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Mutual enlightenment: A toolbox of concepts and methods for integrating evolutionary and clinical toxinology via snake venomics and the contextual stance

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ABSTRACT

Snakebite envenoming is a neglected tropical disease that may claim over 100,000 human lives annually worldwide. Snakebite occurs as the result of an interaction between a human and a snake that elicits either a defensive response from the snake or, more rarely, a feeding response as the result of mistaken identity. Snakebite envenoming is therefore a biological and, more specifically, an *ecological* problem. Snake venom itself is often described as a "cocktail", as it is a heterogenous mixture of molecules including the toxins (which are typically proteinaceous) responsible for the pathophysiological consequences of envenoming. The primary function of venom in snake ecology is pre-subjugation, with defensive deployment of the secretion typically considered a secondary function. The particular composition of any given venom cocktail is shaped by evolutionary forces that include phylogenetic constraints associated with the snake's lineage and adaptive responses to the snake's ecological context, including the taxa it preys upon and by which it is predated upon. In the present article, we describe how conceptual frameworks from ecology and evolutionary biology can enter into a mutually enlightening relationship with clinical toxinology by enabling the consideration of snakebite envenoming from an "ecological stance". We detail the insights that may emerge from such a perspective and highlight the ways in which the high-fidelity descriptive knowledge emerging from applications of -omics era technologies – "venomics" and "antivenomics" – can combine with evolutionary explanations to deliver a detailed understanding of this multifactorial health crisis.

1. Evolutionary ecology and clinical impact of snake venoms—a brief introduction

Venomous snakes share a long co-evolutionary history with primates, including our ancestors: their continuous co-existence across Africa (since ~6.7 My) and more recently (last ~2.5 My) in Asia, has shaped selection pressures for both lineages (Harris et al., 2021; Kazandjian et al., 2021). In our world today, humans and venomous

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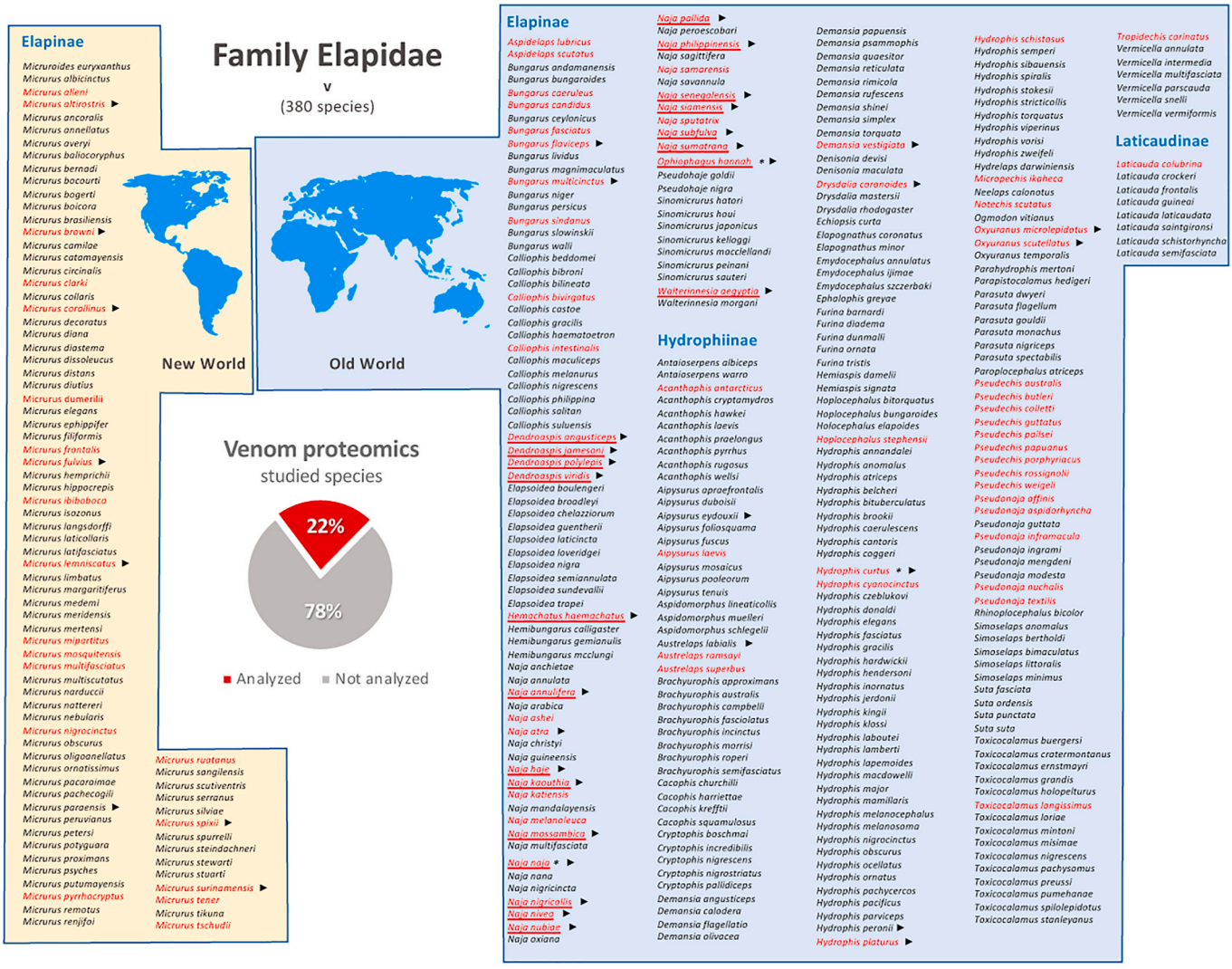

Fig. 1. Snake species of the family Elapidae whose venoms have been studied by using proteomic tools (names indicated in red) among all snake species within this family (371; based on lists provided by www.reptile-database.org). As shown in the inset pie chart, studied species cover 24% of all elapids. ▶ and * denote, respectively, the availability of specific venom gland transcriptome and genome sequence. Species whose venom proteome was gathered using top-down MS are underlined. Last update: April 26, 2021.

snakes coexist in tropical and subtropical rural regions. While a certain level of human ophidian accidents is inevitable, habitat reshaping due to agricultural and other practices, and climate change, contribute to increasing the level of human-snake interactions and, thus, the incidence of snakebites (Gutiérrez, 2020). Legitimate snakebite envenomings inflicted on humans by any of the approximately 110 species included in the World Health Organisation's (WHO) category 1 (highest medical importance) are an occupational hazard and a disease of poverty in many tropical regions (Harrison et al., 2009). The majority of envenoming from these snakes, which either belong to the Viperidae or Elapidae family (WHO, 2017), occur during capricious encounters with people engaged in rural activities in tropical and subtropical regions, especially in Asia and Africa. Such encounters can be described as ecological interactions between snakes and humans (Jackson et al., 2019; see below). Literature reports estimate that 400,000–1,200,000 snakebite envenomings occur annually, causing 81,000–138,000 deaths and many more injuries, such as physical sequelae (stigmatising disfigurements and amputations) and chronic mental morbidity (Gutiérrez et al., 2017a).

Snakebite envenoming affects not only the victims but often their entire families, which may enter a cycle of generational poverty that is difficult to break. In spite of its magnitude, the problem of snakebite envenoming has historically faced a neglect: therapeutic antivenoms are scarce or even unavailable in many regions (Longbottom et al., 2018); knowledge regarding the venoms/toxins and their pathophysiological actions is relatively limited; and modern biotechnological tools such as humanised or fully human antibodies still seem far from being introduced into active therapeutic deployment. Recent international efforts fostered by the WHO, science-funding agencies, and other stakeholders including Médecins Sans Frontières, the Wellcome Trust, the Kofi Annan Foundation, and the Global Snakebite Initiative (Chippaux, 2017; Longbottom et al., 2018; Harrison et al., 2009), are attempting to reverse this neglect and reduce the mortality and disability from snakebite envenoming worldwide by 50% before 2030 (WHO, 2019).

Snakebite envenoming represents a One Health challenge requiring clinical, ecological, and public health expertise (Longbottom et al., 2018). Hence, to comprehensively address this disease burden, the clinical challenge of appropriate case diagnosis needs to be considered within an ecological evolutionary context. Throughout this manuscript we reflect on the meaning, experimental methodologies, application and interpretation of shared or specific concepts of the partly overlapping domains of ecological and clinical toxinology. We argue that increased knowledge of snake behavior and life-history traits are crucial for understanding and predicting the frequency and geographical distribution

