Venom Use in Mammals: Evolutionary Aspects

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Abstract

Mammals are recently accepted as venomous animals, with four orders having venomous representatives. These are Eulipotyphla (solenodons and some shrews), Monotremata (platypus), Chiroptera (vampire bats), and Primates (slow and pygmy slow lorises). Each of them has different strategies for using very diverse mixtures of toxic molecules. Venomous saliva is used by eulipotyphlans to paralyze and cache prey, and by chiropterans to avoid blood clotting in suitable prey, allowing continuous feeding. Monotremata use crural spurs to inject a highly painful secretion as a tool in sexual selection, while Primates lick an elbow gland, loading modified teeth with anaphylaxis-inducing venom. There is no homology between venomous systems in these different orders, making a common origin for all venom in Mammalia unlikely, even considering gaps in the fossil record. An emerging picture of complex interactions between cost of venom producing, specialized teeth for feeding and possible lack of benefits for venom in larger, stronger mammals may be able to justify the rarity of venom in this group. Both basic science and biotechnology are benefited as more knowledge accumulates about mammalian venoms.

Keywords

Mammals • Platypus • Bat • Loris • Shrew

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Introduction

Mammalian venoms may be considered a novelty, but they have not been discovered recently. For centuries, up to the 1940s, shrew bites were regarded as highly painful (and the animal itself was taken as evil and ill intentioned). Comparisons were drawn between shrews and venomous reptiles, including cobra and beaded lizards. However, with advances in microbiology and the advent of antibiotics, effects originally attributed to shrew saliva were attributed to the action of microorganisms from the animal's mouth. It took 50 years for this topic to be rediscovered, or, more accurately, rediscussed. Unfortunately, Dufton's seminal work (Dufton 1992) remained largely ignored by mainstream zoologists (and biologists as a whole). The emergence of more solid evidence on many mammalian venoms, two decades later prompted new investigation of the subject (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

There are four mammalian orders with known venomous representatives, as recognized today. These comprise solenodons and some species of shrews (Order Eulipotyphla), platypuses (Order Monotremata), vampire bats (Order Chiroptera), and slow lorises (Order Primates). The amount of knowledge regarding each class varies greatly. There is also great variation in the strategies in which the venoms are employed. These secretions are used to immobilize prey, to facilitate feeding, for predator defense, and for sexual selection. In this chapter, historical aspects and specifics of the venoms' toxicity will be presented first. Subsequently, evolutionary mechanisms that led to each of these venom-use strategies will be discussed.

Venomous Mammals: Overview

Eulipotyphla

The eulipotyphlans include the majority of venomous mammals. These are the American short-tailed shrew (*Blarina brevicauda*), the Hispaniolan solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*), and the Mediterranean water

shrew (*Neomys anomalus*). Some evidences support venom in the Cuban solenodon (*Solenodon cubanus*) and the Canarian shrew (*Crocidura canariensis*), while circumstantial evidence may point to venom in the European mole (*Talpa europaea*). Despite being omnivores, the eulipotyphlans were formerly included in the "wastebasket" taxon Insectivora (Greek for "eaters of insects"). This was a major misnomer, since these animals prey on varied invertebrates and on vertebrates of the same, or even larger, size as themselves. The venom asset of these mammals is found in their saliva, produced in enlarged granular submaxillary salivary glands. Venomous saliva is also found in vampire bats, albeit of different composition (see below). The most extensively studied eulipotyphlan saliva is the one from *B. brevicauda*, since many factors hinder studies with other species, ranging from difficulties in keeping these animals in captivity (shrews) to their endangered status (solenodons).

The European shrew probably was the first mammal to be historically recorded as venomous. In 1607, Reverend Topsell's "History of Four-footed Beasts" described the animals as cunning and cruel, pretending to be gentle and tame, but desiring to hurt anything with a deep, deadly, bite. In Europe, shrews have been associated with malignancy, depravity, wickedness, and taken as signs of ill omen. The Latin name for the European shrew, *Sorex araneus*, and the French common name, *musaraigne*, derive from the Latin word for spider (*aranea*). In North America, shrews were considered mostly harmless, and their dangerous status was relegated to folklore, while the natives of the Caribbean Islands regarded solenodons as venomous. Solenodons and shrews had their venomous saliva scientifically examined almost simultaneously. In 1877, Gundlach studied bites of Cuban solenodons (*S. cubanus*), comparing them to bites from venomous snakes, while Maynard, in 1889, made a case report on the effects of an American short-tailed shrew (*B. brevicauda*) bite. From 1942 to the 1960s, the saliva toxicity of various eulipotyphlans was tested on animal models. However, this line of research seemed abandoned until 1992, when a major review with new data (Dufton 1992) brought the subject back into the spotlight. In the 2000s, the identification of the *B. brevicauda* toxin (Kita et al. 2004) and the uncovering of fossil shrews with envenomation apparatus (Cuenca-Bescós and Rofes 2007) once again emphasized this aspect of mammalian physiology.

Bites from shrews are considered uncomfortable to humans, with personal perceptions ranging from no detectable effect to an immediate burning sensation, swelling, and impossibility of using the affected member for days (Ligabue-Braun et al. 2012). One of the first observations made about the Cuban solenodon bite was that the lower incisors caused inflammation at wound entry, while the upper incisors had no effect, something considered common by natives. This was the first observation that the submaxillary glands were the production site of venom (Dufton 1992). Solenodons have gutter-like grooves in their inferior incisor teeth that allow saliva flow to the bite-induced wound. Shrews lack this modification. When present (as in the Eurasian shrew), there is only a slight concavity in the incisors (Fig. 1).

Testing *Blarina*, *Neomys*, and *Solenodon* submaxillary extracts on mice, rabbits, and cats established that the venomous saliva causes general depression, breathing disturbance, paralysis, and convulsions. Even though small vertebrate prey (especially mice and voles) are an important part of some eulipotyphlan diets,

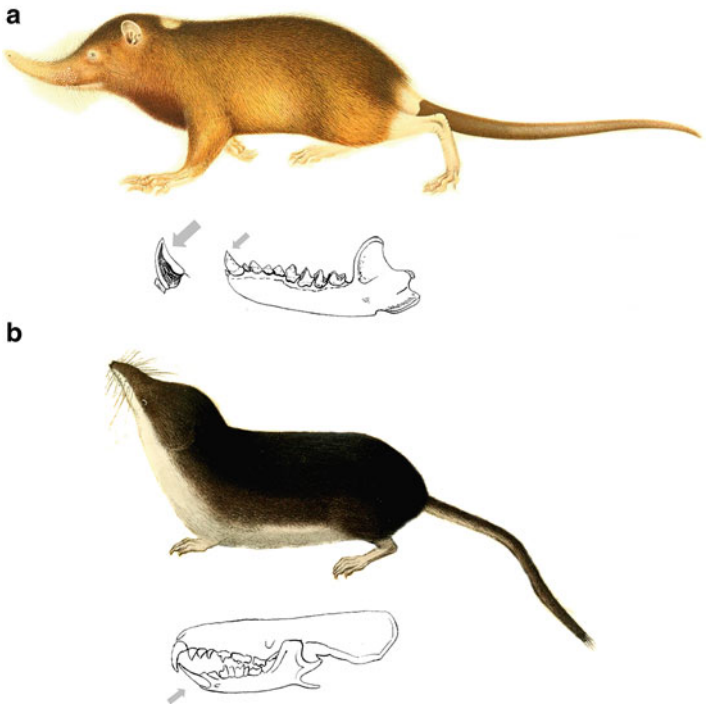

Fig. 1 Venomous Eulipotyphla. (**a**) General aspect of a Solenodon (*Solenodon paradoxus*). The detail highlights the grooved incisor teeth. (**b**) General aspect of a Eurasian shrew (*Sorex araneus*). The detail highlights the slightly concave incisor teeth (Author's own artwork, incorporating images in the public domain from "Solenodon paradoxus" by GM Allen, "Règne animal" by G Cuvier, and "Faune des vertébrés de la Suisse" by V Fatio)

