

Snake venomomics at the crossroads between ecological and clinical toxinology

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Venoms are complex secretions used for predatory and defensive purposes by a wide range of organisms. Venoms and venom production represent fascinating systems to study fundamental evolutionary processes. Understanding the evolution of venom generation demands the integration of the selective interactions and mechanisms, which transformed ordinary genes into deadly toxins, in the context of the natural history of the producing organism. Humans are not prey for any extant venomous creature on Earth, and thus human envenomings result from unexpected encounters with venomous animals, e.g., snakes. Research on snake venoms conducted on mammalian prey from an ecologically informed perspective is conceptually transferable to the clinic, highlighting the mutually enlightening relationship between evolutionary and translational venomomics.

Brief introduction to the evolutionary context of snake venom

Venoms, and their associated venom-delivery systems, represent innovations that have evolved independently in a broad phylogenetic range of animal lineages across all major phyla of the animal kingdom.

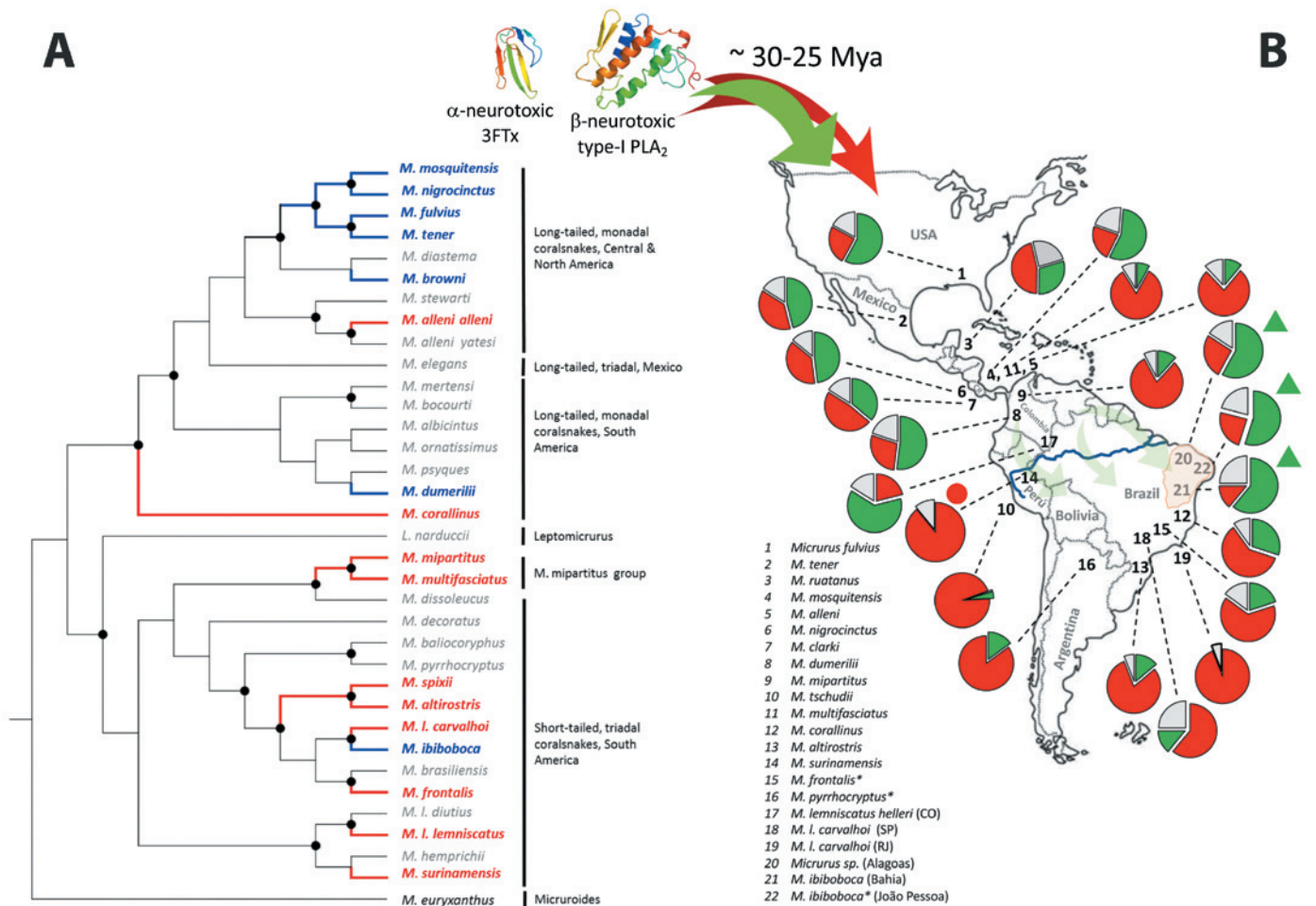
Venom is an intrinsically ecological trait used by more than 250,000 species, for the purpose of subjugating prey, deterring competitors or defending themselves from predators. The reason for possessing toxic weaponry is simple. Ancestral snakes, such as *Titanoboa cerrejonensis* and its living relatives such as the boas, anacondas and pythons, use constriction to crush and eat animals by wrapping their mouths around their prey and swallowing them whole. In contrast, venom represents a more sibilant means by which one can subdue potentially dangerous prey whilst minimizing the risk to self in any struggle.