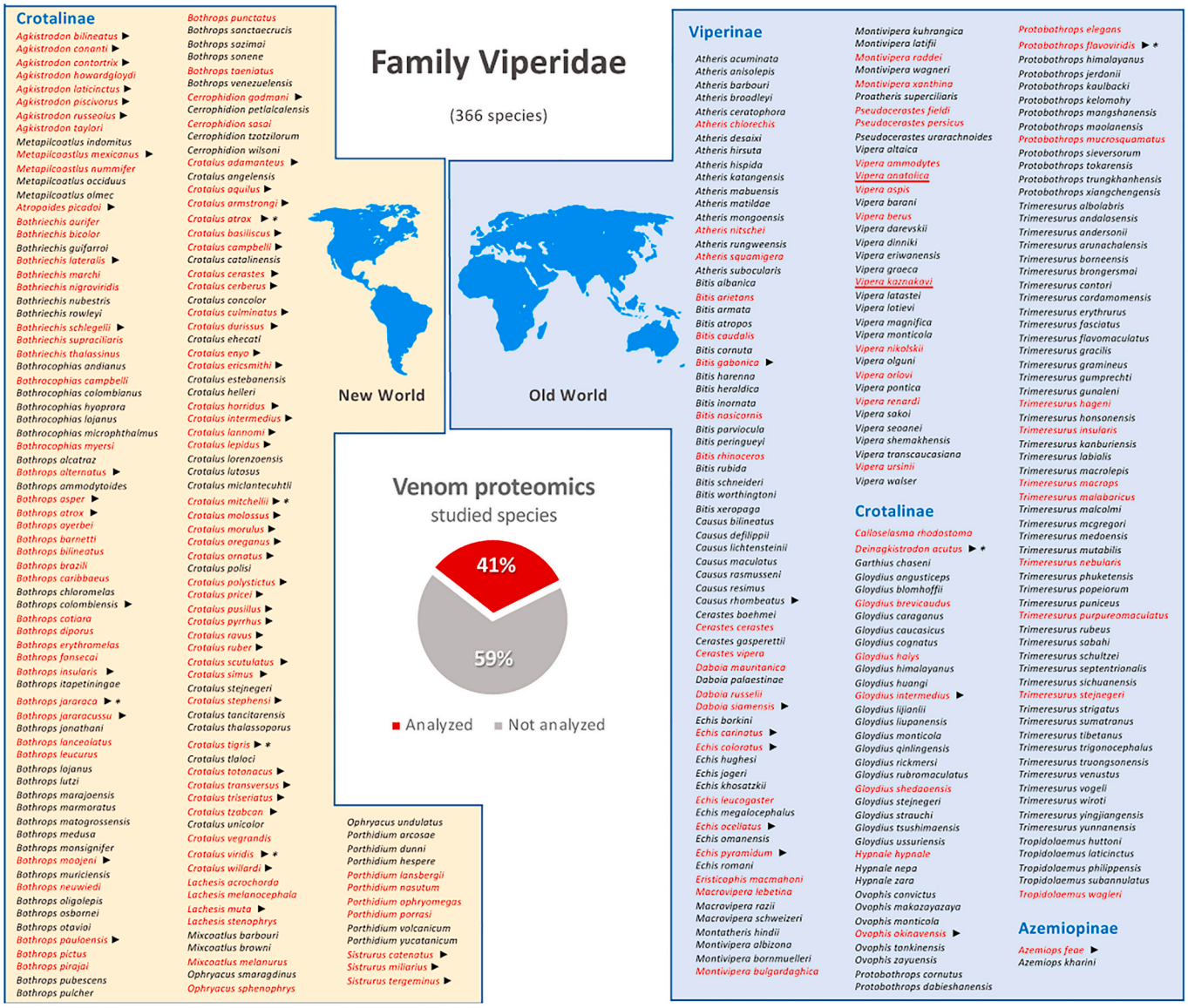

Fig. 2. Snake species of the family Viperidae whose venoms have been studied by using proteomic tools (names indicated in red) among all snake species within this family (355; based on lists provided by www.reptile-database.org). As shown in the inset pie chart, studied species cover 35% of all viperids. ▶ and * denote, respectively, the availability of specific venom gland transcriptome and genome sequence. Species whose venom proteome was gathered using top-down MS are underlined. Last update: April 26, 2021.

of snakebites (Jackson et al., 2019). Furthermore, knowledge of the evolutionary ecology of venomous snakes and the identity of the most relevant toxin molecules in the context of prey capture and human envenoming, are both key for improving our understanding of both the underlying pharmacology and the ability to improve the efficacy of antivenoms—the only effective antidote for envenoming. This remains a major challenge, as nearly half of all snake species capable of inflicting a life-threatening bite do not have antivenoms on the market, and distribution pipelines in the most afflicted countries are often substandard (WHO, 2017).

Venoms are intrinsically ecological traits (Jackson et al., 2019) and their natural history should thus be understood in the context of a network of chance and selection events. Identifying the specific pressures that tailored the composition of extant medically relevant venoms, e.g. by inferring influential nodes across phylogeny (Calvete, 2013, 2019; Pla et al., 2013, 2017a; Gibbs et al., 2013; Lomonte et al., 2014; Blanchet et al., 2017; Calvete et al., 2017; Ainsworth et al., 2018; Sanz et al., 2019a; Zaher et al., 2019; Jackson and Koludarov, 2020; Kazandjian et al., 2021; Holding et al., 2021), may have implications for the clinical treatment of human envenomings. The thesis advocated in this essay is that integration and contextualisation of complementary evolutionary, ecological, and clinical toxinological information represents a powerful holistic approach to tackle the global challenge of snake envenoming.

2. Evolutionary and contemporary contexts of snake venoms

Venoms are bioactive secretions typically consisting of cocktails of secretory proteins collectively referred to as toxins. Venoms have evolved independently in over a hundred animal lineages, where they are key evolutionary innovations that play a wide range of important ecological roles that predominantly involve predation and/or defense (Casewell et al., 2013; Schendel et al., 2019). One such lineage is caenophidian snakes, which explosive radiation in the wake of the Cretaceous–Paleogene mass extinction approximately 60–50 million years ago (Hsiang et al., 2015), was perhaps a consequence of the evolution of

venom (Fry et al., 2006, 2012). Snake venom is thought to have evolved primarily as a trophic adaptation, that is for predatory purposes (Davies and Arbuckle, 2019; Ward-Smith et al., 2020), and among the ca. 3700 extant snakes, at least 800 species (within Elapidae, Viperidae, Colubridae and Atractaspididae) use venom to immobilize their invertebrate or vertebrate prey before swallowing it whole. Venom is also used defensively by many snakes, and several lineages of venomous snakes have evolved specialised defensive venom adaptations (Arbuckle et al., 2017; Calvete, 2017), such as the blindingly cytotoxic venoms of the three lineages of spitting cobra (Kazandjian et al., 2021) and the hyperalgesic venoms of micrurine snakes (Bohlen et al., 2011; Lomonte et al., 2016; Sanz et al., 2019b).

To achieve their predatory and defensive functions, snake venom arsenals comprise mixtures of dozens to hundreds of bioactive compounds that belong to a limited number ($2 < n < 20$) of protein families (Fry et al., 2009; Calvete, 2013; Junqueira-de-Azevedo et al., 2016; Tasoulis and Isbister, 2017; Barua and Mikheyev, 2019), suggesting that relatively few protein folds are amenable for evolving a toxic role (Reeks et al., 2015). As in other venomous lineages, each of these protein toxin families represent molecular phenotypic innovations or novelties that emerged through functional recruitment of single-copy gene products, gene duplication massive structural and functional diversification, or both (Zancolli and Casewell, 2020). Interestingly, venom proteomics evidence gathered from 236+ snake species, mainly within the families Elapidae (Fig. 1) and Viperidae (Fig. 2), has revealed that variation in venom composition -within- and between-species, in-space (geographic) and in-time (ontogenetic)- is a common feature at all taxonomic levels. Much of this variation is due to differences and changes in prey composition, highlighting the influence that trophic ecology has on the evolution of snake venom. Importantly, this influence of ecological factors also extend to other venom-associated traits and characters—the *venom system*— including both morphological and behavioural traits (Kazandjian et al., 2021; Westeen et al., 2020). Extant venomous snakes thus represent fascinating models for studying the emergence of novel functional traits within clear adaptive contexts and across multiple levels of biological complexity that span from molecule to morphology to behavior (Casewell et al., 2013; Schendel et al., 2019; Zancolli and Casewell, 2020; Post et al., 2020; Jackson and Koludarov, 2020).

The genome contains traces of sterile recombination events as well as those that have contributed to the functional genome of the extant species. However, snake genomics is in its infancy. The first genome draft of a venomous snake, the king cobra (*Ophiophagus hannah*), was published in 2013 (Vonk et al., 2013). Since then, the sequences of only a handful of venomous snake genomes have been compiled, including the speckled rattlesnake (*Crotalus mitchellii*) (Gilbert et al., 2014); the five-pacer viper (*Deinagkistrodon acutus*) (Yin et al., 2016); the habu snake (*Protobothrops flavoviridis*) (Shibata et al., 2018); the prairie rattlesnake (*Crotalus viridis*) (Schield et al., 2019); two hydrophiins (*Hydrophis melanocephalus* and *Emydocephalus ijimae*) and two laticaudins (*Laticauda laticaudata* and *L. colubrina*) (Kishida et al., 2019); the Shaw's sea snake (*Hydrophis curtus*) (Peng et al., 2020); the western diamondback rattlesnake (*Crotalus atrox*) (Giorgianni et al., 2020); the Indian cobra (*Naja naja*) (Suryamohan et al., 2020); the tiger rattlesnake (*Crotalus tigris*) (Margres et al., 2021); the olive sea snake (*Aiysurus laevis*) (Ludington and Sanders, 2021), and the jararaca (*Bothrops jararaca*) (Almeida et al., 2021). The foreseeable exponential growth of comparative snake genomics studies in the coming years (Kerkkamp et al., 2016; Drukewitz and von Reumont, 2019) will undoubtedly result in a major leap forward in our understanding of the deep history of venom evolution. In addition, the comprehensive catalog of venom-gland-specific toxin genes complemented with transcriptomics, venomics and toxinological information may pave the way for the generation *a la carte* of synthetic antivenoms.

3. Snakebite and the "ecological stance"

Snakebite envenoming is a biological problem and understanding a biological problem requires a biological lens. Biology is a science of complex functional systems with myriad sub-components of endless variety. Nonetheless, certain basic principles appear to hold across all biological systems. Acknowledgement of these basic principles should therefore be a core component of any attempt to understand biological systems and thereby solve (or mitigate) biological problems. Two of biology's fundamental principles are evolution – all biological systems evolve and have an evolutionary history – and ecology – all biological systems interact with other biological systems. These principles hold at all levels of analysis within biology, from the molecular to the organismal. Evolution and ecology themselves share a common principle – the transcendent role of context. In biology, what things are determined by their context – both their temporal and "genetic" context in an evolutionary lineage, and their extant context in an ecological assemblage. Again, this applies to molecules as much as it applies to organisms (Guttinger, 2018; Jackson and Koludarov, 2020). An appreciation of the role of context thus seems to be a core component of a minimal biological perspective – an evolutionary or ecological stance, akin to the "intentional stance" (Dennett, 1995) or "empirical stance" (van Fraassen, 2008). These "stances" represent heuristic strategies for understanding and getting to grips with phenomena. Here, we will discuss three ways in which the evolutionary and ecological stances, with their shared emphasis on context, may help us get to grips with the problem of snakebite envenoming.