invertebrates account for most of the animals' nutrition. Based on that, tests of *B. brevicauda* with experimental insect prey (such as roaches and crickets) revealed that its saliva has immobilizing effects, with these immobilized insects being stored for later consumption. In natural conditions, *B. brevicauda* caches a varied array of preys in a comatose state. These include, besides insects, snails, earthworms, and small mammals (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

The active compound in the saliva was thought to be a neurotoxin, based mainly in some similarities between shrew and cobra venoms proposed in the late 1940s (Ligabue-Braun et al. 2012). However, no resemblance was found (Lawrence 1945; Dufton 1992). This neurotoxin-targeting search, coupled with difficulties to work with pure *B. brevicauda* submaxillary gland extracts, hindered further research into this topic until the 2000s. In 2004, it was found that the major toxic component in *B. brevicauda* saliva is blarina toxin (BLTX) (Kita et al. 2004). Still, other, unidentified synergistic components may be acting in the venom. BLTX is an N-glycosylated kallikrein-like protease of 253 amino acids, with heterogenous glycoforms. This toxin releases bradykinin from kininogens and would be

responsible for the effects observed in experimental animals (dyspnea, hypotension, hypokinesia), since bradykinin is an inflammation mediator that acts in increasing vascular permeability and lowering blood pressure.

Monotremata

There is only one venomous Monotremata species, the platypus (*Ornithorhynchus anatinus*). This egg-laying mammal has semifossorial, semiaquatic habits, living in rivers and streams in the eastern coast of Australia. Both males and females are born with spurs in their hind legs, but only the former maintain them for life. These keratinized spurs are connected to the crural glands, which produce venom, forming the venom-injecting structure known as the crural system (Grant and Temple-Smith 1998). The crural glands are found in the dorsocaudal sides of the abdomen, and derive from sweat glands (Whittington and Belov 2016; Fig. 2).

Platypus envenomation was first recorded in the scientific literature in 1818, and detailed anatomical description, including venom use and tests on domestic animals, followed. From 1935 to the 1960s, there seems to be no records on this subject. In 1968, however, a major monograph on platypus detailed the toxic properties of the crural secretions. Some envenomation case reports have been published since but have been normally treated more as a curiosity than as a real medical issue (Ligabue-Braun et al. 2012). From 1995 onwards, more biochemical characterizations of the venom became available, and the Platypus Genome Project (Warren et al. 2008) allowed a much more detailed inspection of this secretion.

Platypuses have been hunted for their fur by Australian colonists, who sometimes were victims of envenomation. In humans, all cases involved hands or wrists. The venom, injected by repetitive jabbing of spurs from both hind legs pressed against one another, causes immediate acute pain and swelling and requires anesthetic blockade combined with intravenous narcotic infusion as regular analgesic treatment is ineffective. The envenomation symptoms may persist for a long period, from

Fig. 2 Venomous Monotremata. General aspect of a platypus (*Ornithorhynchus anatinus*). The detail highlights the crural spur (Author's own artwork, incorporating images in the public domain from "Genera mammalium," by A Cabrera)

2 weeks to several months. In test animals, swelling and tenderness at the site of injection were observed, followed by decrease in blood pressure, respiratory distress, and death (Whittington and Belov 2007).

During the mating season, male platypuses are frequently found with punctures in their bodies, despite attacks among platypus being rarely observed. The crural glands show cyclic activity, becoming highly active during the mating season, producing venom to be delivered by the channeled spur (Grant and Temple-Smith 1998). Due to this cyclic activity and the protected status of platypuses, studies on their venom composition have been hindered until recently. The venom is a complex mixture of C-type natriuretic peptides, defensin-like peptides, nerve growth factors, isomerases, hyaluronidases, proteases, and other, uncharacterized proteins (Whittington and Belov 2007).

Four major components of the venoms have had more in-depth characterization (Whittington and Belov 2007; Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015) but their exact functions have not been established (Whittington and Belov 2016). C-type natriuretic peptides differ from A and B natriuretic peptides, which act in controlling blood pressure, by lacking natriuretic activity, suggesting other actions for these peptides. They are the most biologically active peptides in the platypus venom, and may be responsible for envenomation signs (such as hypotension). These peptides seem able to disrupt membranes and interact with putative nociceptors. Defensin-like peptides are structurally similar to β-defensins but lack sequence and functional similarity with them. These are the most abundant peptides in the venom and could cause pain, possibly by synergistic action with venom nerve growth factors. These factors are also devoid of their classical function, having a putative immunogenic effect. C-type natriuretic peptides and defensin-like peptides from platypus venom also show isoforms with either L- or D-amino acids in specific positions. This is due to an L-to-D-amino acid-residue isomerase. Though not confirmed, the function of these D-residues seem to be resistance to proteases while in the crural gland.

Chiroptera

Among Chiropterans, only a subfamily of New World leaf-nosed bats (Phyllostomidae) holds venomous representatives. These are the vampire bats from the Desmodontinae subfamily. They are found from Mexico to southern Argentina and comprise the common vampire bat (*Desmodus rotundus*), the rarer hairy-legged vampire bat (*Diphylla ecaudata*), and white-winged vampire bat (*Diaemus youngi*). The saliva of these bats has anticoagulant properties and is part of many other adaptations that allow these animals to feed on blood only, including razor-sharp teeth (Schondube et al. 2001; Tellgren-Roth et al. 2009; Fig. 3).