Venom emerged as a key evolutionary innovation that underpinned the explosive radiation of caenophidian snakes, in the wake of the Cretaceous–Paleogene (around 66 million years ago). The evolutionary success of venom is highlighted by the fact that venomous animals have arisen in every ecosystem of our planet where organisms compete for resources. Venom is also a useful defensive strategy. Painful venoms are used to deter predators, and there are many examples of Batesian mimicry by which a harmless species protects itself from predators by deceitfully imitating the genuine aposematic warning signal of a noxious species. Venomous predator–prey

interactions generate coevolutionary dynamics through an escalating arms race, characterized by asymmetrical selection between the predator's toxic arsenal and the prey's evolved counter-adaptative resistance mechanisms.

Adaptations to ecosystems require evolutionary changes of both morphological and molecular phenotypic traits that maximize the organism's fitness in local environments, e.g., the success of a snake foraging on preferred prey. Thus, unveiling the evolutionary potential and history of those characters requires a deep knowledge of the patterns of functional variation generated by spatially discrete selection among individuals, populations and species underlying adaptive changes. The genome of an organism contains information of its full repertoire of genes, those that are actively transcribed, but also of the many additional genetic features, such as introns, intergenic regions and regulatory elements, that play pivotal roles in the control of gene expression and in the physiology of the organism.

In addition, the genome also encodes traces of events from its evolutionary history, both from functionally failed recombinations and from those that passed the natural selection filter and contributed to the functional genome of the species. Hence, the sequencing of snake genomes, but especially inter- and intra-specific comparative genomics will uncover a treasure trove of biological information to reconstruct the molecular bases of the evolution of venom genes. Genomic data is tremendously informative for phylogenies. It can be used to estimate rates of speciation and extinction, to



reconstruct historical diversification scenarios, and to link these to ecological and evolutionary factors, such as climate or organismal traits, as well as population genetics (phylogeography) and species delimitation. Merging morphological and molecular datasets for fossil and extant taxa would also give a more complete view of the natural history of snakes. Genomic resources are currently only available for specific snake species; however, the steady growth in annotated snake genomes represents the tip of an ‘-omic’ revolution. These will play a fundamental role in addressing the connection between genotype and phenotype for fitness-related traits and more explicitly—the relative importance of structural changes in proteins, versus gene regulatory changes as the basis for adaptive variation in the venom phenotype.

However, despite being traits of moderate genetic complexity in terms of the number of genes that encode toxin families, within- and between-species venom variability in-space (geographic) and in-time (ontogenetic) seems to be a common feature at all taxonomic levels. The mechanisms that generated such biodiversity remain largely elusive, although genomic reorganizations and post-transcriptional regulation of the expression patterns of messengers encoding toxins

have been reported to be involved. This scenario implies the need to analyse individual venom proteomes, rather than pooled venoms in different contexts, in order to understand the spatio-temporal variability landscape underlying the adaptations that drive intra-specific venom evolution.