3.1. Circumstances of bites

Snakebite itself, the result of an interaction between a snake and a human, is an uncontroversial ecological phenomenon. One of the most important factors in reducing the burden of snakebite envenoming is gaining detailed knowledge of the circumstances in which bites occur. Such knowledge may help prevent many snakebites from occurring, thus reducing the burden on healthcare services. What are the contexts in which snakebites occur? Answering this question requires knowledge of the ways in which snake ecology and human ecology become human-snake ecology. Currently we have much anecdotal evidence on the circumstances of bites, but very little research has been conducted in this space.

Naturally, the context of bites differs considerably from place to place, both regionally and locally. Thus, the circumstances in which bites occur in India may or may not exist in (for example) sub-Saharan Africa. Thus, ecological studies of human-snake interactions must be conducted in all snakebite-afflicted regions. Some examples of simple, testable hypotheses include:

- Do snakebites typically occur when a snake is attempting to defend itself from a human it perceives as a potential threat?
- Are bites to sleeping humans (e.g. by spitting cobras in Africa and kraits in India) a case of mistaken identity – when a confused snake mistakes the smell of a human for that of the mammalian (e.g. rodent) prey it seeks – or can be considered defensive (e.g. when a sleeping human accidently rolls over onto a snake that was seeking warmth)?
- Does carrying a torch at night or wearing appropriate footwear reduce the incidence of snakebite?
- Does clearing away rubbish or overgrown vegetation from around human dwellings reduce the number of human-snake interactions?

Questions like these can be combined to elaborate an educational program to instruct people about the behavior of snakes in their area, which may lead to a significant reduction in snakebites.

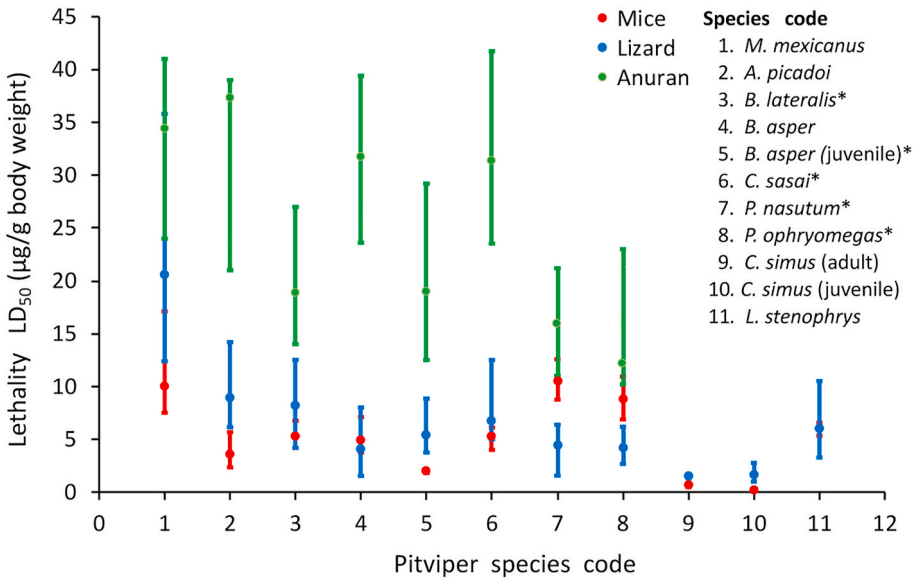

Fig. 3. Median Lethal Dose (LD_{50}) estimates (95% CI, i.p. route) for several Neotropical pitvipers in three models of preys (mouse, *Mus musculus*; lizard, *Hemidactylus frenatus*; anuran, *Engystomops pustulosus*). Species that consume regularly lizards and frogs are highlighted from those that specialize in mammals by asterisks.

3.2. Molecular evolution and function of toxins

The composition and activity of snake venoms is strongly influenced by the feeding ecology of the snakes that deploy them, often resulting in compositional variation across biogeographical regions and developmental stages (Sunagar et al., 2016; Jackson et al., 2019). However, the mechanisms that generate this variation remain largely elusive, partly due to the paucity of both venomous and non-venomous snake genomic data (e.g., Aird et al., 2017). The evolution of this continuous spectrum of venom phenotypes is best understood through the application of quantitative genetic methods (Hansen and Houle, 2008; Walsh and Lynch, 2018), which have yet to be applied to snake venom systems. In addition, identifying the ecological correlates of particular venom mixtures from the current composition of venoms is not straightforward: We must take into account both phylogenetic constraints and the potentially confounding effect of having to use atypical prey species as models to measure venom potency and bioactivities (see below).

Contrasting our lack of knowledge on the mechanisms that underlie the population and developmental variational properties and dynamics of snake venoms, we have a much better knowledge about the processes that shape snake venoms on macroevolutionary scales (between species). For example, we can infer nodes where particularly influential shifts were made in genotype-phenotype space by mapping the venom phenotype profiles of a species clade across its phylogeny (Pla et al., 2013, 2017a; Gibbs et al., 2013; Lomonte et al., 2014; Calvete et al., 2017; Ainsworth et al., 2018; Calvete, 2019; Sanz et al., 2019a; Zaher et al., 2019; Kazandjian et al., 2021). Further, ancestral structure inference may allow the reconstruction of the evolutionary origin of key mutations across the history of individual toxins (Whittington et al., 2018) and functional snake venom clades (Blanchet et al., 2017; Ainsworth et al., 2018; Jackson and Koludarov, 2020; Holding et al., 2021).

3.3. The effectiveness or "ecological validity" of therapeutics

"Ecological validity" is a concept with a long history in psychology and refers to the generalizability of data from one context (e.g. the laboratory) to another (e.g. the world outside the laboratory) (Schmuckler, 2001). Whilst the term might not be commonly used in association with the design and development of snakebite therapeutics, it is a familiar concept within pharmacological studies – a drug may be effective *in vitro* without those promising results being translated to *in vivo* contexts. This may be described as the difference between "efficacy" and "effectiveness", a distinction that has been previously highlighted in research on snake antivenoms (Isbister, 2010). Whether or not a snakebite therapeutic (e.g. an antivenom) is ecologically valid is partly determined by factors including whether or not an antibody that binds a toxin under laboratory circumstances will do so under physiological circumstances; whether binding a toxin translates to neutralisation (or elimination); whether the biodistribution of injected antibodies (or other therapeutics) is comparable to that of their targets (e.g. toxins); and a host of other factors. In addition to these molecular and physiological factors, the ecological validity of a snakebite therapeutic is influenced by the simple fact of whether the therapeutic can be delivered to a bite victim in a timely manner. Thus, as the World Health Organisation's Snakebite Envenoming Roadmap indicates (WHO, 2019), antivenom/drug distribution programs and other aspects of health infrastructure are crucial components in determining the ecological validity of a potential snakebite therapeutic. Much like any other functional trait—tools such as drugs can be considered part of an extended phenotype (Dawkins, 1982) of the human organism—the effectiveness of a snakebite therapeutic is contingent on its deployment in the appropriate context. Studying and engineering (e.g., through the creation of efficient distribution pipelines; the training of clinicians; etc.) this context is part of the landscape of snakebite research.

3.4. The evolutionary ecology of venoms and its impact on snakebite

Being primarily a trophic adaptation, the composition and potency of a snake venom is shaped by complex intrinsic (the genetic propensities of a lineage) and extrinsic (ecological interactions and environmental conditions) factors (Casewell et al., 2014, 2020; Sunagar et al., 2016). Hence, understanding the evolutionary ecology of venom becomes invaluable, not only from an academic perspective but also for designing efficient snakebite therapeutics. Ecological factors that are well-known to affect snake venoms include feeding ecology, ontogeny, predator pressure, inter- and intraspecific competition, and geographic distribution and population ecology.

Variations in snake venoms are believed to be primarily driven by differences in the physiologies of the prey and/or predatory animals against which venom is deployed and are especially pronounced in snake species that are distributed across distinct biogeographical or agroclimatic zone. Dietary shifts, which may result from transitions in

ecological niches, or from the differences in prey abundance from one region to another, have led to the extensive evolutionary fine-tuning of the snake venom cocktail (Daltry et al., 1996; Gibbs et al., 2011). The effect that dietary modifications and food specialisations have on the venom's composition has some restrictions since there appears to be a gradient of susceptibility to venom among different prey types. Thus, anurans appear to be much more resistant to snake toxins than lizards and rodents, sometimes by several orders of magnitude, even in species that feed on them regularly (Fig. 3).

Asiatic kraits *(Bungarus* spp.*)* provide an instructive example of the effects of such dietary shifts and feeding specialisations on snake venoms. Kraits that chiefly feed on mammals secrete some of the most potent and medically important venoms to humans (Sunagar et al., 2021) whereas those that specialize in feeding on reptiles possess venoms with greatly reduced toxicity to mammals (Senji Laxme et al., 2019). Similar shifts in diet from mammals to arthropods (Barlow et al., 2009; Senji Laxme et al., 2019), amphibians (Hutchinson et al., 2007), birds (Mackessy et al., 2006), and eggs of various animals (Li et al., 2005) have significantly influenced the compositions and toxicities of snake venoms and, in some cases, their delivery systems.