Venomous bats satisfy the criteria for producing venom, i.e., a secretion produced in a specialized gland in one animal and delivered to a target animal through the infliction of a wound, containing molecules that disrupt normal physiological processes so as to facilitate feeding or defense by the producing animal (Brodie 2009;

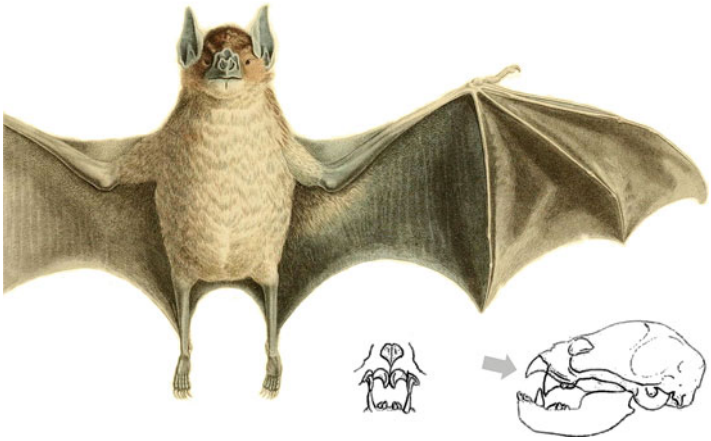

Fig. 3 Venomous Chiroptera. General aspect of a common vampire bat (*Desmodus rotundus*). The detail highlights the modified sharp teeth in front and side view (Author's own artwork, incorporating images in the public domain from "Voyage dans l'Amérique Méridionale," by AD d'Orbigny)

Fry et al. 2009). However, the large majority of vampire preys do not perish from the venom, which causes only a minor discomfort. In this regard, vampire bats resemble parasites in their feeding behavior (Delpietro and Russo 2009), since the physiological disruption facilitates feeding while keeping the prey alive, ensuring continuous nutritional supply for the bats (Fry et al. 2009).

For centuries prior to the discovery of vampire bats, Europe had legends of supernatural blood-sucking entities (Ligabue-Braun et al. 2012). Serendipitous crossing of such folklore with the growing reports on hematophagous bats from South and Central America from 1498 onwards led to the association of these animals with the myth of the vampire, summited by the publishing of "Dracula" (despite the fact that bats are mostly unmentioned in Bram Stoker's book).

Vampire bats have characteristic feeding bites, which are sharply circumscribed, crater-like, 4 mm wounds inflicted onto the attacked animal's bare skin. The anticoagulant saliva allows bats to ingest a continuous flow of blood for up to half an hour, through a piston-like motion of the tongue. In vivo comparisons have shown that a bat-inflicted wound may bleed from 180 to 480 min, while an equivalent, blade-induced wound bleeds for about 15 min (Tellgren-Roth et al. 2009).

The three species of hematophagous bats prey upon different animals (Tellgren-Roth et al. 2009). The common vampire bats (*Desmodus rotundus*) mostly feed on farm animals, such as cattle, horses, goats, pigs, and sheep. Less commonly, they feed on humans, poultry, and wild prey. The white-winged vampire bats (*Diaemus youngi*) feed mostly on birds but are also able to feed on mammals, while the hairy-legged vampire bats (*Diphylla ecaudata*) feed exclusively on birds.

The free bleeding of bat-inflicted wounds led many researchers to propose that some kind of anticoagulant should be present in the saliva. In 1966, one such compound, a plasminogen activator, was identified, followed in the 1990s by molecular characterization of four activators and a factor Xa inhibitor. In the 2010s, yet another plasminogen activator was identified (Ma et al. 2013), along with the molecular characterization of the fXa inhibitor (Francischetti et al. 2013; Low et al. 2013).

Bat venomous saliva has tissue-type plasminogen activators and a lactoferrin, while other components may still be discovered. The plasminogen activators (originally identified in 1966 but still being unfolded into different types) convert the plasmin proenzyme to its active form, which is able to degrade blood clots. While the rarer vampire bats have only one type of plasminogen activator in their genomes, the common vampire bat has five (Tellgren-Roth et al. 2009; Francischetti et al. 2013; Low et al. 2013).

The tissue-type plasminogen activator molecule has five domains: finger, epidermal growth factor, kringle 1, kringle 2, and serine protease. Only the *D. ecaudata* plasminogen activator has all five domains, with the other two species having smaller chains with domain deletions (Tellgren-Roth et al. 2009; Ma et al. 2013). These chain variations, combined with variable glycosylation structures (one *O*- and two *N*-glycosylation sites) alter binding properties of vampire bat plasminogen activators compared to tissue-type plasminogen activators.

The second type of anticoagulant from vampire bat saliva is draculin (Francischetti et al. 2013; Low et al. 2013). This modified lactoferrin is a noncompetitive, tight-binding inhibitor of activated factor X from the coagulation cascade. Factor Xa is the only enzyme that converts prothrombin into thrombin (a key point in the blood coagulation process). Draculin action is dependent on correct *N*- and *O*-glycosylation, and a mixture of draculin glycoforms are proposed to modulate the degree of fXa inhibition. As with many other vampire bat studies, draculin has been inspected only in *D. rotundus* so far.

Since the feeding bites from hematophagous bats are considered painless, it has been proposed that their saliva may also have an anesthetic. However, vampire bats are known to learn how to properly bite prey by trial and error. So far, there is no concrete evidence for this putative anesthetic.

Primates

The nocturnal prosimians slow loris (*Nycticebus coucang*, *N. bengalensis*), Kayan slow loris (*Nycticebus kayan),* and pigmy slow loris (*N. pygmaeus*) are the venomous representatives of the Primates order (Rode-Margono and Nekaris 2015). They inhabit trees in Southeast Asia and Western Indonesia. They are unique in their mode of toxin delivery, since unrelated body parts produce and inject the venom. This venom is synthesized in the brachial gland, located in the ventral, almost hairless, side of the elbow. Then, by licking of the gland, the secretion is mixed with saliva and loaded into the toothcomb, a specialized compression of the needle-like canines

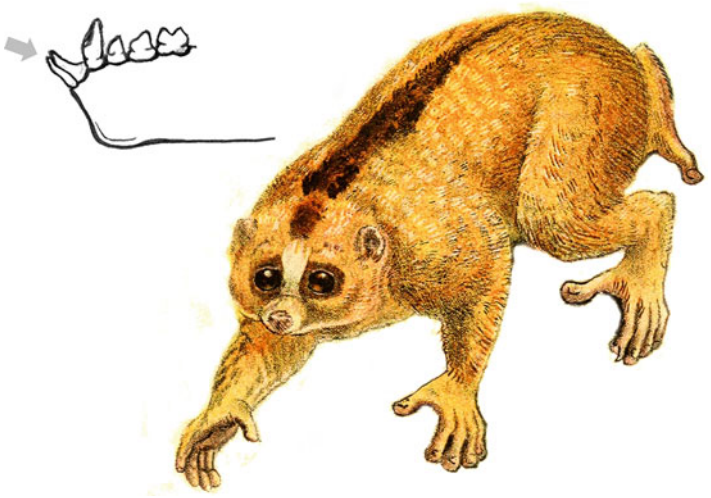

Fig. 4 Venomous Primates. General aspect of a slow loris (*Nycticebus coucang*). The detail highlights the modified tooth comb (Author's own artwork, incorporating images under Creative Commons license by Kathleen Reinhardt, and in the public domain from "A handbook to the primates" by HO Forbes)

and incisors of the loris jaw (Hagey et al. 2007; Fig. 4). Exhibition of the elbow, by positioning of their front hands above the head, and intense spreading of the venom on the head are also taken as indications of venomousness. Most (if not all) generally accepted definitions of a venomous animal state that the venom producing site and the delivery (or injecting) organ must be directly connected. This is not the case with the primates, which are only now becoming accepted as venomous (Ligabue-Braun et al. 2012; Rode-Margono and Nekaris 2015).