Snake venomomics

Venom proteomics, ‘venomics’, began to be applied modestly in the field of toxinology at the turn of the century. To date, the venom proteomes of more than 200 species and subspecies of snakes, particularly from the families Viperidae (340 species of true vipers and pit vipers) and Elapidae (360 species of elapids, for example, cobras, kraits, mambas and sea snakes) (www.reptiledatabase.org) have been provided. The field continues to expand at an accelerated expansion pace, which is mainly due to analytical advances over the last decade. In particular, the combination of next-generation transcriptomics and well-established bottom-up mass spectrometry-based proteomics platforms, has demonstrated capabilities for the rapid identification and relative quantification of toxins in snake venoms in unprecedented detail.

Figure 1: A) Consensus phylogeny of New World coral snakes highlighting the scattered distribution of PLA₂-rich (blue) and the 3FTx-predominant (red) venom phenotypes. **B)** Geographic distribution of currently available micruroid venom proteomes. The pie charts display the relative abundances of 3FTxs (res), PLA₂s (red) and other toxins (grey). Green triangles highlight the outlier venom proteomes of Caatinga coral snakes; the red disc identifies the streamlined venom proteome of the aquatic coral snake, *M. surinamensis*.

However, these figures still represent only approximately 12–27% of the venom proteomes of front-fanged (solenoglypha and proteroglypha) snakes, and the situation is much more extreme regarding the venoms of rear-fanged (opisthoglypha) snakes, where only a handful of venoms have been characterized out of the approximately 700 species that produce venom. This bias may be ascribed to the high incidence of human morbidity and mortality from envenomation by front-fanged snakes, which inject venom into at least 1.8–2.7 million people worldwide per year, with combined upper estimates of mortality ranging from 80,000 to 138,000 deaths. On the other hand, although the large majority of rear-fanged snakes (>2200 species in the family Colubridae) are unable to deliver sufficient quantities of toxin to be lethal, at least three species (*Dispholidus typus*, *Thelotornis capensis* and *Rhabdophis tigrinus*) have caused human fatalities, and bites by two additional species (*Philodryas olfersii* and *Tachymenis peruviana*) have resulted in serious human envenomations, clearly indicating that the study of their venoms should not be further neglected. In addition, rear-fanged snakes are a phylogenetically diverse collection of species that feed on a variety of prey and show varying prey capture strategies, from constriction to envenomation, and prey-specific toxins have been identified. It is hypothesized that venoms of rear-fanged snakes, particularly those of species with highly specialized diets, may contain novel prey-specific toxin genes, moulded by adaptive trophic evolution and exhibit unique pharmacological activities. Clearly, understanding the nature of the venoms of a group of organisms (i.e., organisms that share a common ancestor), which includes the vast majority of ecological variants amongst extant snakes, is fundamental to understanding venom evolution in advanced snakes. However, differences in gland structure and size, as well as fang morphology, have resulted in high-pressure and low-pressure venom delivery systems in front-fanged and rear-fanged snakes. A derived consequence is that, in comparison to front-fanged snakes, venom extraction from rear-fanged snakes is more challenging, time-consuming and generally results in significantly lower venom yields.

Research on venoms has been continuously enhanced by advances in technology. Challenges remain to be solved in order to achieve a compact and automated platform with which to routinely carry out comprehensive quantitative analysis of all toxins present in a venom. However, the introduction in 2015 of highly sensitive and resolute ion-trap spectrometers for top-down venomomics represents a turning point in snake venom analysis. This technology allows the in-depth characterization of proteins by sequentially confining, manipulating and fragmenting whole toxin ions inside the mass spectrometer.

Whether as a stand-alone workflow or in combination

with bottom-up approaches, top-down venomomics is gaining momentum, particularly for the analysis of venoms from species for which a homologous transcriptomic reference database is available. This point is illustrated by the recent transcriptomics-guided bottom-up and top-down study of the changes undergone by the venom of the arboreal rear-fanged brown treesnake, *Boiga irregularis*, which correlate with a pronounced ontogenetic shift in prey preference, from a diet consisting almost exclusively of poikilothermic ectotherms (small lizards) in neonates, to both ectotherms and homeothermic vertebrates (birds and mammals) being consumed by adults.

Identification of evolutionary trends through clade venomomics

The identification of evolutionary trends across whole genera, taxonomic clades and phylogenetic families is of increasing interest in venom analysis.