As venoms play an essential role in prey incapacitation and, perhaps, predigestion, dietary shifts that are associated with developmental stage or ontogeny can alter venom compositions across the lifetime of an individual snake (Mackessy et al., 2003; Gibbs et al., 2011; Jackson et al., 2016). A shift in prey preference, from ectotherms as juveniles to endotherms as adults, is associated with changes in the venom profiles of various species of *Crotalus* snakes (Mackessy et al., 2003). Paedomorphosis (i.e., the retention of larval or juvenile traits into adulthood) has been noted in some species (e.g., *Crotalus durissus* (Calvete et al., 2010); *Pseudonaja modesta* (Jackson et al., 2016)). Dichotomous venom profiles are documented in others, in which the juvenile snakes possess a neurotoxin-rich venom as opposed to the proteolytic venoms of their adult counterparts (e.g. *Crotalus simus* (Durban et al., 2013), *P. textilis* (Jackson et al., 2016; Cipriani et al., 2017)). While the underlying molecular mechanisms remain in most cases uninvestigated, it has been suggested that microRNAs (miRNA) play a role in underpinning such stark transitions in the proteomic compositions of venoms across the ontogeny of the Central American rattlesnake (*Crotalus simus simus*) (Durban et al., 2013). Snake venoms and their molecular targets in prey and/or predators can exert reciprocal selection pressures. The clearest example of selection pressure towards venom resistance is in predators (e.g., opossums (Marsupialia: Didelphidae) and mongoose, *Herpestes ichneumon*) preying on venomous animals (Voss and Jansa, 2012; Drabeck et al., 2015; Holding et al., 2016). On the other hand, the evolution of adaptive resistance in ground squirrels (*Spermophilus beecheyi*) against the venom of the Northern Pacific rattlesnake (*Crotalus viridis oreganus*) represents another example of evolutionary chemical arms races between a venomous snake and its prey (Holding et al., 2016; Margres et al., 2017; Gibbs et al., 2020). While several molecular mechanisms of resistance are clearly understood, the precise impact of venom resistance on venom composition and potency remains elusive.

In addition to biotic interactions, environmental conditions are a primary influence on the abundance of predators and prey in a region and, in turn, influence snake venom cocktails. In addition, many extrinsic factors, including humidity, temperature and altitude can determine the rate of transcription and translation (i.e., RNA and protein synthesis, respectively). Hence, the environment may directly or indirectly influence venom profiles in a multitude of ways. It is unsurprising, therefore, that considerable variation in venom compositions and toxicities have been documented in snakes with large geographical distributions across distinct biogeographic conditions (Strickland et al., 2018; Senji Laxme et al., 2021). Considerable differences in snake venoms have also been described at finer geographical scales (Glenn et al., 1983). While the underlying causes are mostly unknown, this variability may stem from subtle differences in microhabitat, dietary preference, intraspecific competition, and venom resistance in local prey at all

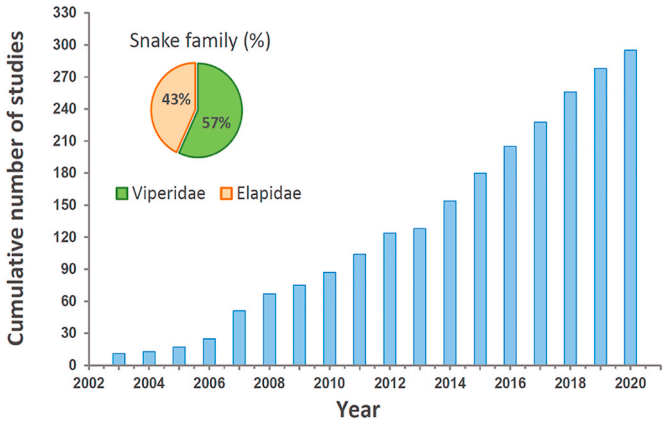

Fig. 4. Cumulative number of published studies dealing with the proteomic characterisation of snake venoms. Starting with pioneer works in 2003, up to mid-2020, a sustained increment in publications is evidenced (light blue bars). The inset pie chart illustrates the proportion of venomic studies that correspond to snake species from Elapidae (43%) (Fig. 1) and Viperidae (57%) (Fig. 2).

taxonomic levels.

As with evolutionary diversification more broadly, venom variation arises from the interaction between a plethora of intrinsic factors with those that are extrinsic (described in the preceding paragraphs). This interaction is an example of the transcendent role of context in evolution, in which an organism's environmental context (i.e., extrinsic factors) influence trait evolution in a manner that is both constrained and facilitated by the organism's phylogenetic context (i.e., factors intrinsic to an organismal lineage). Many genetic mechanisms including gene duplication, domain loss, neofunctionalisation, pseudogenisation, evolutionary tinkering of expression levels, alternative splicing, trans-splicing, and gene co-option have been identified as influencing the molecular diversification of snake venoms (Sunagar et al., 2016; Casewell et al., 2020). While positive Darwinian selection is implicated in rapidly introducing sequence variations in venom coding genes (Lynch, 2007; Juárez et al., 2008; Sunagar et al., 2013), mostly in an episodic or a 'two-speed' fashion (Sunagar and Moran, 2015), venom variation can also arise from the phylogenetic divergence of snakes over time. Cryptic genetic divergence in clinically important species can be detrimental to snakebite therapy, as antivenoms raised against the venom of one lineage may not be efficient in neutralising toxin variants in genetically distinct snakes. The existence of cryptic evolutionary lineages within a single species, therefore, can have tremendous implications for snakebite treatment, as can the existence of phenotypic crypsis in distinct species with overlapping range-distributions (Carbajal-Márquez et al., 2020; Sunagar et al., 2021). More infrequently, evolutionary convergence may result in cryptic lineages which are relatively inconsequential to snakebite therapy, given the convergence of venom composition along with the rest of the phenotype (Ukuwela et al., 2012).

4. What's in a venom?

For more than a century, biochemists and pharmacologists have found snake venoms to be a rich and exciting ground for the discovery of novel molecules, mainly polypeptides, with a broad array of potent and specific bioactivities that explain their toxicity. Research on venoms has been continuously enhanced by technological advances, and a paradigm shift in the study of snake venoms came with the introduction of '-omic' technologies at the turn of third millennium of our calendar (Fig. 4). However, proteomic strategies used to analyze venoms ("venomics") in different laboratories have been diverse and no general consensus has been adopted among molecular toxinologists that would facilitate comparison of results. For the most part, venomics studies have used peptide-centric 'bottom-up' approaches, with pre-mass spectrometry

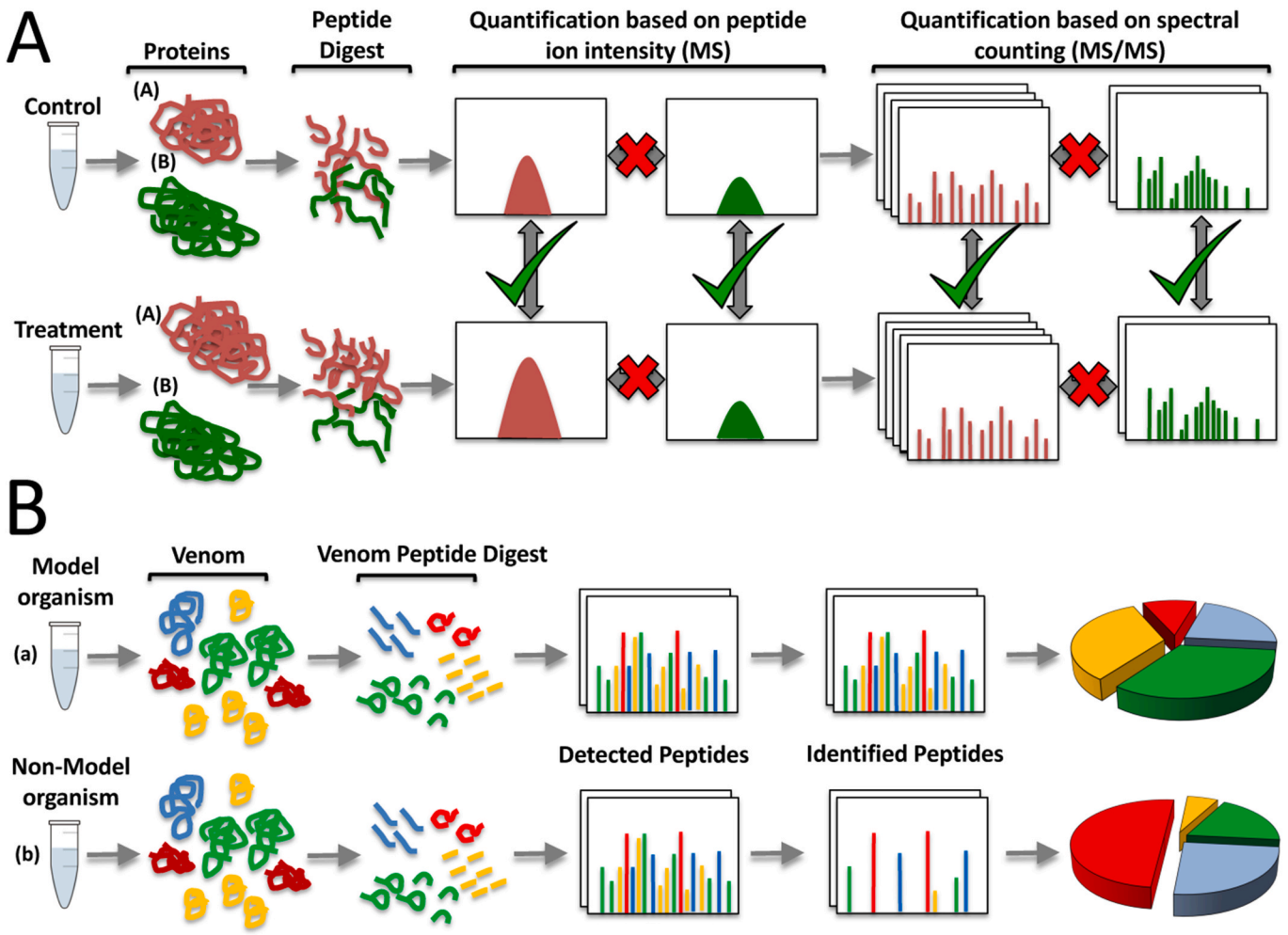

Fig. 5. **Panel A**, generalised representation of label-free shotgun proteomics. Both control and treatment samples are separately prepared and subjected to individual mass spectrometric analysis. In shotgun, or bottom-up proteomics, proteins are enzymatically digested into peptides, often with trypsin, prior to mass spectrometry. Label-free quantification (LFQ) is based on comparison of the areas of the peptide precursor ions of the same peptide, or by counting the number of fragment ion spectra of the same protein, in the different analyses. Appropriate comparisons are highlighted by the green ✓. Red X highlight that label-free strategies are not appropriate for determining the abundances of different proteins in a single sample: in this example, there is no change in abundance for protein B between control and treatment samples, whereas protein A is most abundant in the treated sample. **Panel B**, simulation of the different outcomes of label-free quantification of the same shotgun-generated tryptic venom peptidome when a species-specific genomic/transcriptomic reference database is available (**a**) or not (**b**) to match the tryptic peptide set.