Folklore in Thailand holds lorises as venomous animals capable of causing intense pain and death. However, human envenomation is rarely reported. There are only three records in the medical literature (a man bitten by his pet *N. coucang*, a pregnant zookeeper bitten by a *N. pygmaeus*, and a researcher bitten by a *N. kayan*), and anecdotal evidence from manuals for zookeepers and wildlife caretakers (Madani and Nekaris 2014). The animal bite causes some effect besides the laceration itself – symptoms that do not differ from anaphylaxis. These include pulsating pain, hypotension, extremity cyanosis, and hematuria. Researchers working in close contact with lorises develop allergies to the venom. Despite its ability to cause anaphylactic shock, this seems to be only an incidental effect of the venom (Hagey et al. 2007).

There is ongoing research aiming to define the main physiological role of the loris brachial gland secretion, which may act synergistically with the animal's saliva (Rode-Margono and Nekaris 2015). Venom use for prey capture is not supported, while use in intraspecific competition seems plausible. Predator and ectoparasite defense are the most well-supported ecological roles for the venom.

Despite being a highly complex mixture, containing dozens to hundreds of compounds, the main toxic component of the venom has been identified as the

brachial gland exudate protein (BGE protein) (Krane et al. 2003). It is a heterodimeric protein (17.6 kDa), with the α-chain (7.8 kDa) and β-chain (9.8 kDa) linked by two disulfide bridges. All studied lorises have two BGE isoforms, due to variable β-chains. The BGE protein is highly similar to the major cat allergen, Fel d 1. Both share the uteroglobin protein fold, are disulfide bound, and have alternate β-chains. The uteroglobin fold is related to transport of hydrophobic molecules (such as steroid hormones) and calcium binding. Regardless of being poorly understood, uteroglobins are postulated to act as boxes, being able to open and close according to physiological conditions, loading, carrying, and delivering hydrophobic cargo (Hagey et al. 2007). The similarity to a feline allergen reinforces the possible cross-reactivity of loris venom instead of a de facto venomous role (at least for humans) (Ligabue-Braun et al. 2015).

Mammalian Venom Evolution: Shadows of the Past or Rare Recurrences?

As can be observed from the mammalian phylogenetic tree (Fig. 5), orders with venomous representatives do not cluster together, having no obvious common origin. This observation prompts the question: Why are venomous mammals so rare? Moreover, what is the utility of the extant venom, being so scarce among this animal class? Since each order's venom is different, the following segment will describe peculiarities and strategies in which these mammalian groups employ venoms, discussing current evolutionary hypotheses regarding their origins.

Venomous eulipotyphlans have been compared to venomous reptiles long before the identification of BLTX. Solenodons and shrews were studied taking snakes as reference (Dufton 1992). In 1942, when identifying that shrews had modified salivary glands responsible for the venom production, like snakes, Pearson also noted that in the latter the parotid glands are modified to produce venom, while in the former, the submaxillary glands are the venom source. Despite this important difference between the two cases, there are indeed venomous reptiles with venom-producing submaxillary glands (including the Gila monster *Heloderma suspectum* and the Mexican beaded lizard *H. horridum*). With the purification of BLTX, it was confirmed that these cases were indeed linked (Kita et al. 2004). The Gila toxin (GTX) and BLTX have similar effects on prey and are similar (34% identical). Horridum toxin also has high sequence similarity with BLTX (32%). The reptilian and mammalian kallikreins underwent convergent transitions to venomous ones, with similar, nonhomologous residue insertions that increased the protein flexibility, altering loop lengths, polarity, and surface charges. Both GTX and BLTX started from independent serine proteases that had locally different alterations generating globally similar, toxic, structures (Aminetzach et al. 2009; Fig. 6). Blarinasin, another kallikrein-like protein from shrew saliva is not toxic on tested animals, suggesting that small differences, including glycosylation heterogeneity, may play key roles in their toxicity.

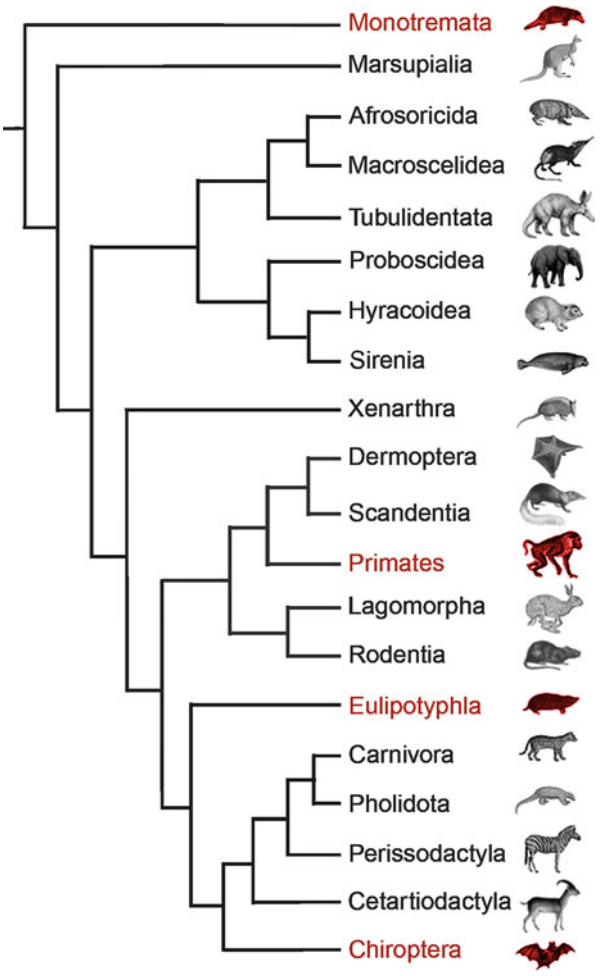

Fig. 5 Mammalian phylogeny highlighting orders with venomous representatives (in *red*). Please note that there are alternate versions for the evolutionary history of Mammalia (Author's own artwork, based on Springer et al. 2004)

Regarding the venom function in Eulipotyphla, Furió et al. (2010) defined an ongoing debate as "hunting big or hoarding small." As part of an adaptive winter profile, shrews cache various preys in a comatose state. Other adaptions include elaborate nests, stable thermal regime for foraging, and reduced activity during periods of cold. Within this framework, venom would be an asset to sustain a living hoard when hunting is difficult, especially in cold winters. The high metabolic rate of shrews would make this ability very relevant. The use of eulipotyphlan venom as a paralyzing, conservative agent is thus taken as support for the "hoarding small" hypotheses. The "hunting big" hypothesis proposes that venom is a tool to overcome bigger prey. According to Dufton (1992), vertebrate food is of major importance for eulipotyphlans, and this kind of prey is larger and more dangerous to subdue than their power-to-weight ratio would allow, thus making venom necessary. There is no specialized venom delivery apparatus in extant shrews, which have only a concavity