Unveiling the origin and phylogenetic distribution of venom traits is key to understanding the underlying evolutionary processes (local adaptation, balancing selection) and reconstructing the historical ecological constraints that moulded snake venoms to their present-day variability. These traits include:

- Paedomorphic traits, for example the retention of juvenile β -neurotoxic heterodimeric PLA₂ crotoxin-rich venom phenotypes in adult South American *Crotalus durissus* species.
- Ontogenetic traits, such as the age-dependent transition from crotoxin-rich (type A) to haemorrhagic PIII-metalloproteases-rich (type B) venom in Costa Rican *Crotalus simus*.
- Dichotomic traits, such as type A versus type B venoms in North American *Crotalus* or PLA₂-rich versus 3FTx-rich venoms across the genus *Micrurus*; dendrotoxin-rich versus 3FTx-predominant venoms in African mambas.

The overall picture, rather than the individual venom proteomes, provides hints for reconstructing the origin of evolutionary trends. Moving from reference proteomes to genus-wide and macroevolutionary venom pattern recognition adds the required extra dimensionality for comparative venomomics to achieve this goal.

At this point, it must be emphasized that the functional evolution of venoms is intimately linked to, and can only be understood in the context of, the organismal ecology and dietary habits. To this end, research questions should be formulated from an ecologically informed perspective. For instance, the physiological state of the prey at the time of the encounter, which is in turn modulated by the ecosystem's climate, may impose restrictions on the selection of the type of venom dominant in the snake population with which

Ecological venomics

(Prey subjugation)

Clinical toxinology

(Defensive bites inflicted to non-prey animals)