(MS) venom decomplexation, or applying direct 'shotgun' approaches (Fox and Serrano, 2008; Calvete, 2014; Zelanis and Tashima, 2014; Eichberg et al., 2015; Lomonte and Calvete, 2017).

An important challenge in the characterisation of venoms (and other complex biological samples) is the inability of any single method to provide a complete view of its proteome. Proteome decomplexation and toxin identification and quantification represent major pillars of current state-of-the-art snake venom proteomics. Sample decomplexation protocols can be categorised as gel-based and gel-free methods (reviewed by Lomonte and Calvete, 2017). Among the multiple hyphenated approaches proposed by different research groups for integrating pre-MS venom proteome decomplexation and the identification and quantification of the individual toxins into a single workflow, arguably the most successful combination has turned out to be the one that takes advantage of venom fractionation to simultaneously quantitate the relative abundances of its different venom components. This strategy, which is usually referred to as 'snake venomics' (Juárez et al., 2004), relies on reverse-phase chromatographic decomplexation and eluate monitorisation at the absorbance wavelength of the peptide bond to estimate the relative abundances of the chromatographically separated fractions by peak area integration (Calvete, 2014, 2018). Among the gel-based approaches, two-dimensional electrophoresis (2-DE) generates an overview of a venom's protein complexity in a single image, including the approximate number of protein species and their apparent molecular masses and isoelectric points. In addition, comparison of non-reduced (IEF)/non-reduced (SDS-PAGE) and non-reduced/reduced 2-DE gels provides information on the quaternary structure of the venom proteins (Rabilloud, 2002; Eichberg et al., 2015). However, small (<3 kDa) peptides, which represent abundant components of some snake venoms, as well as proteins exhibiting extreme isoelectric points, may not be visualised in 2-DE venom reference maps, and there are a number of challenges in 2-DE proteome quantification: some spots tend to have severe tails in either dimension; contrast variations due to stain exposure, geometric distortions due to casting, polymerisation, and the running procedure may confound spot modeling, challenging reliable spot volume quantification.

Gel-free LC-MS shotgun approaches often bypass the initial sample decomplexing step, and instead, the entire protein mixture is enzymatically digested into peptides that are subjected to mass spectrometric analysis (Wolters et al., 2001; Wu and MacCoss, 2002; Zhang et al., 2013). Conceptually, label-free quantification approaches are relatively straightforward, and are typically used to compare abundance changes

of the same protein in different analyses. For example, comparing the abundance changes of protein A in control vs protein A in treated samples (Fig. 5A; Zhu et al., 2010; Neilson et al., 2011). In contrast, mass spectrometry is essentially a non-quantitative technique because proteolytic peptides exhibit a wide range of physicochemical properties that influence their ionisation efficiency, and hence, the quantitative estimations of parent protein abundances by label-free shotgun approaches is not appropriate for determining the abundances of different proteins in a single sample (Neilson et al., 2011; Lee et al., 2019). This point is discussed in more detail below. On the other hand, shotgun venomics, particularly when LC-MS data can be matched against a next generation species-specific venom gland transcriptome, may provide a deep catalogue of the protein/peptide components present in a venom (Rokyta et al., 2011; Aird et al., 2013; Calvete et al., 2018a). However, among the 236+ nominal snake species within families Elapidae and Viperidae that have their venom proteomes characterised through MS-based venomics, only for 114 taxa has the venom gland transcriptome been reported, and venom gland transcriptomes are also available for another 6 species whose venom proteomes have not been analysed (Figs. 1 and 2). In the absence of a reference transcriptomic database, the data gathered through shotgun the relationship of shotgun MS/MS-derived peptides to their intact parent molecules may be lost, or very difficult to reconstruct (e.g., in the case of highly similar isoforms). With the advancement of next-generation approaches to sequence and assemble shotgun omics data, the limitation to generate a transcriptomic database is not technical but has more to do with accessibility to the necessary biological material, e.g. venom gland tissue. Very stringent national wildlife protection laws, notably the Wildlife Protection Act, 1972 in India, a country home to more than 60 species of venomous snakes some of which are sufficiently common to cause severe human envenomings (Whitaker and Captain, 2004), severely hinders the collection of venom and snake tissues for scientific research.

Recent venomics studies have applied standalone 'top-down' (TD) MS or combined top-down and bottom-up approaches (Petras et al., 2015, 2016, 2019; Göçmen et al., 2015; Melani et al., 2016; Ainsworth et al., 2018; Pla et al., 2018; Hempel et al., 2020; Kazandjian et al., 2021). Compared to bottom-up peptide-centric methods, top-down venomics has the capability to resolve the venom proteome to the level of individual proteoforms or closely related isoforms. On the other hand, TD-MS requires higher resolution instrumental configurations with extended mass-to-charge range to isolate and fragment multiply charged monoisotopic ions and interpret the resulting complex fragmentation patterns of daughter ions. A major limitation of current snake venomics arise from the paucity of genomic and venom gland transcriptomic sequence information for venomous snakes in public domain databases (Brahma et al., 2015; Drukewitz and von Reumont, 2019). This limitation is more pressing in the case of TD venomics, where the upper mass limit for efficient fragmentation and the poor understanding of the mechanism of gas-phase dissociation, makes the automatic interpretation of the complex fragmentation spectra of native protein ions impractical without specific database support. Conversely, in bottom-up venomics of orphan organisms, algorithms for aiding in the automated *de novo* interpretation of fragmentation mass spectra are becoming powerful tools. In addition, though demanding a high level of user skill, manual *de novo* sequencing of high-quality fragmentation spectra followed by BLAST analysis of the deduced sequence remains another valid option for low-throughput identification of venom proteins from poorly studied venoms.

5. The evils of quantification

The abundance and specific toxicity of the individual toxins are important features for inferring composition-activity correlations of venoms at any level of organisation, whether isolated from single snakes, within and between populations, or across phylogenetic clades. Mass spectrometry-based proteomics has emerged as a powerful tool for the global characterisation and quantification of proteins from complex biological samples (Cravatt et al., 2007; Han et al., 2008). However, intrinsic differences in the ionisation efficiency and/or detectability of peptide ions limit the applicability of label-free inferences to directly estimate relative parent protein abundances. Precise quantification of proteins generally requires modification of standardised protocols, and is contingent upon the specific scientific question being addressed, the proteome being investigated, and the type of sample being analysed (Megger et al., 2013). Further, the experimental workflow is largely dependent on the availability of comprehensive protein sequence databases. Hence, while ongoing and completed genome sequencing projects have resulted in high-quality reference databases for many model organisms (Collins et al., 2003), the lack of comprehensive protein sequence databases represents a major challenge for proteomic investigations of non-model organisms (Carpentier et al., 2008; Armengaud et al., 2014; Heck and Neely, 2020), such as the majority of venomous snakes. However, this situation may rapidly improve. Thus, not counting subspecies or distinct conspecific populations, there are 114+ venom gland transcriptomes available in the NCBI TSA (Transcriptome Shotgun Assembly Sequence) and/or the SRA (Short Read Archive) (Figs. 1 and 2), and if species clade transcriptomics becomes the trend (Kazandjian et al., 2021; Holding et al., 2021) the snake venom gland transcriptomics database will see significant growth in the immediate future.

The strategy of label-free quantification by spectral counting or ion intensity is based on the assumption that the likelihood of data-dependent precursor ion selection is higher for abundant precursor ions, and that the number of peptide identifications, normalised to account for the fact that larger proteins tend to contribute more peptide/spectra (Powell et al., 2004), represents a proxy of the abundance of the parent protein. A normalised spectral abundance factor (NSAF) is calculated as the number of spectral counts identifying a protein "i" (SpC_i), divided by the protein's length (L_i), divided by the sum of SpC/L for all proteins in the experiment. Despite label-free proteome quantification strategies being developed for model organisms for which comprehensive genomic or transcriptomic databases are available, and thus protein identifications (SpCi) does not represent a limiting factor (Old et al., 2005), there has been increased use of label-free shotgun proteomic techniques to characterize venom compositional patterns and estimate the relative abundance of individual proteins within the proteome of non-model snake venoms, particularly by very active research groups in India (Kalita et al., 2018; Patra et al., 2019; Patra and Mukherjee, 2020) and Malaysia (Zainal Abidin et al., 2016; Tan et al., 2016, 2018, 2019). The conceptual shortcoming of this approach results from the fact when a comprehensive reference database is missing, quantification is biased toward the successful peptide identifications (Liu et al., 2004; Tang et al., 2006; Bantscheff et al., 2007, 2012; Choi et al., 2008; Neilson et al., 2011) (Fig. 5B). Instead, deriving reliable quantitative information from peptide-centric MS data should be based on an unbiased procedure, e.g., where there is complete coverage of the venom toxin transcriptome.