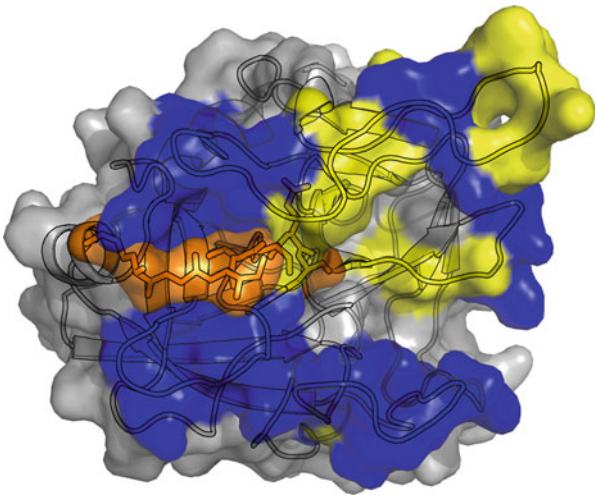

Fig. 6 Structural model of blarinatoxin, BLTX, with regulatory loops colored in *blue* and insertions that convergently evolved towards toxicity in *yellow*. A substrate is shown in *orange*. Model built based on PDB ID 2ZCK, Ménez et al. (2008) (Author's own artwork)

on the surface of their first incisors. However, the discovery of an extinct giant shrew (*Beremendia* sp.) with an envenomation apparatus (grooved incisors similar to those from solenodons) (Cuenca-Bescós and Rofes 2007) seems to favor Dufton's proposal. These grooves would act as a channel, directing saliva from the submaxillary glands to wounds inflicted on the prey, as seen in solenodons. However, alternative explanations, based on paleoenvironmental reconstructions, propose that *Beremendia* lived in a highly unpredictable environment and that their prey consisted mostly on gastropods and coleopterans (Furió et al. 2010). Most likely, venom is used by Eulipotyphla in both ways, including combining them to hoard larger prey. Secondarily, venom use in intraspecific competition has been observed among captive solenodons.

In his model for venomousness in mammals, Dufton (1992) proposed that the earliest eutherian mammals had morphologies that resemble extant hedgehogs and shrews. These early eutherians developed during the late Cretaceous (66–144 millions of years ago), and would form a basal group for extant mammals (Rode-Margono and Nekaris 2015). Eulipotyphlans per se are not ancestral in mammalian phylogeny, but in this proposal, they would be the extant mammals that retained the most from these ancient eutherians. The current distribution of venomous eulipotyphlans, covering Asia, Europe, North and Central America, would support this view of a more widespread occurrence of venomous mammals in their evolutionary past. Since these ancestral mammals were small and not fully homeothermic, foraging efficiency would act as a selective pressure, while the use of venom would bestow a selective advantage on them (Rode-Margono and Nekaris 2015). Dufton also observed that extant venomous eulipotyphlans almost exclusively co-occur with flightless birds. This scenario would somewhat resemble their origins sharing habitats with dinosaurs, suggesting that beyond egg-eating, larger flightless birds (or dinosaurs) could be targets for venom (either predatorily or defensively). Once

their diet shifted to invertebrates, venom would become less useful, being retained in only a few species. This is an especially cumbersome hypothesis, since extant venomous Eulipotyphlan are very successful at using venom to prey on invertebrates (Folinsbee 2013).

Despite the well-supported occurrence of venom in the extinct giant shrew *Beremendia* (and possibly in the solenodon relative *Nesophontes*), other evidences from the fossil record are still being disputed. The discovery of a Palaeocene eutherian mammal with canine grooves, phylogenetically distant from Eulipotyphla, prompted its classification as a venomous mammal (Fox and Scott 2005). This animal, *Bisonalveus browni*, was proposed to use its grooved teeth to deliver toxic saliva, resembling solenodons. However, the occurrence of grooved teeth alone has been deemed insufficient and inadequate to support the occurrence of true venom delivery apparatus in extinct mammals. Orr et al. (2007) and Folinsbee et al. (2007) cited numerous examples of extant mammals with grooved teeth and no sign of venom. These include suiforms, coatis, lemurs, and primates. The grooving in their teeth seems to act as a structural reinforcement of the dentary structure, unrelated to venom delivery. Both works conclude that the traditional comparative method alone could not ascertain if a primitive mammal was venomous without slipping into "false positives," i.e., if the structure (grooved teeth) is related to function (venom delivery), all extant mammals with this anatomy would be expected to be venomous, which is not the case. Additionally, except for solenodons, all other extant venomous mammals lack truly grooved teeth. The mammalian masticatory apparatus, however, is considered highly sophisticated, enabling a wide range of feeding strategies. This, alone, could render venom use redundant (Folinsbee et al. 2007). As observed by Rode-Margono and Nekaris (2015), this seems to be the case, since the venomous eulipotyphlans are exceptions to the general pattern in mammalian diets. While many orders are chiefly herbivorous or insectivorous with small prey relative to predator body mass, the carnivorous orders are large and able to overcome their prey by sheer strength.

In a recent phylogenetic construction of Eulipotyphla phylogeny, Folinsbee (2013) observed that *Neomys*, *Solenodon*, and *Blarina brevicauda* are phylogenetically distant, with the *Solenodon* lineage diverging around 80.5 mya and the ancestors of *Neomys* and *Blarina* diverging around 16.5 mya. The author observed that, if venomousness was an ancestral condition of Eulipotyphla, at least nine convergent losses of venom would be required to explain the obtained tree. On the other hand, if venom evolved convergently in different eulipotyphlans, only three unique acquisitions would be required. Still, venom is rare in this group, occurring in no more than 2% of extant species.

Toxic saliva is not exclusive to shrews and solenodons. Vampire bats use their venomous secretion to be able to feed on the continuous blood flow from a sharply cut wound. Vampire bat saliva, however, is just part of a highly specialized physiology, as a reflection of blood being their sole source of hydration and nutrition. Other adaptions include a modified gastrointestinal tract, which allows the ingested blood to enter the intestines prior to entering their tubular stomachs (due to a T-shaped gastroesophageal-duodenal junction). When reaching around half their

weight in ingested blood, most of the water is eliminated in a process known as instant diuresis, which leads to the highly nitrogenous blood remains being processed with almost no water. Their high capacity for concentrating urea in the urine makes vampire bats physiologically equivalent to desert mammals. Hematophagous chiropterans feed almost exclusively on the proteinaceous moiety of the ingested fluid, with the carbohydrates being almost unused, and sucrase and maltase being absent in their gastrointestinal tracts (Schondube et al. 2001). Another physiological characteristic of these animals is that they have no adipose tissue for storage, making them dependent on daily blood meals and reliant on a highly ordered social system, in which fed animals are able to regurgitate blood into the mouth of individuals that are unsuccessful or unable to prey for themselves. Their sharp teeth and anticoagulant saliva work as to facilitate the tongue-directed flow of blood to the animals' mouth. The tongue does not act licking up the blood, but rather acting like a piston. For this method to work, blood cannot coagulate, as to guarantee free flowing from the prey to the predator.