Natural Selection

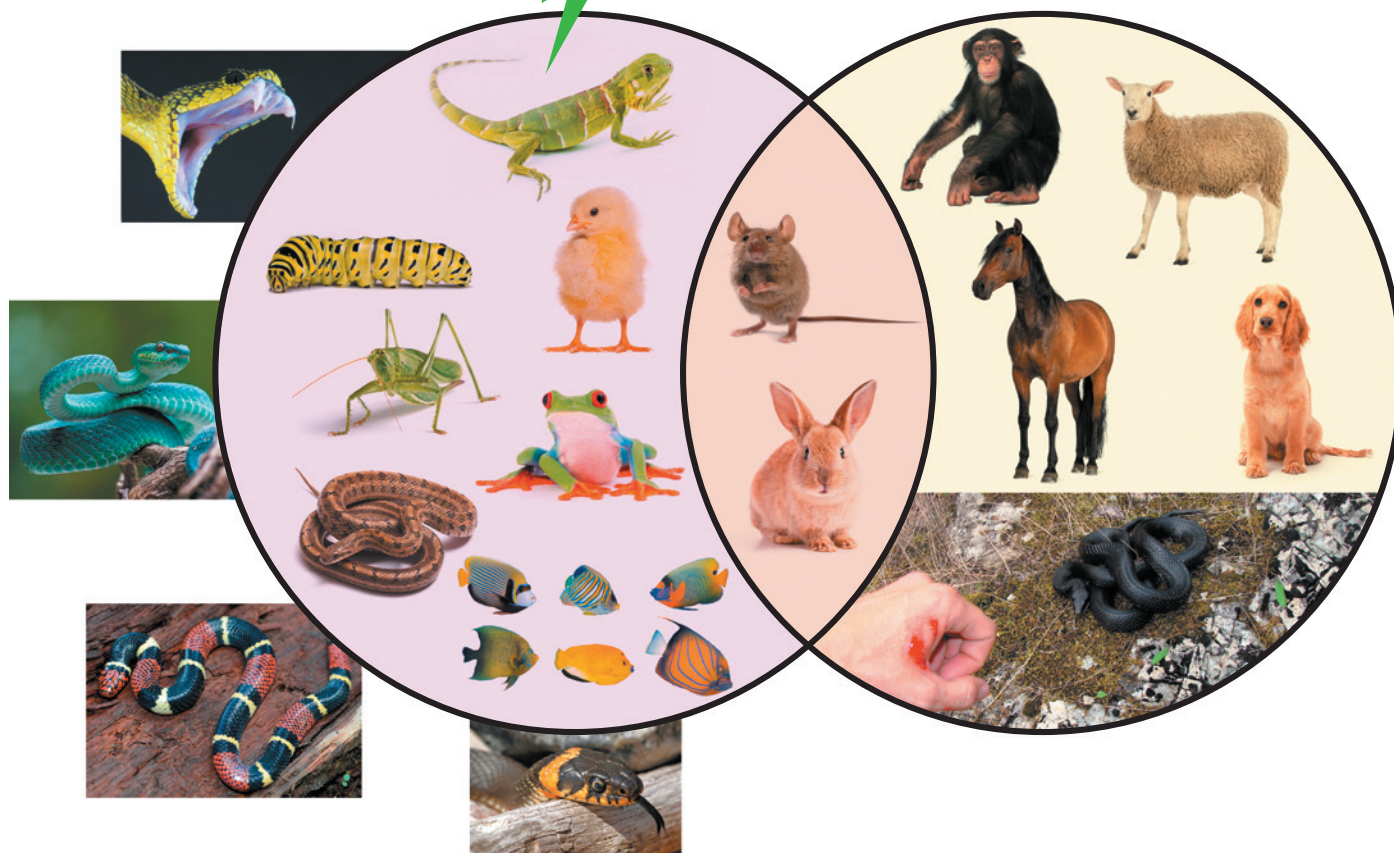


Figure 2. Cartoon of the exclusive and overlapping domains of ecological venomics and clinical toxinology, with an emphasis on the mutually enlightening relationship between evolutionary and translational venomics. The recognition of adaptive variations that have evolved via natural selection and enhance the snake foraging success on preferred prey, belongs exclusively to the field of ecological venomics, whereas the identification of the relevant toxins that should be neutralized to reverse the symptoms of snakebites inflicted to non-prey animals, such as pets, farm animals or humans, falls into the exclusive scope of clinical toxinology.

it has a shared ecological and evolutionary history. Mojave rattlesnakes (*Crotalus scutulatus*) exhibit two distinct venom phenotypes, type A (neurotoxic) and type B (haemotoxic), which are geographically segregated in populations that exhibit no discernible difference in diet. However, strong association has been found between venom type and climate, in which the type A neurotoxic venom was found in regions with cooler winters and higher rainfall. The effectiveness of type B venom appeared to be strongly dependent on metalloproteinases, whose enzymatic activity is rate limited by temperature; on the other hand, the most lethal component of the type A β -neurotoxic venom, namely

the heterodimeric PLA_2 Mojave toxin, acts non-enzymatically and is thus less affected by temperature. Each venom type possesses a slight competitive edge over the other in its distribution range. In areas with milder winters, type B venom may be a higher effective strategy for rattlesnakes, whereas in habitats with more extreme temperature variations, snakes with a rate-limited toxin arsenal may be outcompeted by those using a temperature invariant, neurotoxic strategy.

The venoms from coral snakes of the *Micrurus* genus that have been proteomically characterized to date also exhibit a puzzling phenotypic dichotomy. This is characterized by the toxin arsenal being dominated,

either by pre-synaptically acting PLA₂s or post-synaptic 3FTxs, and a general, but imperfect, distributional pattern of these venom phenotypes along the North-South axis of the American continent. Due to their cryptozoic and fossorial habits, natural history data for most micrurine species, such as diet or feeding behaviour, are scarce. Nevertheless, evidence for strong positive selection for the pre-synaptic 3FTx and post synaptic PLA₂ toxin families has been reported in *M. fulvius* venom, suggesting that dichotomic micrurine venoms may have been shaped through balancing selection.

In this evolutionary scenario, fitness is dependent on the frequencies of the two lethal venom toxin classes involved, with the hetero- (i.e., 3FTx + PLA₂) phenotype having an advantage over the homo-phenotypes. *M. ibiboboca* and micrurine taxa of uncertain taxonomy found within the Brazilian Caatinga biomass express PLA₂-rich venom phenotypes. The finding of PLA₂ outliers within a 3FTx biogeographic spot may indicate the occurrence of local adaptive evolution to the unique Caatinga ecosystem, and highlights the often underappreciated consequences of phenotypic plasticity of balanced phenotypes in ecological speciation (Figure 1).

Conversely, streamlined phenotypes may be indicative of strong selection in snakes that have acquired morphological adaptations towards very specific dietary regimes. An example is *Micrurus surinamensis*, a semi-aquatic coral snake, endowed with an extreme venom arsenal dominated by 3FTxs (>95%), which is highly toxic to banded knifefish, *Gymntus carapo* (LD₅₀ of 0.01 µg of total venom/g of fish body weight), one of the most preferred prey species of this snake. In comparison to terrestrial congeners, *M. surinamensis* exhibits morphological modifications that reflect adaptations to life in an aquatic environment. In line with this hypothesis, similar adaptations have convergently evolved in the Amazon water snake *Hydrops martii* (Colubridae, Dipsadinae), known to consume elongated fishes of the same taxa as *M. surinamensis*.