The "snake venomics" proteome quantification strategy, which uses offline pre-MS two-separation step (reverse-phase HPLC and SDS-PAGE) to decomplex and quantify the venom proteome (Calvete, 2014; Eichberg et al., 2015; Calvete, 2017), fulfills this condition. It is worth mentioning that unlike "label-free" strategies that refer to unitless figures, the "snake venomics" proteome quantification strategy provides an estimate of the relative abundance of the venom's toxin arsenal in percentage (mg/100 mg) of total venom proteins (Calderón-Celis et al., 2016, 2017, 2019). Novel instrumental configurations have been proposed that combine elemental and molecular mass spectrometry platforms to achieve locus-resolved absolute quantification of the venom proteome (Calvete et al., 2017). Implementing top-down and absolute quantification approaches into next-generation mass spectrometry systems promises a quantitative leap, in proteomics in general, and for the study of venoms in particular. Although not discussed in this work, a

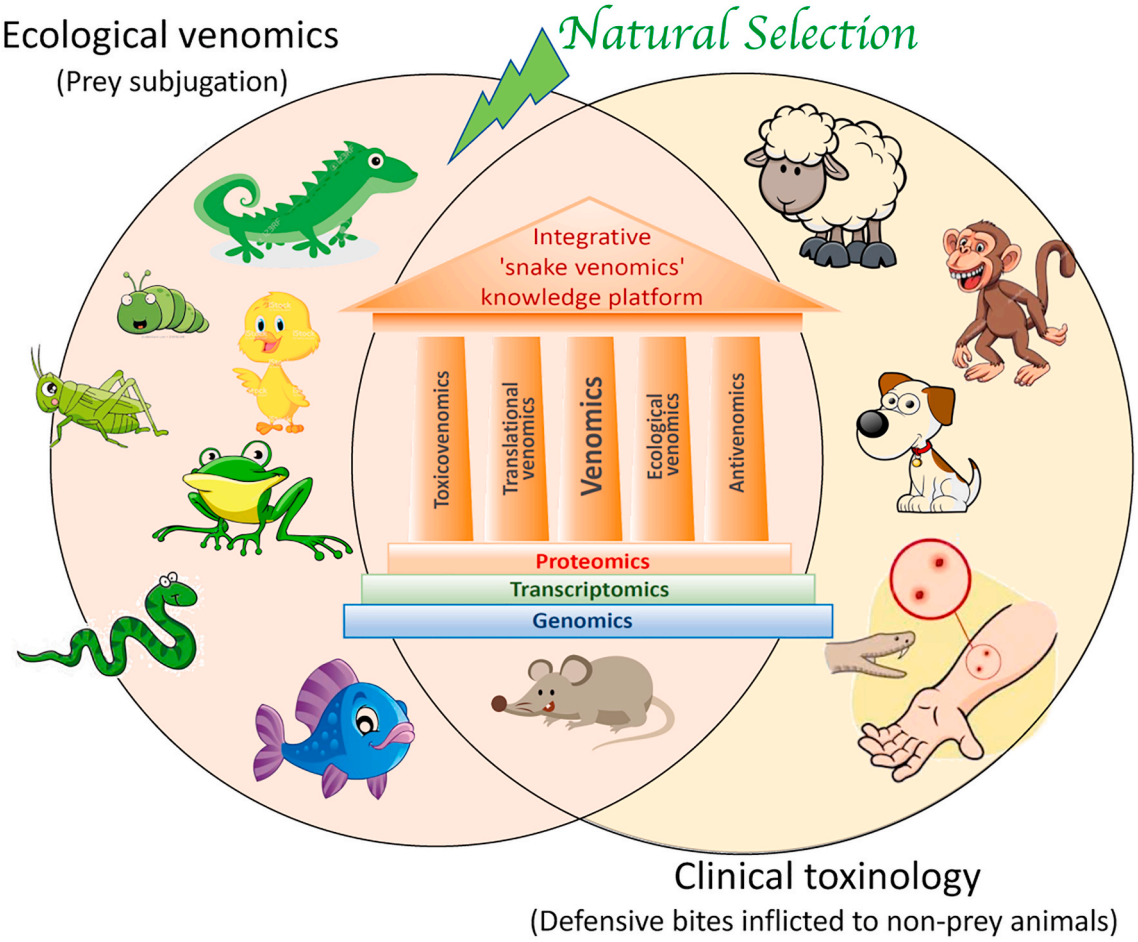

Fig. 6. Cartoon of the exclusive and overlapping domains of Ecological Venomics and Clinical Toxinology, emphasising the mutually enlightening relationship between evolutionary and translational venomics, guided by an integrative knowledge platform on snake venoms, supported by their compositional (venomics, aided by databases provided by transcriptomics and genomics), functional (toxicovenomics), and immunological (antivenomics) characteristics. Studies on 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 neutralised 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 is hoped that the expansion of integrative venomics may facilitate a deeper understanding of biological (ecological venomics) and medical (translational venomics) aspects of snake venoms, as well as contribute to confront the problem of snakebite envenomings by guiding improvements in the design and production of improved antivenoms.

thorough characterisation of venom peptidomes, protein glycosylations, and other post-translational modifications, will be a relevant and necessary task to complete our understanding of 'what's in a venom'.

6. The road to integrative snake venomics

As addressed above, an increasing trend in venom analysis is "clade venomics", the identification of evolutionary trends across whole genera, taxonomic clades, and phylogenetic families. Clade venomics is contingent upon the specific scientific question to be addressed. This in turn implies that concepts, such as median lethal dose (LD_{50}), must be interpreted differently depending on whether the question belongs to the ecology or clinical toxinology domain. Likewise, both ecological venomics and clinical venomics rely on complementary omic platforms that add new approaches to the study of venoms, contributing to the multidisciplinarity of the field. Venomics (*sensu lato*), "antivenomics" (Pla et al., 2017b; Calvete et al., 2018) and "toxicovenomics" (Lomonte, 2015; Lauridsen et al., 2017) are three complementary pillars of the molecular toxinologist's toolbox (Fig. 6). Strategic alliances between clade venomics, antivenomics and toxicovenomics highlight the versatility and adaptability of the original 'venomics' concept to the distinctive research of the different venomics domains.

6.1. Antivenomics provides key information to guide antivenom optimisation

Antivenoms are critical life-saving biologicals consisting of immunoglobulins (or derived F (ab')$_2$ and Fab fragments) elicited by hyperimmunisation of horses or sheep with venom(s) from the most medically relevant snakes in the region where the antivenom is intended to be deployed. A strategy coined 'antivenomics' has been developed to evaluate the preclinical immunorecognition profile of an antivenom towards homologous and heterologous venoms. Successive "generations" of the antivenomics protocol have refined and extended its analytical scope (reviewed by Calvete et al., 2018). First generation consisted of the in-solution immunoprecipitation of antigen-antibody complexes followed by the chromatographic quantification of the uncomplexed venom proteins present in the supernatant (Lomonte et al., 2008). Affinity chromatography-based second generation (2G) antivenomics (Pla et al., 2012) yielded smoother and better resolved chromatographic profiles of both the non-binding and the bound venom proteins. The antivenom's toxin-resolved immunocapture ability can be estimated as the relative ratio of the chromatographic areas of the same protein recovered in the non-retained (NRi) and retained (Ri) affinity chromatography fractions using the equation: %NRi = 100 - [(Ri/(Ri +

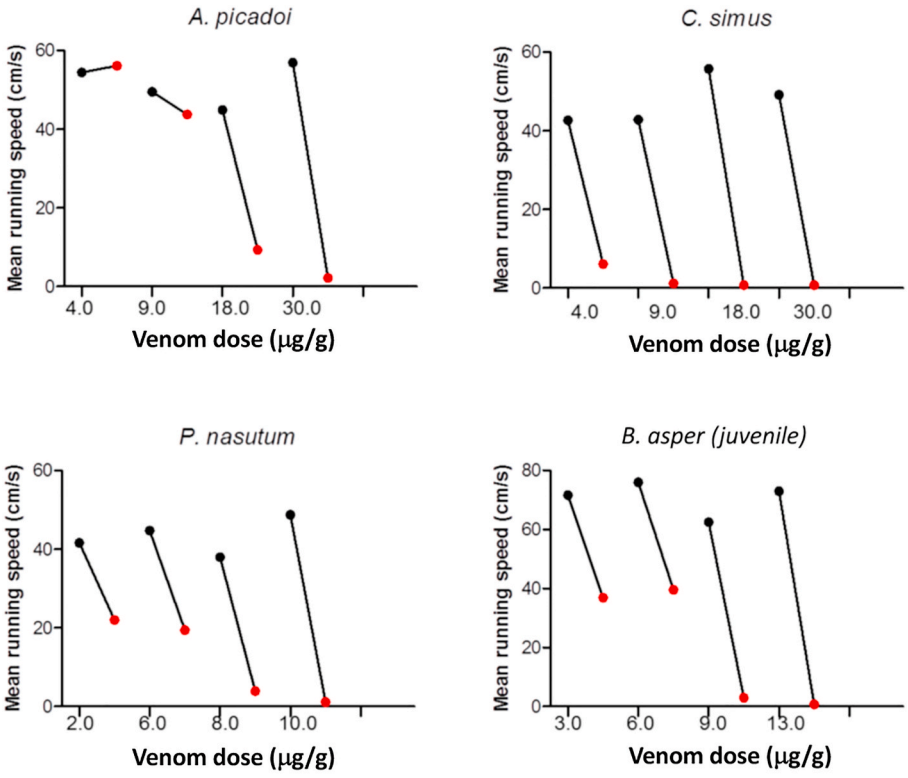

Fig. 7. Changes in running speed after pitviper venom injection in the lizard *Hemidactylus frenatus*. Mean speed for four individuals before (black circle) and after (red circle) injection, tested on a 60 cm race track. Venoms from *Atropoides picadoi*, *Crotalus simus*, *Porthidium nasutum*, and *Bothrops asper* (juvenile) were tested. The first two species specialize in mammals; the last two include lizards in their diets. For any of the doses, lethal or sublethal, a significant decrease in escape velocity is observed few minutes after injection. In an ecological scenario, this reduction alone could represent an adaptive advantage for the snake.

