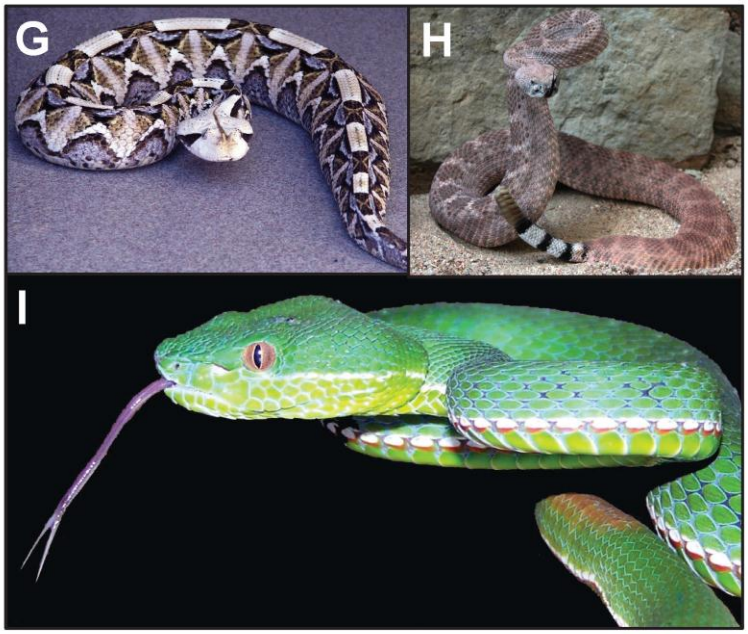

Fig. S1. Representative species of venomous reptiles. The two species of venomous lizards are members of the family Helodermatidae. **A.** Gila Monster, *Heloderma suspectum* – Arizona, USA. **B.** Mexican Beaded Lizard, *Heloderma horridum* – Cuernavaca, México. The family Colubridae is distributed worldwide, with many rear-fanged species, most of which are harmless to humans. **C.** Asian Vinesnake, *Ahaetulla prasina* – Sumatra. **D.** Mangrove Catsnake, *Boiga dendrophila* – Sumatra. The family Elapidae, including coral snakes, Australian front-fanged snakes, cobras, kraits and others, contains many species of dangerously venomous front-fanged snakes. **E.** Yellow-faced Whipsnake, *Demansia psammophis* – Eastern Australia. **F.** Uruguayan Coral Snake, *Micrurus altirostris* – Brazil. The broadly distributed family Viperidae also includes many dangerously venomous front-fanged snakes, such as the true vipers, pitvipers, rattlesnakes and others. **G.** West African Gaboon Viper*, Bitis rhinoceros* - subsaharan Africa. **H.** Western Diamondback Rattlesnake, *Crotalus atrox* – Arizona, USA. **I.** Chinese Green Tree Viper, *Trimeresurus stejnegeri* - Taiwan.