Vampire bats form a subfamily, Desmodontinae, in the Phillostomidae family. This family is considered to have the most diverse feeding habits among all mammal families. They include nectarivory, omnivory, frugivory, carnivory, and hematophagy (the latter deriving from insectivory) (Schondube et al. 2001). The three hematophagous species have different preferred preys, and this reflects the evolution of their salivary anticoagulants. The fibrin specificity and susceptibility to plasminogen activator inhibitor 1 of chiropteran plasminogen activators has been altered by gene duplication, domain losses, and further sequence evolution (Tellgren-Roth et al. 2009; Fig. 7). *D. ecaudata* has a single copy of plasminogen activator, which is similar to the one found in other mammals and feeds only on birds. *D. youngi* plasminogen activator lacks the kringle 2 domain, having increased

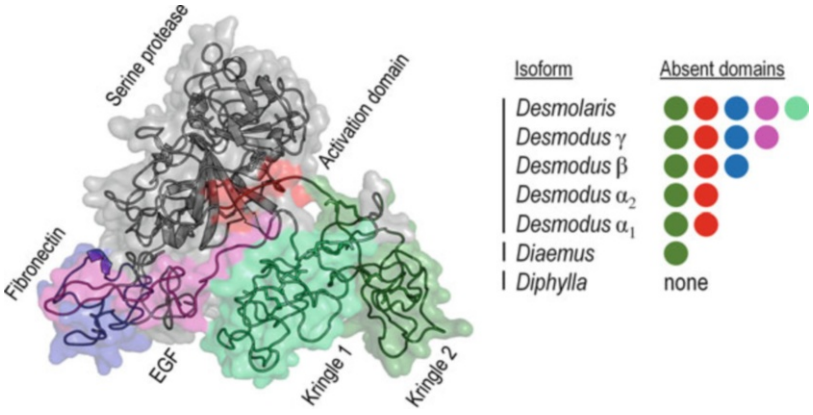

Fig. 7 Structural scheme of plasminogen activators from vampire bats. Absent domains in each protein are color-coded, while isoforms from each species are clustered together. Depicted based on Tellgren-Roth et al. (2009), model built based on PDB ID 4DUR, Law et al. (2012) (Author's own artwork)

fibrin specificity. This bat is more generalist, feeding on birds and mammals. The plasminogen activators in *D. rotundus* went through rounds of gene duplication and domain loss, creating versions with decreased sensitivity to plasminogen activator inhibitor 1 and enhanced ability to feed only on mammalian blood (Tellgren-Roth et al. 2009). Differences in glycosylation also take part in the improved anticoagulatory activity, since the clearance of some chiropteran plasminogen activators are up to four times slower than those observed for tissue plasminogen activators.

Only chickens have been observed to die of hemorrhage after vampire bat attacks. Other prey do not succumb to their bites or saliva, prompting arguments against the inclusion of bats as venomous animals (Ligabue-Braun et al. 2012). Indeed, their saliva facilitates feeding by disrupting regular prey physiology while ensuring its survival for the continuous supply of nutrition for the bat. The highly specialized saliva of Chiroptera is not homologous to Eulipotyphla venom, with the involved teeth being different as well as the molecules involved in the toxicity. Additionally, the emergence of hematophagy in bats is considerably more recent in evolutionary terms than the speciation of solenodons and shrews with envenomation apparatus. These observations highlight the emergence of venom more than once in the history of mammals.

Male platypuses (Monotremata) employ the secretion of their crural system in a different way to the venomous saliva from Eulipotyphla or Chiroptera, which is used in prey acquisition and feeding facilitation, respectively. The use of the glands and spurs has been proposed to take part in multiple behaviors in these animals, from helping climb riverbanks to waterproofing the fur (Grant and Temple-Smith 1998). However, their true usage is to act as a weapon in male-male competition for females, taking part in sexual selection. Adult male platypuses largely avoid each other and have testicular and crural gland size increases in the mating season, in which males become aggressive. In this season, it is common to find males with spur punctures, especially in their tails. Grant and Temple-Smith (1998) used this evidence to propose a polygynous mating system for platypuses. In this system, male interactions direct the access to females, something that would justify the retention of the crural system in former. Only one other Monotremata representative, the short-beaked echidna (*Tachyglossus aculeatus*), had its crural apparatus examined (Krause 2009). Both genders of these animals have degenerate crural spurs, with the males having cyclic growth of the crural gland in accordance to mating season. However, they are unable to use their spurs aggressively or to support the structure on their tibia during attacks. The seasonal growth cycle would suggest a role as scent gland, but its real function is still uncertain.

Monotremata are the sole remaining mammals from the class Prototheria, the first to diverge from other mammals (around 166 Mya). Platypus, in special, have anatomical features that are closely related to reptiles (similar ribs and pectoral girdle), despite being furred homeotherms. These mixed characteristics are reflected in their genome, with a large amount of reptilian-like genes, and taken as a possible link between reptiles and therians (Whittington and Belov 2016). Likewise, platypus venom has many similarities with reptilian ones, via convergent evolution

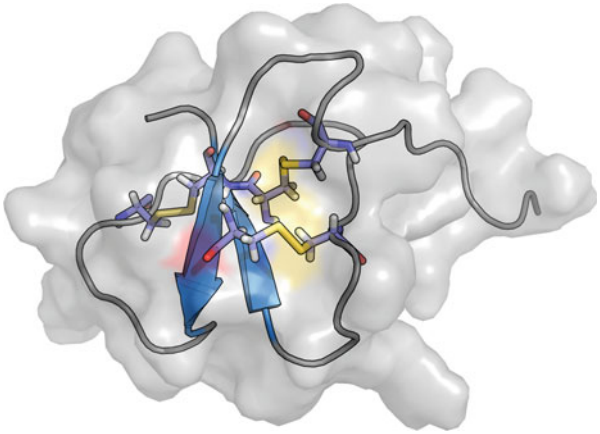

Fig. 8 Structure of defensin-like peptide 2, DLP-2, from platypus venom (PDB ID 1ZUE, Torres et al. 2005). The three characteristic defensin disulfide pairs are highlighted (Author's own artwork)

(Whittington et al. 2008). In both cases, defensin-like peptides, C-type natriuretic peptides, and nerve growth factor gene families were duplicated and then co-opted for toxic purposes (Warren et al. 2008). Many of the proposed "venom genes" are also expressed in non-venom tissues (i.e., outside the crural gland), while the natriuretic peptides and nerve growth factors from venom are also expressed in females, suggesting additional roles for these peptides. As observed in reptiles, there is an ongoing debate on whether putative toxins expressed in multiple tissues constitute true toxins (please see ▶ Chap. 4, "A Critique of the Toxicoferan Hypothesis" for more details). A transcriptome of platypus venom gland labeled 88 toxin genes when filtered against transcriptomes of nonvenomous tissues (Whittington et al. 2010). So far, only defensin-like A (Fig. 8) is considered a crural-gland exclusive peptide (Whittington and Belov 2009).