NRi)) x 100]. The inclusion of (R) in the equation compensates for possible losses during sample handling and chromatographic analysis, resulting in a more accurate quantification of the antivenom's immunorecognition profile than with the immunoprecipitation method. Polyclonal antivenoms exhibit distinct affinity concentration-dependent patterns of maximal binding toward different toxins of the venom. Optimising the immunoaffinity conditions for all venom components seems an impossible mission. However, the maximal immunocapturing capacity of an antivenom can be determined by the parallel running of identical antivenom affinity columns incubated with increasing amounts of venom until saturation is reached. This modified antivenomics protocol has been dubbed third generation (3G) antivenomics (Pla et al., 2017b).

Antivenomics informs on the maximal binding capacity of an antivenom's antibodies towards each of the venom toxins. However, the ability to bind toxins is an essential but not necessarily sufficient molecular property to endow an antivenom with clinical utility. A more relevant preclinical feature, the percentage of lethality neutralising antivenom antibodies (%AV_{neutr}) can be calculated from the combined outcome of antivenomics and *in vivo* neutralisation assay as the ratio [P]/Max_{bind} (Calvete et al., 2018; Sanz et al., 2018; Al-Shekhadat et al., 2019; Sanz et al., 2020). [P] is the antivenom's potency (P [mg venom neutralised per mass unit (mL or mg) that rescue 100% of the test population] = [(n-1)/ED_{50}] x LD_{50}), where "n" is the number of median lethal doses (LD_{50}s) used as challenge dose to determine the median effective dose (ED_{50}) of the antivenom in the murine model) (Araujo et al., 2008; Morais et al., 2010). Max_{bind} stands for the antivenom's maximum toxin binding capacity.

6.2. Toxicovenomics in ecological venomics: LD_{50}, and the relevance of contextualising toxinological studies

Antivenomics reveals information on the relative immunogenicity of the different venom components and quantifies the relative amounts of anti-toxin antibodies of an antivenom. However, venom toxins exhibit variable toxicity and it is the combination of abundance and potency that defines the ecological or clinical relevance of a toxin in the context of the venom injected into the prey or victim. Toxicovenomics aims to rank the different toxins present in a venom according to their contribution to the venom LD_{50}. To this end a Toxicity Score (TS) is calculated by dividing the toxin (TX_i) abundance (in moles/g of total venom proteins) by its median lethal dose (LD_{50}) (in moles TX_i/g animal body weight). The TS contributes to the identification of the most relevant toxins in a given venom (Calvete et al., 2010; Laustsen et al., 2015). The occurrence of taxon-preferent or taxon-specific toxins in certain venoms, particularly within the Colubridae family (Modahl et al., 2018; Modahl and Mackessy, 2019; Calvete et al., 2020), but also described in *Micrurus* (Elapidae) (Da Silva and Aird, 2001), underscores the importance of considering the adequate animal model for the purpose of the research aim (Jackson et al., 2019). Clearly, assessment of toxicity (median lethal dose, LD_{50}) in the wild or in a controlled laboratory setting that mimics the natural ecosystem requires including natural prey in the study. Despite having stood the test of time, the LD_{50} is a somewhat coarse metric, which does not identify some important envenoming subtleties. The outcomes are reported at 24 or 48 h, which completely obscures events that occur during the first minutes of the envenoming. Under natural conditions, this period is crucial to the outcome of predation. The immobilisation or partial reduction of a prey's escape abilities or total paralysis are central aspects of a venom's efficiency (Fig. 7). For this reason, the knock-down time, the time to paralysis, could be a measure with greater biological significance when evaluating toxicity.

The ecological limitations of the LD_{50} can be illustrated in the following example. The Duvernoy's gland secretory proteome of the Costa Rican false coral snake *Rhinobothryum bovallii* comprises a handful of toxins belonging to only three protein families (Calvete et al., 2020). Major monomeric (86.5 mol%) and minor dimeric (2.8 mol%) three-finger toxins (3FTxs) dominate its venom arsenal. The remaining 10.6% of the venom proteome comprises CRISP (8.2%) and PIII-SVMP (2.4%) molecules. Despite the large abundance of 3FTxs, a protein family whose elapid members are largely neurotoxic to mammals, *in vivo* lethality assays showed that *R. bovallii* venom is non-toxic to mice. Even intracerebroventricular injection of 100 μg of whole venom (containing

67.2 μg of 3FTxs) was harmless to mice. Intraperitonal administration of 50–650 μg of *R. bovallii* venom only killed one of two chickens at the maximum inoculated dose (14 μg/g), with death occurring around 18 h post-injection. Instead, a toxicovenomics analysis in *R. bovallii*'s preferred prey, lizards e.g., baby green iguana, showed that both the major monomeric and minor dimeric 3FTxs at doses equivalent to 7.1–14.1 μg venom/g iguana body weight, 1 out of 3 animals was completely and irreversibly paralysed by 3 h post-injection and died overnight, <10 h later. At 450 μg/iguana (25.8–28.1 μg venom/g body weight) 100% (3/3) of the animals were immobilised entirely, but breathing, between 10 and 180 min post-injection. An identical effect was observed at the highest amount of venom injected (900 μg/iguana; 49–56 μg venom/g body weight), but at this dose all (3/3) iguanas collapsed between 10 and 35 min after venom administration, but only died hours later without showing during that time any signs of recovery. From these data, an "LD_{50}″ of 315 μg/iguana was estimated, although this value was associated to broad 95% Confidence Intervals ($CI_{95\%}$, 62–569 μg/iguana). The fact that venom produces an irreversible immobilisation of the prey, which will then be swallowed alive, supports rapid prey immobilisation as the major role for the Costa Rican false coral snake venom, and questions the ecological meaning of LD_{50} in this, and likely other snake venoms. Perhaps it would be appropriate to define the irreversible immobilising activity of a venom as "the minimal dose of venom that induces irreversible immobilisation of the prey".

6.3. Toxicovenomics in preclinical research: allometric dose translation between model animals and human

In preclinical research, toxicovenomics complements antivenomics by uncovering the identity and lethal potential of the individual venom's toxins, thus informing which toxins should be primarily neutralised by an effective antivenom. In this sense, research on the likely toxic activities of venom from a snake species exhibiting a generalised or specialised diet towards endothermic prey, and how it would impact humans will involve the use of a laboratory animal of similar physiology to the victim, i.e. rodents. Making sense of the outcome of animal research in the context of human envenoming requires allometric dose translation between animal and human (West and Brown, 2005; Nair and Jacob, 2016). Allometry is used to describe how traits covary with body size, such as body surface area (BSA) and mass (White and Seymour, 2005). Allometric scaling is also an empirical approach where the exchange of drug dose is based on normalisation of dose to body surface area, and is used in research to predict an approximate dose on the basis of data existing in other species using the equation HED (human equivalent dose [mg/kg]) = animal dose [mg/kg] x [animal Km/human Km], where the correction factor (Km) is estimated by dividing the average body weight (kg) of the species to its body surface area (m^2). For an average human body weight of 65 kg, and a body surface area of 1.75 m^2 (calculated using the formula human body surface area (BSA) = $[(\text{weight [kg] x height [cm]})/3600]^{1/2}$ (Mosteller, 1987; Verbraecken et al., 2006)), the Km factor for human is 65/1.75 = 37, whereas Km for a 20 g mouse is 3 (consult Table 1 in Nair and Jacob, 2016). Thus, allometric scaling of Egyptian cobra (*Naja haje*) i.p. LD_{50} for mice (20 g) of 1.8 μg/g mouse body weight translates into an LD_{50} for human of 0.15 mg/kg or 9.5 mg venom/65 kg person. For an estimated yield of 175–300 mg wet venom (52.5–100 mg dry proteins) (Branch, 1981; Mirtschin et al., 2006), a severe bite may deploy 6–11 median lethal doses into the victim. The amount of venom injected forms part of the "context" of a given snakebite treatment. Thus, for an antivenom dose to be effective in context – i.e. to possess ecological validity – it must be capable of neutralising at least 11 lethal doses of *N. haje* venom.

The venom of the Egyptian Cobra, *N. haje*, comprises a minor α-neurotoxin (αNTxs, 7.3 kDa, 9% of the total venom proteins) and two major cytotoxins (CTx-1, 32% (6.7 kDa) and CTx-2, 44% (6.8 kDa) of the venom proteome). Toxicovenomics analysis revealed disparity in LD_{50}s of 0.4, 6.6, and 6 mg/kg mouse body weight, respectively, for the αNTx and cytotoxins CTx-1 and CTx-2 (JJC, data not published). These data correspond to allometrically calculated human LD_{50}s [mg/human of 65 kg body weight] of 1.95 (αNTxs), 34.5 (CTx-1) and 31.5 (CTx-2). TS for these toxins are 45.6 (αNTxs), 2.38 (CTx-1), and 2.63 (CTx-2), clearly indicating that the major contributor to the venom's lethality are the α-NTXs. In an average 76 mg venom snakebite, the calculated number of LD_{50}s deployed into a 65 kg human victim are unevenly distributed between the α-NTxs (3.5 LD_{50}s) and the cytotoxins (CTx-1, 0.69 LD_{50}s; CTx-2, 1.05 LD_{50}s), highlighting the challenge for an effective antivenom to neutralize all three major 3FTxs present in the venom, but particularly the α-NTxs. According to allometric scaling predictions, only a mild snakebite, consisting of less than 20 mg of injected venom, would deploy sublethal amounts of any toxin into a victim, and in an envenoming where the amount of venom injected is in the range of 20–70 mg, only the α-NTXs would be life-threatening to the envenomed adult person. However, these calculations may vary if various toxins act synergistically.