Toxicovenomics

Recent studies have employed a strategy combining compositional analysis and functional assays, referred to as 'toxicovenomics' at the 18th World Congress of the International Society on Toxinology (IST) held in Oxford in 2015. The essence of the toxicovenomics approach lies in screening the resolved profile of venom fractions provided by the venomics workflow for specific toxic activities. The combination of a toxin's incapacitation potency and abundance into a toxicity score allows a more realistic view of the relevance of particular toxins in prey incapacitation than toxic potency alone.

Applying toxicovenomics on natural prey serves to rank the adaptive potential of individual venom

toxins and identify venoms whose components act additively (e.g., *Aspidelaps* spp.) or synergistically (*Micropechis ikaheka*). In most studies, however, venom toxicity assessments are based upon non-native 'model' laboratory surrogate prey species, which are not consumed in the wild by the venomous predator. Nonetheless, toxicovenomic analyses performed on mammalian prey has something to offer clinical toxinology. Toxins bearing the highest mammalian prey-incapacitation activity are often also the most medically important molecules in the context of a human envenoming, i.e., those toxins that need to be neutralized to reverse the effects of the venom. In this sense, ecological venomics and clinical toxinology can be mutually enlightening, providing that predictions inferred from toxicovenomics evidence gathered from laboratory animals are testable and falsifiable.

To this end, 'antivenomics', a proteomics-based affinity chromatography protocol to quantify the extent of cross-reactivity of antivenoms against homologous and heterologous venoms, is available. In its current format, the so-called 'third-generation antivenomics' platform, antivenomics reveals information on the relative immunogenicity of the resolved venom components, and the maximal capacity of the antivenom antibodies to immunodeplete each of the venom toxins. The combination of antivenomics and *in vivo* neutralization tests (e.g., the Median Effective Dose, ED₅₀) constitutes a powerful toolbox for assessing the neutralizing efficacy of an antivenom, and hence a convenient and easy way to test toxicovenomics predictions.

The overlapping domains of ecological venomics and clinical toxinology

Snakebite envenoming is a disease of poverty, which annually kills more people than any other disease on the neglected tropical diseases (NTDs) list of the World Health Organization (WHO), residing in some of the world's most disadvantaged subsistence farming communities in rural impoverished African, Asian and Latin American regions. These events leave over 300,000 surviving victims with permanent physical disabilities, stigmatizing disfigurements and chronic mental morbidity. Antivenoms constitute the only scientifically validated therapy for snakebite envenomings, provided they are safe, effective, affordable, accessible and administered appropriately.

The wide spectrum of pathological and pathophysiological manifestations of envenomings, due to the concerted actions of the unpredictable venom variability across the phylogeny and distribution range of extant snakes, represents a great challenge for the development and preclinical evaluation of the efficacy of antivenoms.

From a biotechnological standpoint, this goal requires

knowing the phylogeographical patterns of present-day snake venoms, identifying their most medically important molecules in the context of human envenoming, and assessing the specific and para-specific efficacy of current antivenoms against the different medically relevant snake venoms.

From an evolutionary ecology perspective, human snake envenomings result from defensive bites inflicted by sympatric venomous snakes when snakes and humans have a chance encounter in their shared natural environment (Figure 2). In this context, the same ‘-omics’ strategies applied for unravelling the composition of venoms (‘venomics’), the adaptive potential of individual venom toxins towards mammalian prey (‘toxicovenomics’) and the efficacy of antivenoms to

neutralize the deleterious activities of individual venom toxins (‘antivenomics’) have conceptually the same applicability in clinical toxicology as in the ecological context, highlighting the mutually enlightening relationship between evolutionary and translational venomics.

The recognition of adaptive variations that have evolved via natural selection and enhance the snake foraging success on preferred prey, belongs exclusively to the field of ecological venomics, whereas the identification of the relevant toxins that should be neutralized to reverse the symptoms of snakebites inflicted to non-prey animals, such as pets, farm animals or humans, falls into the exclusive scope of clinical toxicology. ■

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