As with eulipotyphlans, fossil evidence has been interpreted as signs that crural spurs were widespread among primitive mammals. The proposed role for the crural system would be in defense against larger predators, dinosaurs in particular (Hurum et al. 2006; Kielan-Jaworowska and Hurum 2006). Once again, this proposition is based on comparisons with extant mammals, of which only one species has a venom-delivery apparatus involving crural glands and spurs. Still, true monotreme fossils are rare and consist of tooth and jaw fragments, not allowing certainty in defining ancestral monotremes as venomous. Considering the conserved (vestigial or functional) crural systems in extant platypuses and echidnas and some shared elements between their glandular secretions, it is possible to propose that their ancestral species were also venomous (Whittington and Belov 2016). The fact that spurs (functional or vestigial) are present in both sexes in extant Monotremata raises the possibility that their original purpose was in defense, especially in confronting large predators, from dinosaurs to large mammals (from the Jurassic to Pleistocene). It has been proposed that, once this selective pressure was no longer present, the crural system was co-opted to a reproductive context. This change would justify the maintenance of energetically expensive venom production as a sexually dimorphic trait (Whittington and Belov 2016).

There are many cross-taxa examples of convergent venom evolution (Fry et al. 2009), and the very unusual mammalian ones are no exception. Most toxins in mammalian venoms (with the possible exception of BGE protein) are products of this type of process, being similar to toxins found in other animal groups. Rapid effect, be it fast prey immobilization or quickly occurring pain, is a major requirement for a venom. This is proposed to be one of the main factors that limit what kinds of protein may be co-opted for toxicity. There is only a small group of proteins that recurrently develop into toxins once their genes are duplicated. However, this is not the sole process acting on mammalian venoms, since mutations in regulatory or coding regions and alternate splicing has been shown for platypus venoms, and alternate glycosylation has been related to variable activity in shrew and vampire bat venoms.

The order Primates displays a unique venom delivery system, unlike any other present in venomous animals. Currently four species in the *Nyctycebus* genus are recognized as being venomous. They load needle-like modified teeth with the secretion of an elbow gland, possibly mixing it with saliva, thus establishing a venom delivery apparatus from unrelated body parts. Many hypotheses have been tested to ascertain the ecological role of this venom, as reviewed by Rode-Margono and Nekaris (2015). Aiding in feeding seems unlikely, since their diet consists of fruits, invertebrates, and small vertebrates, i.e., smaller than the animal itself. Also unlike eulipotyphlans, there is no caching behavior observed, with all food being consumed immediately. Testing the brachial gland exudate on arthropods did not confirm deleterious effects on this type of prey. Predator defense is somewhat unlikely, since the predators are diverse and the loris-predator encounters are rare. However, when testing BGE-saliva mixes on olfactory-oriented predators (leopards, clouded leopards, tigers, sun bears, common palm civets, and binturongs), these were repelled by the venom. In the field, it has been observed that Javan slow lorises move close to palm civets and leopard cats. Visually oriented predators reacted differently when faced with loris venom. Eagles (*Spizaetus*, *Spilornis*) show inconclusive behavior, while orangutans actually eagerly consumed the venom-containing swabs. These visual predators (along with pythons) are known to prey on lorises (Hagey et al. 2007). There is growing evidence that the secretion may act as an antiparasitic, since lorises are conspicuously low in ectoparasites, being slightly more affected in the rainy season. Tests on leeches and tick-related models revealed that the BGE-saliva mixture is able to kill them.

The venom may have a role in intraspecific competition (as observed for solenodons and platypuses). Loris-on-loris bites are severe, affecting large areas with loss of fur, prolonged edema, and are slow-healing (often life-threatening) (Hagey et al. 2007). The chemical complexity of BGE, associated with grooming behaviors, point to the substance being used as a signaling device. The main target, however, may not be predators but the lorises themselves. Aside from fending off some predators, the secretion may alert other lorises about the predator's presence. Since the venom shows species specificity, this may reinforce a communication role. Taking its high similarity to Fel d 1, the major cat allergen, structural modeling suggest that the BGE protein may act as a box (Fig. 9), being able to close its lid on

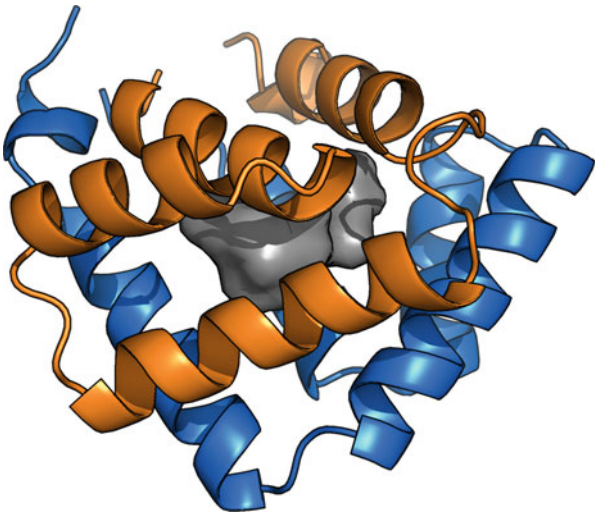

Fig. 9 Fel d 1, a working model for BGE protein. α-chain in *blue*, β-chain in *orange*. The internal cavity, proposed to act as a box, is shown as a *grey* volume (PDB ID 2EJN, Kaiser et al. 2007) (Author's own artwork)

signaling molecules from saliva or BGE. This entrapping and delivery system helped propose the physiological role of Fel d 1 in big and small cats (Ligabue-Braun et al. 2015). It is interesting to note that chimpanzee and human genomes harbor remnants of the BGE protein in the form of pseudogenes, indicating a putative venomous past for apes (Hagey et al. 2007) or a long lost redundant uteroglobin.

Nekaris et al. (2013) proposed an elegant hypothesis for the role of venom in lorises as part of a much broader Müllerian mimicry system. In this proposal, the use of venom to repel olfactory-orientated predators is combined with other features, such as extra vertebra in the spine that allow serpentine movements, aggressive snake-like vocalizations, and long dark dorsal stripes and dark ocular circles, constituting a mimic of cobras (*Naja* spp.). Despite its multipurpose role (including intraspecific competition and parasite defense), the evolution of venom may have been an adaptive strategy against predators when combined with other features, arising in the Miocene, when slow lorises and cobras migrated through Southeast Asia.

Recently, Harris and Arbuckle (2016) used large datasets with comparative phylogenetic methods to inspect patterns of venom and poison evolution in birds, amphibians, reptiles, and mammals. They found that venom biosynthesis evolution is much less dynamic than that of toxin sequestration from the diet. Apart from amphibians, the remaining tetrapods show an association between the evolution of toxins and higher diversification rates. Furthermore, the work found that mammals and reptiles evolve under a similar regime regarding their toxicity/venomousness, with gains and losses of toxicity sparsely and infrequently distributed across the phylogeny. Interestingly, mammals form the only tetrapod lineage in which venom is used for intraspecific competition. Harris and Arbuckle (2016) speculate that, due to frequent social interactions in mammals (compared to other groups), they may be under higher selection pressure to use venom in social situations. To support this,

the authors argue that such behavior is observed in some eusocial hymenopteran insects.