6.4. Allometric scaling in ecological venomics

Continuing with the same species selected to illustrate the concept of allometry in preclinical venomics, in this section we highlight the relevance of allometric scaling in ecology in general, and ecological venomics in particular. The Egyptian cobra ranges across most of North Africa north of the Sahara, across the savannas of West Africa to the south of the Sahara, south to the Congo basin and east to Kenya and Tanzania. Knowledge on its dietary habits is based primarily on anecdotal data, suggesting they prey on a variety of small vertebrates, such as anurans, mammals, tortoises, birds and their eggs, lizards, fish, and occasionally snakes, including conspecifics (Schleich et al., 1996; Filippi and Petretto, 2013). Snake venom potency is generally prey-specific (Mebs, 2001; Healy et al., 2019). However, the potency of Egyptian cobra venom, and that of the vast majority of venomous snakes, has been studied only in the murine model, and venomics studies across its broad range and wide variety of habitats (steppes, dry to moist savannas, arid semi-desert regions with some water and vegetation) have not been reported. Adopting the "contextual stance" of evolution and ecology, this variation in diet and habitat is likely correlated with variation in venom composition and potency, including variations in potency towards distinct prey taxa.

Since the publication of Kleiber's influencial monograph, a great deal of effort has been invested in the investigation of both basal metabolic rate (BMR) scaling of drug doses from experimental animals to human, and the adaptive significance of BMR (Kleiber, 1932). During the last 35 years, investigation of BMR variation has gained prominence in ecology, and many studies have addressed the adaptive variation in BMR by correlating it with traits of interest (White and Seymour, 2005). Morphological and metabolic scaling result from adaptations evolved in the context of both physico-chemical and ecological constraints (reviewed by Glazier, 2005; but read also Magalhlaes Magalhaes Silva et al. (2018) regarding the interesting case of aquatic adaptations in a neotropical aquatic coral snake *Micrurus surinamensis*). Theoretical models of metabolic scaling have been proposed to explain interspecific relationships, including adaptive maximisation of foraging efficiency and maximisation of reproductive fitness. A 'metabolic-level boundaries hypothesis' focusing on two major constraints (surface-area limits on resource/waste exchange processes and mass/volume limits on power production), conceptually similar to the allometric dose translation between model animals and human discussed above, can explain much but not all the models of metabolic scaling with body mass (White et al., 2007).

A debate about the value of the allometric scaling exponent (b) relating metabolic rate (MR) to body mass (M) ($MR = aM^b$) is still ongoing (Farrell-Gray and Gotelli, 2005; Glazier, 2005; White et al., 2007). A meta-analysis of 127 interspecific allometric exponents showed lack of support for a single exponent model (White et al., 2007). The

analysis showed that the effect of size of mass on metabolic rate is significantly heterogeneous and that, on average, this effect is stronger for endotherms than for ectotherms. Significant differences between scaling exponents were also identified between ectotherms and endotherms, as well as between metabolic states (e.g., rest, field, and exercise). The absence of a universal metabolic allometry represents a significant challenge to any model between taxa or between metabolic states. A complete understanding of metabolic scaling will require the identification of both proximate (functional) and ultimate (evolutionary) causes.

6.5. *Integrating biological reality into preclinical evaluation of antivenom efficacy*

Preclinical studies of antivenom efficacy are typically based on elucidating the potency of antivenom against the potential lethal effects of venom in a model laboratory animal, generally mice. WHO considers the (murine) median effective dose (ED_{50}) of antivenom to be a critical preclinical assessment bioassay that should be performed by manufacturers for all antivenoms, and enforced by the National Regulatory Authorities (NRAs) as part of the antivenom licensing procedure (WHO, 2017). The assay measures the dose of antivenom that prevents the death of 50% of test animals and may be reported in a number of ways (e.g.: mg venom/mL antivenom, μl antivenom/mg venom, mg venom/gram antivenom, etc.) (Ainsworth et al., 2020). The relevance to biological reality lies only in the premise that a product that prevents death in an animal model might also do the same in humans, albeit under different dosing, physiological and pharmacokinetic conditions. As we have discussed some venoms do not necessarily have death as primary endpoint; immobilisation, pre-digestion or pain (in the case of predator deterrence) may be the objective. Potency has been linked to prey-specificity and venom yields (the mass of venom the species produces in its venom glands), parameters that are likely associated with spatial environment, body size, metabolism (Healy et al., 2019; De Roodt et al., 2016), phenotypic, geographical and sexual factors (Mirtschin et al., 2002). How a venom is used also influences the type and quantity of venom produced. By relying on neutralisation of lethality in isolation the true biological context of antivenom design is often ignored, resulting in antivenoms that are ineffective in 'field conditions' where the ability to achieve clinical 'cure' (the objective of treatment) is crucial.

No minimum potency specifications currently exist for antivenoms marketed in sub-Saharan Africa (Gutiérrez et al., 2017b). Most products in the market have copied the specifications of FAV-Afrique and claim to neutralize a minimum of 200–250 murine (m) LD_{50} doses of each venom against which efficacy is claimed. The reality, however, is that neither yield of venom, nor mass of injected venom are equal across species. While 250 (m) LD_{50}s/vial against *Echis ocellatus* venom from Ghana (LD_{50}: 26.41 μg/19 g mouse) translates into 6602 μg venom, 250 LD_{50}s/vial against *Naja haje* venom from Ugandan specimens (LD_{50}: 1.90 μg/19 g mouse) is equal to 475 μg. When these figure are allometrically scaled to humans (LD_{50} *E. ocellatus* Ghana: 7.35 mg/65 kg human; LD_{50} *N. haje* Uganda: 0.52 mg/65 kg human), and the average venom yields are considered (*E. ocellatus*: 11.2 mg; *Naja haje*: 314.3 mg), it becomes clear that while 1.7 vials could potentially neutralize 50% of the lethality of *E. ocellatus*, the number needed for *N. haje* could be at least 604 vials (JJC, unpublished). However, current antivenom instructions for use recommend the same (typically small) doses be administered regardless of the species involved (DJW, unpublished). Venom yields obtained during laboratory-based venom extractions are typically much larger than the masses of venom released by snakes delivering defensive bites. For example, the mass of venom injected by the dreaded *Dendroaspis polylepis* during single defensive bites was 68.5% ± 12.5% less than the yield obtained from the same snakes during laboratory extractions (DJW, unpublished). Incorporating biologically relevant data such as mass of injected venom during defensive strikes and calculated human LD_{50} figures into the evaluation of preclinical antivenom efficacy could greatly improve formulation of products to ensure that they are likely to be effective against all of the species they are designed for use against. Similarly greater emphasis should be given to the specific activity of venom and the likely endpoints of envenoming. For example, antivenoms for species that are predominantly dermonecrotic or myotoxic should be assessed for their specific ability to abrogate these effects (WHO, 2017; Gutiérrez et al., 2017a) since lethality is often rare after bites by these species.

7. Concluding remarks and perspectives

Snakebite envenoming is a multifactorial health crisis afflicting the tropical developing world (WHO, 2019), which arises as a consequence of ecological interactions between humans and venomous snakes. The complexity of snakebite envenoming emerges from the reciprocal influence of the many factors involved – an interdependence that is characteristic of biological phenomena at all levels of analysis. Understanding such complex phenomena requires a combination of descriptive and explanatory knowledge. Descriptive knowledge consists of answers to "what?" questions – e.g., "what is there?" and "what is happening?". Explanatory knowledge, on the other hand, consists of answers to "why?" questions – "why are things one way and not another?". The achievement of explanatory understanding is contingent on the amassing of large quantities of descriptive knowledge – only when information about a large and diverse array of particulars is assembled can general patterns begin to be discerned.

In the present essay, we have detailed a number of approaches to the acquisition of highly resolved descriptive knowledge concerning the composition (venomics); activity and potency (toxicovenomics); and neutralisation (antivenomics and neutralisation assays) of snake venoms. In addition, we have provided perspectives, leveraging the contextual stance of ecology and evolutionary biology that provide a pathway towards explanatory knowledge. Mitigating the burden of snakebite envenoming will require strategies as multifactorial as the problem itself, including biotechnological approaches (e.g., knowledge-based refurbishing current antivenoms, creating recombinant antibodies, identifying small molecule inhibitors, etc. -Casewell et al., 2020) and health infrastructure engineering that have not been discussed in detail here. There is much work to be done. The specifics of venom variation and its impact in the clinical management of snakebite envenomings remain completely unknown, and thus our knowledge of the ecological validity of antivenoms against the venom of geographic variants of conspecific populations is extremely limited. Through the treatment of snakebite envenoming as the biological phenomenon that it is, by bringing to bear the resources of contemporary biological science in concert with those of biomedical science, we believe a pathway to deeper understanding is being forged. Understanding the problem will help us to solve it.

Author contributions

All the authors have contributed to the conceptualisation, original draft preparation, review and editing of the final manuscript version. All authors have read and agreed to the published version of the manuscript.

Ethical statement

Authors declare that international ethical guidelines for scientific papers were followed in the preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

BOX

- Integration and contextualisation of ecological and clinical toxinology information can be mutually enlightening for both disciplines. In the present essay we have provided perspectives that should make the reader reflect on the appropriate use of concepts common to both disciplines, as well as on how to translate animal research outcome to humans.
- Abundance and toxicity are conjugate variables in venom pharmacology. However, usage of this binomial is contingent on the appropriate toxinological context. The occurrence of taxon-preferent or taxon-specific venom toxins underscores the importance of considering the adequate animal model for the purpose of the research aim. The concept of venom median lethal dose (LD_{50}) has also different meanings depending on animal species used and field of application, i.e., ecological vs. clinical toxinology.
- Venomics, antivenomics and toxicovenomic are three complementary pillars of the molecular toxinologist's toolbox. The "ecological stance" should be also a vital part of the toxinologist's conceptual toolkit. The incorporation into preclinical antivenom evaluation of relevant snake's ecology data, such as mass of injected venom during defensive strikes of local medically relevant snakes and model animal LD_{50} figures allometrically scaled to human, could greatly impact the formulation of efficient snakebite therapeutics.

the work reported in this paper.

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