Folinsbee (2013) summarized the main (nonmutually exclusive) hypotheses to why venom is rare among extant mammals. Venom may not be adaptive in mammals (i.e., there is no need for it); the production of venom may be constrained by some biological factor; there are high costs associated with production and maintenance of venomous secretions; and venom may be adaptive only in a narrow range of morphologies. Regarding the need for venom, the greater size and masticatory adaptions acquired by mammals may have supplanted the (putative) ancestral venomousness. Still, it is unclear, at least for the diverse Eulipotyphla order (452 extant members), what proportion is really venomous. There is an abundance of untested mammals in this order to be evaluated prior to considering them nonvenomous. Studying them comparatively may confirm (or disprove) multiple origins for venom in these animals. The cost of producing venoms also needs to be evaluated in mammals. Snakes and arachnids carefully measure the amount of venom deployed in each attack, with pitvipers and death adders increasing significantly their metabolic rates when synthesizing venom (Folinsbee 2013; Morgenstern and King 2013). There is still no study regarding venom production costs in sister mammalian taxa that would clarify the energetic costs of using venom for predation. If it is not costly, another constraint must be in place. A likely explanation is that the highly specialized mammalian teeth, intensively used for oral processing of food, may hinder their modification into venom delivering tools, making venom less adaptive. It is possible that a combination of factors makes venom adaptive only for a specific phenotype, namely, small body size with high metabolism (Folinsbee 2013). As becomes clear when one inspects each mammalian order's venom, its uses, and injecting apparatus, there is no homology present (at least, none that can be ascertained without major concessions). Venomousness seems to have independently arisen at least four times among mammals, once in each of the four orders presented in this chapter. Explaining the scarcity of venomous mammals may take into account specificities of each mammalian order.

Anthropocentric Biases: Research Limitations, Exciting Applications

The study of venoms in general suffers from a human-centered perspective. This is understandable, since humans are the ones doing research, basic science is expensive, and resources are scarce. However, this should be avoided as soon as perceived as an obstacle to fully understand venoms and toxinology as a whole. The study of venomous mammals also have these caveats.

Most of the available data reflects venom effects on humans, pets, farm, or experimental animals. Venom effects, however, are context- and taxon-specific. For this reason, using lethal dosage evaluation (LD_{50}), for instance, is problematic, since it is normally based on a single species, ignoring that different species respond

differently to the same compound (Brodie 2009). Only recently there has been an effort to understand the physioecological role of venom in the animals' life history.

Despite mammalian venoms reflecting such bias, there are indeed tests on animals that are more strongly related to the mammals' ecology. For instance, roaches and crickets were tested with Eulipotyphla, in an attempt to mimic their invertebrate diet, and lorises had their venom tested on spiders, maggots, ants, fleas, and caterpillars (Grow et al. 2015).

Much information regarding venom use by mammals originated from observational studies. Still, there is room for more assessments aiming to understand venom use by these animals in their natural habitat. Since the notion of venomousness as a mammalian trait is just beginning to be accepted by the wider zoology community, there are many gaps still waiting to be bridged in respect to these animals. The vast majority of venomous mammals are difficult to maintain in captivity, and even when this is not a limiting feature, the amount of venom is too small to allow in-depth research without straining individual animals or requiring large numbers of individuals. Genomic techniques are rising as possible answer to this conundrum.

Toxic proteins from mammals have served as models to understand mammalian evolution, as well as providing interesting prototypes for new drugs. Anticoagulants from vampire bat saliva have been proposed as promising treatments for myocardial infarction, pulmonary thromboembolism, and stroke, since a key aspect of these events is to keep blood unclotted. BGE protein from lorises may help to assess allergy-related issues in humans, considering its high similarity to cat allergens. In its turn, platypus venom may aid in the study of pain perception and as a model to design novel pain relievers, particularly interesting when one targets long lasting, treatment-unresponsive pain. Isomerases from Monotremata venom may offer tools to develop degradation-resistant peptides with medical application. Other, less studied, toxins from this venom may even work as scaffolds for antineoplastic drugs (Ligabue-Braun et al. 2012, 2015; Rode-Margono and Nekaris 2015; Whittington and Belov 2016). The field of venomics is growing together with toxin-based drug discovery (Calvete 2009). Mammalian venoms are thus rich sources of novel frameworks for drug development.

Conclusion and Future Directions

Mammalian venoms prompt a need to revise the definitions of venomous and poisonous animals. In the traditional definition, venoms are produced and stored in specialized structures (glands), associated with delivery devices, forming the envenomation apparatus with which the venoms is delivered directly to the recipient's body. Thus, venomous animals are actively toxic. Poisons may be available in specialized structures in the toxic animal but lack any special mechanism of delivery. The recipient animal must eat (or at least be in direct contact with) the poisonous animals to be affected. This is considered passive toxicity. There are obvious flaws with these definitions. For instance, the spitting cobra would be considered poisonous and not venomous, when venom is delivered by squirting and not directly onto

the prey. Likewise, the feeding secretions from hematophagous animals are not universally considered as venom, even though they satisfy all requirements to be. It is the fact that these animals depend on the survival of the food source for continuous supply of nutrients that may divert them from traditional definitions, despite their venoms being capable of facilitating feeding by disruption of normal physiological processes of the prey.

Eulipotyphlans and platypuses satisfy the criteria to be taken as venomous. Vampire bats are considered venomous only if hematophagy is enrolled along with traditional venom uses (Fry et al. 2009). Lorises clearly do not satisfy the criteria, since their venom-producing organ is not directly connected to the injury-inflicting and toxin-delivery apparatus. Still, one can no longer argue that these animals cannot act venomously.

Chemical defenses (passive toxicity) are also present in mammals. Pangolins, skunks, the greater long-nosed armadillo (*Dasypus kappleri*), and the striped polecat (*Ictonyx striatus*) emit noxious substances to fend off predators. This form of chemical defense would allow these mammals to be considered poisonous. In the most striking example of chemical defense in mammals so far, the African crested rat (*Lophiomys imhausi*) is able to sequester toxic substances from plants and accumulate them in their manes, forming a protective mantle. These cases have been considered "arguably venomous" (Rode-Margono and Nekaris 2015). Considering that examples of chemical defense in other animal groups are ample (amphibians), understudied (marine turtles) and still being elucidated (birds) (Ligabue-Braun and Carlini 2015), it is possible that mammalian poisons may be much more widespread than mammalian venoms. Hopefully this chapter, along with recent literature, will be able to include mammals in the "hall of venomous animals" from the perspective of both toxinologists and the general public.

Cross-References

- ▶ A Critique of the Toxicoferan Hypothesis
- ▶ Evolution of Resistance to Toxins in Prey
- ▶ Evolutionary Context of Venom in Animals
- ▶ Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins

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